RESEARCH PAPER

Cooperation of three WRKY-domain transcription factors WRKY18, WRKY40, and WRKY60 in repressing two ABA-responsive genes ABI4 and ABI5 in Arabidopsis

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Abstract

Three evolutionarily closely related WRKY-domain transcription factors WRKY18, WRKY40, and WRKY60 in Arabidopsis were previously identified as negative abscisic acid (ABA) signalling regulators, of which WRKY40 regulates ABI4 and ABI5 expression, but it remains unclear whether and how the three transcription factors cooperate to regulate expression of ABI4 and ABI5. In the present experiments, it was shown that WRKY18 and WRKY60, like WRKY40, interact with the W-box in the promoters of ABI4 and ABI5 genes, though the three WRKYs have their own preferential binding domains in the two promoters. WRKY18 and WRKY60, together with WRKY40, inhibit expression of the ABI5 and/or ABI4 genes, which is consistent with their negative roles in ABA signalling. Further, genetic evidence is provided that mutations of ABI4 and ABI5 genes suppress ABA-hypersensitive phenotypes of the null mutant alleles of WRKY18 and WRKY60 genes, demonstrating that ABI4 and ABI5 function downstream of these two WRKY transcription factors in ABA signalling. A working model of cooperation of the three WRKYs in repressing ABI4 and ABI5 expression is proposed, in which the three WRKYs antagonize or aid each other in a highly complex manner. These findings help to understand the complex mechanisms of WRKY-mediated ABA signal transduction.

Key words: ABA-responsive gene, ABA signalling, ABI4, ABI5, Arabidopsis thaliana, WRKY transcription factor, WRKY18, WRKY40, WRKY60.

Introduction

The WRKY family is regarded as one of the 10 largest transcription factor families in higher plants and has been found throughout the green plant lineage (green algae and land plants) (Ulker and Somssich, 2004). The WRKY transcription factor family in Arabidopsis thaliana contains 74 members, which are divided into three subfamilies according to the number of WRKY domains and category of zinc-finger, forming integral parts of signalling webs that modulate many plant processes by means of modification of the expression patterns of WRKY genes and/or changes in their activity (Eulgem and Somssich, 2007; Rushton et al., 2010, 2012; Chen et al., 2012). The defining feature of WRKY transcription factors is their DNA-binding domain, which is the almost invariant WRKY amino acid sequence at the N-terminus (Rushton et al., 1996; Eulgem et al., 2000). The conservation of the WRKY domain is mirrored by a remarkable conservation of its cognate binding site, the W-box (TTGACY) (Rushton et al., 1996; Eulgem et al., 2000). However, the binding site preferences are also partly determined by additional adjacent
DNA sequences outside of the TTGACY core motif (Ciolkowski et al., 2008; Brand et al., 2010).

WRKY transcription factors play an important role in the response of plants to both biotic and abiotic stress and many developmental processes. The function and molecular mechanisms of WRKY transcription factors in biotic stress such as wounding and pathogen infection have been well studied to date. In particular, WRKY factors are key regulators, both positive and negative, of the two partly interconnected branches of plant innate immunity, microbe- or pathogen-associated molecular pattern-triggered immunity and effector-triggered immunity (Jones and Dang, 2006; Eulgem and Somssich, 2007; Rushton et al., 2010). In regard to abiotic stress, various WRKY transcription factors have been reported to be implicated in drought, cold, and thermo stress tolerance in plants (Chen et al., 2012; Rushton et al., 2012).

Abscisic acid (ABA) is an essential hormone in the regulation of plant growth and development, and in the control of plant adaptation to environmental challenges (reviewed in Finkelstein and Rock, 2002; Adie et al., 2007; Fujita et al., 2011). In the past decades, numerous ABA signalling components, including ABA receptors, have been identified (for reviews, see Finkelstein and Rock, 2002; Cutler et al., 2010; Fujita et al., 2011), although many key processes of the highly complex ABA signalling network remain largely unknown. WRKY transcription factors have been suggested to play important roles in ABA signalling pathways (Jiang and Yu, 2009; Ren et al., 2010; Shang et al., 2010; Chen et al., 2010; Chen et al., 2012; Rushton et al., 2012). In Arabidopsis, the expression level of many WRKY transcription factors can be promoted in the presence of exogenous ABA, and some of the WRKY transcription factors have been identified as key components in the ABA signalling pathways (Jiang and Yu, 2009; Ren et al., 2010; Shang et al., 2010; Chen et al., 2010; Chen et al., 2012; Rushton et al., 2012). WRKY proteins may control the complex mechanisms of transcriptional reprogramming via protein–protein interaction and autoregulation or cross-regulation of expression of their genes (Xu et al., 2006; Shen et al., 2007; Rushton et al., 2010, 2012; Chen et al., 2012; Wenke et al., 2012). Previous reports showed that three paralogous WRKY transcription factors, WRKY18, WRKY40, and WRKY60, cooperate to regulate plant defence in Arabidopsis in a complex pattern of overlapping, antagonistic, and distinct roles in response to different types of microbial pathogens (Xu et al., 2006; Shen et al., 2007; Wenke et al., 2012). The three closely related WRKY proteins can form both homocomplexes and heterocomplexes, and the DNA binding activities were significantly shifted according to the WRKY proteins present in these complexes (Xu et al., 2006). It was previously shown that these three WRKY proteins are also involved in ABA signalling, where WRKY40 plays a central role, and function as negative regulators directly downstream of a candidate ABA receptor CHLH/ABAR (Mg-chelatase H subunit/putative ABA receptor) localized to the chloroplast/plastid envelope membrane, and also repress a set of ABA-responsive genes (Shang et al., 2010). At the same time, an independent group reported that WRKY40 negatively regulates ABA signalling, which is consistent with the previous findings, although they found that two other paralogues, WRKY18 and WRKY60, may positively regulate ABA signalling (Chen et al., 2010). However, it still remains unclear whether and how the three paralogous WRKY transcription factors cooperate to regulate expression of ABA-responsive genes, especially ABI4 (Finkelstein et al., 1998) and ABI5 (Finkelstein and Lynch, 2000), which encode two key components in the ABA-mediated signalling pathways in seed germination and post-germination growth. To address this question is of importance in order to understand the highly complex ABA signalling pathways.

In the present experiments, it was shown that WRKY18 and WRKY60, together with WRKY40, interact with the promoters of ABI4 and ABI5 genes, and cooperatively inhibit ABI4 and ABI5 expression, and, further, genetic evidence is provided that ABI4 and ABI5 function downstream of WRKY18 and WRKY60 transcription factors in ABA signalling. A working model of cooperation of the three WRKYs in repressing ABI4 and ABI5 expression is proposed, in which the three WRKYs antagonize or aid each other in a highly complex manner. These findings help to understand the complex mechanisms of WRKY-mediated ABA signal transduction.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) were used as wild-type plants. The three wrky mutants, wrky18, wrky40, and wrky60, were described in a previous report (Shang et al., 2010). Briefly, the wrky40-1 mutant (named wrky40 in this report; stock no. ET5883, with the Ler ecotype as background) was obtained from Cold Spring Harbor Laboratory gene and enhancer trap lines, and contains a T-DNA inserted within the second exon of WRKY40 (Arabidopsis genomic locus tag: At1g08340), and the wrky18-1 mutant [named wrky18 in this report; Arabidopsis Biological Resource Center (ABRC) stock no. SALK 003916] and wrky60-1 mutant (named wrky60 in this report; ABRC stock no. SALK_097006) are T-DNA insertion knockout mutants with a T-DNA insertion within the first exon in the WRKY18 (At4g31800) and WRKY60 (At2g25000) genes, respectively. Both wrky18 and wrky60 mutants were isolated from the Col-0 ecotype. All three mutants were previously identified as null alleles in their respective genes (Xu et al., 2006; Shang et al., 2010). The wrky40-1 mutation was transferred from its background Ler ecotype into the Col-0 ecotype by backcrossing, as previously described (Xu et al., 2006; Shang et al., 2010). Double and triple mutants were generated by genetic crosses and identified by PCR genotyping. The abi4-1 mutant (named abi4 in this report; ABRC stock no. CS8104) was isolated from the Col-0 ecotype (Finkelstein et al., 1998). The mutated abi5-1 gene in the abi5-1 mutant (named abi5 in this report; ABRC stock no. CS8105) was transferred from its background Wassilewskija (Ws) ecotype into the Col-0 ecotype by backcrossing. Double mutants abi4 wrky18, abi4 wrky60, abi5 wrky18, and abi5 wrky60 were created by crossing.

Plants were grown in a growth chamber at 19–20 °C on Murashige and Skoog (MS) medium (Sigma) at ~80 μmol photons m⁻² s⁻¹, or in compost soil at ~120 μmol photons m⁻² s⁻¹ over a 16 h photoperiod.
Real-time PCR for expression of various genes was performed as previously described (Shang et al., 2010). Briefly, the cDNAs encoding these proteins were amplified by PCR using the following primer pairs: forward primer 5'-GGAATTCATGGATCAGTACTC-3' and reverse primer 5'-ACGGCTGCATCATGCTGTTCT-3' for WRKY40; forward primer 5'-GGAATTCTATGGGACGTTTCTCTTTC-3' and reverse primer 5'-ACGGCTGCACTCATGCTGTTCT-3' for WRKY18; and forward primer 5'-GGAATTCTATGGGACGTTTCTCTTTC-3' and reverse primer 5'-ACGGCTGCACTCATGCTGTTCT-3' for WRKY60. The PCR products were digested and cloned into pET48b (+). The cDNA fragments in the plasmids were sequenced to check for errors. The recombinant cDNAs were expressed in E. coli BL21 (DE3) (Novagen) strains as 6xHis-tagged fusion proteins. The E. coli strains expressing the target plasmids were grown at 37 °C in 1 litre of LB medium containing 50 µg/ml of kanamycin until the OD600 was 0.6–0.8. Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM at 16 °C with 150 rotations min⁻¹. After 16 h, the cells were lysed and proteins purified on a Ni²⁺-chelating column as described in the pET system manual.

Quantitative real-time PCR

Real-time PCR for expression of various genes was performed as previously described (Wu et al., 2009; Shang et al., 2010) essentially according to the instructions provided for the BioRad Real-Time System CFX96TM C1000 Thermal Cycler (Singapore). The primers used are as follows: forward primer 5'-GTTAAACATTGTGCTCAGTGG-3' and reverse primer 5'-AACGACCTTAATCTTCATGCTGC-3' for Actin2/8 (an internal control); forward primer 5'-GGGGGACGAAA CGAGGGAAAGT-3' and reverse primer 5'-ACGGCCGTGTTG ATGAGTTATG-3' for ABI4 (At2g40220); and forward primer 5'-CAAAAGAGGAGGATAGCAGAGAG-3' and reverse primer 5'-CGTCACCATTGGCTGTCTCTTCCA-3' for ABI5 (At2g36270).

Yeast one-hybrid assay

Yeast one-hybrid assay was performed essentially as previously described (Shang et al., 2010) with the kit provided by Clontech (Matchmaker™ One-Hybrid Library Construction & Screening Kit catalogue no. 630304) using the AH109 yeast strain according to the manufacturer's instructions. The related cDNAs or promoter DNAs were amplified by PCR using the following primer pairs: forward primer 5'-TCCCCGGGCGCCGCGGA CGGATTCTTTGTGC-3' and reverse primer 5'-CGACCGGTCTTCCA CACCCACCTCTCCA-3' for the ABI5 promoter; forward primer 5'-CGGGAATTCTATGGGACGTTTCTCTTTG-3' and reverse primer 5'-CGGGAATTCTATGGGACGTTTCTCTTTG-3' for the ABI4 promoter; forward primer 5'-CTCCCCCGGGCCGACGCGAA CGGATTCTTTGTGC-3' and reverse primer 5'-CGACCGGTCTTCCA CACCCACCTCTCCA-3' for the ABI5 promoter; forward primer 5'-CGGGAATTCTATGGGACGTTTCTCTTTG-3' and reverse primer 5'-CGGGAATTCTATGGGACGTTTCTCTTTG-3' for the ABI4 promoter; forward primer 5'-GGAATTCATGGGACGTTTCTCTTTTGC-3' and reverse primer 5'-GGAATTCATGGGACGTTTCTCTTTTGC-3' for WRKY40 cDNA; forward primer 5'-GGAATTCATGGGACGTTTCTCTTTTGC-3' and reverse primer 5'-GGAATTCATGGGACGTTTCTCTTTTGC-3' for WRKY18 cDNA; and forward primer 5'-CGGGAATTCTATGGGACGTTTCTCTTTTGC-3' and reverse primer 5'-CGGGAATTCTATGGGACGTTTCTCTTTTGC-3' for WRKY60 cDNA. The promoter fragments of ABI4 and ABI5 were subcloned into the Smal/MluI sites of the pPHIS2 vector. The one-hybrid assays were performed using the AH109 yeast strain according to the manufacturer's instructions. Yeast cells were co-transformed with pHIS2 bait vector that harboured the promoter of target genes and pGADT7 prey vector that carried the ORF of WRKY40, WRKY18, and WRKY60. As negative controls, the yeast cells were transformed with the empty pGADT7 vector with pHIS2 harbouring the corresponding promoter. Transformed yeast cells were first grown in liquid tryptone (Trp) and leucine (Leu)-deficient SD medium (SD- Trp-Leu-His) to ensure that the yeast cells were successfully co-transformed, and then the yeast cells were grown on plates of the Trp, Leu, and histidine (His)-deficient SD medium (SD-Trp-Leu-His) supplemented with 3-amino-triazole (3-AT; Sigma) at 25 mM (for the pHIS2 vector with the ABI5 promoter) or 5 mM (for the pHIS2 vector with the ABI4 promoter). The plates were then incubated at 30 °C for 3 d prior to investigations.

Gel shift assay

Gel shift assay (GSA) was performed essentially as previously described (Brand et al., 2010; Shang et al., 2010) using the recombinant His-WRKY40, His-WRKY18, and His-WRKY60 proteins purified from E. coli as described above. The promoter fragments used for the GSA were amplified by PCR using the following primer pairs: forward primer 5'-GACAAAGAGATGTTGACCTC-3' and reverse primer 5'-GGAGAATTTTCTGACTGAAGG-3' for the ABI5 promoter fragment pABI5-I; forward primer 5'-GACAAAGAGATGTTGACCTC-3' and reverse primer 5'-GGAGAATTTTCTGACTGAAGG-3' for the ABI5 promoter fragment pABI5-II; forward primer 5'-CTGTTTTGCTCAGCTGACG-3' and reverse primer 5'-GTCCTTTTACATCTACG-3' for the ABI3 promoter fragment pABI3-I; forward primer 5'-CTGTTTTGCTCAGCTGACG-3' and reverse primer 5'-GTCCTTTTACATCTACG-3' for the ABI3 promoter fragment pABI3-II. Yeast one-hybrid assays were performed using the pHIS2 vector with the ABI4 promoter fragment pABI4-I; and forward primer 5'-CTGTTTTGCTCAGCTGACG-3' and reverse primer 5'-GTCCTTTTACATCTACG-3' for the ABI4 promoter fragment pABI4-II; and forward primer 5'-CTGTTTTGCTCAGCTGACG-3' and reverse primer 5'-GTCCTTTTACATCTACG-3' for the ABI4 promoter fragment pABI4-III; forward primer 5'-CTGTTTTGCTCAGCTGACG-3' and reverse primer 5'-GTCCTTTTACATCTACG-3' for ABI4 promoter fragment pABI4-IV; and forward primer 5'-CTGTTTTGCTCAGCTGACG-3' and reverse primer 5'-GTCCTTTTACATCTACG-3' for ABI4 promoter fragment pABI4-VI. The boxed letters in the primer sequences indicate mutated nucleotides.

The site-specific mutations in the core sequence of the pABI5-I and pABI5-II fragments of the promoters were introduced into the above promoters by two independent PCRs. Reconstitution was done using equimolar quantities of the two fragments from the initial PCRs for each promoter, which were used as the template for a third PCR. The mutations were verified by sequence analysis. Each of the promoter fragments was labelled in the base T with digoxigenin-dUTP (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Binding reactions were performed in 20 µl of binding buffer composed of 10 mM TRIS-HCl (pH 8.0), 20 mM NaCl, 0.4 mM MgCl₂, 0.5 mM ZnSO₄, 0.25 mM EDTA, 0.25 mM diithiothreitol (DTT), and 10% glycerol, and in the presence of 0.04 mg ml⁻¹ of poly(dI–dC) sodium salt (Sigma). Binding reactions were carried out using 50 ng of His-WRKY fusion proteins and 26 ng of each of the digoxigenin-labelled promoter fragments at room temperature for 30 min. Samples were separated on a 5% denaturing polyacrylamide gel (19:1 acrylamide:bisacrylamide) in 0.25 TRIS-borate-EDTA at 4 °C, transferred onto a nylon filter, exposed under ultraviolet light to cross-link the samples to the filter, washed with SSC buffer twice for 15 min each, blocked with blocking reagent (provided in the kit) for 2 h, incubated with the anti-digoxigenin-AP (alkaline phosphatase) antibody for 1 h before adding the AP substrate into the mixture, and analysed by autoradiography. The WRKY40 protein amounts loaded for the GSA were tested by immuno blotting with both the anti-His serum (Beijing Zhongshan Jinqiao Biotechnology Co. Ltd., China) and an antisera specifically against WRKY40 that was described previously (Shang et al., 2010), and WRKY18 and WRKY60 proteins were assayed using the anti-His serum.
Trans-inhibition of ABI4 promoter and ABI5 promoter activity by WRKYs in tobacco leaves

This assay was performed essentially according to the previously described procedures (Shang et al., 2010). WRKY40, WRKY18, and WRKY60 were used for the construction of the reporter constructs. The cDNAs of the WRKY genes were amplified by PCR using the following primer pairs: forward primer 5'-GCTGTACAGTTGACTCTAGC-3’ and reverse primer 5'-AACCTGTCGAGAATTCGATATGAC-3' for WRKY40; forward primer 5'-GGGTTACCATGAGCAGCTTTGTTTC-3' and reverse primer 5'-ACCCTGTCGAGAATTCGATATGAC-3' for WRKY18; and forward primer 5'-GCTGTACAGTTGACTCTAGC-3’ and reverse primer 5'-GGGTTACCATGAGCAGCTTTGTTTC-3' for WRKY60. The PCR products were fused to the pCAMBIA1300-Myc vector downstream of the Cauliflower mosaic virus (CaMV) 35S promoter and the Myc tag at the XbaI/SalI sites for WRKY40 and WRKY60, and KpnI/SalI sites for WRKY18. The reporter constructs were composed of the ABI5 or ABI4 promoter linked to the luciferase (LUC) gene. The ABI4 promoter was isolated using the forward primer 5'-CGGGGATCCAGCAAGCCGG-3’ and reverse primer 5'-TCCCCTGGAACACTGTCATTAC-3’. The ABI4 promoter was isolated using the forward primer 5'-GGGTTACCATGAGCAGCTTTGTTTC-3' and reverse primer 5'-ACCCTGTCGAGAATTCGATATGAC-3' for WRKY40; forward primer 5'-GGGTTACCATGAGCAGCTTTGTTTC-3' and reverse primer 5'-ACCCTGTCGAGAATTCGATATGAC-3' for WRKY18; and forward primer 5'-GCTGTACAGTTGACTCTAGC-3’ and reverse primer 5'-GGGTTACCATGAGCAGCTTTGTTTC-3' for WRKY60. 

Phenotypic analysis

Phenotypic analysis was done essentially as previously described (Shen et al., 2006; Wu et al., 2009; Shang et al., 2010). Briefly, to assay germination, seeds (dry seeds ~1 month after harvest) were planted on MS medium (Sigma, St. Louis, MO, USA; product #M5524; full-strength MS) that contained 3% sucrose and 0.8% agar (pH 5.9). The medium was supplemented or not with different concentrations of (+)-ABA. The seeds were incubated at 4 °C for 3 d for stratification, and then placed at 20 °C under light conditions, and germination (emergence of radicals) was scored at the indicated times. Early seedling growth was assessed by directly planting the seeds in the (+)-ABA-containing MS medium. The seeds were incubated at 4 °C for 3 d for stratification, and then placed at 20 °C under light conditions to investigate the response of seedling growth to ABA after germination. Early seedling growth was also investigated by transferring germinating seeds to (+)-ABA-containing MS medium, and then placed at 20 °C under light conditions to investigate the response of seedling growth to ABA after germination. Early seedling growth was investigated at the indicated times after the transfer, and the length of primary roots was measured using a ruler.

Accession numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession nos: At1g08080 (WRKY40), At4g31800 (WRKY18), At2g25000(WRKY60), At2g40220 (ABI4), and At2g36270 (ABI5). Germplasm identification numbers for mutant lines and SALK lines are as follows: abi4-1 (ABRC stock no. CS8104), abi5-1 (ABRC stock no. CS8105), and wrky40-1 (stock no. ET5883, Cold Spring Harbor Laboratory gene and enhancer trap lines). wrky18-1 (ABRC stock no. SALK_093916), and wrky60-1 (ABRC stock no. SALK_120706).

Results

WRKY18, WRKY40, and WRKY60 bind the ABI4 promoter and cooperatively inhibit its activity

It was previously observed that WRKY40 binds the promoter of the ABI4 gene (Shang et al., 2010), but it still remains unknown whether it regulates ABI4 expression, and whether WRKY18 and WRKY60 also interact with ABI4 promoter and regulate its expression. ABI4 belongs to a class of Apetala-2 domain transcription factors (Finkelstein et al., 1998), and the ABI4 gene has, in its promoter region, W-box sequences, a
cis-regulatory element to which the WRKY transcription factors bind. Interactions of the WRKYs with the *ABI4* promoter were first tested in a yeast one-hybrid system. Yeast cells co-transformed with both a WRKY-encoding cDNA (*WRKY18, WRKY40, or WRKY60*) and the *ABI4* promoter could grow in a selection SD medium (lacking Trp, Leu, and His nutrients, and containing 3-AT), indicating a potential interaction between the WRKYs and the *ABI4* promoter (Fig. 1A). As a negative control, yeast cells co-transformed with empty vector (pGADT7) and the *ABI4* promoter could not grow in this medium (Fig. 1A), showing that this system was specific for testing protein–promoter interactions. These data suggested that WRKY18 and WRKY60 potentially bind the promoter of *ABI4* (Fig. 1A).

These protein–promoter interactions were confirmed, and interactions of the WRKYs with two different domains of the *ABI4* promoter were further tested in a GSA, in which one domain (domain I, p*ABI4*-I) covers the first W-box and another covers the second and third W-boxes (domain II, p*ABI4*-II) (Fig. 1B; Supplementary Table S1 available at JXB online). The three WRKY proteins were produced and purified from *E. coli* (Supplementary Fig. S1), and it was shown that the purified WRKY18, WRKY40, and WRKY60 proteins all bind domain I (p*ABI4*-I) (Fig. 1C). The mixed proteins WRKY18 plus WRKY40, WRKY18 plus WRKY60, WRKY40 plus WRKY60, or all three WRKYs all showed binding activities to this promoter domain (Fig. 1C).

WRKY40 and WRKY60, but not WRKY18, showed binding activities to domain II of the *ABI4* promoter (p*ABI4*-II), and the mixed proteins pairs WRKY18 plus WRKY40, WRKY18 plus WRKY60, or WRKY40 plus WRKY60 also showed binding activities to this promoter domain, while the three mixed WRKYs showed no binding activity to this domain (Fig. 1D), suggesting that the interactions of the three WRKYs with each other affect their binding to this domain. It is noteworthy that a previous report showed the *in vivo* interaction of WRKY40 with the *ABI4* promoter by ChIP via p*ABI4*-I/II domains (Fig. 1B, and see also Shang et al., 2010), which is consistent with the data of the present GSA.

As negative controls, neither the binding activity of the WRKYs to the *ABI4* promoter domains harbouring a mutated W-box 1 (Fig. 1C) or mutated W-boxes 2 and 3 (Fig. 1D), nor that of 6×His peptide and bovine serum albumin (BSA) to any domain of the *ABI4* promoter (Fig. 1C, D), was observed, showing that the observed binding activities of the WRKYs were specific in this GSA system. Additionally, the loading amounts of the different WRKY proteins in the assays were tested by immunoblotting, which showed that substantially an equal amount of each WRKY protein was loaded in the assays with a single WRKY protein, WRKY18, WRKY40, or WRKY60, while the amounts were equally doubled in the assays with two WRKY proteins, and tripled with three WRKY proteins, indicating that the loss of binding activities of WRKY18 and the mixture of the three WRKYs to the p*ABI4*-II domain (Fig. 1D) was specific, but was not caused by a possible difference in protein loading amount.

Next, the interactions of WRKY18 and WRKY60 with the *ABI4* promoter in the same above-mentioned p*ABI4*-I/II domains were assayed by ChIP analysis combined with PCR and quantitative real-time PCR. It was observed that WRKY18 binds to the promoters of *ABI4* via the p*ABI4*-I domain but not the p*ABI4*-II domain, and that WRKY60 binds the promoters of *ABI4* via both the p*ABI4*-I and p*ABI4*-II domains (Fig. 2A, B; Supplementary Table S1 at JXB online), which is consistent with the data of the above-described GSA (Fig. 1B–D), and reveals that WRKY18 and WRKY60 interact with the *ABI4* promoter in vivo.

Furthermore, the interactions of WRKYs with the *ABI4* promoter were assayed in an *in vivo* system by co-transforming tobacco leaves with the WRKY genes and the *ABI4* promoter linked to LUC. The results showed that all the three WRKYs interact with the *ABI4* promoter in vivo, and WRKY40 and WRKY60 inhibit the promoter activity reported by LUC-produced fluorescence (Fig. 3A). It was observed that co-expression of WRKY18 and WRKY40 suppressed the repressive effect of WRKY40, and co-expression of WRKY40 and WRKY60 partly suppressed the repressive effect of the single WRKY40 or WRKY60 on the *ABI4* promoter activity (Fig. 3B). The co-expression of the three WRKYs completely suppressed the repressive effect of the each single WRKY on the *ABI4* promoter activity (Fig. 3C), which is consistent with the inability of the mixture of the three WRKYs to bind to domain II of the *ABI4* promoter observed in the GSA (Fig. 1D). As internal controls, the ActIn2 protein was co-expressed with the *ABI4* promoter–LUC and/or with the WRKYs in a virtually equal amount for each assay, which did not change the activity of *ABI4* promoter–LUC (Fig. 3D) or any observations (Fig. 3A–C), and the expression levels of each WRKY protein, assayed by immunoblotting with the antiserum against their Myc tag, showed no significant difference among them in any assay (Fig. 3A, B), indicating that the observed differences in the fluorescence intensity were not caused by possible differences in the WRKY levels. These controls showed that the observations of these WRKY–*ABI4* promoter interaction assays are specific and reliable. Additionally and importantly, it was further shown that WRKY40 and WRKY60 did not inhibit the activity of the *ABI4* promoter harbouring W-box 2 and 3 double mutations (Fig. 4A), revealing that the WRKY proteins bind the *ABI4* promoter through the W-box motif.

**WRKY18, WRKY40, and WRKY60 bind the *ABI5* promoter and cooperatively inhibit its activity**

It was previously shown that WRKY40 binds the promoter of *ABI5* and represses its expression (Shang et al., 2010), but it still remains unknown whether WRKY18 and WRKY60 interact with the *ABI5* promoter and regulate its expression. *ABI5* is a member of the basic leucine zipper transcription factor family (Finkelstein and Lynch, 2000), and there are six W-boxes in the promoter region of the *ABI5* gene. The same assays as described above for the *ABI4* promoter was performed to test interactions of WRKYs with the *ABI5* promoter. It was shown that, as previously reported for WRKY40 (Shang et al., 2010), the two other WRKY members WRKY18 and WRKY60 interact with the *ABI5* promoter in the yeast one-hybrid system (Fig. 5A). For assaying these protein–promoter interactions by the GSA system, three domains in the *ABI5* promoter were isolated: domain I (p*ABI5*-I) covers the first and second W-boxes, domain II (p*ABI5*-II) covers the fourth and fifth W-boxes, and
Fig. 1. Interaction of WRKYs with the \textit{ABI4} promoter. (A) Yeast one-hybrid assay. The prey vector harbouring WRKY40, WRKY18, and WRKY60 (indicated by +WRKY18, +WRKY40, and +WRKY60, respectively) and the bait vector pHIS2 harbouring the \textit{ABI4} promoter (indicated by p\textit{ABI4}-pHIS2) were used to co-transform yeast cells. The transformation with the empty vector pGADT7 (indicated by + pGADT7) was taken as a negative control. The experiments were repeated three times with the same results. (B) The promoter structure of the \textit{ABI4} gene. W1, W2, and W3 denote each W-box numbered from left to right with sequence sites relative to the start code. The top bars with the p\textit{ABI4}-1 and p\textit{ABI4}-2 domains indicate the sequence fragments used in the ChIP assay with the antiserum specifically against WRKY40, which was described in a previous report (Shang \textit{et al.}, 2010). The bottom bars with the first domain (p\textit{ABI4}-I) and second domain (p\textit{ABI4}-II) indicate the sequence fragments used in the GSAs described in (C) and (D). (C) GSA shows that WRKY18, WRKY40, WRKY60, WRKY18 plus WRKY40 (WRKY18+40), WRKY18 plus WRKY60 (WRKY18+60), WRKY40 plus WRKY60
domain III covers the sixth W-box (Fig. 5B). W-box 3 was not assayed due to a technical difficulty with its isolation (Fig. 5B). It was shown that WRKY18 and WRKY40, but not WRKY60, bind domain I (pABI5-I) and domain II (pABI5-II) (Fig. 5C, D), and that WRKY18 and WRKY60, but not WRKY40, bind domain III (pABI5-III) of the ABI5 promoter (Fig. 5E). The binding of WRKY18 to domain II disappeared when it was mixed with WRKY60 (Fig. 5D), and binding of WRKY 18 and WRKY60 to domain III disappeared when the two WRKYs were mixed together (Fig. 5E), suggesting a mutual antagonistic effect of WRKY18 and WRKY60 in these binding processes to domains II and III of the ABI5 promoter. However, WRKY18 (WRKY40+60), and WRKY40 plus WRKY18 plus WRKY60 (WRKY40+18+60) all bind the pABI4-I fragment (left panel). The 6×His tag peptide (6His), which was fused to WRKY protein for WRKY protein production, and BSA protein (left panel) and the promoter fragments harbouring the mutated W-box 1 (W1) (right panel), served as negative controls. W-box mutation at W1: TTGAC→TTCAC (see the Materials and methods). The experiments were repeated five times with substantially the same results. (D) GSA shows that WRKY40, WRKY60, WRKY40 plus WRKY18 (WRKY40+18), WRKY18 plus WRKY60 (WRKY18+60), and WRKY40 plus WRKY60 (WRKY40+60) bind the pABI4-II fragment, but WRKY18 and WRKY40 plus WRKY60 plus WRKY60 (WRKY40+18+60) do not (left panel). The 6×His tag peptide (6His), which was fused to WRKY protein for WRKY protein production, and BSA protein and the promoter fragments harbouring the mutated W-boxes 2 and 3 (W2/W3), served as negative controls (right panel). W-box mutations at W2 and W3: TGAC→TTAC (see the Materials and methods). The experiments were repeated five times with substantially the same results. In (C) and (D), loading protein amounts were tested by immunoblotting with anti-6His serum and an antiserum specifically against WRKY40 (Shang et al., 2010), and relative band intensities, which are normalized relative to the intensity of the loading sample of the assay with WRKY40 alone (indicated by 100), are indicated by numbers above (for ‘WRKY40’ bands) or below the bands (for ‘Ant-6His’ bands).
Fig. 3. Test of the interactions of WRKYs with the ABI4 promoter in vivo in tobacco leaves. Tobacco leaves were transformed with the following constructs together with a control construct harbouring the Actin-encoding open reading frame, called ‘K’: (A) with pABI4-LUC plus K, pABI4-LUC plus WRKY40 and K, pABI4-LUC plus WRKY18 and K, or pABI4-LUC plus WRKY60 and K; (B) with pABI4-LUC plus K, pABI4-LUC plus WRKY40 and WRKY18 and K, pABI4-LUC plus WRKY18 and WRKY60 and K, or pABI4-LUC plus WRKY40 and WRKY60 and K; (C) with pABI4-LUC plus K, or pABI4-LUC plus WRKY40 and WRKY18 and WRKY60 and K; and (D) with pABI4-LUC alone and pABI4-LUC plus K (as controls). The prefix ‘p’ indicates promoter. Left panels in (A), (B), (C), and (D): fluorescence imaging. Right panels in (A), (B), (C), and (D): fluorescence intensities estimated by fluorescence counts. The expression levels of the internal control Actin protein (K) and the Myc-tagged WRKY18/WRKY40/WRKY60 protein were assayed by immunoblotting with antiserum against Actin and anti-Myc serum, respectively, and are displayed below the fluorescence count columns. Relative protein
WRKYs regulate *ABI4* and *ABI5* expression

Fig. 4. Test of the interactions of WRKYs with the W-box-mutated *ABI4* and *ABI5* promoters in vivo in tobacco leaves. The *ABI4* promoter harbours W-box 2 and 3 double mutations (still named pABI4), and the *ABI5* promoter harbours W-box 1, 2, and 4 triple mutations (still named pABI5). The tobacco leaves were transformed with the following constructs together with a control construct harboring the Actin-encoding open reading frame (K): (A) with pABI4-LUC plus K, pABI4-LUC plus WRKY40 and K, pABI4-LUC plus WRKY18 and K, or pABI4-LUC plus WRKY60 and K; (B) with pABI5-LUC plus K, pABI5-LUC plus WRKY40 and K, pABI5-LUC plus WRKY18 and K, or pABI5-LUC plus WRKY60 and K. Left panels in (A) and (B): fluorescence imaging. Right panels in (A) and (B): fluorescence intensities estimated by fluorescence counts. The expression levels of the internal control Actin protein (K) and the Myc-tagged WRKY18/WRKY40/WRKY60 protein were assayed by immunoblotting with antiserum against Actin and anti-Myc serum, respectively, and are displayed below the fluorescence count columns. Relative protein band intensities, which are normalized relative to the intensity of the sample from no-transgenic portions (NT) of the same transgenic leaves for K (Actin), or to that of the pABI4- (A) or pABI5-WRKY40 treatment (B), are indicated by numbers below the bands. Each value for the columns in the right panels is the mean ±SE of five independent determinations, and different letters indicate significant differences at $P < 0.05$ (Duncan’s multiple range test).
Fig. 5. Interaction of WRKYS with the ABI5 promoter. (A) Yeast one-hybrid assay. The prey vector harbouring WRKY40, WRKY18, and WRKY60 (indicated by +WRKY40, +WRKY18, and +WRKY60, respectively) and the bait vector pHIS2 harbouring the ABI5 promoter (indicated by pABI5-pHIS2) were used to co-transform yeast cells. The transformation with empty vector pGADT7 (indicated by +pGADT7) was taken as a negative control. The experiments were repeated three times with the same results. (B) The promoter structure of the ABI5 gene. W1–W6 denote each W-box numbered from left to right with sequence sites relative to the star code. The top bars with the pABI5-1 to ABI5-5 domains indicate the sequence fragments used in the ChIP assay with the antiserum specifically against WRKY40, which was described in a previous report (Shang et al., 2010). The bottom bars with the first domain (pABI5-I),
and WRKY60 did not show antagonistic interaction in their binding to domain I (Fig. 5C). The mixed proteins WRKY18 plus WRKY40 and WRKY40 plus WRKY60 showed binding activities to all three domains I, II, and III (Fig. 5C, D, E). The mixture of the three WRKYs was shown to bind domain III (Fig. 1E), but not domains I or II (Fig. 3C, D). These data suggest, as described above for the ABI4 promoter, that the interactions of the WRKYs with each other affect their binding to the ABI5 promoter domains.

Neither the binding activity of the WRKYs to the negative control ABI5 promoter domains harbouring various mutated W-boxes, nor that of 6×His peptide and BSA to any domain of the ABI5 promoter (Fig. 5C, D, E), was observed, showing that the observed binding activities of the WRKYs were specific. The loading amounts of the different WRKY proteins in the assays, tested by immunoblotting, were shown to be substantially equal for each WRKY protein in the assays with a single WRKY protein, and the amounts were equally doubled in the assays with two WRKY proteins, and tripled with three WRKY proteins, indicating that the loss of binding activities of the different WRKYs or mixtures (Fig. 5C–E) was specific, but was not caused by a possible difference in the protein loading amount.

The above-described binding activity of WRKY18 to all three domains (pABI5-I/II/III) of the ABI5 promoter and that of WRKY60 to the pABI5-III domain of the ABI5 promoter (Fig. 5B–E) were confirmed by the ChIP analysis (Fig. 2A, B; Supplementary Table S1 at JXB online), revealing that WRKY18 and WRKY60 interact with the ABI5 promoter in vivo.

It is noteworthy that the in vivo interaction of WRKY40 with the ABI5 promoter by the ChIP assay via pABI5-3/4 domains (Shang et al., 2010) that cover the pABI5-III domain of the present experiment (Fig. 5B), but not via the pABI5-5 domain (Shang et al., 2010) that covers the pABI5-III domain of the present experiment (Fig. 5B), was previously shown, which is consistent with the data of the above-described GSA (Fig. 5D, E). However, the previous report did not detect any binding activity of WRKY40 to the pABI5-1/2 domains (Shang et al., 2010) that cover the present pABI5-I domain (Fig. 5B), which is inconsistent with the data of the above-described GSA (Fig. 5C).

These experiments were conducted repeatedly, and the results were confirmed. The reason for this inconsistency is unknown. Nevertheless, the present GSA-detected in vitro interaction of WRKY40 with the ABI5 promoter is essentially consistent with the ChIP-detected in vivo data.

Furthermore, using the in vivo system by co-transforming tobacco leaves with the WRKY genes and the ABI5 promoter, it was shown that WRKY18 and WRKY40 interacted with the ABI5 promoter in vivo, and inhibited the promoter activity, while WRKY60 showed a reduced in vivo effect (Fig. 6A). This is globally consistent with the GSA data in which WRKY60 does not bind to the ABI5 promoter (Fig. 3C, D). It was further observed that co-expression of WRKY18 and WRKY60 suppressed the repressive effect of WRKY18 on the ABI5 promoter activity (Fig. 6B), which is consistent with the antagonistic effect of WRKY60 on WRKY18 binding to domains II and III of the ABI5 promoter observed in the GSA (Fig. 5D, E). As mentioned above for the ABI4 promoter (Fig. 3C), co-expression of the three WRKYs completely suppressed the repressive effect of each single WRKY18 or WRKY40 on the ABI5 promoter activity (Fig. 6C), which is consistent with the inability of the mixture of the three WRKYs to bind domain I or II of the ABI5 promoter observed in the GSA (Fig. 5C, D).

In the internal controls, it was observed that the Actin2 protein with the ABI5 promoter–LUC and/or with the WRKYs co-expressed in a substantially equal amount did not change the activity of the ABI5 promoter–LUC (Fig. 6D) or any observations (Fig. 6A–C), and the expression levels of each WRKY protein, assayed by immunoblotting with the antiserum against their second domain (pABI5-II), and third domain (pABI5-III) indicate the sequence fragments used in the GSAs described in (C), (D), and (E). (C) GSA shows that WRKY18, WRKY40, WRKY40 plus WRKY18 (WRKY40+18), WRKY18 plus WRKY60 (WRKY18+60), and WRKY40 plus WRKY60 (WRKY40+60) bind the pABI5-I fragment, but WRKY60 and WRKY40 plus WRKY18 plus WRKY60 (WRKY40+18+60) do not (left panel). The 6×His tag peptide (6His), which was fused to WRKY protein for WRKY protein production, and BSA protein and the promoter fragments harbouring the mutated W-boxes 1 and 2 (W1, W2) or the W1/W2 double mutation, served as negative controls (right panel). W-box mutation at W1: TTGAC→TTCAC, and at W2: GTCAA→CTCAA (see the Materials and methods). The experiments were repeated five times with substantially the same results. (D) GSA shows that WRKY40, WRKY18, WRKY40 plus WRKY18 (WRKY40+18), and WRKY40 plus WRKY60 (WRKY40+60) bind the pABI5-II fragment, but WRKY60, WRKY18 plus WRKY60 (WRKY18+60), and WRKY40 plus WRKY18 plus WRKY60 (WRKY40+18+60) do not (top panel). The 6×His tag peptide (6His), which was fused to WRKY protein for WRKY protein production, and the promoter fragments harbouring the mutated W-box 4 (W4), served as negative controls (bottom panel). W-box mutation at W4: GTCA→GTTA (see the Materials and methods). The experiments were repeated five times with substantially the same results. (E) GSA shows that WRKY18, WRKY60, WRKY40 plus WRKY18 (WRKY40+18), WRKY40 plus WRKY60 (WRKY40+60), and WRKY40 plus WRKY18 plus WRKY60 (WRKY40+18+60) bind the pABI5-III fragment, but WRKY40 and WRKY18 plus WRKY60 (WRKY18+60) do not (top panel). The 6×His tag peptide (6His), which was fused to WRKY protein for WRKY protein production, and BSA protein and the promoter fragments harbouring the mutated W-box 6 (W6), served as negative controls (bottom panel). W-box mutation at W6: GTCAA→CTCAA (see the Materials and methods). The experiments were repeated five times with substantially the same results. In (C), (D), and (E), loading protein amounts were tested by immunoblotting with anti-6His serum and an antiserum specifically against WRKY40 (Shang et al., 2010), and relative band intensities, which are normalized relative to the intensity of the loading sample of the assay with WRKY40 alone (indicated by 100), are indicated by numbers above (for ‘WRKY40’ bands) or below the bands (for ‘Ant-6His’ bands).
Fig. 6. Test of the interactions of WRKYs with the ABI5 promoter in vivo in tobacco leaves. The tobacco leaves were transformed with the following constructs together with a control construct harbouring the Actin-encoding open reading frame, called 'K': (A) with pABI5-LUC plus K, pABI5-LUC plus WRKY40 and K, pABI5-LUC plus WRKY18 and K, or pABI5-LUC plus WRKY60 and K; (B) with pABI5-LUC plus K, pABI5-LUC plus WRKY40 and WRKY18 and K, pABI5-LUC plus WRKY18 and WRKY60 and K, or pABI5-LUC plus WRKY40 and WRKY60 and K; (C) with pABI5-LUC plus K, or pABI5-LUC plus WRKY40 and WRKY18 and WRKY60 and K; and (D) with pABI5-LUC alone and pABI5-LUC and K (as controls). The prefix ‘p’ indicates promoter. Left panels in (A), (B), (C), and (D): fluorescence imaging. Right panels in (A), (B), (C), and (D): fluorescence intensities estimated by fluorescence counts. The expression levels of the internal control Actin protein (K) and the Myc-tagged WRKY18/WRKY40/WRKY60 protein were assayed by immunoblotting with the antisera against...
Myc tag, showed no significant difference among them in any assay (Fig. 6A, B), indicating that the observed differences in the fluorescence intensity were not caused by possible differences in the WRKY levels. These controls showed that the observations of these WRKY–ABI5 promoter interaction assays are specific and reliable. It was further shown that WRKY18, WRKY40, and WRKY60 did not inhibit the activity of the ABI5 promoter harbouring W-box 1, 2, and 4 triple mutations (Fig. 4B), revealing that the WRKY proteins bind the ABI5 promoter through the W-box motif.

Disruption of WRKY18, WRKY40, or WRKY60 enhances ABI4 or ABI5 expression in germinating seeds

It was previously shown that, in 2-week-old seedlings, disruption of either WRKY18 or WRKY40 decreased ABI4 expression, but increased ABI5 expression, and disruption of both the WRKY genes increased expression of both ABI4 and ABI5, while disruption of WRKY60 increased ABI4 expression, but decreased ABI5 expression (Shang et al., 2010). Given that ABI4 and ABI5 are mainly expressed in early developmental processes and regulate seed germination and post-germination growth (Finkelstein et al., 1998; Finkelstein and Lynch, 2000), the expression levels of these two genes were tested in germinating seeds of different wrky mutants 36 h and 90 h after stratification. The results showed that the expression levels of both ABI4 and ABI5 increased in the wrky40 single mutant and the wrky18 wrky40 and wrky18 wrky60 double mutants in comparison with those in wild-type plants at both 36 h and 90 h after stratification. The results showed that the expression levels of both ABI4 and ABI5 increased in the wrky40 single mutant and the wrky18 wrky40 and wrky18 wrky60 double mutants in comparison with those in wild-type plants at both 36 h and 90 h after stratification (Fig. 7). The data in the wrky18 wrky40 double mutant and those regarding ABI5 expression in the wrky40 mutant are consistent with those obtained in 2-week-old mutant plants (Shang et al., 2010), but the up-regulation of ABI4 expression in the wrky40 mutant was not observed in the 2-week-old mutant plants, in which ABI4 was down-regulated (Shang et al., 2010).
as mentioned above. In the \textit{wrky18} single mutant and \textit{wrky18, wrky40, wrky60} triple mutant, expression of \textit{ABI5} increased, but that of \textit{ABI4} was not significantly affected at both 36 h and 90 h after stratification (Fig. 7). In contrast, in the \textit{wrky60} single mutant and \textit{wrky40, wrky60} double mutant, expression of \textit{ABI4} increased at both 36 h and 90 h after stratification, but that of \textit{ABI5} was not significantly affected at 36 h after stratification; however, at 90 h after stratification, \textit{ABI5} was up-regulated in the two mutants (Fig. 7). The data regarding \textit{ABI4} expression in the \textit{wrky60} mutant and \textit{ABI5} expression in the \textit{wrky18} mutant are consistent with those obtained in 2-week-old mutant plants (Shang et al., 2010), but \textit{ABI4} in the \textit{wrky18} mutant and \textit{ABI5} in the \textit{wrky60} mutant were shown to be down-regulated in the 2-week-old mutant plants (Shang et al., 2010), which is different from the expression levels in the germinating seeds (Fig. 7). These findings showed that the regulation of expression of \textit{ABI4} and \textit{ABI5} by the three WRKY transcription factors is developmental stage dependent. Previous reports did not observe differences in \textit{ABI4} and \textit{ABI5} expression in the \textit{wyky18} mutant in 5-week-old mature plants (Pandey et al., 2010; Wenke et al., 2012), suggesting that WRKY18 (or also the other two WRKYs) does not function in the regulation of these two ABA-related genes during late developmental stages, which is consistent with the very early expression profile of \textit{ABI4} and \textit{ABI5} mainly in germinating seeds and young seedlings (1–5 d after stratification; Finkelstein et al., 1998; Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001) and with the present observations that the three WRKYs' function to regulate early developmental processes including seed germination and early seedling growth in response to ABA.

Fig. 8. The \textit{abi4} and \textit{abi5} mutations suppress ABA-hypersensitive phenotypes of the \textit{wrky18} and \textit{wrky60} mutants in ABA-induced seed germination inhibition. Seed germination rate of the wild-type plants (Col) and mutants \textit{abi4, wrky18, and abi4 wrky18} (A), \textit{abi5, wrky18, and abi5 wrky18} (B), \textit{abi4, wrky60, and abi4 wrky60} (C), or \textit{abi5, wrky60, and abi5 wrky60} (D) was recorded in ABA-free medium (0 µM ABA) and ABA-containing medium (0.5, 1, and 3 µM) from 48 h to 72 h after stratification. Each value is the mean ±SE of five independent biological determinations and different letters indicate significant differences at $P < 0.05$ (Duncan's multiple range test) when comparing values within the same ABA concentration and the same time point after stratification.
Introduction of the **abi4** or **abi5** mutation into the **wrky18** and **wrky60** mutants suppresses ABA hypersensitivity of these mutants

It was previously reported that WRKY40 functions directly upstream of ABI5 and also probably upstream of ABI4 in ABA signalling (Shang et al., 2010), but it remains unclear whether WRKY18 and WRKY60 cooperate with WRKY40 to regulate ABA signalling upstream of ABI4 and ABI5. In the present experiment, it was first confirmed that the null mutation in the **WRKY18** or **WRKY60** gene results in ABA-hypersensitive phenotypes in seed germination and post-germination growth, though the ABA-hypersensitive phenotypes are weaker in the **wrky60** mutant than in the **wrky18** mutant (Figs 8–10). The **wrky60** mutant showed wild-type ABA responses sometimes, especially at higher ABA concentrations for seed germination (Fig. 8C, D) or at lower ABA concentrations for post-germination growth (Fig. 9C, D), but in no case did this mutant show ABA-insensitive phenotypes. The ABA-hypersensitive phenotypes of the **wrky18** mutant were virtually always significant (Figs 8A, B, 9A, B, 10). It is noteworthy that post-germination growth was assayed with two different systems: one in which...
the seeds were directly planted in ABA-containing medium, and post-germination growth was investigated 7 d after stratification (Fig. 9), and another system in which the germinating seeds were transferred ~46 h after stratification from ABA-free medium to ABA-containing medium, and post-germination growth was recorded 10 d after the transfer (Fig. 10). Substantially the same
results of ABA-hypersensitive phenotypes in post-germination growth were obtained with these two systems for both wrky18 and wrky60 mutants (Figs 9, 10). These data verify previous observations that both WRKY18 and WRKY60 negatively regulate ABA signalling (Shang et al., 2010), and support the notion that WRKY60 balances the negative roles of WRKY18 and WRKY40 in ABA signalling.

The abi4 wrky18 and abi4 wrky60 double mutants showed substantially the same intensities of ABA-insensitive phenotypes as those in the abi4 single mutant, and the abi5 wrky18 and abi5 wrky60 double mutants as those of the abi5 single mutant, in ABA-induced seed germination inhibition (Fig. 8A–D) and post-germination growth arrest (Figs 9A–D, 10A–D), which provides genetic evidence that ABI4 and ABI5 function downstream of WRKY18 and WRKY60 in ABA signalling, consistent with the idea that ABI4 and ABI5 are the targets of these two WRKY transcription factors.

Discussion

WRKY18 and WRKY60 target ABI4 and ABI5 genes in the ABA signalling pathway

Although it was previously demonstrated that WRKY40 functions in ABA signalling by repressing the ABI5 gene and interacting with the ABI4 gene (Shang et al., 2010), it has been unclear whether its two paralogues, WRKY18 and WRKY60, target the ABI4 and ABI5 genes. In the present study, this question was answered by providing evidence that ABI4 and ABI5 are downstream targets of these two WRKY transcription factors to regulate ABA signalling. First, it was shown that WRKY18 and WRKY60 proteins interact with the W-box in the promoters of the ABI4 and ABI5 genes, as evidenced by yeast one-hybrid assay, GSA, and ChIP analysis (Figs 1, 2, 5). Secondly, WRKY18 and WRKY60 were shown to inhibit the expression of the ABI5 gene, and WRKY60 inhibits the expression of the ABI4 gene,
Fig. 12. The models to explain ABA sensitivities and expression levels of ABI4 and ABI5 genes in different wrky mutants. ‘ABA sensitivity’ indicates the intensity of the ABA-hypersensitive phenotypes of different wrky mutants in comparison with wild-type plants as described in the present study and a previous report (Shang et al., 2010); the larger red area indicates higher ABA sensitivity (stronger ABA-hypersensitive phenotype); the blue area indicates wild-type ABA sensitivity in seed germination and post-germination growth. ‘ABI5 level’ and ‘ABI4 level’ indicate the approximate averages (at 36 h and 90 h after stratification) of the expression levels of these two genes in the different wrky mutants as described in the present experiment in Fig. 7. In the wrky18 wrky40 wrky60 triple mutant, a negative feedback mechanism of the high ABI5 level on ABI4 is postulated to explain the low ABI4 expression, which is mediated by additional, unknown factors (indicated by a question mark). See the text for a detailed explanation.
as evidenced by both the in vivo assay of co-expression of the WRKYs with the ABI4 and ABI5 promoters (Figs 3, 4, 6) and expression analysis of ABI4 and ABI5 in various wrky mutants (Fig. 7), which is consistent with their negative roles in ABA signalling. Finally, genetic evidence was provided that the mutations of ABI4 and ABI5 suppress ABA-hypersensitive phenotypes of the null mutant alleles of WRKY18 and WRKY60 genes (Figs 8–10), demonstrating that ABI4 and ABI5 function downstream of these two WRKY transcription factors in ABA signalling.

As regards the negative roles of WRKY18 and WRKY60 in ABA signalling, it is noteworthy that, in a previous report, the wrky40 single mutant and wrky40 wrky18 double mutant were shown to display ABA-hypersensitive phenotypes (Chen et al., 2010), which is consistent with the present (Figs 8–10) and previous observations (Shang et al., 2010). However, Chen and co-workers showed that the wrky18 and wrky60 single mutants and wrky18 wrky60 double mutants displayed ABA-insensitive phenotypes in seed germination and post-germination growth, and the wrky40 wrky60 double mutant showed ABA insensitivity in seed germination but a wild-type ABA response in post-germination growth (Chen et al., 2010). These data suggest the possible positive roles of WRKY18 and WRKY40 in ABA signalling (Chen et al., 2010), which is inconsistent with the present (Figs 8–10) and previous observations (Shang et al., 2010). In the present experiment with considerable biologically independent repetitions, previously reported data (Shang et al., 2010) showing that null mutation of either WRKY18 or WRKY60, like null mutation of WRKY40, results in ABA hypersensitivity, but not ABA insensitivity, in ABA-induced seed germination inhibition and post-germination growth arrest were reproduced well (Figs. 8–10).

These discrepancies are probably due to weak ABA-related phenotypes of the wrky18 and wrky60 mutants, which may be affected more easily by environmental conditions. It was previously shown that the double mutant wrky40 wrky18 displayed the strongest ABA-hypersensitive phenotypes, which was followed by the wrky40 single mutant, and that the wrky18 and wrky60 mutants showed relatively weaker ABA-hypersensitive phenotypes (Shang et al., 2010). This may partly explain why the ABA-hypersensitive phenotypes of the wrky40 single and wrky18 wrky60 double mutants were, but those of the wrky18, wrky60, and relative double mutants were not, detected by Chen et al. (2010). The weak ABA-related phenotypes of the wrky18 and wrky60 mutants may be due to antagonistic effects of different WRKY members (as described in the present experiment) on the one hand, and, on the other, may probably be explained by functional redundancy, because multiple WRKY transcription factors are involved in ABA signalling as negative regulators (Shang et al., 2010). For instance, in addition to WRKY40 (Chen et al., 2010; Shang et al., 2010), two other reports have identified WRKY2 and WRKY63 as further transcription repressors in ABA signalling (Jiang and Yu, 2009; Ren et al., 2010). The different environmental conditions may have an impact on the weak phenotypes of ABA sensitivity of the wrky18 and wrky60 mutants, thus possibly creating the discrepancies. In this regard, the seed storage conditions may also be of importance for showing proper phenotypes, and non-optimal storage conditions of seeds may affect the investigation of ABA sensitivity.

Additionally and importantly, it was noted that Chen and co-workers transferred 4-day-old seedlings from ABA-free medium to 2 µM ABA-containing medium to assay post-germination growth in response to ABA (Chen et al., 2010), which is different from the techniques used in this study where the assays was performed by directly planting seeds in ABA-containing medium (ABA concentrations <0.7 µM) or transferring the germinating seeds to ABA-containing medium (ABA concentrations <1–2 µM) 46–48 h (but not 4 d) after stratification. In the latter system, the early transfer (<48 h after stratification) of germinating seeds to ABA-containing medium is important for assaying ABA responses of these wrky mutants, because the ABA-related phenotypes disappeared when the transfer was carried out >48 h after stratification. In contrast to the observations described in the previous report (Chen et al., 2010), in the conditions used in the present study, ABA-insensitive phenotypes were never observed in these wrky mutants when seedlings were transferred 4 d after stratification from ABA-free to 2 µM ABA-containing medium, and ABA-hypersensitive phenotypes even disappeared except for the wrky40 and wrky40 wrky18 mutants that showed weakly ABA-hypersensitive phenotypes (data not shown). The same phenomenon of this short developmental window (<48 h) of the ABA response as observed in the present (Figs 9, 10) and previous assays (Shang et al., 2010) was described by an independent group for the wrky2 ABA-hypersensitive mutant in ABA-induced post-germination growth arrest (Jiang and Yu, 2009). This may be due to a post-germination developmental arrest checkpoint mediated by temporal expression of ABI5 (Lopez-Molina et al., 2001), which may be a central player downstream of these WRKYs.

Taken together, previous (Shang et al., 2010) and present biochemical and genetic experiments consistently demonstrate that the WRKY18 and WRKY60 transcription factors, cooperatively working with WRKY40, inhibit expression of two important, positive ABA signalling components, ABI4 and ABI5, and thus are negatively involved in the ABA signalling pathway.

How do WRKY18, WRKY40, and WRKY60 transcription factors cooperate to repress ABI4 and ABI5 genes?

It has been known that one single WRKY may function in the cell signalling process as a negative or positive regulator, the underlying mechanisms of which may involve complex autoregulation or cross-regulation via protein–protein interaction (Eulgem et al., 2000; Xu et al., 2006; Eulgem and Somssich, 2007; Pandey and Somssich, 2009; Chen et al., 2012; Rushton et al., 2010, 2012). Consistently, in the current experiments, it was shown, through biochemical and genetic experiments, that WRKY18, like WRKY40, represses the ABI5 gene as one of the key regulators (Figs 1–7, and for WRKY40, see also Shang et al., 2010), and that WRKY60 plays essentially an antagonistic role to balance the functions of WRKY18 and WRKY40 in binding of the ABI4 and ABI5 promoters and inhibiting the expression of ABI4 and ABI5 genes (Figs 1–7), though WRKY60 itself is a repressor of ABI4 and ABI5 genes (Figs 3, 7) and functions genetically as a weakly negative regulator of ABA signalling (Figs 8–10, and
see also Shang et al., 2010). The expression level of WRKY60 was previously shown to be low (Shen et al., 2007; Wenke et al., 2012), which, however, suggests that the functional WRKY40 and WRKY18 are of special importance for plant development and the proper response to environmental challenges such that their antagonists such as WRKY60 should be repressed. Interestingly, however, the regulation of ABI4 and ABI5 expression involves much more complex interactions of the three WRKYs with each other (Figs 1–7), in which one WRKY may play either an agonistic or antagonistic role to other WRKYs in different situations. For instance, WRKY18 antagonizes WRKY40 in the in vivo tested expression of the ABI4 gene (Fig. 3B), whereas WRKY18 aids WRKY40 in repressing both ABI4 and ABI5 genes in planta (Fig. 7); and the repressive effects of a single WRKY on the promoter binding (Figs 1, 5) and expression (Figs 3, 6) of ABI4 and ABI5 genes may be completely abolished when the three WRKYs meet together.

Synthesizing the experimental data of the promoter binding (Figs 1, 5) and expression analysis in the in vivo systems (Figs 2–4, 6) and especially in planta expression analysis in various wrky mutants (Fig. 7), a working model is proposed of cooperation of the three WRKYs, which bind to different domains of the ABI4 and ABI5 promoters (Fig. 11A, B), in repressing ABI4 and ABI5 expression (Fig. 11C, D). In this model, expression of ABI4 and ABI5 may be regulated by different mechanisms. For the ABI4 gene, the WRKY40 and WRKY60 transcription factors repress its expression, while the mutual antagonistic effects occur between WRKY40 and WRKY60, and WRKY18 antagonizes WRKY40 to balance the repressive functions on the ABI4 gene (Fig. 11C). For the ABI5 gene, however, the two WRKYs, WRKY18 and WRKY40, work alone or together to repress ABI5, and WRKY60 represses ABI5 but at the same time antagonizes the repressive effect of WRKY18 and the WRKY18–WRKY40 heterodimer to balance the repressive effects on the ABI5 gene (Fig. 11D).

This model may partly explain the expression profile of ABI4 and ABI5 genes in wrky mutants (Fig. 7) and the ABA-hypersensitive phenotypes of these wrky mutants (Figs 8–10, and see also Shang et al., 2010), which is summarized in Fig. 12. Disruption of WRKY18 and WRKY40 in the wrky18 wrky40 double mutant derepresses expression of both the ABI4 and ABI5 genes to the greatest degree, given that WRKY60 alone present in this mutant has no substantial repressive effect on the ABI5 gene and a weaker repressive effect on the ABI4 gene. It is noteworthy that loss of function of WRKY18 and WRKY40 may also induce other unknown processes to stimulate ABI4 and ABI5 expression. The wrky40 mutant follows the wrky18 wrky40 double mutant in terms of its higher expression levels of both ABI4 and ABI5 genes, because ABI5 is, and ABI4 is partly, derepressed according to the working model. After the wrky40 mutant, it is interesting to note that the wrky18 wrky40 wrky60 triple mutant has the highest ABI5 level like the wrky18 wrky40 double mutant, but a wild-type low level of ABI4. This is essentially consistent with its intensity of ABA hypersensitivity, which follows that of the wrky18 wrky40 double mutant and the wrky40 single mutant (Shang et al., 2010). The low ABI4 level in the triple mutant may be explained by a complex negative feedback mechanism of the high ABI5 level on ABI4 expression, which may involve other unknown factors when none of the three WRKY transcription repressors is present. These unknown factors may be, for example, WRKY2 and/or WRKY63, which may act both upstream and downstream of ABI4 and ABI5 (Jiang and Yu, 2009; Ren et al., 2010; Rushton et al., 2012), and may repress the ABI4 gene in this situation of a high ABI5 level due to loss of function of the three WRKY repressors. This negative feedback loop of the WRKY members on the ABI4 and ABI5 genes (WRKY18/40/60–ABI5–WRKY2/63–ABI4) may also exist in other wrky mutants and the wild-type plants: the feedback effect of WRKY18/40/60-dependent ABI5 repression on ABI4 could partly explain the observations that the expression level of ABI4 tends to be higher when the ABI5 level is lower (Figs 7, 12).

ABI4 and ABI5 expression in other wrky mutants (wrky18, wrky60, wrky18 wrky60, and wrky40 wrky60) may also be partly explained by this model. However, much more complex mechanisms than this simplified model concerning mainly post-translational interaction of the three WRKYs must be involved in the regulation of expression of ABI4 and ABI5, where the three WRKYs and even WRKY2 and WRKY63 may constitute only one part of the entire network. First, and most importantly, the three WRKY transcription factors may repress expression of their own encoding genes by auto- and/or cross-regulation (Pandey and Somssich, 2009; Pandey et al., 2010), and so up- or down-regulation of any of the three WRKYs may induce a feedback regulation of its own gene and the two other genes. Secondly, in addition to the three members of the WRKY transcription factor superfamily, other WRKY members and other classes of transcription factors must participate in this transcription regulation. As mentioned above, two examples are WRKY2 and WRKY63 (Jiang and Yu, 2009; Ren et al., 2010; Rushton et al., 2012), which may potentially cross-talk with WRKY18, WRKY40, and WRKY60 to mediate ABA signalling upstream of ABI4 and ABI5. Thirdly, the WRKY18 and/or WRKY40 transcription factors are involved in many other signalling processes such as reactive oxygen species, cytokinin, salicylic acid, ethylene, and jasmonic acid signalling, through which the WRKYs may regulate ABA-related genes (Pandey et al., 2010; Argueso et al., 2012; Wenke et al., 2012). Finally, WRKYs may regulate gene expression by complex mechanisms by acting on chromatin remodelling, interacting with small RNA, and even preparing the promoter for activation or repression (Turck et al., 2004; Pandey and Somssich, 2009). Therefore, it is most likely that the highly complex mechanisms as described above may interact to form a regulation network to determine the expression of ABA-related genes such as ABI4 and ABI5, in which the three WRKY transcription factors may participate to constitute a key step. In this transcriptional regulation network, disruption of any of the three WRKYs may induce complex feedback or feed-forward consequences.

Additionally, it is noteworthy that ABI4 was reported to act upstream of ABI5 (Bossi et al., 2009) or possibly to function in a different pathway from that of ABI5 in ABA signalling (Ren et al., 2010). Therefore, the transcriptional regulation of ABI5 by WRKY40/60 may be realized to some extent indirectly through regulation of ABI4 by these WRKYs. The function of ABI4 was shown to be independent of WRKY63/ABO3 (Ren et al., 2010), suggesting that, while probably sharing ABI5 as a common downstream target, the WRKY18/40/60-mediated signalling
may be partly different from the WRKY63-mediated signalling, which may diverge at ABI4 from each other. These findings also suggest that ABI5 is likely to be a common and central player downstream of the WRKYs at least including WRKY18, WRKY40, WRKY60, and WRKY63.

Consistently, the intensities of the ABA-hypersensitive phenotypes of the wrky mutants, which were described in a previous report (Shang et al., 2010) and in the present experiments (wrky18 and wrky60 mutants, see Figs 8–10), may be partly explained by the expression levels of ABI5 (Fig. 10). However, the expression level of ABI4, which tends to be higher when the ABI5 level is lower, is generally not correlated with the ABA sensitivities of the wrky mutants except for the wrky18 wrky40 double mutant that shows the highest ABI4 level with the highest ABA sensitivity. Particular exceptions are the wrky18 wrky60 and wrky40 wrky60 double mutants: the former shows higher expression levels of both ABI4 and ABI5, and the latter has the same levels of ABI4 and ABI5 as the wrky60 ABA-hypersensitive mutant, but both mutants show a wild-type ABA response, which, however, supports the function of WRKY60 as a central balancer of WRKY18 and WRKY40. Overall, the present observations are consistent with the idea that the three WRKYs function by WRKY18 and WRKY40. The latter has the same levels of both ABI5 and ABI4 and the latter has the same levels of ABI5. However, the expression level of ABI4, which tends to be higher when the ABI5 level is lower, is generally not correlated with the ABA sensitivities of the wrky mutants except for the wrky18 wrky40 double mutant that shows the highest ABI4 level with the highest ABA sensitivity.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Purification of the E. coli-expressed WRKY18, WRKY40, and WRKY60 proteins from E. coli extracts.

Table S1. Information for detecting the WRKY18-, WRKY40-, and WRKY60-binding promoter sequences by PCR in the chromatin co-immunoprecipitation (ChIP) assay with the antibody against GFP and by gel shift assay (GSA).

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