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AITRL, an evolutionarily conserved plant specific transcription repressor regulates ABA response in Arabidopsis

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Expression of stress response genes can be regulated by abscisic acid (ABA) dependent and ABA independent pathways. Osmotic stresses promote ABA accumulation, therefore inducing the expression of stress response genes via ABA signaling. Whereas cold and heat stresses induce the expression of stress response genes via ABA independent pathway. ABA induced transcription repressors (AITRs) are a family of novel transcription factors that play a role in ABA signaling, and *Drought response gene (DRG)* has previously been shown to play a role in regulating plant response to drought and freezing stresses. We report here the identification of DRG as a novel transcription factor and a regulator of ABA response in Arabidopsis. We found that the expression of DRG was induced by ABA treatment. Homologs searching identified AITR5 as the most closely related Arabidopsis protein to DRG, and homologs of DRG, including the AITR-like (AITRL) proteins in bryophytes and gymnosperms, are specifically presented in embryophytes. Therefore we renamed DRG as AITRL. Protoplast transfection assays show that AITRL functioned as a transcription repressor. In seed germination and seedling greening assays, the *aitrl* mutants showed an increased sensitivity to ABA. By using qRT-PCR, we show that ABA responses of some ABA signaling component genes including some *PYR1*-likes (*PYLs*), *PROTEIN PHOSPHATASE 2Cs* (*PP2Cs*) and *SUCROSE NONFERMENTING 1 (SNF1)-RELATED PROTEIN KINASES 2s* (*SnRK2s*) were reduced in the *aitrl* mutants. Taken together, our results suggest that AITRLs are a family of novel transcription repressors evolutionarily conserved in embryophytes, and AITRL regulates ABA response in Arabidopsis by affecting ABA response of some ABA signaling component genes.

As one of the five classic plant hormones, abscisic acid (ABA) regulates multiple aspects of plant growth and development, such as seed development, seed germination, bud dormancy and stomatal movement^{1–5}. Most importantly, ABA is a key hormone in regulating plant responses to abiotic environmental stresses including drought, salinity, cold and heat^{3–8}. Regulation of plant abiotic stress responses by ABA is usually related to osmotic stresses caused by drought and other water limiting conditions, which can promote ABA accumulation^{1,9–11}, whereas ABA signal transduction lead to the activation/repression of stress related genes, thereby affecting plants response to abiotic stresses^{3,6–8,12–14}.

ABA signaling is mediated by a few key regulators including the Pyrabactin resistance 1/*PYR1*-likes/Regulatory component of ABA receptors (*PYR1*/*PYLs*/*RCARs*) receptors^{15–17}, the A-group *PROTEIN PHOSPHATASE 2Cs* (*PP2Cs*) phosphatases^{18,19}, the *SUCROSE NONFERMENTING 1 (SNF1)-RELATED PROTEIN KINASES 2s* (*SnRK2s*) protein kinases²⁰, and the ABA-responsive element binding protein/*ABRE*-binding factor/*ABA* *INSENSITIVE 5* (*ABF*/*AREB*/*ABI5*)-type basic region leucine zipper (*bZIP*) transcription factors^{21,22}.

At the absence of ABA, *PP2Cs* phosphatases interact with, and inhibit the function of *SnRK2s* kinases. Perception of ABA molecules by *PYR1*/*PYLs*/*RCARs* receptors allows them to interact with *PP2Cs*, therefore lead to the release and self-activation of *SnRK2s*. Activated *SnRK2s* are able to phosphorylate and activate *ABF*/*AREB*/*ABI5*-type *bZIP* transcription factors, resulting in the activation/repression of ABA response genes, and plant responses to abiotic stresses^{4,5,8,13,14,18–20,23}.

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The expression of hundreds and thousands of stress related genes is regulated by ABA^{14,23,24}. However, functions of many of them remained largely unknown. In an attempt to identify novel players in ABA signaling transduction, we previously identified ABA induced transcription repressors (AITRs) as a novel family of transcription factors⁸. We found that the expression of *AITRs* is up-regulated by ABA, and AITRs play a role in regulating ABA signaling transduction⁸. Most importantly, we found that AITRs are conserved in angiosperms, and may have been evolved from AITR-like proteins in bryophytes and gymnosperms⁸.

Regulation of stress related genes by ABA affects plant responses to abiotic stresses^{1,4,5,9–11}. It has been shown that the conserved PyACGTGG/TC ABRE in the promoter regions of ABA regulated stress related genes is the major *cis*-element for binding of ABF/AREB/ABI5-type bZIP transcription factors^{11,25}. However, expression of some stress related genes can be regulated in an ABA independent pathway. Analysis of the promoter regions of these genes has identified the conserved A/GCCGAC dehydration-responsive element (DRE) *cis*-element, as a binding site for APETALA 2/ETHYLENE RESPONSE FACTORS (AP2/ERF) transcription factor DREB1 and DREB2, whose expression was induced by cold and osmotic stress, respectively²⁶. In addition, some NAM, ATAF, and CUC (NAC) transcription factors can also regulate stress related gene expression in an ABA independent pathway via binding directly to the CACG NAC recognition sequence (NACR) *cis*-element in the promoter regions of some stress related genes²⁷.

It has been previously reported that the expression of *Drought response gene* (*DRG*) was induced by drought treatment, and *DRG* is involved in the regulation of plant responses to drought and freezing stresses²⁸. We found in this report that the expression of *DRG* was induced by ABA, and the most closely related protein to *DRG* in Arabidopsis is AITR5. However, *DRG* is more closely related to the AITR-like (AITRL) proteins in bryophytes and gymnosperms, we therefore renamed it as AITRL. We found that AITRL is involved in the regulation of plant response to ABA, but have opposite functions with AITRs. We also found that AITRLs may represent a family of novel transcription repressors, and are evolutionally conserved in embryophytes.

Materials and methods

Plant materials and growth conditions. The Columbia-0 (Col-0) wild type Arabidopsis was used for protoplast isolation and plant transformation. The transgenic plants overexpressing *AITRL* were generated by transform the Col wild type plants with the 35S:*AITRL* construct. The T-DNA insertion lines of SALK_022729²⁸ and SALK_203161 were obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus, OH, USA), and used to identified homozygous *aitrl-1/drg*²⁸ and *aitrl-2* mutants, respectively. The ABA-deficient mutant *aba1-5* was obtained from ABRC and used to examine the expression of *AITRL*.

For RNA isolation, seed germination and seedling greening assays, seeds of the Col wild type, the 35S:*AITRL* transgenic plants, and the *aitrl* mutants were surface sterilized and sown on plates with solidified 0.5 × MS salts containing 1% sucrose. The plates were kept at 4 °C and in darkness for 2 days, and then transferred into a plant growth room.

For protoplast isolation and plant transformation, seeds of the Col wild type were germinated directly in soil pots and grew in a growth room. The temperature at the growth room was set at 22 °C, and the light/dark cycle at 16 h/8 h with light density at ~ 120 μmol/m²/s.

Bioinformatics analysis of AITRLs. Homologs of AITRL in other plant species were identified by using “Protein Homologs” on Phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html#>). Full-length amino acid sequences of the AITRs and AITRLs in selected plant species were obtained on phytozome, and used for amino acid sequence alignment by using BioEdit, and for phylogenetic analysis by using “One Click” mode on phylogeny (http://www.phylogeny.fr/simple_phylogeny.cgi) with default settings.

Gene numbers of the *AITRs* and *AITRLs* were obtained by using *AITR5* and *AITRL*, respectively for “Gene Ancestry” assays on phytozome. Average gene number per plant species was calculated for different catalogs of the angiosperms.

ABA treatment, RNA isolation and quantitative RT-PCR (qRT-PCR). To examine the expression of *AITRL* in response to ABA in the Col wild type seedlings, and the expression of ABA signaling component genes in the Col wild type seedlings, the 35S:*AITRL* transgenic plants and the *aitrl* mutants, 12-day-old seedlings were treated with 50 μM ABA in darkness for 4 h, frozen in liquid N₂ and used for RNA isolation. Seedlings treated with methanol were used as a control. To examine the expression of *AITRL* in *aba1-5* mutant, 12-day-old seedlings were frozen in liquid N₂ and used for RNA isolation.

Total RNA was isolated by using an EasyPure plant RNA kit (Transgen Biotech), and 2 μg of the total RNA isolated was subjected to first-strand cDNA synthesis by using an EasyScript First-strand DNA Synthesis Super Mix (TransGen Biotech). Synthesized cDNA was used as templates for RT-PCR or qRT-PCR analysis. The expression of *ACT2* was used as an inner control. The primers used for RT-PCR analysis of *AITRL* are 5'-CAACATATGATAAAGATACTCAACCCCA-3' and 5'-CAAGAGCTCCTATCTGCGGTCAGTGGTTG-3'. The primers used for qRT-PCR analysis of ABA signaling component genes were as described previously^{8,29,30}.

Constructs. The LD-VP activator, the Gal4 DNA binding domain (GD) control effector, the NLS-RFP nuclear indicator, and the *LexA-Gal4:GUS* reporter constructs for protoplast transfection were described previously^{31–33}. The 35S:*GFP* construct was provided by Prof. Zheng-Yi Xu (Northeast Normal University).

To generate the *GD-AITRL* construct for protoplast transfection assays, the full length open reading frame (ORF) sequence of *AITRL* was amplified by RT-PCR as described above for the examination of the ABA response of *AITRL*, and cloned into the *pUC19* vector with a N-terminal GD tag under the control of the 35S promoter^{32,33}. The *GFP-AITRL* construct was generated by replacing the GD tag in the *GD-AITRL* construct with a GFP tag.

To generate the 35S:*AITRL* construct for plant transformation, the GD tag in the *GD-AITRL* construct was replaced with a HA tag, and then digested and subcloned into the binary vector *pPZP211*³⁴.

Plant transformation and transgenic plant selection. The Col wild type plants ~5-week-old and with several mature flowers were used for plant transformation, and the plants were transformed via *Agrobacterium tumefaciens* GV3101 mediated transformation by using floral dip method³⁵.

To select transgenic plants, T1 seeds were collected and germinated on plates with solidified 50 µg/ml Kanamycin and 100 µg/ml Carbenicillin containing 0.5 × MS salts. To select transgenic plants with a single T-DNA insertion, T2 seeds collected from transgenic T1 plants were germinated on plates with solidified 25 µg/ml Kanamycin containing 0.5 × MS salts. To identify T3 homozygous overexpression plants, T3 seeds collected from T2 plants were selected on plates with solidified 25 µg/ml Kanamycin containing 0.5 × MS salts, and the expression levels of *AITRL* in the transgenic plants were examined by RT-PCR. Two confirmed homozygous overexpression lines were used for the experiments.

Plasmid DNA isolation, protoplast isolation and transfection. Plasmid DNA of the reporter and the effectors was isolated from transformed *E. coli* cells by using a GoldHi EndoFree Plasmid Maxi Kit (OMEGA BIO-TEK), and the concentration of the plasmid DNA was measured by using a NanoDrop (Thermo, USA).

Protoplasts were isolated from rosette leaves collected from 3- to 4-week-old Col wild type plants, and used for protoplast transfection by following the procedure with co-transfection of 35S:*Luciferase* as an inner control as described previously^{8,33,36,37}.

For protein subcellular location assay, plasmids of *GFP-AITRL* and *NLS-RFP* were co-transfected into the protoplasts isolated, and co-transfection of *GFP* and *NLS-RFP* was used as a control. For transcriptional activity assays, plasmids of the *LexA-Gal4:GUS* reporter, the *LD-VP* activator and the *GD-AITRL* effector or the *GD* control genes were co-transfected into protoplasts. The transfected protoplasts were incubated at room temperature for 18–22 h in darkness, then GFP and RFP fluorescence were examined under a fluorescent microscope (Olympus, Japan), and GUS activities were measured by using a Synergy HT fluorescence microplate reader (BioTEK, USA).

ABA sensitivity assays. ABA inhibited seed germination and seedling greening were assayed as described previously^{30,38,39}. Briefly, Surface sterilized seeds of the Col wild type, the 35S:*AITRL* transgenic plants and the *aitrl* mutants were sown on plates with solidified 0.5 × MS salts in the presence or absence 0.5 µM ABA, kept at 4 °C and in darkness for 3 days, and then transferred to a growth room. Germinated seeds were counted every 12 h after the transfer. Pictures were taken 12 days after the transfer, and seedlings with green cotyledons were counted. The experiments were repeated at least three times.

Data analysis. Data obtained was analysis in Excel, and student *t* test (<https://www.graphpad.com/quickcalcs/ttest1/>) was used for statistic analysis.

Results

DRG is an AITR-like protein. In the process to identify novel players in regulating ABA signaling as described previously⁸, we found that the expression level of *DRG*, a gene previously reported to regulate drought and freezing tolerance in Arabidopsis²⁸, was increased in response to ABA treatment³⁸. Protein homologs analysis for *DRG* on Phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html#>) shows that its most closely related protein in Arabidopsis is AITR5, a member of the AITRs family that have been show to play a feed-forward regulating role in ABA signaling⁸.

Our previously results showed that AITRs are a family of novel transcription repressors conserved in angiosperms, and may have been evolved from AITR-like proteins found in bryophytes and gymnosperms⁸. Phylogenetic analysis shows that *DRG* and its homologs in both soybean and tomato, two dicot species that are either less or more closely related to Arabidopsis in the phelogenetic analysis⁸, rather than AITR5, are in the clade formed by the AITR-like proteins and AITRs from early evolved angiosperms (Fig. 1a). These results suggested that *DRG* is more closed related to AITR-like proteins, rather than AITR5, therefore we renamed *DRG* as *AITRL*. Amino acid alignment shows that *AITRL* shares high amino acid identity and similarity with its homologs from soybean and tomato (Fig. 1b), but less with AITR5. Amino acid sequence BLAST on NCBI (<https://blast.ncbi.nlm.nih.gov>) shows that *AITRLs* are plant specific proteins, and gene ancestry analysis for *AITRL* on Phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html#>) shows that *AITRLs* are present only in embryophytes. These results indicate that *AITRLs* are a novel family of proteins.

Expression of *AITRL* is up-regulated by ABA. Considering that AITRs are involved in the regulating of ABA signalling⁸, it is likely that *AITRL* may also play a role in regulating plant response to ABA. To examine if that is the case, we first wanted to confirm the expression of *AITRL* is regulated by ABA. To do that, total RNA was isolated from Arabidopsis seedlings treated with ABA, and used to examine the expression of *AITRL* by RT-PCR. As shown in Fig. 2a, the expression level of *AITRL* in Arabidopsis seedlings increased more than 300 folds in response to ABA treatment, indicating that similar to *AITRs*, *AITRL* is an ABA response gene. To further examine if *AITRL* is an ABA responsive gene, we examined the expression level of *AITRL* in the ABA deficient mutant *aba1-5*, and a more than tenfold decrease was observed in the *aba1-5* mutant seedlings when compared with the wild type seedlings (Fig. 2b).

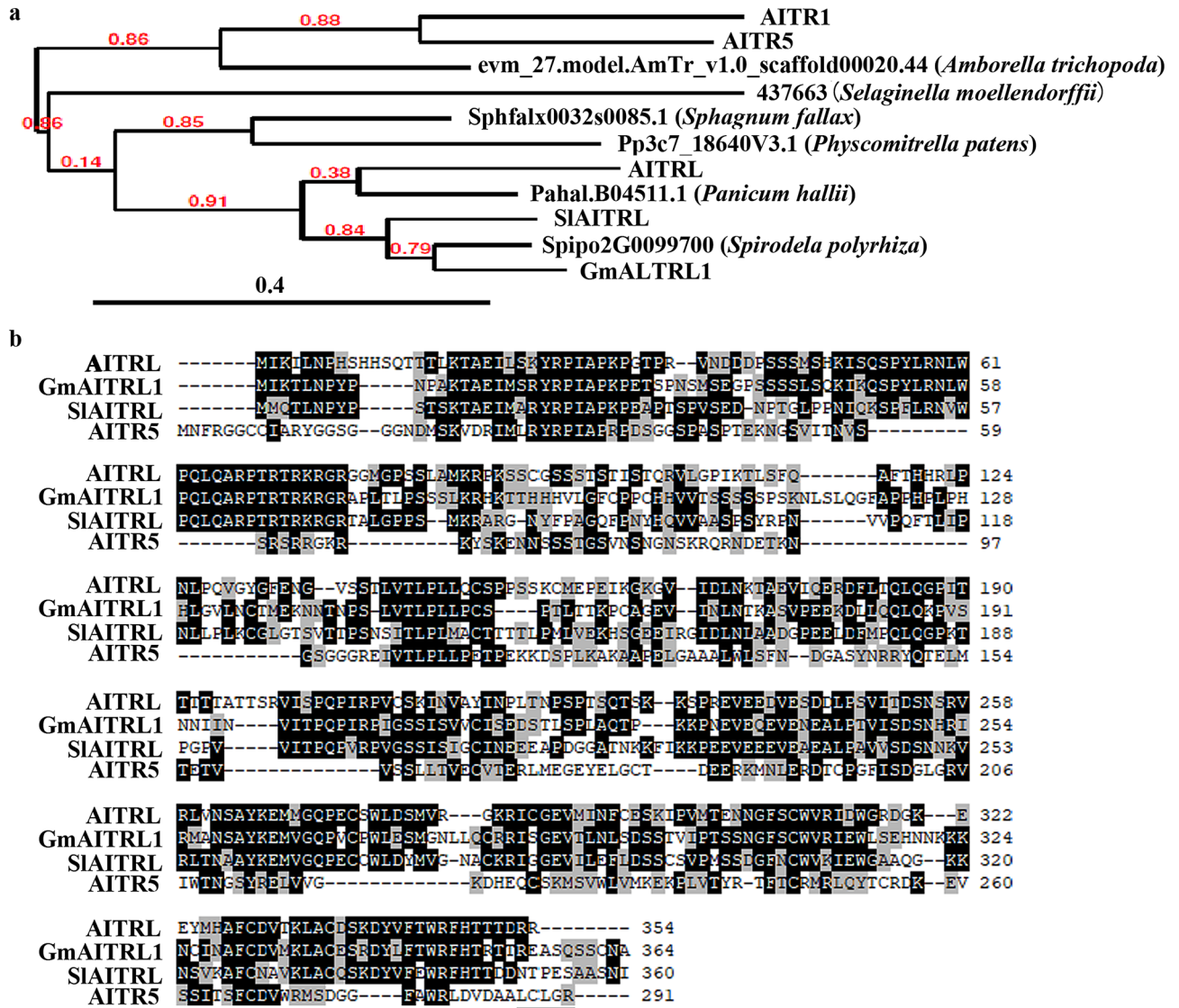


Figure 1. Phylogenetic analysis of AITRs and AITRLs, and amino acid alignment of AITRL, GmAITRL1, SIAITRL and AITR5. (a) Phylogenetic analysis of AITRs and AITRLs. AITRs and AITRLs in the plant species selected were identified on phytozome v12 (<https://phytozome.jgi.doe.gov/pz/portal.html#>), and their full-length amino acid sequences were used for phylogenetic analysis by using “One Click” mode on phylogeny (http://www.phylogeny.fr/simple_phylogeny.cgi) with default settings. Branch support values are indicated above the branches. (b) Amino acid alignment of AITRL, GmAITRL1, SIAITRL and AITR5. Full-length amino acid sequences of AITRL, GmAITRL1, SIAITRL and AITR5 were obtained on phytozome and used for sequence alignment by using BioEdit 7.0 (<https://bioedit.software.informer.com/7.0/>). The identical amino acids in AITRL, GmAITRL1, SIAITRL and AITR5 were shaded in black, and the similar ones in gray. Underline indicates the partial conserved LxLxL motif in AITR5.

AITRL is a transcription repressor. After confirmed that the expression of *AITRL* was induced by ABA (Fig. 2), we examined if *AITRL* may functions as a transcription repressor. It has been shown that *AITRL* is a nuclear protein²⁸. To confirm this by using Arabidopsis protoplast transit transfection assays, plasmid DNA of *GFP-AITRL* was transfected into Arabidopsis protoplasts, and GFP fluorescence was observed under a cofocal microscope. As shown in Fig. 3a, GFP fluorescence was specifically observed in the nucleus. As a control, no specific subcellular localization was observed for GFP alone (Fig. 3a).

Arabidopsis protoplast transit transfection assays were also used to examine the transcriptional activities of *AITRL*. Plasmids of the *LexA-Gal4:GUS* reporter, the *LD-VP* activator and the *GD-AITRL* effector or the *GD* control genes were co-transfected into Arabidopsis protoplasts, and GUS activities were measured by using a microplate reader. As shown in Fig. 3b, GUS activity activated by the *LD-VP* activator was repressed by the co-transfection of *GD-AITRL*, suggesting that *AITRL* functions as a transcription repressor.

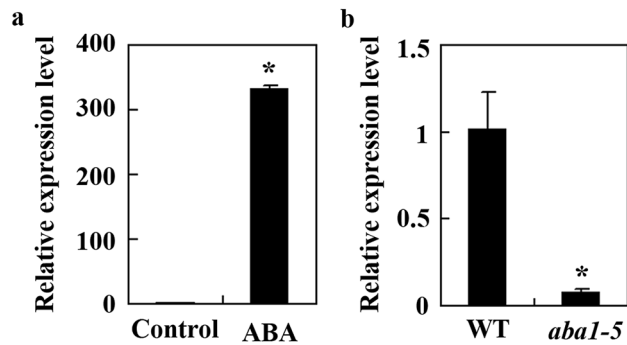


Figure 2. Expression of *AITRL* in response to ABA treatment. **(a)** Expression of *AITRL* in response to ABA. Twelve-day-old Col wild type seedlings were treated with 50 μ M ABA or solvent methanol as a control for 4 h. Total RNA was isolated and qRT-PCR was used to examine the expression of *AITRL*. The expression of *ACT2* was used as an inner control, and the expression level of *AITRL* in the control sample was set as 1. Data represent the mean \pm SD of three replicates. *Significantly different from the control ($p < 0.0001$). **(b)** Expression of *AITRL* in the *aba1-5* mutants. Total RNA was isolated from 12-day-old Ler wild type and *aba1-5* mutant seedlings, and qRT-PCR was used to examine the expression of *AITRL*. The expression of *ACT2* was used as an inner control, and the expression level of *AITRL* in the wild type seedlings was set as 1. Data represent the mean \pm SD of three replicates. *Significantly different from the wild type ($p < 0.005$).

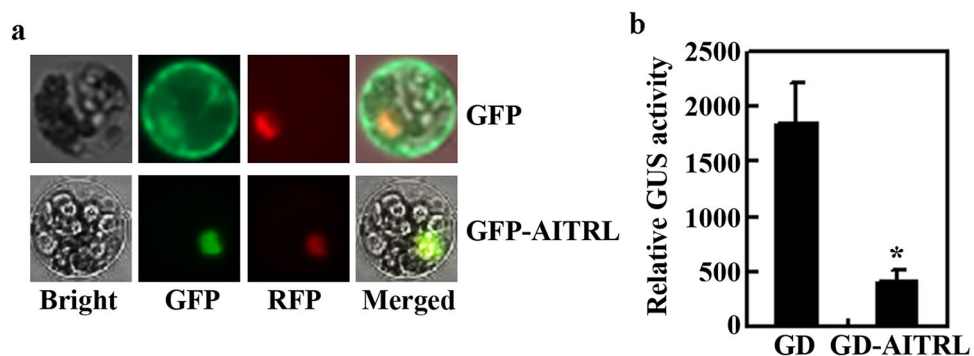


Figure 3. *AITRL* is a transcription repressor. **(a)** Subcellular localization of *AITRL*. Plasmids of the *GFP-AITRL* effector gene and the *NLS-RFP* nuclear marker gene were co-transfected into Arabidopsis protoplasts isolated from leaves of the Col wild type. The transfected protoplasts were incubated in darkness at room temperature for 16–18 h. The GFP and RFP fluorescence was observed under a fluorescence microscope. Co-transfection of *GFP* effector gene and the *NLS-RFP* nuclear marker gene was used as a control. **(b)** Transcriptional activities of *AITRL*. Plasmids of the *LexA-Gal4:GUS* reporter gene, the *LD-VP* transcription activator gene and the *GD-AITRL* effector gene were co-transfected into Arabidopsis protoplasts isolated from leaves of the Col wild type plants. Co-transfection of the *GD* effector gene was used as a control. The transfected protoplasts were incubated in darkness at room temperature for 20–22 h. GUS activities were measured by using a microplate reader. Data represent the mean \pm SD of three replicates. *Significantly different from the GD ($p < 0.005$).

The *aitrl* mutants are hypersensitive to ABA. Having shown that the expression of *AITRL* was induced by ABA and *AITRL* functions as a transcription repressor, we further examined the function of *AITRL* in regulating ABA response by using ABA inhibited seed germination and seedling greening assays.

In seed germination assays, we found that even though slightly reduced germination rate was observed for the seeds of the *aitrl* mutants 24 h after the plates were transferred into a growth room, all the seeds including that of the Col wild type, the 35S:*AITRL* transgenic plants, and the *aitrl* mutants on the control plates germinated 36 h after the transfer (Fig. 4). However, on the ABA-containing plates, when compared with seeds of the Col wild type plants, lower germination rate for seeds of the *aitrl* mutants was observed at most of the time points examined (Fig. 4), indicating that the *aitrl* mutants are more sensitivity to ABA treatment.

Similar, increased sensitivity to ABA treatment in the *aitrl* mutants was observed in seedling greening assays (Fig. 5a). Quantitative assays showed that the green cotyledon rate of the *aitrl* mutant plants was $\sim 75\%$, compared with $\sim 95\%$ of that in the Col wild type plants (Fig. 5b). On the other hand, the green cotyledon rate of the 35S:*AITRL* transgenic plants was largely indistinguishable from that of the Col wild type plants (Fig. 5b).

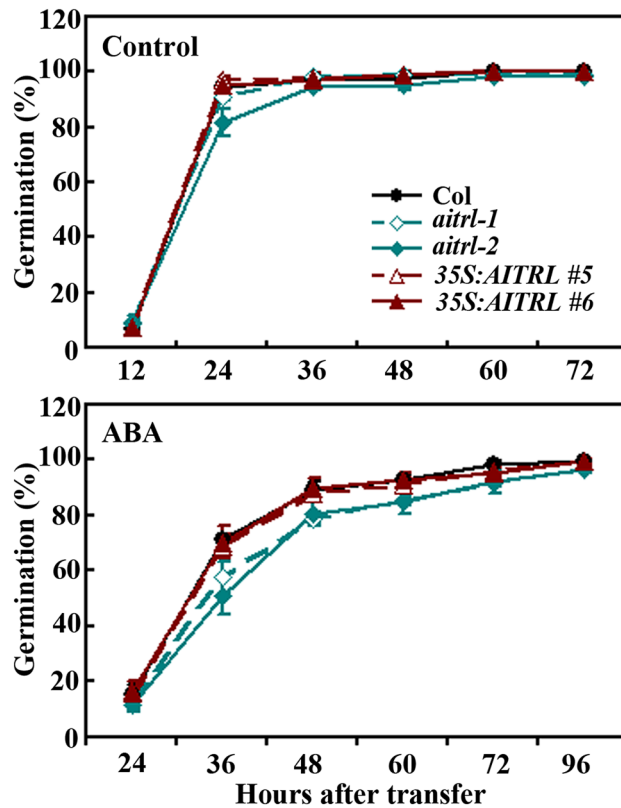


Figure 4. Effects of ABA on seed germination of the Col wild type, the 35S:AITRL transgenic plants and the *aitrl* mutants. Seeds of the Col wild type, the 35S:AITRL transgenic plants and the *aitrl* mutants were sterilized and sown on solidified 0.5 × MS salts plates in the presence or absence of 0.5 μM ABA. The plates were kept at 4 °C in darkness for 3 days before transferred to a growth room. Germinated seeds were counted every 12 h after the transfer till all the seeds were germinated, and percentage of seed germination was calculated. Data represent the mean ± SD of three replicates.

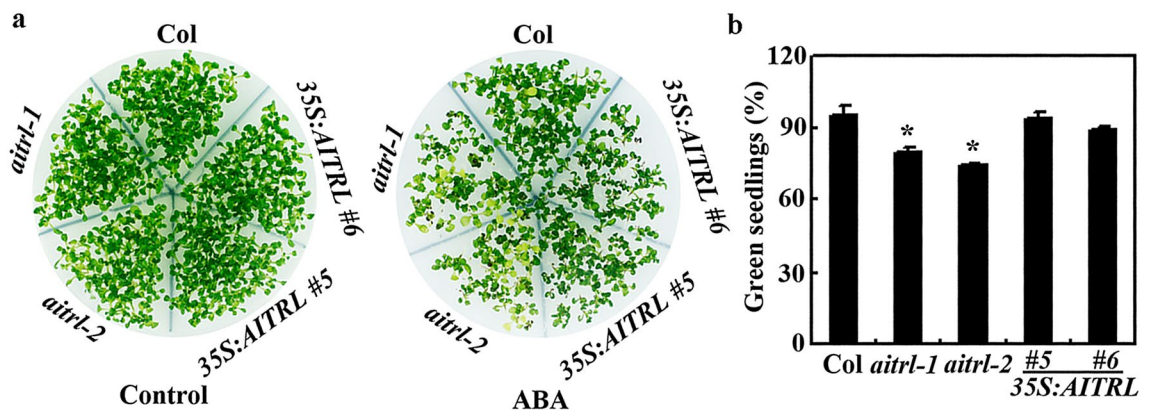


Figure 5. Effects of ABA on seedling greening of the Col wild type, the 35S:AITRL transgenic plants and the *aitrl* mutants. (a) Seedling greening of the Col wild type, the 35S:AITRL transgenic plants and the *aitrl* mutants in response to ABA treatment. Seeds were sterilized and sown on plates with solidified 0.5 × MS salts in the presence or absence 0.5 μM ABA. The plates were kept at 4 °C in darkness for 3 days before transferred to a growth room. Pictures were taken 12 days after the transfer. (b) Percentage of green seedlings of the Col wild type, the 35S:AITRL transgenic plants and the *aitrl* mutants in response to ABA treatment. Seedlings with green cotyledons were counted 12 days after the transfer, and the percentage of green seedlings was calculated. Data represent means ± SD of three replicates. *Significantly different from the wild type (p < 0.05).

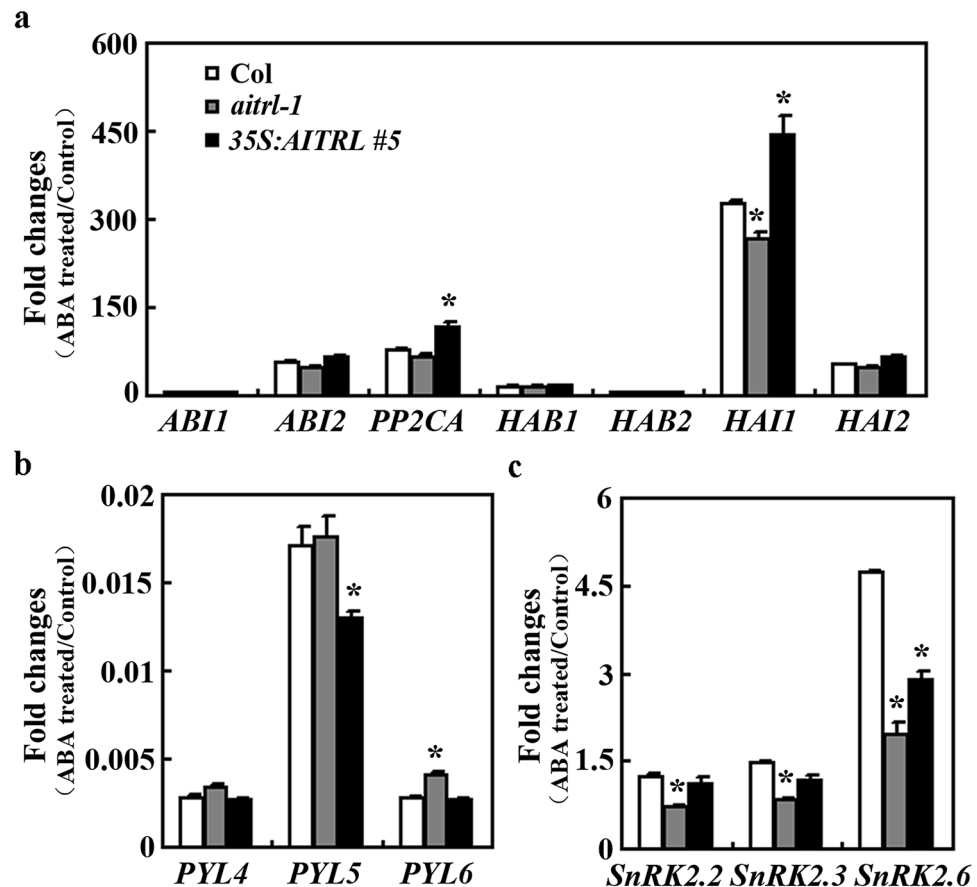


Figure 6. Expression of ABA signaling component genes in the Col wild type, the *35S:AITRL* transgenic plants and the *aitrl* mutants. Expression of *PP2C* genes (a), *PYL* genes (b) and *SnRK2* genes (c) in response to ABA in the Col wild type, the *35S:AITRL* transgenic plants and the *aitrl* mutants. Twelve-day-old seedlings were treated with 50 μ M ABA or solvent methanol as a control for 4 h. Total RNA was isolated and qRT-PCR was used to examine the expression of the ABA signaling component genes. The expression of *ACT2* was used as an inner control. Fold changes were calculated by comparing the expression levels of the corresponding genes in ABA treated and the control seedlings. Data represent the mean \pm SD of three replicates. *Significantly different from the wild type ($p < 0.05$).

Expression of ABA genes was affected in the *aitrl* mutants. We have previously shown that AITRs regulate the expression of some ABA signaling component genes⁸, having shown that ABA sensitivity was increased in the *aitrl* mutants (Figs. 4, 5), we then examined if the expression of ABA signaling component genes was regulated by AITRL. As expected, the expression of some *PP2C* genes was dramatically induced by ABA in the Col wild type seedlings, however, the fold changes of the expression of the *PP2C* gene *HAI1* in response to ABA were reduced in the *aitrl* mutants, and increased in the *35S:AITRL* transgenic plant seedling (Fig. 6a). On the other hand, the expression of *PYL* genes including *PYL4*, *PYL5* and *PYL6* was dramatically repressed by ABA in the Col wild type seedlings, however, decrease in ABA inhibition of the expression of *PYL6* was observed in the *aitrl* mutants, whereas enhance of *PYL5* was observed in the *35S:AITRL* transgenic plant seedling (Fig. 6b). We also observed that ABA induced expression of some *SnRK2* genes including *SnRK2.2*, *SnRK2.3* and *SnRK2.6* was reduced in the *aitrl* mutant seedlings, but also reduced or remained unchanged in the *35S:AITRL* transgenic plant seedling (Fig. 6c). These results suggest that AITRL may regulate ABA responses in Arabidopsis by regulating the expression of ABA signaling component genes.

AITRLs are evolutionarily conserved in plants. Similar to AITRs, the expression of AITRL was induced by ABA (Fig. 2), and AITRL functions as a transcription repressor (Fig. 3). However, unlike the *aitrs* mutants which showed a reduced ABA sensitivity⁸, ABA sensitivity was increased in the *aitrl* mutants (Figs. 4, 5), and opposite effects for AITRL and AITRs on the expression of ABA signaling component genes were also observed⁸ (Fig. 6). Considering that it is likely that AITRs are evolved from AITRLs⁸, and AITRLs are presented in most recently evolved plants including Arabidopsis (Fig. 1), we examined the distribution of AITRL and AITR genes in angiosperms. We found that grass plants have an average of more than 2 AITRL genes, but only 1 AITR gene, while eudicot plants have 1 AITRL gene, but nearly 4 AITR genes (Fig. 7). We also found that the number of

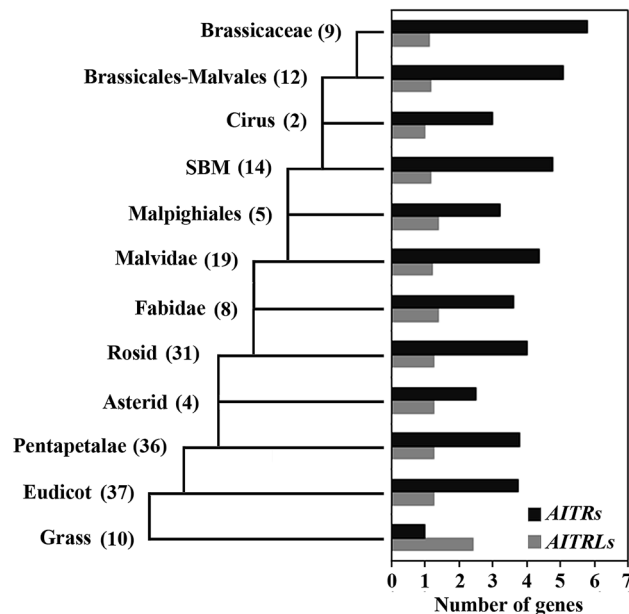


Figure 7. Changes of *AITRs* and *AITRLs* gene numbers during evolution of angiosperm. Numbers of *AITRs* and *AITRLs* genes were obtained by using *AITR5* and *AITRL* respectively, for “Gene Ancestry” assays on phytozome. Average gene numbers per plant species were calculated for different catalogs of angiosperm. Numbers in the brackets indicate total plant species available on phytozome in the indicated catalogs.

AITRL genes remained largely unchanged, however, the number of *AITR* genes increased slowly during the evaluation process of eudicot plants, as a result, brassicaceae have an average of more than 6 *AITR* genes (Fig. 7).

Discussion

We have previously shown that *AITRs* are a family of novel transcription repressors that play a feed-forward regulating role in ABA signaling, *AITRs* may evolved from *AITR*-like proteins in bryophytes and gymnosperms and are conserved in angiosperms⁸. We identified here *DRG* as an ABA inducible *AITRL* gene, we found that *AITRL* plays a role in regulating ABA responses, and *AITRLs* are an evolutionally conserved family of novel transcription repressors in embryophytes.

Expression of stress related genes can be regulated by ABA dependent and independent pathways^{1,9–11,26,27}. *DRG* has previously been reported to be a drought response gene²⁸, we found that its expression was up-regulated by ABA (Fig. 2). Phylogenetic analysis shows that *AITRL* is closely related *AITR*-like proteins in bryophytes and gymnosperms (Fig. 1), amino acid sequence BLAST results show that *AITRLs* are plant specific proteins, and are evolutionally conserved in embryophytes (Fig. 7).

Even though both *AITRL* and *AITRs* are up-regulated by ABA⁸ (Fig. 2), and similar to *AITRs*⁸, *AITRL* functioned as a transcription repressor (Fig. 3), our results show that *AITRL* and *AITRs* have opposite functions in regulating ABA responses in Arabidopsis. In both the seed germination and seedling greening assays, the *aitrl* mutants showed an increased sensitivity to ABA (Figs. 4, 5), whereas *aitrs* mutants showed a decreased sensitivity to ABA⁸. In addition, ABA responses of some ABA signaling component genes were reduced the *aitrl* mutants (Fig. 6), but increased in the *aitrs* mutants⁸. Consistent with their opposite functions in regulating ABA response, *drg/aitrl-1* mutants showed a decreased tolerance to abiotic stresses including drought and freezing²⁸, whereas *aitrs* mutants showed enhanced tolerance to abiotic stresses such as drought and salt stresses⁸. Considering that both *AITRL* and *AITRs* are presented in angiosperms, it is very likely that antagonism functions of *AITRLs* and *AITRs* in regulating ABA signaling in plants fine turn the plant responses to abiotic stresses. It is should be noted that closely related transcriptional regulators have opposing effects in regulating ABA signaling and plant responses to abiotic stresses have been observed in different transcription factor families. For example, both of the bHLH transcription factor genes *ABA-INDUCIBLE BHLH-TYPE TRANSCRIPTION FACTOR (AIB)* and *ANDROGEN-INDUCIBLE GENE 1 (AtAIG1)* are ABA response genes, but *AIB* positively, and *AtAIG1* negatively regulate ABA response^{40,41}. Whereas the ERF transcription factor AP2-like ABA repressor 1 (*ABR1*) and *ATERF15* also have opposing effects in regulating both ABA response and abiotic stress response in Arabidopsis^{42,43}.

As discussed previously, the appearance of *AITRs* during evolution may lead to reduced abiotic stress tolerance in plants⁸. Our evolutionary comparison analysis showed that there are more *AITRL* genes in grass plants, but more *AITR* genes in eudicot plants, and the numbers of *AITRLs* remained largely unchanged, where as that of *AITRs* increased during the evolution of eudicots (Fig. 7), further suggest that reduced tolerance in plants during evolution may be caused by the appearance of *AITRs*, and that *AITRLs* may play a positive role in regulating plant tolerance to abiotic stresses.

As mentioned above, both *AITRL* and *AITRs* are ABA responsive transcription repressor genes, but have opposite functions in regulating plant responses to ABA and abiotic stresses, and ABA response of some ABA signaling component genes. We noted that *AITRs* have a full or partial conserved LxLxL motif at the C-terminal⁸, an ERF-associated amphiphilic repression (EAR) repression motif initially identified in class II ERFs⁴⁴. However, *AITRL* does not (Fig. 1).

It has been shown that EAR motif-containing proteins mediated transcriptional repression represent the main form of transcriptional repression in plants^{45,46}. They can mediate transcription repression in at least two different ways⁴⁵, one is epigenetic modification by recruiting a histone deacetylase (HDAC) and interacting with co-suppressors to form a HDAC complex. For example, ERF7 can interact with SIN3 to recruit HDA19 to form a HDAC complex⁴⁷. Another is, similar to other transcription repressors, interference of the activities of other transcription factors via directly or indirectly binding. For example, OFP1 and OFP4 interact with KNAT7 to enhance its repression activities⁴⁸. It is likely that the different functions observed for *AITRL* and *AITRs* may be caused by different ways they are mediating transcriptional repression. Never the less, it will be of interest to examine if the L × L × L motif is responsive for the different functions observed for *AITRL* and *AITRs*, therefore to figure out how *AITRL* may mediate transcription repression.

ABA signaling lead to the activation of ABF/AREB/ABI5-type bZIP transcription factors, which in turn activate/repress the expression of ABA response genes^{4,5,13,14,18–20}. We showed that the expression of *AITRL* was up-regulated by ABA (Fig. 2), it is worthwhile to examine if the expression of *AITRL* is regulated by ABF/AREB/ABI5-type bZIP transcription factors. On the other hand, the expression of *AITRL* was up-regulated by drought²⁸. Considering that NAC and AP2/ERF transcription factor DREB1 and DREB2 are able to activate drought response genes via directly binding to the DRE *cis*-element^{26,27}, it will be also of interest to examine if DREB1 and DREB2 may regulate the expression of *AITRL* in an ABA independent way.

In summary, we found that *AITRL* is an ABA response gene, *AITRL* negatively regulates ABA responses in Arabidopsis, *AITRLs* are a novel family of transcription repressors conserved in embryophytes, and antagonism functions of the *AITRLs* and *AITRs* in regulating ABA signaling in plants may fine turn the plant responses to abiotic stresses.

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Author contributions

S.W. conceived the study. S.W., H.T. and T.W. designed the experiments. Y.M., H.T., R.L., W.W., N.Z., S.H., W.Y., C.Z., and G.Z. performed the experiments. Y.M. and S.W. analyzed the data. S.W. and Y.M. drafted the manuscript. All the authors participated in the revision of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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