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ORIGINAL ARTICLE

AtDIV2, an R-R-type MYB transcription factor of *Arabidopsis*, negatively regulates salt stress by modulating ABA signaling

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Abstract

Key message ***AtDIV2* integrates ABA signaling to negatively regulate salt stress in *Arabidopsis*.**

Abstract *AmDIV* (DIVARICATA) is a functional MYB transcription factor (TF) that regulates ventral identity during floral development in *Antirrhinum*. There are six members of DIV homologs in *Arabidopsis*; however, the functions of these proteins are largely unknown. Here, we characterized an R-R-type MYB TF *AtDIV2*, which is involved in salt stress responses and abscisic acid (ABA) signaling. Although universally expressed in tissues, the nuclear-localized *AtDIV2* appeared not to be involved in seedling development processes. However, upon exposure to salt stress and exogenous ABA, the transcripts of *AtDIV2* are markedly increased in wild-type (Wt) plants. The loss-of-function mutant *div2* displayed much more tolerance to salt stress, and several salt-responsive genes were up-regulated. In addition, the *div2* mutant showed higher sensitivity to ABA during seed germination. And the germination variance between the Wt and *div2* mutant cannot be rectified by treatment with both ABA and sodium tungstate at the same time. ELISA results showed that the endogenous ABA content in the *div2* mutant is clearly increased than that in Wt plants. Furthermore, the transcriptional expressions of several ABA-related genes, including *ABA1* and *ABI3*, were elevated. Taken together, our results suggest that the R-R-type MYB TF *AtDIV2* plays negative roles in salt stress and is required for ABA signaling in *Arabidopsis*.

Keywords *DIV* · Salt stress · MYB · Transcription factor · ABA

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Qing Fang and Qiong Wang contributed equally to this work.

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Introduction

Various abiotic stresses, such as high salinity, are easily posing a threat for plants. As a severe environmental factor, high salinity limits diverse plant growth and development processes, including reducing seed germination, inhibiting root elongation, and hence leading to substantial damages for crops (Kaneko et al. 2015; Mittler 2006). In general, high salinity not only leads to K^+/Na^+ imbalance inside the cytosol, but also changes gene expression to produce protective or adaptive physiological responses in plants (Britto et al. 2010; Munns 2002; Sun et al. 2009). Given the huge impact on crops caused by high salinity and other stresses (Qin et al. 2011; Tilman et al. 2011), there is a central significance to further shed light on the plant responses to these environmental challenges.

Upon exposure to salinity stress, plants coordinate biochemical and physiological variances with gene expression (Covarrubias and Reyes 2010; Kumar et al. 2004; Wang et al. 2003; Zhu 2002). Many studies have revealed that

transcriptional control is a critical step during these responses, and in plants numerous transcription factors (TFs) have been characterized. MYB TFs, with conserved MYB DNA-binding domains and comprising the largest gene family in plants, have been examined since the first plant MYB gene *C1* was identified (Dubos et al. 2010; Paz-Ares et al. 1987; Schmidt et al. 2013; Tominaga-Wada and Wada 2014; Wang and Chen 2014). For instance, the R2R3 MYB TF *AtMYB2* regulates salinity and dehydration responses (Baek et al. 2013; Yoo et al. 2005). In rice, the *OsMYB2*, which was isolated by a salt-treated rice microarray, plays roles in salt, cold and dehydration stresses; and the rice that overexpressing *OsMYB2* accumulated more soluble sugars and proline than wild-type plants; these also suggest that there are enhancing activity of several genes related to these processes (Yang et al. 2012).

MYB TFs also integrate hormone biosynthesis and/or signaling to mediate environmental challenge, particularly for the ABA, a well-known stress-associated hormone (Barrero et al. 2005; Kohli et al. 2013; Umezawa et al. 2010). For instance, the *SRM1*, a salt-related MYB1, can directly regulate the expression of *NCED3/STO1*, a key ABA biosynthesis gene, resulting in changed ABA levels in the overexpressing lines (Wang et al. 2015). *MYB96* has recently been characterized as a master regulator in seed germination and environmental responses integrating ABA signaling (Lee et al. 2015a, b; Lee and Seo 2015; Seo et al. 2009). In poplar, several MYB TFs also showed function in ABA signaling to mediate plant tolerance to salt stress (Chen et al. 2017; Fang et al. 2017).

In *Antirrhinum*, the *AmDIV* (DIVARICATA) encodes a 307-aa MYB protein and promotes ventral identity during flower zogomorphy (Galego and Almeida 2002). DIV and DIV-like proteins belong to the R-R-type clade of the MYB family (Gao et al. 2017; Howarth and Donoghue 2009; Yanhui et al. 2006). In *Arabidopsis*, there are nine R-R-type MYB proteins, in which six members are regarded as homologs of the DIV and DIV-like proteins. Here, the function of one member, *At5g04760*, previously named *AtDIV2* (Machemer et al. 2011) was investigated. Our results indicate that the loss-of-function mutant of *AtDIV2* exhibited improved salt tolerance, enhanced sensitivity to exogenous ABA, and markedly increased the ABA content compared with Wt plants. In addition, transcript levels of several ABA signaling genes were obviously increased. We conclude that the R-R type MYB TF *AtDIV2* is required for ABA signaling and functions as a negative regulator of salt stress in *Arabidopsis*.

Materials and methods

Plant materials and growth condition

Wild-type *Arabidopsis thaliana* Columbia (Col-0) ecotype and *div2* mutant (Salk_059505) plants were used in the present study. The T-DNA insertion line Salk_059505 was obtained from the ABRC Institute (https://abrc.osu.edu/), and homozygous mutant plants were identified by PCR with specific primers listed in the Supplementary Table 1. The Wt and *div2* mutant seedlings with similar size generated from surface-sterilized seeds were maintained on 1/2 MS medium supplemented with different concentrations of ABA and salt in a light-controlled chamber. The culturing photoperiod was set with a regime of 16-h light at 22 °C and 8-h dark at 18 °C. The samples used to measure the ABA content were obtained from 2-week-old seedlings in soil-containing pots under similar conditions as those used for plate culturing.

Isolation of homozygous mutant

For isolation of homozygous T-DNA insertion mutant, Wt (Col-0, used as a negative control) and mutant seeds (salk_059505) were surface-sterilized and germinated on 1/2 MS medium, 10-day-old seedlings were then transferred to soil-containing pots for further culture. Three primers (BP, for T-DNA specific primers; LP and RP, for the *AtDIV2* gene-specific primers) were synthesized and PCR based on the analysis of genomic DNA with primer pairs (LP/RP and BP/RP) was conducted according to previous methods (Missihoun et al. 2012) with a little modification, detailed as that the previous primers (BP and RP) were used for *div2* mutant, and the later primers (LP with RP) were used for the Wt plants, and after the examination by two consecutive PCR assay, the homozygous mutant was further identified by RT-PCR.

Sequence analysis

BLASTP was performed using NCBI and EBI to retrieve the amino acid sequences using the AtDIV2 sequence as a query. The screened MYB-like protein sequences were further aligned by the Clustal W program (Larkin et al. 2007). Then the phylogenetic tree was developed using the neighbor-joining method with parameters of pairwise deletion and P-distance model with 1000 replicates in the Molecular Evolutionary Genetic Analysis software MEGA 6.0 package (Tamura et al. 2013).

Seed germination and stress treatment

For the germination assay, Wt and *div2* mutant seeds were surface-sterilized and then plated on 1/2 MS agar (1%) medium as previous methods (Duan et al. 2015). The dishes were maintained at 4 °C for 72 h and then transferred into a light-controlled chamber to examine germination. During the period, the seeds displaying obvious primary roots with breaking seed coat were scored. To examine the effects of stress-related factors on germination, the sterilized seeds

were directly sown on the medium supplemented with different concentrations of ABA, salt, and sodium tungstate. To examine the growth phenotype, 4–5-day-old Wt and *div2* mutant seedlings of similar sizes were transferred to plates supplemented with ABA or salt and maintained for several days. Controls were coordinately set without ABA and salt added in the medium.

RNA preparation and PCR analysis

For total RNA isolation, 10-day-old Wt and *div2* mutant seedlings from 1/2 MS agar medium or 2-week-old seedlings grown in the soil pots were sampled for RNA extraction as previously reported (Duan et al. 2015). Then, 500 ng of total RNA was digested with DNase I to remove DNA contamination prior to reverse transcription. The first-strand cDNA was synthesized using the Primescript RT Reagent Kit (TAKARA, Dalian, China) according to the manufacturer's instructions. Real-time PCR was performed using StepOne Plus standard method. The real-time PCR was set as 95 °C for 2 min, followed by 40 cycles of 5 s at 95 °C, 5 s at 58 °C, 15 s at 72 °C, and followed by 65–95 °C melting curve detection. The expression level for each target gene was normalized with *Act8* as a reference. For the mean level of each treatment, three biological replicates were performed. Primers used in the assays were specially synthesized, and the detailed sequences are listed in the Supplementary Table 1.

Subcellular localization

To examine cellular localization, full-length *AtDIV2* cDNA was amplified and fused to the C-terminus of the *GFP* ORF in the vector pCX-DG (Duan et al. 2015), generating a *GFP-DIV2* fusion gene under the control of the CAMV 35S promoter. The constructed vector was sequenced, and a transient transformation assay of onion epidermal cells mediated by *Agrobacterium* strain EHA105 was performed. Then, after incubating in 1/2 MS medium for 24–48 h at room temperature, the samples were examined using a fluorescence microscope. Epidermal cells transformed with EHA105 harboring empty vector expressing GFP were used as a negative control. DAPI staining was carried out as reported previously (Duan et al. 2015).

Determination of ABA content

ELISA was performed as previously described (Fang et al. 2017) for the characterization of the ABA content in Wt and *div2* mutant plants. Briefly, 50 mg of fresh samples from 2-week-old seedlings were finely ground and then subjected to extraction (80% methanol, v/v) for 16 h at 4 °C. To harvest the supernatant, the leaching liquid was centrifuged through a CNWBOND LC-C18 cartridge (CNW Technologies GmbH, Germany) at 10,000*g* for 20 min at 4 °C. The products were dried and resuspended in 20 µl cold solution from the kit for ABA-testing (MLBIO, Shanghai Enzyme-linked Biotechnology Co., Ltd. Shanghai China). The ABA standard and 50× dilutions from extracted samples were then subjected to ELISA according to the manufacturer's instructions. The GENios microplate reader (Tecan Co. Ltd., Switzerland) was used to determine the ABA content at 450 nm of the optical density (OD).

Growth phenotype

For comparison of the growth between Wt and *div2* mutant plants, the primary root and bolting shoot of the plants during treatment with stress factors were directly measured using a rectilinear scale. Flowers from the same age on the Wt and *div2* mutant plants were examined and images were recorded with digital camera on the stereo fluorescence microscope Nikon SMZ25.

Statistical analysis

Comparisons were made between the groups of Wt and *div2* mutant plants with SPSS13.0 software, and the data are present as means ± SE (standard error). The mean levels from each assay in all experiments of the present study were from three replicates.

Accession numbers

The proteins and genes referred in the present study can be retrieved from GenBank and EBI according to the accession numbers listed in the supplementary table 2.

Results

AtDIV2 is an R-R-type MYB transcription factor localized in nucleus

In *Arabidopsis*, the locus *At5g04760* encodes a putative TF from the R-R-type clade in the MYB-like subfamily. This TF was named *AtDIV2* (Machemer et al. 2011), as a homolog of *AmDIV*, which is a key factor involved in dorsoventral asymmetry regulation (Almeida et al. 1997; Galego and Almeida 2002; Perez-Rodriguez et al. 2005). The putative AtDIV2 protein possesses 215 amino acids and a molecular mass of 24.4 kDa. Figure 1a shows the amino acid sequence alignment of the four MYB-like proteins, AmDIV, AtDIV1, AtDIV2 and AtMYBL (Zhang et al. 2011). Similar to the other three MYB-like proteins, two MYB-like DNA-binding domains (DNA-BD I and II) are found in the N and C-terminus of AtDIV2 protein, respectively; the DNA-BD I in the

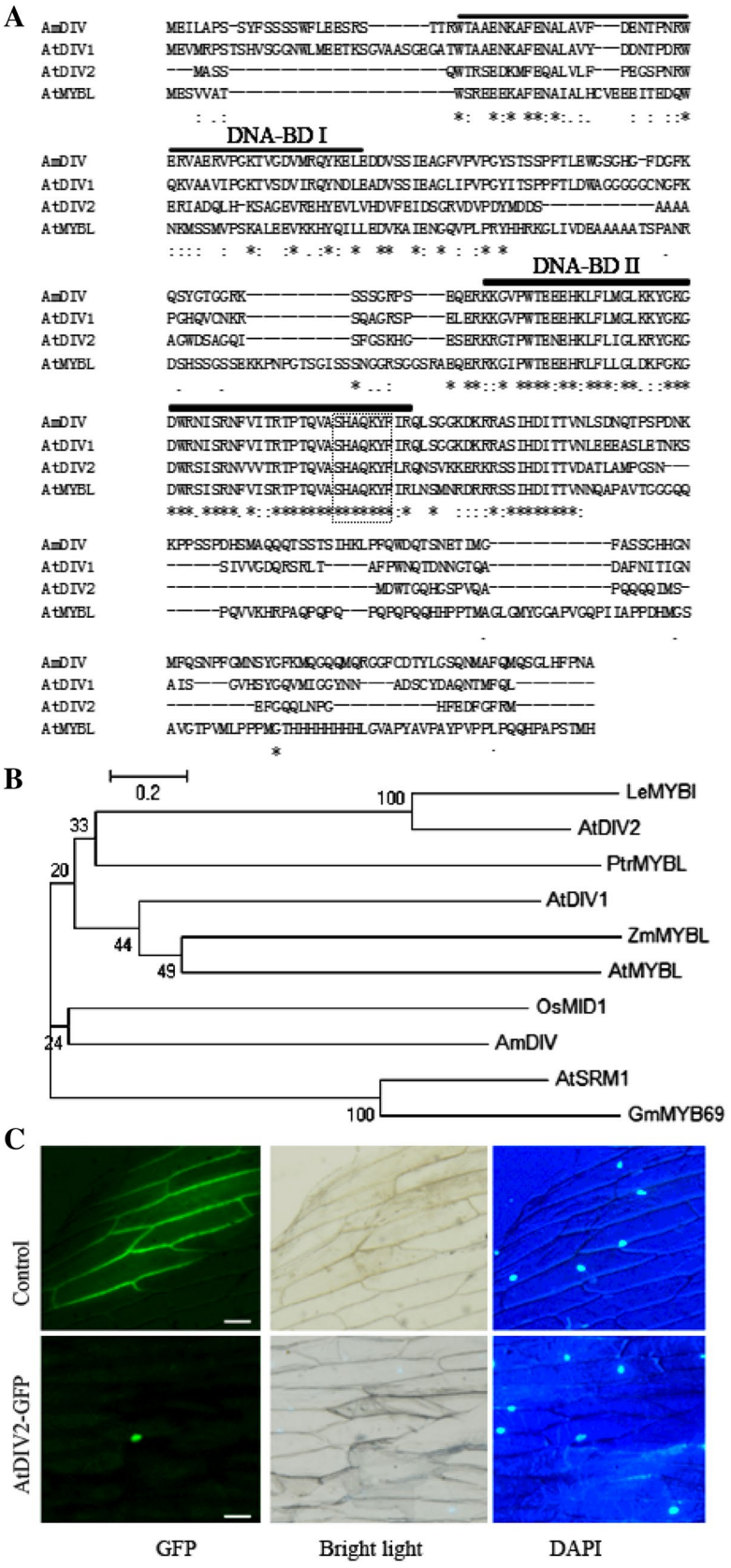

Fig. 1 Amino acid sequence alignment, phylogenetical tree and the nuclear localization of AtDIV2 protein. **a** Alignment of amino acid sequences of four MYB proteins, AmDIV, AtDIV1, AtDIV2 and AtMYBL. Lines on the sequences indicate two putative DNA-binding domains (DNA-BD I, 6–57, and II, 96–147), and the white box shows the conserved "SHAQKYF" motif in the DNA-BD II of the four proteins. **b** Phylogenetic analysis of the MYB-like proteins in plants. AtDIV2 was grouped in a subclade close to LeMYB1. The tree was constructed with plant MYB proteins using software MEGA 6.0. The neighbor-joining method was selected in the program. **c** The results of transient expression assays indicated that the R-R-type MYB protein AtDIV2 is a nuclear-localized transcription factor. Bar = 200 μm

N-terminus is less conserved than the DNA-BD II domains in the C-terminus; and in all DNA-BD II domains of the four proteins, there are conserved "SQHKYF" motifs (Fig. 1a), which is one of the main characteristics that distinguishes from the R2R3 MYB proteins (Rose et al. 1999).

The full-length AtDIV2 protein shows different levels of identity to LeMYBl (53.4%), PtrMYBL (52.0%) and AtDIV1 (33.7%). LeMYB1 was previously identified as a MYB-like I-box-binding protein and is likely involved in the activation of the RBCS promoters, which function in the photosynthetic carbon dioxide fixation (Rose et al. 1999). Figure 1b shows that the AtDIV2, LeMYBl and PtrMYBL (EEF02663) belong to a separate subclade in the phylogenetic tree, which are distinct from the subclade of AmDIV and AtMYBL. AtMYBL was recently characterized as another R-R-type MYB TF that is involved in salinity regulation through the induction of the ABA signaling cascade integrated with senescence (Zhang et al. 2011). These results suggest that the functional DIV homologs evolved in their roles.

To detect the subcellular localization, the *AtDIV2* encoding region was fused in-frame to the *GFP* C-terminus leading to the fusion under the control of the Cauliflower Mosaic Virus 35S (CaMV) promoter. As shown in Fig. 1c, the DIV2-GFP fluorescence was exclusively observed in nucleus versus that in the control sample, in which the GFP fluorescence was dispersed throughout the cell. These findings indicate that *AtDIV2* is an R-R-type MYB TF localized in nucleus.

AtDIV2 specific responses to salt and ABA

To investigate the roles of *AtDIV2*, we first assessed its transcription profiles under normal conditions. *AtDIV2* is universally expressed in *Arabidopsis* tissues, including root and flowers (Fig. 2a). The results were consistent with the detectable expression in microarrays (eFP browser; http://bar.utoronto.ca/efp) and previous transcriptome analyses (Wang et al. 2008; Winter et al. 2007), while the precise function or mechanism mediated by this TF during floral development remain unknown. In contrast, upon salt and ABA treatment, the transcripts of *AtDIV2* are dramatically increased in a short time. Time-course experiments for the NaCl induction revealed that the transcripts of *AtDIV2* were continually increased and peaked in the first 60 min, followed by a slight decline in the second 60 min (Fig. 2b). Upon ABA treatment, the transcripts were increased in the first 30 min (Fig. 2c). These findings implied that *AtDIV2* functions in salt and ABA-related responses.

The homozygous T-DNA insertion mutant *div2* (Salk_059505) (Fig. 3a) was subsequently isolated by PCR with specific primers (Fig. 3b). To screen the obvious distinction between Wt and *div2*, young seedlings were cultured on same plates. However, there were indistinguishable changes between these plants in the considered time (Fig. 3c). The flowers of Wt and *div2* plants were also examined; however, no noticeable alteration appeared during two testing phases (Supplementary Fig. 1). These results suggested that the loss-of-function of *AtDIV2* may not directly affect normal plant growth and flower development.

Loss of *AtDIV2* seedlings show improved tolerance to salt stress

The characterized expression pattern implied that the R-R-type TF *AtDIV2* likely mediates salt stress in *Arabidopsis*. We initially found that the germination rate of *div2* mutant was obviously slower than that of Wt plants (Fig. 4a), subsequently we conducted salt stress assays to explore the responses. Wt and *div2* mutant plants were sown on 1/2 MS agar plates supplemented with different concentrations of NaCl. As shown in Fig. 4b and Supplementary Fig. 2A, *div2* mutant plants grown on medium without NaCl were indistinguishable from Wt seedlings (control); in contrast, on salt-added plates, *div2* seedlings showed better growth than Wt plants. After culturing for 10 days, the survival of *div2* mutant was 1.6 and 3.2-fold that of Wt plants on media supplemented with 150 and 180 mM NaCl, respectively (Fig. 4c); and the primary roots of *div2* mutant were clearly longer than those of Wt plants (Supplementary Fig. 2A and B). These results implied that the loss of *AtDIV2* improved salt tolerance in *Arabidopsis*. We also examined the salt-responsive genes. As shown in Fig. 4d, except for *ABF3*, the other four genes, *ABF4, CBL1, SOS2* and *SOS3* (Albrecht et al. 2003; Choi et al. 2000; Uno et al. 2000; Zhu 2002) were clearly elevated.

Function of *AtDIV2* is tightly linked with ABA

Under stress conditions, the activity of endogenous phytohormones, particularly ABA, was adjusted to support plant survival as well as gene expression (Umezawa et al. 2010). Given the ABA-induced expression pattern of *AtDIV2* in Wt plants (Fig. 2c), we first performed seed germination assays to assess the ABA-related processes in *div2* mutant. As shown in Fig. 5a, when no ABA present (control), a slight inhibition of germination was observed in the *div2* mutant compared with that in Wt at earlier testing periods; and the germination distinction could be strengthened at the later period (Fig. 5a, control); in contrast, upon ABA treatment, the *div2* seed germination rate was clearly slower than that of Wt plants. For example, at 72 h upon 1 μM ABA, only 39% of the *div2* mutant seeds were germinated, while approximately 82% of the Wt seeds were germinated (Fig. 5a).

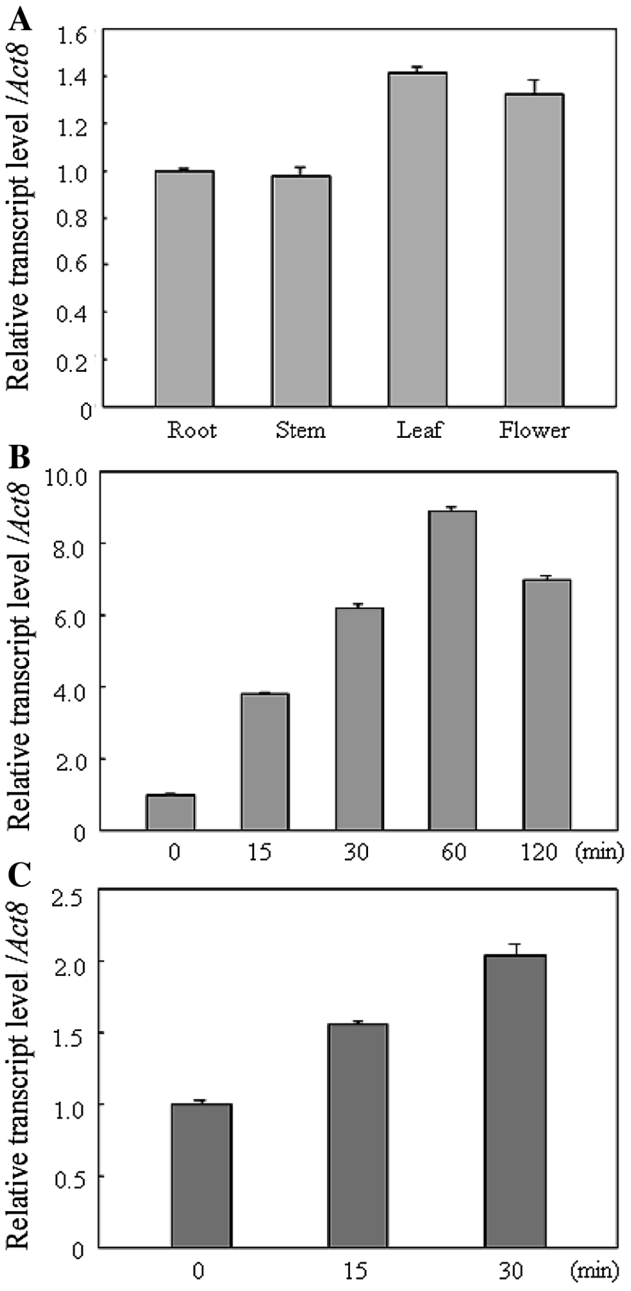

Fig. 2 Characterization of the transcript level of *AtDIV2*. **a** qRT-PCR results indicated that *AtDIV2* was expressed in various tissues under normal conditions. The transcripts in leaves and flowers were relative abundance compared with those in roots and shoots. **b, c** *AtDIV2* transcript is induced by salt and ABA treatment, respectively. During the testing periods, the transcripts of *AtDIV2* is clearly induced by the treatment of salt and ABA. The levels are expressed relative to the expression of *Act8*

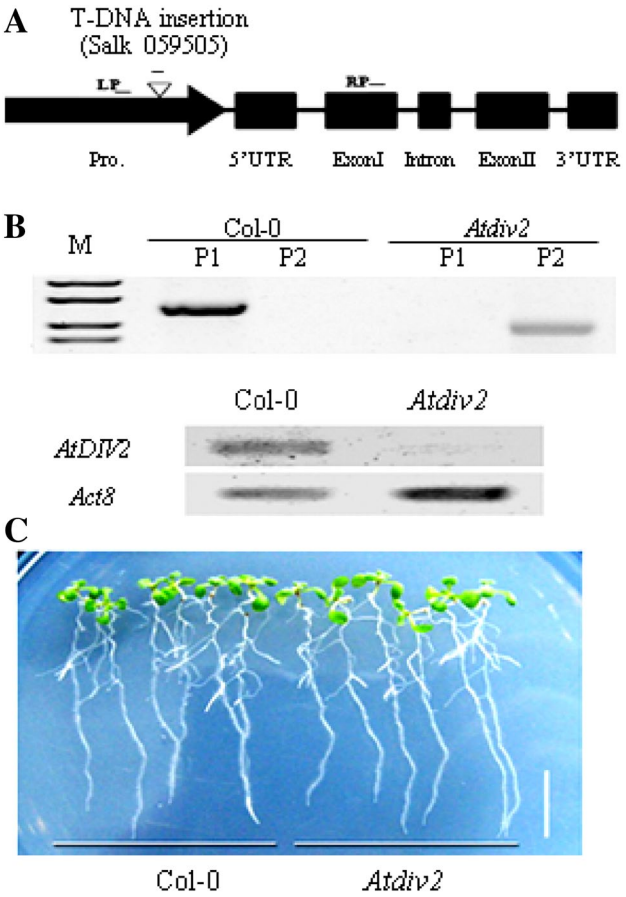

Fig. 3 Isolation the homozygous mutant of the R-R-type MYB transcription factor *div2* using PCR and comparison the seedling growth with Wt (Col-0) plants. **a** T-DNA insertion mutation of *AtDIV2*. The promoter, 5′ and 3′ UTR, exons and introns are indicated. LP and RP with BP (specific primer for the T-DNA sequence) are the primers used for the characterization of the homozygous T-DNA insertion mutant. **b** The characterization the *div2* homozygous mutant by PCR based on the analysis of genomic DNA (above) and total RNA (below). Two primer pairs, P1 (LP and RP) and P2 (BP and RP) were used in these assays (above), and the RT-PCR results demonstrated that the *AtDIV2* transcriptional expression level was markedly reduced in *div2* mutants compared with that in Wt plants, and *Act8* is used as a control (below). **c** The 8-day-old seedlings of *div2* mutant compared with Wt plants. Similar size seedlings of Wt and *div2* mutant were cultured in the testing time in one plates and results showed there were no distinction between them. Bar = 10 mm

The germination variance could be rescued by treatment with only sodium tungstate (1.0 or 5.0 mM) (Fig. 5b), but when treated with sodium tungstate and ABA at the same time (ABA 0.5 μM and sodium tungstate 1.0 mM, or ABA 1.0 μM and sodium tungstate 5.0 mM), the distinction between *div2* mutant and Wt plants was kept (Fig. 5c) similar to the phenotype under the treatment with only ABA (Fig. 5a, b, the controls). These suggested that the processes down-stream of ABA biosynthesis had been disturbed in the *div2* mutant. And we also found that the growth of *div2* seedlings, particularly the bolting shoots, was shorter than that of Wt plants when the culture period was prolonged in the presence of 1.5 μM ABA (Supplementary Fig. 3A and B). To assess the ABA level in *div2*,

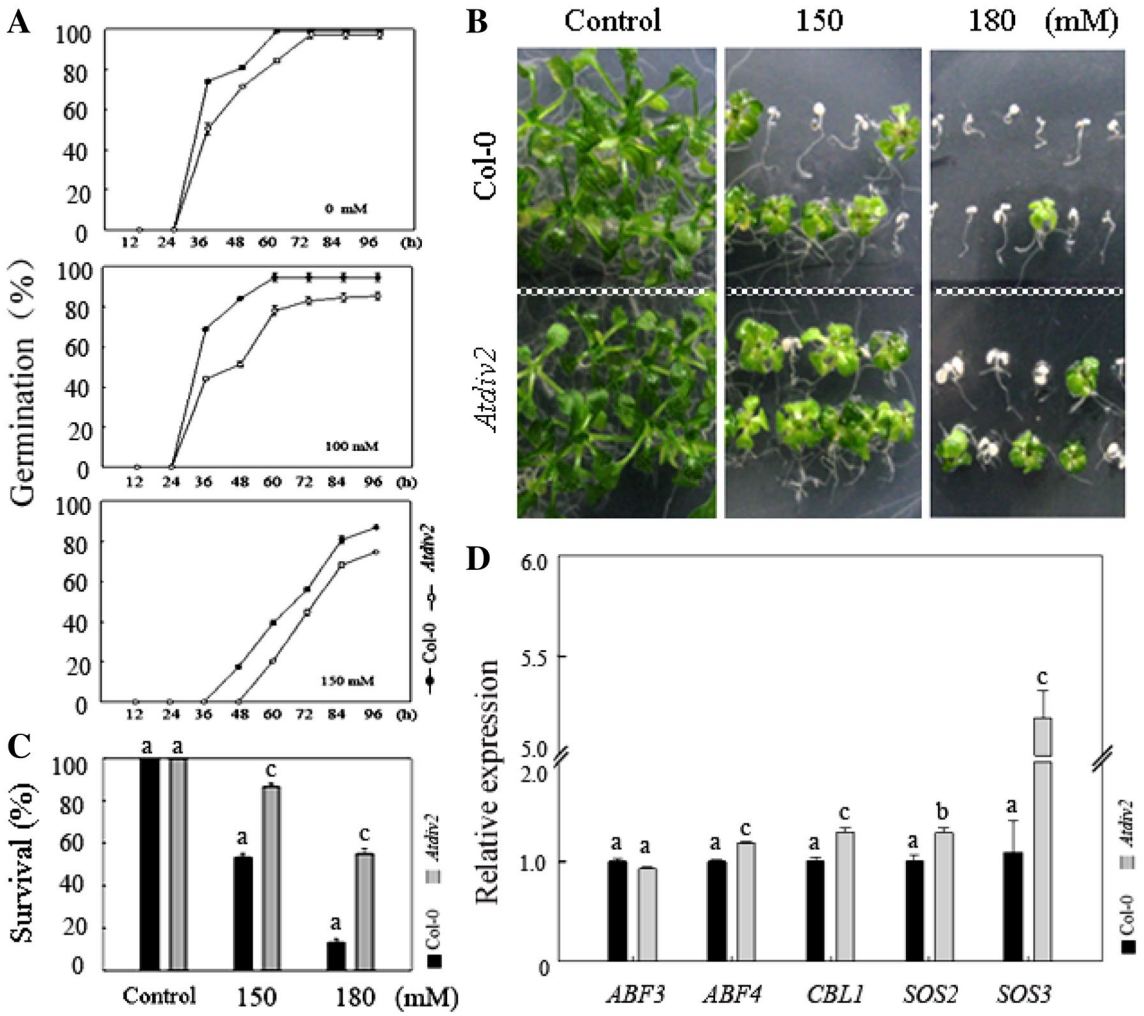

Fig. 4 Characterization the seed germination and salt responses of the loss of *AtDIV2* mutant. **a** Seed germination of Wt plants (Col-0) and *div2* mutant under salt stress conditions. During the testing time, germination of div2 mutant was slower. For each level, three times replicates were conducted to infer the mean level of seed germination in div2 mutant and Wt plants. **b** *div2* mutant shows more tolerance to salt stress than did Wt plants. The similar-sized seedlings of 4-day-old Wt and div2 mutant plants were maintained for another 8 days on the same plates with 1/2 MS agar media supplemented with NaCl at 0 (used as a control), 150 and 180 mM. **c** The survival rates of seedlings, the scores are form the mean levels in three times replicates. Bars show SE ($n=20$), and different letters indicate a significant difference between Wt and *div2* mutant plants, based on *t* test ($P<0.01$). **d** qRT-PCR analysis the salt-related genes expression. Relatively, except for the *ABF3*, the four genes, *ABF4, CBL1, SOS2* and *SOS3* were clearly elevated in the transcript level in div2 mutant compared with Wt plants. The levels are expressed relative to *Act8* using as an endogenous reference gene in *Arabidopsis*

ELISA was performed. As shown in Fig. 6, the endogenous ABA content in *div2* mutants was approximately twofold that in Wt plants. Altogether, the results suggested that the function of *AtDIV2* is tightly linked with ABA.

Transcriptional expression of ABA-related genes are altered in the *div2* mutant

We propose that the alteration of salt responses and ABA sensitivity might result from the adjustment of ABA-related genes in the *div2* mutant. The results of qRT-PCR indicated that several ABA biosynthesis and signaling genes, such as *ABA1* and *ABI3*, were clearly up-regulated in the *div2* mutant compared with those in Wt plants (Fig. 7). *ABA1* (Barrero et al. 2005; Xiong et al. 2002) encodes zeaxanthin epoxidase and controls the first step of ABA biosynthesis. The up-regulated expression of *ABA1* is consistent with the increased level of endogenous ABA content (Fig. 6). The B3 domain-containing TF, *ABI3*, which functions as a positive regulator in ABA signaling (Lopez-Molina et al. 2002; Park et al. 2011), is closely

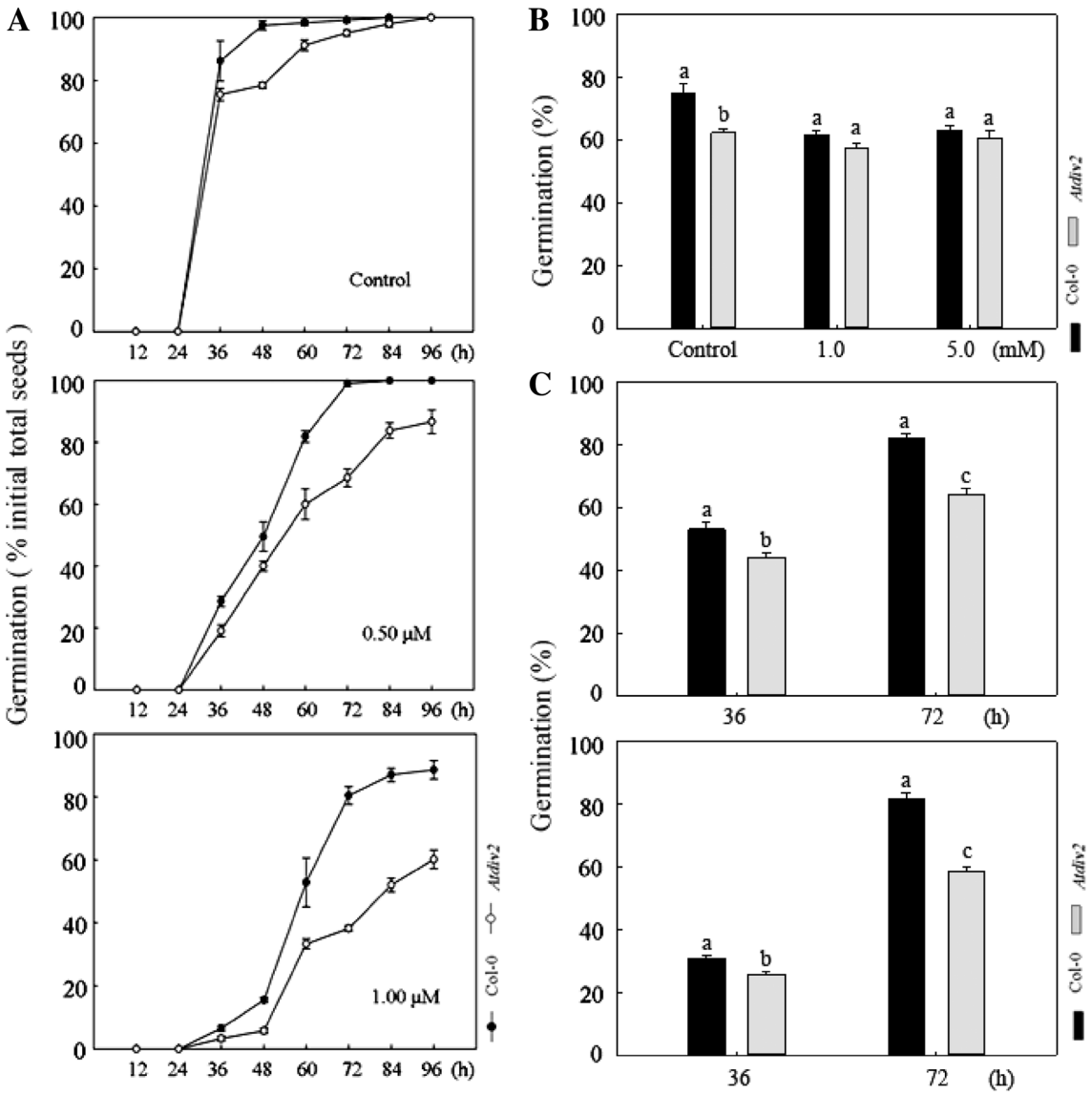

Fig. 5 Characterization the ABA responses during seed germination in the plants. **a** The seed germination of *div2* mutant is distinct from that of Wt (Col-0) plants, whether the ABA present in the medium (0.5 and 1 μM) or not (control). The ABA inhibition was more clear in *div2* mutant than that in Wt when comparing the germination rate (%). Bars show SE ($n=40$). **b** Treatment with sodium tungstate during germination rectified the variance between Wt and *div2* mutant plants. The germination rates (%) were cheked under the condition of two different levels (sodium tungstate, 1 and 5 mM). The plates without sodium tungstate added were set as control. **c** The seed gemination distinction could not be rescued by treatment with both sodium tungstate and ABA at the same time in the medium in our testing time. Two periods (36 and 72 h) were checked in the two different levels (ABA 0.5 μM and sodium tungstate 1 mM, above; and ABA 1 μM and sodium tungstate 5 mM, down). The scores are from the mean levels in three times replicates. Bars show SE ($n=40$), and different letters (a, b or a, c) in **b, c** indicate the level ($P<0.05$ or $P<0.01$) between Wt and *div2* mutant plants

linked with seed physiology (Rohde et al. 2000). Given the expression of the genes and the examined ABA content, the delayed germination in *div2* mutant is reasonable (Fig. 5a). We also checked other genes, such as *ABI1* and *ABI4* (Finkelstein et al. 1998; Leung et al. 1998; Murata et al. 2001; Ramon et al. 2007), but they were not significantly affected.

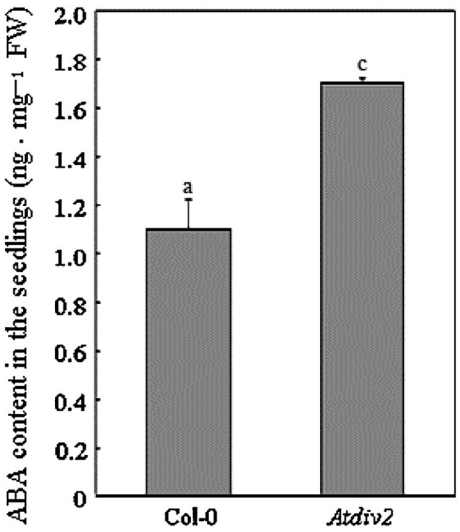

Fig. 6 Comparison the ABA content in *div2* mutant and Wt (Col-0) plants under normal condition. Measurement of endogenous free ABA by ELISA. Fresh levels of 2-week-old plants were sampled for ABA content measurement. Bars show the SE ($n=3$). Different lower letters indicate the difference between the plants based on *t* test ($P<0.01$)

Discussion

In the present report, we provide evidence for the involvement of *AtDIV2*, an R-R-type MYB TF, in salt responses and ABA signaling. Compared with Wt plants, the loss-of-function mutant *div2* displayed obvious salt tolerance (Fig. 4b, c), higher degree of ABA sensitivity during seed germination (Fig. 5), and increased endogenous ABA content (Fig. 6). In addition, qRT-PCR indicated that the transcription levels of ABA-related genes, as well as stress-related genes, such as *ABA1, ABI3* and *P5CS2* were up-regulated in the *div2* mutant (Fig. 7). These noticeable alterations are likely contributed by the loss of function of *AtDIV2*.

Generally, based on the number of conserved MYB domain repeats, plant MYB gene family is grouped into three major clades, referred to as R2R3-MYB, 3R(R1R2R3)-MYB, and MYB-like subgroups (Dubos et al. 2010); and five subclades, the CCA1-like, CPC-like, TBP-like, I-box-binding-like as well as the R-R-type MYB TFs, belong to the MYB-like subgroup (Zhang et al. 2011). In *Arabidopsis*, there are nine R-R-type MYB proteins, in which six proteins are regarded as homologs of the DIV and DIV-like proteins (Gao et al. 2017). A few studies have indicated that the R-R-type TF members in this clade possess diverse functions. For instance, *AtMYBL* has been implicated in leaf senescence and abiotic stress regulation (Zhang et al. 2011). In rice, the *MID1* (*MYB important for drought response 1*) encodes a putative R-R-type MYB TF involved in drought stress responses, and the overexpression of *MID1* improves rice yield under drought conditions (Guo et al. 2016).

In the present study, although there are detectable transcripts in flowers (Fig. 2a), further evidence is required to determine the precise roles of *AtDIV2* in floral development. However, upon salt treatment, the transcript level of *AtDIV2* was markedly increased (Fig. 2b), and the *div2* mutant showed improved salt tolerance compared with Wt plants (Fig. 4b, c). These indicated that in Wt plants the *AtDIV2* responds to salt treatment, and plays negative role in the regulation of salt stress. Generally, three steps are required for plants to cope with high salinity and other abiotic stresses, including recognizing the stress conditions, signaling and altering gene expression to result in protective and adaptive physiological responses (Guo et al. 2016; Huang et al. 2012; Zhu 2002). In view of the nuclear localization and the changed expression of salt-related genes, there could be potential targets to be regulated by this R-R-type MYB TF. The *AtDIV2*-mediated regulation would be involved in alternative processes in plants under salt stress, and it might decrease plants resistance to abiotic stresses, such as high salinity. Alternatively, there might be more central processes in which the *AtDIV2* were involved, such as flowering, hormone balance for growth and development (Davis 2009; Kohli et al. 2013; Peleg and Blumwald 2011; Su et al. 2013). We noticed the changed seed germination and endogenous ABA content, and hence concluded that *AtDIV2* is involved in salt stress regulation, which appears to be tightly associated with ABA.

As a well-known stress-related hormone, ABA coordinates gene activities to mediate stress challenges in plants. Numerous members of the MYB gene family, such as *MYB96* and *SRM1*, have been implicated in stress responses. MYB96 mediates drought stress by directly binding to conserved sequence motifs present in the promoters of genes encoding very-long-chain fatty acid-condensing enzymes, which contributes to cuticular wax biosynthesis (Lee et al. 2016; Seo et al. 2011). In addition, *MYB96* is highly expressed in embryos, mediating seed germination through the regulation of *ABI4* (Lee et al. 2015a, b). The *SRM1* mutation results in salt tolerance in *Arabidopsis* (Wang et al. 2015), and further evidence indicated that SRM1 directly activates *NCED3*, which is a key ABA biosynthesis gene (Jensen et al. 2013; Ruggiero et al. 2004).

By comparison, the effect on salt stress as a result of *AtDIV2* mutation is similar to that by mutation of *srm1*. However, there could be a complex mechanism in *Arabidopsis*. In the *div2* mutant, the transcription levels of two key ABA biosynthesis genes, *ABA1* (*ABSCISIC ACID DEFICIENT1*) and *ABA2* (Barrero et al. 2005; Gonzalez-Guzman et al. 2002; Ozfidan et al. 2012), were elevated (Fig. 7). The increased ABA content in *div2* (Fig. 6) likely resulted from the effect on these two genes. In addition, the transcripts

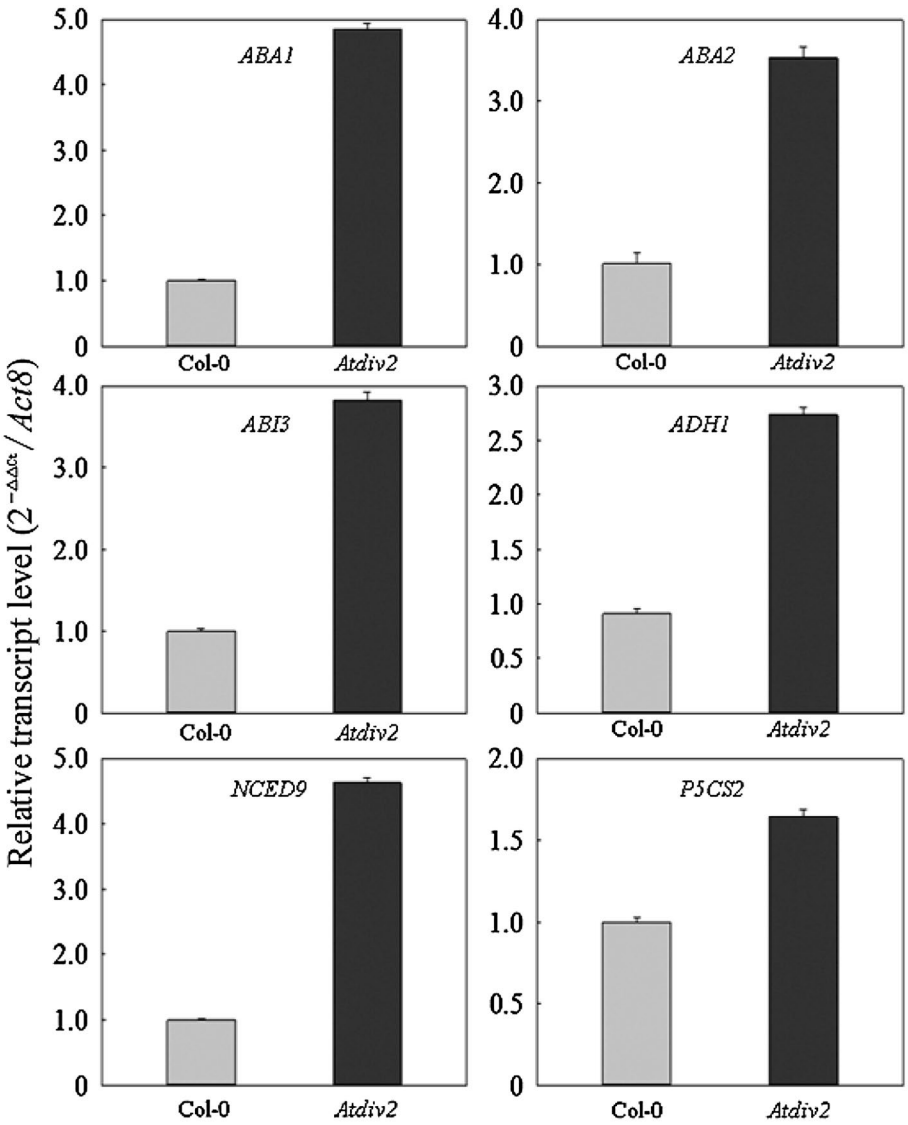

Fig. 7 Relative transcript level of ABA-related genes were elevated in *div2* mutant. ABA-related genes in *div2* mutant were up-regulated compared with those in Wt (Col-0) plants. The qRT-PCR analyses were performed with gene-specific primers, and *Act8* was used an endogenous control

of *ABI3* were increased approximately threefold in *div2* mutant compared with that in Wt plants. As a key regulator in the ABA signaling pathway, the B3 domain-containing TF, *ABI3* (ABSCISIC ACID INSENSITIVE3) has diverse functions at different stages in plant life cycle, including seed development, dormancy inception and even in response to stress conditions (Bies-Etheve et al. 1999; Crowe et al. 2000; Khandelwal et al. 2010; Parcy et al. 1994; Rohde et al. 2000). The seed germination was obviously altered as loss of function of *AtDIV2* (Fig. 5). This finding implied that proper *ABI3* activity might be modulated by *AtDIV2* in Wt plants. In fact, several reports on proteins that interact with *ABI3* have indicated that the functions of *ABI3* are not restricted in different stages of plant life; hence, there is a complex network constituted by ABI3 and its co-actors (Ding et al. 2014; Duong et al. 2017; Kurup et al. 2000; Zeng et al. 2013; Zhang et al. 2005), and the R-R-type Myb TF *AtDIV2* could be a novel repressor upstream of the global regulator, *ABI3*.

Compared with Wt plants, no noticeable changes were observed during flowering, or further assays would be required for revealing the details. The regulation of flower development is complex, or other strategies may compensate for the mutation of *AtDIV2* (Duong et al. 2017; Gao et al. 2013; Schneider et al. 2016; Zeng et al. 2013). Several studies recently have indicated the delicate links between ABA signaling and flower development (Davis 2009; Fitzpatrick et al. 2011; Wang et al. 2013); for instance, the *ABI5* and *ABFs* (abscisic acid-responsive element (ABRE)-binding factors) could inhibit flowering by elevating the transcriptional expression of *FLC* (*FLOWERING LOCUS C*) (Wang et al. 2013). The detectable transcripts of *AtDIV2* (Fig. 2a) with dynamic changes in flower development (Wang et al. 2008; Winter et al. 2007) suggested that *AtDIV2* likely

mediates ABA signaling or expression in vivo to coordinate flowering. Upon loss of *AtDIV2* activity, the tolerance to salt is elevated for survival in the *div2* mutant (Fig. 4b, c). This finding may present an additional effect, and to some extent, this effect is likely dependent on *AtDIV2*. For plants, this strategy is smart; because survival under stress conditions is also urgent as well as flowering for generation. In view of this hypothesis, we infer that *AtDIV2* is a functional mediator of plant survival under salt stress, which might coordinate flower-related processes.

Without NaCl and ABA treatment, seed germination of the *div2* mutant was clearly slower than that of Wt during the later testing period (Figs. 4a, 5a). The ABA levels in vivo are likely unbalanced as a result of the loss of *AtDIV2*. The elevated endogenous ABA has affected seed germination. This finding suggested that the R-R-type MYB TF *AtDIV2* might function in normal seed germination in an ABA-dependent manner. Upon exogenous NaCl and ABA, the inhibition of seed germination was observed both in Wt plants and *div2* mutant, but the *div2* mutant showed more sensitivity than Wt plants (Figs. 4a, 5a). We also found that when treated with both sodium tungstate and ABA at the same time, the germination rate kept similar to that of treatment with only ABA. It centrally implied that *AtDIV2* functions down-stream of ABA biosynthesis in *Arabidopsis*. With extended ABA treatment period, the *div2* seedlings showed more resistance compared with Wt plants (Supplementary Fig. 3A and B). We propose that an overlapping effect resulted from *AtDIV2* mutation and exogenous ABA are present in these processes; and for plant cells, being with or without the overlapping effect might be distinct, in view of their responses. Altogether, for plants, response to ABA and survival under severe conditions are important aspects through their life. The results suggest that the R-R-type MYB TF *AtDIV2* is a negative player in salt responses and involved in ABA signaling in *Arabidopsis*. It is helpful to gain more information on plant life concerning the interplay of ABA and MYB TFs in vivo and even might reveal an interesting connection between ABA signaling and floral transition mediated by the *AtDIV2*. In future, to reveal the answer requires diverse skills and assays.

Author contribution statement QF and KL conceived and designed research. QF, QW, HM, JX, YW, HH, SH, JT and CC conducted experiments; QF, QW and HM performed the phenotypic identification and analyzed the data; CC, GT, XW, XL and CZ contributed devices and materials; QF and QW wrote the manuscript. All the authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors have declared that no conflict of interest exists.

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