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Arabidopsis ABRE-binding factors modulate salinity-induced inhibition of root hair growth by interacting with and suppressing RHD6

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ABSTRACT

Soil salinity causes crop losses worldwide. Root hairs are the primary targets of salt stress, however, the signaling networks involved in the precise regulation of root hair growth and development by salinity are poorly understood. Here, we confirmed that salt stress inhibits the number and length of root hairs in Arabidopsis. We found that the master regulator of root hair development and growth, the RHD6 transcription factor, is involved in this process, as salt treatment largely compromised root hair overaccumulation in *RHD6*-overexpressing plants. Yeast-two-hybrid and co-immunoprecipitation analyses revealed that RHD6 physically interacts with ABF proteins, the master transcription factors in abscisic acid signaling, which is involved in tolerance to several stresses including salinity. Phenotypic analyses showed that ABF proteins, which function upstream of RHD6, positively modulate the salinity-induced inhibition of root hair development. Further analyses showed that ABF3 suppresses the transcriptional activation activity of RHD6, thereby regulating the expression of genes related to root hair development. Overexpression of *ABF3* reduced the root hair-overgrowing phenotype of *RHD6*-overexpressing plants. Collectively, our results demonstrate an essential signaling module in which ABF proteins directly suppress the transcriptional activation activity of RHD6 to reduce the length and number of root hairs under salt stress conditions.

1. Introduction

Root hairs are tubular-shaped unicellular extensions of epidermal cells that facilitate nutrient acquisition, as well as being involved in the response to stress and interactions with soil microorganisms (Grierson et al. 2014). Root hair development and growth have three critical steps; root hair cell fate determination, root hair initiation, and root hair elongation (Bibikova and Gilroy, 2003). Among these steps, root hair cell fate determination is the core process controlled by a developmental program involving an array of transcription factors that activate or suppress the expression of the gene encoding the homeodomain protein GLABRA 2 (GL2) in hair cells (H-type cells) and non-hair cells (N-type cells), respectively (Ishida et al. 2008; Ryu et al. 2005; Song et al. 2011; Schiefelbein et al. 2014; Balcerowicz et al. 2015). In N-type cells, *GL2* is activated by the MYB-bHLH-WD40 (MBW) transcriptional complex, and then GL2 suppresses the transcription of the master regulator gene, *ROOT HAIR DEFECTIVE 6* (*RHD6*), to restrict root hair development (Lin et al. 2015). In H-type cells, the activation of *GL2* is compromised due to the competitive replacement of an R3 MYC-domain transcription factor, WERWOLF (WER), in the MBW complex by another R3 MYC-domain transcription factor, CAPRICE (CPC). This forces the cell into the root hair cell destiny program through the simultaneous activation of *RHD6* and its homologs (Menand et al. 2007; Pires et al. 2013).

In Arabidopsis, RHD6, a class I member of the Group VIII subfamily of basic-helix-loop-helix (bHLH) transcription factors, plays an essential role in triggering root hair initiation (Masucci and Schiefelbein, 1994; Menand et al. 2007; Bruex et al. 2012). RHD6 along with its closest homolog RHD6-LIKE1 (RSL1) activates the expression of the secondary

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members of the Group VIII subfamily of bHLH, such as RHD6-LIKE 2 (RSL2) and RHD6-LIKE 4 (RSL4), to trigger the differentiation of root hairs and their subsequent polarized tip growth (Karas et al. 2009; Yi et al. 2010; Bruex et al. 2012). These secondary members of the bHLH family of transcription factors play an active role in the process of root hair elongation (Masucci and Schiefelbein, 1994; Yi et al. 2010; Bruex et al. 2012; Shibata and Sugimoto, 2019). RSL4 is crucial for root hair elongation and determines the final root hair cell size in Arabidopsis, while RSL2 modulates the accumulation of reactive oxygen species and root hair growth (Zhu et al. 2020; Rymen et al. 2017). The application of phytohormones such as ethylene (ETH) and jasmonate can stimulate root hair growth in a RHD6-dependent manner. Specifically, ETH promotes root hair elongation via its key transcription factor (EIN3) associating with RHD6 to coactivate the expression of *RSL4* and other related genes (Feng et al. 2017). Jasmonate also stimulates root hair development. Genetic evidence indicates that JA signal transduction mediated by COI1/JAZ is necessary for root hair elongation, which occurs in a RHD6-dependent manner (Han et al. 2020). The results of those studies reveal that RHD6 acts as an essential node that integrates multiple root hair development and growth-regulating signals to allow plants to acclimate and adapt to stress.

Salt stress is one of the most severe stresses faced by plants during their lifecycle. Soil salinity leads to growth inhibition and developmental changes, as well as remodeling of root system architecture in plants (Munns et al. 2012; Zou et al. 2022). Roots are the most sensitive plant organs, and they can coordinate cellular stress signals and readjust their growth direction after sensing various environmental stimuli (Lamers et al. 2020; Muthert et al. 2019). Various changes, such as redirection of root growth and alterations in root length, branching, and root hair density occur dynamically in response to biotic and abiotic stresses (Zou et al. 2022). Root hairs are the most sensitive part of the root and can serve as an index of salt stress. Salt stress strongly modulates root hair plasticity, affecting both the density and elongation of root hairs in Arabidopsis (Wang et al. 2008). In sorghum, SbbHLH85 negatively regulates salt tolerance through increasing the number and length of root hairs (Song et al. 2022). Although root hairs are the primary targets of salt stress, little is known about the molecular mechanism controlling their length and density in salt-treated Arabidopsis.

In this study, we confirmed that salinity negatively modulates root hair development in Arabidopsis. The transcription factor RHD6 is involved in the inhibition of root hair development under salt stress, as demonstrated by the salt-induced inhibition of the overgrowing root hair phenotype of a line overexpressing RHD6 (*RHD6-OE*). Further analyses of the molecular mechanism indicated that RHD6 physiologically interacts with ABF proteins, the master transcription factors involved in abscisic acid (ABA) signaling during stress responses. Compared with wild type (Col-0), the loss-of-function *abf* mutants displayed a lower level of root hair development impairment under high-salt conditions, suggesting that ABF transcription factors are required for the salt-induced inhibition of root hair development. Genetic analyses showed that ABF functions genetically upstream of RHD6 during root hair development. Further analyses showed that ABF3 suppresses the transcriptional activation of RHD6 to regulate *RSL4* expression. *ABF3* overexpression largely suppressed the root hair-overgrowing phenotype of *RHD6-OE* plants. Collectively, our results reveal a molecular framework in which salt stress restricts root hair development by interfering with the ability of RHD6 to activate the transcription of *RSL4*. Our results provide the molecular basis for a gene editing strategy to produce more salt-tolerant plants to increase crop yield on salinized agricultural land.

2. Methods and materials

2.1. Materials and plant growth conditions

The wild-type and mutant *Arabidopsis thaliana* plants used in this study were in the Columbia (Col-0) genetic background. The mutants *rhd6 rsl1* and transgenic *Pro$_{35S}$:RHD6-GFP* plants were gifts from Hongwei Guo (Southern University of Science and Technology, Shenzhen, China). The same *abf134*, *abf234*, *abf1234* mutants used in the study of Nambara et al. (2002) were used in this study. To generate transgenic plants overexpressing *ABF3*, the full-length cDNA of *ABF3* fused with a *HA-tag* was cloned into the binary vector pOCA30 in the sense orientation under the control of the cauliflower mosaic virus 35 S promoter (*Pro$_{35S}$*). Two stable transgenic lines, *Pro$_{35S}$:ABF3-HA-L5* and *Pro$_{35S}$: ABF3-HA-L6*, were selected for phenotypic analysis. *Pro$_{35S}$: RHD6-GFP /Pro$_{35S}$:ABF3-HA* and *abf3 abf4 rhd6 rsl1* were generated by genetic crossing using standard techniques. Arabidopsis seeds were sown on half-strength Murashige and Skoog (MS) medium and stratified at 4 °C for 1 d before germination. Plants were grown in an artificial growth chamber at 22 °C under a 16-h-light (100 mE m^{-2} s^{-1}, white fluorescent bulbs, full-spectrum light), 8-h-dark photoperiod.

For NaCl treatment, Arabidopsis seeds were grown on half-strength MS medium supplemented with 50 mM NaCl or 70 mM NaCl for 7 days. For the control, seeds were grown on half-strength MS medium without NaCl.

2.2. Microscopy

All root hairs within the same root range (the first 2 mm of the zone of maturation) were counted under an SZX16 microscope (Olympus, Tokyo, Japan) as described previously (Han et al. 2020). To measure root hair length, root hairs on both sides of the root within the same range were measured. A total of 12 roots of similar size were evaluated. Olympus software (Moon et al., 2019) was used to measure root hair length. To quantify root hair cells in the root epidermis, the same root range (the first 2 mm of the zone of maturation) of 12 roots was observed. A cell was scored as a root hair cell if any protrusion was visible, regardless of the root hair length. Four replicates of each genotype with or without NaCl treatment were analyzed. The mean root hair length and the number of hair cells were compared among treatments/genotypes using analysis of variance (ANOVA).

2.3. RNA extraction and RT-qPCR analysis

Total RNA (for each sample, roots were collected) was extracted from roots using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and qRT-PCR was performed as described previously (Yang et al. 2021; Han et al. 2022). Briefly, 1.0 μg DNase-treated root total RNA was reverse-transcribed in a 20-μL reaction volume using Moloney murine leukemia virus reverse transcriptase (Fermentas, Waltham, MA, USA) with oligo (dT) 18 primers. Then, 1 μL cDNA was used in a qRT-PCR reaction with the SYBR Premix Ex Taq kit (Takara, Dalian, China) on a Roche Light Cycler 480 real-time PCR machine. At least three biological replicates for each sample were used for RT-qPCR analysis and at least three technical replicates were analyzed for each sample. The *ACTIN2* (AT3G18780) gene was used as the reference gene. The gene-specific primers used for the RT-qPCR are listed in Supplemental Table 2.

2.4. Yeast two-hybrid assays

The full-length coding sequence (CDS) of *RHD6* was fused to the bait vector pGBKT7 (Clontech, Palo Alto, CA, USA) to produce BD-RHD6, which contained the Gal4 DNA-binding domain (BD). The full-length CDSs of *ABF1*, *ABF2*, *ABF3,* and *ABF4* were separately inserted into pGADT7 (Clontech) to generate prey vectors (AD-ABF) containing the Gal4 activation domain (AD). To identify specific regions critical for interactions, several truncated forms of RHD6 were cloned into pGBKT7, and truncated forms of ABF3 were cloned into pGADT7. The yeast two-hybrid (Y2H) assays were performed as described previously (Jiang and Yu, 2016). The bait and prey vectors were co-transformed into the yeast

strain AH109 and physical interactions were indicated by the ability of cells to grow on dropout medium lacking Leu, Trp, His, and Ade for 4 days after plating. The primers used for cloning are listed in Supplemental Table 1.

2.5. Bimolecular fluorescence complementation assays

The cDNA sequences of the N-terminal 173-amino acid-enhanced yellow fluorescent protein (nYFP) and C-terminal 64-amino acid YFP (cYFP) fragments were PCR-amplified and individually cloned into tagged pFGC5941 plasmids to produce pFGC-nYFP and pFGC-cYFP, respectively (Kim et al. 2008). The full-length CDS of *RHD6* was inserted into pFGC-cYFP to generate a C-terminal in-frame fusion with cYFP (RHD6-cYFP). Each of the full-length CDSs of *ABF1*, *ABF2*, *ABF3,* and *ABF4* was fused with nYFP to generate ABF-nYFP. The obtained plasmid was transformed into *Agrobacterium tumefaciens*, which was then then injected into wild tobacco (*Nicotiana benthamiana*) leaves. Infected leaves with YFP and 4,6-diamidino-2-phenylindole (DAPI) fluorescence were observed at 40–52 h after infiltration under a confocal laser-scanning microscope (Olympus). These bimolecular fluorescence complementation (BiFC) experiments were performed at least four times using different batches of wild tobacco plants. For each biological replicate, more than 12 tobacco plants were infiltrated, and more than 600 cells were observed. The primers used for cloning are listed in Supplemental Table 1.

2.6. Co-immunoprecipitation assays

Co-immunoprecipitation (Co-IP) assays were conducted confirm the physical interactions between RHD6 and ABF3. Total proteins were extracted from *Pro$_{35S}$:RHD6-GFP*, *Pro$_{35S}$:ABF3-HA*, and *Pro$_{35S}$:RHD6-GFP Pro$_{35S}$:ABF3-HA* seedlings in an extraction buffer consisting of 50-mM Tris–HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 10% (v/ v) glycerol, 0.1% (v/v) Triton X-100, 1 mM PMSF, and 1 × Roche Protease Inhibitor Cocktail. The Co-IP experiments were performed with GFP-trap beads (gtma-20, ChromoTek, Munich, Germany) following the manufacturer's protocol. In brief, cell lysates were incubated with the GFP-trap beads at 4 °C overnight in extraction buffer. The beads were washed four times extensively with the extraction buffer and the co-immunoprecipitated protein was then detected by immunoblotting using an anti-HA antibody (1:5000 dilution; Abmart, Shanghai, China).

2.7. Transient transcriptional activation assays

The full-length CDSs of *RHD6*, *ABF3*, and *GFP* were each cloned into the pGreenII 62-SK vector as effectors. The putative promoter sequence of *RSL4* was amplified by PCR and cloned into the pGreenII 0800-LUC vector (Biovector Science Lab, Beijing, China) as the reporter (Hellens et al. 2005). Combinations of plasmids were transformed into the wild-type and/or *abf1 abf2 abf3 abf4* mutant Arabidopsis leaf mesophyll protoplasts according to the Sheen laboratory protocol (Sheen, 2001). Transfected cells were cultured for 16–18 h, and then relative LUC activity was analyzed using a Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA), which measured the activities of firefly LUC and the internal control *Renilla reniformis* LUC (REN). The primers used for cloning are listed in Supplemental Table 1.

3. Results

3.1. Salinity inhibits RHD6-mediated root hair development and growth

Previous studies have shown that salt stress suppresses the density and elongation of root hairs in Arabidopsis (Wang et al. 2008). To investigate the molecular mechanisms by which salinity inhibits root hair development, we first confirmed the regulatory role of salt stress. Consistent with the results of a previous study (Wang et al. 2008), root hair elongation was significantly reduced in wild-type Col-0 plants growing on medium containing 50 mM NaCl (Fig. 1A and 1B). We examined the density of visible roots hairs in the same root range (the first 2 mm of the zone of maturation) and found that the total number of root hairs was lower in NaCl-treated Col-0 plants than in those in the control (Fig. 1C). To explore the basis of this phenotype, we performed RT-qPCR analyses to determine the transcript levels genes involved in root hair growth in Col-0 seedlings with or without NaCl treatment. As shown in Fig. 1D–F and Supplementary Fig. S1, genes related to root hair growth were downregulated by NaCl treatment in Col-0 seedlings.

As mentioned before, RHD6 is the master regulator of root hair growth in Arabidopsis (Masucci and Schiefelbein, 1994; Menand et al. 2007). Compared with wild-type plants, *RHD6-OE* plants have a noticeably higher density of longer root hairs (Menand et al. 2007). Given that NaCl treatment largely inhibits root hair development, we wondered whether NaCl treatment inhibits RHD6-mediated root hair growth. To test this, we investigated the responses of Col-0 and *RHD6-OE* seedlings to NaCl treatment. Interestingly, the elongation of root hairs was suppressed, and their density was reduced in *RHD6-OE* seedlings treated with NaCl (Fig. 2A–C). Consistent with this phenotype, the basal and NaCl-inhibited transcript levels of several root hair-related genes were much lower in *RHD6-OE* than in Col-0 (Fig. 2D–E; Supplementary Fig. S2). Taken together, these results demonstrate that the RHD6 transcription factor is involved in the salt-induced inhibition of root hair growth in Arabidopsis.

3.2. RHD6 and RSL1 physically interacts with ABF proteins

RHD6 plays a decisive role in root hair cell fate determination and is involved in the salt-induced inhibition of root hair development. To explore the precise regulation of this process, we performed Y2H screening to identify proteins that may interact with RHD6. In these assays, we fused the RHD6 to the Gal4 DNA-binding domain (BD) and used it as bait (BD-RHD6) to screen a homogenized cDNA library of Arabidopsis. Sequencing revealed that the seven positive clones contained the same insert sequence, encoding the N-terminal region of ABF3. To confirm the interaction between RHD6 and ABF3, we fused the full-length CDS of *ABF3* with the Gal4 activation domain (AD). Since there are three close homologs of ABF3 in Arabidopsis (ABF1, ABF2, and ABF4), we also fused the full-length CDS of *ABF1, ABF2,* and *ABF4* with the Gal4 AD and conducted Y2H assays. As shown in Fig. 3A, RHD6 physically associated with ABF1, ABF3, and ABF4 in the Y2H system. Moreover, RSL1 is the closest homolog of RHD6 in Arabidopsis, and they always function redundancy during root hair development. Thus, we detected the interactions between RSL1 and ABF proteins. Unlike RHD6, RSL1 only interacted with ABF1, but not with ABF3 and ABF4 in yeast (Supplementary Fig. S3A).

To further confirm the interaction between ABFs and RHD6 in plant cells, we conducted BiFC assays. The full-length CDSs of *ABFs* were each ligated with the sequence encoding the C terminal of YFP driven by *Pro$_{35S}$* to generate ABF1-cYFP, ABF2-cYFP, ABF3-cYFP and ABF4-cYFP respectively, while the full-length CDS of RHD6 was fused with the sequence encoding the N terminal of YFP to produce RHD6-nYFP. When each pair (ABF1-cYFP with RHD6-nYFP, ABF3-cYFP with RHD6-nYFP, and ABF4-cYFP with RHD6-nYFP) was transiently coexpressed in leaf cells of *N. benthamiana*, strong YFP fluorescence was detected in the nucleus of the transformed cells after staining with DAPI (Fig. 3B). As controls, the pairs ABF2-cYFP with RHD6-nYFP, and ABF3-cYFP with nYFP were coexpressed transiently in leaf cells of *N. benthamiana.* As expected, no YFP fluorescence was detected in the nucleus of the cells expressing these pairs (Fig. 3B). Consistent with the Y2H and BiFC results, in a CoIP assay, RHD6 was immunoprecipitated by anti-HA conjugated-agarose in Arabidopsis coexpressing *ABF3-HA* and *RHD6-GFP*, but not in Arabidopsis expressing *ABF3-HA* alone (Fig. 3C). Additionally, ARF1 and ABF4 were also immunoprecipitated by the anti-YFP conjugated-agarose in *N. benthamiana* leaves coexpressing pairs of

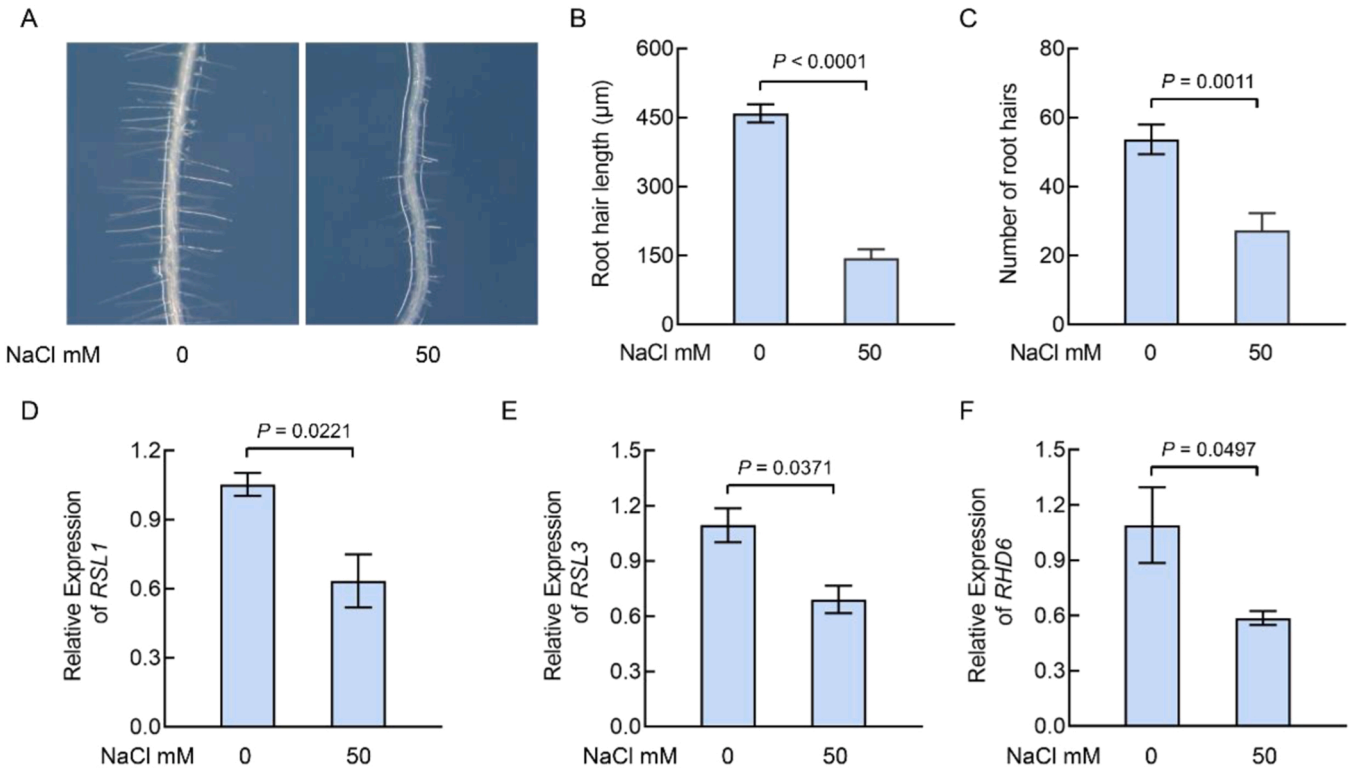

Fig. 1. Salt stress inhibits root hair development. (A) Images of root hairs of untreated Col-0 control plants (Mock) and plants treated with 50 mM NaCl. Experiments were performed more than three times with different batches of seeds, with similar results. (B) and (C) Quantitative analysis of root hair length and proportion of visible hair cells out of total epidermal cells in response to NaCl treatment. Arabidopsis seeds were grown on half-strength MS medium supplemented with 50 mM NaCl for 7 d before examination. Values are means ± s.e.m ($n = 12$ roots); analyzed by one-way ANOVA. (D)–(E) Transcript levels of genes encoding root hair-related bHLH transcription factors and their targets under NaCl treatment. Arabidopsis seeds were grown on half-strength MS medium supplemented with 50 mM NaCl for 7 d before analysis. Total RNA was extracted from at least three batches of roots as biological replicates, and at least three technical replicates were analyzed for each sample. Values are means ± s.e.m; analyzed by one-way ANOVA. *ACTIN2* was used as the normalization control.

ABF1-Myc and *RHD6-YFP*, *ABF4-Myc* and *RHD6-YFP,* but not in *N. benthamiana* leaves coexpressing negative pairs of *ABF1-Myc* and *YFP, ABF4-Myc* and *YFP* (Fig. 3D). Interestingly, ARF1, ABF3, and ABF4 were immunoprecipitated by RSL1-YFP (Fig. 3E), even there were no positive results of ABF3 and ABF4 with RSL1 in the Y2H assays (Supplementary Fig. S3A). These findings provide further evidence for the association between ABFs with RHD6 and RSL1.

Together, these results demonstrate that ABF1, ABF3, and ABF4 physically interact with RHD6 and RSL1, implying that stress-responsive ABFs may be involved in modulating the salt-induced inhibition of root hair development.

3.3. *N-terminal region of RHD6 and C1 to C3 domains of ABF3 are critical for the interactions of RHD6 and RSL1 with ABF3*

To determine which region(s) of the RHD6 protein are required for the interaction with ABFs, we performed additional directed Y2H analyses. RHD6 was divided into the N-terminal region ($RHD6^{1–193}$) and the C-terminal portion including the bHLH DNA-binding domain ($RHD6^{194–298}$). The N-terminal fragments of RHD6 were confirmed to interact with ABFs in yeast, because removing the N-terminal part eliminated these interactions (Supplementary Fig. S3B). Further mapping revealed that the amino acids 1–193 of RHD6 ($RHD6^{1–193}$) are sufficient for the interaction (Supplementary Fig. S3B).

Similarly, we identified fragments of ABF3 that are necessary for physical associations with the RHD6 and RSL1 protein. ABF3 was divided into the N-terminal-containing C1 domain ($ABF3^{1–114}$), C2 and C3 domains ($ABF3^{115–201}$), middle fragment ($ABF3^{202–348}$), and the C-terminal fragment containing bZIP and C4 domains ($ABF3^{348–454}$). As shown in Supplementary Fig. S3C, deletion of the C1, C2, and C3 domains of ABF3 abolished the interaction with RHD6 and RSL1 in yeast. In contrast, deletion of the C-terminal residues of ABF3 did not affect the interactions. These results demonstrate that the N-terminal region containing the C1, C2, and C3 domains of ABF3 is essential for the interactions with RHD6 and RSL1.

3.4. *ABF proteins are required for salinity-induced inhibition of root hair development*

ABF proteins are members of the bZIP family and their expression is induced by high salinity, dehydration, osmotic stress, and ABA treatment (Kang et al., 2002; Sakuma et al., 2002; Fujita et al., 2005; Lee et al., 2010; Yoshida et al. 2010; Yoshida et al. 2015; Wang et al. 2019). Considering that ABFs respond to salt treatment and physically interact with the RHD6 transcription factor, we speculated that they might be required for the salt-induced inhibition of root hair development. To test this hypothesis, we first tested the transcriptional and protein levels of ABF3 responding to salt stress. As shown in Supplementary Fig. S4A, ABF3 protein accumulated after 24 h salt treatment. In line with this, the transcriptional level of *ABF3* induced by salt treatment (Supplementary Fig. S4B). Then, we investigated the responses of Col-0 and the *abf1 abf3 abf4* (*abf134*), *abf1 abf2 abf3* (*abf234*), and *abf1 abf2 abf3 abf4* (*abf1234*) mutants to NaCl treatment. Treatment with NaCl clearly inhibited root hair elongation and root hair density in Col-0 seedlings, whereas the NaCl-induced inhibition of root hair development was obviously disrupted in the *abf* mutants, especially the *abf1234* quadruple mutant (Fig. 4A–C).

To further support the positive role of ABF transcription factors in NaCl-induced inhibition of root hair development, we selected two stable *ABF3*-overexpressing (*ABF3-OE*) lines for analysis. As expected,

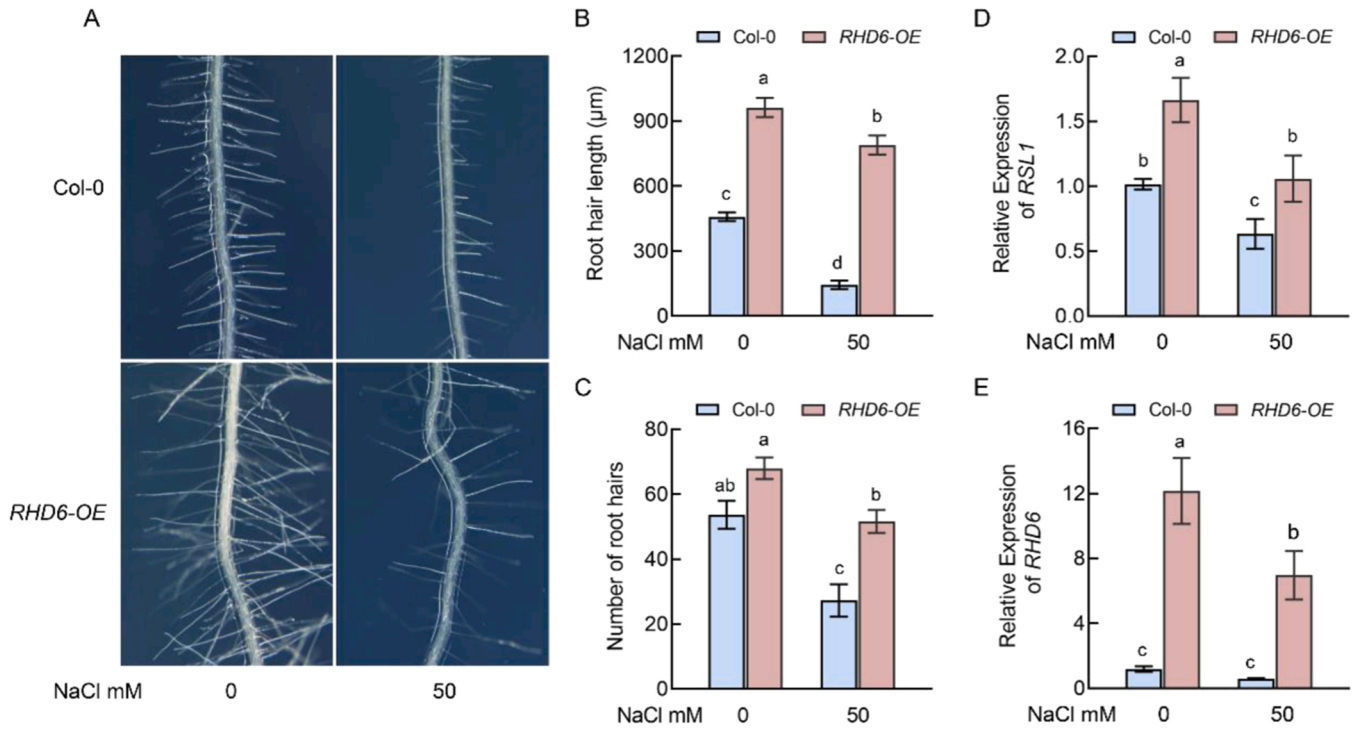

Fig. 2. RHD6 is involved in salinity-induced inhibition of root hair development. (A) Images of root hairs of untreated Col-0 and *RHD6*-overexpressing *(RHD-OE)* control plants (Mock) and plants treated with 50 mM NaCl. Experiments were performed more than three times with different batches of seeds, with similar results each time. (B) and (C) Quantitative analysis of root hair length and proportion of visible hair cells out of total epidermal cells in response to NaCl treatment. Arabidopsis seeds were grown on half-strength MS medium supplemented with 50 mM NaCl for 7 d before examination. Values are means ± s.e.m ($n = 12$ roots). Different letters indicate significant difference ($p < 0.05$); analyzed by two-way ANOVA. (D)–(E) Transcript levels of genes encoding rroot hair-related bHLH transcription factors and their targets under NaCl treatment. Arabidopsis seeds were grown on half-strength MS medium supplemented with 50 mM NaCl for 7 d before analysis. Total RNA was extracted from at least three batches of roots as biological replicates, and at least three technical replicates were analyzed for each sample. Values are means ± s.e.m ($n = 3$ biological replicates). Different letters indicate signfiicant difference ($p < 0.05$); analyzed by two-way ANOVA. *ACTIN2* was used as the normalization control.

compared with Col-0 seedlings, *ABF3-OE* seedlings formed fewer and shorter root hairs under NaCl treatment (Fig. 5A–C). Consistent with this phenotype, genes related to root hair development exhibited much lower transcript levels in *ABF3-OE* seedlings than in Col-0 under salt stress (Supplementary Fig. S5). Collectively, these findings indicate that ABF transcription factors are necessary for NaCl-induced inhibition of root hair growth.

3.5. ABF proteins exert genetic effects upstream of RHD6

Because our results showed that NaCl-induced inhibition of root hair development is dependent on ABFs and they physically interact with RHD6, we wondered whether RHD6 and its closest homolog RSL1 have a clear up- or down-stream regulatory relationship with ABF proteins. To test this possibility, we crossed *abf134* with *rhd6 rsl1* to construct the *abf134 rhd6 rsl1* mutant. Unfortunately, we only obtained the *abf3 abf4 rhd6 rsl1* quadruple mutant in this generation. Unlike the *abf* mutants, the *abf3 abf4 rhd6 rsl1* quadruple mutant showed a defective root-hair development phenotype similar to that of the *rhd6 rsl1* double mutant under normal growth conditions and under NaCl treatment (Fig. 6A–C). These results demonstrate that ABF proteins function upstream of RHD6 and RSL1 to positively modulate NaCl-induced inhibition of root hair development.

3.6. ABF3 suppresses the ability of RHD6 to activate RLS4 transcription

Having demonstrated that ABF transcription factors interact with RHD6 protein and are required for NaCl-induced inhibition of root hair growth, we selected ABF3 as a representative to further examine its regulatory effect on RHD6. We used a dual luciferase (LUC) reporter method to determine the regulatory effect of the ABF3 protein on the transcriptional-activation function RHD6 in wild-type Arabidopsis mesophyll protoplasts (Yoo et al. 2007). The effector constructs contained *GFP*, *ABF3*, or *RHD6* under the control of Pro_{35S} (Fig. 7A). As mentioned before, the *RSL4* gene is a direct target of RHD6, and it is crucial for root hair elongation and determination of the final root hair cell size in Arabidopsis (Yi et al. 2010). Thus, its promoter (Pro_{RSL4}) was fused to LUC to produce a reporter construct (Fig. 7A). Consistent with the results of a previous study, RHD6 obviously increased *LUC* expression driven by the *RSL4* promoter (Fig. 7B; (Han et al. 2020). In contrast, coexpression of RHD6 with ABF3 decreased *LUC* expression compared with the expression of RHD6 with GFP (Fig. 7B). To obtain further evidence that ABF3 suppresses the transcriptional activation function of RHD6, we coexpressed *RHD6* with *LUC* driven by the *RSL4* promoter in protoplasts of Col-0 and the *abf* quadruple mutant. As shown in Fig. 7C, loss-of-function of *abf* significantly compromised the effect of RHD6 to activate *RSL4* expression.

To further explore how ABF3 suppresses the ability of RHD6 to activate the transcription of *RSL4* during salt stress, we crossed *ABF3-OE* with *RHD6-OE* to obtain the double-overexpressing mutant *ABF3-OE RHD6-OE*. Phenotypic analysis showed that *ABF3-OE* largely compromised the overgrowing root hair phenotype of *RHD6-OE* plants under salt treatment (Fig. 7D). Under salt treatment, root hairs were shorter and less abundant in *ABF3-OE RHD6-OE* seedlings than in *RHD6-OE* seedlings (Fig. 7E–F). Together, these results indicate that ABF3 inhibits the ability of RHD6 to transcriptionally activate *RSL4* under salt stress conditions, and this inhibition relies on their physical interaction.

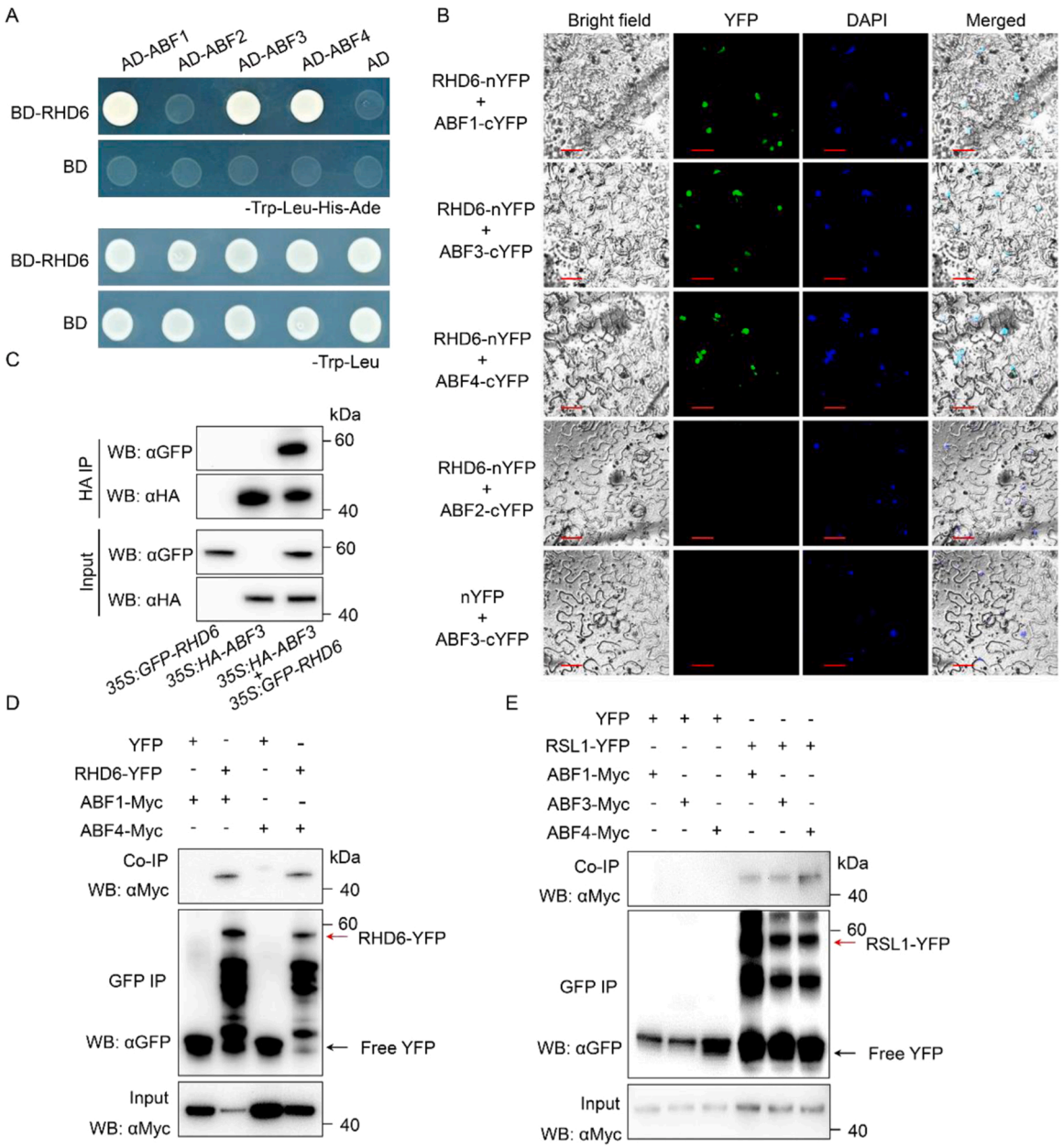

Fig. 3. RHD6 physically interacts with ABF proteins. (A) RHD6 interacts with ABF1, ABF3, ABF4 in yeast. Interactions of RHD6 with full-length ABFs are indicated by ability of yeast cells to grow on dropout medium lacking Leu, Trp, His, and Ade for 4 d after plating. pGBKT7 (BD) and pGADT7 (AD) were used as negative controls. (B) BiFC assays. Fluorescence was observed in the nuclear compartment of transformed *N. benthamiana* cells, resulting from the complementation of RHD6-nYFP with ABF1-cYFP, ABF3-cYFP or ABF4-cYFP. No signal was obtained for the negative controls in which RHD6-nYFP was coexpressed with ABF2-cYFP and nYFP was coexpressed with ABF3-cYFP. Nuclei are indicated by DAPI staining. Scale = 20 μm. (C) Co-immunoprecipitation analyses in Arabidopsis transgenic plants. GFP-RHD6 fusion proteins were immunoprecipitated using an GFP-trap beads, and the coimmunoprecipitated protein was detected using an anti-HA antibody. IP, immunoprecipitation. (D) ABF1 and ABF4 were co-immunoprecipitated by RHD6. *N. benthamiana* leaves expressing combinations of RHD6-YFP and ABF1-Myc, YFP and ABF1-Myc, RHD6-YFP and ABF4-Myc, and YFP and ABF4-Myc were incubated in darkness for 48 h. Total proteins were extracted,.
and then immunoprecipitated with GFP-trap beads. The coimmunoprecipitated proteins were detected by anti-Myc antibody. (E) ABF1, ABF3, and ABF4 were co-immunoprecipitated by RSL1. *N. benthamiana* leaves expressing combinations of RSL1-YFP and ABF1-Myc, RSL1-YFP and ABF3-Myc, RSL1-YFP and ABF4-Myc, YFP and ABF1-Myc, YFP and ABF3-Myc, YFP and ABF4-Myc were incubated in darkness for 48 h. Total proteins were extracted, and then immunoprecipitated with GFP-trap beads. The coimmunoprecipitated proteins were detected by anti-Myc antibody. Experiments were repeated three times with similar results.

4. Discussion

Several studies have shown that salt stress strongly affects root hair development (Wang et al. 2008; Song et al. 2022). However, the underlying molecular mechanisms by which root hair growth is precisely regulated by salinity are poorly understood. In this study, we confirmed that both the length and density of root hairs are significantly inhibited by salt treatment (Fig. 1A–1 C). Further analyses revealed that the decisive factor for root hair growth, RHD6, is involved in this regulation. *RHD6-OE* seedlings displayed a root hair-overaccumulation phenotype under normal growth conditions, and this phenotype was clearly attenuated under salt-stress conditions (Fig. 2). These findings indicate that salt stress inhibits RHD6-mediated root hair development.

Previous studies have shown that RHD6 together with its homologs, which are regulated by an array of upstream protein complexes, are the main factors determining root hair cell fate (Yi et al. 2010; Bruex et al.

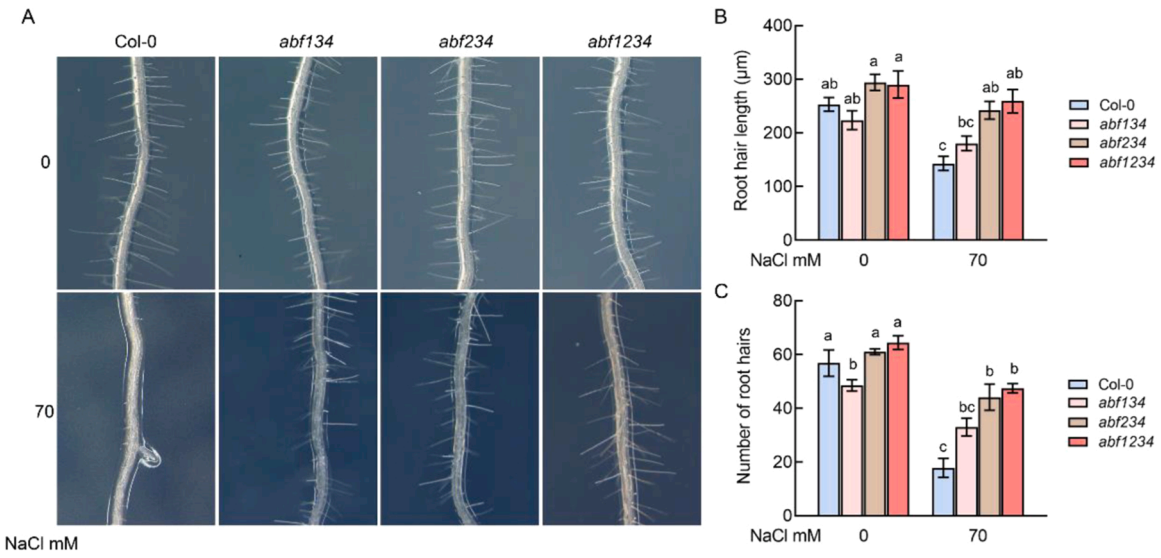

Fig. 4. Salinity-induced inhibition of root hair development and growth are impaired in loss-of-function *abf* mutants. (A) Images of root hairs of untreated Col-0, *abf134*, *abf234*, and *abf1234* control plants (Mock) and plants treated with 70 mM NaCl. Experiments were performed more than three times with different batches of seeds, all with similar results. (B) and (C) Quantitative analysis of root hair length and proportion of visible hair cells out of total epidermal cells in response to NaCl treatment. Arabidopsis seeds were grown on half-strength MS medium supplemented with 70 mM NaCl for 7 d before examination. Values are means ± s.e.m ($n = 12$ roots). Different letters indicate significant difference ($p < 0.05$); analyzed by two-way ANOVA.

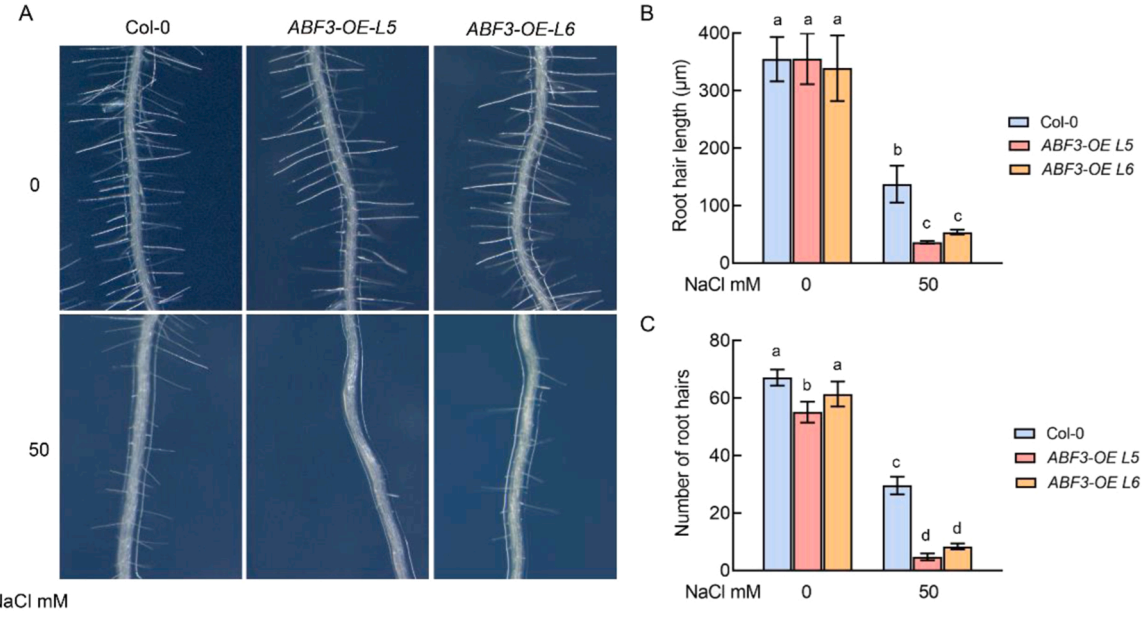

Fig. 5. Salinity-induced inhibition of root hair development and growth are enhanced in *ABF3-OE* plants. (A) Images of root hairs of untreated Col-0 and *ABF3-OE* control plants (Mock) and plants treated with 50 mM NaCl. Experiments were performed more than three times with different batches of seeds, all with similar results. (B) and (C) Quantitative analysis of root hair length and proportion of visible hair cells out of total epidermal cells in response to NaCl treatment. Arabidopsis seeds were grown on half-strength MS medium supplemented with 50 mM NaCl for 7 d before examination. Values are means ± s.e.m ($n = 12$ roots). Different letters indicate significant difference ($p < 0.05$); analyzed by two-way ANOVA.

2012; Grierson et al. 2014; Lin et al. 2015). As a core regulator, RHD6 interacts with multiple key components of other important signaling pathways and integrates different signals to mediate root hair growth. For example, the ethylene-activated transcription factor ETHYLENE-INSENSITIVE 3 (EIN3) associates with RHD6 to coordinately promote root hair elongation (Feng et al. 2017). Additionally, EIN3 physically interacts with TTG1, which competitively binds with the MYB–bHLH–WD40 (WER–GL3–TTG1) transcriptional complex that controls the transcription of *GL2* to determine root hair differentiation (Qiu et al. 2021). Jasmonate also promotes root hair development. Key repressors of the jasmonate signaling pathway, JAZ proteins, interact with RHD6 and suppress its transcriptional activity to modulate jasmonate-stimulated root hair growth (Han et al. 2020). Interestingly, in this study, we found that RHD6 physically interacts with several ABF proteins. Our analyses show that the N-terminal region of RHD6 and the C1 motif of ABF3 are required for their interaction (Fig. 3; Supplementary Fig. S1). These results suggest that ABF proteins are involved in the salt-induced inhibition of root hair development through forming a protein complex with RHD6.

ABFs, including ABF1, ABF2/AREB1, ABF3 and ABF4/AREB2, are

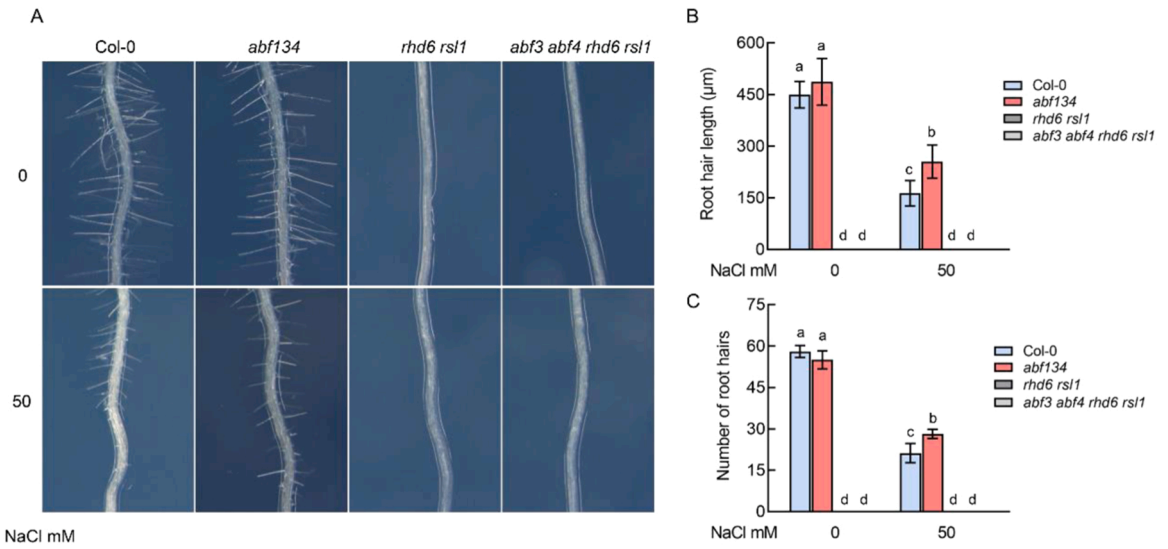

Fig. 6. ABFs function upstream of RHD6 and RSL1 during root hair development under salt stress conditions. (A) Images of root hairs of untreated Col-0, *abf134*, *rhd6 rsl1*, and *abf3 abf4 rhd6 rsl1* control plants (Mock) and plants treated with 50 mM NaCl. Experiments were performed more than three times with different batches of seeds, all with similar results. (B) and (C) Quantitative analysis of root hair length and proportion of visible hair cells out of total epidermal cells in response to NaCl treatment. Arabidopsis seeds were grown on half-strength MS medium supplemented with 50 mM NaCl for 7 d before examination. Values are means ± s.e.m ($n = 12$ roots). Different letters indicate significant difference ($p < 0.05$); analyzed by two-way ANOVA.

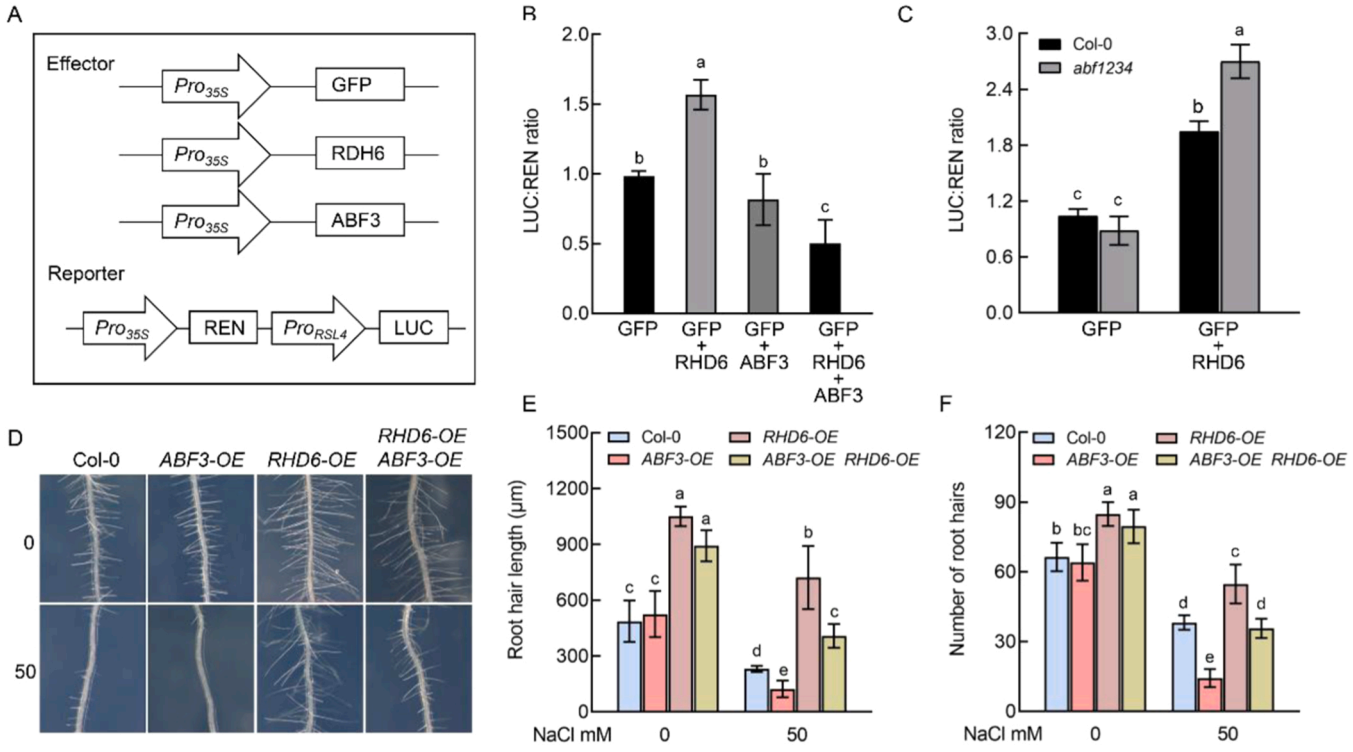

Fig. 7. ABF3 suppresses the ability of RHD6 to transcriptionally activate *RLS4*. (A) Schematic of effectors and reporters used in transient transactivation assays. *Pro35S*, CaMV 35S promoter; *ProRSL4*, *RSL4* promoter. (B) Transient dual-luciferase reporter assays. ABF3 interferes with ability of RHD6 to activate expression of *RSL4*. Error bars indicate s.e.m from three biological replicates using different batches of plants. (C) Transient transcriptional activity assays. Activation of *RSL4* promoter by RHD6 is compromised in the *abf1234* mutant plants. Error bars indicate s.e.m from five independent biological replicates using different batches of plants. Different letters indicate significant difference ($p < 0.05$); analyzed by ANOVA. (D) Images of root hairs of untreated Col-0 and RHD6 control plants (Mock) and plants treated with 50 mM NaCl. Experiments were performed more than three times with different batches of seeds, all with similar results. (E) and (F) Quantitative analysis of root hair length and proportion of visible hair cells out of total epidermal cells in response to NaCl treatment. Arabidopsis seeds were grown on half-strength MS medium supplemented with 50 mM NaCl for 7 d before examination. Values are means ± s.e.m ($n = 12$ roots). Different letters indicate significant difference ($p < 0.05$); analyzed by two-way ANOVA.

members of the bZIP transcription factor family and have largely overlapping functions downstream of SnRK2s (Yoshida et al. 2015; Yoshida et al. 2010; Qin et al. 2011). The quadruple mutant defective in all four *ABF* genes displays an ABA-insensitive phenotype (Yoshida et al. 2010; Yoshida et al. 2015), suggesting that ABF/AREBs are the key transcription factors involved in ABA signaling. The results of some studies imply that ABF transcription factors cooperatively regulate ABRE-dependent gene expression for ABA signaling in response to osmotic stresses, such as drought and high salinity (Yoshida et al. 2015; Yoshida et al. 2010). In the present study, we found that the inhibition of root hair development under high salinity relies on ABF transcription factors. Both the length and number of root hairs were decreased in *abf* mutants, especially the *abf1234* quadruple mutant (Fig. 4). However, the *ABF3-OE* plants also showed significantly impaired root hair development (Fig. 5). These finds demonstrate that ABF transcription factors play positive roles during root hair development under salt stress conditions. ABA can also inhibit root hair growth in Arabidopsis (Schnall and Quatrano, 1992; Rymen et al. 2017). Further research should determine whether ABF transcription factors, which are key regulators of ABA signaling, are required for ABA-induced repression of root hair growth, and whether they integrate endogenous ABA signals and exogenous salinity cues to coordinately modulate root hair development.

As typical transcription factors, ABFs accumulate in the promoter sequences of their downstream target genes to regulate their transcription. For instance, ABF proteins directly bind to the promoter sequences of group A *PP2C* genes and rapidly induce their expression to achieve feedback ABA signaling under exogenous ABA treatment (Wang et al. 2019). In this study, we found that ABF3 functions upstream of RHD6 during root hair development under high-salinity conditions, as the *rhd6 rsl4* double mutant almost totally restored the phenotype of *abf* mutants (Fig. 6). Further biochemical evidence showed that ABF3 suppresses the ability of RHD6 to activate the transcription of *RSL4* (Fig. 7). However, it is still unknown whether ABF3 binds to, and accumulates in, the promoter sequence of *RSL* to directly modulate root hair development.

5. Conclusion

Based on the results of this and other studies, we developed a simplified model to show how ABF proteins regulate the transcriptional activation function of RHD6 under salt stress conditions. In the presence of salt, ABF proteins are activated and form a protein complex with RHD6 to suppress its transcriptional activation activity, which reduces root hair development and enhances the ability of plants to withstand harsh environmental conditions. However, under suitable growing conditions, the inhibitory effect of ABF on RHD6 is disrupted, which facilitates normal root hair growth for better nutrient uptake from soil. Our results not only explain the regulatory relationship between ABFs and the RHD6 transcription factor during root hair growth under high-salinity conditions, but also show how plants integrate different cues to coordinate their adaptation to stress.

Accession numbers

The genes discussed in this article can be found in the Arabidopsis Genome Initiative database as follows: RHD6, AT1G66470; ABF1, AT1G49720; ABF2, AT1G45249; ABF3, AT4G34000; ABF4, AT3G19290; RSL1, AT5G37800; RSL3, AT2G14760; RSL4, AT1G27740; RSL5, AT5G43175; EXPA7, AT1G12560; LRX1, AT1G12040; RHS10, AT1G70460; ACTIN2, AT3G18780.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2023.111728.

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