Research article

The non-DNA binding bHLH transcription factor Paclobutrazol Resistances are involved in the regulation of ABA and salt responses in Arabidopsis

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A R T I C L E   I N F O

Keywords:
Paclobutrazol resistances
bHLH transcription factor
Abscisic acid
Abiotic stress
Salt tolerance

A B S T R A C T

Abscisic acid (ABA) is the key hormone that regulating plant responses to abiotic stresses. Several basic helix-loop-helix (bHLH) transcription factors have been reported to regulate ABA signaling in Arabidopsis. Paclobutrazol Resistances (PREs) are non-DNA binding bHLH transcription factors involved in the regulation of plant response to several different plant hormones including gibberellin, brassinosteroid and auxin. Here, we show that PREs are involved in the regulation of ABA and salt responses in Arabidopsis. Quantitative RT-PCR results showed that the expression levels of PRE6 as well as several other PRE genes were reduced in response to ABA treatment, but increased in salt treatment. Seed germination assays indicated that ABA sensitivity is reduced in the pre6 mutants, but increased in transgenic plants overexpressing PRE6. On the other hand, the 3SS:PRE6 transgenic plants showed enhanced tolerance to salt, whereas little, if any changes were observed in the pre6 mutants. Similar responses to ABA and salt treatments were observed in the pre2 mutants and the transgenic plants overexpressing PRE2, and a slight increased resistance to ABA in seed germination was observed in the pre2 pre6 double mutants. Taken together, our results suggest that at least some of the PRE genes are ABA responsive genes, and PREs may function redundantly to regulate ABA and salt responses in Arabidopsis.

1. Introduction

As one of the largest transcription factor families in Arabidopsis (Bailey et al., 2003; Zhao et al., 2012), the basic helix-loop-helix (bHLH) transcription factors regulate several different aspects of plant growth and development including seed germination (Oh et al., 2004; Park et al., 2011), cell fate determination (Nesi et al., 2000; Payne et al., 2000; Bernhardt et al., 2003; Pillitteri and Torii, 2007; Zhao et al., 2012) and flowering time (Ito et al., 2012), as well as plant response to environmental stimuli such as light signaling (Halliday et al., 1999; Soh et al., 2000; Huq and Quail, 2002; Leivar et al., 2012). The bHLH transcription factors in Arabidopsis also regulate hormone signaling including jasmonate signaling (Kazan and Manners, 2013), brassinosteroid signaling (Wang et al., 2009; Zhang et al., 2009), and abscisic acid (ABA) signaling (Tian et al., 2015).

Several bHLH transcription factors have been reported to regulate ABA signaling via different ways. For example, INDUCER OF CBF EXPRESSION 2 (ICE2) and bHLH122 regulate ABA biosynthesis and catabolism, respectively (Kurbidaeva et al., 2014; Liu et al., 2014), bHLH129 regulates the expression of some ABA signaling component genes (Tian et al., 2015), whereas ANDROGEN-INDUCIBLE GENE 1 (AtAIG1), AtMYC2 and AtMYB2 regulate the expression of some ABA-responsive genes (Abe et al., 2003; Kim and Kim, 2006). On the other hand, ABA affects the expression of some bHLH genes. For example, ABA-INDUCIBLE BHLH-TYPE TRANSCRIPTION FACTOR (AIB) and AtAIG1 are ABA up-regulated genes, whereas bHLH129 is an ABA down-regulated gene (Kim and Kim, 2006; Li et al., 2007; Tian et al., 2015).

Paclobutrazol Resistances (PREs) are atypical bHLH transcription factors that lack the DNA binding domain (Lee et al., 2006; Mara et al., 2010). Similar to the typical bHLH transcription factors, PREs are involved in the regulation of plant growth and development, plant response to environmental stimuli, as well as hormone signaling (Lee et al., 2006; Wang et al., 2009; Zhang et al., 2009; Mara et al., 2010; Bai et al., 2012; Castelain et al., 2012; Hao et al., 2012; Iked a et al., 2012, 2013; Zheng et al., 2017).
So far all the six PREs in Arabidopsis have been shown to regulate plant growth and development likely via regulating gibberellin signaling (Hyun and Lee, 2006; Lee et al., 2006; Mara et al., 2010; Bai et al., 2012; Oh et al., 2014; Zheng et al., 2017). All but PRE4 have been shown to regulate brassinosteroid and light signaling (Hyun and Lee, 2006; Wang et al., 2009; Zhang et al., 2009; Mara et al., 2010; Bai et al., 2012; Castelain et al., 2012; Oh et al., 2014). It should be noted that the pre4 mutants showed light signaling related phenotypes, indicating that PRE4 is also involved in the regulation of light signaling (Mara et al., 2010). In addition, PRE3 and PRE6 have been shown to be involved in the regulation of auxin signaling (Schlereth et al., 2010; Castelain et al., 2012; Oh et al., 2014; Zheng et al., 2017). However, it is unclear if PREs are involved in the regulation of ABA signaling.

In this study, we report the identification of some of the PRE genes as ABA responsive genes, and we found that PREs may function redundantly to regulate ABA and salt responses in Arabidopsis.

2. Materials and methods

2.1. Plant materials and growth conditions

The Columbia-0 (Col) wild type Arabidopsis was used for protoplast isolation, plant transformation and gene cloning. The T-DNA insertion lines for PRE2, SALK_135685 and CS122628 were obtained from the ABRC, and designated as pre2-1 and pre2-2 respectively. The pre6 mutants and the 35S::PRE6 transgenic plants have been described previously (Gommers et al., 2017; Zheng et al., 2017). The pre2 pre6 double mutants were generated by crossing pre2-1and pre6-1 single mutants. All the mutants and transgenic plants are in Col background.

To generate plants for plant transformation and protoplast isolation, seeds of Col wild type Arabidopsis were germinated and grown in pots filled with soil. To generate seedlings for RNA isolation, ABA and salt response analyses, seeds of the Col wild type, mutants and transgenic plants were surface sterilized and plated on solidified ½ MS (Murashige and Skoog) medium containing 1% w/v sucrose and 0.6% w/v phytagar.

All the plants were grown in a growth chamber at 22 °C, with a photodensity at ∼125 μmol m⁻² s⁻¹ and a photoperiod of 16 h light/8 h dark.

2.2. RNA isolation and quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from seedlings or different tissues and organs by using EasyPure Plant RNA Kit (TransGen Biotech) and following the manufacturer’s instructions. To examine the expression level of PRE genes in response to ABA and salt treatment, 14-day-old Col seedlings were treated with 50 μM ABA and 150 mM NaCl, respectively for 4 h. To examine the expression of genes involvement in ABA responses, 14-day-old seedlings of Col, pre2-1, pre2-2, pre6-1, pre6-2, pre2 pre6, 35S::PRE2-1, 35S::PRE2-2, 35S::PRE6-1 and 35S::PRE6-2 were treated with 50 μM ABA or mock treated for 4 h.

Two μg of total RNA was subjected complementary DNA (cDNA) synthesis by using EasyScript First-Strand DNA Synthesis Super Mix Kit (TransGene Biotech). Synthesized cDNA was used for qRT-PCR reactions, and data was normalized to the expression level of ACTIN2 (ACT2) (Zheng et al., 2016). The primers used for qRT-PCR examination of PRE1 to PRE6, ABF1, ABF2, ABF3, ABI1, ABI2, SnRK2.2, SnRK2.3, SnRK2.6, NACED3, RD29A, RD29B, DREB2A, Em1, Em6, ABF3, ABF4 and ABF5 have been described previously (Xu et al., 2013; Feng et al., 2014; Tian et al., 2015, 2017; Huang et al., 2016; Zheng et al., 2017).

2.3. Constructs

The effector constructs GD, GD-PRE6, LD-VP and the reporter construct LexA-Gal4::GUS for protoplast transfection have been described previously (Tiwari et al., 2003; Wang et al., 2005, 2007; Zheng et al., 2017). To generate constructs for protoplast transfection, the full-length open reading frame (ORF) of PRE1, PRE2, PRE3, PRE4, and PRE5 were amplified by RT-PCR using RNA isolated from 10-day-old Col seedlings as described previously (Tiwari et al., 2003; Wang et al., 2015; Zheng et al., 2017), cloned in frame with a N-terminal GD tag into pUC19 vector under the control of the double CaMV 35S promoter.

To generate 35S::PRE2 for plant transformation, the full-length ORF of PRE2 was amplified by RT-PCR, cloned in frame with an N-terminal HA tag into pUC19 vector under the control of the double CaMV 35S promoter, and then digested with PstI and SacI and subcloned into the binary vector pZZP211 (Hajdukiewicz et al., 1994).

2.4. Plasmid DNA isolation, protoplast isolation and transfection

Reporter and effectors plasmid DNA were isolated using the GoldHi Endo Free Plasmid Maxi Kit (CWBO). Protoplasts were isolated from 4-week-old Col wild type plants and transfected as reported previously (Tiwari et al., 2003; Wang et al., 2005, 2007; 2015; Zheng et al., 2016). For the transcriptional activity assay of PREs, plasmids of the effector genes GD, GD-PREs, LD-VP and reporter gene LexA-Gal4::GUS were co-transfected into protoplasts, and then incubated at room temperature in dark for 20–22 h. GUS activity were then measured using a SynergyTM HT microplate reader.

2.5. Plant transformation and transgenic plants selection

The Col wild type plants were transformed with the 35S::PRE2 construct in pZZP211 by using flower dipping via Agrobacterium tumefaciens strain GV3101 (Clough and Bent, 1998). Transgenic plants were selected as described previously (Zheng et al., 2017). A total of 16 independent transgenic plants with similar phenotypes were obtained and 2 different lines of transgenic plants were used for the experiments.

2.6. ABA and salt sensitivity assays

Seed germination assays were performed as described previously (Tian et al., 2017). Briefly, Arabidopsis seeds were surface sterilized and sown on ½ MS medium containing 1 μM ABA. The plates were kept at 4 °C for 2 days in darkness and then transferred to a growth room. The number of seeds germinated was counted every 12 h after transfer, and the germination rate was calculated.

For cotyledon greening assays, Arabidopsis seeds were surface sterilized and sown on ½ MS medium with or without 150 mM NaCl. The plates were kept at 4 °C for 2 days in darkness and then transferred to a growth room. Seedlings with green cotyledons were counted 10 days after transfer, and percentage of green seedlings was calculated.

3. Results

3.1. The expression of some PREs is regulated by ABA and salt

Some bHLH transcription factors are involved in the regulation of ABA signaling, and the expression of several bHLH genes is regulated by ABA (Tian et al., 2015). PREs have been shown to be involved in the regulation of signaling of several different plant hormone, including gibberellin, brassinosteroid and auxin (Zheng et al., 2017). To examine if PREs are also involved in the regulation of ABA signaling, we first examined the expression of PREs in response to ABA by using qRT-PCR. As shown in Fig. 1A, the expression level of PRE6 in Arabidopsis seedlings reduced ∼3 folds in response to ABA treatment. The expression levels of PRE1 and PRE2 reduced ∼1/3 when compared with that in the Col wild type seedlings. However, little if any decrease on the expression level was observed for PRE3 and PRE5. On the other hand, the expressive level of PRE4 increased ∼2 folds in response to
ABA treatment.

We also examined if the expression of PREs is altered in response to abiotic stresses, we found that the expression levels of all the PREs but PRE3 and PRE4 increased in response to salt treatment, with a more than 10 folds increase for PRE1 and PRE2, and ∼4 and 2 folds for PRE5 and PRE6, respectively (Fig. 1B).

3.2. PREs function as transcription repressors

Previously experiment results indicate that PREs functionally redundantly to regulate plant growth and development and plant response to hormone and light signaling (Lee et al., 2006; Hyun and Lee, 2006; Wang et al., 2009; Zhang et al., 2009; Mara et al., 2010; Schlereth et al., 2010; Bai et al., 2012; Castelain et al., 2012; Oh et al., 2014; Zheng et al., 2017). Our recent results showed that PRE6 function as a transcription repressor (Zheng et al., 2017), therefore we wanted to examine other PREs may also function as transcription repressors.

Arabidopsis mesophyll protoplast transfection system was used to examine the transcriptional activities of PREs. The transcription activator plasmids LD-VP, the effector plasmid GD-PREs and the reporter plasmid LexA-Gal4:GUS were co-transfected into protoplasts, and GUS activities were assayed after overnight incubation of the transfected protoplasts. As shown in Fig. 2, co-transfection of the control effector gene GD, the activator gene LD-VP, and the reporter gene LexA-Gal4:GUS led to activation of the reporter gene, whereas the activities were reduced when any of the GD-PREs was co-transfected into protoplasts, suggesting that all the PREs function as transcriptional repressors.

3.3. ABA sensitivity is decreased in the pre mutants, but increased in the transgenic plants overexpressing PRE genes

The above results that some of the PREs are ABA responsive genes and all the PREs function as transcription repressors indicate the PREs may function redundantly to regulate ABA signaling in Arabidopsis. PRE6 and PRE2 were used to examine if that is the case. PRE6 was chosen because PRE6 is the most down-regulated PRE gene (Fig. 1), and we have already had pre6 mutants and transgenic plants overexpressing PRE6 (Zheng et al., 2017). PRE2 was chosen because it also showed decreased express level in response to ABA treatment, and there are T-DNA insertion lines available from Arabidopsis Biological Resource Center (ABRC, https://abrc.osu.edu).

To examine the functions of PRE2 and PRE6 in ABA signaling, we first examined the expression patterns of PRE2 and PRE6 in Arabidopsis by using qRT-PCR. We found that PRE2 and PRE6 have shared similar expression patterns in most of the tissues and organs examined, i.e., both PRE2 and PRE6 have relative lower expression levels in roots, rosette leaves, cauline leaves and stems, but were highly expressed in flowers. However, PRE6 was also highly expressed in shoots and siliques (Fig. 3).

We then further examined if PREs are involved in regulating ABA

![Fig. 1. Expression of PREs in response to ABA and salt treatments. Fourteen-day-old Col wild type seedlings were mock-treated or treated with 50 μM ABA (A) or 150 mM NaCl (B) for 4 h, total RNA was then isolated and used for qRT-PCR analysis. ACT2 was used as a reference gene. The expression level of the corresponding PRE gene in mock-treated seedlings was set as 1. Data represent the mean ± SD of three replicates.](image)

![Fig. 2. Transcriptional activities of PREs. Protoplast transfection assay was used to examine the ability of PREs to repress transcription of the LexA-Gal4:GUS reporter gene activated by the transcription activator LD-VP16. Plasmids of the effector genes GD, GD-PREs, LD-VP and reporter gene LexA-Gal4:GUS were cotransfected into protoplasts isolated from rosette leaves of 3–4 weeks old Arabidopsis plants. The transfected protoplasts were then incubated at room temperature in dark for 20–22 h before GUS activities were measured. Data represent the mean ± SD of three replicates.](image)

![Fig. 3. Expression pattern of PRE2 and PRE6. RNA was isolated from different tissues of the Col plants and used for qRT-PCR analysis. Shoots and roots were harvested from 2-week-old plants. Stems, rosette leaves, and cauline leaves were harvested from 6-week-old plants. Flowers and siliques were harvested from 8-week-old plants. ACT2 was used as a reference gene. The expression level of PRE2 in shoots was set as 1. Data represent the mean ± SD of three replicates.](image)
3.4. Expression level of ABI3 is increased in the 35S:PRE2 and 35S:PRE6 transgenic plants

To further examine how PREs may regulate ABA response in Arabidopsis, we examined the expression of some ABA signaling component genes and ABA responsive genes in pre mutant and transgenic plants overexpressing PRE genes by using qRT-PCR.

We found that the basal expression level of ABI3 in the 35S:PRE2 and 35S:PRE6 transgenic plant seedlings were increased to ~2 folds when compared with that in the Col wild type seedlings (Fig. 5A). However, ABA response of ABI3 remained largely unchanged when compared with that in the Col wild type, i.e., an ~2 folds increased in response to ABA was observed in both Col wild type and the 35S:PRE2 and 35S:PRE6 transgenic plant seedlings (Fig. 5A). On the other hand, although the basal expression level of ABI3 remained largely unchanged in the pre2, pre6 and pre2 pre6 mutant seedlings, its response to ABA was reduced (Fig. 5A). We also found that no changes of the basal expression level was observed for gene DREB2A in either the pre mutants or the transgenic plant overexpressing PRE genes, but its ABA response in the transgenic plants was increased (Fig. 5B).

3.5. Salt tolerance is increased in the 35S:PRE2 and 35S:PRE6 transgenic plants

As ABA is key hormone that regulates plant response to abiotic stresses, after showing that PREs are involved in the regulation of ABA response in Arabidopsis, we further examined if PREs may regulate plant response to abiotic stresses. Because bHLHs have been shown to regulate salt tolerance in Arabidopsis, we examined salt response of the pre mutants and the transgenic plant overexpressing PRE genes. We found that salt tolerance is enhanced in both the 35S:PRE2 and 35S:PRE6 transgenic plants (Fig. 6A). Quantitative analysis results showed that the percentages of green seedlings of both the 35S:PRE2 and 35S:PRE6 transgenic plants (Fig. 6A). Quantitative analysis results showed that the percentages of green seedlings of both the 35S:PRE2 and 35S:PRE6 transgenic plants were ~3 folds of that of the Col wild type plants (Fig. 6B). However, the pre2, pre6 and pre2 pre6 mutants showed a near wild type response to salt treatments (Fig. 6B).

4. Discussion

Accumulated evidences suggest that the atypical bHLH transcription factors PREs are involved in the regulation of plant hormone signaling including gibberellin signaling (Hyun and Lee, 2006; Bai et al., 2012; Oh et al., 2014), brassinosteroid signaling (Hyun and Lee, 2006; Wang et al., 2009; Zhang et al., 2009; Mara et al., 2010; Bai et al., 2012; Castelain et al., 2012; Oh et al., 2014), and auxin signaling (Schlereth et al., 2010; Castelain et al., 2012; Oh et al., 2014; Zheng et al., 2017). Two lines of experimental evidence generated in the research indicate that PREs are also involved in the regulation of ABA signaling. First, some of the PREs are ABA responsive genes. Second, loss-of-function and overexpression of PRE2 and/or PRE6 affected plant sensitivities to ABA.

Previous experiments have shown that the expression of PREs is regulated by several different hormones. For example, the expression of PRE1 is regulated by gibberellin, auxin, and brassinosteroid (Lee et al., 2006; Zhang et al., 2009), the expression of PRE1, PRE2, PRE5 and PRE6 are induced by auxin (Zhang et al., 2009; Oh et al., 2014; Zheng et al., 2017). By examining the expression of PREs in response to ABA treatment, we found that the expression levels of several PREs, including PRE1, PRE2 and PRE6 were dramatically reduced in ABA response by using seed germination assays. As shown in Fig. 4, the germination rate of the 35S:PRE2 and 35S:PRE6 transgenic plant seeds was reduced, whereas that of the pre2 and pre6 mutant seeds was increased at all the time points examined. These results suggest that PREs positively regulate ABA response in Arabidopsis. A slightly increased germination rate was observed in the pre2 pre6 double mutant seeds when compared with the pre2 and pre6 single mutant seeds (Fig. 4), indicating that PRE2 and PRE6 function redundantly in regulating ABA response in Arabidopsis.
dependent and independent pathways to regulate salt tolerance in transgenic plants overexpressing Arabidopsis (Fig. 7).

PRE1, PRE2 and PRE5 were also observed in ABA treated seedlings (Fig. 1A). Seed germination assays indicate that ABA sensitivities increased in transgenic plants overexpressing PRE2 or PRE6, but decreased in pre2 and pre6 mutants (Fig. 4). Slightly increased tolerance to ABA was observed in pre2 pre6 double mutants when compared with that in the pre2 or pre6 single mutants (Fig. 4), indicating that PREs may have function redundancy in regulating ABA response in Arabidopsis. Consistent with this conclusion, all the PREs inhibited reporter gene expression in protoplast transfection assays (Fig. 2).

ABA plays an important role in regulating plant response to abiotic stresses such as cold, drought and salinity (Shang et al., 2010; Umezawa et al., 2010; Rushton et al., 2012; Xu et al., 2013; Tian et al., 2017). In addition to ABA, we found that salt also regulate the expression of PRE genes. However, different from ABA, salt treatment induced the expression of PRE1, PRE2, PRE5 and PRE6 (Fig. 1B). Similar, opposite results in green seedlings assays were also observed for salt when compared with that of ABA, i.e., increased salt tolerance was observed in transgenic plants overexpressing PRE2 or PRE6, but decreased in pre2 and pre6 mutants (Fig. 6). Considering that the expression levels and/or ABA response of both ABA signaling gene ABI3 and ABA independent abiotic stress resistance regulator gene DREB2A were changed in transgenic plants overexpressing PRE2 or PRE6 (Fig. 5). These results indicate that it is very likely that PREs may function through both ABA dependent and independent pathways to regulate salt tolerance in Arabidopsis (Fig. 7).

It should be noted that, in contrary to other PREs, the expression level of PRE4 was increased in response to ABA treatment (Fig. 1A), and PRE2 and PRE6 showed different expression patterns (Fig. 3). Considering that all the PREs function as transcription repressors (Fig. 2), and previous experiments have been shown that PREs function redundantly to regulate plant growth and development, as well as plants response to hormone and light signaling (Hyun and Lee, 2006; Lee et al., 2006; Mara et al., 2010; Bai et al., 2012; Oh et al., 2014), it is very likely that PREs function in a high redundant manner to regulate plant growth and development, as well as plant response to environmental stimuli. Different expression pattern and/or different hormone responses of different PRE genes may affect the expression levels of in different tissue and organs, as well as in response to different stimuli, therefore serve as a fine turn to regulate the functions of PREs.

As mentioned above, our experiment results indicate that PREs are able to activate the expression ABA signaling gene ABI3, and enhance ABA response of the ABA independent abiotic stress resistance regulator gene DREB2A (Fig. 5), therefore play a positive role in regulating ABA singling and salt tolerance in Arabidopsis (Fig. 7). On the other hand, we found the expression levels of some the PREs, in general were increased in response to salt treatment, but decreased in response to ABA treatment (Fig. 1). Several feedback regulatory loops in ABA signaling have been identified (Tian et al., 2017), inhibition of PREs expression by ABA may serves as another negative feedback regulatory loop in ABA signaling. Considering that PREs are involved in the regulation of plant growth and development (Hyun and Lee, 2006; Lee et al., 2006; Mara et al., 2010; Bai et al., 2012; Oh et al., 2014; Zheng et al., 2017), ABA-PREs regulatory network may play an important role in balance...
Fig. 7. A model showing the roles of PREs in ABA signaling and salt tolerance. Salinity induces the activation of ABI3 and other ABA-activated transcription factors via ABA-dependent pathway, and the activation of DREB2A and related transcription factors via ABA-independent pathway. The activated transcription factors then regulate the expression of stress-responsive genes, therefore affect plant responses to abiotic stresses. Salinity induces the expression of some PRE genes, PREs, in turn regulate ABA singling and salt tolerance via activating ABI3 and enhancing ABA response of DREB2A. Inhibition of the expression of some PRE genes by ABA may serve as a negative feedback loop in this regulatory network.

plant growth and development and plant response to abiotic stresses.

Nevertheless, our results suggest that some of the PREs are ABA responsive genes, and PREs are involved in the regulation of plant response to ABA and salt tolerance.

Author contribution statement

SW conceived the study. SW and KZ designed the experiments. KZ andYW performed the experiments. KZ, YW and SW analyzed the data. SW conceived the study. SW and KZ designed the experiments. KZ, YW and SW analyzed the data. SW and KZ drafted the manuscript. All the authors participated in the revision of the manuscript.

Acknowledgement

This work was supported by the National Key R&D Program of China (2016YFD0101902). The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript, and the authors declared no conflict of interest.

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