Appetite PED2 positively modulates plant drought stress resistance

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Abstract  Abscisic acid (ABA) is an important phytohormone that functions in seed germination, plant development, and multiple stress responses. Arabidopsis Peroxisome defective 2 (AtPED2) (also known as AtPeroxin14, AtPEX14), is involved in the intracellular transport of thiolase from the cytosol to glyoxysomes, and peroxisomal matrix protein import in plants. In this study, we assigned a new role for AtPED2 in drought stress resistance. The transcript level of AtPED2 was downregulated by ABA and abiotic stress treatments. AtPED2 knockout mutants were insensitive to ABA-mediated seed germination, primary root elongation, and stomatal response, while AtPED2 over-expressing plants were sensitive to ABA in comparison to wide type (WT). AtPED2 also positively regulated drought stress resistance, as evidenced by the changes of water loss rate, electrolyte leakage, and survival rate. Notably, AtPED2 positively modulated expression of several stress-responsive genes (RAB18, RD22, RD29A, and RD29B), positively affected underlying antioxidant enzyme activities and negatively regulated reactive oxygen species (ROS) level under drought stress conditions. Moreover, multiple carbon metabolites including amino acids, organic acids, sugars, sugar alcohols, and aromatic amines were also positively regulated by AtPED2. Taken together, these results indicated a positive role for AtPED2 in drought resistance, through modulation of stress-responsive genes expression, ROS metabolism, and metabolic homeostasis, at least partially.

Keywords: Abscisic acid; Arabidopsis; drought stress; metabolite; oxygen species; PED2; reactive


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INTRODUCTION

Environmental stresses largely affect plant growth and development, and severely limit crop production worldwide (Seki et al. 2007; Qin et al. 2011). Among them, drought stress is one of the most common environmental stresses (Seki et al. 2007; Qin et al. 2011). Although plants have developed complex biochemical and physiological strategies to respond to sudden environmental changes, the plant hormone abscisic acid (ABA) is the most essential in plant drought stress responses (Seki et al. 2007; Fujii et al. 2009; Cutler et al. 2010; Harb et al. 2010; Hirayama and Shinozaki 2010; Qin et al. 2011). When drought stress is applied, both ABA signaling and endogenous ABA syntheses are quickly and largely activated (Cutler et al. 2010; Harb et al. 2010). In the absence of ABA, type 2C protein phosphatases (PP2Cs) (ABI1, ABI2, HAB1, HAB2, AHG1, and PP2CA/AHG3) interact with SNF1-related protein kinases 2 (SnRK2s) to inhibit their autophosphorylation and downstream activation, while in the presence of ABA, ABA receptors (RCAR/PYR/PYLs) disrupt the interaction between the PP2Cs and SnRK2s to prevent the PP2Cs-mediated dephosphorylation of the SnRK2s and result in the activation of the SnRK2s that promote transcription of ABA-responsive genes (Fujii et al. 2009; Cutler et al. 2010; Harb et al. 2010).

Arabidopsis Peroxisome defective 2 (AtPED2), also known as AtPEROXIN14 (AtPEX14/At5g62810), encodes a peroxisomal membrane protein that assists in importing proteins into the peroxisome matrix (Monroe-Augustus et al. 2011; Burkhart et al. 2013). Mutations in peroxisome biogenesis proteins (peroxins) result in serious developmental deficiencies in various eukaryotes (Monroe-Augustus et al. 2011; Burkhart et al. 2013). PEX14 and PEX13 are important peroxins involved in docking cargo-receptor complexes in the peroxisomal membrane, thus facilitating the transport of the cargo into the peroxisomal matrix (Monroe-Augustus et al. 2011; Burkhart et al. 2013). The ped2 mutant was first isolated as a defect in the intracellular transport of thiolase from the cytosol to glyoxysomes (Hayashi et al. 1998, 2000; Lopez-Huertas et al. 1999). Additionally, the ped2 mutant was insensitive to (2,4-dichlorophenoxy)acetic acid (2,4-D), and indole-3-butyric acid (IBA), and could not grow without sucrose (Hayashi et al. 1998, 2000). Further study showed that ped2 mutants were viable, fertile, and displayed residual peroxisome matrix protein import (Monroe-Augustus et al. 2011; Burkhart et al. 2013). However, the involvement of AtPED2 in abiotic stress response remains unclear, and the underlying physiological and molecular mechanisms are also unknown.

In this study, the expression pattern and in vivo role of AtPED2 were characterized under abiotic stress conditions. Using AtPED2 overexpressing plants and knockout mutants, we assigned a new role for AtPED2 in ABA signaling and drought stress resistance. Additionally, the expression levels...
of ABA-responsive genes, reactive oxygen species (ROS) metabolism, and metabolic homeostasis modulated by AtPED2 expression under control and drought stress conditions were also investigated to gain insight into the possible mechanisms of AtPED2-mediated stress responses. All of these results indicated that AtPED2 was a positive regulator of ABA-mediated drought stress responses.

RESULTS

The expression pattern of AtPED2 in response to stress treatments

Through quantitative real-time polymerase chain reaction (PCR) analysis, we found that the expression levels of AtPED2 were significantly downregulated after ABA, cold, dehydration, and salt stress treatments for 6 h, 12 h, and 24 h (Figure 1). However, hydrogen peroxide (H2O2) stress for 3 h, 6 h, 12 h, and 24 h had no significant effect on the transcript level of AtPED2 (Figure 1). These results suggested the possible correlation between AtPED2 and stress treatments, and indicated the possible involvement of AtPED2 in abiotic stress responses in Arabidopsis.

Isolation of AtPED2 overexpression plants and knockout mutants

To further investigate the in vivo role of AtPED2, we isolated the T-DNA mutants of AtPED2 [ped2-1 (SALK_007441) and ped2-2 (SALK_072373)] (Figure 2A–D), and constructed AtPED2 overexpressing transgenic plants (Figure 2E, F). As shown in Figure 2B, C, the expression level of AtPED2 was largely inhibited in ped2-1 and ped2-2 mutants, with about 10% of AtPED2 transcripts in comparison to WT plants. When grown in soil, ped2-1 and ped2-2 mutants were smaller than WT plants, were fertile and produced viable seeds as Monroe-Augustus et al. (2011) described. Moreover, the AtPED2 overexpressing transgenic plants exhibited significantly higher AtPED2 transcripts than WT plants, especially line 2 and line 3 (with about 14-folds of AtPED2 transcripts than WT (Figure 2E). Therefore, the homozygous transgenic plants (line 2 and line 3) were chosen for further analysis. However, soil-grown AtPED2 overexpressing transgenic plants displayed no significant

Figure 1. The expression levels of AtPED2 after different stress treatments by quantitative real-time polymerase chain reaction (PCR)

For real-time PCR assay, 14-day-old Arabidopsis plant seedlings were transferred to fresh MS liquid medium containing 50 μM abscisic acid (ABA), or 200 mM NaCl, or 20 mM H2O2, or subjected to cold stress (4 °C), dehydration (un-covering the plate in the growth chamber), and plant samples were collected at 0, 3, 6, 12, and 24 h after treatments. The expression level of AtPED2 at 0 h of treatment was normalized as 1.0. The results shown are the means ± SEs (n < 3), and asterisks (*) indicate the significant difference of P < 0.05 in comparison to 0 h of treatment.
difference in plant growth in comparison to WT plants (Figure 2F).

Modulation of AtPED2 expression positively affects plant sensitivity to ABA
Since the expression of AtPED2 is downregulated by ABA treatment, responses of WT, ped2 mutants, and AtPED2 overexpressing plants to ABA were further compared on Murashige and Skoog (MS) plate. Germination of AtPED2 overexpressing plant seeds was severely affected by exogenous ABA treatment, as evidenced by less emerged radical (Figure 3A, B), less green cotyledon (Figure 3A, C), and shorter primary root (Figure 3D, E) in comparison to those of WT. On the contrary, ped2-1 and ped2-2 mutants exhibited more green cotyledons (Figure 3A, C) and longer primary root (Figure 3D, E) on ABA-supplied MS plate than those of WT. Consistently, the stomatal responses of AtPED2 knockout mutants were less sensitive to ABA than those of WT, while those of AtPED2 overexpressing plants were more sensitive to ABA (Figure 3F). These results indicated that modulation of AtPED2 expression positively affects ABA sensitivity in seed germination stage, post-germination growth stage, and stomatal response, suggesting that AtPED2 might be a positive regulator of ABA responsive pathway.

AtPED2 modulates the expression of ABA-responsive genes in Arabidopsis
To investigate the link between AtPED2-mediated ABA sensitivity and AtPED2-mediated drought stress response, quantitative real-time PCR was performed to analyze the expression of several ABA-responsive genes. Under control conditions, the ped2-1 mutant displayed significantly lower RAB18 and RD22 transcripts, while AtPED2 overexpressing plants showed significantly higher RAB18 transcript than WT (Figure 4A, B). When drought stress was applied, the ped2-1 mutant displayed significantly lower RAB18, RD29A and RD29B transcripts, while AtPED2 overexpressing plants showed significantly higher RD22, RD29A, and RD29B transcripts in comparison to those of WT (Figure 4A-D). Moreover, we assayed the effect of AtPED2 expression on endogenous ABA concentration under control and drought stress conditions, but no significant difference was examined among WT, AtPED2 overexpressing lines and knockout mutants (Figure 5). These results further indicated that AtPED2 might function as a positive regulator of ABA-responsive genes’ expression, without modulating the endogenous ABA concentration.

AtPED2 modulates drought stress resistance in Arabidopsis
To investigate the potential effect of AtPED2 expression on drought stress resistance, 14-day-old WT, ped2 mutants, and AtPED2 overexpressing plants in pots were subjected to drought stress treatment by withholding water for 21 d and then re-watering for 4 d. After drought stress treatment, ped2-1 and ped2-2 mutants exhibited significantly higher electrolyte leakage (EL), lower survival rate, while AtPED2 overexpressing plants displayed significantly lower EL and higher survival rate than those of WT (Figure 6A-C). Consistently, the AtPED2 overexpressing plants showed significantly lower water loss rate, while the ped2-1 and ped2-2 mutants exhibited significantly higher water loss rate from 2 h to 8 h after detachment than WT plants (Figure 6D). These results indicated AtPED2 positively regulated drought stress resistance.

Effects of AtPED2 expression on ROS accumulation and antioxidant enzyme activities under drought stress condition
Under control conditions, the ped2-1 and ped2-2 mutants displayed significantly higher levels of H$_2$O$_2$ and superoxide radical (O$_2^-$.•) than WT, while AtPED2 overexpressing plants showed no significant difference in comparison to WT (Figure 7A, B). When subjected to drought stress conditions, the ped2-1 and ped2-2 mutants showed higher levels of H$_2$O$_2$ and O$_2^-$.•, while AtPED2 overexpressing plants displayed lower levels of H$_2$O$_2$ and O$_2^-$.• than those of WT plants (Figure 7A, B). Consistently, AtPED2 overexpressing plants exhibited higher activities of superoxide dismutase (SOD) and catalase (CAT) under drought stress conditions, whereas the ped2-1 and ped2-2 mutants displayed lower activities of these enzymes than WT plants (Figure 7C, D).

Modulation of metabolic homeostasis by AtPED2 in Arabidopsis
To gain more insights into AtPED2-mediated drought stress response, gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) reproductively identified 54 metabolites including 16 amino acids, 13 organic acids, 18 sugars, five sugar alcohols, and two aromatic amines in WT, ped2 mutants, and AtPED2 overexpressing plants (Figure 8 and Table S1). Among these metabolites, about half of these metabolites exhibited lower concentrations in ped2-1 mutant, and almost all of these metabolites except threonine, ascorbic acid, galactose, gentiobiose in AtPED2 overexpressing plants exhibited higher concentrations than WT plants (Figure 8 and Table S1). These results indicated that modulation of AtPED2 affected homeostasis of downstream primary carbon metabolites including amino acids, organic acids, sugars, sugar alcohols, and aromatic amines.

DISCUSSION
Drought stress is one of the most serious environmental stresses that adversely impact agriculture production...
Figure 3. Modulation of AtPED2 expression positively affects plant sensitivity to abscisic acid (ABA)
(A) The photograph showing Arabidopsis plant seeds after 10 d of ABA treatment. (B, C) Germination rate of plant seeds in the presence of different concentrations of ABA. Germination rates were determined as developmental of emerged radicals (B) and green cotyledons (C). (D–E) Root elongation of 5-d-old plant seedlings in the presence of different concentrations of ABA for 7 d. (F) The stomatal responses of WT, AtPED2 knockout mutants, and AtPED2 overexpressing plants to ABA. The results shown are the means ± standard errors (SEs) (n < 3), and asterisks (*) indicate the significant difference of P < 0.05 in comparison to WT.
Involvement of AtPED2 in drought stress resistance

To date, there are three efficient approaches for improvement of plant drought stress resistance: one approach is screening drought-tolerant varieties from various plant varieties; the other approach is exogenous application of several chemicals such as ABA; and the third approach is genetic engineering via modulating several core genes’ expression especially the ABA signaling genes (Yu et al. 2008; Du et al. 2010; Li et al. 2011; Qin et al. 2011; Yue et al. 2012; Cao et al. 2014; Liu et al. 2014; Xie et al. 2014). In this study, the new roles for AtPED2 in ABA signaling and drought stress resistance were assigned based on physiological, molecular, and phenotypic characterizations.

The expression pattern of AtPED2 after ABA treatment indicated the possible role of AtPED2 in ABA signaling (Figure 1). Furthermore, ABA-repressed seed germination, primary root elongation, and ABA-induced stomatal closure were chosen as selection criteria to investigate the involvement of AtPED2 after ABA treatment which indicated the possible role of AtPED2 in ABA signaling. After obtaining AtPED2 knockout mutants and AtPED2 overexpressing plants (Figure 2), we found that AtPED2 positively modulated plant sensitivity to ABA (Figure 3). These results indicated that AtPED2 might function in ABA-dependent pathway. In accordance with the insensitive phenotype to ABA treatment (Figure 3), the ped2-1 and ped2-2 mutations exhibited decreased drought stress resistance, as also evidenced by the changes of water loss rate, EL, and survival rate (Figure 6). On the contrary, AtPED2 overexpressing plants were sensitive to ABA and displayed improved drought stress resistance (Figures 3, 6). These data demonstrated that modulation of ABA sensitivity by AtPED2 expression might be connected with drought stress resistance, and AtPED2-mediated stomatal closure after ABA treatment might be responsible for the leaf water loss status.

**Figure 4.** Effect of AtPED2 expression on abscisic acid (ABA)-responsive gene expression in Arabidopsis under control and drought stress conditions (A–D) The expression levels of RAB18 (A), RD22 (B), RD29A (C), and RD29B (D) in Arabidopsis plants under control and drought stress conditions for 14 d. The expression level of each gene of WT under control condition for 14 d was normalized as 1.0. The results shown are the means ± standard errors (SEs) (n < 3), and asterisks (*) indicate the significant difference of P < 0.05 in comparison to WT.

**Figure 5.** The effect of AtPED2 expression on endogenous abscisic acid (ABA) concentration in Arabidopsis under control and drought stress conditions

For the assay, 14-d-old Arabidopsis plants of different lines in pots in the soil were subjected to control condition (well-watering) and drought condition (withholding water) for 14 d, and plant leaf samples were harvest after 14 d of treatments. The results shown are the means ± standard errors (SEs) (n < 3).
In response to drought stress, plants have evolved various adaptive and protective alterations, especially ABA signaling perception, which led to the activation of several downstream gene expressions and physiological changes (Seki et al. 2007; Fujii et al. 2009; Cutler et al. 2010; Harb et al. 2010; Hirayama and Shinozaki 2010; Qin et al. 2011). Thus, drought stress responses in plants are initiated through both ABA-dependent and ABA-independent signal transduction pathways (Cutler et al. 2010; Harb et al. 2010; Qin et al. 2011).

RAB18, RD22, RD29A, and RD29B are widely known ABA-dependent stress-responsive genes, and overexpression of some stress-responsive genes such as RAB18 and RD29A conferred drought stress resistance in transgenic plants (Lang and Palva, 1992; Yamaguchi-Shinozaki and Shinozaki 1994a, 1994b, 2006; Liu et al. 1998; Nakashima et al. 2000). In this study, the higher ABA-responsive transcripts (RAB18, RD22, RD29A, and RD29B) in Arabidopsis overexpressing plants under control and drought stress conditions might be directly linked to improved drought stress resistance, while lower ABA-responsive transcripts in the ped2-1 and ped2-2 mutants were consistent with decreased drought stress resistance in comparison to WT (Figures 4, 6). Moreover, less sensitive stomatal responses of AtPED2 knockout mutants ABA and more sensitive stomatal responses of AtPED2 overexpressing plants to ABA might be directly connected to AtPED2-mediated water loss rate and drought resistance (Figures 3, 6). These results suggested that AtPED2 might positively regulate drought stress resistance in an ABA-dependent pathway.

Under almost all stress conditions, ROS accumulation (including H$_2$O$_2$ and O$_2$•-) and related oxidative damage could be largely activated, in accordance with unstabilization of plasma membrane and serious cell damage even to cell death (Apel and Hirt 2004; Miller et al. 2010; Mittler et al. 2011). In this study, modulation of AtPED2 expression negatively modulated H$_2$O$_2$ and O$_2$•- contents, but positively regulated SOD and CAT activities in response to drought stress (Figure 7). SOD is directly responsible for catalyzing O$_2$•- into H$_2$O$_2$, and CAT are essential for converting H$_2$O$_2$ into H$_2$O and O$_2$ (Apel and Hirt 2004; Miller et al. 2010; Mittler et al. 2011). Thus, the positive modulation of AtPED2 expression in the ROS detoxification system might be directly connected with drought stress-triggered ROS accumulation, as well as AtPED2-mediated drought stress resistance. Moreover, ROS including H$_2$O$_2$ and O$_2$•- are important second messengers in ABA signal transduction (Miao et al. 2006; Cutler et al. 2010). ABA upregulates endogenous ROS concentration, and Arabidopsis glutathione peroxidase 3 (GPX3) plays dual roles in H$_2$O$_2$ homeostasis and transduction in ABA and drought stress responses (Miao et al. 2006). The modulation of AtPED2 expression in ABA sensitivity, ROS accumulation and drought resistance further indicated the dual crosstalks among these pathways.
Involvement of AtPED2 in drought stress resistance

Figure 7. Modulation of AtPED2 expression affects reactive oxygen species (ROS) accumulation in Arabidopsis under control and drought stress conditions

(A, B) Quantifications of H$_2$O$_2$ content (A) and O$_2^- \cdot$ content (B) under control and drought stress conditions for 14 d. (C, D) Quantifications of superoxide dismutase (SOD) activity (C) and catalase (CAT) activity (D) under control and drought stress conditions for 14 d. The relative SOD and CAT activities of WT plants under control conditions were normalized as 1.0. The results shown are the means ± standard errors (SEs) (n < 3), and asterisks (*) indicate the significant difference of P < 0.05 in comparison to wild type (WT).

Metabolic profiling has been widely performed in the assays of functional genomic endeavors, genotype distinction and chemotyping (Krasensky and Jonak 2012; Witt et al. 2012; Barchet et al. 2014; Shi et al. 2014a, 2014b). Sugars are the important energy source for the biosynthetic processes in plants (Klotke et al. 2004). Higher level of most carbohydrates is primed for carbon gain and growth-maintaining under stress conditions, the while lower level of them is a limiting factor for plant growth under stress conditions (Kerepesi and Galiba 2000). As Krasensky and Jonak (2012) reviewed, higher levels of proline and some carbohydrates especially multiple sugars and sugar alcohols provide significant beneficial effects in response to drought stress. As shown in Figure 8 and Supplementary Table S1, about half of these metabolites exhibited lower concentrations in ped2-1 mutant, and almost all of these metabolites in AtPED2 overexpressing plants exhibited higher concentrations than WT plants. Thus, the modulation of AtPED2 expression in the accumulation of compatible solutes such as proline, arabinose, allose, dulcitol, fructose, galactinol, lactulose, lactose, mannose, sucrose, tagatofuranose, talose, etc., conferred significant advantage in modulating cell membrane stability and balancing osmotic pressure under drought stress conditions. On the contrary, lower concentrations of these compatible solutes in ped2-1 mutant might be related with the decreased drought stress resistance. Among these amino acids, the ped2-1 mutant showed lower concentrations of many amino acids, whereas AtPED2 overexpressing plants displayed significantly higher concentration of all the assayed amino acids except threonine, indicating the modulation of AtPED2 in regulating amino acid pools. Asparagine accumulation indicates that nitrogen re-distribution and mobilization are important features of AtPED2 overexpressing plants (Maaroufi-Dguimi et al. 2011), and asparagine is an amino group donor for the synthesis of the photorespiratory intermediate glycine, and this is also a good indicator of drought stress resistance in wheat cultivars with different stress resistance (Nagy et al. 2013). Moreover, the modulation of these metabolites by AtPED2 expression suggested common changes of carbon metabolism might contribute to the affected stress resistance. In the carbon metabolism, secondary carbon fixation is transferred to sugars, and glycolysis provides energy such as ATP and generates precursors for anabolism such as fatty acids and amino acids, which might be directly related to stress resistance (Plaxton 1996; Kerepesi and Galiba 2000; Selwood and Jaffe 2011). Although ABA-mediated metabolic profiling in plants remains unclear, the relationship among various stress treatments, primary metabolites, and stress resistance has been widely revealed (Krasensky and Jonak 2012; Witt et al. 2012; Barchet et al. 2014; Shi et al. 2014a, 2014b). The extensive modulation of AtPED2 expression on the primary metabolites indicated that AtPED2 might be involved in reorientation of carbohydrate and nitrogen metabolisms.

AtPED2/AtPEX14 is widely known as a peroxisomal membrane protein that assists in importing proteins into the peroxisome matrix (Monroe-Augustus et al. 2011; Burkhardt et al. 2013). Peroxisomes are highly dynamic organelles in morphology and metabolism, and plant peroxisomes are involved in numerous processes, including development,
stress responses, and in multiple metabolic reactions, many of which are related to the β-oxidation of fatty acids or fatty acid-related metabolites (Aung and Hu 2011; Hu et al. 2012; Quan et al. 2013; Cassin-Ross and Hu 2014). Coordination of the biosynthesis, biochemical activity, import, and degradation of peroxisomal proteins provides highly dynamic responses of peroxisomal metabolism to meet the needs of a plant especially in biomass production and stress tolerance (Aung and Hu 2011; Hu et al. 2012; Quan et al. 2013; Cassin-Ross and Hu 2014). Mutations in peroxisome biogenesis proteins (peroxins) result in serious developmental deficiencies and stress sensitivities in various eukaryotes (Aung and Hu 2011; Monroe-Augustus et al. 2011; Hu et al. 2012; Burkhart et al. 2013; Cassin-Ross and Hu 2014). AtPED2/AtPEX14 is an important peroxin involved in docking cargo-receptor complexes in the peroxisomal membrane, thus facilitating the transport of the cargo into the peroxisomal matrix (Monroe-Augustus et al. 2011; Burkhart et al. 2013), and the ped2 mutant is fertile, dwarfed, and there is a defect in the intracellular transport of thiolase from the cytosol to glyoxysomes (Hayashi et al. 1998, 2000; Lopez-Huertas et al. 1999; Monroe-Augustus et al. 2011; Burkhart et al. 2013). Modulation of the expression of AtPED2/AtPEX14 might affect the import of some peroxisomal proteins, as well as peroxisome-mediated development, stress responses, and metabolic reactions including β-oxidation of fatty acids. Thus, the role of AtPED2/AtPEX14 as a peroxisomal membrane protein in dynamic responses of peroxisomal metabolism might be directly linked to its modulation in ABA sensitivity, ROS accumulation and multiple primary metabolites. Additionally, peroxisomes are the sole site of fatty acid β-oxidation in plant cells and are involved in generating the phytohormone of indole-3-acetic acid (IAA) and 2, 4-D from IBA and 2,4-dichlorophenoxybutyric acid (2,4-DB) (Aung and Hu 2011; Hu et al. 2012; Quan et al. 2013; Cassin-Ross and Hu 2014). Due to its defect in importing proteins into the peroxisome matrix and β-oxidation, the ped2 mutant was
insensitive to 2,4-D and IBA and could not grow without sucrose (Hayashi et al. 1998, 2000; Burkhart et al. 2013), indicating the possible role of AtPED2/AtPEX14 in regulation of auxin synthesis and signaling pathways. Considering the effect of auxin content in ABA sensitivity and drought stress resistance in Arabidopsis, the possible link between AtPED2/AtPEX14 and auxin might be also contributed to AtPED2/AtPEX14-mediated drought stress resistance.

Taken together, this study demonstrated the possible novel role for AtPED2 in ABA signaling and drought resistance. AtPED2 played an important role in ABA-mediated seed germination, seedling development and drought stress resistance, through regulation of ABA-responsive genes expression, ROS metabolism, and metabolic homeostasis, at least partially.

**MATERIALS AND METHODS**

**Plant materials and growth conditions**

The Arabidopsis thaliana seeds in the background of Columbia ecotype were used in this study. Plant seeds were first sterilized with 70% (v/v) ethyl alcohol, 10% (w/v) NaClO solution with 0.05% (v/v) Triton X-100, and thoroughly washed five times with sterile water. After stratification at 4 °C for 3 d in darkness, plant seeds were then sown in soil or on MS medium containing 0.8% agar (w/v) and 3% sucrose (w/v) in a growth chamber. The growth chamber was controlled at 23 °C and in 16 h light and 8 h dark cycles. Nutrient solution was watered twice in the bottom of the pots with plants twice every week to maintain good growth conditions. The ped2-1 (SALK_007441) and ped2-2 (SALK_072373) mutants were obtained from the Arabidopsis Biological Resource Center.

**RNA isolation, Semi-quantitative reverse transcription (RT)-PCR, and quantitative real-time PCR**

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instruction, and RQ1 RNase-free DNase (Promega, Madison, WI, USA) was added for digestion of possible genomic DNA contamination. Then, 2 μg of total RNA was used for the synthesis of first-strand cDNA using reverse transcriptase (TOYOBO, Osaka, Japan) according to the manufacturer’s instruction. Semi-quantitative RT-PCR was performed as Shi et al. (2013b, 2013c, 2014c) described. Quantitative real-time PCR was performed using CFX96 Real Time System (BIO-RAD, Hercules, CA, USA) with SYBR Green Super mix (BIO-RAD) as Shi et al. (2013b, 2013c, 2014c, 2014d) described. The expression level of every gene was normalized with ubiquitin 10 (UBQ10) according to the comparative ΔΔCT method. The primers of AtPED2 for semi-quantitative RT-PCR and quantitative real-time PCR were: AtPED2F: 5’-TCATTTCACTTGGAGGCCATGGCAACTCTATCCAGAACAAAC-3’, AtPED2R: 5’-AGGATCCGATTCGTACACCCCTTAGTTGACTTCCTGCGTCGTA-3’. After sequencing confirmation, the recombinant construct was transformed into Agrobacterium tumefaciens strain GV3101 and introduced into Columbia-0 plants using the floral dip method (Clough and Bent 1998). Homozygous transgenic plants were selected on MS medium using basta resistance and were confirmed by PCR analysis.

**Seed germination and root elongation assays of ABA response**

Plant ABA sensitivity was determined as Shi et al. (2013b, 2014c) previously described. Briefly, for seed germination assay, Arabidopsis seeds of different lines were stratified at 4 °C for 3 d and sown on MS medium plates containing various concentrations of ABA. Germination ratios were scored as emerged radicals and green cotyledons after 10 d in a growth chamber. For the root elongation assay, 4-day-old plant seedlings grown on MS medium were transferred to fresh MS medium containing various concentrations of ABA for the next 7 d. Primary root length was determined for each root using a ruler. The stomatal response to ABA was assayed as Shi et al. (2013c) described. Briefly, plant leaves were incubated in stomatal opening buffer (5 mM 2-(N-morpholine)-ethanesulfonic acid (MES), 10 mM KCl, 50 mM CaCl2, pH 6.15) for 2 h, and then were moved to water solution containing 0 μM and 5 μM ABA for 2 h. After treatment, stomatal aperture width was photographed and quantified and at least 50 stomata were tested for each line.

**Quantification of ABA content**

For ABA quantification, samples from Arabidopsis plant leaves were extracted and determined as Xiong et al. (2001) described. The endogenous ABA concentration in the leaf samples was determined using Phytodetek ABA Test Kit (Sigma, San Francisco, CA, USA), based on Melatonin Enzyme Linked Immunosorbent Assay (ELISA) assay.

**Plant drought stress treatment and drought stress resistance assay**

For drought stress treatment, 14-d-old Arabidopsis plants of different lines in pots in the soil were subjected to control conditions (well-watering) and drought conditions (withholding water) for 21 d. More than three pots of each variety were used in each independent experiment, and all of these pots with plants were rotated daily during drought stress treatment to minimize the environment effect. The EL of stressed plants was assayed after 21 d of control and drought stress treatments as Shi et al. (2013a, 2013b, 2013c, 2014a, 2014b, 2014c, 2014d) described. The survival rate of stressed plants was recorded after recovery for 4 d from 21 d of drought stress treatment.

**Determination of relative leaf water loss rate in vitro**

Relative leaf water loss rate in vivo was determined as Shi et al. (2013b, 2013c) described, and was expressed as percentage change in leaf fresh weight (FW) in vitro.
Determination of ROS accumulation and antioxidant enzymes activities

H$_2$O$_2$ content was determined using the titanium sulfate reduction method that formed peroxide-titanium complex, and O$_2$. content was determined using plant O$_2$. ELISA Kit (10-40-488, Beijing Dingguo, Beijing, China) based on antibody-antigen-enzyme-antibody complex, according to Shi et al. (2013a, 2013b, 2013c, 2014a, 2014b, 2014c, 2014d) described previously.

The activities of SOD (EC 1.15.1.1) and CAT (EC 1.11.1.6) were determined using Total SOD Assay Kit (S0102, Beyotime, Haimen, China) and CAT Assay Kit (S0051, Beyotime), respectively, according to the manufacturer’s instructions as Shi et al. (2013a, 2013b, 2013c, 2014a, 2014b, 2014c, 2014d) described previously. The protein concentration was quantified using Bradford method (Bradford 1976). The relative SOD and CAT activities of WT plants under control conditions were normalized as 1.0.

Quantification and cluster analysis of metabolites

The extraction and derivatization of metabolites were performed as Lisec et al. (2006) described. Then the derivatized extracts was injected into a DB-5MS capillary (30 m × 0.25 mm × 0.25 μm, Agilent J&W GC column, Palo Alto, CA, USA) using GC-TOF-MS (Agilent 7890 A/5975C) as Shi et al. (2014b) described. After identification using retention time index specific masses and reference spectra in mass spectral libraries (NIST 2005, Wiley 7.0), the metabolites was quantified based on the pre-added ribitol (internal standard).

The concentration of every metabolite in WT plants was normalized as 1.0 for hierarchical cluster analysis, according to Cluster 3.0 software in CLUSTER program (http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/) and Java Treeview (http://jtreeview.sourceforge.net/).

Statistical analysis

All the experiments were repeated at least three times, and the data shown are the average means ± SEs of three independent experiments. Asterisks (*) indicate the significant difference of P < 0.05 in comparison to wild type (WT).

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Involvement of AtPED2 in drought stress resistance


SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web-site. Table S1. Concentrations of 54 metabolites in different Arabidopsis lines under control and drought stress conditions for 14 d. The concentrations of metabolites were expressed as μg g⁻¹ FW. The results shown are the means ± standard errors (SEs) (n < 3).
Dear Author,

During the copyediting of your paper, the following queries arose. Please respond to these by annotating your proofs with the necessary changes/additions using the E-annotation guidelines attached after the last page of this article.

We recommend that you provide additional clarification of answers to queries by entering your answers on the query sheet, in addition to the text mark-up.

<table>
<thead>
<tr>
<th>Query No.</th>
<th>Query</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1</td>
<td>Please confirm that given names (red) and surnames/family names (green) have been identified correctly.</td>
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<tr>
<td>Q2</td>
<td>Please check whether “unstabilization” is correct. Or should it be “destabilization”?</td>
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<td>Q3</td>
<td>Please check whether ” exacted” is correct. Or should it be “extracted”?</td>
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<td>Q4</td>
<td>Please check whether ” regent” is correct.</td>
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USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 8.0 or above). (Note that this document uses screenshots from Adobe Reader X)

The latest version of Acrobat Reader can be downloaded for free at: http://get.adobe.com/reader/

Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:

This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We’ve picked out some of these tools below:

1. **Replace (Ins) Tool** – for replacing text.
   - Strikes a line through text and opens up a text box where replacement text can be entered.
   - How to use it:
     - Highlight a word or sentence.
     - Click on the Replace (Ins) icon in the Annotations section.
     - Type the replacement text into the blue box that appears.

2. **Strikethrough (Del) Tool** – for deleting text.
   - Strikes a red line through text that is to be deleted.
   - How to use it:
     - Highlight a word or sentence.
     - Click on the Strikethrough (Del) icon in the Annotations section.

3. **Add note to text Tool** – for highlighting a section to be changed to bold or italic.
   - Highlights text in yellow and opens up a text box where comments can be entered.
   - How to use it:
     - Highlight the relevant section of text.
     - Click on the Add note to text icon in the Annotations section.
     - Type instruction on what should be changed regarding the text into the yellow box that appears.

4. **Add sticky note Tool** – for making notes at specific points in the text.
   - Marks a point in the proof where a comment needs to be highlighted.
   - How to use it:
     - Click on the Add sticky note icon in the Annotations section.
     - Click at the point in the proof where the comment should be inserted.
     - Type the comment into the yellow box that appears.
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

5. **Attach File Tool** – for inserting large amounts of text or replacement figures.

   Inserts an icon linking to the attached file in the appropriate pace in the text.

   **How to use it**
   - Click on the **Attach File** icon in the Annotations section.
   - Click on the proof to where you’d like the attached file to be linked.
   - Select the file to be attached from your computer or network.
   - Select the colour and type of icon that will appear in the proof. Click OK.

6. **Add stamp Tool** – for approving a proof if no corrections are required.

   Inserts a selected stamp onto an appropriate place in the proof.

   **How to use it**
   - Click on the **Add stamp** icon in the Annotations section.
   - Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
   - Click on the proof where you’d like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).

7. **Drawing Markups** Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

   Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks.

   **How to use it**
   - Click on one of the shapes in the **Drawing Markups** section.
   - Click on the proof at the relevant point and draw the selected shape with the cursor.
   - To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
   - Double click on the shape and type any text in the red box that appears.

For further information on how to annotate proofs, click on the **Help** menu to reveal a list of further options: