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Research article

Overexpression of *Actinidia deliciosa pyruvate decarboxylase 1* gene enhances waterlogging stress in transgenic *Arabidopsis thaliana*

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ABSTRACT

Ethanolic fermentation is classically associated with waterlogging tolerance when plant cells switch from respiration to anaerobic fermentation. Pyruvate decarboxylase (PDC), which catalyzes the first step in this pathway, is thought to be the main regulatory enzyme. Here, we cloned a full-length *PDC* cDNA sequence from kiwifruit, named *AdPDC1*. We determined the expression of the *AdPDC1* gene in kiwifruit under different environmental stresses using qRT-PCR, and the results showed that the increase of *AdPDC1* expression during waterlogging stress was much higher than that during salt, cold, heat and drought stresses. Overexpression of kiwifruit *AdPDC1* in transgenic *Arabidopsis* enhanced the resistance to waterlogging stress but could not enhance resistance to cold stress at five weeks old seedlings. Overexpression of kiwifruit *AdPDC1* in transgenic *Arabidopsis* could not enhance resistance to NaCl and mannitol stresses at the stage of seed germination and in early seedlings. These results suggested that the kiwifruit *AdPDC1* gene is required during waterlogging but might not be required during other environmental stresses. Expression of the *AdPDC1* gene was down-regulated by abscisic acid (ABA) in kiwifruit, and overexpression of the *AdPDC1* gene in *Arabidopsis* inhibited seed germination and root length under ABA treatment, indicating that ABA might negatively regulate the *AdPDC1* gene under waterlogging stress.

1. Introduction

Plants often meet with flooding or waterlogging conditions caused by heavy rain or irrigation in natural and agricultural ecosystems. Unanticipated flooding reduces oxygen supply and affects plant growth and development (Hinz et al., 2010; Luo et al., 2008; Voesenek and Bailey-Serres, 2015). Plants have evolved several metabolic and morphological adaptations to survive in transient periods of complete or partial submergence and escape hypoxia (Sairam et al., 2009; Thirunavukkarasu et al., 2013; Yin et al., 2009). Oxygen deprivation acts as a primary signal in the response to flooding (Jackson and Colmer, 2005; Milroy and Bange, 2013). Plant alcohol fermentation is activated under low oxygen stress conditions: pyruvate decarboxylase (PDC, EC 4.1.1.1) firstly converts pyruvate to acetaldehyde, and then alcohol dehydrogenase (ADH, EC 1.1.1.1) converts acetaldehyde to ethanol.

In plants, PDC plays an important role in response to abiotic stresses such as anoxia, cold, salt and wounding (Tadege et al., 1999). *PDC* is a multigene family, and there are four *PDC* genes in *Arabidopsis* (Kürsteiner et al., 2003) and rice (Rivoal et al., 1997). The expression of *PDC1*, *PDC2* and *PDC4* is also strongly up-regulated during flooding in rice, which improves tolerance under long-term anoxia (Rivoal et al., 1997). Hypoxia and anoxia significantly induced the expression of *Arabidopsis PDC1* and *PDC2*, and these two genes play key roles in the tolerance to submergence by mutant and transgenic experiments (Mithran et al., 2014). Overexpression of *PDC1* in *Arabidopsis* enhances the low-temperature sweetening tolerance in the transgenic potato (Pinhero et al., 2011). *PDC* have been significantly induced to express during cold and mannitol treatments in *Arabidopsis* (Conley et al., 1999; Dolferus et al., 1997; Kürsteiner et al., 2003) and cold treatments in rice and maize (Christie et al., 1991).

Abbreviations: ABA, Abscisic acid; CaMV, Cauliflower mosaic virus; CDS, coding sequence; DEG, Differentially expressed unigene; ERF, Ethylene response factor; EST, Expression sequence tag; LOES, Low-O_2 escape syndrome; LOQS, Low-O_2 quiescence syndrome; MS, Murashige and Skoog; ORF, Open reading frame; PDC1, *Pyruvate decarboxylase 1* gene; PI, Isoelectric point; qRT-PCR, Real-time quantitative RT-PCR; WT, Wild-type.

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Overall, the *PDC* gene family regulates sugar metabolic processes in response to environmental stress in plants. However, Kürsteiner et al. (2003) reported that the *AtPDC1* gene of *Arabidopsis* is required during anoxia but not any other environmental stresses. Thus, different *PDC* genes from different species may have distinct functions.

Kiwifruit plants are particularly sensitive to soil waterlogging. Enhancement of waterlogging tolerance in kiwifruit can potentially increase its fruit production and extend the shelf-life of the fruit. Previously, we generated 95,945,496 bases of high-quality sequence from kiwifruit roots after 4 days of waterlogging treatment using Illumina sequencing technology, and demonstrated de novo assembly and annotation of genes (Zhang et al., 2015a); 14,843 transcripts were identified as differentially expressed unigenes (DEG) in two samples. Among these unigenes, 5,697 DEGs were found to be induced by waterlogging, and 9,146 DEGs decreased in abundance (Zhang et al., 2015a). Two out of three kiwifruit *ADH* genes were significantly up-regulated in roots following waterlogging treatment and both *PDC* genes were also significantly up-regulated, suggesting that ethanolic fermentation was activated in kiwifruit roots following waterlogging treatment (Zhang et al., 2015a).

In order to analyze the function of the *AdPDC1* gene, the complete coding sequence (CDS) was isolated from *Actinidia deliciosa* (Jinkui) in this study. The expression pattern of the *AdPDC1* gene was analyzed in kiwifruit after treatment with waterlogging, salt, cold, heat, drought and abscisic acid (ABA) using real-time quantitative RT-PCR (qRT-PCR). Moreover, the *AdPDC1* gene was transformed into *Arabidopsis* to investigate whether *AdPDC1* overexpression could enhance resistance to waterlogging and other biotic stresses.

2. Materials and methods

2.1. Plant materials and growth conditions

Two year-old *A. deliciosa* (Jinkui) cutting seedlings were grown in nutrient soil in a greenhouse with air temperature of 25–28 °C during the day and 20–25 °C during the night for the treatments with waterlogging, salt and drought stresses. *A. deliciosa* plants were subcultured in vitro in Murashige and Skoog (MS) media supplemented with 6-benzylaminopurine (6-BA, 3.0 $mg.L^{-1}$) and naphthalene acetic acid (NAA, 0.2 $mg.L^{-1}$) under conditions of a 16 h light (23 ± 1 °C)/8 h dark (23 ± 1 °C) cycle for the treatments with ABA, heat and 4 °C.

Arabidopsis thaliana (ecotype Columbia, Col-0) was used for experiments involving transformation and various abiotic stress conditions. *Arabidopsis* seedlings were grown in half MS medium containing 3% sucrose and 0.8% (w/v) agar in a chamber at 22/20 °C (night/day), with a photoperiod of 16/8 h (day/night) for 10 days, then transplanted into nutrient soil. The seeds used in the experiment were harvested at the same stage, and stored in the same way to ensure that they all had the same vitality.

2.2. Treatment of A. deliciosa with abiotic stresses

For waterlogging, salt and drought stresses, the cutting seedlings of *A. deliciosa* were grown for 2 weeks in a greenhouse as mentioned earlier. The waterlogging treatment was performed as described previously (Yin et al., 2009). The pots were flooded by standing in a 28 cm × 14 cm × 14 cm container filled with tap water to 2.5 cm above the level of the soil surface. The tap water had a pH of 7.3. The water temperature was held at ~25 °C. Roots were sampled at 0, 24, 48, and 96 h after treatment, frozen in liquid nitrogen and stored at −80 °C. For drought stress, watering was withheld and plants were allowed to wilt (which took another 14 days). Control plants were well watered till leaves were collected. Leaf samples from wilted and well-watered plants were frozen in liquid nitrogen and stored at −80 °C. For salt stress, the cutting seedlings were soaked at high salinity (200 mM NaCl) for 4, 12 and 48 h. For heat and freezing stresses and ABA treatments, *A. deliciosa* in vitro plants were grown on Petri dishes containing half-strength MS medium supplemented with indole butyric acid (IBA, 0.6 $mg.L^{-1}$) for 4 weeks. Heat stress was imposed by placing the Petri dishes containing seedlings at 48 °C for 2 h and 4 h. The plates were subsequently incubated at 23 ± 1 °C for another 6 h. To induce freezing stress, the plates were placed at 4 °C for 4, 12 and 48 h. ABA (0.01 mM, Sigma) was spared onto the leaves of plants for 4, 12 and 48 h. The leaves from salt stress, heat stress, freezing stress, ABA and control treatments were flash frozen in liquid nitrogen and stored at −80 °C. The experiment was repeated three times and there were five plants per treatment in each replication. qRT-PCR was performed to study the responses of the *AdPDC1* gene to different stresses.

2.3. BLAST-based searches and in silico cloning

High-throughput DNA sequencing (Illumina) techniques have been performed previously in kiwifruit after treatment with waterlogging stress for 4 days, and showed that the expression level of *AdPDC1* was up-regulated strongly (Zhang et al., 2015a). In order to obtain the CDS of *AdPDC1*, the NCBI BLASTn program (**Error! Hyperlink reference not valid.**www.ncbi.nlm.nih.gov/blast) was used to search for the *AdPDC1* gene in the GenBank, nr and expression sequence tag (EST) databases. The partial sequence of *AdPDC1* (comp104616) was used as an initial query sequence for the *Actinidia* ESTs. Additional iterative BLAST searches identified other related sequences to search for EST fragments for gene assembly. EST fragments (accession numbers FG481401, FG482125, FG482146, FG482349 and FG453505) were identified from the EST database and assembled using DNAMAN software to obtain the *AdPDC1* full-length cDNA sequence. The open reading frame (ORF) was analyzed using the NCBI ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gor.html).

2.4. Total RNA isolation and cDNA synthesis

Total RNA was isolated according to the method reported previously (Cai et al., 2008). Reverse transcription of mRNA was achieved with a PrimeScript™ RT Reagent Kit with gDNA eraser (Perfect Real Time, TaKaRa, cat. # RR047Q, Dalian, China) with 1.0 μg mRNA. cDNA samples were diluted 1: 10 with sterile double-distilled water and stored at −20 °C before being used.

2.5. PCR amplification for the complete coding sequence of AdPDC1

The cDNA sample from the kiwifruit root treatment with waterlogging for 4 days was used to amplify the complete CDS of *AdPDC1*. Gene-specific primers were designed based on the assembled sequence of *AdPDC1*. The PCR reaction mixture (25 μL) contained 1 μL reverse transcribed first strand of cDNA and 0.4 pM each of the gene-specific primers (Table S1). PCR was conducted with an initial denaturation step at 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, 55 °C for 45 s, 72 °C for 2 min, and a final extension at 72 °C for 20 min. The PCR product was separated on 1.2% (w/v) agarose gels stained with ethidium bromide. The DNA fragment was cloned into pMD19-T (TaKaRa, China) and sequenced by the Shanghai Invitrogen Biotechnology Co. Ltd (China).

2.6. Sequence analysis and phylogenetic tree construction

The nucleotide and deduced amino acid sequences were compared with those in GenBank using the BLAST program. Protein sequence alignment was performed using the ClustalW program. Molecular weight and isoelectric point (PI) were obtained using online analysis software (http://web.expasy.org/compute_pi/). Phylogenetic trees of the aligned kiwifruit AdPDC1 protein sequences were constructed using MEGA version 5.0 (Tamura et al., 2011) (http://www.megasoftware.net) via the neighbor-joining method with the following parameters: Poisson correction, pairwise deletion and bootstrap (1,000 replicates; random seed).

2.7. Gene expression analysis of AdPDC1 in kiwifruit using qRT-PCR

qRT-PCR was carried out on an Applied Biosystems 7300 Real Time PCR System with a 20 μL reaction volume, containing 1 μL 10-fold diluted cDNA, 0.3 μL (10 pM) of each primer (Table S1), 10 μL SYBR® *Premix Ex Taq*™ (Perfect Real Time, TaKaRa, code: DRR041A) and 8.4 μL sterile double-distilled water. The PCR conditions consisted of denaturation at 94 °C for 4 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 20 s and 72 °C for 43 s. The specificity of the individual PCR amplification was checked using a heat dissociation curve from 55 to 95 °C following the final cycle of PCR. Kiwifruit actin was used as the housekeeping gene to monitor cDNA abundance (Yin et al., 2012). To ensure gene-specific amplification, normal PCR was performed to amplify the *AdPDC1* or *AdActin* genes, respectively. A single PCR fragment with the expected size was amplified, suggesting that the primers were suitable for qRT-PCR analysis. The resulting PCR product was cloned and sequenced to confirm the expected fragment of the *AdPDC1* or *AdActin* gene. All samples were harvested and three biological replicates were run independently. The relative levels of genes to control *AdActin* mRNAs were analyzed using 7300 system software and the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001). Data analyses were conducted using SPSS version 17.0 statistical software. For all analyses, the level of significance between different time points was set at $P < 0.05$.

2.8. Vector construction and Arabidopsis transformation

The coding sequence of the *AdPDC1* gene was amplified using primers (AF2 and AR2, Table S1), and cloned into a binary vector pCAMBIA1301 under control of the cauliflower mosaic virus (CaMV) 35S promoter. The resulting vector was mobilized into *Agrobacterium tumefaciens* strain EHA105. Transformation of *Arabidopsis* plants was performed by the flower dip method (Bechtold and Pelletier, 1998). Transgenic plants were first screened on half MS medium supplemented with 50 $mg.L^{-1}$ hygromycin. Seeds from each T_1 plant were individually collected. Selected T_2 plants were propagated. T_3 progeny homozygotes were obtained for further analysis.

2.9. Analysis of transgenic lines for tolerance to waterlogging

Five week-old *A. thaliana* homozygous T_3 seedlings of transgenic overexpression lines and wild-type (WT) seedlings were used for the waterlogging treatment. The waterlogging treatment was performed for 2 weeks, as described previously (Yin et al., 2009), and then seedlings were returned to normal growth conditions for 1 week. The phenotypic traits were observed 2 weeks after waterlogging treatment and 1 week after recovery. Statistical aspects of the root length, fresh mass and dry mass of the plants were measured after recovery. The experiment was repeated independently three times. The data were subjected to statistical analysis. Analysis of variance was carried out with SPSS, version 17.0.

2.10. Analysis of transgenic lines for tolerance to NaCl, mannitol and ABA

2.10.1. Seed germination tests

For seed germination analysis, sterilized seeds were planted on MS agar medium containing 3% sucrose supplemented with NaCl (100 mM and 200 mM), ABA (2 μM and 10 μM) or mannitol (100 mM and 300 mM), respectively. The germination rate was expressed as a percentage of the total number of seeds plated. A seed was regarded as germinated when the radical protruded through the seed coat. The number of germinated seeds was counted at 7 days. The phenotypic traits were graphed at 10 days. Three replicates were used for each treatment to ensure reproducibility of the data. Each replicate contained at least 50 seeds. The data were subjected to statistical analysis. Analysis of variance was carried out with SPSS, version 17.0.

2.10.2. Stress tolerance tests in seedlings

Four day-old seedlings of WT and transgenic plants which were germinated just from MS agar medium were transferred to MS medium containing NaCl (100 mM), ABA (2 μM) or mannitol (300 mM), respectively. The phenotype and root length were measured after 7 days. The experiments were carried out on three replicates of 30 seedlings. The results from one set of experiments are shown. The data were subjected to statistical analysis. Analysis of variance was carried out with SPSS, version 17.0.

2.11. Analysis of transgenic lines for tolerance to cold

Five week-old *A. thaliana* homozygous T_3 seedlings of transgenic overexpression lines and WT seedlings were used for cold treatment. The plates were placed at −20 °C for 5 h then returned to normal growth conditions for 16 h. The phenotypes were observed. The experiment was repeated independently three times.

3. Results

3.1. Cloning and sequence analysis of AdPDC1

A 1931 bp full-length cDNA sequence was obtained by in silico cloning and sequencing from *A. deliciosa* Jinkui root and has been submitted to GenBank (accession no. KU095879). The CDS contains an 1821 bp ORF, which encodes a protein of 606 amino acids with a predicted molecular weight of about 65.25 kDa and a PI of 5.81.

Amino acid sequence comparisons showed that AdPDC1 had high identity with *Erythranthe guttatus* PDC1 (92% identity, XP 012832203), *Sesamum indicum* PDC1 (89% identity, XP 011095042) and *Theobroma cacao* PDC (87% identity, XP 007026634.1). The amino acid comparison of the predicted AdPDC1 protein with other higher plant PDCs is shown in Fig. S1. The kiwifruit putative AdPDC1 protein contains seven conserved amino acids which were reported in other plant PDC proteins (Moyano et al., 2004). A phylogenetic tree was constructed on the basis of the sequence similarities of PDC proteins, and it showed that the PDC proteins were divided into two groups (I and II) (Fig. 1). Group I consisted exclusively of dicot plants and group II consisted exclusively of monocot plants. These results indicated that the *PDC* genes were highly conserved during evolution and had similar evolutionary trends in species.

3.2. Expression of AdPDC1 in response to stress

We analyzed the behaviors of *AdPDC1* transcripts of kiwifruit

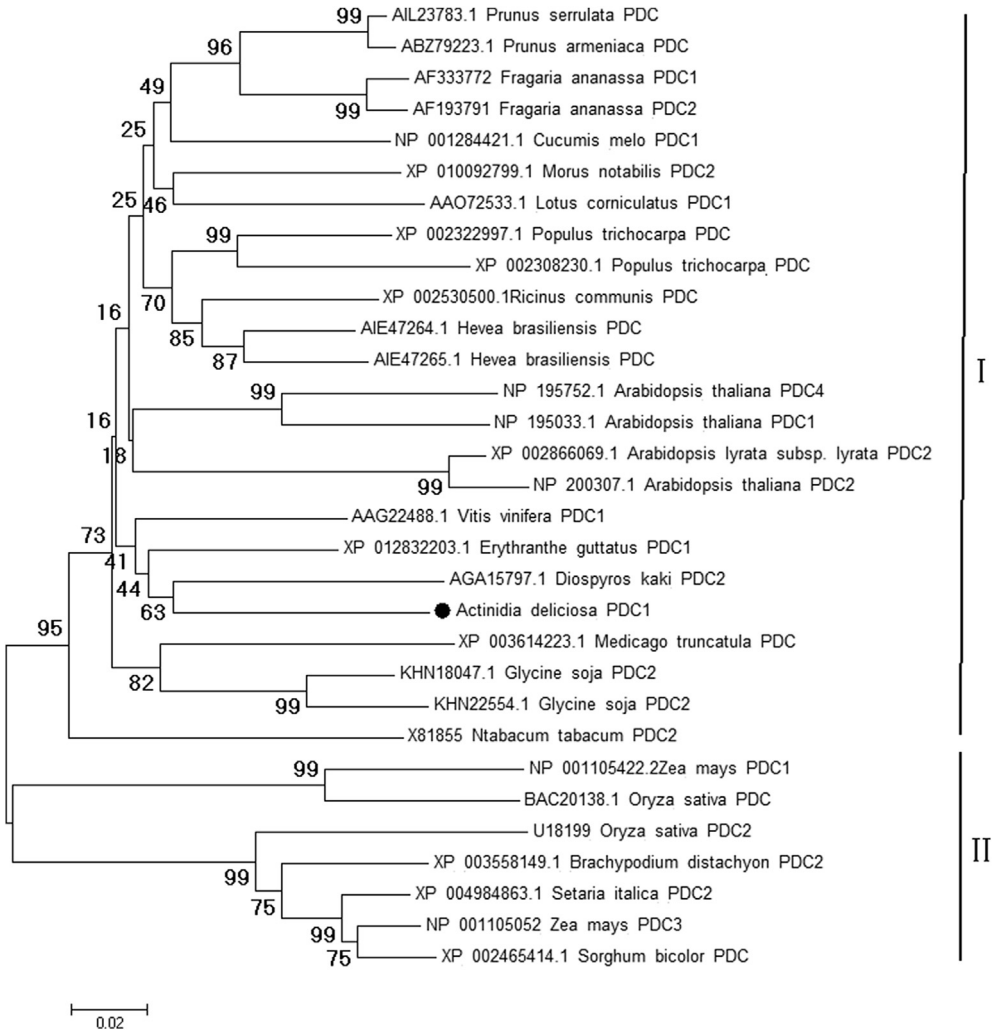

Fig. 1. Phylogenetic analysis of amino acid sequences of *AdPDC1* and other related PDCs. A neighbor-joining tree was constructed using MEGA version 5.0 (Tamura et al., 2011) (http://www.megasoftware.net) based on the full-length amino acid sequences. Bootstrap analysis with 1000 replicates was performed to obtain confidence levels for the branches of the tree. Selected species are from GenBank or UniProtKB/Swiss-Prot (accession number in parentheses).

subjected to a range of abiotic stresses using qRT-PCR (Fig. 2). For waterlogging stress, expression of *AdPDC1* was initially induced at 24 h (~270.60-fold), reaching the highest level at 48 h (~883.48-fold) and then decreased at 96 h (~228.33-fold). For NaCl stress, expression of *AdPDC1* reached the highest level at 4 h (~4.52-fold) and then decreased. Low temperature and drought could not induce expression of *AdPDC1*. Heat stress enhanced the *AdPDC1* expression level, which peaked (~15.03-fold) early during treatment (2 h), and then decreased to a low level. Compared with other environmental stresses, *AdPDC1* is strongly induced by waterlogging. However, the expression of *AdPDC1* was decreased after treatment with ABA, and was at the lowest level at 48 h (~0.33-fold) during the first 48 h of treatment.

3.3. Overexpression of AdPDC1 enhances tolerance to waterlogging stress in Arabidopsis

In order to detect the function of kiwifruit *AdPDC1*, transgenic *Arabidopsis* plants overexpressing the *AdPDC1* gene were generated. Five week-old *A. thaliana* homozygous T_3 seedlings of transgenic lines and WT plants were used for waterlogging stress experiments. After 2 weeks of waterlogging stress, the transgenic *AdPDC1* lines had a higher waterlogging stress tolerance than WT plants (Fig. 3). As shown in Fig. 3b, the WT plants became wilted and stopped growing, and the leaves became yellow after waterlogging stress, but the transgenic *AdPDC1* lines showed more open, growing leaves and continual growth. Following the waterlogging stress treatment, plants were returned to normal growth conditions for 1 week (Fig. 3c). Growth vigor of the transgenic *AdPDC1* lines was much better than that of WT plants (Fig. 3c). Root length (Fig. 3d), fresh mass (Fig. 3e) and dry mass (Fig. 3f) of the transgenic *AdPDC1* lines and WT plants were measured after recovery, and the results showed that root length, fresh mass and dry mass of the transgenic *AdPDC1* lines were significantly higher than those of WT plants. These results indicated that overexpression of kiwifruit *AdPDC1* in *Arabidopsis* enhances waterlogging stress tolerance.

3.4. Overexpression of the AdPDC1 gene could not enhance resistance to salt and mannitol stress at seed germination and seedling stages

To determine whether the overexpression of *AdPDC1* in *Arabidopsis* enhances salinity resistance, the seed germination rate of transgenic *Arabidopsis* plants was examined under different levels of salt stress (Fig. 4a and b). There was no difference in the germination rates between WT and PDC1-1, PDC1-2, and PDC1-3

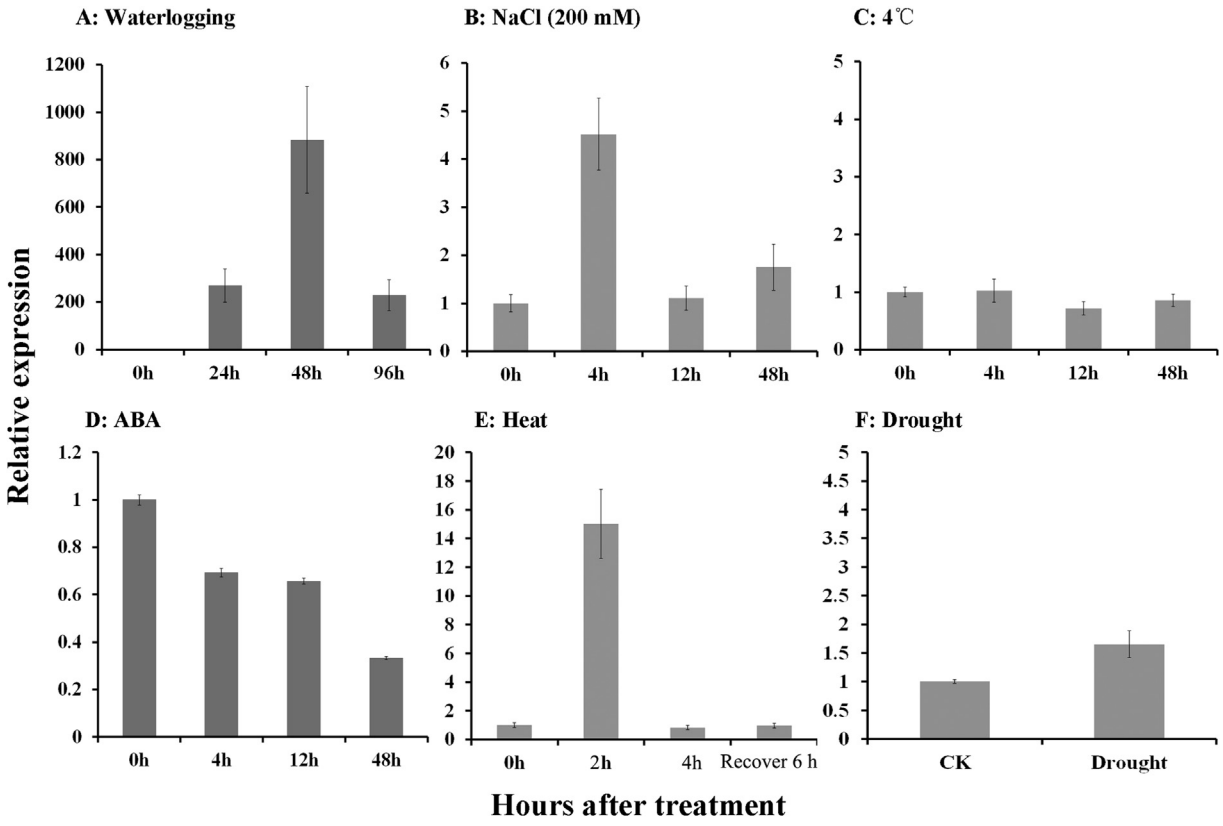

Fig. 2. Expression patterns of *AdPDC1* in roots under waterlogging stress (a) and in leaves under NaCl (b, 200 mM), 4 °C (c), ABA (d), heat (e) and drought (f) stresses were performed using qRT-PCR. *AdActin* was used as an internal control. The experiments were repeated in triplicate with similar results.

transgenic *Arabidopsis* lines grown on the control MS medium and MS medium supplemented with NaCl (100 mM and 200 mM), indicating that overexpression of the *AdPDC1* gene does not regulate seed germination and plant tolerance to salt stress at the seed germination stage. T_3 seedlings of the transgenic *Arabidopsis* lines were analyzed for salinity tolerance at the seedling stage. Four day-old seedlings WT and transgenic seedlings which were germinated just from MS agar medium were transferred to MS medium

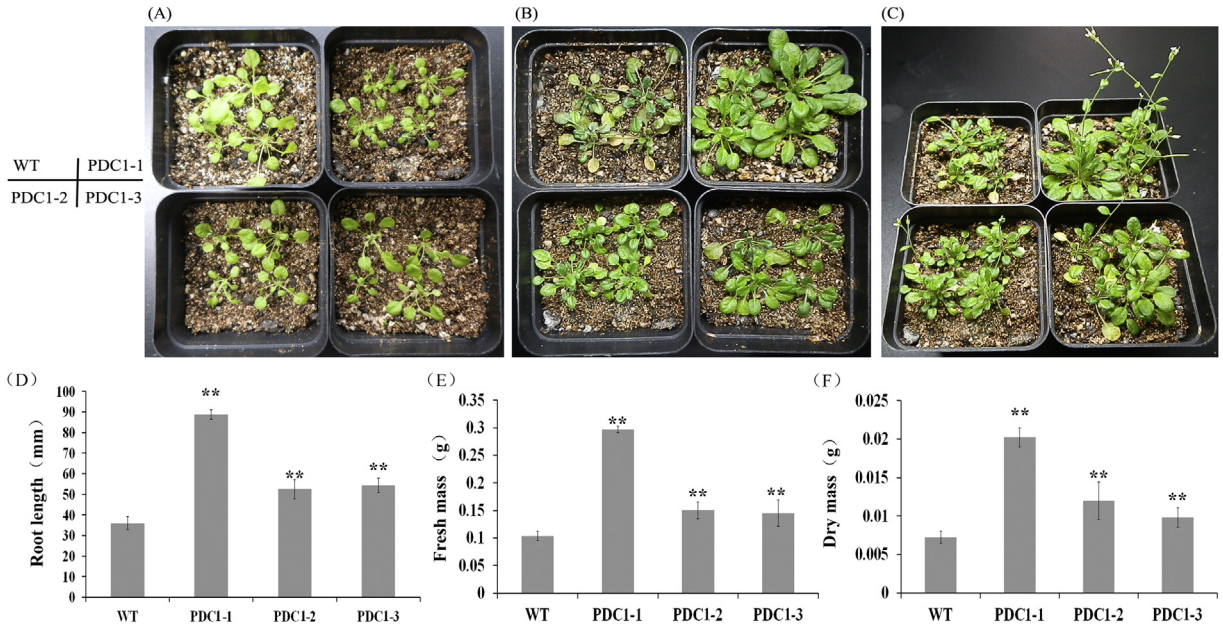

Fig. 3. Phenotypes of *AdPDC1* transgenic and WT plants under waterlogging stress. Waterlogging tolerance assay of the *AdPDC1* overexpression lines PDC1-1, PDC1-2, PDC1-3 and wild-type (WT). Five week-old plants were grown in nutrient soil before waterlogging assay (a) and waterlogging for 2 weeks (b), and then returned to normal growth conditions for 1 week (c). Similar results were observed in three independent experiments. Root length (d), dry mass (e) and fresh mass (f) of the transgenic *AdPDC1* lines and WT plants under waterlogging stress. The data presented are the mean ± SD; double asterisks indicate significant differences compared with WT line values ($P < 0.01$). Similar results were observed in three independent experiments.

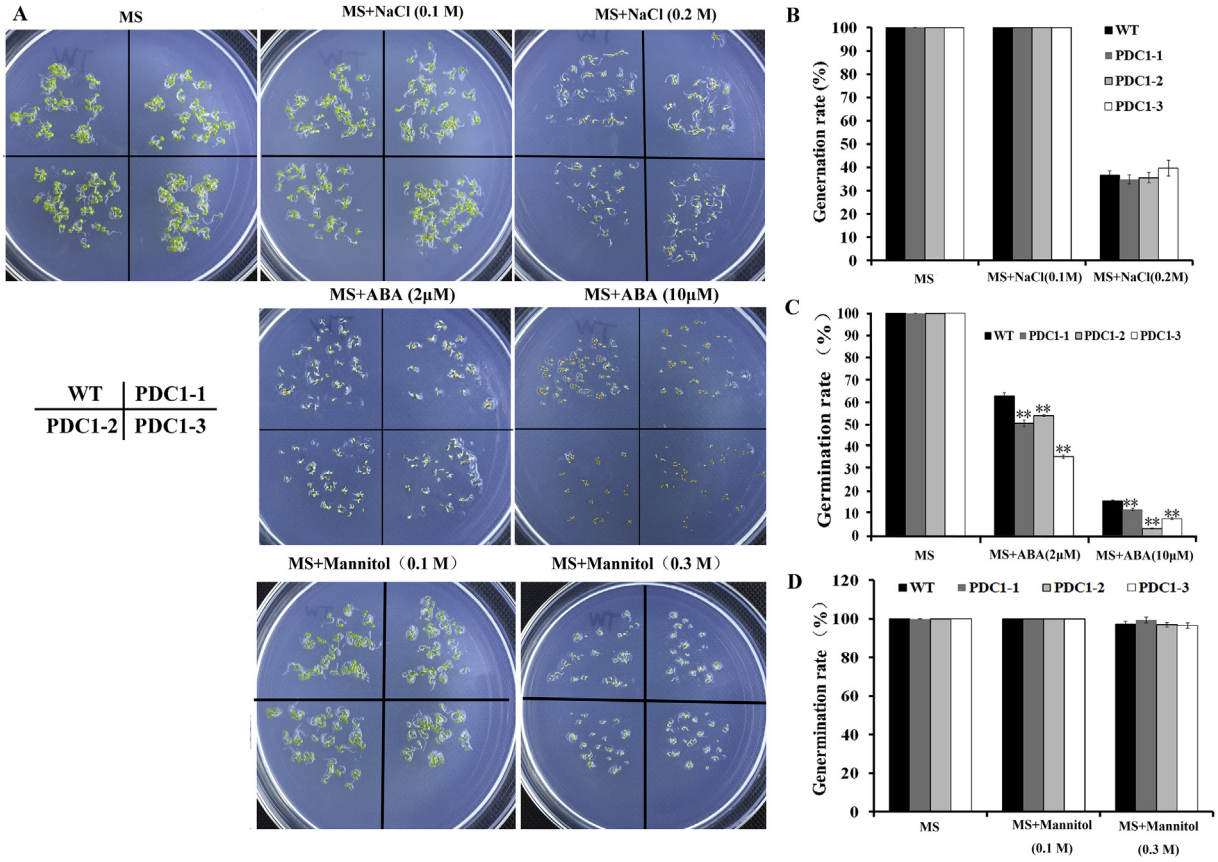

Fig. 4. Comparison of germination rates between transgenic (PDC1-1, PDC1-2 and PDC1-3) and wild-type (WT) lines under NaCl, ABA and mannitol stresses. Photographs were taken 10 days after seed germination of WT and transgenic *Arabidopsis* plants in MS medium containing various concentrations of NaCl, ABA and mannitol (a). Germination rates in WT and transgenic plants grown in MS (control) or MS supplemented with various concentrations of NaCl (b), ABA (c) and mannitol (d) for 7 days. Results are presented as mean ± SD from three independent experiments (50 seeds of each line were grown for each experiment). Double asterisks indicate significant differences compared with WT line values ($P < 0.01$).

containing NaCl (100 mM). The phenotypes and root lengths were measured after 7 days. Almost all of the transgenic and WT *Arabidopsis* seedlings had a similar appearance (Fig. 5a), and the root lengths were not significantly different (Fig. 5b), indicating that overexpression of the *AdPDC1* gene does not regulate plant tolerance to salt stress at the seedling stage.

For the mannitol stress treatment assay, no differences in seed germination were observed when seeds from WT and transgenic *Arabidopsis* lines were germinated in MS supplemented with 100 mM or 300 mM mannitol (Fig. 4a and d). In addition, transgenic lines and WT *Arabidopsis* seedlings had a similar appearance (Fig. 5a), and the root lengths were not significantly different after the transgenic and WT *Arabidopsis* seedlings were treated with 150 mM mannitol (Fig. 5b). These results suggested that overexpression of the *AdPDC1* gene does not regulate plant tolerance to mannitol stress at seed germination and seedling stages.

3.5. Overexpression of the AdPDC1 gene in Arabidopsis inhibits seed germination and root length under ABA treatment

Seed germination of transgenic *Arabidopsis* plants was assessed under different concentrations of ABA (Fig. 4a and c). When the concentration of ABA was increased to 2 μM, seed germination rates of PDC1-1, PDC1-2 and PDC1-3 were 51%, 54% and 36%, respectively, significantly lower than that of WT (63%; $P < 0.01$; Fig. 4c). However, 10 μM ABA resulted in serious inhibition of seed germination. Germination rates of PDC1-1, PDC1-2 and PDC1-3 were 12%, 3% and 8%, respectively, significantly lower than that of WT plants (15%; $P < 0.01$). The root lengths of PDC1-1, PDC1-2 and PDC1-3 lines were shorter than those of WT after treatment with 2 μM ABA at the seedling stage (Fig. 5). These results showed that overexpression of the *AdPDC1* gene in *Arabidopsis* inhibited seed germination and root length under ABA treatment.

3.6. Overexpression of the AdPDC1 gene could not enhance resistance to cold stress at the seedling stage

In order to determine whether overexpression of *AdPDC1* in *Arabidopsis* enhances cold resistance, 5 week-old transgenic *Arabidopsis* plants were examined under cold stress (Fig. 6). The results showed that all the transgenic *Arabidopsis* lines and WT plants died after −20 °C treatment for 5 h then return to normal growth conditions for 16 h (Fig. 6), indicating that overexpression of the *AdPDC1* gene could not enhance resistance to cold stress at the seedling stage.

4. Discussion

4.1. The kiwifruit AdPDC1 gene is required during waterlogging

Ethanolic fermentation is classically associated with flooding tolerance when plant cells switch from respiration to anaerobic fermentation. PDC, which catalyzes the first step in this pathway, is thought to be the main regulatory enzyme. Overexpression of a

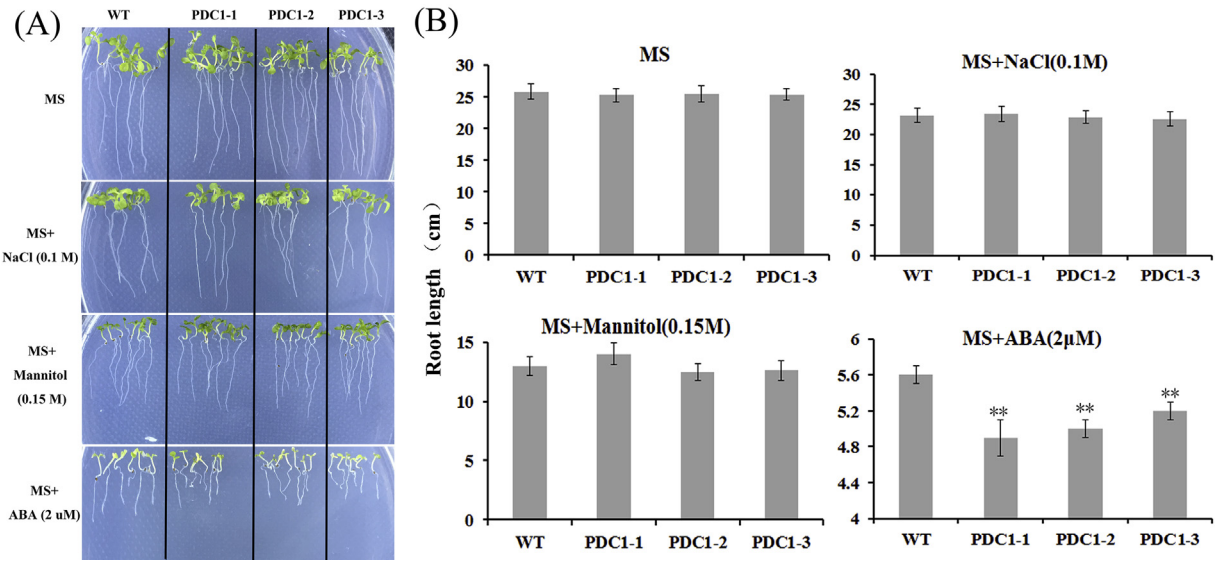

Fig. 5. Comparison of root length between transgenic (PDC1-1, PDC1-2 and PDC1-3) and wild-type (WT) lines under NaCl, ABA and mannitol stresses. Four day-old seedlings of WT and transgenic seedlings which were germinated just from MS agar medium were transferred to MS medium containing NaCl (100 mM), ABA (2 μM) or mannitol (150 mM), respectively. The phenotype (a) and root length (b) were measured after 7 days. Double asterisks indicate significant differences compared with WT line values ($P < 0.01$). The experiments were carried out on three replicates of 30 seedlings. The results from one set of experiments are shown.

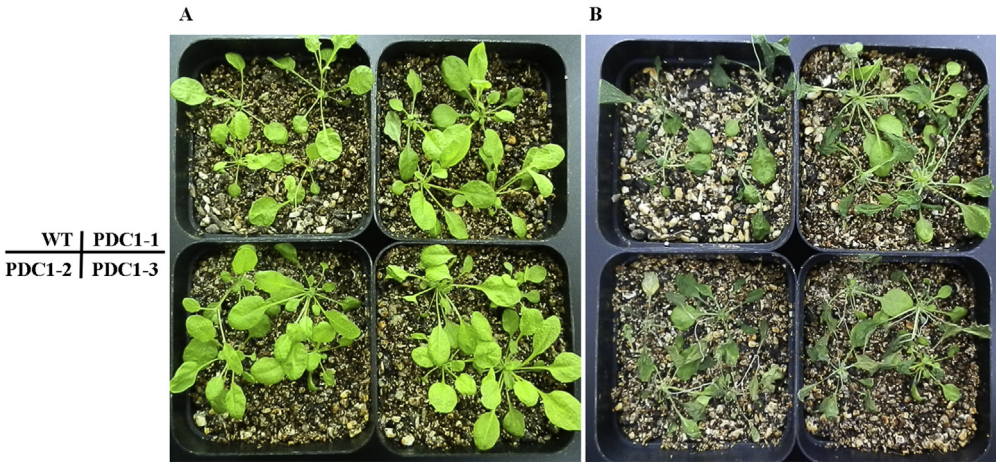

Fig. 6. Analysis of transgenic lines for tolerance to cold. Five week-old *Arabidopsis thaliana* homozygous T_3 seedlings of transgenic overexpression lines and wild-type (WT) seedlings (a) were placed at −20 °C for 5 h then returned to normal growth conditions for 16 h (b). The experiment was repeated independently three times.

bacterial *PDC* in transgenic tobacco resulted in constitutive high and active protein levels under both normoxic and anoxic conditions (Bucher et al., 1994). Overexpression of *Arabidopsis* genes for the enzymes ADH and PDC improved the tolerance of *Arabidopsis* roots to low oxygen conditions (Dennis et al., 2000; Shiao et al., 2002). *AtPDC1* is the only gene induced under oxygen limitation among the *PDC* gene family and a *pdc1* null mutant is comprised in anoxia tolerance but no other environmental stresses in *Arabidopsis* (Kürsteiner et al., 2003). Cotton *PDC* gene was up-regulated by waterlogging stress in both roots and shoots (Zhang et al., 2015b). In our study, the expression level of the *AdPDC1* gene significantly induced by waterlogging stress (Fig. 2), and overexpression of the *AdPDC1* gene in *Arabidopsis* enhanced the resistance to waterlogging stress (Fig. 3). Those results suggested that, as with the function of the *Arabidopsis AtPDC1* gene (Kürsteiner et al., 2003), the kiwifruit *AdPDC1* gene is required during waterlogging stress.

Waterlogging caused O_2 deprivation and ethylene elevation in soil (Bailey-Serres and Voesenek, 2008; Yamauchi et al., 2014). *RAP2.12*, which is one of the ERF-VII genes in *A. thaliana*, is constitutively expressed and further up-regulated by ethylene or O_2 deprivation (Voesenek and Bailey-Serres, 2015). Reduction of atmospheric O_2 below 10% increases the nuclear localization of RAP2.12 (Kosmacz et al., 2015). As O_2 concentrations decline, RAP2.12 is relocalized from the plasma membrane to the nucleus, concomitant with increased accumulation of hypoxia-responsive mRNAs (Kosmacz et al., 2015). Nuclear RAP2.12 is associated with increased core hypoxia-responsive gene transcript levels (Gibbs et al., 2011; Licausi et al., 2011), including PDC1 and hypoxia-responsive ERF1/2 (HRE1/2). HRE2 transcriptionally activates PDC1 (Voesenek and Bailey-Serres, 2015).

4.2. ABA might negatively regulate the AdPDC1 gene under waterlogging stress

Waterlogging-tolerant plants have evolved a continuum of survival strategies including the low-O_2 escape syndrome (LOES)

and low-O_2 quiescence syndrome (LOQS) (Bailey-Serres and Voesenek, 2008, 2010; Voesenek and Bailey-Serres, 2013). Some plants from flood-prone environments have evolved the ability to elongate their porous shoots when underwater to facilitate a LOES (Voesenek and Bailey-Serres, 2015). Both rice and the dicot *Rumex palustris* involve the conserved molecular triumvirate that includes ethylene, ABA and gibberellic acid (GA), and a hormonal hierarchy involving ethylene as an elicitor, ABA as a repressor and GA/auxin as promoters are associated with modulation of underwater elongation growth (Voesenek and Bailey-Serres, 2015). Petioles of the related *R. acetosa* from rarely flooded riparian sites show a reduced elongation rate when submerged. This is caused by an incapability to reduce ABA concentrations, which appears to be a requirement for underwater elongation growth (Benschop et al., 2005; van Veen et al., 2013). Variation between ecotypes in submergence-induced petiole elongation occurs at the level of ethylene-controlled down-regulation of ABA in *R. palustris*. In *R. palustris* ecotypes that rapidly elongate, submerged petioles display stronger submergence-induced declines in ABA content than slowly elongating ecotypes (Chen et al., 2010). ABA concentrations declined by 80% within 1 h of submergence in *Rumex*. The strong reduction of petiole elongation in submerged plants that received exogenous ABA demonstrates the importance of ABA-dependent regulation (Benschop et al., 2005, 2006). ABA-8-hydroxylase was induced as early as 1 h after submergence, and could convert ABA to inactive phaseic acid (Voesenek and Bailey-Serres, 2015). The *PDC1* gene is required during anoxia stress (Kürsteiner et al., 2003). In this study, expression of the *AdPDC1* gene was down-regulated by ABA in kiwifruit, and overexpression of the *AdPDC1* gene in *Arabidopsis* inhibited seed germination and root length under ABA treatment (Figs. 4a, c and 5). These results suggested that ABA might negatively regulate the *AdPDC1* gene under waterlogging stress.

4.3. Kiwifruit AdPDC1 is not required for cold stress and for osmotic stress on early growth

Increase of *PDC* expression has been reported by cold and mannitol treatments in *Arabidopsis* (Conley et al., 1999; Dolferus et al., 1997; Kürsteiner et al., 2003) and by cold treatments in rice and maize (Christie et al., 1991). Low temperature could induce the expression of *HbPDC1* in bark and leaves of the rubber tree (Long et al., 2015). In addition, the overexpression of *AtPDC1* results in low-temperature sweetening tolerance in the transgenic potato (Pinhero et al., 2011). Mannitol treatments could induce the expression of *AtPDC1* in *Arabidopsis*, but no considerable differences in the germinating rate under mannitol stress conditions were observed between WT and *PDC1* mutant plants, indicating that germination under osmotic stress conditions is presumably independent of *PDC1* (Kürsteiner et al., 2003). In the present study, low temperature and drought could not induce the expression of the *AdPDC1* gene in kiwifruit (Fig. 2), and overexpression of the *AdPDC1* gene in transgenic *Arabidopsis* did not enhance resistance to mannitol stress at stage of seed germination and early seedlings (Figs. 4 and 5) and cold stress at 5 weeks old seedlings (Fig. 6). Thus, kiwifruit *AdPDC1* is not required for cold stress and for osmotic stress on early growth.

4.4. Kiwifruit AdPDC1 is induced by salt and heat stresses

The *AtPDC1* gene could be induced by salt in *Arabidopsis* (Kürsteiner et al., 2003). Our results showed that expression of the *AdPDC1* gene was induced in kiwifruit after treatment with salt (Fig. 2b) and heat (Fig. 2e) stress, but the increase of expression was much lower than that during waterlogging stress. These results suggested the involvement of ethanolic fermentation in response to salt and heat stress. However, overexpression of the *AdPDC1* gene could not enhance resistance to salt stress at the seed germination and early seedling stages (Figs. 4 and 5), indicating that *PDC1* seems not to be essential in salt stress on early growth. Thus, ethanolic fermentation might be a part of salt stress adaptation, but how much it contributes to salt stress tolerance has to be investigated.

In summary, the results showed that the increase of *AdPDC1* expression during waterlogging stress was much higher than that during other environmental stresses and that the kiwifruit *AdPDC1* gene is required during waterlogging but might not be required during other environmental stresses. Expression of the *AdPDC1* gene was down-regulated by ABA in kiwifruit, and overexpression of the *AdPDC1* gene in *Arabidopsis* inhibited seed germination and root length under ABA treatment, indicating that ABA might negatively regulate the *AdPDC1* gene under waterlogging stress.

Authors' contributions

ZJY and GZR designed the experiments and wrote the manuscript. ZJY and HSN performed the experiments. ZJY, WG and XJP analyzed data. ZJY supervised the gene expression data analyses. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2016.05.009.

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