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Activated expression of *EsHD1* enhances drought tolerance in tobacco plants via mitigation of reactive oxygen species-mediated membrane damage

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Abstract: It is well documented that the homeodomain-leucine zipper (HD-Zip) transcription factors play diverse roles during plant growth and development. The Class I HD-Zip genes are shown to be involved in the regulation of abiotic stress responses and tolerance. Herein, a novel Class I HD-Zip gene, *EsHD1*, was isolated from *Eutrema salsugineum*, and an investigation was performed of its physiological functions in response to drought stress. The analyses of gene expression profiles revealed that the *EsHD1* transcripts quickly accumulated upon exposure to various abiotic stress conditions or abscisic acid treatments. Furthermore, the overexpression of *EsHD1* in tobacco plants conferred improved tolerance to drought stress. The *EsHD1*-overexpressing lines had lower levels of reactive oxygen species (ROS), ion leakage, and malondialdehyde, but they manifested higher activities of antioxidant enzymes and the transcription of pathogenic-related genes as compared to wild-type plants under drought stress conditions. Therefore, our findings demonstrated that *EsHD1* positively regulated plant drought tolerance via alleviation of ROS-mediated membrane damage.

Key words: *Eutrema salsugineum*, HD-Zip, drought stress, membrane stability, transgenic tobacco

1. Introduction

In nature, plants as sessile organisms cannot change their location and escape adverse effects from multiple environmental challenges including extreme temperature, high salinity, and drought (Zhu, 2001; Yu et al., 2008). To survive these challenges, plants have developed efficient strategies to activate a wide array of adaptive responses at the molecular, cellular, and whole-plant levels (Mittler, 2006; Yu et al., 2008).

Drought is one of the major obstacles that severely impede plant growth and agricultural production worldwide. Drought stress often induces overproduction of reactive oxygen species (ROS) including singlet oxygen ($^1O_2$), superoxide radicals ($O_2^{\cdot-}$), hydroxyl radicals (.OH), and hydrogen peroxide (H_2O_2), which cause damage to membrane structures via oxidization of lipids and proteins (Gill and Tuteja, 2010). Meanwhile, plants have also evolved enzymatic and nonenzymatic antioxidant systems to alleviate ROS-associated membrane injuries. Previous studies have shown that enzymatic systems such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) play pivotal roles in the stabilization of membranes by scavenging cellular ROS (Perl et al., 1993; Yu et al., 2008; Zhou et al., 2015). In addition to antioxidant enzymes, cell membrane stability can be achieved by promoting the synthesis of osmotic solutes (such as soluble sugar and proline) and other proteins in plants under various stresses conditions (Bartels and Sunkar, 2005; Umezawa et al., 2006). Intriguingly, pathogenic-related (PR) proteins have been found to mediate abiotic stress responses and stabilize membrane structures in plants. A large number of studies have reported that the overexpression of *PR* genes, such as pepper *PR1* and *Arabidopsis PR2* and *PR4*, confers tolerance of transgenic plants to various abiotic stresses (Sarowar et al., 2005; Cabello and Chan, 2012). Furthermore, several transcription factors (TFs) have been demonstrated to enhance abiotic stress tolerance in plants through upregulated expression of *PR* genes. The overexpression of *HaB1* and *AtHB13* obviously enhanced the tolerance to salt and drought stresses by the induction of *PR* genes, including *PR2*, *PR4*, and *Glu*

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(Cabello and Chan, 2012). Concurrently, these transgenic plants exhibited better membrane stability as compared to wild-type (WT) plants, indicating that the stabilization of membranes is essential to plant adaption to adverse stress conditions.

Eutrema salsugineum is a model plant for studying abiotic stress because of its extreme tolerance to cold, salt, and drought stress (Inan et al., 2004). In a previous study, a homolog HD-Zip gene was differentially expressed in a full-length cDNA library constructed from abiotic stress-treated *Eutrema* plants (Taji et al., 2008), implying that this gene may participate in stress responses. However, there was no information about the roles of the HD-Zip gene from *E. salsugineum*. Herein, the main aims of our study were to investigate the functions of the HD-Zip gene from *E. salsugineum*, designated as *HD1*, under drought stress conditions. The overexpression of *EsHD1* in plants substantially enhanced the tolerance to drought stress via increased antioxidant enzymatic activities and transcription levels of several *PR* genes.

2. Materials and methods

2.1. Plant materials, growth conditions, and stress treatments

Seeds of *Nicotiana tabacum* and *Eutrema salsugineum* were utilized in our experiments. After initial sterilization with 0.1% (w/v) $HgCl_2$ for 5 min, followed by three rinses with sterilized water, the seeds were placed and grown on MS agar medium with 3% sucrose at 23 °C under a 16-h light/8-h dark cycle in a greenhouse. To shorten the flowering time of *E. salsugineum*, 2-month-old seedlings were moved from 23 °C to 4 °C for 6 weeks for vernalization (Zhu et al., 2014). For abiotic stress or abscisic acid (ABA) treatments, 15-day-old *E. salsugineum* seedlings were exposed to 4 °C, 30% PEG 6000, 300 mM NaCl, or 100 μM ABA for different time periods (0 h, 6 h, 12 h, 24 h, and 48 h).

2.2. Gene cloning, plasmid construction, and plant transformation

The coding sequence of *EsHD1* was cloned from *E. salsugineum* (accession no. AK353311) through polymerase chain reaction (PCR). The PCR-amplified products were separated and inserted into the pGEM-T Easy Vector (Promega, Madison, WI, USA). *EsHD1* was then digested by XbaI and KpnI and ligated into a plant binary vector, pSuper1300, that contained a constitutive promoter consisting of the octopine synthase (ocs) transcriptional activating element affixed to the mannopine synthase 2' (mas2') transcriptional activating element and a minimal promoter (Lee et al., 2007).

The resulting vector was transferred into *Agrobacterium tumefaciens* (GV3101) and the positive cloning was selected to transform tobacco plants via the leaf-disc transformation method. The transformed plants were screened on MS agar medium containing 50 mg/L hygromycin, and fragments of *EsHD1* were amplified and employed as selection markers. The primers used in the above experiments are listed in Supplementary Table 1 (on the journal's website).

2.3. Drought-tolerant assays

To assess the performance of WT and transgenic plants under drought stress, the tobacco plants were initially grown in plots with soil for 5 weeks under well-watered conditions. Subsequently, the 5-week-old tobacco plants were subjected to water withholding for 10 days and 16 days, respectively. Furthermore, the untreated and drought stress-treated plants were used for the investigation of several important physiological parameters.

2.4. Gene expression analyses

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the contaminated DNA was digested by RNase-free DNase I (Invitrogen). Aliquots (500 ng) of total RNA were then reversely transcribed into cDNA as the template of quantitative real-time (qRT)-PCR analyses. The qRT-PCR reactions were carried out on an ABI 7500 real-time PCR system as recently described by Zhu et al. (2014). *EsActin2* or *NtActin* was used as an internal control gene to normalize the expression levels of targeted genes. The primers exploited in the above experiments are listed in Supplementary Table 1.

2.5. Determination of physiological parameters

The relative water content (RWC) was determined according to the method described by Zhu et al. (2014). The amount of chlorophyll and photosynthetic efficiency (Fv/Fm) were measured as previously described by Zhang et al. (2009). The leaf water potential (LWP) was assessed as recently reported by Lu et al. (2013). The levels of malondialdehyde (MDA) and ion leakage (IL) were monitored as previously described by Zhou et al. (2012). The levels of the two major types of ROS (H_2O_2 and $O_2^{\cdot-}$) were quantified as described by Yadav et al. (2012). Antioxidant enzymatic activities including SOD, CAT, POD, and APX were examined according to the method reported by Xie et al. (2008).

2.6. Histochemical staining analyses

In vivo localization of H_2O_2 and $O_2^{\cdot-}$ in tobacco leaves was determined by 3,3'-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining, respectively. The NBT and DAB staining was performed according to a method described by Yadav et al. (2012). In addition, trypan blue staining was used to assess the membrane integrity of plant cells as reported by Miller et al. (2010).

2.7. Statistical analysis

Statistical analyses were performed by Duncan's multiple range test. The mean values ± SD of at least three replicates

are shown, and different letters above the bars in figures indicate significant differences between the WT and each transgenic line within well-watered or drought treatments at $P < 0.05$.

3. Results

3.1. Isolation and sequence characterization of *EsHD1*

The full-length cDNA of *EsHD1* was isolated from *E. salsugineum* plants. The *EsHD1* cDNA was 1194 bp in length, which contains a 218-bp 5' untranslated region (UTR), an 888-bp open reading frame (ORF), and an 88-bp 3' UTR. The ORF of this gene encoded a predicted polypeptide of 285 amino acids with a molecular mass of 33.46 kDa and a theoretical pI of 5.25. Multiple alignments by the ClustalW2 program revealed that the EsHD1 protein shared high identity with other HD-Zip genes from different plant species. Additionally, the analysis of conserved domains by the SMART program showed that EsHD1 contained one conserved HD domain and one leucine zipper region (Figure 1a). To analyze the evolutionary relationship between EsHD1 and other Class I–IV HD-Zip TFs from other plant species, a phylogenetic tree was constructed using MEG 4.0 software (Figure 1b). The phylogenetic analyses clearly evidenced that EsHD1 was clustered into the same subgroup of AtHB13, HaB1, and Zmhdz10, which played regulatory roles in abiotic stress response and tolerance in *Arabidopsis* plants. These findings implied that EsHD1 was a member of the Class I HD-Zip TF family and was possibly involved in abiotic stress response.

3.2. Expression profiles of *EsHD1* in response to abiotic stresses

To examine the expression profile of *EsHD1* in response to abiotic stress, qRT-PCR was performed to detect its transcripts in 15-day-old *E. salsugineum* seedlings exposed to treatments with 4 °C, PEG 6000, NaCl, or ABA. Our data

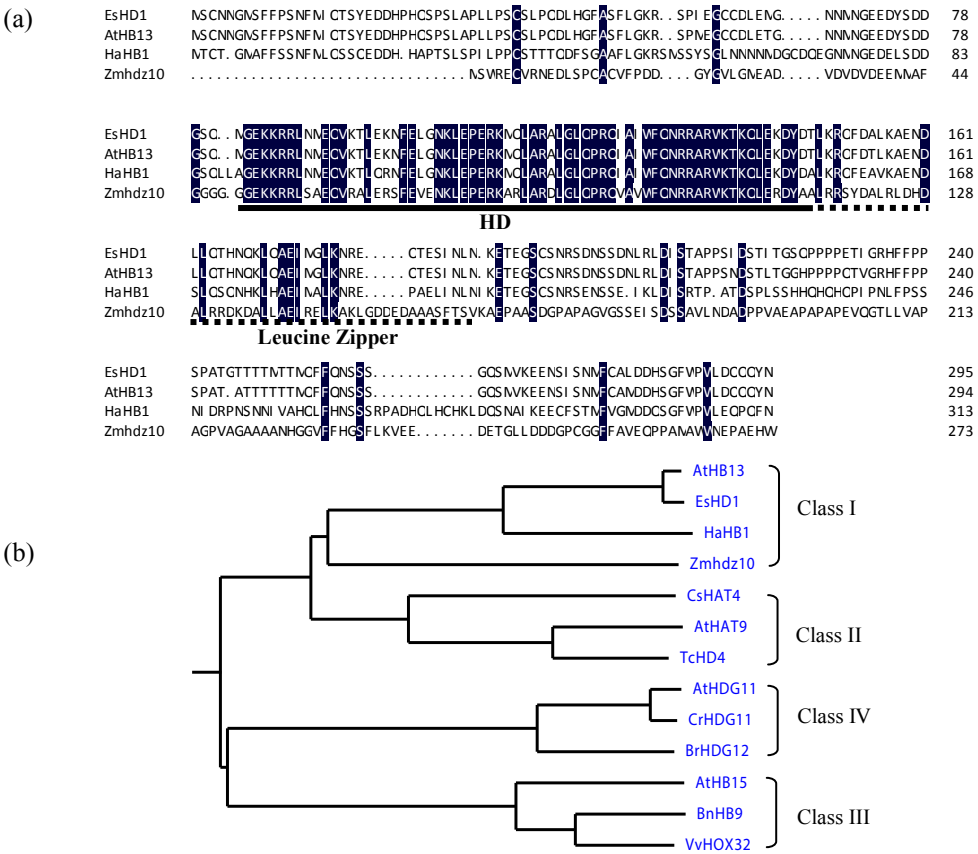

Figure 1. Sequence alignment and phylogenetic relationship of EsHD1 with HD-Zip TF family. (a) Sequence alignment of EsHD1 and several Class I HD-Zip TFs from different plant species. The HD and leucine zipper domains in EsHD1 are underlined by solid and dashed lines, respectively. (b) A phylogenetic tree of EsHD1 and other plant Class I–IV HD-Zip transcription factors was constructed using the neighbor-joining method by MEGA software (version 4.0). Locus names of all HD-Zip genes are as follows: EsHD1 (XP_006390971), CrHDG11 (XP_006301097), HaHB1 (AAA63765), Zmhdz10 (AFT92045), AtHB13 (NP_177136), AtHAT9 (AEC07356), AtHB15 (AEE32762), TcHD4 (XP_007047635), BrHAT22 (XP_009133094), AtHDG11 (NP_177479), BrHDG12 (XP_009110377), CsHAT4 (XP_006477139), BnHB9 (CDY02297), and VvHOX32 (XP_002281868).

indicated that the expression of *EsHD1* was considerably elevated when plants were subjected to treatments with various abiotic stresses and ABA. The expression of *EsHD1* was apparently upregulated by the 4 °C treatment within 24 h and was then sharply decreased up to 48 h as compared to that of the control plants (0 h) (Figure 2a). After exposure to PEG 6000, the *EsHD1* transcripts accumulated rapidly in 6 h, followed by an obvious decline in the 24-h duration (Figure 2b). In the treatments with 300 mM NaCl, the transcription level of *EsHD1* reached its maximum value in the 12-h treatment and thereafter decreased up to 48 h (Figure 2c). In addition, the treatments with ABA resulted in the transient induction of *EsHD1* in the 6-h duration and the expression of *EsHD1* was markedly upregulated by the tendency of a gradual increase until 12 h after the ABA treatments; subsequently, a quick reduction was observed after 24 h of ABA treatments (Figure 2d).

3.3. Overexpression of *EsHD1* enhances drought tolerance in transgenic tobacco

To explore whether the overexpression of *EsHD1* in plants increased drought resistance, we developed 8 independent transgenic tobacco lines carrying the coding sequence of *EsHD1* under the control of a superpromoter (Figure 3a). These T2 transgenic lines were further confirmed by PCR amplification of *EsHD1* fragments (Figure 3b). The expression levels of *EsHD1* in transgenic plants were determined by qRT-PCR analyses. As shown in Figure 3c, the *EsHD1* transcripts were detected in these transgenic lines, but not in WT plants. The two transgenic lines L3 and L6 displayed higher transcription levels than the other lines, and L3 and L6 were selected for drought tolerance assays.

To further assess the performance of *EsHD1*-overexpressing lines under drought stress, WT plants and transgenic lines (L3 and L6) were used for drought tolerance assays. The seedlings of WT and transgenic lines were regularly watered for 5 weeks and were then subjected to drought stress imposed by withholding irrigation. Under well-watered conditions, the transgenic lines exhibited no obvious phenotypic differences during plant growth and development as compared to WT plants. However, the transgenic lines manifested more tolerance to drought stress than WT plants (Figure 4). After 10 days of irrigation deprivation, curly and wilting leaves were more evident in the WT plants than in L3 and L6 (Figure 4). Upon exposure to 16 days of drought treatments, most leaves of WT plants displayed more severe symptoms of wilting and yellowing than those of L3 and L6.

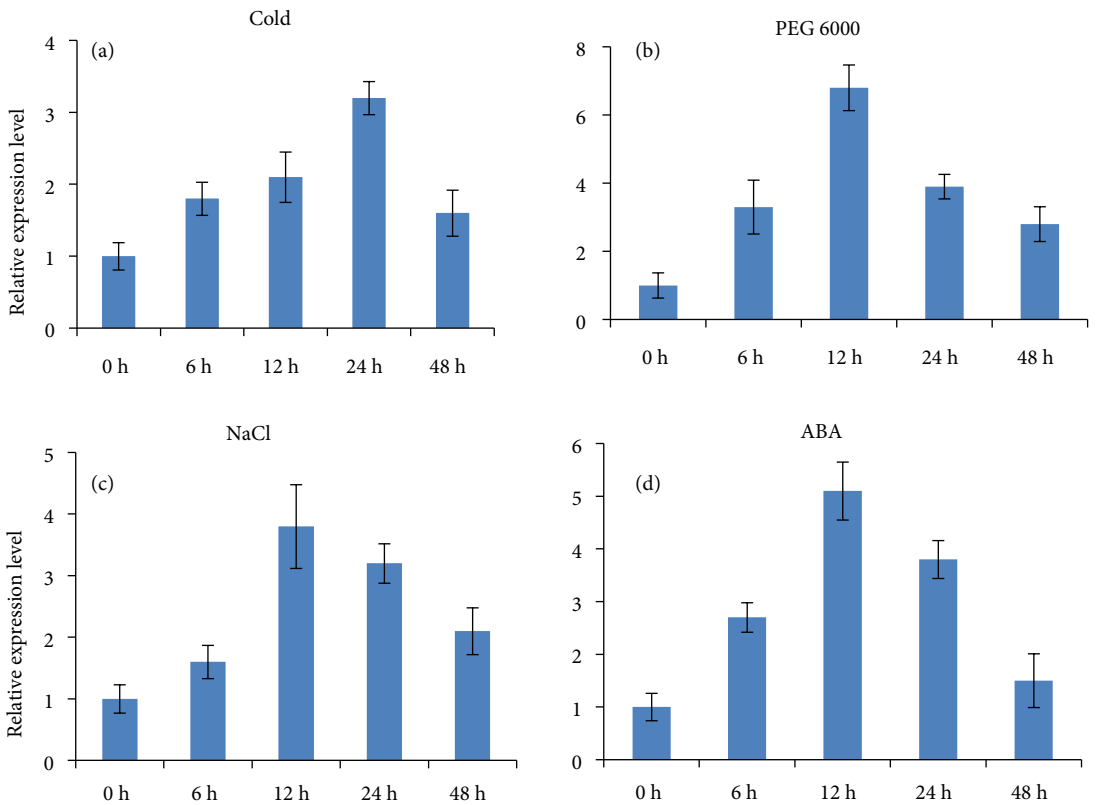

Figure 2. qRT-PCR analyses of expression patterns of *EsHD1* in response to abiotic stress or ABA treatments. Fifteen-day-old *E. salsugineum* seedlings exposed to cold (4 °C) (a), PEG 6000 (30%) (b), NaCl (300 mM) (c), or ABA (100 μM) (d) for different time points (0, 6, 12, 24, and 48 h) were sampled to extract total RNA. *EsActin2* was used as an internal reference. Vertical bars indicate standard deviation of mean.

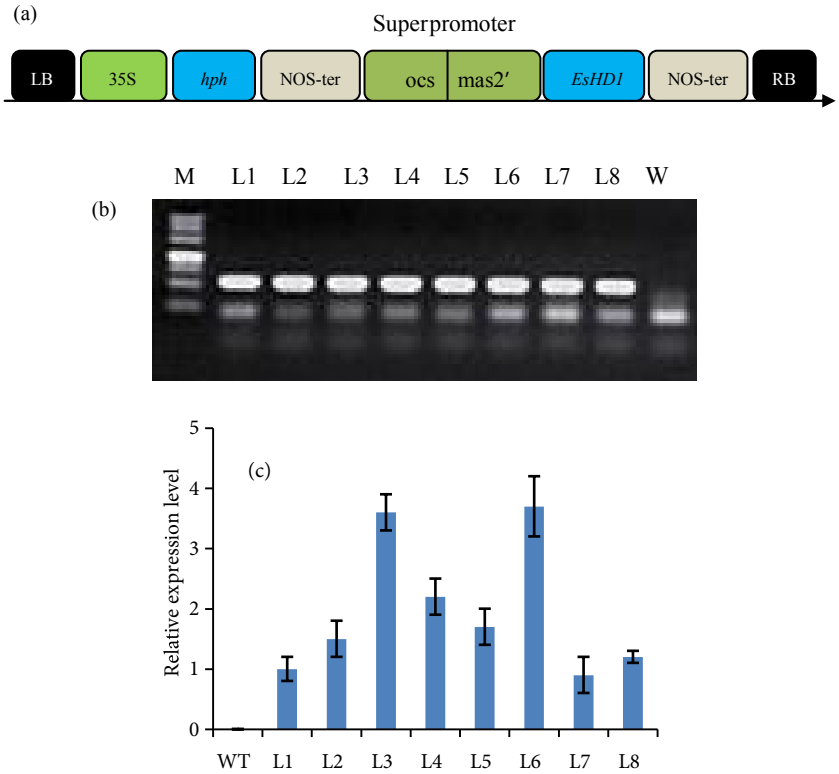

Figure 3. Molecular characterization of transgenic tobacco plants harboring *EsHD1*. (a) Schematic diagram of plant binary vector pSuper1300::*EsHD1*. (b) Genomic PCR amplification of *EsHD1* in WT and transgenic plants. (c) qRT-PCR analyses of the *EsHD1* transcripts in WT and transgenic lines. M, DNA maker; L1–L8: T2 transgenic lines; W: WT plants. Vertical bars indicate standard deviation of mean.

3.4. Transgenic plants exhibit better physiological status under drought

To evaluate the ability of transgenic plants to conserve water under drought stress, the RWC and LWP in leaves from WT and transgenic lines (L3 and L6) were measured. Our data showed that there was no significant difference between the values of RWC of WT and transgenic lines under well-watered conditions. Drought stress caused a considerable decrease in the RWC of WT and transgenic lines, while the transgenic lines exhibited higher RWC than WT plants. As shown in Figure 5a, the RWC values of L3 and L6 were substantially higher than those of WT plants after 10 days or 16 days of drought treatments. Furthermore, a similar tendency was observed in the change of LWP. After 10 days or 16 days of withdrawing water, the LWP values of WT plants were remarkably lower than those of L3 and L6, respectively (Figure 5b).

The chlorophyll content is an important indicator for assessing abiotic stress tolerance in plants (Charrier et al., 2012; Lü et al., 2013). In this work, the amount of chlorophyll displayed evident alterations with a gradual decrease accompanied by duration of drought treatments. As shown in Figure 5c, L3 and L6 had higher chlorophyll content than WT plants after 10 days or 16 days of exposure to drought stress. To further measure the photosynthetic potential of transgenic lines, the chlorophyll fluorescence was determined in the WT and transgenic lines. Our data revealed that the Fv/m ratio in WT plants was distinctly lower than that in L3 and L6, but no differences were observed under well-watered conditions (Figure 5d).

3.5. Transgenic plants display lower levels of ROS, IL, and MDA with better membrane integrity

The *EsHD1*-overexpressing lines manifested phenotypic characterization of improved drought tolerance, implying that the transgenic lines might experience lower oxidative injuries imposed by drought stress in comparison to WT plants. In this work, the levels of H_2O_2 and $O_2^{\cdot-}$ in WT and transgenic lines (L3 and L6) were detected. Localization of H_2O_2 and $O_2^{\cdot-}$ in the leaves from WT and transgenic lines was determined by histochemical staining with DAB and NBT. Our data indicated that L3 and L6 exhibited similar DAB and NBT staining patterns as those observed in WT plants under well-watered conditions, whereas the WT plants displayed more staining spots than L3 and L6 under drought stress (Figure 6a and 6b). Moreover, the content of H_2O_2 and $O_2^{\cdot-}$ in the leaves from WT and transgenic lines was quantified (Figure 6c and 6d). After 10 days or

Figure 4. Effects of *EsHD1* overexpression on transgenic tobacco plants and phenotypic characterization of WT and transgenic tobacco lines (L3 and L6). Five-week-old WT and transgenic plants were deprived of water irrigation for 10 days and 16 days, respectively. BD, before drought; AD-10 d or -16 d , after 10 days or 16 days of exposure to drought treatments.

16 days of exposure to drought stress, L3 and L6 produced higher amounts of H_2O_2 and $O_2^{\cdot-}$ than did WT plants. These findings demonstrated that the overexpression of *EsHD1* obviously reduced drought stress-induced ROS accumulation in plants.

The reduced accumulation of ROS in the transgenic plants implied that the overexpression of *EsHD1* alleviated oxidative damages to cell structures under drought stress. The levels of IL and MDA have been taken as pivotal stress indicators of physical damage to cell membranes (Yadav et al., 2012; Zhou et al., 2015). To examine the degree of oxidative injury in the transgenic lines, the levels of IL and MDA were measured. Our results showed that the IL in L3 and L6 was markedly lower than that in WT plants under drought stress (Figure 7a). As shown in Figure 7b, L3 and L6 displayed lower values of IL and MDA than WT plants after 10 days or 16 days of exposure to water withdrawal. Nevertheless, no observable differences were detected between the levels of IL and MDA in the WT and each transgenic line. Furthermore, we analyzed the cell membrane integrity of the WT and transgenic lines under well-watered and drought stress conditions via trypan blue staining. As shown in Figure 7c, fewer blue staining spots were observed in L3 and L6 than in WT plants under drought stress.

3.6. Overexpression of *EsHD1* enhances antioxidant enzymatic activities and the expression of the PR-related gene

Transgenic plants experienced milder membrane injuries under drought stress, which contributed to the augmented elimination of ROS and the biosynthesis of certain proteins for relieving ROS-mediated damages. In our experiments, the activities of SOD, CAT, and APX in L3 and L6 were significantly higher than those in WT plants, but there was no obvious difference in POD activity under drought stress (Figures 8a–8d).

Many recent studies reported that the abundant accumulation of PR proteins is conducive to improving abiotic stress resistance in some different plant species via the stabilization of cell membranes (Sarowar et al., 2005; Cabello and Chan, 2012). To investigate whether *EsHD1* could regulate the expression of *PR* genes in tobacco plants, several *PR* genes, such as *PR1* and *PR2*, were selected for analysis of their expression levels. As shown in Figures 8e and 8f, the transcription of *PR1* and *PR2* was obviously higher in the two transgenic lines (L3 and L6) than in the WT plants. Taken together, these results indicated that the overexpression of *EsHD1* was sufficient to enhance the antioxidant enzymatic activities and upregulate the expression of *PR* genes in transgenic tobacco plants.

4. Discussion

Upon exposure to abiotic stress, plants can perceive stress signals and then can be transduced to activate the expression of TFs for regulating a wide array of downstream genes that participate in the response to the stress. It has been shown that some members of the TF family such as MYB, NAC, and HD-Zip are involved in mediating abiotic stress response and tolerance (Yu et al., 2008; Nakashima et al., 2012; Zhu et al., 2014; Zhou et al., 2015). Among these TFs, the HD-Zip TFs are plant-specific modular genes that contain a homeodomain associated to a leucine zipper (Mukherjee and Burglin, 2006; Magnani and Barton,

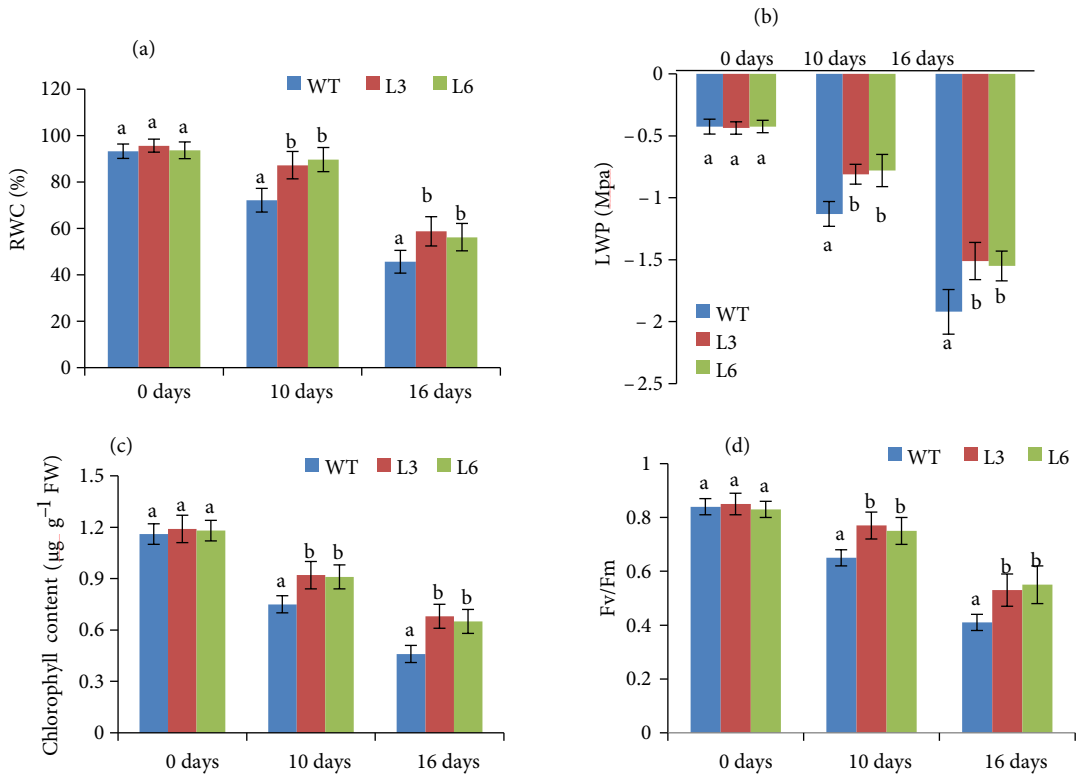

Figure 5. Overexpression of *EsHD1* improved water status and increased chlorophyll content and photosynthetic efficiency in transgenic tobacco lines (L3 and L6). Five-week-old WT and transgenic plants were deprived of water irrigation for 10 days and 16 days, respectively. These untreated and drought stress-treated plants were sampled to determine RWC (a), LWP (b), chlorophyll content (c), and average values of Fv/Fm (d) of WT and transgenic lines (L3 and L6). Vertical bars indicate standard deviation of mean. Different letters show statistically significant ($P < 0.05$) difference.

2011). Many reports have confirmed that the HD-Zip TFs play diverse roles in plant growth and development, and in response to abiotic stresses. Increasing evidence has recently demonstrated that the enhanced expression of some genes encoding Class I HD-Zip TFs significantly elevates the ability of transgenic plants to tolerate freezing, high salinity, and drought stress (Cabello and Chan, 2013; Belamkar et al., 2014; Zhu et al., 2014).

The HD-Zip proteins consist of four subfamilies (HD-Zip I–IV), which were defined based on their conserved regions: a homeodomain (HD) associated with a Leu zipper domain (ZIP), a steroidogenic acute regulatory-related lipid transfer domain (START), and a MEKHLA domain (Schena and Davis, 1992; Mukherjee and Burglin, 2006). Through the analysis of conserved domains, we observed that a putative HD-Zip domain occurs in the N-terminal region of the EsHD1 protein. In addition to the putative HD-Zip domain, other conserved regions, including START and MEKHLA, which only belong to the Class III subgroup of HD-Zip TFs, were not observed in the EsHD1 protein. Furthermore, EsHD1 shared high identity with its counterparts from other plant species including *Arabidopsis thaliana*, *Helianthus annuus*, and *Zea mays*. Moreover, the analysis of the phylogenetic tree revealed that EsHD1 was clustered into the Class I subgroup of HD-Zip TFs from other plant species. Altogether, these findings strongly indicated that EsHD1 was a member of the Class I HD-Zip TF family.

It has previously been reported that *EsHD1* was differentially expressed in the full-length cDNA library generated from *E. salsugineum* plants treated with abiotic stress (Taji et al., 2008), implying that this gene might be involved in the mediation of abiotic stress responses. In our experiments, the transcription levels of *EsHD1* were transiently upregulated after exposure to the treatments with cold, high salinity, and PEG 6000. In addition, its expression also rapidly elevated upon exposure to ABA treatment. The observation was similar to the expression patterns of *Zmhdz10* presented in a recent report by Zhao et al. (2014). The *Zmhdz10* gene encoding a Class I HD-Zip TF was significantly induced by drought, high salinity, and ABA treatments (Zhao et al., 2014). It was thus inferred that *EsHD1* participated in ABA-dependent signaling and played crucial roles in abiotic stress tolerance.

To investigate the physiological roles of *EsHD1* in response to drought stress, we developed transgenic plants

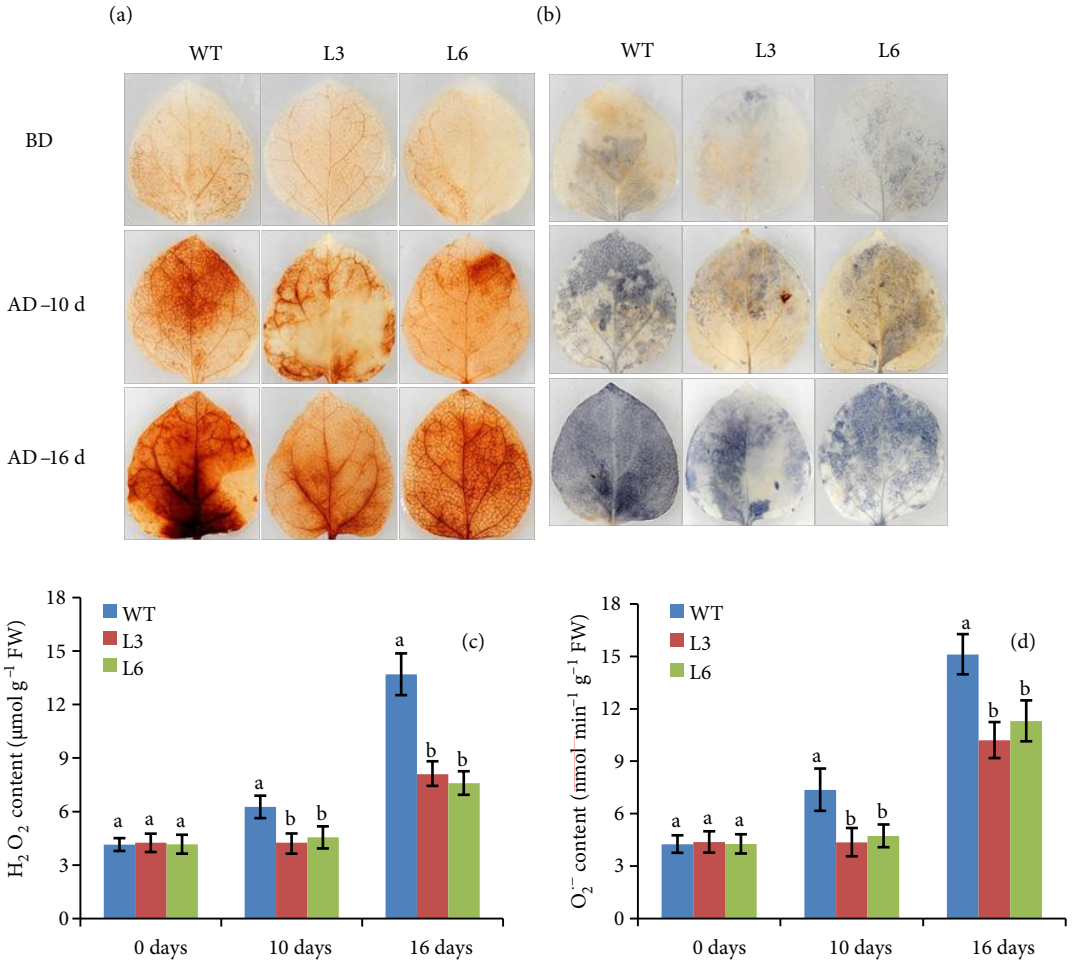

Figure 6. Detection of two major types of ROS (H_2O_2 and $O_2^{\cdot-}$) accumulation in WT and *EsHD1*-overexpressing tobacco lines (L3 and L6). Five-week-old WT and transgenic plants were deprived of water irrigation for 10 days and 16 days, respectively. Untreated and drought stress-treated plants were then used to determine the levels of ROS. (a) In situ detection of H_2O_2 by DAB. (b) In situ detection of $O_2^{\cdot-}$ by NBT. (c) Quantification of H_2O_2. (d) Quantification of $O_2^{\cdot-}$. BD, before drought; AD-10 d or -16 d , after 10 days or 16 days of exposure to drought treatments. Vertical bars indicate standard deviation of mean. Different letters show statistically significant ($P < 0.05$) difference.

harboring the coding sequence of *EsHD1*, which was driven by the constitutive promoter. In this work, it was observed that the expression of *EsHD1* was drastically upregulated in the *EsHD1*-overexpressing tobacco lines. Our transgenic lines exhibited improved the ability to tolerate drought stress as compared to WT plants. The experimental data were consistent with those of recent reports on some members of Class I HD-Zip TFs such as Zmhdz10, HaHB1, and AtHB13 conferring tolerance to adverse environmental conditions (Cabello and Chan, 2013; Zhao et al., 2014). Moreover, to elucidate mechanisms of *EsHD1* conferring plant drought resistance, some experiments were performed to detect alteration in physiological processes. In this work, the values of RWC and LWP in the transgenic tobacco lines were notably higher than those of WT plants under drought stress, suggesting that the transgenic plants had efficient means to acclimate to water-deficient environments. In addition, the chlorophyll content and photosynthetic potential in the transgenic lines was markedly higher than those in WT plants. These results indicated that the change in physiological parameters investigated in the transgenic plants contributed to plant drought tolerance.

Drought stress has been shown to induce oxidative stress that results in peroxidation of membrane lipids and proteins (Gill and Tuteja, 2010). The values of MDA and IL are extensively used as an indicator for assessing the extent of membrane injury in plants (Yadav et al., 2012; Zhou et al., 2015). In this work, the *EsHD1*-overexpressing lines accumulated lower levels of MDA and IL than those of WT plants under drought stress. Thus, the *EsHD1*-transgenic lines had higher cell membrane stability as compared to

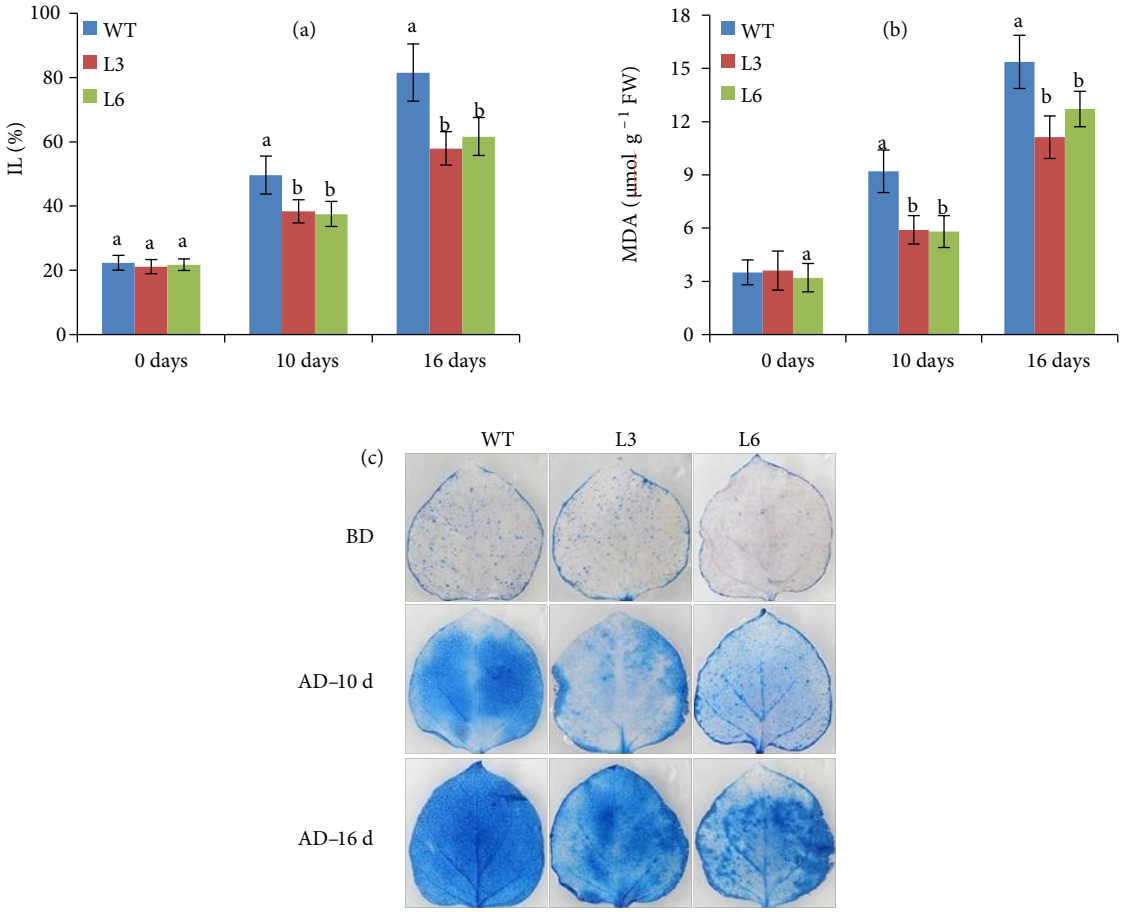

Figure 7. Analyses of IL, MDA, and membrane integrity in WT and *EsHD1*-overexpressing tobacco lines (L3 and L6). Five-week-old WT and transgenic plants were deprived of water irrigation for 10 days and 16 days, respectively. Untreated and drought stress-treated plants were then used to measure IL, MDA, and cell death. (a) IL. (b) MDA. (c) Trypan blue staining. BD, before drought; AD-10 d or -16 d , after 10 days or 16 days of exposure to drought treatments. Vertical bars indicate standard deviation of mean. Different letters show statistically significant ($P < 0.05$) difference.

that of WT plants under drought stress. The stability and integrity of cell membranes are considered as the primary indices that are tightly associated with the ability of plants to tolerate adverse conditions. The results of trypan blue staining further indicated that the transgenic lines were subjected to slighter membrane injuries caused by the overaccumulation of cellular ROS.

To scavenge cellular ROS, plants can adopt complex antioxidant systems in which some important enzymes directly participate in eliminating ROS (Gill and Tuteja, 2010). In this work, the *EsHD1*-overexpressing lines accumulated less ROS than WT plants under drought stress, implying that the overexpression of *EsHD1* enhanced the antioxidant defense systems. As observed, the activities of antioxidant enzymes such as SOD, CAT, and APX were substantially elevated in transgenic tobacco plants under drought stress. Besides the antioxidant systems, several PR proteins have also been shown to improve plant abiotic stress tolerance and membrane stability. The overexpression of plant *PR* genes in transgenic plants obviously increases the resistance to various abiotic stresses via increased stabilization of membranes (Sarowar et al., 2005; Cabello and Chan, 2012). Recently, it has been shown that the overexpression of *HaB1* and *AtB13* in *Arabidopsis* exhibited higher transcription levels of several *PR* genes that stabilize the membranes under abiotic stress conditions. In this work, it was also found that the overexpression of *EsHD1* led to higher expression levels of *PR1* and *PR2* in the transgenic lines as compared to those of WT plants. Taken together, these results suggested that the overexpression of *EsHD1* supported the defense mechanisms of the transgenic lines against ROS-mediated damages imposed by drought stress via increased the membrane stability.

In conclusion, we isolated and characterized a Class I HD-Zip family transcription factor, EsHD1. The expression levels of *EsHD1* were significantly upregulated after exposure to various abiotic stresses or

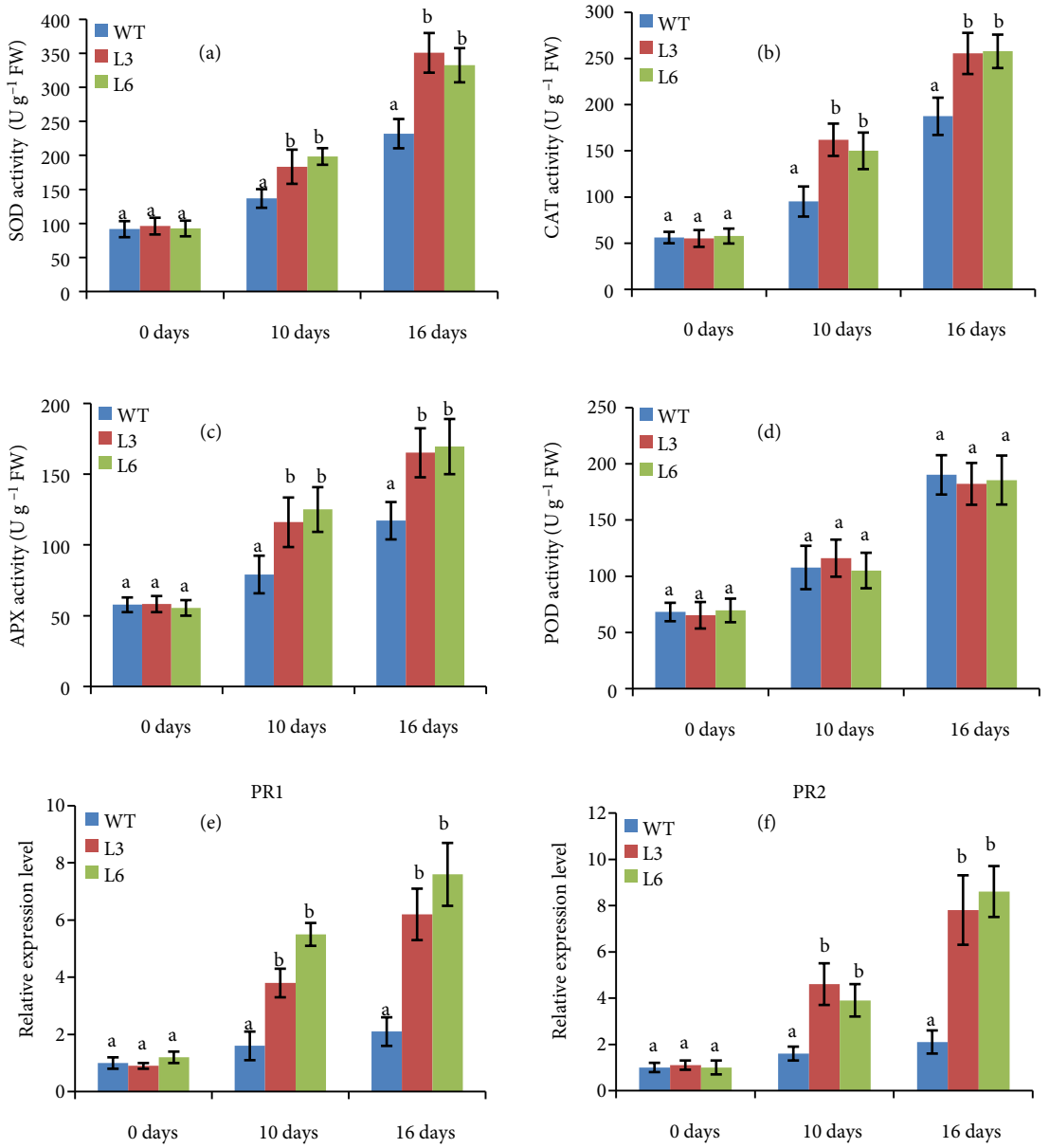

Figure 8. Analyses of antioxidant enzymatic activities and the expression of *PR* genes in WT and *EsHD1*-overexpressing tobacco lines (L3 and L6). Five-week-old WT and transgenic plants were deprived of water irrigation for 10 days and 16 days, respectively. Untreated and drought stress-treated plants were then used to examine the activities of SOD (a), CAT (b), APX (c), and POD (d) and the expression levels of *PR1* (e) and *PR2* (f). Vertical bars indicate standard deviation of mean. Different letters show statistically significant ($P < 0.05$) difference.

ABA treatments. Furthermore, heterologous expression of *EsHD1* significantly enhanced drought tolerance in the transgenic tobacco plants. This work will lead the way to the utilization of *EsHD1* in increasing drought resistance in crop plants via transgene technology.

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Supplementary Table 1. Primers used in this study.

Gene name	Usage	Sequence
EsHD1	Gene overexpression	5'-TCTAGAATGTCTTGTAATAATGGAAT-3' 5'-GGTACCTTAATTGTACTGTTGCTGAT-3'
EsHD1	qRT-PCR or genomic PCR	5'-GCCGGAGACAATCGGTAGAC-3' 5'-TCAAGCCATGGCCAAAAACC-3'
EsActin2	qRT-PCR	5'-CGAGTGTTGTTGGTAGG-3' 5'-ATTGGGTATTTCAAGGT-3'
NtActin	qRT-PCR	5'-TGATGGTGTGAGTCACAC-3' 5'-GGAGCCAAGGCGGTGAT-3'
NtPR1	qRT-PCR	5'-CCCAAAATTCTCAACAA-3' 5'-TAGTATGGACTTTCGCC-3'
NtPR2	qRT-PCR	5'-GGCTTTCTTGCAGCTGC-3' 5'-TCCAAAGTGTTTCTCTG-3'