Gene 692 (2019) 26–34

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Overexpression of a protein kinase gene *MpSnRK2.10* from *Malus prunifolia* confers tolerance to drought stress in transgenic *Arabidopsis thaliana* and apple

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ARTICLE INFO

Keywords:
SnRK2
Abiotic stress
ABA

ABSTRACT

Members of the sucrose non-fermenting-1-related protein kinase 2 (SnRK2) family play central roles in the abscisic acid (ABA) signaling pathway and in mediating osmotic stress signaling and tolerance in plants. Previously, 12 full-length coding sequences that belong to *SnRK2* gene family were identified and cloned in wild apple species *Malus prunifolia*. In this study, one of the members, *MpSnRK2.10*, was overexpressed in *Arabidopsis* and apple to investigate its potential function in response to drought stress. The results showed that overexpression of this gene did not affect plant growth under normal conditions. However, the transgenic plants showed enhanced tolerance to drought, as indicated by the amelioration in phenotype appearance and physiological indices related to drought stress damage. Additionally, transgenic apple plants overexpressing *MpSnRK2.10* exhibited greater sensitivity to ABA compared to wild-type (WT) plants. Moreover, expressions of three stress response genes, *MdRAB18*, *MdRD22,* and *MdRD29B*, were more strongly induced in transgenic apple plants than in the WT when subjected to ABA and mannitol treatments. Taken as a whole, our study suggests that *MpSnRK2.10* has a role in the enhancement of ABA signal transduction in response to stress, and that the manipulation of *MpSnRK2.10* expression could be a feasible approach for improving abiotic stress tolerance in apple and other important crops.

1. Introduction

Because of their immobility, plants are especially vulnerable to unfavorable or stressful growth conditions, e.g. drought, salinity, extreme temperatures, strong light, heavy metals, and hypoxia. Such conditions determine the geographic distribution of plant species and limit agricultural productivity, with drought being the most pervasive and economically damaging (Zhu, 2002, 2016). In order to survive and develop, plants have evolved elaborate mechanisms to perceive, transmit, and respond to stress signals at the molecular, cellular, and physiological levels. In eukaryotes, one of the major mechanisms for the transduction of signals is reversible protein phosphorylation, which is catalyzed by protein kinases and phosphatases (Halford and Hey, 2009). The primary signal caused by drought is hyperosmotic stress (often referred to simply as osmotic stress), which is characterized by a decreased turgor pressure and water loss (Boudsocq and Laurière, 2005). Under osmotic stress conditions, several protein kinases are rapidly activated, for example, mitogen-activated protein kinase (MAPK), calcium-dependent protein kinase (CDPK), calcineurin B-like (CBL) -interacting protein kinase (CIPK), and the sucrose non-fermenting-1 (SNF1) related protein kinase 2 (SnRK2) (Mikołajczyk et al., 2000; Monks et al., 2001; Boudsocq et al., 2004; Kobayashi et al., 2004; Boudsocq and Laurière, 2005).

SnRK2s are monomeric protein kinases containing a short characteristic patch of acidic amino acids (aspartic/glutamic acid-rich) in their C-terminal domains, with a molecular weight of approximately 40 kDa (Halford and Hardie, 1998). They are assigned to the SNF1-related kinase (SnRK) family, a large group of plant serine-threonine protein kinases that are related to the classical SNF1-type kinases from yeast and to adenosine monophosphate (AMP)-activated protein kinases (AMPK) from animals (Hrabak et al., 2003). Protein kinases of SnRK family consists of three distinct subfamilies: SnRK1, SnRK2, and SnRK3. Members of the SnRK1 subfamily possess remarkable homology and functional similarity with SNF1/AMPK, which act as key sensors of

Abbreviations: 6-BA, 6-benzyl aminopurine; IAA, indoleacetic acid; IBA, indole butyric acid; SAPK, stress activated protein kinase

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https://doi.org/10.1016/j.gene.2018.12.070
Received 17 September 2018; Received in revised form 23 December 2018; Accepted 30 December 2018
Available online 11 January 2019
0378-1119/

cellular energy status to play central roles in the regulation of metabolism (Baena-González and Hanson, 2017). Diverging further from SNF1/AMPK than SnRK1s, SnRK2s and SnRK3s appear to be unique to plant kingdom; they are believed to have arisen from the gene duplication of *SnRK1* and then diverged during evolution, taking on new roles to enable plants to link metabolic and various stress signaling (Halford and Hey, 2009). SnRK3s are also known as calcineurin B-like proteins (CBLs; a plant-specific group of calcium sensors)-interacting protein kinases (CIPKs), and the CBL-CIPK complexes have been identified as key regulators of ion homeostasis in plant responses to salt and nutrient stress in soil (Zhu, 2016). Members of the SnRK2 subfamily, such as SnRK2.6/Open Stomata1 (OST1) and the tobacco homolog of *Arabidopsis* serine/threonine kinase1 (ASK1), were implicated in plant abiotic stress-responsive signaling in light of their osmotic or ABA-dependent activation in early studies (Mikołajczyk et al., 2000; Yoshida et al., 2002). There is now compelling evidence that SnRK2s are major players in plant response to osmotic stress and in ABA signaling pathway (Kulik et al., 2011).

Ten SnRK2 encoding genes were isolated in both *Arabidopsis* (SnRK2.1–2.10; Hrabak et al., 2003) and *Oryza sativa* (SAPK1–10; Kobayashi et al., 2004) genome. Based on phylogeny, SnRK2s have been categorized into three subclasses, denoted as subclasses I, -II, and -III (Kobayashi et al., 2004). Comprehensive analysis by protoplast transient expression assays revealed that all SnRK2s, except SnRK2.9, can be rapidly activated by hyperosmolarity, but that each subclass has a distinct activation pattern in response to abscisic acid (ABA). SnRK2s in subclass I (SnRK2.1, -2.4, -2.5,-2.9, and -2.10 in *Arabidopsis*; SAPK4, -5, -6, and -7 in rice) are not activated by ABA, and members in subclass II are weakly (SnRK2.7 and -8) or not (SAPK1, -2, and -3) activated by ABA. In contrast, three members of subclass III, SnRK2.2, -2.3, and -2.6, as well as their rice orthologs (SAPK8, -9, and -10) are strongly activated by ABA (Boudsocq et al., 2004; Kobayashi et al., 2004). These ABA-activated SnRK2s have been reported to associate with Group A protein phosphatase 2Cs (PP2C) and ABA receptor proteins pyrabactin resistance1/pyrabactin resistance1-like/regulatory components of the ABA receptor (PYR/PYL/RCAR). Together, these proteins make up a central regulatory module in ABA signaling (Fujii et al., 2009), which regulates various developmental and physiological processes including seed maturation, dormancy, germination, seedling development, stomatal behavior, flowering time, and the switch between stress responses and regrowth (Fujii and Zhu, 2009; Fujita et al., 2009; Nakashima et al., 2009; Wang et al., 2013; Wang et al., 2018). In the *snrk2.2/3/6* triple mutant, all examined ABA responses are blocked. This strengthens the theory that ABA-activated SnRK2s are essential components in plant ABA signaling pathway (Fujii and Zhu, 2009). In addition, the rest of SnRK2s are supposed to play key roles in plant ABA-independent osmotic responses, as evidenced by a clear disruption of normal osmotic stress responses in the *Arabidopsis snrk2* decuple mutant lacking all ten SnRK2s. Moreover, even when nine of the *SnRK2s* are knocked out, the severe deficiency in the osmotic responses of the decuple mutant could be largely rescued as long as one of ABA-activated SnRK2s is intact (Fujii et al., 2011). Therefore, based on the above, SnRK2s have been identified as key components of plant osmotic responses both in ABA-dependent and ABA-independent pathways (Kulik et al., 2011; Fujii and Zhu, 2012).

Furthermore, SnRK2s have been proven to function in transgenic plants in responses to various abiotic stresses. Overexpression of *SnRK2.8* enhances drought tolerance in *Arabidopsis* (Umezawa et al., 2004), while ectopic expression of *TaSnRK2.8* from wheat (*Triticum aestivum*) affords tolerance to multiple abiotic stresses in *Arabidopsis* (Zhang et al., 2010). Similarly, *ZmSAPK8* from maize (*Zea mays*) elevates salt stress tolerance in transgenic *Arabidopsis* (Ying et al., 2011). *BdSnRK2.9* from *Brachypodium distachyon* assists in increasing tolerance to drought and salt stresses in transgenic tobacco (Wang et al., 2015), and overexpression of *GhSnRK2.6* in upland cotton (*Gossypium hirsutum*) improves the salt tolerance (Su et al., 2017). This conserved activity of SnRK2s reveals their potential value for engineering abiotic stress tolerance across crop species.

Apple (*Malus domestica*) is one of the most widely cultivated fruit crops in the world. As perennial plants, apple growth and fruit production are continuously impacted by various environmental stresses. To investigate the molecular mechanisms underlying stress response in apple, we previously identified and cloned the sequences of *SnRK2* gene family in *Malus prunifolia*, a wild apple species that is highly tolerant to drought and other abiotic stresses, and that is widely used apple rootstock species in northwest China. The expression of several *MpSnRK2*s was up-regulated under stress treatments, suggesting their involvement in abiotic stress response (Shao et al., 2014). One of these, *MpSnRK2.10*, was induced by drought and ABA treatment, and was found to be highly similar to *Arabidopsis SnRK2.6/OST*1 in sequence (over 88% at amino acid level), implying its positive role in drought tolerance of apple.

In this study, we introduced *MpSnRK2.10* into *Arabidopsis* and apple, in order to further characterize its function in drought response in plants. Our results revealed that *MpSnRK2.10* serves as a positive regulator in drought stress response and ABA signaling, suggesting its potential for genetic improvement of drought tolerance in crops.

2. Materials and methods

2.1. Construction of plant-overexpressing vector for MpSnRK2.10

The Gateway binary vector pGWB411 (P_{35S}-attR1-attR2-FLAG-T_{NOS}) was used for constructing the overexpression (OE) vector. The vector was driven by the Cauliflower mosaic virus (CaMV) 35S promoter and carried the P_{NOS}: *NPTII*: T_{NOS} (kanamycin-resistance) marker. The coding region of *MpSnRK2.10* was introduced into pGWB411 by BP and LR reactions, using pDONR222 as an intermediate donor vector, as specified by the Gateway Technology protocol (Invitrogen). Oligonucleotide primers used in the process are listed in Supplementary Table S1. After confirmation by sequencing, the target plasmid was transferred into *Agrobacterium tumefaciens* strain EHA105 for plant transformation.

2.2. Generation of transgenic Arabidopsis thaliana and apple plants

Wild type (WT) *Arabidopsis thaliana* (Columbia-0) plants were grown in pots at 22 °C under long-day (16-h light) photoperiods and subjected to *Agrobacterium*-mediated transformation via the floral dip method (Zhang et al., 2006). For screening of primary transformants (T_1), mature seeds were surface-sterilized as described by Zhang et al. (2006), and then distributed onto Murashige-Skoog (MS) medium supplemented with 50 mg L^{-1} kanamycin (Kan). Kan-resistant plantlets were transferred into pots and further maintained in a growth chamber for polymerase chain reaction (PCR) confirmation and seed production. Afterward, seeds from selected T_1 plants were screened further on Kan-containing medium to select homozygous lines. Seeds from three independent T_3 homozygous lines with relatively high expression levels (Supplementary Fig. S1) and WT plants were used for subsequent experiments.

The apple cultivar 'Royal Gala' derivative line GL-3 was used for transformation. This genotype has a high regeneration capacity and is susceptible to *Agrobacterium* (Dai et al., 2013). Transformation was carried out as previously described (Wang et al., 2017b). Regenerated buds were transferred to MS medium supplemented with 25 mg L^{-1} Kan and 250 mg L^{-1} cefotaxime for selection, and those showing normal growth were subcultured to develop into green shoots with Kan resistance. Subsequently, PCR was performed to verify positive transgenic seedlings, and quantitative real-time PCR (qRT-PCR) assays were performed to verify the transcription of the exogenous gene. The identified positive lines and non-transformed GL-3 (hereafter referred to as WT) were further propagated and rooted for subsequent treatments.

2.3. RNA extraction, RT-PCR and qRT-PCR

Total RNA was extracted according to a modified CTAB method (Gambino et al., 2008). Residual DNA was removed by RNase-free DNase I (#EN0521, Thermo Scientific, USA). First-strand cDNA was synthesized with the RevertAid First Strand cDNA Synthesis Kit (#K1622, Thermo Scientific, USA) using 2 μg of total RNA for each sample. PCR was performed using the Phusion High-Fidelity PCR Kit (#E0553S, NEB, USA) with cDNA template. qRT-PCR was performed on a Real-Time PCR System (CFX-96, Bio-Rad) using the SYBR®Premix Ex Taq™ II Kit (Code No. RR820A, TaKaRa, Japan). All procedures were carried out according to the manufacturer's instructions. Relative quantification of target genes was determined through the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using *Arabidopsis ACTIN2* (Fujii et al., 2011; Zhao et al., 2016) and/or apple *MALATE DEHYDROGENASE* (*MDH*) (Perini et al., 2014) as internal controls. The specific primers used in the process are listed in Supplementary Table S1.

2.4. Plant materials and treatments

Seeds of WT and transgenic *Arabidopsis* were suspended in 0.05% agarose and stratified in darkness at 4 °C for 3 d, and then spread on potting mix in plastic pots (7 × 7 × 7 cm). The seedlings were cultured in a growth chamber (22 °C, 100 μmol photons $m^{-2} s^{-1}$, 70% relative humidity, and 16-h light photoperiods) with regular watering for three weeks. Drought stress was induced by withholding water for two weeks after a full irrigation. Plants that continued to receive normal irrigation served as the controls. After two weeks, the dehydrated plants were re-watered for recovery. At least 12 and 80 plants per line were used to measuring the physiological indices and survival rates, respectively. The experiments were performed with three biological replicates.

Tissue-cultured WT and transgenic apple plants were initially propagated on MS medium supplemented with 0.3 mg L^{-1} 6-BA and 0.2 mg L^{-1} IAA at 24 °C, with a light level of 60 μmol photons $m^{-2} s^{-1}$ and a 14-h light photoperiods. Root primordia were induced after two weeks of further propagation on MS medium supplemented with 0.5 mg L^{-1} IBA and 0.5 mg L^{-1} IAA. For the osmotic stress treatment, plants with root primordia were transferred to MS medium supplemented with 0 or 150 mM mannitol. For the ABA treatment, plants with root primordia were transferred to MS medium supplemented with 0, 5 or 10 μM ABA. Both osmotic and ABA treatments lasted for one month, and were performed in triplicate using at least 12 plants per line for each replicate. Photographs were taken after the treatments and samples were collected at certain times (see details in the corresponding Figure Legends) for further analysis.

For the drought treatment, apple plants with root primordia were subsequently maintained on MS medium for one month to promote root elongation. Afterwards, the rooted plants were transferred to small plastic pots filled with culture substrate (Pindstrup, Denmark) in a phytotron set at 25 °C, with 150 μmol photons $m^{-2} s^{-1}$ and 16-h light photoperiods. One month later, they were transplanted to larger pots (30 × 26 × 22 cm) filled with a mixture of forest soil, sand and organic substrate (5:1:1 by volume) and grown in a greenhouse. Three months later, WT and transgenic plants were selected for drought treatment. After one-time sufficient irrigation, water supply was interrupted to induce drought stress. Plants that continued to receive regular irrigation served as the controls. Six days later, plants subjected to drought were re-watered for recovery. Drought stress treatment was performed in triplicate with at least five plants per line for each replicate.

2.5. Physiological assays

Malondialdehyde (MDA) content was determined by the thiobarbituric acid reactive substances (TBARS) assay (Jambunathan, 2010). Electrolyte leakage (EL) was measured with a conductivity meter (FE38-Standard, METTLER TOLEDO), as described by Wang et al. (2017a). Accumulation of O_2^- and H_2O_2 were detected by histochemical staining using nitroblue tetrazolium (NBT) and diaminobenzidine tetrahydrochloride (DAB), respectively (Jambunathan, 2010). Chlorophyll was extracted with 80% acetone, and the concentration was measured spectrophotometrically (Li et al., 2013). Root activity was evaluated by the triphenyltetrazolium chloride (TTC) method, and was expressed as equivalents of the reductive product (Comas et al., 2000). Relative water content (RWC) of leaves was calculated using the following formula: RWC (%) = (FW − DW) / (TW − DW) × 100 (FW, fresh weight; DW, dry weight; TW, turgid weight). Photosynthetic rate was measured using a portable photosynthesis system (Li-6400; LICOR) on sunny days (9:00 to 10:00 a.m.). Parameters for measurement included a photosynthetic photon flux density of 1000 μmol $m^{-2} s^{-1}$ and a constant airflow rate of 500 μmol s^{-1}. The concentration of cuvette CO_2 was 400 $cm^3 m^{-3}$ and the temperature was 28 ± 2 °C.

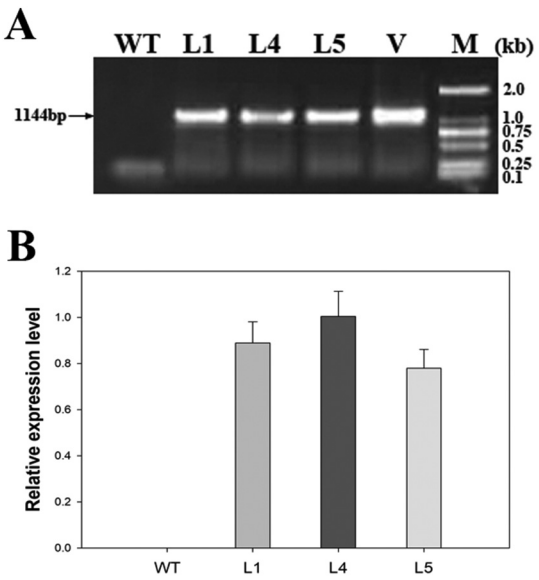

Fig. 1. Ectopic expression of *MpSnRK2.10* in *Arabidopsis*. (A) Verification of transgene presence by PCR. M, molecular marker DL2000; V, positive vector containing *MpSnRK2.10* sequence; WT, non-transformed wild type; L1, L4, and L5, three representative transgenic lines. (B) qRT-PCR analysis of *MpSnRK2.10* expression in transgenic lines. *Arabidopsis ACTIN2* served as the reference gene for normalization. The highest expression level in the transgenic lines (L4) was defined as 1.0. Data are means of three replicates ± standard deviation (SD).

2.6. Statistical analysis

Experimental data were presented as means ± standard deviation (SD). Statistical analysis was performed via one-way analysis of variance (ANOVA) using the IBM SPSS Statistics 20 software. Differences between values were assessed by Duncan analyses ($P < 0.05$).

3. Results

3.1. Ectopic expression of MpSnRK2.10 enhances drought tolerance in Arabidopsis thaliana

As a first step to investigate a potential function related to drought tolerance, we assessed the effect of *MpSnRK2.10* on drought tolerance when expressed constitutively in transgenic *Arabidopsis*. Three transgenic lines showing relatively high expression of *MpSnRK2.10* were selected for subsequent analyses: L1, L4, and L5 (Fig. 1 and Supplementary Fig. S1). No obvious differences in growth were observed between WT and transgenic plants under normal conditions (Supplementary Fig. S2). However, when three-week-old *Arabidopsis* were

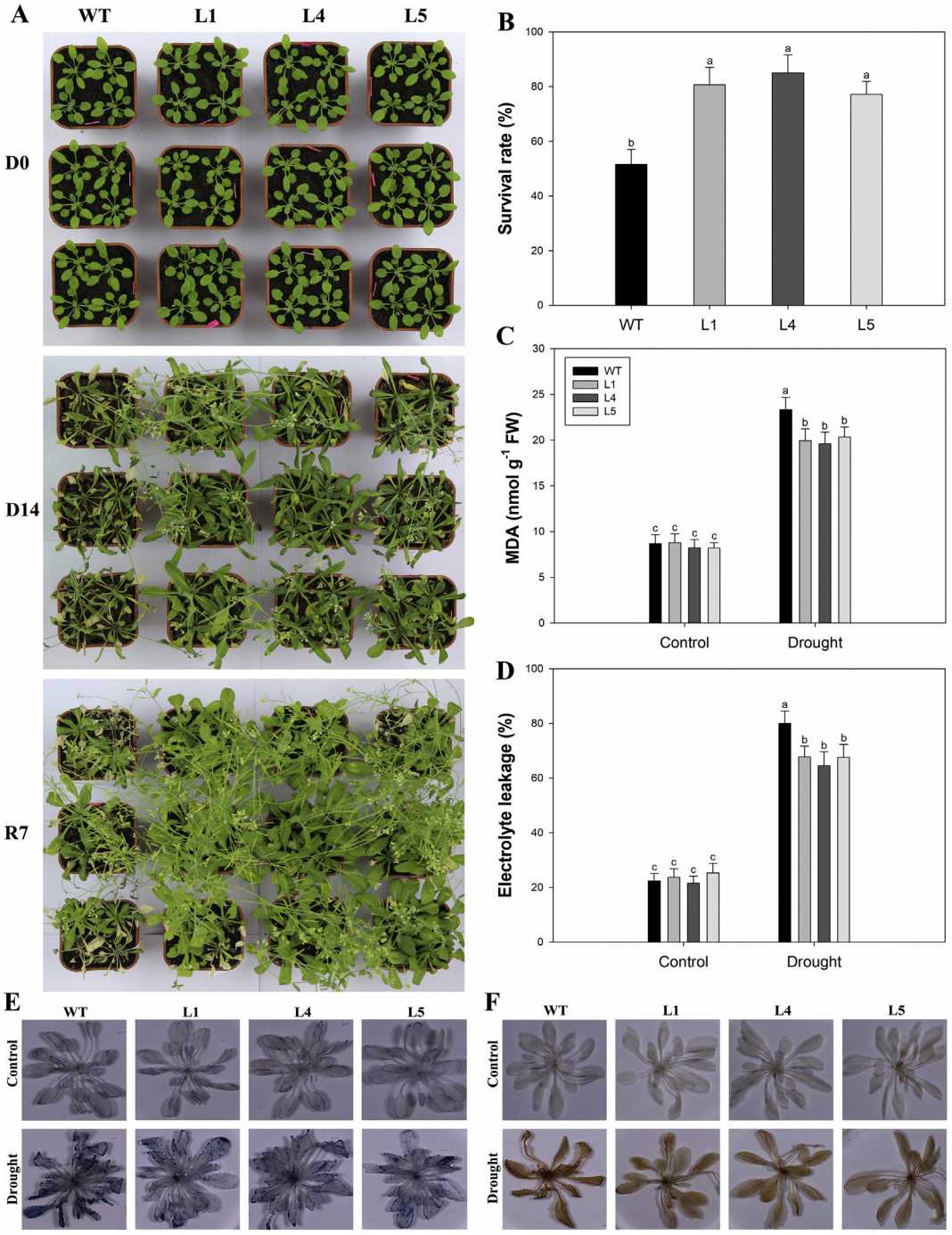

Fig. 2. Ectopic expression of *MpSnRK2.10* enhances drought tolerance in transgenic *Arabidopsis*. (A) Phenotype comparisons between WT and transgenic lines before drought (D0), after drought treatment for 14 days (D14), and seven days after re-watering (R7). Seedlings were three weeks old when drought treatment was initiated. (B) Survival rate for WT and transgenic lines at R7. (C and D) MDA content (C) and electrolyte leakage values (D) of WT and transgenic lines at D14. (E and F) Histochemical staining assay with NBT (E) and DAB (F) to detect O_2^- and H_2O_2 accumulation in rosettes, respectively, on D14. Data are means of three replicates ± standard deviation (SD). Values not followed by the same letters are significantly different according to Duncan's multiple range test ($P < 0.05$).

subjected to withholding water for two weeks, WT plants showed severe symptoms of dehydration, including shriveled leaves, whereas transgenic plants showed only slightly withered phenotypes (Fig. 2A). After re-watering for one week, most of the transgenic plants survived (77–85%), while only about half of the WT plants survived (Fig. 2B).

Drought leads to hyperosmotic stress as well as oxidative stress in plant cells (Zhu, 2016). Accumulation of reactive oxygen species (ROS), assays of electrolyte leakage, and MDA content are common indicators of the degree of cellular damage (Verslues et al., 2006). Under normal conditions, the detected MDA content and relative electrolyte leakage (REL) exhibited little difference between transgenic and WT plants. Drought stress treatment resulted in obviously higher MDA content and REL value, especially in WT plants, at the end of drought stress treatment (Fig. 2C and D). In addition, the accumulation of two kinds of ROS molecules, O_2^-, and H_2O_2 did not change with the overexpression of *MpSnRK2.10* in *Arabidopsis* under normal conditions. However, transgenic plants accumulated less ROS than WT plants did when exposed to drought stress, as indicated by the weaker blue in NBT staining (Fig. 2E) and weaker brown in DAB staining (Fig. 2F) in transgenic plants. These results suggested that overexpression of *MpSnRK2.10* led to enhanced

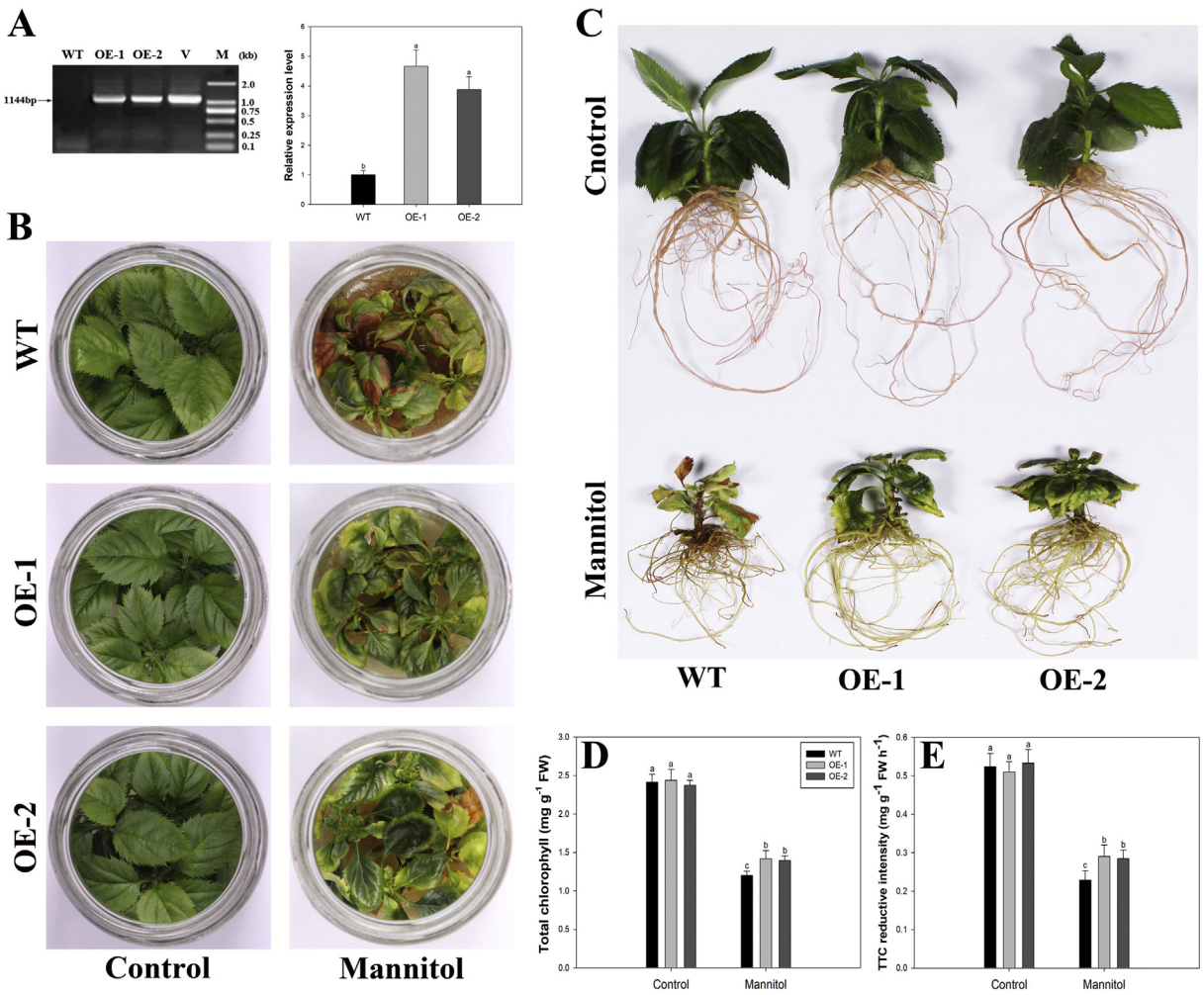

Fig. 3. Overexpression of *MpSnRK2.10* enhances osmotic tolerance in transgenic apple. (A) Overexpression of *MpSnRK2.10* in apple. Left panel: Verification of transgene by PCR. M, molecular marker DL2000; V, positive vector containing the *MpSnRK2.10* sequence; WT, non-transformed GL-3; OE-1 and OE-2, two transgenic apple lines. Right panel: qRT-PCR analysis of apple *SnRK2.10* expression in WT and transgenic lines. Apple *MDH* served as the reference gene for normalization. The expression level in the WT was defined as 1.0. (B and C) Phenotype comparisons between WT and transgenic lines grown on standard MS medium (control) or MS medium supplemented with 150 mM mannitol for one month. Before treatment, the subcultured in vitro shoots were maintained on rooting medium for two weeks to induce root primordia. (D and E) Total chlorophyll content (D) and Root activity (expressed as equivalents of the TTC reduction) (E) of WT and transgenic lines after one month of treatment. Data are means of three replicates ± standard deviation (SD). Values not followed by the same letters are significantly different according to Duncan's multiple range test ($P < 0.05$).

drought stress tolerance in *Arabidopsis*.

3.2. Overexpression of MpSnRK2.10 confers tolerance to osmotic stress in transgenic apple

To gain more insight into the function of *MpSnRK2.10* in drought tolerance, we generated transgenic apple plants overexpressing *MpSnRK2.10*. After RT-PCR and qRT-PCR verification, two over-expressing lines, OE-1 and OE-2, were employed in further experiments (Fig. 3A). Expression analysis showed that *SnRK2.10* transcripts increased by 3.6 and 4.8 times in OE-1 and OE-2, respectively (Fig. 3A). Under normal conditions of in vitro culture, no abnormality in growth or morphology was observed in the transgenic lines (Fig. 3B and C). However, when they were grown on culture medium containing 150 mM mannitol for one month, WT plants displayed obvious chlorosis and necrosis in leaves, and browning in stems and roots, while transgenic plants exhibited milder symptoms (Fig. 3B and C). Meanwhile, chlorophyll content was higher in transgenic plants than in WT, and root activity was greater in transgenic plants than in WT (Fig. 3D and E). These results indicated that overexpression of *MpSnRK2.10* in apple alleviated the damage caused by osmotic stress.

3.3. Overexpression of MpSnRK2.10 in apple enhances drought tolerance

Further experiments were performed with potted plants to investigate the effect of *MpSnRK2.10* on drought tolerance in apple. Little differences in phenotype or physiological indices were observed between WT and transgenic apple plants in the absence of stress (Fig. 4A, top panel). However, when plants were exposed to drought treatment for 6 days, leaves of transgenic plants showed mild wilting than the WT. In addition, the detected RWC was higher in transgenic plants than in WT (Fig. 4B), whereas MDA levels and REL values were lower in transgenic plants than in WT (Fig. 4C and D). When stained with NBT and DAB, leaves of transgenic plants displayed lighter staining, while leaves of WT displayed dark blue staining with NBT and dark brown staining with DAB, indicating that transgenic plants accumulated less ROS under drought stress (Fig. 4F). After re-watering, the majority of leaves of transgenic plants appeared healthy, while widespread necrosis was apparent in leaves of WT plants (Fig. 4A). Moreover, we monitored the rate of photosynthesis (Pn), an important indicator of physiological status, at 3-day intervals. On Day 3 and Day 6 of drought treatment, Pn values were decreased dramatically compared with Day 0 for all plants without differences among each line. However, 3 days after re-

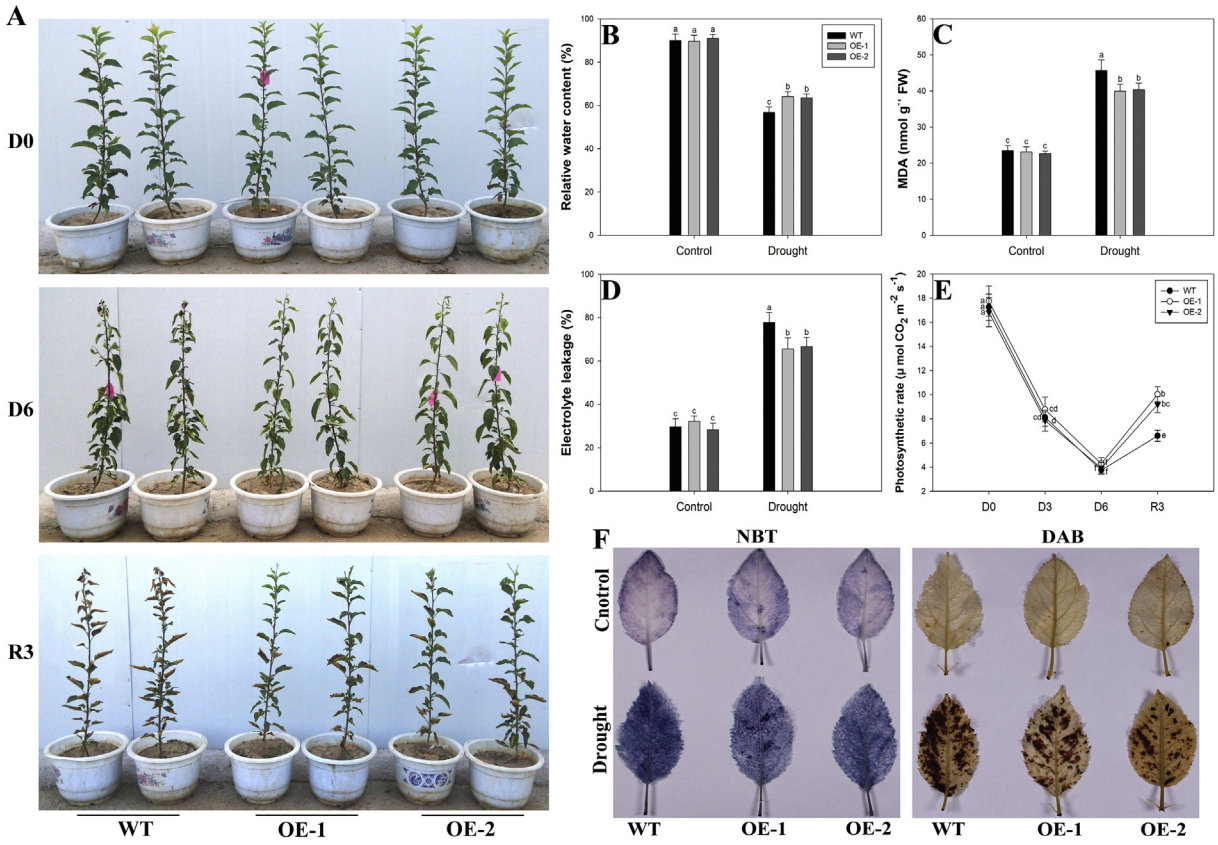

Fig. 4. Overexpression of *MpSnRK2.10* enhances drought tolerance in transgenic apple. (A) Phenotype comparisons between WT and transgenic lines prior to drought treatment (D0), after drought treatment for six days (D6) and three days after re-watering (R3). Potted plantlets were grown in a greenhouse for three months prior to withholding water for 6 days and then re-watering. (B–D) Relative water content (B), electrolyte leakage (C), and MDA content (D) in leaves of WT and transgenic lines on D6. (E) Photosynthetic rate of WT and transgenic lines on D0, D3, D6, and R3. (F) Histochemical staining assay with NBT and DAB to detect O_2^- and H_2O_2 accumulation, respectively, in leaves on D6. Data are means of three replicates ± standard deviation (SD). Values not followed by the same letters are significantly different according to Duncan's multiple range test ($P < 0.05$).

watering, Pn values of the OE-1 and OE-2 lines were approximately 1.5 and 1.4 times higher than those of WT, respectively (Fig. 4E). All of these results indicated that overexpression of *MpSnRK2.10* conferred improved drought stress tolerance in apple.

3.4. Overexpression of MpSnRK2.10 increases ABA sensitivity in transgenic apple

Members of the SnRK2 that belong to subclass III are important regulators of ABA signaling (Kulik et al., 2011), suggesting a similar role for *MpSnRK2.10* in apple. Although *MpSnRK2.10*-overexpressing lines displayed a similar phenotype to WT under standard conditions of in vitro culture (above), transgenic lines exhibited enhanced dwarfing and diminished root growth when they were exposed to exogenous ABA treatments (Fig. 5A and B). Under 5 μM and 10 μM ABA treatments, the average heights of WT plants were 25.9 and 14.2 mm, respectively, whereas the average heights of transgenic plants were 19.0 and 8.9 mm, 25% and 36% shorter than WT (Fig. 5C). Meanwhile, the average root dry weights of WT plants were 27.8 and 16.6 mg, respectively, while the average root dry weights of transgenic plants were 22.3 and 11.6 mg, 20% and 30% less than WT (Fig. 5D). These results suggested that overexpression of *MpSnRK2.10* increased sensitivity to ABA in apple.

3.5. Overexpression of MpSnRK2.10 alters expression of stress-responsive genes

Previous studies demonstrated that the elevated stress tolerance of *SnRK2*-overexpressing plants was accompanied by up-regulation of a suite of stress-responsive genes, including *RESPONSIVE TO COLD-REGULATED 15A* (*COR15A*), *RESPONSIVE TO ABA 18* (*RAB18*), *RESPONSIVE TO DESICCATION 29A* (*RD29A*), *RESPONSIVE TO DESICCATION 29B* (*RD29B*), and *Nicotiana tabacum ABA RESPONSIVE ELEMENT BINDING PROTEIN 1* (*NtAREB1/NtABF2*) (Umezawa et al., 2004; Ying et al., 2011; Wang et al., 2015). Here, we analyzed the expression of *MdAREB2*, *MdRAB18*, *MdRD22* and *MdRD29B*, to investigate whether overexpression of *MpSnRK2.10* induced the expressions of these stress-responsive genes in apple. We found that the expression levels of all detected genes were not altered by the overexpression of *MpSnRK2.10* under non-stress conditions (Fig. 6), indicating that SnRK2.10 does not function upstream of these genes in the absence of stress. However, the expressions of the detected genes, except for *MdAREB2*, showed increased induction in transgenic plants in response to both ABA and mannitol (Fig. 6). These results indicated that overexpression of *MpSnRK2.10* leads to additional up-regulation of some stress-responsive genes in apple under abiotic stresses.

4. Discussion

Identification and expression analyses of the apple *SnRK2* gene family have provided a baseline for further characterization of the function of these genes in stress responses (Shao et al., 2014). In this study, we demonstrated that one of the members, *MpSnRK2.10*, participates in the ABA signaling pathway and confers tolerance to drought stress in transgenic *Arabidopsis* and apple. Under non-stress conditions, we observed no significant phenotypic effects of *MpSnRK2.10*-

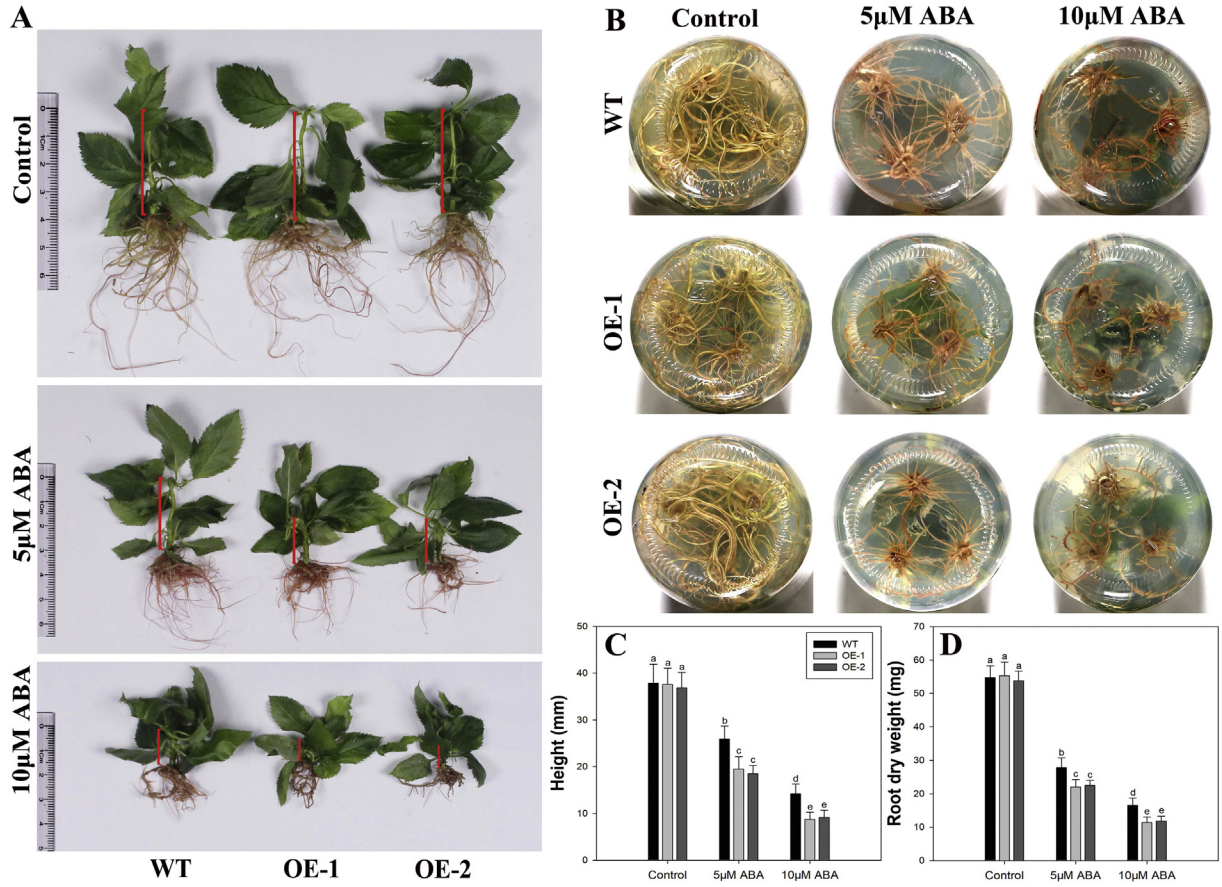

Fig. 5. Overexpression of *MpSnRK2.10* increases ABA sensitivity in transgenic apple. (A and B) Phenotype comparisons between WT and transgenic lines grown on standard MS medium (control), or MS medium supplemented with 5 μM or 10 μM ABA, for one month. The red lines indicate the stem length of plants. Before treatment, the subcultured in vitro shoots were maintained on rooting medium for two weeks to induce root primordia. (C and D) Height (stem length) (C) and root dry weight (D) of WT and transgenic lines after one month of treatment. Data are means of three replicates ± standard deviation (SD). Values not followed by the same letters are significantly different according to Duncan's multiple range test ($P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

overexpression in either *Arabidopsis* or apple, similar to observations of *SAPK4*, *ZmSAPK8*, and *BdSnRK2.9*-overexpressing plants (Diédhiou et al., 2008; Ying et al., 2011; Wang et al., 2015). These results might be explained by the osmotic stress and/or ABA dependent activation of SnRK2 (Fujii and Zhu, 2012). However, in our stress treatment experiments, the transgenic plants exhibited milder symptoms of stress damage and following better restorations compared to the corresponding WT plants in their phenotype appearance. These observations suggested that overexpression of *MpSnRK2.10* conferred drought stress tolerance at the whole-plant level.

Severe dehydration caused by osmotic stress results in structural damage and metabolic dysfunction at the cellular level, which can be indicated by accumulation of ROS and ROS-induced oxidative damage (Verslues et al., 2006). Electrolyte leakage allows relatively quick assessment of the intactness of cell membranes. Accumulation of ROS can be presented visually by the histochemical staining techniques, and the most common measure of ROS-induced damage is lipid peroxidation that can be estimated by the colorimetric assay of MDA generation (Jambunathan, 2010). In the current study, the values of these examined physiological indicators were lower in transgenic plants than in the WT under drought treatments. In addition, loss of chlorophyll content and root activity, other common measurements that quantify cellular damage, were declined in transgenic plants in the mannitol treatment experiment. In summary, detection of these physiological indicators showed that the stress-caused chemical damage was ameliorated in *MpSnRK2.10*-overexpressing plants. All of these results indicated that overexpression of *MpSnRK2.10* conferred tolerance to drought stress in plants at the cellular level, which constituted a foundation for their stress-tolerant phenotype.

Photosynthetic capacity serves as a key index not only for plant growth and biomass estimation, but also for assessing plant health and the influence of environmental stressors. Drought can reduce photosynthesis either directly (e.g., stomatal closure and metabolic alteration) or indirectly, such as the impairment of photosynthetic apparatus due to ROS accumulation (Chaves et al., 2009). It is interesting to note that the decline in photosynthetic rate (Pn) was similar between the transgenic and WT apple plants after 6 days of exposure to drought treatment (Fig. 4E), in spite of an amelioration in phenotype appearance and physiological status for transgenic lines under the same conditions. This finding may be attributed to the enhanced transduction of stress response signals in *MpSnRK2.10*-overexpressing plants. According to a very recent study (Wang et al., 2018), reciprocal regulation of the ABA core signaling components and the Target of Rapamycin (TOR) complex represents a critical mechanism that balances growth and stress response for plants. Based on phylogeny, *MpSnRK2.10* belongs to subclass III, members of which are known as essential components of ABA signaling (Fujita et al., 2013). Excessively activated SnRK2.10 in transgenic plants may facilitate the switch from growth to stress response, thus leading to a rapid decrease in Pn. We also noticed a significantly higher value of Pn in the transgenic plants than in the WT after release of the drought stress. This apparent resilience of photosynthesis in the transgenic lines, in conjunction with the observed

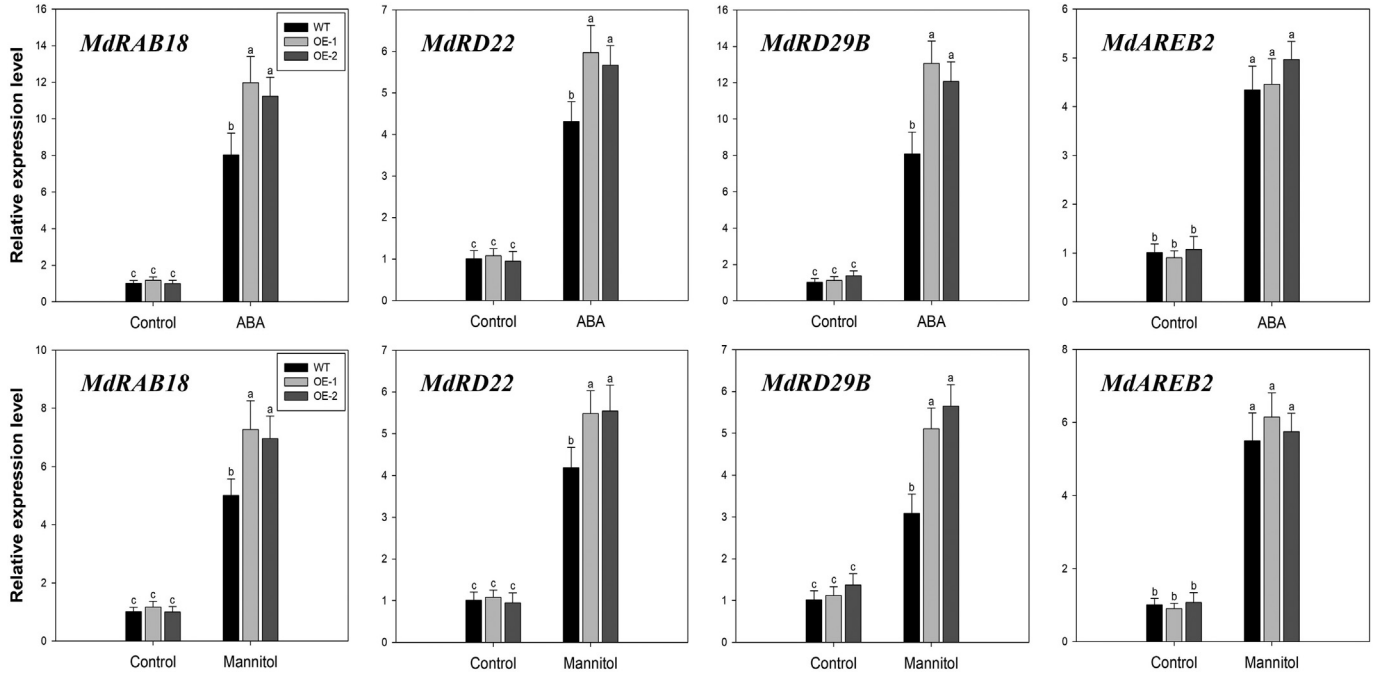

Fig. 6. Overexpression of *MpSnRK2.10* affects expression of stress-responsive genes in transgenic apple. Samples were collected from the WT and transgenic-in vitro apple plants after 10 day treatment with 10 μM ABA or 150 mM Mannitol. Total RNA were extracted to examine the expression of four stress-responsive genes (*MdAREB2*, *MdRAB18*, *MdRD22,* and *MdRD29B*) with qRT-PCR. Apple *MDH* served as the reference gene for normalization. The expression level in the WT under normal conditions was defined as 1.0. Data are means of three replicates ± standard deviation (SD). Values not followed by the same letters are significantly different according to Duncan's multiple range test ($P < 0.05$).

amelioration of stress-induced morphological and cellular damage, reconfirmed the enhanced tolerance to drought by the overexpression of *MpSnRK2.10* in plants.

The observation that *MpSnRK2.10*-overexpressing apple plants have increased sensitivity to ABA in terms of ABA induced growth retardation implicated *MpSnRK2.10* as a positive regulator of the ABA signaling pathway in apple. One of several possible mechanisms by which this might be affected is through the AREB/ABF class of transcription factors, which are known to have a conserved function downstream of SnRK2 kinases in ABA signaling in response to osmotic stress in land plants (Yoshida et al., 2015; Fujita et al., 2013). *MpSnRK2.10* is highly homologous to SnRK2.6, and overexpression of *SnRK2.6* increases ABA sensitivity during seed germination and seedling growth (Zheng et al., 2010). In addition, overexpression of *MdAREB2* increases ABA sensitivity in apple (Ma et al., 2017). Thus MdAREB2 could be putative substrate of *MpSnRK2.10*, and overexpression of *MpSnRK2.10* might enhance the ABA signal output in response to abiotic stress in apple.

In *Arabidopsis*, *RD29B* and *RAB18* are well-known stress-inducible marker genes, and are direct target genes of AREB/ABFs (Fujita et al., 2005). Subclass III SnRK2s, as the major positive regulators in ABA signaling, phosphorylate and control AREB/ABFs to activate AREB-dependent gene expression (Fujita et al., 2009; Fujita et al., 2013). In addition, another well-known dehydration-responsive gene, *RD22,* is controlled by Myc and Myb TFs, and the activity and/or expression of Myc and Myb TFs is presumed to require SnRK2s-mediated protein phosphorylation (Abe et al., 2003; Fujii and Zhu, 2009). In apple, few suitable marker genes for stress have been reported. A recent study showed that transcripts of apple *MdAREB2* increase greatly with ABA treatment, and that expression of its target gene *MdRAB18* is upregulated in *MdAREB2*-overexpressing apple plants (Ma et al., 2017). In addition, the homologous gene of *Arabidopsis RD22* and *RD29B* in apple, *MdRD22* and *MdRD29B*, have been identified in our laboratory (unpublished). In the examination of expression of these stress-responsive genes under ABA or mannitol treatments, enhanced induction of *MdRAB18*, *MdRD22*, and *MdRD29B* in transgenic apple plants suggested that stress responses were strengthened with the overexpression of *MpSnRK2.10*. This effect might be due to the *MpSnRK2.10*-mediated intensive ABA signal output in response to stress, and thus was in concert with the increased sensitivity to ABA of transgenic plants. The LEA class of proteins is believed to play crucial roles in cellular tolerance under conditions of dehydration and cold stress (Battaglia et al., 2008). Both *RD29B* and *RAB18* belong to the LEA class of proteins. Thus, the elevated induction of these genes under stress may contribute to the enhanced drought tolerance of *MpSnRK2.10*-overexpressing apple plants. Although the expression of *MdAREB2* was not altered in transgenic plants under stress treatments, MdAREB2 may still function through association with *MpSnRK2.10*; SnRK2-mediated phosphorylation is required for full activation of AREB/ABFs, and such post-translational modifications play crucial roles in the ABA signaling pathway (Fujita et al., 2013).

Apple is one of the most economically important fruits in the world. In China, the Loess Plateau is one of the main apple-producing areas, and drought is a major environmental factor limiting tree growth and fruit yield in this region (Liu et al., 2013). The adverse effects of drought are exacerbated by global climate change, which has been predicted to result in an increased frequency of extreme weather (Zhu, 2016). Therefore, clarifying the molecular mechanisms underlying stress responses, and increasing tolerance to abiotic stress in apple trees are important goals for sustainable development of the apple industry. Genetic engineering has being widely used in plants to enhance abiotic stress tolerance, and some transgenic apple plants have shown improved stress tolerance-phenotypes (Li et al., 2010; Li et al., 2013; Wang et al., 2014; Wang et al., 2017a). Here, we demonstrated that overexpression of *MpSnRK2.10* conferred enhanced drought tolerance in transgenic plants without growth retardation under normal conditions, implying that the manipulation of its expression could be a feasible approach toward improving abiotic stress tolerance in important crops.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2018.12.070.

Acknowledgements

This work was supported by the Earmarked Fund for China Agriculture Research System (CARS-27), and the National Natural Science Foundation of China (No. 31601737). The authors are grateful to Zhihong Zhang for providing materials of *M. domestica* genotype 'GL-3'.

Declaration of interests

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

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