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Research article

An apple (*Malus domestica*) NAC transcription factor enhances drought tolerance in transgenic apple plants

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ABSTRACT

Plant NAC proteins constitute one of the largest transcription factor families. They play pivotal functions during responses to various abiotic stresses. However, knowledge on roles of NAC proteins in abiotic stress tolerance as well as corresponding mechanisms has not been fully studied in perennial woody plants, including domesticated apple (*Malus domestica*). In the present study, we characterized the role of apple MdNAC1 transcription factor in response to drought stress. Apple plants overexpressing *MdNAC1* gene exhibited promoted tolerance to drought stress, as evident by reduced water loss and electrolyte leakage in leaves, and maintenance of photosynthesis and photosynthetic pigments content under drought conditions. In addition, the levels of malondialdehyde (MDA) and reactive oxygen species (ROS) were significantly lower for transgenic apple lines than those for non-transgenic plants under drought conditions. This was accompanied by higher activities of several antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), as well as increased expression of the associated genes in transgenic lines. Together, our results indicate that overexpression of the apple *MdNAC1* gene enhances drought stress tolerance in apple plants by promoting higher photosynthesis and activities of ROS-scavenging enzymes.

1. Introduction

Drought stress is one of the major abiotic stresses, which limits plant growth and crop productivity. It can lead to striking alteration of morphology, physiology, biochemistry, and gene expression in plants. Drought stress may cause imbalances of osmotic homeostasis and redox, impaired photosynthesis, and depletion of cellular energy (Golldack et al., 2014). It also increases the generation of reactive oxygen species (ROS), leading to membrane lipid peroxidation (Ippolito et al., 2011; Li et al., 2011).

Many symptoms caused by drought stress can be observed in plant leaves, such as drooping, yellowing, wilting, and death (Jaleel et al., 2009; Sapeta et al., 2013). Furthermore, when exposure to drought conditions, plants exhibit decreased photosynthetic rate and lower chlorophyll content (Manivannan et al., 2007; Li et al., 2015). In addition, prolonged drought stress results in oxidative damage due to increased accumulation of ROS, and this disrupts cellular physiological homeostasis (Smirnoff, 1993; Miller et al., 2010). Excess ROS can also result in extensive cell damage and cell death (Asada and Takahashi, 1987; Dat et al., 2000).

However, plants have evolved several systems that allow them to adapt or survive under water deficit conditions, such as readjusting homeostasis, reducing water loss, and accumulation of low-molecular-weight osmolytes (de Carvalho, 2008; Farooq et al., 2012). In addition, several anti-oxidative mechanisms in plants can be deployed to reduce generation of ROS under drought conditions. The major enzymatic ROS-scavenging mechanisms include a series of antioxidant enzymes, that is, ascorbate peroxidase (APX), superoxide dismutase (SOD), peroxidase (POD), glutathione peroxidase (GPX), and catalase (CAT) (Bowler et al., 1992; Willekens et al., 1997; Noctor and Foyer, 1998; Passardi et al., 2004; Miller et al., 2010; Huang et al., 2013). In plants, H_2O_2 and O_2^- are ubiquitous forms of ROS. Antioxidant enzyme of SOD can convert O_2^- to H_2O_2, while H_2O_2 can be deoxidized into H_2O by POD under abiotic stress (Matamoros et al., 2010; Huang et al., 2013). CAT is also a main anti-oxidative enzyme that converts H_2O_2 to H_2O and O_2 in plants during resistance to drought stress (Pastori and Trippi, 1993; Mittler et al., 2004).

The plant-specific NAC transcription factors (TF) proteins have

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pivotal regulatory functions in plant development, senescence, and in adaptation to abiotic and biotic stresses (Olsen et al., 2005; Nakashima et al., 2012; Liu et al., 2018; Tak et al., 2018). In *Arabidopsis thaliana*, three NAC TFs (ANAC072, ANAC055 and ANAC019) were identified and were reported to have the ability to bind the promoter region of *ERD1* (Tran et al., 2004). Meanwhile, these NAC genes can be induced by high salinity, drought, abscisic acid (ABA), and methyl jasmonate (Tran et al., 2004). Overexpression of the abiotic stress-induced NAC gene, *ATAF1*, in Arabidopsis plants conferred enhanced drought tolerance, suggesting its regulatory function in controlling abiotic stress tolerance (Wu et al., 2009). *ANAC016* was found to promote tolerance to drought stress by suppressing the expression of *AREB1*, a negative regulator in ABA signaling pathway (Sakuraba et al., 2015). In rice (*Oryza sativa*), the participation of several NAC proteins in abiotic stress tolerance has been examined. Overexpression of *ONAC045* in transgenic rice plants significantly enhance plant tolerance to salinity and drought stresses (Zheng et al., 2009). Rice plants overexpressing *OsNAC10* improved grain yield and drought tolerance under field drought conditions (Jeong et al., 2010). NAC genes of *GmNAC20* and *TaRNAC1* were reported to enhance drought or other abiotic stresses by overexpressing in soybean (*Glycine max*) and wheat (*Triticum aestivum*), respectively (Hao et al., 2011; Chen et al., 2018). In addition, several recent research papers have reported that NAC transcription factor genes, *MusaNAC68*, *MusaNAC042*, and *MusaSNAC1*, from the important fruit crop of banana (*Musa x paradisiaca*) play positive roles in responses to salt or drought stresses in transgenic banana plants (Negi et al. 2016, 2018; Tak et al., 2017).

Together, these studies reveal that NAC proteins play vital functions during responses to drought or other abiotic stresses. However, the specific physiological or molecular mechanisms remain to be clarified, especially in non-model plants. Previously, we found that an apple NAC protein, MdNAC1 (MF401514.1), functioned as a transcription factor with transcriptional activity (Jia et al., 2018). In this study, we found that MdNAC1 also plays an important role in response to drought stress. These results support the idea that MdNAC1 and NAC proteins in general may provide an opportunity for improving stress tolerance for apple and other woody perennial crops.

2. Materials and methods

2.1. Plant materials and treatments

Three previously generated *MdNAC1*-overexpressing transgenic apple lines, OE-1, OE-2, and OE-3, were used in these experiments (Jia et al., 2018). The nontransgenic control plants were GL-3 genotype, which was also the genetic background for these transgenic lines. To prepare plant materials, buds of transgenic overexpressing (OE) lines and nontransgenic wild-type (WT) were grafted on uniform, two-year-old apple rootstocks (*Malus hupehensis*. var. Pingyiensis, hereafter Pingyiensis) in late March 2017. These grafted plants were grown in plastic pots which contained a mixture of 1:5 (*v*:*v*) organic substrate and soil. Potted seedlings were well watered to maintain soil water content with 75%–85% of the field capacity (FC) for four months, and they were supplemented with 1/2 Hoagland solution once a week.

For drought treatment, uniform grafted seedlings for each line were randomly divided into two groups: drought treatment group and well-watered control group. One day before the beginning of the treatment, all plants were watered thoroughly. During the treatment period, the soil water content of the control groups was maintained with a FC of 75%–85% by irrigating daily, whereas water for the treatment groups was withheld for six days, and then resupplied thoroughly for another two days. The field experiments were conducted in a greenhouse in late July in 2017, at Northwest A & F University (34°20′ N, 108°24′ E), Yangling, China. Thirty plants of each line were used for each drought or control group.

2.2. Gene expression analysis

To analyze *MdNAC1* expression characteristics in response to drought stress, leaf samples were collected from one-year-old cv. ‘Golden Delicious’ apple (*Malus domestica*) (hereafter Golden Delicious) plants grafted on two-year-old apple rootstocks of Pingyiensis during exposure to drought treatment similar to that used for transgenic and GL-3 nontransgenic line (above). To analyze *MdNAC1* expression profiling to salt stress, the same plants (above) exposure to salt stress (200 mM NaCl solution irrigating the potted plants every four days) were used to collect leaf samples. Leaf total RNA was isolated with the method as described previously (Chang et al., 1993) and cDNA was synthesized using PrimeScript® Reverse Transcriptase Kit (Takara; Dalian, China). Gene expression was analyzed by quantitative real-time PCR (qRT-PCR) reaction with SYBR® Premix Ex Taq (Takara). Apple *MdACTIN* (EB136338.1) was used as internal reference. The results were analyzed according the method of Livak and Schmittgen (2001). Primers information is shown in Supplementary Table S1. Primer specificities and corresponding melting curves were confirmed prior to the experiment. Each experiment was carried out with four replicates.

To know the developmental expression pattern of *MdNAC1*, root sample was collected from Pingyiensis, and samples of stem, leaf, flower and fruit were collected from Golden Delicious plants. RNA isolation, cDNA synthesis, qRT-PCR analysis and gene expression calculation were carried out according to the same methods as described above.

To analyze the expression pattern of *MdCAT*, *MdPOD*, and *MdSOD*, which encode the key ROS-scavenging enzymes of CAT, POD, and SOD, respectively, we identified these genes by BLAST analysis using an ORF product database of apple genome (GDR, https://www.rosaceae.org/) with corresponding homologous protein sequences from *Prunus persica*, *Pyrus pyrifolia*, or *Malus xiaojinensis* (Supplementary Table S2). Similarity among these homologous proteins was identified using DNAMAN software (Lynnon Biosoft, San Ramon, USA). Gene expression analysis was performed using leaf samples collected from plants of control or treatment groups on Day 6.

2.3. Measurement of relative water content and relative electrolyte leakage

To know the effects of drought stress on plant performance, relative water content (RWC), and relative electrolyte leakage (REL) of leaves for each group were measured during the treatment period. For measurement of RWC, leaves were excised and their weights were recorded. Then immersed in deionized water overnight and their weights were recorded. Finally, those samples were dried at 85 °C in an oven for three days and then weighted. RWC of leaves was monitored using previous method (Gaxiola et al., 2001). Leaf REL was determined as described previously (Dionisio-Sese and Tobita. 1998). Each experiment was performed with four replicates.

2.4. Determination of photosynthetic characteristics

Plant leaf photosynthetic indexes, including photosynthetic rate (Pn), stomatal conductance (Gs), intercellular CO_2 concentration (Ci) and transpiration rate (Tr) for each group were monitored. These values were recorded from 9:00 to 11:00 a.m. during the treatment period with a photosynthetic apparatus (Li-6400; LICOR, Huntington Beach, USA). These parameters of this machine were set as the same as previous report (Li et al., 2015). Well expanded plant leaves at the same positions from randomly selected plants were used. Eight measurements were performed for each group.

2.5. Content of photosynthetic pigments

Leaf chlorophylls (total chlorophyll, chlorophyll *a* and *b*) as well as carotenoids for each group were detected according to the method of

Arnon (1949). Each measurement was carried out for with four repeats.

2.6. Content of malondialdehyde, H_2O_2, O_2^- and nitro blue tetrazolium staining of O_2^-

Leaves of each group were used for determining content of malondialdehyde (MDA), O_2^-, and H_2O_2. MDA was measured with the method as described by Heath and Packer (1968). H_2O_2 was quantified using the method of Patterson et al. (1984). O_2^- was monitored according to the method of Wang and Luo (1990). Each experiment was performed with four repeats.

For detection *in-situ* O_2^-, leaves were harvested on day 6 of the treatment and incubated in 0.1 mg mL^{-1} NBT dissolved in 100 mM HEPES buffer (pH = 7.5). After incubation for one night, the leaves were soaked in 75% ethanol for 3 h to remove chlorophyll and then the samples were photographed.

2.7. Extraction and assays of antioxidant enzymes

For each group, crude enzyme liquid was extracted from 0.1 g frozen leaf sample with the method as reported by Yin et al. (2013). These supernatant for different groups was further used to determine activities of antioxidant enzymes (CAT, POD, and SOD). CAT activity was determined with the method as decreased by Chance and Maehly (1955). POD activity was estimated according the method of Wu et al. (2016). SOD activity was determined by the method of Fatima and Ahmad (2005). Each experiment was performed with four replicates.

2.8. Statistics

All data are shown as mean ± standard deviation. The significant differences of the data were detected using one-way ANOVA and Tukey's multiple comparison test (significant: 0.05). Statistical analysis of data was performed with SPSS software (version 20; SPSS Inc.; Chicago, USA).

3. Results

3.1. MdNAC1 is induced by drought stress

During drought stress treatment, the relative expression of the *MdNAC1* gene in leaves of Golden Delicious was significantly induced by Day 2 but had returned to basal levels by Day 4 (Fig. 1 A). However, the *MdNAC1* expression was not effected significantly by salt stress (Supplementary Figure S1). *MdNAC1* was expressed relatively strongly in leaf, flower and in fruit, and was relatively weakly expressed in root and stem (Fig. 1 B).

3.2. Overexpression of MdNAC1 enhanced drought stress tolerance

When exposure to drought treatment for six days, leaves of both transgenic plants overexpressing *MdNAC1* and nontransgenic control plants showed wilting phenotype, but apical leaves of transgenic lines did not wither and remained viable (Fig. 2 A).

Leaf RWC showed similar values for nontransgenic wide-type (WT) and transgenic overexpresing (OE) lines at the beginning of the drought stress, and both WT and OE lines showed declining RWC until Day 6 (Fig. 2 B). However, OE plants showed significantly higher RWC than WT plants from Day 2 to until Day R2 (2 days later after been resupplied by water) (Fig. 2 B). On Day 6, the RWC in the leaves of WT plants was ~50%, while values for transgenic OE lines were between 55% and 57% (Fig. 2 B).

Conversely, whereas REL values in leaves of WT and OE seedlings were similar prior to drought treatment, and increased until Day 6 in both WT and OE plants, and transgenic plants exhibited significantly lower REL values than nontransgenic plants (Fig. 2 C). On Day 6 of the treatment, the REL value was 55% for the WT plants, while that for the OE lines ranged from 24% to 31% (Fig. 2 C).

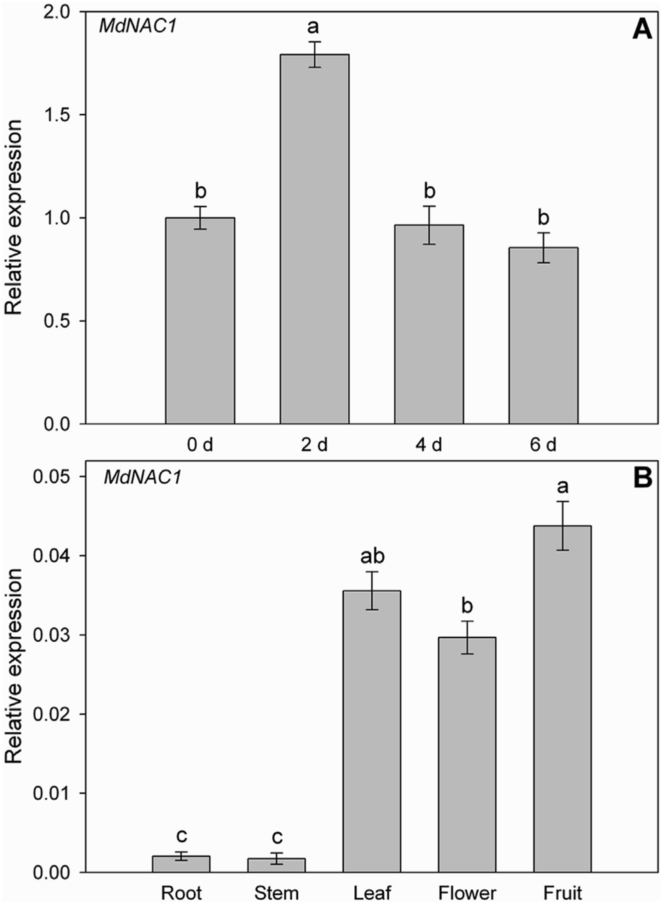

Fig. 1. *MdNAC1* expression during response to drought treatment (A) and in different tissues of *Malus* plants (B). Values are presented as mean ± standard deviation. The significant differences of the data are shown by lowercase letters above the bars.

3.3. Photosynthetic characteristics and photosynthetic pigments contents

To know whether *MdNAC1* alters photosynthetic capacity in response to drought stress in overexpressing plants, we compared four parameters of photosynthetic capacity - Pn, Ci, Gs and Tr - between the WT and OE plants during the drought treatment. Nontransgenic and *MdNAC1*-overexpressing lines showed similar values for all four parameters prior to drought (Fig. 3). Pn decreased in both nontransgenic and transgenic lines throughout the drought treatment, with values being significantly higher for transgenic plants than for nontransgenic plants (Fig. 3 A). On Day 6 of the drought treatment, value of Pn for WT plants (0.83 μmol CO_2 m^{-2} s^{-1}) was less than half of that for the OE lines (1.92–2.26μ mol CO_2 m^{-2} s^{-1}) (Fig. 3 A). Values for Gs and Tr showed similar trends to Pn (Fig. 3 B, D). For Ci, there were no significant differences between nontransgenic and transgenic plants in the early stage of the treatment (until Day 4). However, Ci values for the OE lines were significantly lower than those for the WT plants in the late period of the treatment (between Day 4 and R2) (Fig. 3 C).

The content of photosynthetic pigments was also determined on Day 6 of the treatment. Prior to the drought treatment, the levels of total chlorophyll, chlorophyll a, chlorophyll b, and carotenoids were almost the same between OE and WT plants. However, while under drought conditions, levels of those were higher in transgenic lines compared with in nontransgenic plants (Fig. 4).

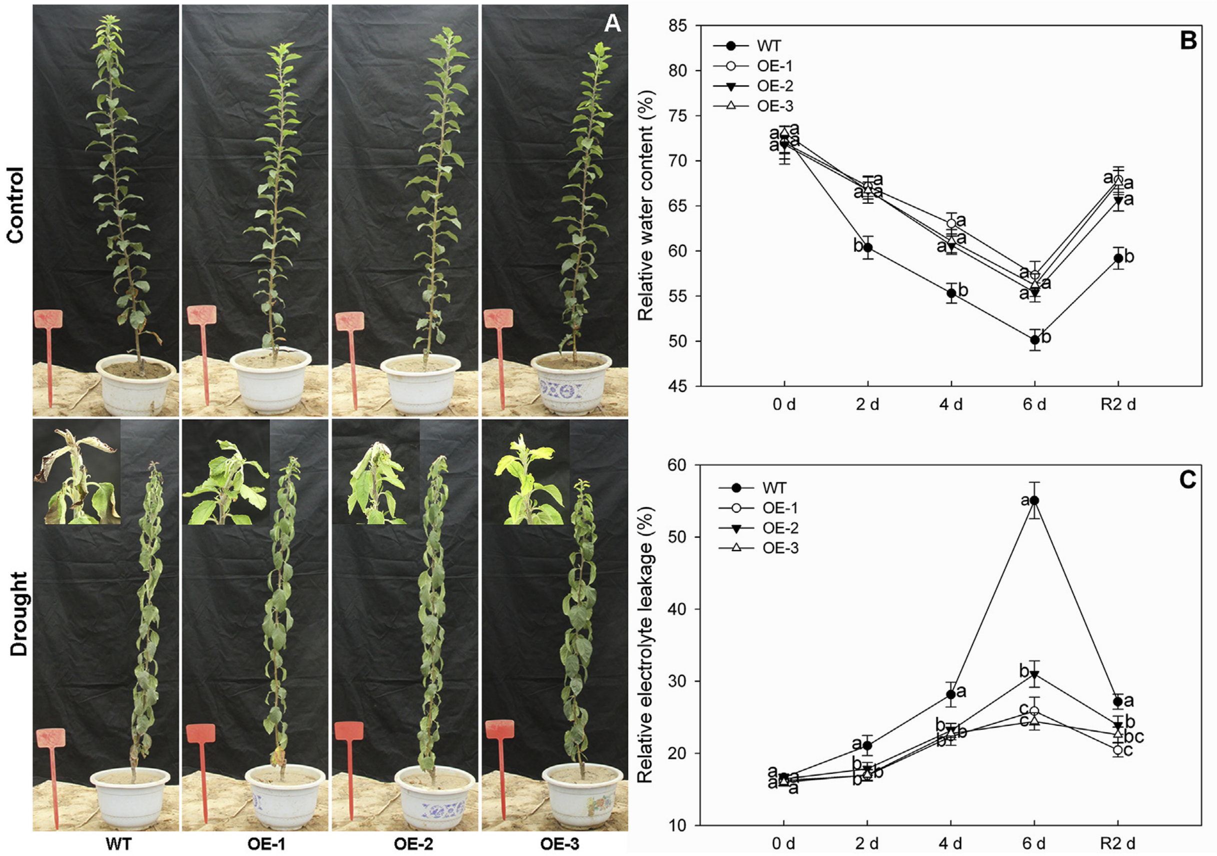

Fig. 2. Phenotypes and physiological characteristics of nontrangenic GL-3 and *MdNAC1* overexpressing lines. (A) Phenotypes of *MdNAC1* overexpressing (OE) lines and nontransgenic wide-type (WT) plants under drought conditions on Day 6. Relative water content (B) and relative electrolyte leakage (C) of leaves during period of drought treatment. Values are presented as mean ± standard deviation. The significant differences of the data are shown by lowercase letters beside the bars.

3.4. *Lipid peroxidation and production of ROS*

To determine whether overexpression of *MdNAC1* could alleviate peroxidation of membrane lipids or reduce the accumulation of ROS in leaves under drought conditions, we measured the levels of MDA and ROS (H_2O_2 and O_2^-) in the leaves of OE and WT plants. MDA obviously increased during the stress period for both transgenic and nontransgenic lines. However, MDA content was significantly lower in the transgenic plants compared with the nontransgenic plants throughout the treatment (Fig. 5 A). For example, the content of MDA in the WT plants was 19.9 μmol g^{-1} FW on Day 6, which was almost 1.4-fold higher than that in the OE lines at the same time (Fig. 5 A).

Drought stress also increased levels of H_2O_2 in transgenic and nontransgenic lines. However, the values were significantly lower in the OE lines than that in the WT plants after Day 2 (Fig. 5 B).

In addition, in both transgenic and nontransgenic lines, O_2^- increased during drought treatment. However, accumulation in OE lines was much slower than in the WT (Fig. 5 C). By Day 6, O_2^- in the OE lines was between 195 and 236 nmol g^{-1} FW, significantly lower than the 412 nmol g^{-1} FW in nontransgenic plants (Fig. 5 C). Accordingly, NBT staining in leaves indicated that the transgenic lines accumulated less O_2^- in the leaves than did the nontransgenic plants under drought conditions on Day 6 (Fig. 5 D).

3.5. *Activities of antioxidant enzymes and corresponding gene expression*

We further examined the activities of several antioxidant enzymes, including CAT, POD and SOD. Both transgenic and nontransgenic plants showed enhanced activities of CAT under drought conditions (Fig. 6 A). The CAT activities of the OE plants were significantly higher comparing with WT plants under drought stress, almost the same as the control plants after being re-watered (Fig. 6 A). POD activity was also significantly higher in the transgenic plants than that in the nontransgenic plants from Day 0 to Day 4. The POD activities decreased in OE lines after Day 4, and were much lower than in the WT plants from Day 6 to Day R2 (Fig. 6 B). Furthermore, the activities of SOD dramatically increased in the early stage of the drought treatment (from Day 0 d to Day 2) and then decreased, with SOD activities of OE lines significantly higher than WT plants (Fig. 6 C).

In addition, we analyzed relative gene expression of the *MdCAT*, *MdPOD*, and *MdSOD*, which encode CAT, POD, and SOD, respectively. These results indicated that expression of all these three genes was induced by drought treatment on Day 6 of the treatment compared with plants under non-stressed conditions (Fig. 6 D, E, and F).

Under drought conditions, the expression level of *MdCAT* was almost two-fold higher in the three OE lines compared with the WT plants (Fig. 6 D). Although there were no significant differences for the expression level of *MdPOD* between the OE and the WT plants under drought conditions on Day 6 (Fig. 6 E), the expression of *MdSOD* was significantly higher for OE-1 and OE-3 than that in WT plants under drought conditions (Fig. 6 F).

4. Discussion

Although a wealth of knowledge has been acquired since the discovery of NAC TFs (Shao et al., 2015; Liu et al., 2018), research on NAC TFs is still in its infancy, especially for the roles of NAC TFs in perennial woody plants such as apple. In this study, we focused on the function of the apple *MdNAC1* gene during response to drought stress. These results showed that it conferred increased tolerance to drought stress in

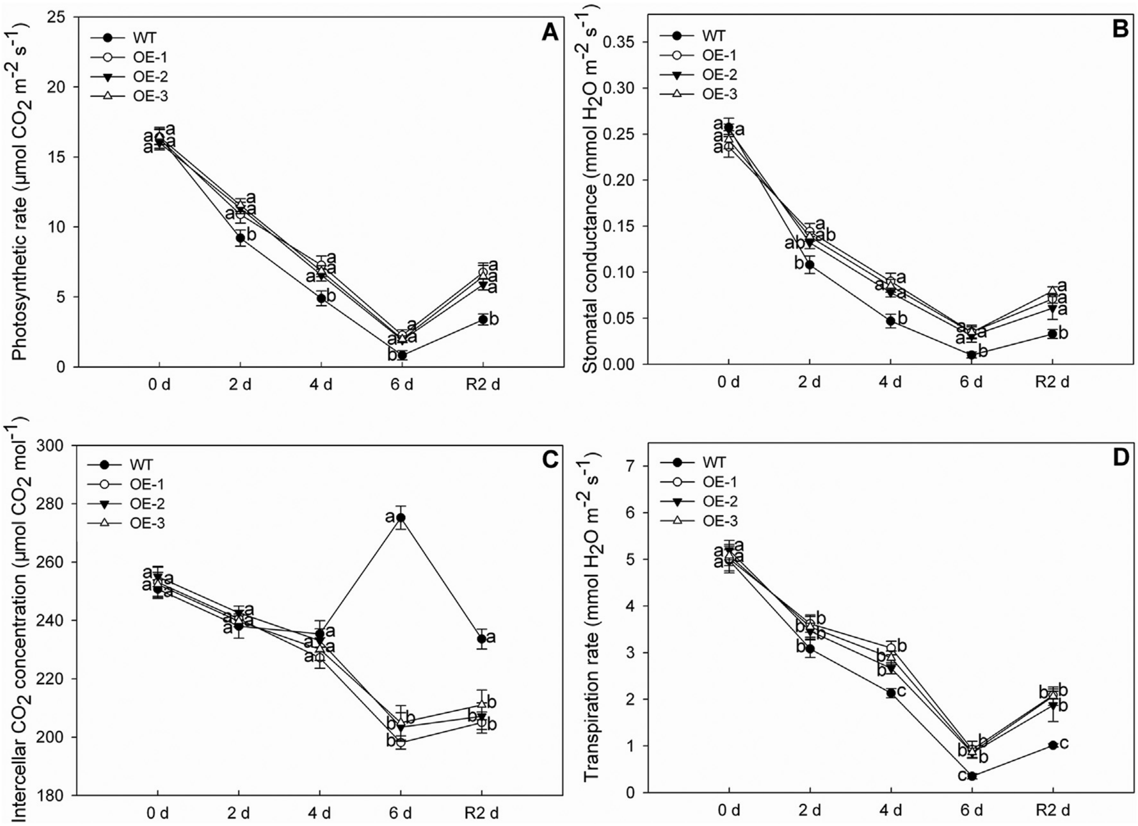

Fig. 3. Parameters of photosynthetic capacity for wild-type (WT) and *MdNAC1* overexpressing (OE) lines under drought conditions. (A), (B), (C), (D) represent values of leaf photosynthetic rate (Pn), stomatal conductance (Gs), intercellular CO2 concentration (Ci), and transpiration rate (Tr), respectively. Values are presented as mean ± standard deviation. The significant differences of the data are shown by lowercase letters beside the bars.

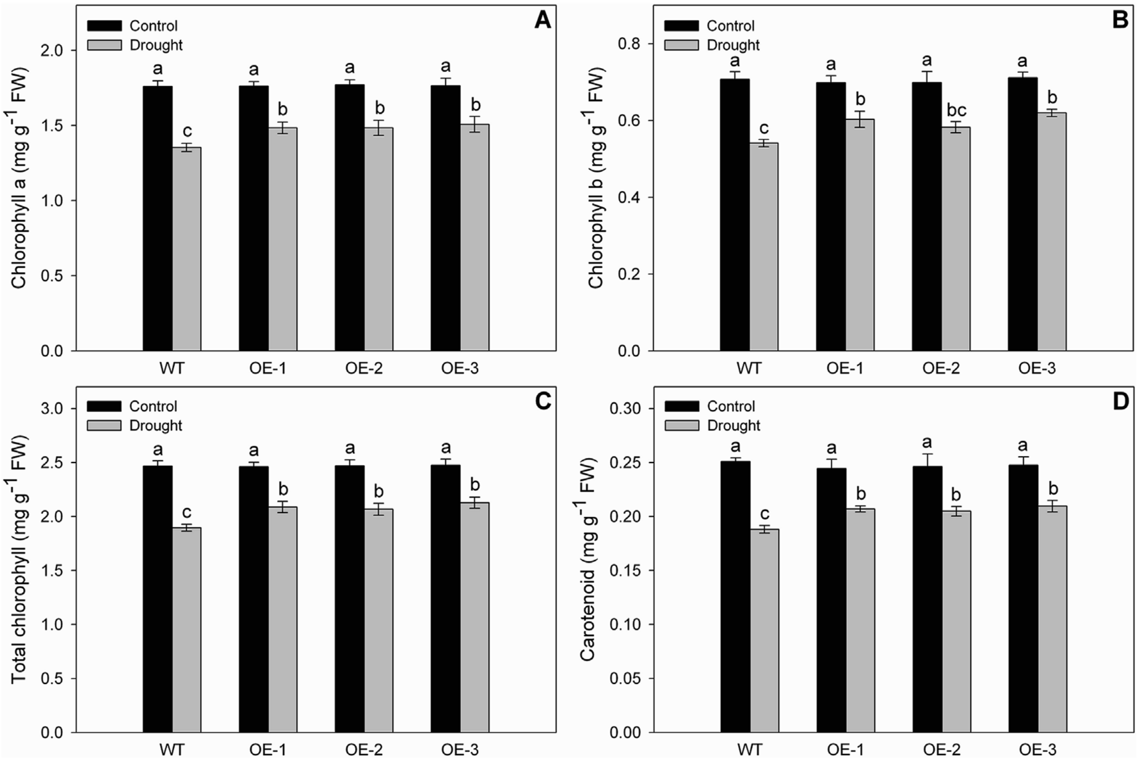

Fig. 4. Levels of chlorophyll a (A), chlorophyll b (B), total chlorophyll (C), and cartenoids (D) for overexpressing (OE) and wild-type (WT) lines on Day 6 of the treatment. Values are presented as mean ± standard deviation. The significant differences of the data are shown by lowercase letters above the bars.

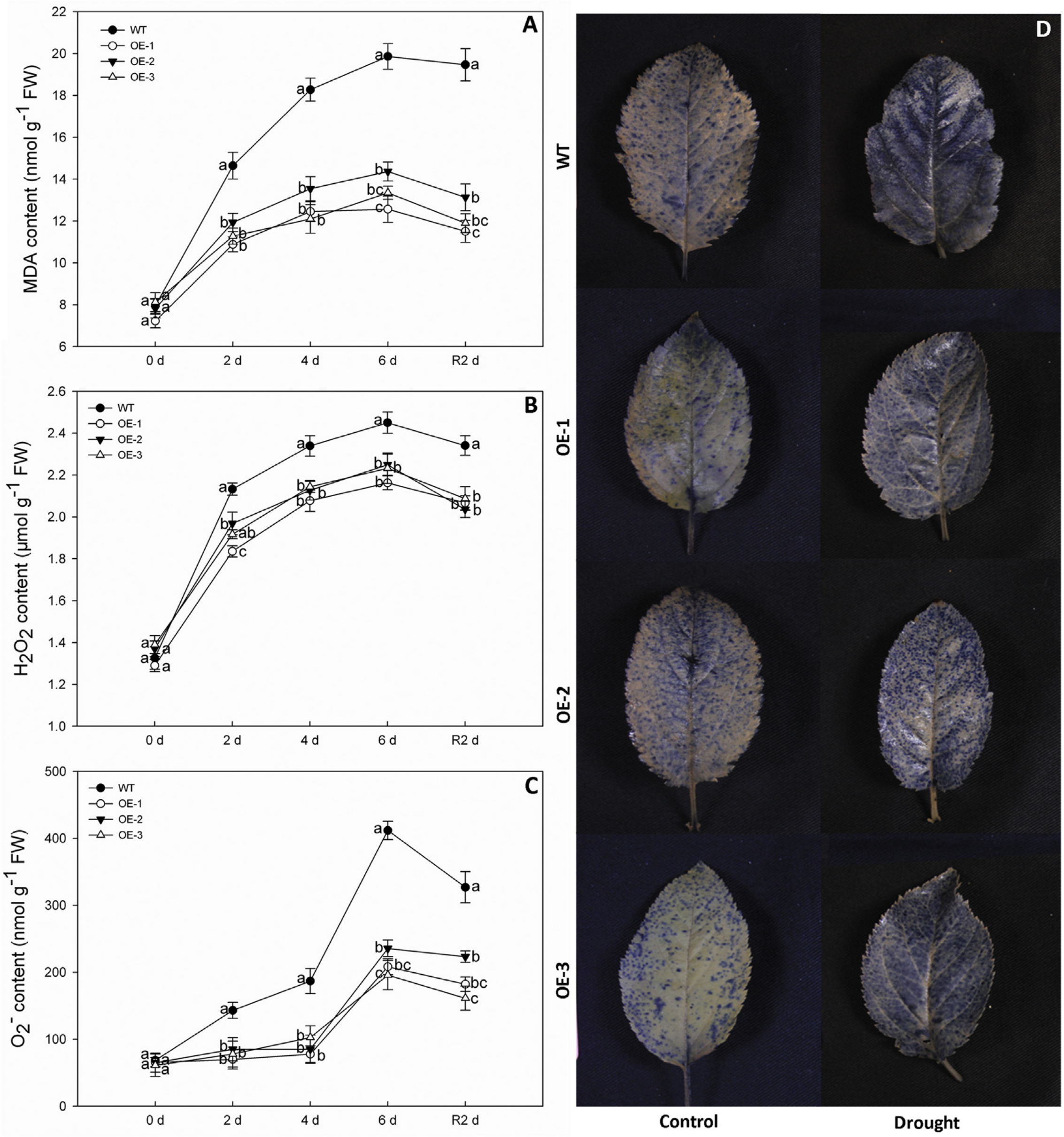

Fig. 5. Content of MDA (malondialdehyde) and reactive oxygen species (ROS) in leaves of overexpression lines (OE) and wild-type (WT) plants. (A) MDA content. (B) H_2O_2 content. (C) O_2^- content. (D) *In-situ* staining of O_2^- (blue spots) on Day 6 of the treatment. Values are presented as mean ± standard deviation. The significant differences of the data are shown by lowercase letters beside the bars. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

transgenic apple plants.

Drought stress negatively impacts several physiological processes (Flexas et al., 1999). Maintenance of optimal rate of photosynthesis is crucial for crop productivity, especially in arid or semi-arid areas. The present data obtained show that photosynthesis in apple plants is sensitive to drought stress. For example, Pn decreased dramatically after the initiation of the drought treatment. However, when comparing with the nontransgenic plants, the *MdNAC1*-overexpressing plants maintained higher values of Pn during the drought treatment period. Similarly, although Gs also deceased obviously under drought conditions, its values in the transgenic lines were higher than that in the nontransgenic plants. A similar effect was also observed in a study of the enhanced drought stress resistance of apple plants conferred by treatment with melatonin (Li et al., 2015). The present results indicate that overexpression of *MdNAC1* promoted plant photosynthesis rate under drought conditions, accompanied by higher Gs.

Furthermore, plant photosynthetic capacity depends in part on the levels of photosynthetic pigments (Ashraf and Harris, 2013). In this study, the content of chlorophyll significantly declined in both transgenic and nontransgenic apple plants under drought conditions, but the transgenic plants maintained higher levels throughout the drought treatment. The ability of the *MdNAC1*-overexpressing lines to reduce chlorophyll loss may at least partly explain their better photosynthetic performance. In addition, the maintenance of higher levels of carotenoids may contribute to enhanced photosynthesis rate in the transgenic plants, since carotenoids play positive roles in photosynthesis, for example, in light harvesting, photoprotection, and singlet oxygen ($^1O_2$) scavenging (Frank and Cogdell, 1996).

Plants are sensitive to water deficit (Rampino et al., 2006; Li et al., 2009), and the increasing antioxidant capacity is a major adaptive mechanism (Li et al., 2009). Adverse environment may result in oxidative stress and increased accumulation of ROS, including O_2^-, $^1O_2$, hydroxyl radical (·OH), and H_2O_2 (Mittler, 2002; Li et al., 2015). ROS can react with lipids or proteins, leading to damage to plant cell, and

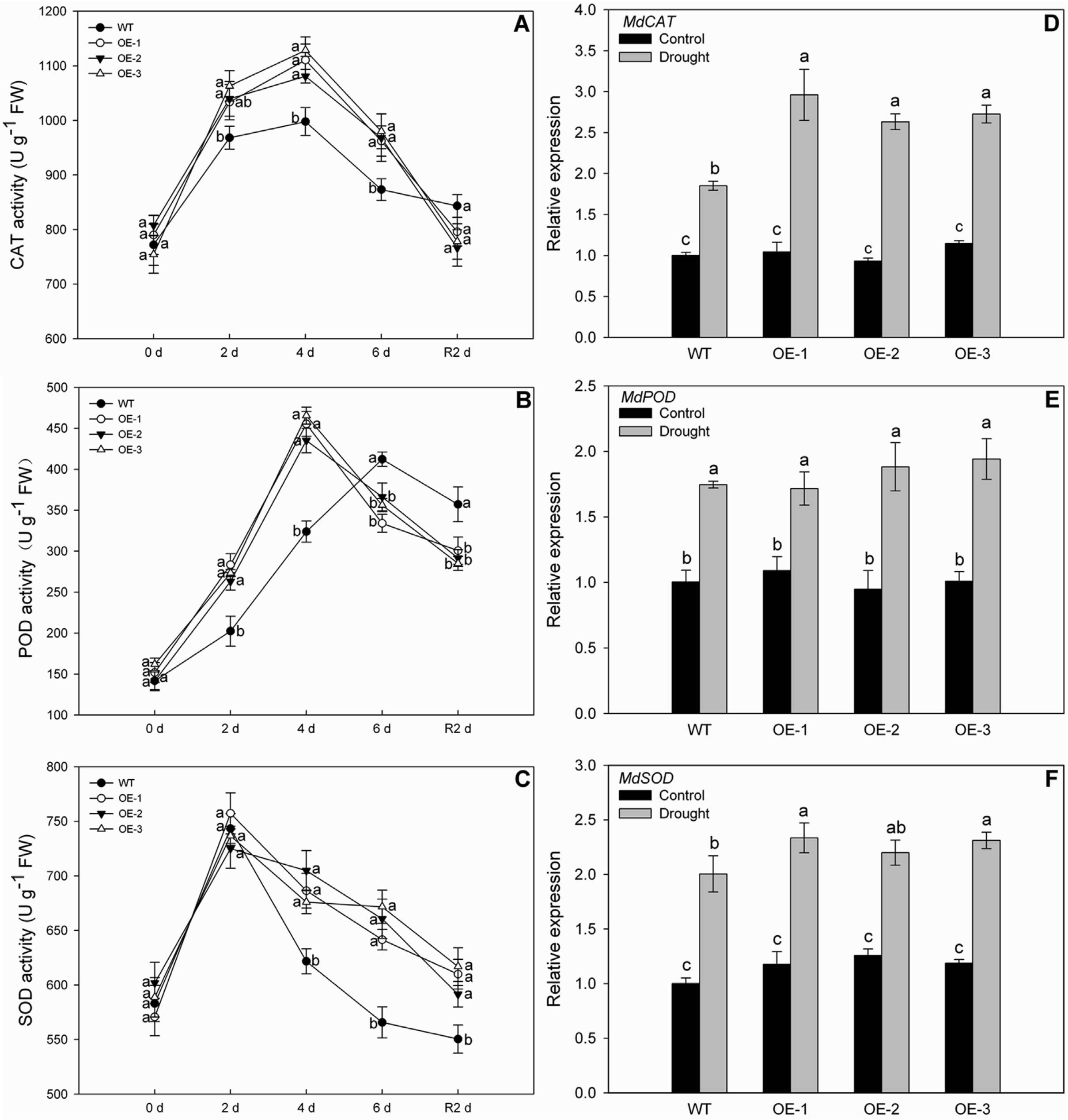

Fig. 6. Activities of antioxidant enzymes and related gene expression. Activities of CAT (A), POD (B), and SOD (C). Relative gene expression of *MdCAT* (D), *MdPOD* (E), and *MdSOD* (F) on Day 6 of the treatment. Values are presented as mean ± standard deviation. The significant differences of the data are shown by different lowercase letters beside (A, B, and C) or above (D, E, and F) the bars.

inhibiting photosynthesis and metabolism (Apel and Hirt, 2004; Miller et al., 2010). Under drought conditions, the leaves of *MdNAC1*-overexpressing plants accumulated lower levels of O_2^- and H_2O_2 when compared with nontransgenic plants, indicating that overexpression of *MdNAC1* reduces oxidative stress. Consistent with this, transgenic Arabidopsis plants that express the *Miscanthus* NAC TF MlNAC12 accumulated less O_2^- and H_2O_2 under drought conditions and they exhibited enhanced tolerance to drought stress (Yang et al., 2018). Also, overexpression of the Arabidopsis NAC TF JUB1 in tomato (*Solanum lycopersicum*) increased drought tolerance, accompanied by higher RWC and reduced H_2O_2 levels (Thirumalaikumar et al., 2018).

In addition, MDA, which is used as an indicator for estimating membrane lipids peroxidation, accumulated much lower in *MdNAC1* overexpressing lines in comparison with nontransgenic plants under drought conditions, indicating *MdNAC1* could alleviate membrane damage in response to drought stress. Similar reduction in MDA levels was also observed in Arabidopsis plants expressing *Miscanthus MlNAC9* (Yang et al., 2018), and in transgenic tomato plants expressing Arabidopsis NAC gene, *JUB1* (Thirumalaikumar et al., 2018).

Abiotic stresses, including salinity and drought stress, impose osmotic stress in plants, leading to over-accumulation of ROS (Miller et al., 2010; Krasensky and Jonak, 2012). However, multiple enzymatic or non-enzymatic antioxidants can function as vital ROS scavengers (Apel and Hirt, 2004). For example, in chloroplasts of plant leaves, the Mehler reaction during photosynthesis produces superoxide as a side product. This ROS can be rapidly catalyzed by SOD, producing H_2O_2. H_2O_2 is then converted to water by APX (Asada, 1999). Overexpression of a *Poncirus trifoliate* bHLH TF protein in lemon (*Citrus limon*) enhanced cold tolerance at least partly by positively regulating the POD-mediated ROS removal system (Huang et al., 2013). Therefore, we evaluated the activities of ROS-scavennging CAT, POD, and SOD. Consistent with the reduced accumulations of ROS (H_2O_2 and $O_2{}^-$) in leaves of transgenic plants, the activities of these ROS-scavenging enzymes were significantly higher in most cases for the stressed groups. Similar case was also reported in transgenic Arabidopsis plants that overexpress *MlNAC9* under drought and salt stresses (Zhao et al., 2016).

In addition to the decreased accumulation of ROS and the increased ROS-scavenging capability for the *MdNAC1*-overespressing plants under drought conditions, overexpression of *MdNAC1* also enhanced the expression of *MdCAT* and *MdSOD*. Their increased expression likely underlies the higher activities of CAT and SOD.

Our experiments indicated the positive roles of *MdNAC1* in transgenic apple plants in response to drought treatment. However, some studies have reported that multiple NAC transcription factors act with dual functions, such as regulating plant development and abiotic stress tolerance (Jeong et al., 2010; Redillas et al., 2012). In banana, overexpression of *MpSNAC67* reduced leaf size in transgenic plants (Tak et al., 2018). Kato et al. (2010) reported that overexpression of *ANAC036* in transgenic Arabidopsis also showed growth inhibition. Our previous study showed that *MdNAC1* overexpressing plants with their own roots also exhibited growth inhibition (Jia et al., 2018). However, in our present study, when grafted on apple root stocks, no apparent growth penalty was observed for the *MdNAC1* overexpressing plants compared to the grafted wild-type control plants. Those results may imply the functional diversity for some transcription factors.

5. Conclusions

Our present research systematically investigated the functions of MdNAC1 during response to drought stress. The apple plants overexpressing *MdNAC1* altered plant physiological status, biochemical characters, and gene expression profile under drought conditions. This enhanced drought tolerance was evident by higher RWC and decreased membrane lipids peroxidation, higher photosynthetic capacity, lower accumulation of ROS, and higher activities of ROS-scavenging enzymes in plant leaves. Thus, apple MdNAC1 may positively regulate drought stress tolerance by maintaining photosynthesis and up-regulating activities of ROS-scavenging enzymes. Therefore, *MdNAC1* may serve as a potential tool for the breeding of new drought-tolerant apple cultivars.

Conflicts of interest

The authors declare no conflict of interest.

Contributions

D. Jia performed most of the experiments and wrote the paper. Q. Jiang assisted with the measurements of physiological and biochemical indexes. S. van Nocker organized the paper structure and revised the manuscript. X. Gong provided intellectual input in the analysis of the results. F. Ma helped with the experiment design and provided all financial support for the study.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.plaphy.2019.04.011.

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