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Research Article

Overexpression of the RNA binding protein MhYTP1 in transgenic apple enhances drought tolerance and WUE by improving ABA level under drought condition

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ABSTRACT

MhYTP1 is involved in post-transcriptional regulation as a member of YT521-homology (YTH) domain-containing RNA-binding proteins. We previously cloned *MhYTP1* and found it participated in various biotic and abiotic stress responses. However, its function in long-term moderate drought has not been verified. Thus, we explored its biological role in response to drought. Under drought condition, the net photosynthesis rate (P_n) and water use efficiency (WUE) were significantly elevated in *MhYTP1*-overexpressing (OE) apple plants when compared with the non-transgenic (NT) controls. Further analysis indicated *MhYTP1* expression was associated with elevated ABA content, increased stomatal density and reduced stomatal aperture. In addition, to gain insight into the function of stem-specific expression of *MhYTP1*, grafting experiments were performed. Interestingly, lower transpiration rate (T_r) and higher WUE were observed when transgenic plants were used as scions as opposed to rootstocks and when transgenic rather than NT plants were used as rootstocks, indicating *MhYTP1* plays crucial roles in grafted plants. These results define a function for *MhYTP1* in promoting tolerance to drought conditions, and suggest that *MhYTP1* can serve as a candidate gene for future apple drought resistance breeding with the help of biotechnology.

1. Introduction

Plants are inevitably challenged by various environmental stresses such as drought, ultra-violet light, and extreme temperatures. Drought stress, in particular, is gaining attention due to its potential to greatly limit future crop production [1,2]. In vascular plants, only a minor portion of the water taken up by roots is used for photosynthesis; most water returns to the environment by transpiration via stomata [3]. During water stress, transpiration can be minimized by constricting stomatal aperture [4]. The importance and benefits of controlling stomatal behavior for transpiration, photosynthesis and water use efficiency to plants have been previously reported in a large number of studies [5–8]. When plants encounter water deficit, cells trigger a network of signaling events that reprogram their biochemical and physiological processes. Phytohormones are highly responsive to drought [9]. For example, ABA content is significantly increased under drought condition, and this promotes stomatal closure and expression of genes that allow the plant to cope with the stress [10–14].

RNA binding proteins (RBPs) participate in gene expression regulation at the transcriptional and post-transcriptional levels [15], interacting with target RNAs via RNA binding domains (RBDs). The YT521-B homology (YTH) RBD was first described in the rat (*Rattus norvegicus*) [16]. Related to hypoxia stress, YT521-B is a splicing factor that influences splice site selection in a concentration-dependent manner [16,17]. Recently, researchers are focusing on interactions between YTH domain-containing RNA binding proteins (YTPs) and target RNAs that affect the translation status and lifetime of mRNA [18,19]. Some researchers are also examining and proving that YTPs

Abbreviations: RBP, RNA binding protein; YTH, YT521-B homology; YTP, YTH domain-containing RNA binding protein; ABA, abscisic acid; RBD, RNA binding domain; *M. hupehensis*, *Malus hupehensis*; *MhYTP1*, *Malus hupehensis YTP1*

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play an important role in conferring tolerance to biotic and abiotic stresses in plant [20–25].

Abscisic acid (ABA) plays an important role in abiotic stress response and tolerance of plants, especially drought stress [26]. ABA promotes the closure of stomata in guard cells to diminish transpiration [27]. Initiation of ABA signaling is dependent on pyrabactin resistance/pyrabactin resistance-like/regulatory component of ABA receptor (PYR/PYL/RCAR) proteins, which function as ABA receptors [28,29]. The downstream events of ABA perception are protein phosphorylation and dephosphorylation, associated with Suc nonfermenting 1-related subfamily 2 (SnRK2) protein kinases and type 2C protein phosphatases (PP2Cs), respectively. SnRK2 protein kinases, including SnRK2.2, SnRK2.3, and SnRK2.6 (OST1), positively regulate ABA signaling. Conversely, Clade A PP2Cs are key repressors of ABA signaling [30]. PYR/PYL/RCAR proteins bind to and inhibit most members of the clade A PP2Cs [29,31,32]. Drought leads to osmotic stress and accumulation of ABA, which subsequently induces ABA-dependent responses. Increased ABA levels are perceived by PYR/PYL/RCAR proteins, which in turn inhibit protein phosphatase-kinase interactions, leading to the release of SnRK2 protein kinases. Apple *PYL4* and *PYL9* are two genes encoding PYL receptors which have been previously shown to be responsive to drought stress [33]. These SnRK2-type kinases promote specific gene expression and ion channel activation via phosphorylation of target proteins, including transcription factors and SLAC1 anion channels, thereby enabling plants to adapt to adverse environments. Osmotic stress also activates ABA-independent responses through SnRK2s, and PYLs antagonize ABA-independent activation of SnRK2s by osmotic stress [34].

Grafting is one way of avoiding or reducing losses in production caused by environmental stresses in fruits and vegetables for the physiological changes during the integrative reciprocal process of scion and rootstock, including increasing water permeability of the cells and organic matter accumulation [35]. Grafting has been shown to enhance plant vigor, extend the harvest period [36], prolong postharvest life [37], improve yield and fruit quality [38–40] and increase water use efficiency [41]. In addition to these commercially important benefits, grafting has been shown to increase tolerance to various stressors, including low and high temperatures [42,43], salinity and heavy metals [44–48], drought and flooding [44,49], diseases and insects [36,50–53] and weeds [50,54].

Apple (*Malus domestica*), is one of the most widely cultivated woody plants in temperate regions and is the fourth most economically important fruit tree after Citrus, grape, and banana [55]. One of the world's largest apple production regions is the Loess Plateau of China. Here, production is frequently limited by drought conditions. Typically, commercial apple cultivars are maintained as grafts to stress-resistant rootstocks, and the drought-tolerant wild species, *Malus hupehensis* (Pamp.) Rehd, is commonly used as an apple rootstock in China. Identifying the genes responsible for drought tolerance and manipulating their expression in genetically modified crops is becoming a critical focus of molecular breeding programs in China and other drought-prone regions. Previously, we found that transgenic apple plants overexpressing a YTP gene from *M. hupehensis*, designated *MhYTP1*, are more tolerant than non-transgenic plants to simulated drought conditions [23]. To further investigate the function of *MhYTP1* in apple plants upon long-term moderate drought stress, we studied various aspects of drought responses in the previously-generated transgenic apple plants that overexpresses *MhYTP1*. Our results highlight a potential function for *MhYTP1* in drought tolerance in apple, which underscore the importance of further studies of the YTP gene family for the purposes of engineering drought-tolerant crops.

2. Materials and methods

2.1. Plant materials, growth conditions and sampling

The *Agrobacterium*-sensitive *Malus* genotype 'GL-3' ('Royal Gala') was obtained from Dai et al. [56]. Transgenic and nontransgenic (NT) *Malus domestica* cv. 'Roya Gala' plants are the same as we previously used in leaf senescence experiments [24]. Tissue-cultured transgenic and nontransgenic *Malus domestica* cv. 'Roya Gala' plants were initially grown on MS agar media containing 0.3 mg L^{-1} 6-benzylaminopurine (6-BA) and 0.2 mg L^{-1} indoleacetic acid (IAA). They were cultured under conditions of 23 °C, 60 μmol m^{-2} s^{-1} and 14-h photoperiods. After rooting on MS agar media containing 0.5 mg L^{-1} indole butyricacid (IBA) and 0.5 mg L^{-1} IAA, transgenic and NT plantlets with similar sizes were transferred to small plastic pots (8.5 × 8.5 × 7.5 cm) containing a mixture of soil/perlite (1 : 1, v : v). After 30 days of adaptation in a growth chamber, the plants were moved to large plastic pots (30 × 26 × 22 cm) filled with a mixture of forest soil/sand/organic substrate (5 : 1 : 1, v : v : v) and grown in a glasshouse at the College of Horticulture, Northwest A&F University, Yangling (34°20'N, 108°24'E), Shaanxi Province, China. They were watered regularly and supplied with half-strength Hoagland's nutrient solution (pH 6.0) once a week. After three months of growth under these conditions, healthy and uniformly sized plants were assigned to two treatment groups. Half were subjected to drought stress by withholding water maintaining at 45–50% field capacity, while the other (well-watered control) continued to receive daily irrigation maintaining a saturated soil water content. On days 0, 40 and 80 of this experiment, between 10:00 and 11:00 h, the ninth to twelfth leaves from the base of a stem (fully mature leaves) were sampled from five trees per treatment. They were rapidly frozen in liquid nitrogen and stored at −80 °C for further analysis.

For grafting experiments, NT and *MhYTP1* transgenic plants were cultivated for one year in a glasshouse. In the spring of the second year, buds from NT and *MhYTP1* transgenic plants were grafted onto the NT and *MhYTP1* transgenic plants in four combinations: NT/NT, OE-36/NT, NT/OE-36 and OE-36/OE-36 (scion/rootstock). The treatments were identical with the transgenic and NT plants above after being moved to large plastic pots.

2.2. Physiological measurements

SPAD values were monitored using a SPAD-502 (Konika-Minolta). Electrolyte leakage was determined from leaves as described by Dionisio-Sese and Tobita [57] with an electrical conductivity meter (DSS-307; SPSIC, Shanghai, China). P_n and T_r were monitored using a Li-6400 portable photosynthesis system (LiCor, Huntington Beach, CA, USA), at a constant air flow rate of 500 μmol s^{-1}, with vapor pressure deficit of 2.0–3.4 kPa, cuvette CO_2 concentration of 400 ± 5 cm^3 m^{-3} and temperature of 28 ± 2 °C. Data were obtained from light-exposed, fully-expanded leaves from the base of selected plant stems on sunny days between 09:00 and 10:00 h. The rate of P_n and T_r were obtained from four plants for each line. Measurements were made at a photosynthetic photon flux density of 1000 μmol m^{-2} s^{-1}, as provided by a Q-Beam (blue and red diode) light source. For biomass accumulation analysis, fresh weight and dry weight of roots, stems and leaves were measured for transgenic and NT plants at the beginning and end of drought treatment on day 0 and day 80, respectively. Data were obtained from four plants of each line.

2.3. Stomatal analysis

Stomata were observed via scanning electron microscopy (SEM) after cultivation for 80 days under well-watered and drought conditions. On sunny days between 09:00 and 10:00 h, eight to twelve light-exposed and fully expanded leaves from the base of four plants stems

were collected for each line. The samples were immediately fixed with a 4 % glutaraldehyde solution in 0.1 M phosphate-buffered saline (PBS; pH 6.8) to avoid any alterations during sample preparation. After rinsing five times with PBS (for 5, 10, 15, 20, and 30 min), they were dehydrated in a graded ethanol series, vacuum-dried, and gold-coated. They were then viewed with a scanning electronic microscope (JSM-6360LV; JEOL Ltd., Tokyo, Japan). The pictures were collected as JPEG digital files. A total of 72 images were obtained for each line, and the number of stomata in each image was recorded using Image J software and converted into final stomatal density. At least 36 stomatal apertures for each line were measured.

2.4. Measurements of ABA content

Abscisic acid was extracted by a method modified from that of Müller and Munné-Bosch [58]. Frozen leaf material (approximately 200 mg FW) was ground in liquid nitrogen and transferred to 2-mL Eppendorf tubes before being extracted with 1 mL of solvent (methanol : isopropanol, 20 : 80 (v/v) with 1% glacial acetic acid), using ultra sonication (4 to 7 °C). After centrifugation (12,000 rpm, 10 min, 4 °C), the supernatants were collected and the pellets were re-extracted with 0.5 mL of solvent. These extractions were repeated three times. Afterward, the supernatants were combined and passed through a 0.22-μm PTFE filter (Waters, Milford, MA, USA). The concentrations of ABA were determined with a Dionex Ultimate 3000 HPLC system (Dionex, http://www.dionex.com) coupled to a Qtrap 5500 triple quadrupole hybrid ion trap mass spectrometer (MDS Sciex, http://www.absciex.com), according to the protocol described by Faix et al. [59].

2.5. RNA extraction, quantification, and gene expression analysis

Total RNAs of the collected samples were extracted by the CTAB method [60]. The cDNA was reverse transcribed from total RNA using a PrimeScript® RT reagent Kit with gDNA Eraser (Perfect Real Time, Takara). Real-time quantitative PCR (Q-RT-PCR) was conducted on an ABI StepOne Plus instrument (Life Technologies, USA) using SYBR® Premix Ex Taq™ II (TliRNaseH Plus, Takara) according to the manufacturer's instructions. The expression level of apple *ELONGATION FACTOR1α* (*EF-1α*; DQ341381) was used as the internal control. The relative quantity of target gene transcript was determined by applying the $2^{-\Delta\Delta CT}$ method [61]. All experiments comprised three biological replicates. Primers used for Q-RT-PCR are listed in Appendix A.

2.6. Statistical analysis

Data were subjected to a one-way ANOVA, and mean differences were assessed by Duncan analyses ($P < 0.05$).

3. Results

3.1. MhYTP1 overexpression lines enhanced the tolerance to long-term moderate drought

To investigate the role of *MhYTP1* in long-term moderate drought, we used three *MhYTP1* overexpressing apple lines, designated OE-36, OE-52 and OE-58, which were previously generated in our laboratory. Under well-watered control conditions, the phenotypes of the transgenic plants did not differ significantly from those of NT plants (Fig. 1A and C). However, after 40 and 80 days of drought treatment, transgenic plants showed significantly lower relative electrolyte leakage, representing the extent of cell damage, than NT plants (Fig. 1B). On Day 40 and Day 80 of treatment, the SPAD values, indicating the total chlorophyll content, in transgenic plants were significantly higher than that of NT plants (Fig. 1D). These data suggested that overexpression of *MhYTP1* resulted in less physiological damage under long-term moderate drought conditions.

3.2. MhYTP1 overexpression lines showed higher rates of photosynthesis and lower rates of transpiration under long-term moderate drought conditions

When stomata close in response to drought stress, the rate of photosynthesis (P_n), which indicates the assimilation efficiency of CO_2, and transpiration (T_r), which reveals the loss rate of water, are directly decreased. To examine the tolerance phenotype of transgenic plants in this respect, we monitored their gas and water exchange parameters. For well-watered plants, P_n in transgenic plants was marginally higher than in NT plants after 80 days (Fig. 2A). In contrast, under simulated drought conditions for 40 or 80 days, P_n in transgenic plants was substantially (~1.21-1.29 times) higher than in NT plants (Fig. 2B). The rate of transpiration was lower in transgenic plants than in NT plants under both well-watered and long-term moderate drought conditions (Fig. 2C and D). These gas and water exchange data suggested that plants overexpressing *MhYTP1* could maintain a better photosynthetic and transpiration system under long-term moderate drought conditions.

3.3. MhYTP1 overexpression lines produced more biomass under both well-watered and long-term moderate drought conditions

To examine the effect of *MhYTP1* on biomass production under long-term drought stress, transgenic and NT apple plants with similar sizes were selected and grown under well-watered and moderate drought conditions (Fig. 3A and D). After 80 days, the biomass production of transgenic and NT plants was measured, including the fresh and dry weight for their leaves, stems and roots, respectively (Fig. 3B, C, E, F). Regardless of the treatment types, the biomass production was higher in transgenic plants than in NT plants.

3.4. MhYTP1 overexpression improved water use efficiency under both well-watered and long-term moderate drought conditions

Improving water use efficiency (WUE) is a critical way for plants to adapt to water deficiency [62]. To examine the effect of *MhYTP1* on WUE, we calculated the instantaneous water use efficiency (WUE_I, the value of P_n / T_r) under both well-watered and long-term moderate drought conditions. As shown in Fig. 4A and B, after 40 days and 80 days of cultivation, WUE_I in transgenic plants was higher than in NT plants. We also calculated the long-term WUE (WUE_L, the value of biomass accumulation / the total amount of irrigated water). After 80 days of cultivation, transgenic plants showed significantly higher WUE_L than NT plants under both well-watered and drought conditions (Fig. 4C). The results indicate that overexpressing *MhYTP1* improved WUE. The growth phenotype of NT and transgenic plants after 80 days cultivation under drought condition was shown in Appendix C.

3.5. Overexpression of MhYTP1 affected stomatal behavior

The function of *MhYTP1* in controlling water losses prompted our investigation into the operation of stomatal behavior, a major factor affecting water-holding capacity in plant leaves. The stomatal characters of NT and transgenic plants were checked using scanning electron microscopy after 80 days of cultivation under both well-watered and drought conditions (Fig. 5A). The transgenic plants showed increased stomatal density and greater stomatal closure than NT plants after 80 days cultivation under both well-watered and long-term moderate drought conditions (Fig. 5B and C). These findings indicated that, the stomata of the transgenic plants responded to growth better than did the NT plants.

3.6. Overexpression of MhYTP1 increased ABA content and affected expression of ABA signaling-related genes

Stomatal closure is one of the most important ABA-mediated

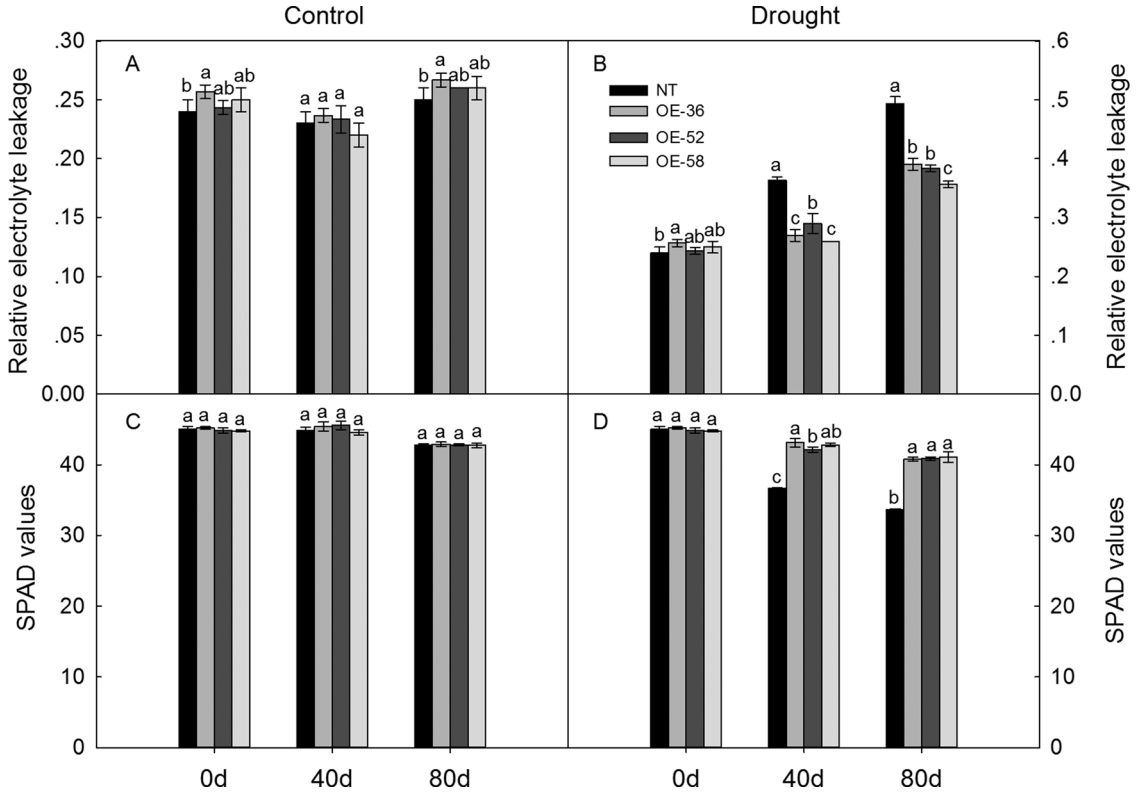

Fig. 1. Changes in relative electrolyte leakage and SPAD values of NT and transgenic plants on Day 0, 40 and 80 of treatment. (A) Relative electrolyte leakage in NT and transgenic plants under well-watered conditions. (B) Relative electrolyte leakage in NT and transgenic plants under long-term drought treatment conditions. (C) SPAD values in NT and transgenic plants under well-watered conditions. (D) SPAD values in NT and transgenic plants under long-term drought treatment conditions. Data are means of three replicates with SD. Different letters indicate significant differences between NT and transgenic plants on the same day of different treatments, according to one-way ANOVA Duncan's multiple range tests ($P < 0.05$).

physiological responses. To determine if the observed promotive effect of *MhYTP1* on drought tolerance might be mediated via ABA and ABA-responsive gene expression, we first directly measured ABA levels. ABA content did not show significant differences between transgenic plants and NT plants under well-watered conditions. However, after 40 days and 80 days of drought treatment, the ABA content of transgenic plants was substantially higher than in NT plants (Fig. 6). Two ABA receptor genes, *PYL4* (MDP0000228470) and *PYL9* (MDP0000284624), were expressed to similar levels between transgenic and NT plants under well-watered conditions (Fig. 7A and C). By contrast, under drought condition, these two genes were expressed to lower levels in transgenic plants compared with NT plants (Fig. 7B and D). *ABI1* (MDP0000437033), *ABI2* (MDP0000231674) and *OST1* (MDP0000224969), the genes for encoding phosphatase 2C (PP2C), were up-regulated after 40 and 80 days cultivation under moderate drought conditions (Fig. 7F, H, P). Transgenic plants showed higher expression of *ABI1* than NT under well-watered conditions, whereas *ABI2* and *OST1* did not show significant differences between transgenic plants and NT plants (Fig. 7E, G, O). The ABA-responsive gene *ABF3* (MDP0000701734 and MDP0000248567) had higher transcription level in transgenic plants under both well-watered and drought conditions (Fig. 7I, J, K, L). For the *RD22* gene (MDP0000268523), which serves as a marker gene for monitoring ABA and stress responses in plants, transgenic plants had lower transcript levels than NT plants under well-watered conditions (Fig. 7M). However, after 40 and 80 days of treatment, higher transcript levels were observed in transgenic plants (Fig. 7N). These findings suggested that the observed increase in drought tolerance conferred by expression of *MhYTP1* is at least partly mediated via ABA and ABA-responsive gene expression.

3.7. *MhYTP1* overexpression lines behaved better as scions than as rootstocks

We previously demonstrated that *MhYTP1* was expressed most strongly in the apple shoot apex [63]. *Arabidopsis* seedlings expressing GUS from the *MhYTP1* promoter showed strong staining only within the vasculature of the hypocotyl (Appendix B). To determine the biological effect of ectopic *MhYTP1* expression in various parts of apple, we conducted grafting experiments using transgenic lines overexpressing *MhYTP1* or NT either as rootstock or as scion (Fig. 8A). The SPAD values, relative electrolyte leakage, and P_n did not show a significant difference when transgenic plants were used as scions or as rootstocks after 60 days treatment under well-watered conditions (Fig. 8B–D). However, under drought condition, SPAD and P_n values were significantly higher when transgenic plants were used as scions rather than rootstocks (Fig. 8B and D). The SPAD was higher after 60 days cultivation under drought condition when transgenic plants were used as rootstocks than NT plants were used as rootstocks (Fig. 8B). Relative electrolyte leakage was significantly lower when transgenic plants were used as scions rather than rootstocks (Fig. 8C). Under both well-watered and drought conditions, the T_r was lower and WUE_I was higher after 60 days cultivation when transgenic plants were used as scions rather than rootstocks and when transgenic plants were used as rootstocks than NT plants were used as rootstocks (Fig. 8E and F). This demonstrated that transgenic plants have better growth and drought tolerance as scions than rootstocks and show similar effect when transgenic rather than NT plants were used as rootstocks.

4. Discussion

Environmental abiotic stresses such as drought, heat, cold and high

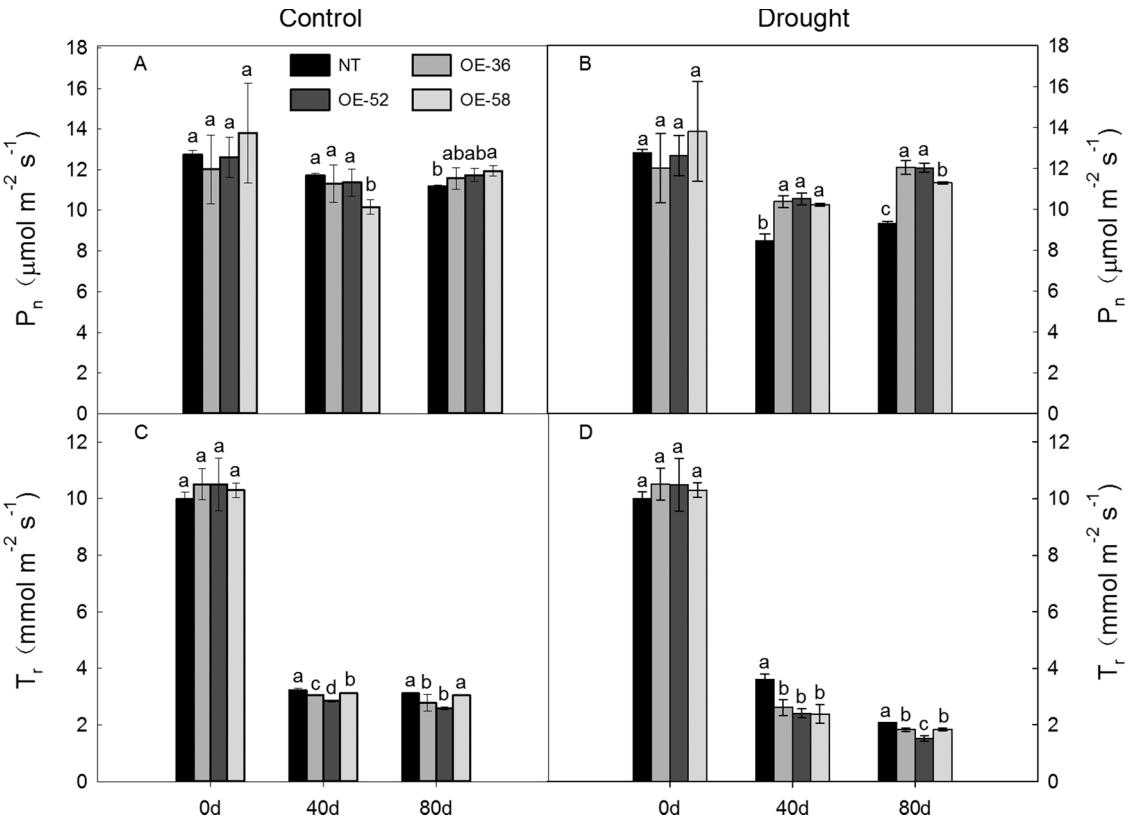

Fig. 2. Changes in photosynthesis (P_n) and transpiration (T_r) of NT and transgenic plants on Day 0, 40 and 80 of treatment. (A) P_n in NT and transgenic plants under well-watered conditions. (B) P_n in NT and transgenic plants under long-term drought treatment conditions. (C) T_r in NT and transgenic plants under well-watered conditions. (D) T_r in NT and transgenic plants under long-term drought treatment conditions. Data are means of three replicates with SD. Different letters indicate significant differences between NT and transgenic plants on the same day of different treatments, according to one-way ANOVA Duncan's multiple range tests ($P < 0.05$).

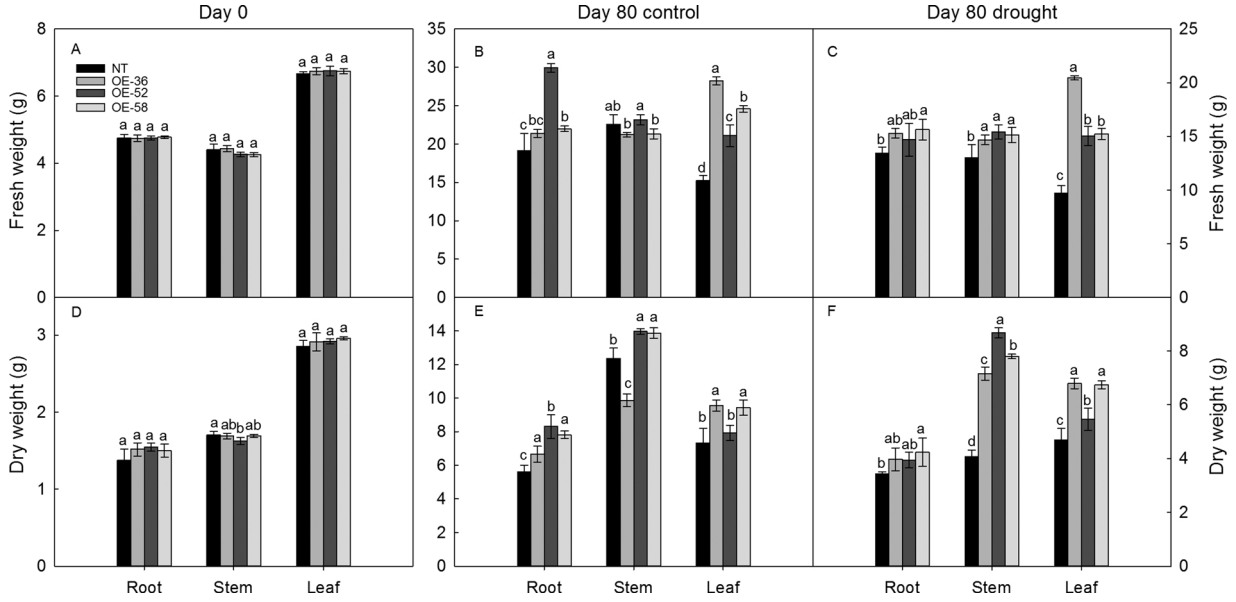

Fig. 3. Biomass accumulation of NT and transgenic plants after 80 days cultivation under well-watered and long-term drought treatment conditions. (A) Fresh weight of NT and transgenic plants on day 0 of treatment. (B) Fresh weight of NT and transgenic plants at the end of well-watered treatment. (C) Fresh weight of NT and transgenic plants at the end of long-term drought treatment. (D) Dry weight of NT and transgenic plants on day 0 of treatment. (E) Dry weight of NT and transgenic plants at the end of well-watered treatment. (F) Dry weight of NT and transgenic plants at the end of long-term drought treatment. Data are means of three replicates with SD. Different letters indicate significant differences between NT and transgenic plants on the same day of different tissues, according to one-way ANOVA Duncan's multiple range tests ($P < 0.05$).

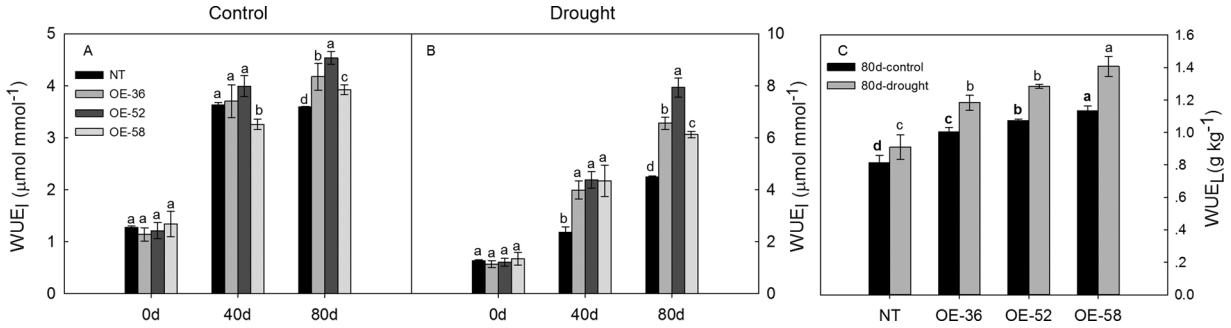

Fig. 4. Instantaneous water use efficiency (WUE_I) of NT and transgenic plants on Day 0, 40 and 80 of treatment and long term water use efficiency (WUE_L) of NT and transgenic plants after 80 days cultivation under well-watered and long-term drought treatment conditions. (A) WUE_I for NT and transgenic plants under well-watered conditions. (B) WUE_I for NT and transgenic plants under long-term drought treatment conditions. (C) WUE_L for NT and transgenic plants at the end of well-watered and long-term drought treatment. Data are means of three replicates with SD. Different letters indicate significant differences between NT and transgenic plants on Day 80 of different treatments, according to one-way ANOVA Duncan's multiple range tests ($P < 0.05$).

salinity affect plant growth, productivity and distribution. Survival in response to abiotic stress involves various physiological, biochemical and genetic responses [64]. RNA binding proteins contribute to transcriptional and post-transcriptional regulation of gene expression important for stress responses. Here, we focused on evaluating the biological roles of *MhYTP1*, one member of apple YT521-B homology domain-containing RNA binding protein family isolated from *M. hupehensis*, in apple plants upon long-term moderate drought stress.

Stomata are present on the leaf surface and allow plants to regulate gas exchange and water loss. Stomatal development and activity is regulated both by external environmental cues and by endogenous genes [65]. Water limitation of plants causes stomatal closure to prevent water loss by transpiration. Drought resistance is enhanced when stomatal density is decreased or stomatal aperture is smaller. Stomatal behavior is mediated by ABA for coping with water deficits [66–72]. We found that the ABA content and stomatal density of transgenic

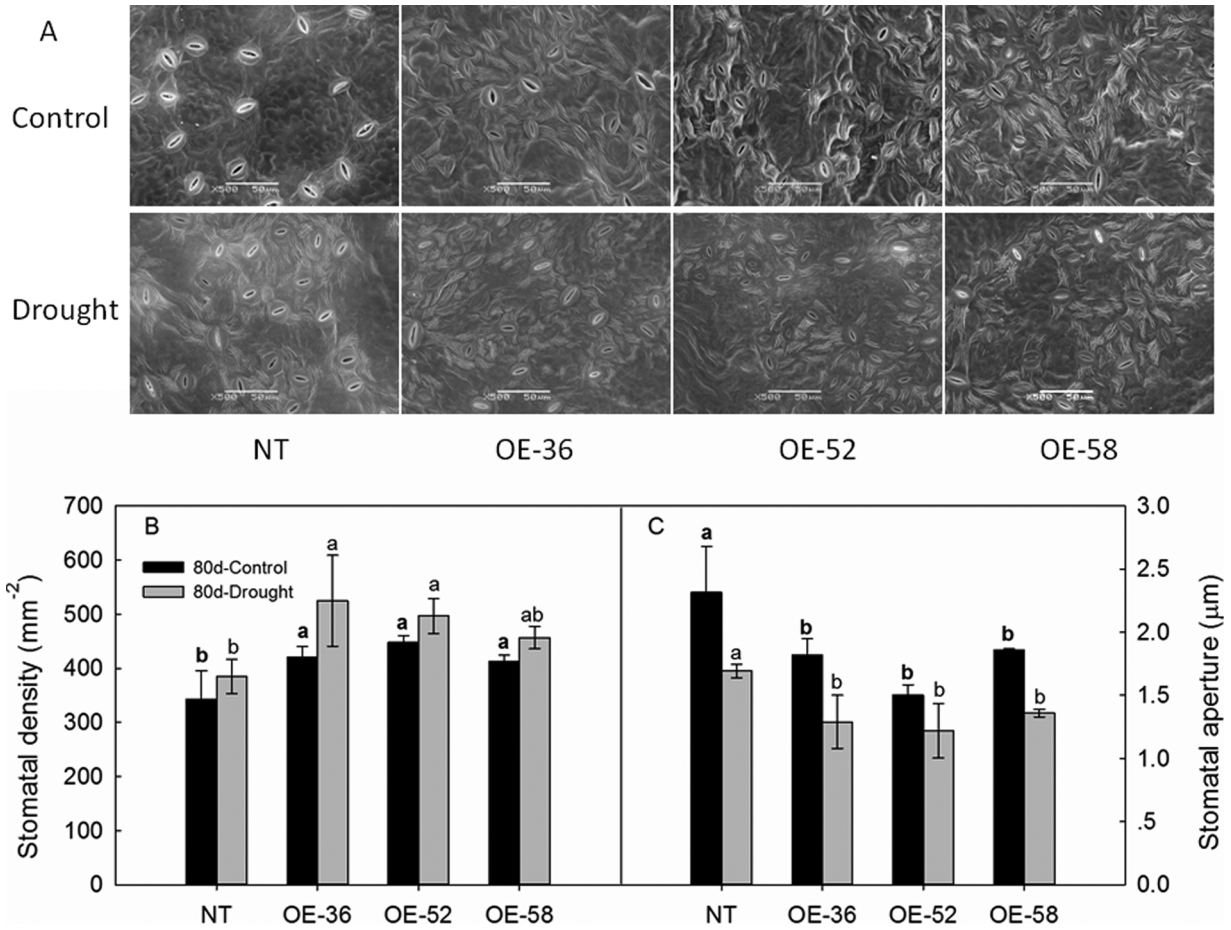

Fig. 5. Stomatal behavior of NT and transgenic plants after 80 days cultivation under well-watered and long-term drought treatment conditions. (A) Stomatal guard cells of NT and transgenic plants were observed at the end of treatment via scanning electron microscopy. Representative photographs for stomata from NT and transgenic lines. Stomatal density (B) and aperture (C) of NT and transgenic lines was observed at the end of treatment. Data are means of three replicates with SD. Different letters indicate significant differences between NT and transgenic plants on Day 80 of different treatments, according to one-way ANOVA Duncan's multiple range tests ($P < 0.05$).

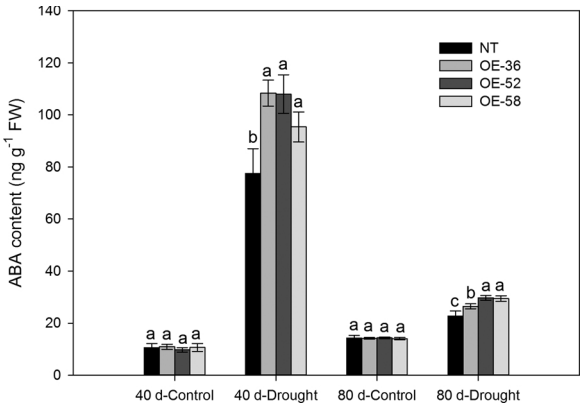

Fig. 6. Changes in ABA content of NT and transgenic plants on Day 40 and 80 of treatment. Data are means of three replicates with SD. Different letters indicate significant differences between NT and transgenic plants on the same day of different treatments, according to one-way ANOVA Duncan's multiple range tests ($P < 0.05$).

plants were increased to a greater extent than in NT plants, and that stomatal aperture was smaller in transgenic plants than in NT plants. Therefore, we conclude that *MhYTP1* influences ABA-mediated stomatal behavior. The water loss by transpiration caused by increased stomatal density may be replenished by smaller stomatal aperture.

Furthermore, numerous studies have focused on ABA not only because of its regulation of leaf stomata during response to water deficit, but also its role in increasing WUE in agriculture [73]. Improved WUE in the presence of elevated ABA levels has been demonstrated in two transgenic tomato (*Solanum lycopersicum*) lines in which there was overproduction of ABA [74]. Similar results have been reported in *Arabidopsis thaliana*, poplar (*Populus davidiana*), bean (*Phaseolus vulgaris*), sugar beet (*Beta vulgaris*) and corn (*Zea mays*) [75–77]. Improving WUE usually comes at the cost of reduced assimilation and slower growth. However, we found that overexpression of *MhYTP1* improved WUE even while increasing biomass production. This increase in biomass is consistent with the elevated P_n rate of the transgenic plants.

Upon ABA perception, the soluble ABA receptors interact with and inhibit clade-A protein phosphatase 2Cs (PP2Cs): ABA-insensitive 1 (ABI1), ABA-insensitive 2 (ABI2), hypersensitive to ABA 1 (HAB1), and protein phosphatase 2CA (PP2CA); this interaction relieves inhibition of the serine-threonine kinase OPEN STOMATA 1 (OST1) [78]. *OST1* encodes an SNF1-related protein kinase 2 (SnRK2)-type protein kinase that participates in ABA-mediated stomatal closure [10]. OST1 activates the anion channel SLAC1 through phosphorylation, thereby regulating guard cell turgor and stomatal apertures [79,80]. Phosphorylation-based channel regulation then occurs, mediated by OST1 as well as several other classes of protein kinases, including Ca^{2+}-dependent kinases (CPKs) and mitogen-activated protein kinases (MPKs) [81–85]. We found that transcript accumulation for two ABA receptor genes, *PYL4* and *PYL9*, was distinctly suppressed in transgenic plants under drought condition. This result differed from the ABA-dependent signaling in response to osmotic stress in plants. PYL-mediated ABA signaling inhibits osmotic stress activation of ABA-independent SnRK2s [34]. Thus ABA-independent SnRK2 activity may be playing more important role than ABA-dependent signaling at this time. Meanwhile, the expression of genes encoding a group of Ser/Thr PP2Cs, including *ABI2* and *OST1*, was increased in those plants under drought condition. *ABI1* expression was increased in those plants under well-watered and drought conditions. The ABA-responsive gene, *ABF3*, had higher transcript levels in transgenic plants under both well-watered and drought conditions. Moreover, *RD22*, which serves as one of marker genes for

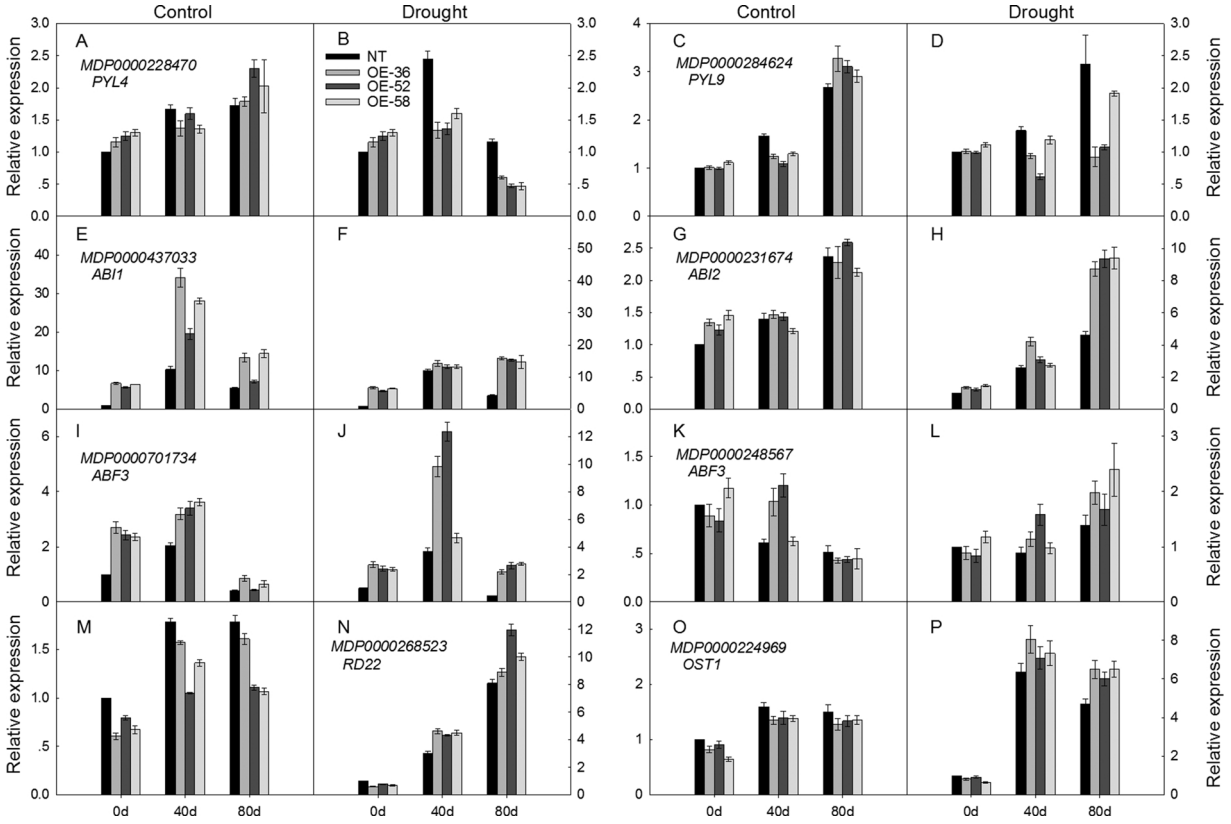

Fig. 7. Analysis of expression of ABA-signaling-related genes in NT and transgenic plants under well-watered and drought conditions. Total RNA was isolated from leaf samples collected at the indicated times, and expression levels were calculated relative to expression of *Malus EF-1α* mRNA. Data are means of three replicates with SD.

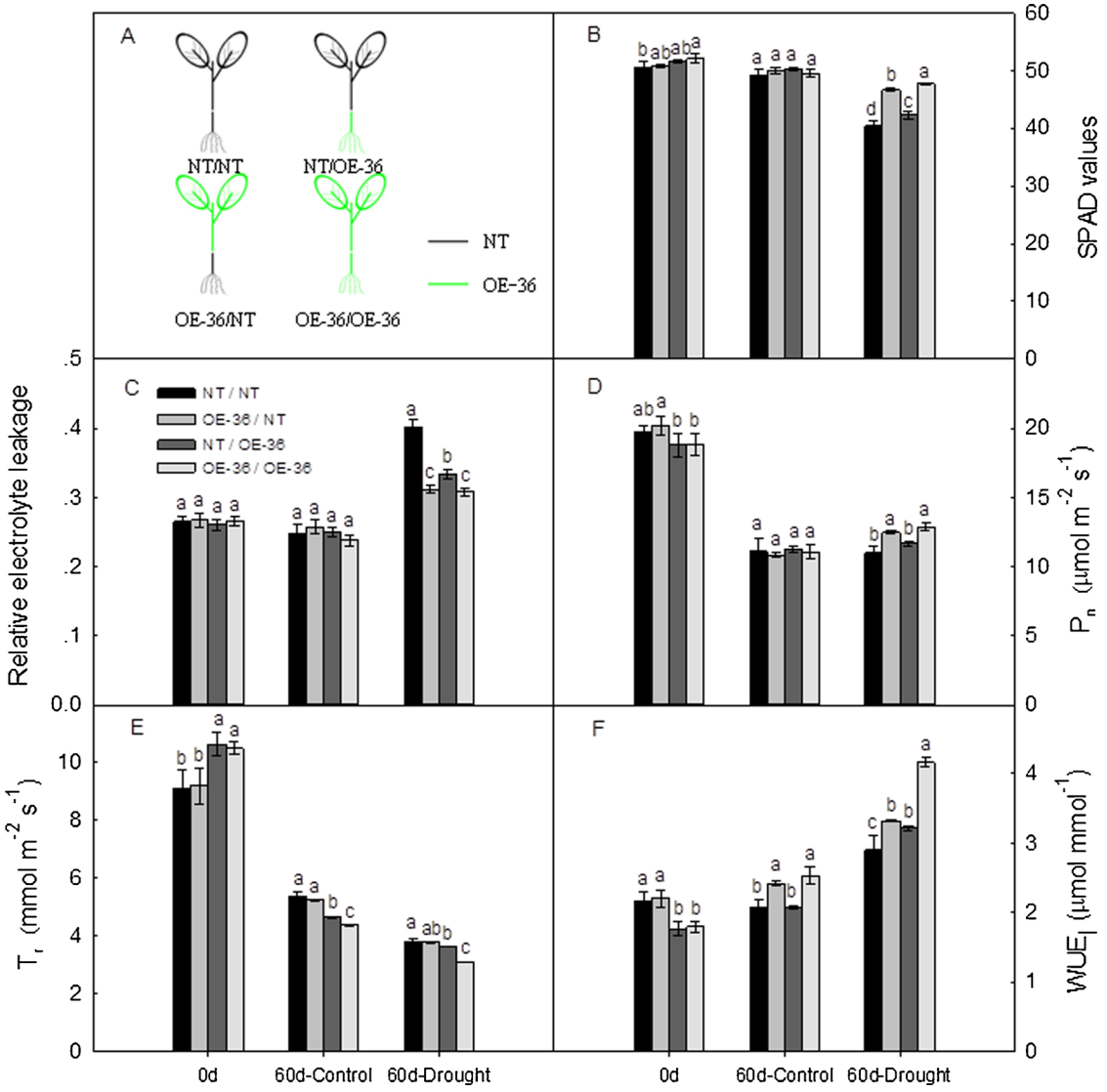

Fig. 8. Grafting experiments demonstrate that transgenic plants have better growth and drought tolerance as scions than rootstocks. (A) Diagrams illustrate the four grafting combinations between scion and rootstock. (B-F) Analysis of grafted apple plants after 60 days cultivation under well-watered and drought treatment conditions: (B) SPAD; (C) Relative electrolyte leakage; (D) P_n; (E) T_r; (F) WUE_I. Data are means of three replicates with SD. Different letters indicate significant differences between the four grafting combinations on the same day of different treatments, according to one-way ANOVA Duncan's multiple range tests ($P < 0.05$).

monitoring ABA and stress responses in plants, showed lower expression in transgenic plants than NT plants under well-watered conditions. However, higher transcription level was observed in transgenic plants than NT plants under drought condition. In addition, *MhYTP1* overexpression increased ABA content under drought condition. Thus, *MhYTP1* appears to positively affect the ABA signaling pathway.

Grafting is an ancient technology used in horticulture and agriculture to help plants gain beneficial properties, such as stress tolerance, increase in production, and improve fruit quality [36,86]. Drought is a major constraint on apple production, and breeding new apple scions or rootstocks with enhanced drought resistance and WUE would be beneficial for this industry. The *MhYTP1* promoter was strongly active in the vasculature of the hypocotyl when expressed in transgenic *Arabidopsis*. Expression of *MhYTP1* in apple stems might play important roles in enhancing WUE of grafted apple plants. To gain insight into the stem-specific expression of *MhYTP1*, grafting experiments were performed. Our data showed that, when used as scions, *MhYTP1* transgenic lines conferred higher WUE_I values than when used as rootstocks, indicating the functional specialization of *MhYTP1* in scions. At the same time, the plants have better growth and drought tolerance when transgenic rather than NT plants were used as rootstocks, indicating the functional of *MhYTP1* to a certain extent in rootstocks. This gives us an opportunity to understand the role of *MhYTP1* in the grafted plants and serves as a basis for further research that will help enhance drought tolerance and apple breeding.

In conclusion, we have demonstrated that *MhYTP1* confers tolerance to long-term, moderate drought conditions when expressed transgenically in apple. This is at least partly due to the role of *MhYTP1* in mediating ABA level, which affected stomatal behavior and expression of a suite of ABA signaling-related genes. Furthermore, we found *MhYTP1* transgenic plants have better growth and drought tolerance as scions than rootstocks and show similar effect when transgenic comparing NT plants were used as rootstocks. These findings provide a promising perspective for future efforts in crop breeding.

Author Contributions

Changhai Liu and **Fengwang Ma:** experimental design and implementation, composition and review of manuscript, financial support for experiments and laboratory apparatus; **Qingmei Guan** and **Steven**

van Nocker: composition and review of manuscript; **Yangchun Xue:** experimental implementation; **Na Wang:** experimental design and implementation; **Tianli Guo:** experimental design and implementation, manuscript composition.

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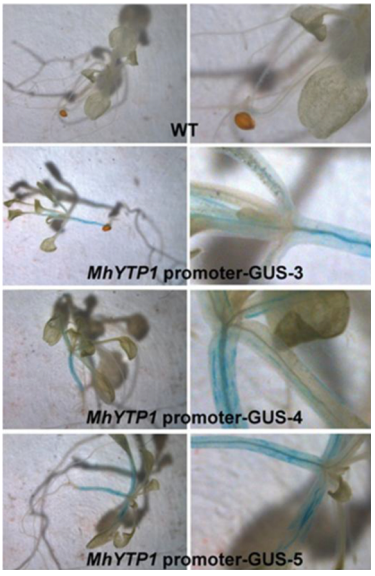
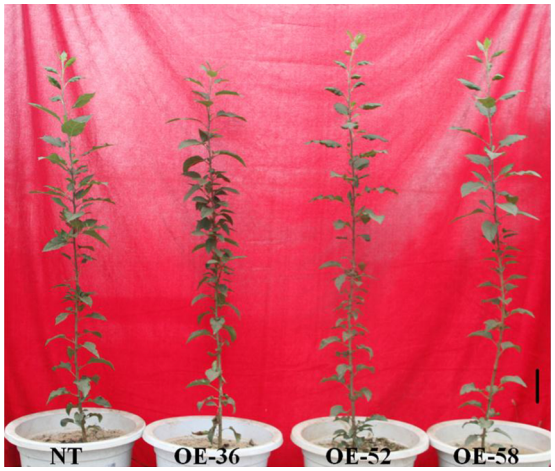
Appendix A. Gene information and primers used for real-time quantitative PCR

Primers name	sequences
RT-*PYL4*-F	CGGCGTCGTCGCAGTACCAA
RT-*PYL4*-R	TCCTGAGTCACGGCGGAGCA
RT-*PYL9*-F	TACATAAGGAGGCACCACAG
RT-*PYL9*-R	CACCAAGGACCAGACGAGAT
RT-*ABI1*-F	GGGAGGAACAACAAGGGA
RT-*ABI1*-R	AAGAAATGAACGGGTGAGAT
RT-*ABI2*-F	GACGACGAATGCCTAATT
RT-*ABI2*-R	TCTTGTGCCAGAGGAGTA
RT-*ABF3(34)*-F	AATGCTCAGTTGGGTAGTCC
RT-*ABF3(34)*-R	TTCGCAGGTGAAGGCGTC
RT-*ABF3(67)*-F	CGAACGCTTAGTCAGAAA
RT-*ABF3(67)*-R	AAAGTCCTCCAAAGTCATC
RT-*RD22*-F	GACATGCGTCCTGGAACAAC
RT-*RD22*-R	ATTTCTGGCAGCTTGTTGGA
RT-*OST1*-F	AGCACCTGAAGTCCTATC
RT-*OST1*-R	ACTAAGAATCCGCCCAAT
RT-*EF1*-F	ATTCAAGTATGCCTGGGTGC
RT-*EF1*-R	CAGTCAGCCTGTGATGTTCC

Appendix B. GUS histochemical assays of wild type and *pro-MhYTP1*-GUS transferred *Arabidopsis* plants. The images in bottom panel are enlargements from images shown in the upper panel



Appendix C. Growth phenotype of NT and transgenic plants after 80 days cultivation under drought condition, bar, 10 cm



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