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Physiological and transcriptomic analyses of roots from *Malus sieversii* under drought stress

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Abstract

Water deficit is one of the main limiting factors for apple growth and production. Root architecture plays an important role in drought tolerance of plants. However, little is known about the molecular basis of root system in apple trees under drought. In this study, we compared root morphology of two widely used rootstocks of apple (R3 and *Malus sieversii*) under drought. Our results suggested that *M. sieversii* is more tolerant to drought than R3, since *M. sieversii* had a higher ratio of root to shoot as well as root hydraulic conductivity under long-term drought conditions. We then performed whole-genome transcriptomic analysis to figure out the molecular basis of root responses in *M. sieversii* under drought. It was found that genes involved in transcription regulation, signaling or biosynthesis of hormones, and oxidative stress were differentially expressed under drought. Consistent with the gene expression profile, roots of *M. sieversii* had higher activities of peroxidase (POD) and superoxide dismutase (SOD) under drought, as well as higher content of abscisic acid (ABA) and lower content of auxin. Taken together, our results revealed the physiological and transcriptomic analyses of *M. sieversii* roots in response to drought.

Keywords: *Malus sieversii*, root architecture, drought stress, RNA-seq

1. Introduction

Drought is considered as one of the key challenges on fruit trees including apple trees since drought often leads to

slow growth and reduced productivity (Condon *et al.* 2002). Although great progress has been made in understanding apple responses under drought (Wu *et al.* 2014; Li *et al.* 2015; Zhou *et al.* 2015; Tan *et al.* 2017), so far, most efforts have targeted above-ground responses.

Roots are essential for uptake of nutrient and water, thus are essential for plant growth and productivity. Nonetheless, roots were less studied due to the difficulty of phenotype observation, let alone the molecular basis of root system in response to drought. By using suppression subtractive hybridization, Bassett *et al.* (2014) identified both up- and down-regulated genes in roots of *Malus domestica* (cv. Royal Gala) during water deficit. MzPIP2;1, an encoded plasma membrane intrinsic protein, improved lateral root growth of *Arabidopsis* under drought (Wang *et al.* 2015).

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In addition, ectopic expression of *MdVHA-A* or *MdMYB1* in tobacco also promotes lateral root system and root growth under drought (Wang *et al.* 2012; Dong *et al.* 2013). Moreover, research conducted by Liao *et al.* (2017) suggested that *MsDREB6.2* is critical for apple root hydraulic conductance and drought tolerance.

When plants suffered from drought stress, a variety of responses had been occurred on roots to resist drought, including increased root to shoot ratio, enhanced fine root growth, deeper taproots and accumulation of solutes (Pimentel *et al.* 1999; Querejeta *et al.* 2003; Tuberosa 2012; Claeys and Inzé 2013). These changes allow increase of water uptake from roots to balance water loss during drought. Unlike herbaceous plants, roots of woody plants generally have secondary growth, which can extend far deeper and grow far thicker to absorb and transport more water to aerial part, indicating that trees like apple trees might respond to drought by increasing root to shoot ratios and root depth (Kozłowski and Pallardy 2002).

Drought also breaks the balance between reactive oxygen species (ROS) production and their elimination, which normally results in an increase in levels of ROS such as hydrogen peroxide (H_2O_2) and singlet oxygen (Cruz de Carvalho 2013). The balance between ROS production and their elimination influences many physiological processes through the redox-dependent regulation of hormone signaling. For example, H_2O_2 takes a part in control of auxin, salicylic acid, and jasmonate responses by thiol regulation linked to the glutathione (Han *et al.* 2013a, b; Gao *et al.* 2014). Hence, the accumulation of ROS may result in an imbalance of hormone signaling. When plants are subjected to severe drought stress, physiological damages caused by ROS accumulation will emerge, thus interfering with the subcellular homeostasis, so that the cells will become dysfunctional (Dat *et al.* 2000). Peroxidase (POD) and superoxide dismutase (SOD) decompose ROS to inhibit damages such as membrane lipid peroxidation, hence protect plants from drought stress (Gill and Tuteja 2010; Bela *et al.* 2015).

The major stress-responsive hormones produced by plants are abscisic acid (ABA), auxin and gibberellins (GAs) (Bari and Jones 2009). ABA is known as a major regulator in mediating plant abiotic stress response (Bari and Jones 2009), such as responses to drought, cold, heat and salinity. Under drought stress, increased ABA induces stomatal closure (Popko *et al.* 2010; Wilkinson and Davies 2010) and reduces lateral root formation and elongation while promoting primary root elongation to access water deep underground (Malamy 2005; Xiong *et al.* 2006). Auxin is a universal partner in hormonal interactions, promoting or inhibiting root growth in different hormonal pathways (Benková and Hejálko 2009). In addition, auxin regulates lateral root formation in *Arabidopsis*. Regulation of root

architecture by auxin suggests that auxin should play pivotal roles in plant drought stress tolerance (Kohli *et al.* 2013). When plants suffered from drought stress, auxin negatively regulates *DEEPER ROOTING 1* (DRO1) that is involved in cell elongation and geotropism (Uga *et al.* 2013). In addition, auxin, in consort with cytokinin, could inhibit ABA-induced stomatal closure under drought (Tanaka *et al.* 2006), playing as an inhibitor of drought induced ABA response. GA could cross talk with other stress-responsive hormone such as ABA, and regulates drought stress response by interacting with DELLA repressors. GA deficiency enhanced drought tolerance in tomato, finger millet (*Eleusine coracana*), and Tef (*Eragrostis tef*) (Nir *et al.* 2014; Plaza-Wüthrich *et al.* 2016).

Adjustment of xylem conducting system is important for plants to maximize their water uptake ability and adapt to drought stress (Sperry *et al.* 2002; Maseda and Fernández 2006). Xylem conducting system is composed of vessel network from roots to leaves, supplying water and nutrients to above ground. Conductivity of this network is determined by the number and diameter of vessels. Diameter is a major factor determining hydraulic conductivity because of the fourth-power relationship described by the Hagen-Poiseuille's law (Tyree and Ewers 1991). Therefore, even a minor difference in mean diameter of vessels will lead to a huge difference in conductivity.

Apple is one of the most important fruit crops worldwide. However, one of the major limiting factors of apple production worldwide is water deficit. Rootstocks are widely used in horticulture for reproduction of fruit trees (Ikinci *et al.* 2014). Selection of rootstocks is usually based on the compatibility between the scion and rootstock, soil type and stress tolerance of rootstocks (Gregory *et al.* 2013). One of the widely-used drought-tolerant rootstocks of apple is *Malus sieversii* (Wang *et al.* 2012; Li *et al.* 2015). *M. sieversii*, however, is difficult to work on because it is a vigorous stock. Dwarfing rootstocks such as M26 and M9-T337 are easier to manipulate but are usually vulnerable to drought because of shallow root system. For that reason, molecular basis of root responses of *M. sieversii* under drought is critically essential for breeders to develop new dwarfing rootstocks with drought tolerance.

To discover the importance and molecular basis of drought resistance of *M. sieversii* roots, we examined root morphology of *M. sieversii* under drought with dwarfing rootstock of R3 as control and performed whole-genome transcriptomic analysis. Because RNA-seq results revealed that genes involved in transcription regulation, signaling or biosynthesis of hormones, and oxidative stress were differentially expressed under drought, we also compared activities of POD and SOD, contents of ABA, indole-3-acetic acid (IAA), and GA in roots of *M. sieversii* and R3 under

drought. Our results provided the evidence for importance of root architecture, endogenous hormones, and antioxidant enzyme in drought tolerance of *M. sieversii*.

2. Materials and methods

2.1. Plant materials for long-term drought treatment

Experiments were conducted at Northwest A&F University, Yangling, China (34°20'N, 108°24'E). Seeds of *M. sieversii* and R3 were grown in pots (5 cm×5 cm×10cm) filled with local loess soil:sand medium (5:1, v:v). Pots were placed in a greenhouse under natural illumination, with temperature of 20–35°C and humidity of 50–75%. Sixty seedlings of each rootstock were transferred to bigger pots (30 cm×18 cm) filled with local loess soil:wormcast-based medium (5:1, v:v) in June, 2016. In July, seedlings of each rootstock were divided into two groups (each group contains 30 seedlings): well-watered group and long-term drought group. Seedlings in well-watered group were daily irrigated to maintain field capacity (FC) of 75–85% and seedlings in long-term drought group were daily irrigated to maintain a FC of 45–55%. The treatments lasted for 2 months. At the end of treatment, roots from 10 uniform trees in each rootstock were harvested for morphology and vessel analysis. Roots from 20 other uniform trees in each rootstock were harvested for measurement of root scan, antioxidant enzyme and hormone. Five biological replicates were performed for each treatment and each biological replicate included two trees.

2.2. Plant materials and simulated-drought treatment for RNA-seq

Thirty *M. sieversii* seedlings were transferred to plastic containers containing 20 L of Hoagland solution in June. The hydroponic culture was performed in a greenhouse under natural illumination, with a temperature of 20–35°C and humidity of 50–75%. In July, seedlings were treated with 20% PEG6000 (Sigma, USA) for 0, 6, 12, or 24 h. At the end of each treatment, roots were washed and quickly frozen in liquid nitrogen, stored at –80°C for RNA-seq and quantitative real-time PCR (qRT-PCR) analysis. Primers used were listed in Appendix A.

2.3. Root morphology analysis

Shoot height, diameter of the stem and dry weight of root and shoot were measured directly after harvesting. Total root length, root surface area, root volume and average diameter were measured by Winrhizo 2002 (Regent Corporation, Canada). Five biological replicates were performed for each measurement.

2.4. Vessel analysis

Roots with diameter of 2–6 mm were selected for vessel analysis. Five root segments taken at 5 cm underground were cut and fixed in FAA stationary solution (5% formalin, 5% acetic acid, and 90% ethyl alcohol) for 24 h, then transferred into 18% ammonia at 65°C for 90 min for dissociation. The root segments were subsequently dehydrated in ethyl alcohol with various concentrations (30, 50, 75, 85, 95, and 100%, twice) for 3 h one by one. Transparent roots obtained from sequential xylene treatment were embedded in paraffin. Then embedded blocks were sectioned with a rotary microtome (RM2125RTS, Leica, Germany) and observed with a light microscope (80i, Nikko, Japan). Photos were taken with a digital camera (CFI60, Nikko, Japan) mounted on the microscope. The theoretical maximum hydraulic conductivity was calculated with the equation described in a first approximation by Hagen-Poiseuille's law (eq. (1)). Because cross-section of the vessel is ellipse, a modified equation (Nobel *et al.* 2005) was used for calculation of hydraulic conductivity of apple roots (eq. (2)).

$$K_s^{\text{theo}} = \sum \left(\frac{\pi \rho}{8 \eta} \right) r_{\text{ves}}^4 / A_{\text{xyl}} \quad (1)$$

$$r_{\text{ves}}^4 = D_{\text{max}}^3 D_{\text{min}}^3 / 8 (D_{\text{max}}^2 + D_{\text{min}}^2) \quad (2)$$

Where, K_s^{theo} , the root-specific theoretical conductivity ($\text{kg m}^{-2} \text{s}^{-1} \text{MPa}^{-1}$), ρ is the density of water at 20°C (998.205 kg m^{-3}), η is the viscosity of water at 20°C ($1.002 \times 10^{-9} \text{MPa} \cdot \text{s}$), r_{ves} is the vessel radius, A_{xyl} is the area of specific root xylem, D_{max} and D_{min} are the maximal and minimum axes of vessel.

2.5. Activity of ROS scavenging enzyme and soluble protein in roots

Crude enzyme solution was prepared according to the methods described by Chen *et al.* (2013). Activity of superoxide dismutase (SOD) was measured by the SOD inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) (Dhindsa *et al.* 1981). Peroxidase (POD) activity was measured by the increase in absorbance at 470 nm because of the guaiacol oxidized by H_2O_2 (Maehly and Chance 1955). Soluble protein was measured by Bradford method (Bradford 1976). Three technical replicates for each of the three biological replicates were performed for each measurement.

2.6. Endogenous hormone content analysis

The content of ABA, gibberellin A3 (GA_3) and IAA were analyzed with the methods described by You *et al.* (2016). Samples for RNA-seq described in 2.2 section were analyzed.

2.7. RNA extraction from roots of *M. sieversii* and RNA-seq analysis

Root RNA from *M. sieversii* was extracted using RNA prep Pure Plant Kit (Polysaccharideas & Polyphenols-rich, #DP441; Tiangen, China). RNA-seq was performed with Illumina Hiseq 4500 platform by Novogene Bioinformatics Technology Co., Ltd. (Novogene, Beijing, China) with the methods described by Zhang *et al.* (2015).

2.8. qRT-PCR analysis

qRT-PCR analysis was performed according to Guan *et al.* (2013). Primers used were listed in Appendix A.

2.9. Data availability

The NCBI accession number for RNA-seq is PRJNA379354.

3. Results

3.1. Morphologic responses of roots under long-term drought stress

As the first part of plants to sense water deficit, root development plays a key role in plant adaptation to drought stress (Janiak *et al.* 2016). To understand the root morphology of apple rootstocks (R3 and *M. sieversii*) in response to drought stress, we measured dry weight of root and shoot, root to shoot ratio, height, and diameter of stems after a 2-month drought treatment. It was obvious that growth of both roots and shoots was significantly affected by drought (Fig. 1). The root dry weight was significantly reduced in R3 but not in *M. sieversii* after long-term drought. In addition, *M. sieversii* had higher root dry weight than R3 under drought (Fig. 1-A). Dry weight of shoots in both R3 and *M. sieversii* were significantly decreased after drought

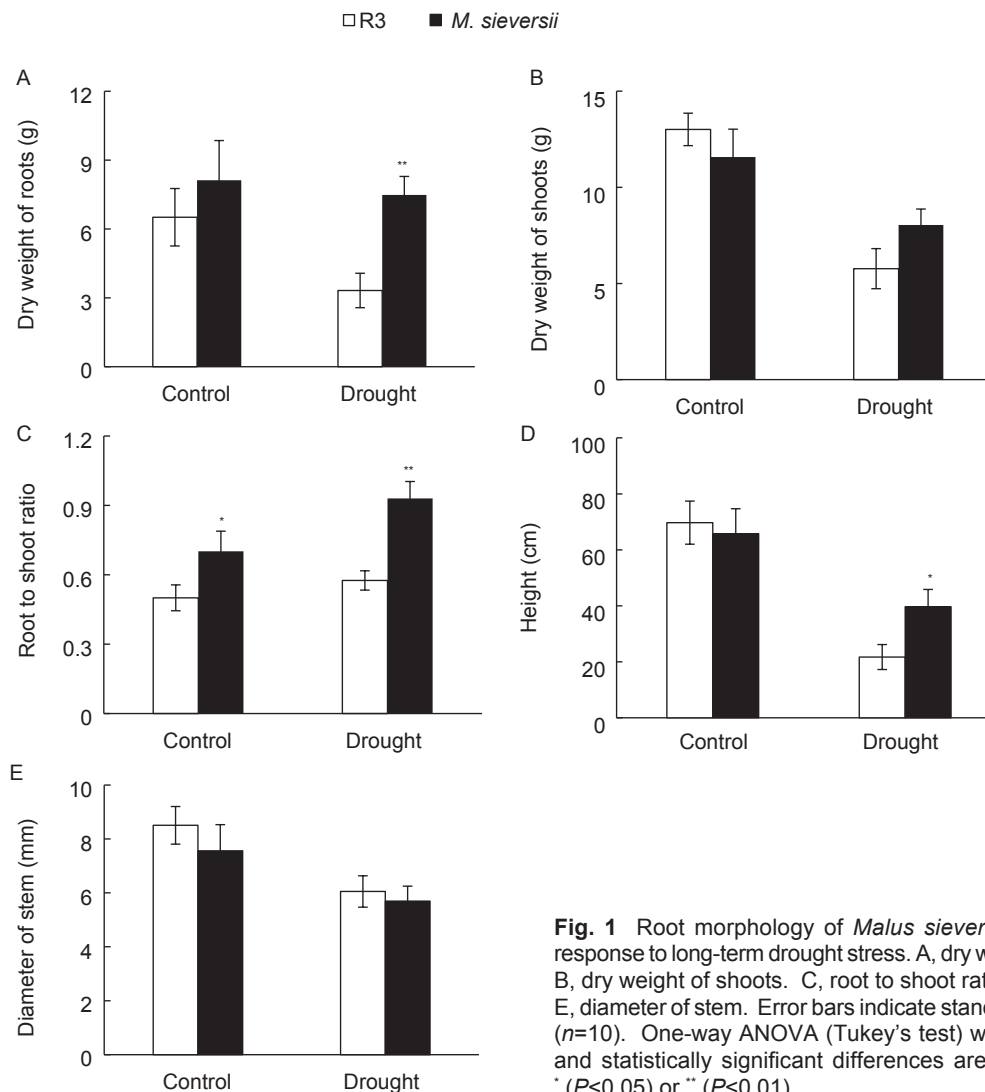


Fig. 1 Root morphology of *Malus sieversii* and R3 in response to long-term drought stress. A, dry weight of roots. B, dry weight of shoots. C, root to shoot ratio. D, height. E, diameter of stem. Error bars indicate standard deviation ($n=10$). One-way ANOVA (Tukey's test) was performed and statistically significant differences are indicated by * ($P < 0.05$) or ** ($P < 0.01$).

(Fig. 1-B), but there was no difference between R3 and *M. sieversii* after drought. As a result, root to shoot ratio in *M. sieversii* was much higher than that in R3 after drought (Fig. 1-C). Data of root dry weight, height and root to shoot ratio suggested that roots of *M. sieversii* were more resistant to drought stress (Fig. 1-A, C and D). Moreover, stem diameter did not show significant difference between R3 and *M. sieversii* under drought stress (Fig. 1-E) though they were dramatically decreased by drought in both rootstocks.

We also scanned the root system in both rootstocks to obtain more details of root morphology in response to drought (Fig. 2). Total root length, total surface area of roots, total root volume and average root diameter were measured to uncover the different responses to drought stress between R3 and *M. sieversii*. The total root length of R3 was declined from 15383 to 4419 cm after drought (Fig. 2-A), total surface area of roots was decreased from 1537 to 460 cm² (Fig. 2-B), and total root volume was reduced from 16.87 to 4.95 cm³ (Fig. 2-D), suggesting a significant drop of root length, surface area and volume of R3 after drought. However, no significant decrease of root length, surface area, average root diameter and volume were observed in *M. sieversii* after drought (Fig. 2-A–D). These data further confirmed that drought did not tremendously affect root growth of *M. sieversii*.

A deeper root usually indicates a stronger capacity of water absorption (Lagerwerff et al. 1961). In order to

understand the cause behind the different root morphologic responses between R3 and *M. sieversii* after drought, we analyzed xylem in two rootstocks with cross sections of roots (Appendix B). Drought decreased vessel density of *M. sieversii* but did not significantly affect that in R3. Compared to R3, *M. sieversii* had a higher level of vessel density under control and long-term drought treatment, though the difference was not significant (Fig. 3-A). Drought also decreased lumen area in *M. sieversii* but increased lumen area in R3. However, *M. sieversii* had more lumen area than R3 under drought (Fig. 3-B). Drought had different effects on xylem area of R3 and *M. sieversii*. Drought decreased the xylem area of R3 but did not affect that in *M. sieversii*, resulting in a lower xylem area in R3 after drought (Fig. 3-C). We also observed an increased length of major axis (D_{\max}) and minor axis (D_{\min}) in *M. sieversii* after drought (Fig. 3-D and E). Hence, the theoretical maximum hydraulic conductivity (K_s^{theo}) is higher in *M. sieversii* than that in R3 under control and drought (Fig. 3-F), though drought did not significantly influence the K_s^{theo} in both rootstocks.

3.2. Differentially expressed genes in roots of *M. sieversii* under simulated drought stress

Since data of root morphology indicated that roots of *M. sieversii* were more resistant to drought stress, we examined expression levels of drought stress-responsive

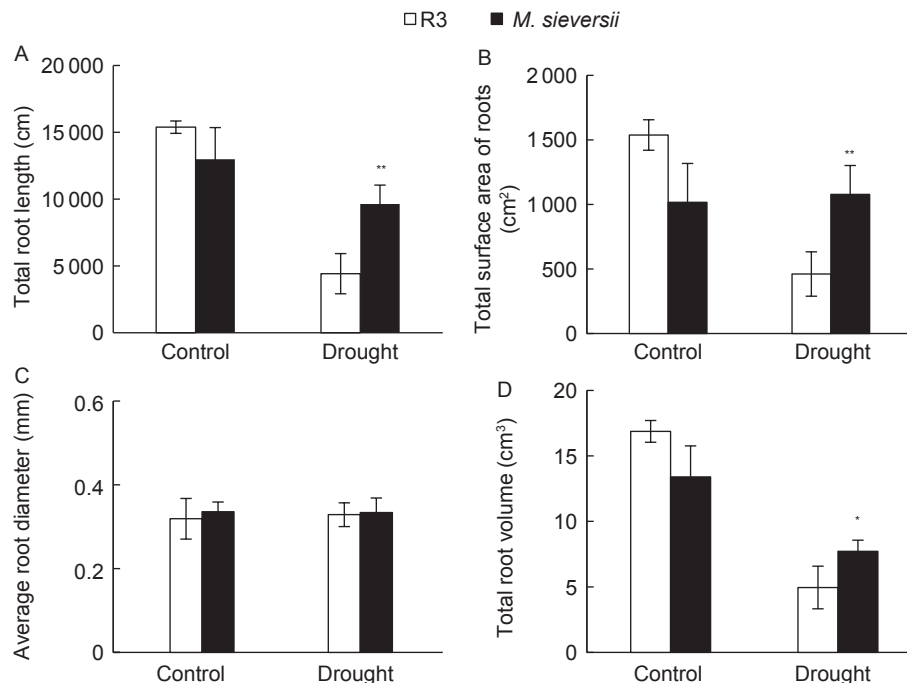


Fig. 2 Root scanning analysis of R3 and *Malus sieversii* under control and long-term drought stress. A, total root length. B, total surface area of roots. C, average root diameter. D, total root volume. Error bars indicate standard deviation ($n=20$). One-way ANOVA (Tukey's test) was performed and statistically significant differences are indicated by * ($P<0.05$) or ** ($P<0.01$).

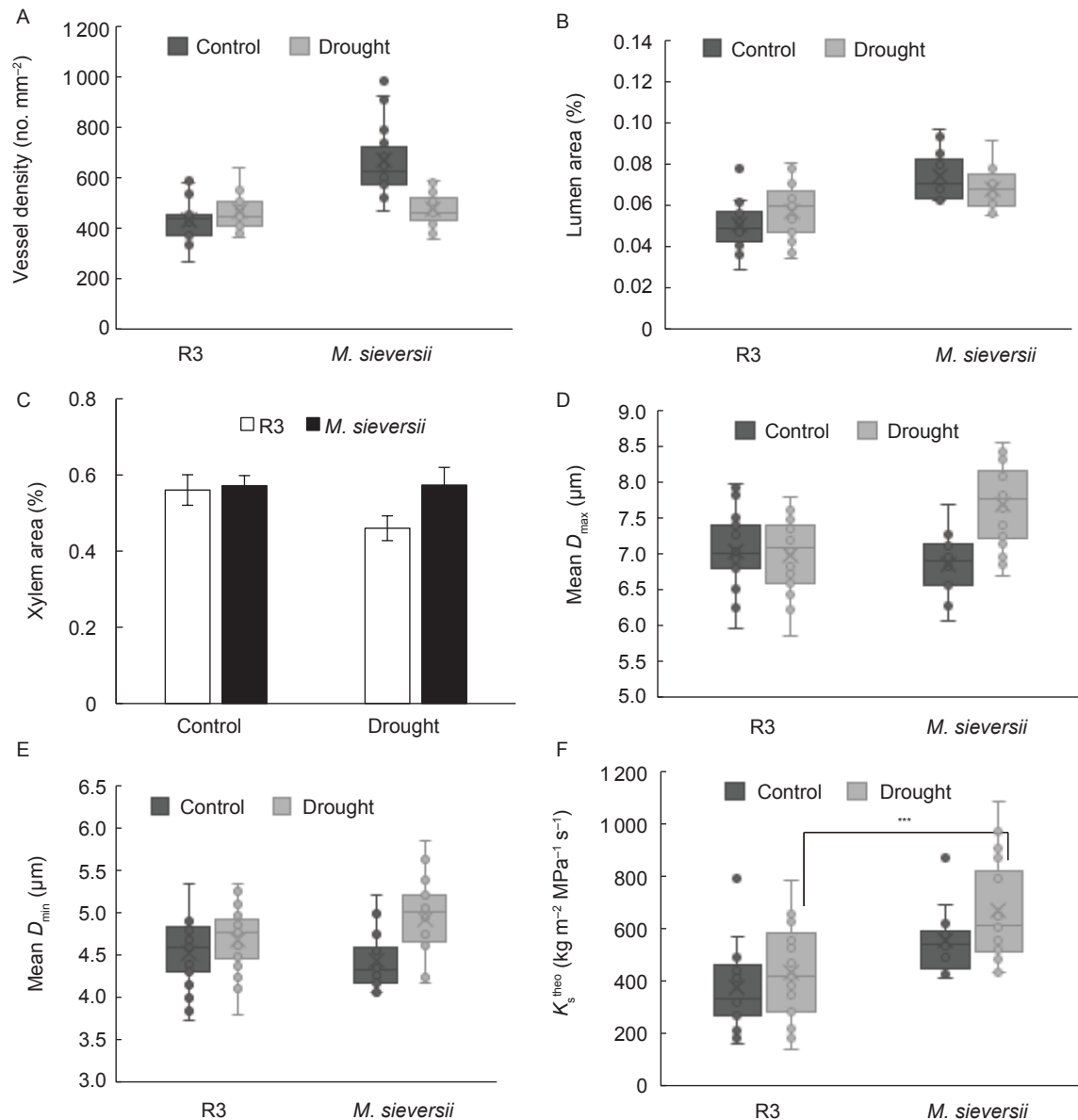


Fig. 3 Root vessel development of R3 and *Malus sieversii* under control and long-term drought conditions. A, vessel density. B, lumen area, shown as a percentage to xylem area. C, xylem area, shown as a percentage to total cross-sectional area. D, mean D_{max} , average length of major axis of vessels. E, mean D_{min} , average length of minor axis of vessels. F, K_s^{theo} , the root-specific theoretical conductivity. Error bars indicate standard deviation ($n=10$). Student's t -test was performed for data in F. One-way ANOVA (Tukey's test) was performed and statistically significant differences are indicated by * ($P<0.05$) or ** ($P<0.01$).

genes in *M. sieversii* roots cultured hydroponically with 20% PEG treatment which can induce water deficit and cause simulated drought stress (Jung *et al.* 2014). qRT-PCR with RNA from *M. sieversii* roots showed that expression of *MsNCED3* was induced significantly after PEG treatment for 6 or 12 h, while expression level of *MsRD22* was increased after 6 h treatment but decreased after 12 h treatment (Appendix C). We then further detected whole-genome gene expression levels in roots of *M. sieversii* using RNA-seq analysis under PEG treatment for 0 and 6 h.

Our RNA-seq analysis generated a total of 371 million raw reads (Appendix D). After removing contaminated reads, clean reads were mapped to the annotated apple genome (MDPs, from Gene Database of Rosease³¹) accounted for 56, 65 and 62% under the control conditions (Appendix E-a-c) and 67, 64 and 65% under the PEG treatment conditions (Appendix E-d-f). In all mapped reads under control conditions, 87.3, 87.7 and 87.8% were mapped to exon, 11.3, 10.8 and 11.0% were mapped to intron while 1.4, 1.4 and 1.3% were mapped to intergenic

area (Appendix E-a–c). In all mapped reads under PEG treatment conditions, 88.9, 87.7 and 88.7% were mapped to exon, 11.0, 9.7 and 10.0% were mapped to intron; 1.3, 1.4 and 1.3% were mapped to intergenic area (Appendix E-d–f). In addition, a high degree of correlation coefficients was found in each sample. Pearson correlation in control samples was higher than 0.93, while in treatment samples, it was higher than 0.95, indicating that gene expression was similar in each biological replicate and our RNA-seq dataset was really robust (Appendix E-g).

To estimate the transcript abundance level and identify the differentially expressed genes (DEGs) after treatment, FPKM and DESeq R package (1.18.0) were used. Significantly up- or down-regulated genes (fold change ≥ 2 or fold change ≤ 2 and P -value < 0.05) were selected for further analysis. Compared with control, 2004 significantly differentially expressed genes were found in treatment samples. Among them, 778 genes were up-regulated by treatment, while 1226 genes were down-regulated (Appendices F and G).

We carried out GO enrichment analysis to examine the function of DEGs. We found that many genes involved in synthesis or signaling of 6-benzylamino adenine, abscisic acid, salicylic acid, gibberellin, and ethylene were up-regulated after simulated drought while genes involved in the signaling or synthesis of jasmonate and auxin, receptor

kinase, calcium regulation, G-proteins, light, glutaredoxin were mainly down-regulated (Fig. 4). We also noticed that some genes involved in development, biotic stress, cell wall and secondary metabolism were significantly up-regulated by drought (Appendix H), whereas most of down-regulated genes were involved in transcription regulation, protein degradation, protein modification, receptor kinase and ethylene, fermentation, terpenes, and development (Fig. 4, Appendices H and I).

3.3. Verification of DEGs from RNA-seq analysis

To verify our RNA-seq data, we selected eight genes including *MDP0000248981* encoding gibberellin 20-oxidase 2; *MDP0000251424* encoding zinc induced facilitator-like 1; *MDP0000808163* encoding growth-regulating factor 4; *MDP0000270237* encoding protein phosphatase 2CA; *MDP0000247000* encoding FERONIA; *MDP0000647167* encoding ralf-like 32; *MDP0000125095* encoding late embryogenesis abundant protein and *MDP0000161440* encoding FERONIA. With qRT-PCR, we confirmed four up-regulated genes (*MDP0000248981*, *MDP0000251424*, *MDP0000808163* and *MDP0000125095*) and four down-regulated (*MDP0000270237*, *MDP0000247000*, *MDP0000647167* and *MDP000016144*), suggesting that our RNA-seq data are reliable (Fig. 5).

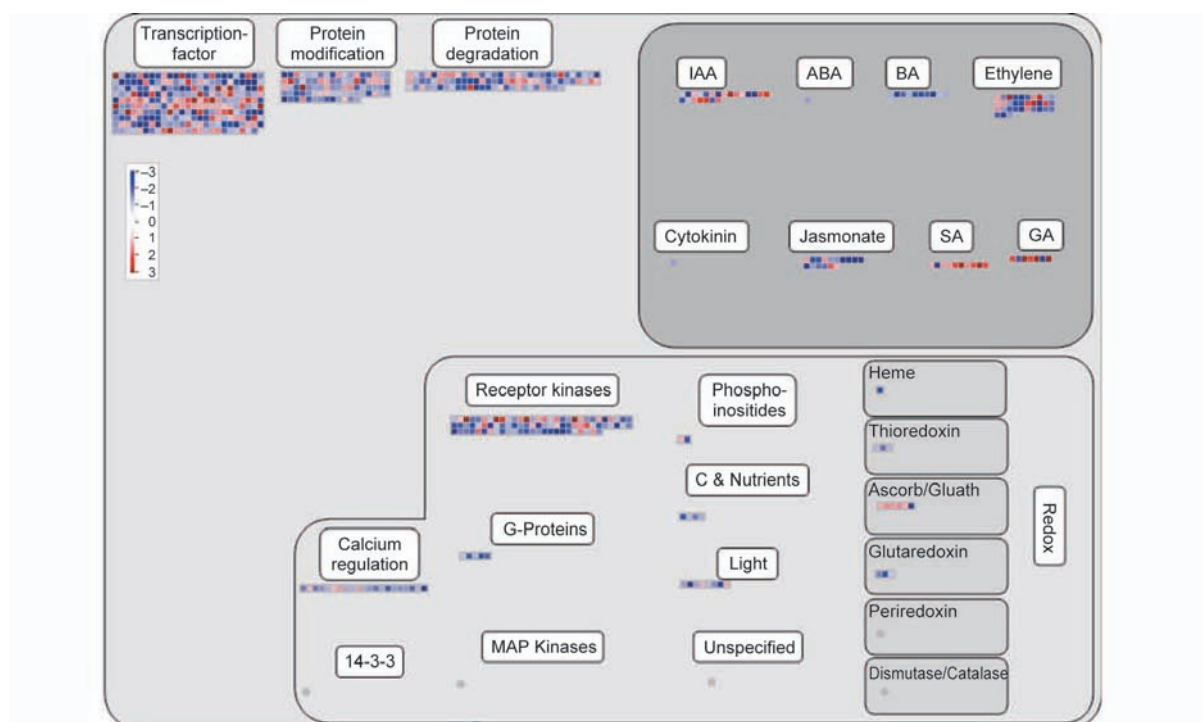


Fig. 4 Differentially expressed genes in *Malus sieversii* roots after simulated drought treatment. Grids represent individual genes. Up- and down-regulated genes are indicated in red and blue, respectively. Color brightness represents the degree of difference. IAA, indole-3-acetic acid; ABA, abscisic acid; BA, benayl aminopurine; SA, salicylic acid; GA, gibberellin.

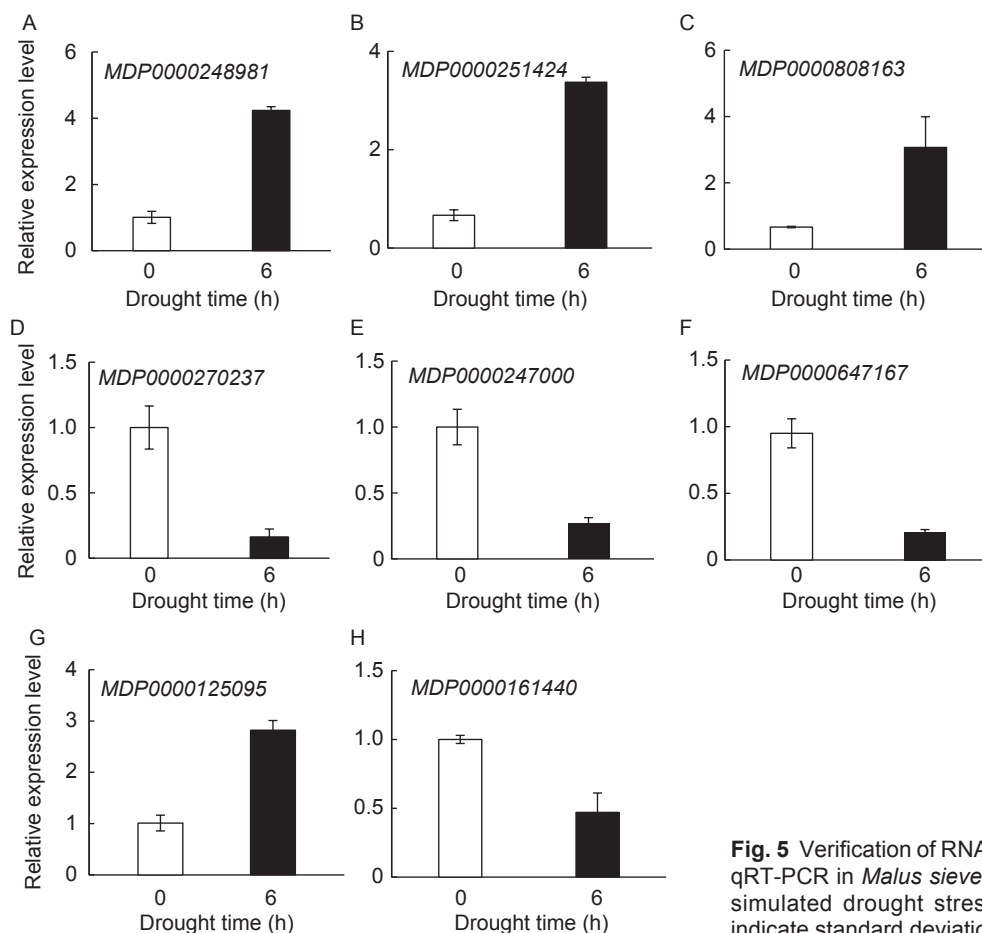


Fig. 5 Verification of RNA-seq results by qRT-PCR in *Malus sieversii* roots under simulated drought stress. Error bars indicate standard deviation (n=3).

3.4. Responses of antioxidant enzyme in roots under long-term drought stress

Our RNA-seq data revealed that some genes encoding ROS detoxification enzyme were among the DEGs up-regulated under drought (Appendix G). For example, *MDP0000802295* and *MDP0000572590* were two genes encoding peroxidase superfamily protein, and *MDP0000162215* was a gene encoding glutathione S-transferase. To unveil the potential roles of ROS detoxification enzyme in the drought tolerance of *M. sieversii* roots, we measured the activities of POD and SOD in roots of *M. sieversii* and R3 under control and drought conditions. Our data showed that activity of antioxidant enzyme was rapidly increased after drought treatment, and *M. sieversii* had a much higher activity than R3 after drought (Fig. 6-A and B). Two-fold increase of POD activity was observed in R3 and more than four-fold increase in *M. sieversii* was detected after drought (Fig. 6-A). SOD activity of R3 was increased by about 1.4 times (from 74.3 to 103.2 U mg⁻¹ protein), whereas SOD activity of *M. sieversii* was increased by nearly two times (from 97.2 to 193.7 U mg⁻¹ protein). In addition, SOD activity of *M. sieversii* was slightly higher than that of R3 under control conditions, and soluble

protein content in *M. sieversii* was much higher than that in R3 under drought stress conditions (Fig. 6-B and Appendix J). The enzyme activity and RNA-seq data about ROS scavenging enzyme were consistent with root morphology of *M. sieversii* and R3 under drought stress, indicating that ROS scavenging enzyme might contribute to the drought tolerance in *M. sieversii* roots. In addition, soluble protein content in *M. sieversii* was much higher than that in R3 under drought stress conditions (Appendix J).

3.5. Endogenous hormone content in roots of *M. sieversii* and R3 under long-term drought

We also identified a number of hormone related genes in RNA-seq data. To understand the potential roles of hormones in the drought response of *M. sieversii* roots, we determined the content of endogenous hormones (IAA, GA₃ and ABA) in *M. sieversii* and R3 under control and drought conditions. Drought decreased IAA content but increased amount of GA₃ and ABA in both rootstocks (Fig. 7). As expected, *M. sieversii* had a dramatically higher ABA content than R3 after drought (Fig. 7-C), whereas similar amount of GA₃ (Fig. 7-B) in both rootstocks under drought were

observed. In addition, *M. sieversii* had a lower amount of IAA than R3 under control and drought conditions (Fig. 7-A).

3.6. Simulated drought stress-responsive transcription factors

Transcription factors (TFs) play important roles in plant drought stress tolerance. We selected all TFs among DEGs for heat map analysis (Fig. 8). The TFs from our RNA-seq data were classified into eight families, with MYB, C2C2 and AP2-EREBP as the three largest TF families. TFs in WRKY and bHLH families were mainly down-regulated, except that one TF (*MDP0000179719*) in WRKY family and four TFs (*MDP0000641681*, *MDP0000534784*, *MDP0000323291*, and *MDP0000205358*) in bHLH family were up-regulated.

4. Discussion

In this study, we examined root morphology of R3 and *M. sieversii* under drought stress conditions. We found that

under drought stress, *M. sieversii* developed longer roots, had a higher root to shoot ratio (Figs. 1 and 2). Vessel analysis suggested that roots of *M. sieversii* had a higher lumen area, xylem area and root hydraulic conductivity (Fig. 3). We then performed RNA-seq using roots of *M. sieversii* under simulated drought stress.

4.1. Roots of *M. sieversii* were developed better than those of R3 under long-term drought conditions

Compared with their control plants, root growth of R3 was tremendously inhibited under drought treatment. On the contrary, root growth of *M. sieversii* was not affected a lot by drought while shoot growth was decreased under drought. As a result, *M. sieversii* had higher root-to-shoot ratio under long-term drought conditions (Fig. 2). Our data indicated that root growth of *M. sieversii* was less sensitive to drought stress than that of R3, which was also consistent with other studies (Wang *et al.* 2012; Shao Y *et al.* 2014).

Root scanning analysis further explained this conclusion.

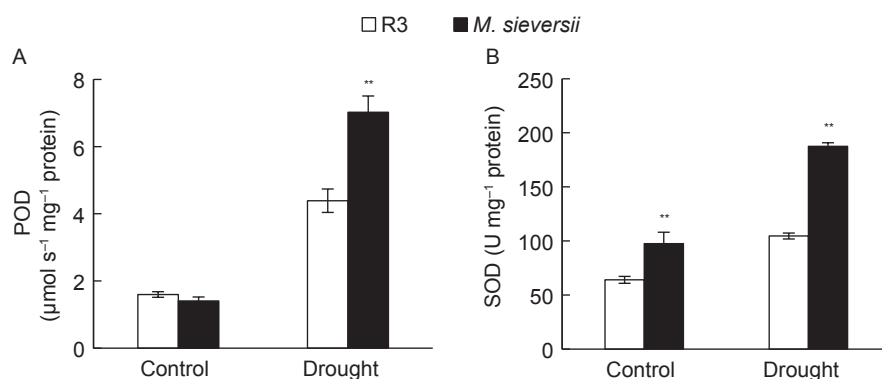


Fig. 6 Activities of reactive oxygen species (ROS) scavenging enzyme in roots of R3 and *Malus sieversii* in response to long-term drought stress. A, peroxidase (POD) activity. B, superoxide dismutase (SOD) activity. Error bars indicate standard deviation ($n=10$). One-way ANOVA (Tukey's test) was performed and statistically significant differences are indicated by * ($P<0.05$) or ** ($P<0.01$).

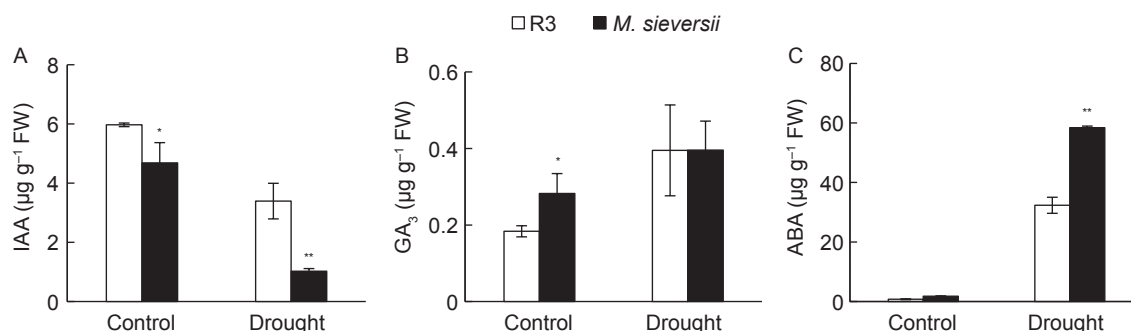


Fig. 7 Hormone content in roots of R3 and *Malus sieversii* under control and drought treatment. A, indole-3-acetic acid (IAA) content. B, gibberellin A₃ (GA₃) content. C, abscisic acid (ABA) content. Error bars indicate standard deviation ($n=3$). One-way ANOVA (Tukey's test) was performed and statistically significant differences are indicated by * ($P<0.05$) or ** ($P<0.01$).

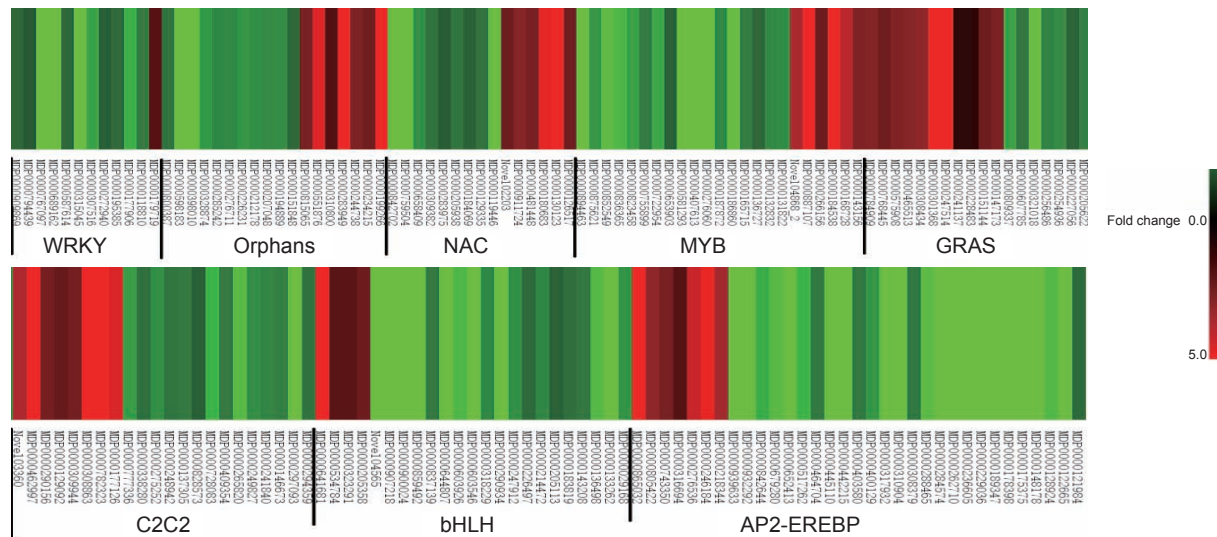


Fig. 8 Heat map for transcription factors in *Malus sieversii* roots after simulated drought treatment. Significantly differentially expressed transcription factors were visualized in HemI. Up- and down-regulated genes are indicated in red and green, respectively. Color brightness represents the degree of difference.

Total root length, root surface and root volume of R3 were significantly decreased by drought. By comparison, *M. sieversii* maintained growth level of total root length, root surface, average root diameter and root volume under long-term drought conditions (Fig. 2-A–D). Root scanning results also indicated that root of R3 had lower wood density compared with *M. sieversii* because *M. sieversii* had less root surface and root volume but larger root dry weights. Lower wood density might indicate that root of R3 had lower content of lignin (Björhager *et al.* 2010).

In addition, vessel analysis explained more about what actually happened during drought treatment. Under drought, no significant change was observed on vessel density, vessel diameter, and lumen area of R3 (Fig. 3-A–E), indicating that the total vessel crossing area was not influenced by drought. However, because drought inhibited xylem growth in R3, area of xylem was decreased, which resulted in a decreased K_s^{theo} in R3 under drought (Fig. 3-C–F). Oppositely, a significant increase was observed on vessel diameter of *M. sieversii* after drought, which led to a higher K_s^{theo} (Fig. 3-D–F). K_s^{theo} is an indicator to evaluate the hydraulic conductivity of roots (Köcher *et al.* 2012). Lower hydraulic conductivity of roots leads to a drought-sensitive phenotype of plants, while higher K_s^{theo} indicated higher hydraulic conductivity of roots, and drought-resistant phenotype of plants (Comas *et al.* 2013).

We also checked the expression level of some drought related genes in *M. sieversii* and R3 (Fig. 5 and Appendix K). Expression level of four up-regulated and two down-regulated genes were analyzed. Under drought

conditions, changes of expression level of drought response genes in R3 were more mildly or no response compared with *M. sieversii*. These might be the reason of different phenotypes between R3 and *M. sieversii* after drought stress.

Moreover, activities of antioxidant enzyme also supported our conclusion above. When suffered from drought, both R3 and *M. sieversii* had increased activities of POD and SOD (Fig. 6). However, compared with R3, *M. sieversii* had a higher level of activities of POD and SOD under drought, suggesting that *M. sieversii* was more able to detoxify the ROS generated by drought.

Finally, we measured hormone content (IAA, ABA, and GA_3) of R3 and *M. sieversii* under control and drought conditions. Under drought conditions, IAA content was decreased in both rootstocks, and *M. sieversii* had a lower level of content of IAA compared with R3 (Fig. 7-A). ABA content was increased in both rootstocks, and *M. sieversii* had a higher level of ABA compared with R3 (Fig. 7-C). IAA is reported as a directly negative regulator of root growth, its accumulation can inhibit the elongation of root cells (Kohli *et al.* 2013). On the contrary, endogenous ABA is reported as a necessary signal to maintain the growth of roots in maize seedlings under low water potentials (Saab *et al.* 1990; Spollen *et al.* 2000; LeNoble *et al.* 2004; Uga *et al.* 2013). The suppression of primary root growth by high salinity can also be released by ABA in *Arabidopsis* (Geng *et al.* 2013). Hence, different contents of IAA and ABA in *M. sieversii* and R3 under drought can lead to the different root morphology of these two rootstocks. No significant

difference of GA₃ content was observed between both rootstocks (Fig. 7-B), suggesting that GA₃ may not play an important role in the response of roots in two rootstocks to drought. Taken together, our data above suggest that roots of *M. sieversii* are developed better and more tolerant to drought compared with roots of R3.

4.2. Genes involved in drought response and development were found in *M. sieversii* roots after drought

Our RNA-seq analysis identified numbers of genes involved in root development. MDP0000144734 is homologous to GASSHO1 in *Arabidopsis*, a transmembrane receptor protein tyrosine kinase expresses in root and is involved in root development. GASSHO1 plays a positive role in regulation of cell multiplication, differentiation of root cells in *Arabidopsis*. Mutants of GASSHO1 and GASSHO2 in *Arabidopsis* have root growth defects (Racolta et al. 2013). MDP0000738420 is a homolog of CEPR2 in *Arabidopsis*, which is reported as a leucine-rich repeat receptor kinase. Mutants of CEPR2 in *Arabidopsis* enhance lateral root elongation (Tabata et al. 2014). In our results, MDP0000144734 was up-regulated and MDP0000738420 was down-regulated by drought. Hence, different expression of these two genes may be related to better development of roots in *M. sieversii* under drought.

We also identified homologous genes which might be drought-related in roots of *M. sieversii* under drought. Among the up-regulated genes after drought treatment (Appendix I), MDP0000381531, a gibberellin synthetase, can increase the gibberellin content (Albacete et al. 2014; Bidadi et al. 2014). MDP0000802295, predicted as a homolog of peroxidase superfamily protein, is a class III plant peroxidase. Class III peroxidases not only are involved in peroxide decomposition but also play an important role in lignification of the cell wall (Marjamaa et al. 2009; Fagerstedt et al. 2010). MDP0000185253, predicted as a homolog of auxin response factor 19 (ARF19), is a positive regulator of lateral root development in *Arabidopsis* (Kang et al. 2013). MDP0000271305 and MDP0000278588, predicted as homologs of tonoplast intrinsic protein (TIP4), are water channel proteins and play important roles in root water uptake (Javot et al. 2003). TIP4 are also reported to express in epidermal and cortical cells of the differentiation zone of *Arabidopsis* roots (Gattolin et al. 2009).

Among the down-regulated genes by drought in roots of *M. sieversii* (Appendix I), MDP0000270237 is predicted as a homolog of protein phosphatase 2CA (PP2CA), which is a negative regulator of ABA signaling, drought and salt stress tolerance (Cui et al. 2013; Rodrigues et al. 2013). MDP0000232116 is predicted as a homolog of auxin

response factor 17 (ARF17), which is a target of miR160 and a negative regulator of adventitious rooting in *Arabidopsis* (Gutierrez et al. 2012). MDP0000557979 is predicted as a homolog of early responsive to dehydration 15, which plays important roles in ABA response and drought tolerance (Kariola et al. 2006; Ziaf et al. 2011; Aalto et al. 2012; Shao H H et al. 2014).

We identified 289 differentially expressed TFs (Fig. 8), which were classified into eight TF families. A number of TFs were up-regulated in roots of *M. sieversii* under drought, with some of them are positive regulators of drought. For instance, MDP0000887107 is predicted as a homolog of AtTT2. Mutants of *Arabidopsis* tt2 decreased germination rate under osmosis, salt stress and ABA treatment (Chen et al. 2012). MDP0000865032 is predicted as a homolog of AtRAP2.4. Overexpression of AtRAP2.4 enhanced drought tolerance of *Arabidopsis*, whereas mutation of AtRAP2.4 increased sensitivity to drought (Lin et al. 2008). Some development-regulated TFs were also induced by drought in roots of *M. sieversii*. MDP0000252726 is predicted as a homolog of AtALF5, a multidrug and toxic compound extrusion (MATE) family protein. alf5 mutant plants in *Arabidopsis* defected in lateral root formation (Diener et al. 2001). MDP0000168728 is predicted as a homolog of AtMYB15 controlling lignification of cell wall appositions under biotic and abiotic stress (Chezem et al. 2017; Kim et al. 2017). MDP0000808163 is predicted as a homolog of AtGRF4. Silencing of OsGRF4 in rice results in dwarfism, delayed growth and inflorescence formation (Kuijt et al. 2014), suggesting that GRF4 is a positive regulator of development and growth. These three development-related genes might play important roles in roots of *M. sieversii* in response to drought by promoting root growth.

Increased drought tolerance of *M. sieversii* roots might be due to the decreased expression of some negative regulators related to root development and drought. For example, MDP0000177906 and MDP0000689162, predicted as a homolog of AtWRKY40 and AtWRKY18, respectively, were down-regulated in *M. sieversii* roots under drought. Both genes are two negative regulators of a cysteine-rich receptor-like protein kinase, AtCRK5, which confers drought tolerance in *Arabidopsis* (Lu et al. 2016). Another example is MDP0000836365, a homolog of AtMYB68 which is a negative regulator for root development, was also found down-regulated by drought (Feng et al. 2004).

5. Conclusion

Based on the discussion above, the presence of differentially expressed genes of root development- and stress-

related genes under drought treatment explained at some extent different phenotypes, for instance, different root growth under drought between *M. sieversii* and R3 under drought. Resistant to drought in *M. sieversii* roots could be a result of up-regulation of genes including *MDP0000185253*, *MDP0000802295*, and *MDP0000381531* and down-regulation of genes including *MDP0000232116*. These differentially expressed genes may enhance the development of adventitious rooting and lateral roots so that allow roots to take more water from soil and promote dry matter accumulation.

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Appendices associated with this paper can be available on <http://www.ChinaAgriSci.com/V2/En/appendix.htm>

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