Activated Expression of *PHT* Genes Contributes to Osmotic Stress Resistance under Low Phosphorus Levels in *Malus*

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ABSTRACT. Osmotic adjustments play a fundamental role in plant responses to water deficit. For apple (*Malus domestica*) trees growing in the primary production areas of China, drought and low phosphorus (P) levels are the main sources of abiotic stress. Although tolerance to drought and low P are important breeding goals for cultivar improvement, there is little information on natural variation within *Malus* for these traits or the molecular mechanisms that may mediate tolerance. In this study, it was found that in plants grown under conditions of osmotic and low P stress, electrolyte leakage and photosynthetic parameters were significantly higher, but chlorophyll concentrations were lower compared with nonstressed plants. These physiological indicators revealed that, under low P condition, the order of osmotic stress resistance (high to low) was *Malus sieversii* (Ms) → *Malus prunifolia* (Mp) → *Malus hupehensis* (Mh). Expression of the phosphorus transporter genes *PHT1;7*, *PHT1;12*, and *PHT2;1* in the roots and *PHT1;12* and *PHT4;5* in the leaves was positively correlated with plant osmotic resistance. It is proposed that the highly expressed *PHT* genes might improve P absorption and transport efficiency, resulting in the high osmotic stress resistance under low P level conditions in *Malus* species.

As changes in the global climate intensify and natural environments continue to deteriorate, the impact of drought on crops has become increasingly prominent (Varshney et al., 2011). Erratic rainfall and low soil P nutrition are specific problems and considered the main factors of reduced food production in rain-fed dry land areas, leading to decreased production (Amberger, 2000; Fageria and Baligar, 1997). Apple is the fourth most economically significant fruit crop after sweet orange (*Citrus sinensis*), grape (*Vitis vinifera*), and banana (*Musa sapientum*) (Hummer and Janick, 2009). China is the world's largest producer of apples, with production centered around the Bohai Gulf and the Northwest Loess Plateau. In the northwestern region of China, drought and low P levels are the most challenging abiotic stresses for apple trees (Hayano-Kanashiro et al., 2009). Phosphorus is an important nutrient because it helps drive apple flowering, as well as fruit set, quality, and yield. This element also promotes cell turgidity by maintaining high leaf water potential, which in turn increases stomatal conductance and rates of photosynthesis under drought conditions (Hassan and Sahrin, 2012).

Rootstocks have been used for propagating temperate fruit trees for more than 2000 years (Webster, 1995). Because rootstocks influence the performance and survival of a cultivar, choosing the most suitable rootstock is vital for the establishment of successful orchards (Kviklys et al., 2017). Fruit trees rely on their root systems for acquiring mineral nutrients. Fruit-producing apple trees comprise both scion and rootstock, it is critical that growers develop the best grafting combination for a particular environment. The primary role of a rootstock is the uptake and transport of water and minerals to the scion (Kviklys et al., 2017). Because of its selectivity in nutrient uptake, selection of a proper rootstock genotype is crucial for growers creating orchards under unfavorable soil conditions (Fazio et al., 2013). As a key nutrient for plant growth and development (Raghothama, 1999), P is also an essential component of fertilizers used to sustain modern agriculture (Turner and Leytem, 2004). This important biomolecule is available in various forms (e.g., phosphate, monophosphate, adenosine triphosphate, adenosine diphosphate, or adenosine monophosphate) and is crucial for energy transfer and metabolic regulation. Phosphorus promotes root growth and maintains high leaf water potential, which results in improved uptake of water and nutrients (Hassan and Sahrin, 2012).

Osmotic adjustments play a fundamental role in plant responses to water stress (Osakabe et al., 2013). Water deficits influence various physiological and biochemical processes (Chaves et al., 2003), restricting normal growth and reproduction (Hackenberg et al., 2015; Seki et al., 2003) because of cell

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dehydration, compromised plasma membrane integrity, and irreversible chloroplast damage (Lötter et al., 1985). The P concentration in the plant cell cytoplasm is generally greater than 10 mM (Raghothama, 1999). Plants use P transporters to uptake P from soil into cells against a large concentration gradient at the root–soil interface. Genome sequence analyses and experimental evidence have indicated the presence of numerous P transporter families in plants, including PHT1 through PHT5 (Guo et al., 2008; Knappe et al., 2003; Liu et al., 2011; Mimura, 1999; Rausch and Bucher, 2002; Schachtman et al., 1998; Zhang et al., 2016). The first high-H⁺/Pi phosphate transporter identified in higher plants was AtPT1 from *Arabidopsis thaliana* (Muchhal et al., 1996). This gene has significant roles in the uptake of P from soil (Lopez-Arredondo et al., 2014). Its overexpression increases P uptake in *A. thaliana*. In response to drought treatment, expression of *PHT1;12* and *PHT2;1* is up-regulated in apple roots, whereas *PHT1;12* and *PHT4;5* are induced in the leaves (Sun et al., 2017). This indicates that *PHTs* are involved not only in low P but also in drought resistance.

Polyethylene glycol 6000 (PEG6000) is frequently used in experiments to simulate osmotic stress (Bhargava and Paranjpe, 2004; Radhouane, 2007). With a molecular weight ≥ 6000 , PEG6000 molecules are inert, nonionic, and cell-impermeable. They are small enough to influence osmotic pressure, but large enough to avoid being absorbed by plants (van den Berg and Zeng, 2006). Here, three kinds of rootstocks were investigated in a hydroponic system for their resistance to osmotic stress under low P conditions. To study the drought resistance of three commonly used rootstocks of apple under different P concentrations, in addition, expression levels of *PHT* genes were also analyzed to determine how they might contribute to osmotic stress resistance when plants receive less P supply.

Materials and Methods

PLANT MATERIALS AND EXPERIMENTAL DESIGN. All experiments were conducted at Northwest A&F University, Yangling, China (lat. 34°20' N, long. 108°24' E). Three genotypes of apple rootstocks—Ms → Mp → Mh—were collected from their native regions in China (Table 1).

For the hydroponic experiments, seeds were stratified in sand at 0 to 4 °C for 60 d. As many as three times more seeds were prepared to select enough similarly sized seedlings for low P and/or osmotic treatments. Afterward, the seedlings were planted in individual plastic pots (12 × 12 cm) filled with sand and then placed in a greenhouse. Beginning at the second-true-leaf stage, the seedlings were irrigated every 4 d with a one-half-strength Hoagland nutrient solution (Hoagland and Arnon, 1950). After 60 d outdoors, selected seedlings with similar sizes (with six to eight leaves) were transferred to black plastic basins (52 × 37 × 15 cm), each containing 13 L of a one-half-strength Hoagland nutrient solution. The basins were placed in a greenhouse under natural light and at day/night temperatures of 23 to

25 °C/15 to 18 °C. The nutrient solution was aerated each hour with an air pump and the dissolved oxygen concentration was maintained at 8.0 to 8.5 mg·L⁻¹. The pH of the nutrient solution was adjusted to 6.0 ± 0.1 by adding diluted H₂SO₄, and the solution was refreshed every 4 d. After 10 d of such precultivation, stress treatments were initiated. Seedlings of Ms, Mp, and Mh were randomly assigned to one of four treatments (n = 54 plants per treatment): 1) control (CK), standard one-half-strength Hoagland nutrient solution supplemented with 500 μM KH₂PO₄; 2) osmotic stress (O), induced by adding PEG6000 to the one-half-strength Hoagland solution and adjusting the osmotic potential (ψ_s) to -0.75 MPa; 3) combination of osmotic and low-P stresses (OLP), one-half-strength Hoagland nutrient solution, 5 μM KH₂PO₄, and the ψ_s adjusted to -0.75 MPa. Each treatment type had three biological replicates (18 plants per replicate). On day 30 of the experimental period, roots and leaves were harvested separately.

ASSAYS OF PLANT GROWTH AND INVESTIGATION OF ROOT ARCHITECTURE. To evaluate their dry weights (DWs), the shoot and root portions were oven-dried individually at 105 °C for 15 min and at 70 °C for 72 h. The whole-plant DWs (shoot + root) and the ratio of root DW to shoot DW (R/S) were calculated from those data.

The fresh root systems were carefully cleared of substrates with tap water and further rinsed with distilled water. After they were arranged for image capture with a scanner, their architecture was studied with an image analysis system (WinRHIZO V4.1C; Regent Instruments, Quebec, QC, Canada). Afterward, the total lengths, surface area, and volume; plus the numbers of root tips and forks and the average diameters for roots were determined in different size categories.

ASSAYS OF ELECTROLYTE LEAKAGE, HYDROGEN PEROXIDE, AND MALONDIALDEHYDE CONTENT. Electrolyte leakage (EL) in the leaves was measured according to the methods of Dionisio-Sese and Tobita (1998) by placing 10 uniformly sized pieces (1 × 1 cm) in a test tube containing 10 mL of distilled water. The initial electrical conductivity (EC₀) was determined by using another test tube that contained 10 mL of distilled water but no leaf tissue. All EL measurements were made with an electrical conductivity analyzer (DDS-307; Shanghai Precision Scientific Instrument Co. Shanghai, China). After 3.5 h of incubation in a water bath at room temperature (RT), the second round of electrical conductivity (EC₁) of the medium was measured. Samples were then autoclaved at 100 °C for 20 min to release all electrolytes and then cooled to RT before measuring the final electrical conductivity (EC₂). Afterward, the percentage of electrolyte leakage was calculated as $EL = (EC_1 - EC_0) / (EC_2 - EC_0) \times 100\%$.

The H₂O₂ concentration was measured according to Patterson et al. (1984). Lipid peroxidation was measured by the determination of malondialdehyde (MDA) concentration by using the 2-thiobarbituric acid method, as described in Heath and Packer (1968).

DETERMINATION OF LEAF CHLOROPHYLL CONCENTRATIONS AND LEAF PHOTOSYNTHESIS. Leaves were collected from 10 plants per treatment to analyze various physiological indexes. Using 80% acetone to extract the chlorophyll (Chl) and determined the Chl concentrations spectrophotometrically as described by Arnon (1949).

The net photosynthetic rate of leaf was monitored on sunny days between 0900 and 1100 HR with a portable infrared gas analyzer (LI-6400; LI-COR, Lincoln, NE), using the 9th to 12th

Table 1. Genotype, species, and origin information of the three apple rootstocks used in this study.

Code	Genotype	Species	Origin in China
Ms	Xinjiangyepingguo	<i>Malus sieversii</i>	Yili, Xinjiang
Mp	Fupingqiuzi	<i>Malus prunifolia</i>	Fuping, Shaanxi
Mh	Pingyitiancha	<i>Malus hupehensis</i>	Pingyi, Shandong

leaves up from the base of selected plant stems. All photosynthetic measurements were taken at a constant airflow rate of 500 $\mu\text{mol}\cdot\text{s}^{-1}$. The concentration of CO_2 was $400 \pm 5 \text{ cm}^3\cdot\text{m}^{-3}$, and the temperature was $28 \pm 2 \text{ }^\circ\text{C}$. The instantaneous water-use efficiency (WUEi) is the ratio of the net photosynthetic (Pn) to transpiration rate (Tr), and the measurement was conducted on five individual plants per treatment.

QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION ANALYSIS OF PHT EXPRESSION. Total RNA was extracted from the roots and leaves by the CTAB method (Chang et al., 1993). The cDNA was reverse-transcribed from total RNA using a PrimeScript[®] RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Shiga, Japan). All quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) protocols were performed with SYBR[®] Premix Ex Taq II (TliRNaseH Plus) (Takara) and an RT-PCR System machine (Bio-Rad iQ 5; Bio-Rad, Hercules, CA). The following gene-specific primers for qRT-PCR were designed with Primer Premier 6 software (Biosoft International, Palo Alto, CA). Transcripts of the *Malus* elongation factor 1 alpha gene were used to standardize the cDNA samples for different genes. Gene-specific primers were designed for these amplifications (Table 2). These qRT-PCR experiments were repeated three times, based on three separate RNA extracts from three samples.

EFFICIENCY OF PHOSPHATE ABSORPTION AND UTILIZATION. For measuring the concentrations of plant P, roots, shoots, leaves, and whole-plant samples were washed three times with distilled water. They were fixed at $105 \text{ }^\circ\text{C}$ for 30 min, dried at $80 \text{ }^\circ\text{C}$ for 48 h, and ground into powder; 0.1 g of powdered material from each tissue type was put into individual 100-mL digestion tubes, to which 5 mL of concentrated sulfuric acid was added. The tubes were placed in an electric digestion furnace (HYP-1040; Xianjian, Shanghai, China) and heated at $370 \text{ }^\circ\text{C}$ for ≈ 4 h, adding 15 drops of H_2O_2 per hour. After cooling, deionized water was added up to the tube's scale mark before 5 mL of clear liquor was taken to determine the total amount of P with a segmented flow analyzer (AA3 HR Nutrient Autoanalyzer; Seal Analytical, Norderstedt, Germany).

Phosphate absorption efficiency (PAE) was defined as general plant P-uptake on a dry weight basis, calculated with the following equation (Yan and Zhang, 1997):

$$\begin{aligned} \text{PAE (milligrams per plant)} \\ &= \text{P concentration (milligrams per gram)} \\ &\quad \times \text{DW (grams per plant)} \end{aligned}$$

The efficiency of P utilization was defined here according to the amount of biomass produced by a plant, phosphate

utilization efficiency (PUE), based on the value calculated for PAE (Yan and Zhang, 1997):

$$\begin{aligned} \text{PUE (grams per milligram)} \\ &= \text{plant DW (grams)/uptake of phosphorus (milligrams)} \end{aligned}$$

EXTRACTION AND MEASUREMENT OF ENZYME ACTIVITY. For enzyme extracts and assays, 10 seedlings were used to provide an adequate amount of root and leaf tissues in each experimental replicate ($n = 3$). Fresh roots (0.1 g) and leaves (0.1 g) were ground separately in liquid nitrogen, and then suspended in 1 mL of solution containing 10 mM phosphate buffer (pH 7.0). The homogenate was centrifuged ($4 \text{ }^\circ\text{C}$, 1500 g , 10 min) and the resulting supernatant was collected. The activities of superoxide dismutase [SOD (A001-4)], catalase [CAT (A007-1)], peroxidase [POD (A084-3)], and acid phosphatase [ACP (A060-1)] were determined with commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All data were obtained with a spectrometer (ultraviolet-1750; Shimadzu, Kyoto, Japan).

STATISTICAL ANALYSIS. All experiments were conducted in triplicate ($n = 3$), and statistical analysis of the data from plants in the control and stress treatments was performed by one-way analysis of variance, using SPSS software (version 20.0; IBM Corp., Armonk, NY). To examine the effects of rootstocks and different treatments on experimental variables, all variables were analyzed by two-way analyses of variance with SPSS 20.0. A probability value of $P < 0.05$ indicated that the difference between treatments was statistically significant. The data are presented as the mean plus standard deviation (SD) for three replicates.

Results

GROWTH PARAMETERS. After 30 d of treatment, total DW accumulations were decreased by stress treatments, but those reductions varied among rootstocks (Table 3). For example, the DW value for osmotic-stressed Mh plants was 53.8% of that measured in the CK treatment for that genotype. Likewise, the DW in stressed Mp was 60.3% of the CK level, whereas the DW for stressed Ms was 80.5% of the CK samples. Under OLP conditions, the DW value for Mp was 56.8% of the normal CK level; for Mh, 47.1% of CK; and for Ms, 60.9% of CK biomass production. Overall, the percentages of biomass production, when compared with normal CK levels, were Mh (63.5%) $\rightarrow$ Mp (67.6%) $\rightarrow$ Ms (82.9%) (Table 3). Therefore, Ms appeared to be the least affected by osmotic stress, based

on changes in biomass production from normal conditions.

Under O and OLP stress treatments, the R/S values increased in all three rootstocks, with Ms showing the greatest change (Table 3).

ROOT ARCHITECTURE. When compared with the normal control and the O treatment alone, most root growth parameters were significantly improved for stressed plants that received supplemental P, as shown by their higher values for root length and volume, numbers of tips and forks, and total surface area

Table 2. Primers used for quantitative real-time polymerase chain reaction analyses of *PHT* genes in apple.

Gene name	Primers (5' – 3')
<i>PHT1</i> ;7 (MDP0000261121)	F: 5'-CTTCCCAGCAAGGTTGAGGT-3' R: 5'-TGCCGAAGAAGTTGATGACG-3'
<i>PHT1</i> ;12 (MDP0000075043)	F: 5'-AGTTTGCCCTCATAATGTTTCG-3' R: 5'-CATAAGGACCATGCCGTAGA-3'
<i>PHT2</i> ;1 (MDP0000685160)	F: 5'-CCTCGGTGTTTCTATTTGCTTC-3' R: 5'-CCTCACGGTTTCTGCTCTAACTCTA-3'
<i>PHT4</i> ;5 (MDP0000256650)	F: 5'-CCATCGTCCCTCTTGCTGCTAAAA-3' R: 5'-CACACCCCATGCCATCACTCTCTT-3'
<i>EF-1a</i> (DQ341381)	F: 5'-ATTCAAGTATGCCTGGGTGC-3' R: 5'-CAGTCAGCCTGTGATGTTCC-3'

(Table 4). For example, those increases over CK levels were 153.9%, 114.8%, and 114.6% (root lengths); 208.7%, 158.7%, and 147.8% (number of root tips); and 187.7%, 173.1%, and 131.3% (number of root forks) for Ms, Mp, and Mh plants, respectively, under O treatment. Under OLP condition, compared with CK, the increases for Ms, Mp, and Mh plants were 156.0%, 154.2%, and 141.2% (root lengths), 208.7%, 178.0%,

and 175.7% (number of root tips), and 160.0%, 157.0%, and 131.0% (number of root forks), respectively.

In contrast, average root diameters were smaller in the stressed plants than in the control group (Table 4). Furthermore, the values for total root volume, number of root forks, and total surface area were higher under OLP conditions than under O treatment for all three rootstocks.

DETERMINATION OF RELATIVE ELECTROLYTE LEAKAGE, H₂O₂ CONTENT, AND MALONDIALDEHYDE CONTENT. EC, H₂O₂, and MDA contents were significantly higher in leaves from osmotic-stressed samples than that from the CK (Table 5). The order of the stress response, based on values of EL, H₂O₂, and MDA contents, from high to low was OLP → O → CK for all tested rootstocks.

Under the osmotic condition, EL values of Ms increased to 115.2% compared with CK, whereas Mp increased to 121.5% and Mh increased to 123.4%, respectively. Under the OLP condition, the EL value increased to 125.6%, 135.3%, and 137.5% for Ms, Mp, and Mh plants, respectively, compared with CK. Similarly, H₂O₂ and MDA contents in three rootstocks also quickly increased under O and OLP conditions (Table 5).

DETERMINATION OF LEAF CHLOROPHYLL CONCENTRATIONS AND LEAF PHOTOSYNTHESIS. Compared with the status under control conditions, Chl concentrations decreased in the leaves of stressed plants, with those declines being most severe in all three rootstocks in response to the combination of osmotic stress and low-P treatment (Table 6). The rootstock showing the greatest resistance to such treatment was Ms, for which Chl

Table 3. The dry weight and the ratio of root and shoot in three apple rootstocks under different treatments.

Rootstock ^z	Treatment ^y	Dry wt [g/plant (%)] ^x	Root-to-shoot ratio
		(mean ± SD)	
Ms	CK	0.82 ± 0.06 a ^w (100)	0.86 ± 0.02 b
	O	0.66 ± 0.06 b (80.5)	1.54 ± 0.21 a
	OLP	0.50 ± 0.05 c (60.9)	1.56 ± 0.12 a
Mp	CK	1.11 ± 0.06 a (100)	0.63 ± 0.03 b
	O	0.67 ± 0.05 b (60.3)	1.12 ± 0.05 a
	OLP	0.63 ± 0.06 b (56.8)	1.13 ± 0.07 a
Mh	CK	1.04 ± 0.09 a (100)	0.84 ± 0.06 b
	O	0.56 ± 0.04 b (53.8)	1.15 ± 0.12 a
	OLP	0.49 ± 0.09 b (47.1)	1.17 ± 0.17 a

^zDetails are described in Table 1.

^yCK = control; O = osmotic stress; OLP = combination of osmotic and low-P supply.

^x% = dry weight of different treatments/the dry weight of CK.

^wDifferent letters in the same column indicate significant differences according to Tukey's multiple-range test ($P < 0.05$).

Table 4. The root length, surface area, diameter volume, tips, and forks of three apple rootstocks under different treatments.

Rootstock ^z	Treatment ^y	Length (cm)	Surface area (cm ²)	Diam (mm)	Vol (cm ³)	Tips (no.)	Forks (no.)
		(mean ± SD)					
Ms	CK	336.61 ± 31.05 b ^x	39.06 ± 9.26 b	0.40 ± 0.02 a	0.36 ± 0.04 b	902.81 ± 81.51 b	1,724.80 ± 196.51 c
	O	518.21 ± 41.38 a	62.48 ± 9.71 a	0.38 ± 0.05 a	0.61 ± 0.05 a	1,714.10 ± 154.70 a	3,238.50 ± 300.62 a
	OLP	525.36 ± 40.20 a	65.02 ± 9.43 a	0.37 ± 0.06 a	0.66 ± 0.05 a	1,884.50 ± 168.19 a	2,769.74 ± 134.19 b
Mp	CK	446.47 ± 40.38 c	48.81 ± 5.20 c	0.42 ± 0.02 a	0.43 ± 0.03 b	1,060.66 ± 130.13 b	1,595.41 ± 130.14 b
	O	512.69 ± 56.67 b	71.16 ± 5.10 b	0.39 ± 0.035 a	0.68 ± 0.02 a	1,683.88 ± 133.51 a	2,761.83 ± 145.82 a
	OLP	688.87 ± 57.97 a	86.41 ± 6.09 a	0.37 ± 0.03 a	0.69 ± 0.07 a	1,887.67 ± 175.58 a	2,510.17 ± 209.63 a
Mh	CK	369.18 ± 47.084 b	46.50 ± 6.40 b	0.45 ± 0.04 a	0.48 ± 0.08 a	945.85 ± 84.55 b	1,772.15 ± 204.14 b
	O	423.68 ± 34.30 ab	64.78 ± 3.18 a	0.40 ± 0.03 a	0.53 ± 0.05 a	1,398.78 ± 193.48 a	2,522.22 ± 156.89 a
	OLP	521.65 ± 41.76 a	69.57 ± 7.07 a	0.39 ± 0.02 a	0.55 ± 0.04 a	1,662.14 ± 134.20 a	2,328.29 ± 161.89 a

^zDetails are described in Table 1.

^yCK = control; O = osmotic stress; OLP = combination of osmotic and low-P supply.

^xDifferent letters in the same column indicate significant differences according to Tukey's multiple-range test ($P < 0.05$).

Table 5. Relative electrolyte leakage, the content of H₂O₂ and the determination of malondialdehyde (MDA) in three apple rootstocks under different treatments.

Rootstock ^z	Treatment ^y	Relative electrolyte leakage	H ₂ O ₂ content (μmol·g ⁻¹ fresh wt)	MDA content (nmol·g ⁻¹)
		(mean ± SD)		
Ms	CK	25.93 ± 1.40 a ^x	1.70 ± 0.07 a	18.21 ± 1.01 a
	O	29.87 ± 1.25 b	2.49 ± 0.14 b	41.52 ± 1.98 b
	OLP	32.58 ± 1.24 c	3.01 ± 0.20 c	54.21 ± 2.71 c
Mp	CK	23.02 ± 1.29 a	1.71 ± 0.15 a	18.35 ± 1.22 a
	O	27.98 ± 0.86 b	2.61 ± 0.14 b	45.36 ± 2.52 b
	OLP	31.16 ± 1.26 c	3.31 ± 0.16 c	58.36 ± 3.43 c
Mh	CK	24.22 ± 0.91 a	1.72 ± 0.13 a	18.18 ± 0.91 a
	O	29.88 ± 1.01 b	2.83 ± 0.20 b	46.21 ± 2.89 b
	OLP	33.31 ± 1.29 c	3.52 ± 0.17 c	62.31 ± 2.97 c

^zDetails are described in Table 1.

^yCK = control; O = osmotic stress; OLP = combination of osmotic and low-P supply.

^xDifferent letters in the same column indicate significant differences according to Tukey's multiple-range test ($P < 0.05$).

Table 6. Total chlorophyll of leaves and determination of leaf photosynthesis in three kinds of apple rootstocks under different treatments.

Rootstock ^z	Treatment ^y	Total chlorophyll (mg·g ⁻¹ fresh wt)	Pn [CO ₂ (μmol·m ⁻² ·s ⁻¹)] ^x	Tr [H ₂ O (mmol·m ⁻² ·s ⁻¹)] ^x	WUEi (μmol·mmol ⁻¹) ^x	g _s [H ₂ O (mol·m ⁻² ·s ⁻¹)] ^x	Ci [CO ₂ (μmol·mol ⁻¹)] ^x
		(mean ± sd)					
Ms	CK	3.22 ± 0.27 a ^w	14.53 ± 0.52 a	5.78 ± 0.38 a	2.13 ± 0.14 b	0.33 ± 0.01 a	377.53 ± 22.86 a
	O	2.34 ± 0.20 b	7.78 ± 0.68 b	3.93 ± 0.30 b	2.73 ± 0.13 a	0.21 ± 0.02 b	336.37 ± 15.25 b
	OLP	2.21 ± 0.21 b	7.45 ± 0.49 b	3.46 ± 0.37 c	2.31 ± 0.10 b	0.17 ± 0.02 b	327.93 ± 17.32 b
Mp	CK	3.27 ± 0.13 a	15.73 ± 0.72 a	4.24 ± 0.46 a	2.28 ± 0.16 b	0.29 ± 0.02 a	273.01 ± 20.14 a
	O	2.25 ± 0.20 b	7.85 ± 0.47 b	2.34 ± 0.32 b	2.83 ± 0.27 a	0.17 ± 0.02 b	250.64 ± 17.16 b
	OLP	2.09 ± 0.15 b	7.14 ± 0.44 b	2.07 ± 0.11 c	2.22 ± 0.17 b	0.15 ± 0.03 b	245.14 ± 21.76 b
Mh	CK	3.28 ± 0.31 a	16.73 ± 1.02 a	5.47 ± 0.59 a	2.42 ± 0.17 b	0.27 ± 0.03 a	378.28 ± 34.07 a
	O	2.19 ± 0.12 b	8.15 ± 0.51 b	3.33 ± 0.30 b	2.89 ± 0.17 a	0.16 ± 0.02 b	305.31 ± 32.68 b
	OLP	1.98 ± 0.19 b	6.44 ± 0.43 c	3.12 ± 0.41 b	2.25 ± 0.27 b	0.13 ± 0.01 b	296.66 ± 16.81 b

^zDetails are described in Table 1.^yCK = control; O = osmotic stress; OLP = combination of osmotic and low-P supply.^xPn = net photosynthetic rate; Tr = transpiration rate; WUEi = instantaneous water-use efficiency; g_s = stomatal conductance; Ci = intercellular CO₂ concentration.^wDifferent letters in the same column indicate significant differences according to Tukey's multiple-range test (*P* < 0.05).

concentrations were less altered by O or OLP conditions. Those levels were also lower in all three rootstocks under OLP compared with under O treatment.

Leaf photosynthetic parameters were affected by osmotic stress and OLP stress (Table 6). The net photosynthetic rate was slower than for the CK samples from all three rootstocks. Under osmotic stress, the decline in Pn was less for Ms than for Mp or Mh, Pn values were lower for all three rootstocks under OLP conditions than under O treatments. For all rootstocks, the changes in Tr, Gs, and Ci were similar to those found for net photosynthesis.

Under osmotic stress and OLP condition, the order of WUEi, from high to low, for the three rootstocks was Ms → Mp → Mh.

QRT-PCR ANALYSIS OF PHTs EXPRESSION. The expression of genes involved in P transport in roots and leaves was analyzed. *PHT1;7* (Fig. 1A) and *PHT4;5* (Fig. 1F) were significantly up-regulated in response to osmotic stress. Both genes were also strongly induced under OLP conditions. Interestingly, under both OLP and O treatments, expression of *PHT4;5* in leaves was higher in Ms than in other two rootstocks.

Both *PHT1;12* (Fig. 1B and E) and *PHT2;1* (Fig. 1C) also responded to osmotic stress. Under the O and OLP treatments, the expression of *PHT1;12* in roots and leaves was also higher in Ms than in other two rootstocks.

EFFICIENCIES OF PHOSPHATE ABSORPTION AND UTILIZATION. In the rootstocks the concentration of total P was significantly affected by osmotic stress and the amount of P within the external environment (Table 7). Under O or OLP conditions, the plant P concentration was lower than that measured in the CK group. For all three rootstocks, leaves had the highest P concentrations while the shoots had the lowest. This pattern of distribution was the same regardless of the degree of stress. Under osmotic stress, the total P concentration of Ms, Mp, and Mh plants were 48.9%, 38.2%, and 40.8%, respectively, compared with the CK. Under OLP conditions, the total P concentration of Mp was 37.4% of the CK level; for Mh, 29.1% of CK; and, for Ms, 23.1% of the total P of CK.

Figure 2 shows that the order of PAE for the three rootstocks, from high to low, followed Mp → Mh → Ms. Under osmotic stress, the capacity for P absorption was lower than under CK conditions, with PAE values declining by 37.8% for Ms, by 23.0% for Mp, and by 21.9% for Mh. Under OLP treatment, the

PAE dropped significantly; i.e., by 22.8%, 21.1%, and 18.5% for Ms, Mp, and Mh, respectively (Fig. 2).

PUE refers to the plant body unit P production of biomass (Fig. 2). Drought can improve the rootstock PUE; compared with CK, the P utilization efficiency of plants was improved and significantly increased in Ms (68.2%), Mp (54.2%), and Mh (31.8%). Under the OLP condition, the rootstock PUE was also increased but was lower than osmotic stress.

ENZYME ACTIVITY. The activity of ACP was obviously higher in the roots than in the leaves for all stress treatments (Table 8). Under the osmotic condition, the activity of ACP in apple roots was 1.82 times in Ms, 1.69 times in Mp, and 1.63 times in Mh compared with that of the control. Under OLP stress, the activity of ACP in apple roots was lower than under osmotic conditions. The activity of ACP in Ms roots was 1.69 times than the control, Mp was 1.64 times than the control and Mh was 1.61 times than CK. The magnitude of response, from high to low activity, followed in the order of Ms → Mp → Mh.

The activities of SOD, POD, and CAT in all three rootstocks were increased by stress treatment, albeit to varying degrees (Table 8). Activity was higher in the roots than in the leaves, and absolute changes in activity due to stress were also greater in the roots. Furthermore, enzyme activity was lower under OLP conditions compared with the osmotic treatment groups, and the magnitude of response, from high to low activity, followed in the order of Ms → Mp → Mh, which is in consistent with the activity of acid phosphatase tested above.

Discussion

Drought is a major limitation to the productivity of agricultural systems and food production worldwide. Water deficit is often accompanied by nutrient deficiencies—especially of P because the soils are often inherently low in P and solubility (Haefele et al., 2006). Our current study clearly demonstrates that low P level affects plant osmotic resistance, although different genotypes behaved differently.

The root-to-shoot ratio of the three rootstocks increased under osmotic stress, possibly because shoot growth is more inhibited by such conditions. In some cases, the absolute root biomass of plants in drying soil may increase relative to that in well-watered conditions (Sharp and Davies, 1985). Possible

Fig. 1. Relative expression of *PHT1;7*, *PHT1;12*, and *PHT2;1* in apple roots; and *PHT1;12* and *PHT4;5* in leaves under different treatments (CK = control, O = osmotic stress, OLP = combination of osmotic and low-P stresses, Ms = *Malus sieversii*, Mp = *M. prunifolia*, Mh = *M. hupehensis*; Ms-R, Mp-R, and Mh-R stand for the root of different apple rootstocks; Ms-L, Mp-L, and Mh-L stand for the leaves of different apple rootstocks). Values not followed by the same letters indicate significant differences according to Tukey's multiple-range test ($P < 0.05$). Probability values obtained from the two-way analyses of variance for rootstocks (R), different treatments (T), and their interactions (R $\times$ T) are indicated; **significant positive correlation between any two variables at the 0.01 level (two tailed).

Table 7. Phosphorus concentration of roots, stems, leaves, and total phosphorus concentration in apple rootstocks under different treatments.

Rootstock ^z	Treatment ^y	P uptake of each organ (mg·g ⁻¹)			Total P concn (mg·g ⁻¹ dry wt)
		Root	Stem	Leaves	
		(mean ± sd)			
Ms	CK	3.44 ± 0.26 a ^x	2.68 ± 0.05 a	3.80 ± 0.09 a	3.74 ± 0.31 a
	O	1.28 ± 0.09 c	1.03 ± 0.04 b	2.12 ± 0.41 b	1.83 ± 0.11 b
	OLP	1.49 ± 0.01 b	0.65 ± 0.24 b	0.87 ± 0.02 c	1.40 ± 0.12 c
Mp	CK	4.23 ± 0.04 a	2.61 ± 0.69 a	4.61 ± 0.09 a	4.50 ± 0.41 a
	O	1.55 ± 0.19 b	1.13 ± 0.02 b	1.79 ± 0.09 b	1.72 ± 0.11 b
	OLP	1.34 ± 0.02 c	0.69 ± 0.05 c	1.06 ± 0.03 c	1.31 ± 0.13 c
Mh	CK	4.33 ± 0.06 a	2.45 ± 0.05 a	4.80 ± 0.05 a	4.68 ± 0.21 a
	O	1.18 ± 0.09 b	0.96 ± 0.07 b	1.89 ± 0.07 b	1.91 ± 0.16 b
	OLP	1.02 ± 0.05 c	0.53 ± 0.09 c	1.10 ± 0.02 c	1.08 ± 0.16 c

^zDetails are described in Table 1.

^yCK = control; O = osmotic stress; OLP = combination of osmotic and low-P stresses.

^xDifferent letters in the same column indicate significant differences according to Tukey's multiple-range test ($P < 0.05$).

explanations might include a limit on the amount of water and nutrients that can be transported to the shoots as well as the induction of hormonal signals in roots when they encounter water deficit (Sharp and Davies, 1985). Therefore, it is critical

response to biotic and abiotic stresses (Davey et al., 2005). Under O and OLP stresses, the H₂O₂ and MDA contents of three kinds of rootstocks were increased compared with the CK. It is not surprising that the contents of H₂O₂ and MDA

that roots continue to grow during periods of drought (Dhanda et al., 1995). Under O and OLP stress, the dry weight of three apple rootstocks was reduced; Ms was reduced the least, whereas Mh decreased the most. Under such stress, the root-to-shoot ratio of Ms was the highest and Mh the lowest.

Root traits can moderate the effects of drought either by increasing the rate of water uptake by promoting root-length densities or by enabling more water to be extracted when longer roots can penetrate to greater soil depths (Manschadi et al., 2006; Passioura, 1983). Although water deficits significantly reduce shoot growth and the number and length of the roots that are produced, the addition of P can mitigate those negative effects and improve shoot and root development (Sawwan et al., 2000). Various traits of root architecture determine the in situ, space-filling properties of a root system. It was found that root lengths, surface areas, root volume, and the numbers of root tips and root forks increased in our three rootstocks under O and OLP stresses, with Ms showing the best response, followed by Mp and Mh.

Electrolyte leakage is a typical parameter used for assessing resistance to abiotic stresses in crops (Dhanda et al., 2004). Measuring EL from leaf tissues indicates the integrity of cell membranes because lower electrical conductance means that a plant is more resistant to drought (Agarie et al., 1995). EL values were higher in plants under osmotic stress than in the control group. However, the electrolyte leakage was enhanced when decreasing P supply. In particular, rootstocks under O treatment showed less leakage compared with plants in the OLP group.

H₂O₂ is one of the reactive oxygen species (ROS) generated as a by-product in plant tissues during normal metabolism as well as under different stress conditions (Blokhina et al., 2003). MDA is also a widely used marker of oxidative lipid injury whose concentration varies in

Fig. 2. Efficiencies of phosphate absorption and utilization by apple rootstocks under different treatments (CK = control; O = osmotic stress; OLP = combination of osmotic and low-P stresses; Ms = *Malus sieversii*; Mp = *M. prunifolia*; Mh = *M. hupehensis*). Values not followed by same letters indicate significant differences according to Tukey's multiple-range test ($P < 0.05$).

were the highest under OLP condition in three tested apple rootstocks.

Ommen et al. (1999) reported that drought stress is associated with a decline in leaf-Chl concentrations. Likewise, Manivannan et al. (2007) showed that the levels of Chl a, Chl b, and total Chl are reduced in the leaves of drought-stressed *Helianthus annuus*. This study also found that total Chl concentrations were diminished in all three rootstocks, and the extent of damage was more severe when the P supply was decreased. Under O and OLP stresses, the order of Chl content of the three root stocks, from high to low, was Ms → Mp → Mh.

Water deficits limit the process of photosynthesis by inducing stomatal closure or impairing metabolic limitations (Hu et al., 2009). Among the three rootstocks tested here, Ms had the best growth performance, followed by Mp, when plants were exposed to PEG6000. This was reflected by their smaller changes in Chl concentrations and more stable rates of photosynthesis. Under osmotic stress and low-P conditions, damage was most severe for Mh. Phosphorus is essential for energy storage and transfer, photosynthesis, and sugar metabolism (Hu and Schmidhalter, 2001). Thus, increasing the supply of P in an available form can help alleviate the negative impact of osmotic stress on plant growth and development (Garg et al., 2004).

The family of P transporters mediates the uptake and translocation of Pi inside the plants. Few studies have focused

on the relationship between *PHTs* expression and drought tolerance. Zhang et al. (2016) reported that the expression of *PtPHT1.2* and *PtPHO9* in poplar (*Populus trichocarpa*) was increased under drought conditions, irrespective of the phosphate levels. The expression of apple *PHT* genes could also be elevated by drought. For example, in our previous study, 23 *MdPHT* genes in leaves and 12 *MdPHT* genes in roots were up-regulated by drought, suggesting that the aerial parts were influenced more than the underground parts (Sun et al., 2017).

Under O and OLP stresses, the physiological indicators showed that Ms had the best resistance, followed by Mp and Mh. To elucidate the relationship between OLP resistance and *PHT* gene expression in apple, several typical apple *PHT* genes were selected to analyze. The results from current study indicate that the combined OLP treatment induced the expression of *PHT* genes, in the following order (from high to low transcript levels): Ms → Mp → Mh; this pattern was also noted in their rankings for stress resistance and total-P concentrations. The analysis showed that expression of *PHT1;7*, *PHT1;12*,

and *PHT2;1* in the roots was positively correlated with overall plant resistance. Moreover, expression was enhanced in response to the OLP treatment, again following the trend in degree of resistance and total-P concentrations in each of the three rootstocks. Greater amounts of P transporter proteins mean that plants can absorb more P from the soil. Boosting the supply of P helps cells maintain pressure and reduce protein hydrolysis under osmotic stress, thereby increasing their resistance to harmful conditions (Nelsen and Safir, 1982). Here, enhanced expression of *PHT1;7*, *PHT1;12*, and *PHT2;1* in the roots and *PHT1;12* and *PHT4;5* in the leaves was positively correlated with greater resistance to osmotic stress (Supplemental Figs. 1 and 2). These results indicated that differential expression of *PHT* genes in apple may contribute to osmotic stress resistance when low level of P is supplied.

The P content, PAE and PUE for the three tested apple stocks were decreased under O and OLP conditions compared with the CK condition. However, Ms had the least and Mh had the greatest reduction, compared with that of CK. This might be attributed to the relatively higher expression of *PHT* genes in Ms compared with Mp and Mh under the OLP stress condition. Drought stress and associated reductions in soil moisture can decrease the availability and uptake of nutrients due to mineralization (Fierer and Schimel, 2002) and through reduced nutrient diffusion and mass flow in the soil (Chapin, 1991; Lamers et al., 2008). For example, microshoot concentrations of K, Ca, and P are significantly lower in *Cucumis sativus* plants

Table 8. The enzyme activities of acid phosphatase, catalase, peroxidase, and peroxidase in three apple rootstocks' root and leaves under different treatments.

Rootstock ^z	Treatment ^y	ACP (U/g protein) ^x		CAT [protein (mg·mL ⁻¹)] ^x		POD (U/mg protein) ^x		SOD (U/mg protein) ^x	
		Root	Leaves	Root	Leaves	Root	Leaves	Root	Leaves
		(mean ± sd)							
Ms	CK	290.12 ± 18.23 c ^w	109.15 ± 10.25 b	26.17 ± 2.62 b	12.36 ± 1.19 b	20.15 ± 2.02 b	16.17 ± 1.51 b	205.71 ± 14.56 b	105.15 ± 12.15 b
	O	529.64 ± 22.14 a	222.01 ± 30.55 a	39.54 ± 2.06 a	19.17 ± 1.94 a	25.65 ± 2.69 a	21.21 ± 3.52 a	235.81 ± 13.89 a	130.83 ± 12.54 a
	OLP	490.86 ± 23.21 b	245.20 ± 22.65 a	38.31 ± 2.75 a	18.98 ± 1.52 a	25.02 ± 2.48 a	20.93 ± 1.59 a	234.21 ± 12.14 a	119.23 ± 12.44 ab
Mp	CK	226.42 ± 19.61 b	95.51 ± 10.21 b	22.81 ± 1.48 b	11.36 ± 1.05 b	18.65 ± 1.74 b	14.62 ± 1.52 b	206.26 ± 12.87 b	107.21 ± 11.99 b
	O	383.38 ± 23.14 a	185.86 ± 16.32 a	31.81 ± 3.29 a	16.85 ± 1.25 a	23.25 ± 2.15 a	18.85 ± 1.35 a	233.96 ± 12.56 a	120.04 ± 12.11 a
	OLP	372.3 ± 16.48 a	199.36 ± 20.36 a	30.54 ± 2.58 a	14.59 ± 1.58 a	22.94 ± 2.01 a	17.91 ± 1.74 a	232.15 ± 12.09 a	114.12 ± 12.40 ab
Mh	CK	251.84 ± 9.87 b	96.48 ± 7.52 b	22.56 ± 2.44 b	10.28 ± 0.86 b	17.62 ± 1.84 b	13.63 ± 1.45 b	204.12 ± 13.12 b	104.28 ± 11.86 a
	O	412.12 ± 18.77 a	133.63 ± 11.02 a	30.93 ± 2.84 a	15.05 ± 1.49 a	21.66 ± 2.11 a	17.16 ± 1.71 a	230.92 ± 13.21 a	118.42 ± 11.88 a
	OLP	407.96 ± 22.87 a	141.36 ± 12.85 a	28.84 ± 2.22 a	13.02 ± 2.04 a	20.03 ± 2.69 a	16.26 ± 1.54 a	229.09 ± 10.15 a	116.22 ± 12.58 a

^zDetails are described in Table 1.^yCK = control; O = osmotic stress; OLP = combination of osmotic and low-P stresses.^xACP = activity of acid phosphatase; CAT = activity of catalase; POD = activity of peroxidase; SOD = activities of superoxide dismutase.^wDifferent letters in the same column indicate significant differences according to Tukey's multiple-range test ($P < 0.05$).

under osmotic stress (Abu-Romman and Suwwan, 2012). Although the efficiency of P utilization was increased in our stressed rootstocks, their absorption efficiency was generally decreased. However, increasing the level of P in the hydroponic solution helped improve the amount of P measured in the plants. Elevating the level of P is an effective means of increasing the ψ_s of cell sap when water deficits exist (Sawwan et al., 2000).

Activity of antioxidant enzymes is stimulated in plant cells as a response to environmental stresses. Drought condition can promote the production of reactive oxygen species, which then adversely affect crop yield and quality (Rahimizadeh et al., 2007). ROS scavenging enzymes such as SOD, CAT, and POD have key roles in plant defenses against oxidative stress (Rahimizadeh et al., 2007). Although all three of our rootstocks showed greater activity by those enzymes under stress treatment, Ms had the strongest response. The higher *PHT* gene expression of Ms under low P conditions probably resulted in the stronger P transport and absorption ability, which could be one of the factors that enhanced Ms ROS scavenging ability.

Low availability of P, which is common in many types of soil, can stimulate phosphatase activity in plants (delPozo et al., 1999). For example, ACP activity is increased in drought-stressed *Medicago sativa* (Ehsanpour and Amini, 2003). It was also found in this study that activity by this enzyme was higher in all three rootstocks under osmotic stress, especially in their roots.

Clearly, under O and OLP conditions, ROS scavenging enzyme activity increased (Table 7). However, under osmotic stress, an appropriate increase in P concentrations can help to alleviate associated damage to plants. This is supported by a report by Garg et al. (2004), which showed that, under drought conditions, additional fertilizer applications can improve plant resistance to such stress.

Under OLP stress, the expression of *PHT* genes in three rootstocks were activated, with fold induction order (from high to low transcript levels) of Ms → Mp → Mh, which probably leads to different P contents in rootstocks and different osmotic resistance under low P supply conditions.

Conclusion

Under our simulated water deficit conditions, biomass production, photosynthetic rates, and Chl concentrations were diminished to varying degrees, according to rootstock genotype. However, the root-to-shoot ratios, root lengths, root surface areas, and the enzyme activities of ACP, CAT, POD, and SOD were increased. When osmotic treatment was combined with low P, Ms showed the strongest osmotic resistance, followed by Mp and Mh. Expression of the P transporter genes *PHT1;7*, *PHT1;12*, and *PHT2;1* in the roots and *PHT1;12* and *PHT4;5* in the leaves was positively correlated with plant osmotic resistance. We conclude that differential *PHT* gene expression among three tested rootstocks may contribute to their different osmotic resistance under a condition of low P supply.

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Supplemental Fig. 1. Regression analysis between apple physiological parameters and *PHT* gene expression under osmotic and low-P conditions. (A–C) Regression analysis between the relative root length and the expression of *PHTs* in roots under osmotic and low-P conditions. (D and E) Regression analysis between the relative total chlorophyll and the expression of *PHTs* in leaves under osmotic and low-P condition (CK = control; OLP = combination of osmotic and low-P stresses; Ms = *Malus sieversii*; Mp = *M. prunifolia*; Mh = *M. hupehensis*). Relative root length = root length of different rootstocks under OLP condition/the root length of different rootstocks under CK. Relative total chlorophyll = total chlorophyll of different rootstocks under OLP condition/the total chlorophyll of different rootstocks under CK condition; R^2 = coefficient of determination of the regression analysis.

Supplemental Fig. 2. The regression analysis between physiological parameters and *PHT* genes expression under osmotic stress condition. (A–C) Regression analysis between the relative root length and the expression of *PHTs* in roots under osmotic stress condition. (D and E) Regression analysis between the relative total chlorophyll and the expression of *PHTs* in leaves under osmotic stress (CK = control; O = osmotic stress; Ms = *Malus sieversii*; Mp = *M. prunifolia*; Mh = *M. hupehensis*). Relative root length = root length of different rootstocks under osmotic condition/the root length of different rootstocks under CK. Relative total chlorophyll = total chlorophyll of different rootstocks under osmotic condition/the total chlorophyll of different rootstocks under CK condition. R^2 = coefficient of determination of the regression analysis.