



# Comparison of drought resistance of rootstocks 'M9-T337' and 'M26' grafted with 'Huashuo' apple

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## Abstract

Drought stress is one of the main limiting factors in apple (*Malus domestica* Borkh.) cultivation. Rootstock plays an important role in the drought tolerance of apple plants. 'M.9-T337' is a novel apple rootstock that was recently introduced and widely cultivated in China. In this study, we selected the new, widely promoted Chinese apple variety 'Huashuo' as the scion and grafted it onto 'M.9-T337' (HM9). Another combination, 'Huashuo'/'M.26'/*Malus robusta* Rehd. (HM26), served as the experimental control to analyse drought resistance in the two hybrids. We believe that this empirical approach is more representative than merely studying rootstock seedlings. After sustained drought stress for over 1 month, the leaf relative water content had decreased in both types of plants, but to a lesser extent in HM26 than in HM9. The SPAD values increased in both plants, but without significant difference. Drought stress reduced photosynthetic activity in both plants, and the net photosynthetic rate was higher in HM26 than in HM9. The observed changes in the chlorophyll fluorescence parameters indicated that drought had damaged the PSII activity centres of both plants, photosynthetic electron transfer was inhibited, and excessive excitation energy accumulated. However, compared to HM26, HM9 displayed lower maximal PSII quantum photochemical efficiency and potential PSII activity. Moreover, HM9 showed lower antioxidant enzyme activity than HM26 under drought stress. A membership function analysis confirmed that 'M.9-T337' was less drought resistant than 'M.26'. Nevertheless, 'M.9-T337' could still recover after prolonged drought stress, indicating it also had good drought resistance.

**Keywords** Antioxidant enzyme · Apple · Drought stress · Dwarf rootstock · Photosynthetic efficiency · Reactive oxygen species

## 1 Introduction

High-density apple orchard planting systems consisting of dwarf rootstocks are widely used in the major apple-producing countries (Robinson and Lakso 1991; Wang et al. 2019). Key dwarf rootstocks used in this system include 'Malling 9' ('M.9') and 'Malling 26' ('M.26'). The latter is extensively used as an interstock in China. However, the most popular rootstocks used in developed countries include self-rooting varieties of 'M.9' and 'M.9-T337' (Ma et al.

2010; Robinson 2011). Unlike interstocks, rootstocks propagate by layering and therefore exhibit uniform post-grafting growth and easy mechanisation (Gao et al. 2012). Apple production has rapidly expanded in the Loess Plateau and Xinjiang Region of China over the last decade. However, it has been significantly challenged by water shortages. Hence, the mechanisms of drought resistance in apple plants merit investigation. In recent years, the 'M.9-T337' apple rootstock has been increasingly used owing to its ease of mechanisation and uniform post-grafting growth. However, few studies have examined its adaptability or that of other 'M.9' apple rootstocks in China. Most research has focused on introduction planting (Mushtaq et al. 2018, 2019). It is believed that the 'M.9-T337' apple rootstock has inferior drought resistance compared to that of the 'M.26' rootstock. Nevertheless, there is insufficient empirical evidence to corroborate this theory (Jensen et al. 2012; Wang et al. 2019).

Of all plant organs, the leaves are the most sensitive to drought stress (Hao et al. 2015; Shao et al. 2016). Leaf

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morphology and physiological parameters such as leaf water content, gas exchange, and chlorophyll fluorescence are effective indicators of plant drought adaptation and resistance (Shao et al. 2016; Wang et al. 2012). Longenberger et al. (2009) used chlorophyll fluorescence to identify drought tolerance in upland cotton. Centritto et al. (2011) investigated the interaction between high growth temperature and water stress on the gas exchange properties of *Populus nigra* saplings. Drought stress affects water use efficiency (WUE) in fruit trees. Liu et al. (2012a) found a close relationship between WUE and drought resistance and reported that these characteristics markedly differed among apple cultivars. For example, under long-term drought conditions, WUE was higher for the ‘Qinguan’ cultivar than for the ‘Changfu-2’ cultivar (Zhou et al. 2015).

Drought stress disrupts cell membrane osmotic potential, causes leaf wilting, and induces the production of reactive oxygen species (ROS) such as superoxide radical and hydrogen peroxide (Ren et al. 2016). ROS are scavenged by enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and those in the ascorbate–glutathione cycle. They are also non-enzymatically scavenged by ascorbate, glutathione, carotenoids, and tocopherol (Sofa et al. 2005; Ren et al. 2016; Kaur et al. 2013). SOD occurs in various cell compartments and scavenges  $O_2^-$  (Asada 2006) by catalysing its dismutation into  $H_2O_2$  and  $O_2$ . CAT and POD then convert the  $H_2O_2$  product to water and oxygen (Apel and Hirt 2004). In numerous plant species, antioxidant enzyme activity is an indicator of drought resistance (Sofa et al. 2005; Sharma and Dubey 2005; Abedi and Pakniyat 2010; Fazeli et al. 2007). Malondialdehyde (MDA) is a by-product of membrane lipid peroxidation; moreover, membrane damage increases with MDA content. Thus, MDA levels are an indirect measure of the degree of membrane damage and plant stress resistance (Chandra and Dubey 2008). Studies have shown that prolonged drought stress causes continuous accumulation of ROS and gradual increase of MDA content (Šircelj et al. 2005; Wang et al. 2012).

The aim of this study was to assess drought resistance in scions of ‘Huashuo’ apple, a novel Chinese cultivar, grafted onto ‘M.9-T337’ or ‘M.26’ rootstocks by measuring the relative water content (RWC) of the leaves and soil, gas exchange and chlorophyll fluorescence parameters, and antioxidant enzyme activity. We believe that this approach was more representative than merely studying rootstock seedlings. We endeavoured to elucidate the physiological mechanisms of drought resistance in apple trees to improve cultivation of this crop in China. The exhaustion of water resources by global warming is a major concern; thus, it is important to study various recovery processes after drought stress to determine the effective use of water resources.

## 2 Materials and methods

### 2.1 Experimental design

All experiments were performed in a glass greenhouse at the Zhengzhou Fruit Research Institute of the Chinese Academy of Agricultural Sciences (34°42′48″ N, 113°42′45″ E; 47 m a.s.l.) in Henan Province, China. The plants were placed in the greenhouse to avoid the impact of rain on drought treatment. The temperature and humidity in the glasshouse were approximately 28 °C and 70%, respectively. Two-year-old ‘Huashuo’/‘M.26’/*Malus robusta* Rehd. (dwarfing interstock) and ‘Huashuo’/‘M.9-T337’ saplings that we cultivated were transplanted into large pots (diameter, 70 cm; depth, 45 cm). Each of these contained 75 kg of a mixed medium, consisting of 1:1:3 (v/v) vermiculite:perlite: peat soil, and garden soil in a 1:2 (v/v) ratio. Each treatment comprised six pots and was repeated in triplicates. Routine management practices were performed until the treatments began. The following treatments were applied: (1) ‘Huashuo’/‘M.26’/*Malus robusta* Rehd. (IC) normally irrigated and kept the soil relative water content above 80%. (2) ‘Huashuo’/‘M.9-T337’ (SC) normally irrigated like IC. (3) ‘Huashuo’/‘M.26’/*Malus robusta* Rehd. (ID) had not been watered for 35 d. (4) ‘Huashuo’/‘M.9-T337’ (SD) had not been watered for 35 d. The treatment groups were rehydrated when the drought lasted for 36 d. Full irrigation was supplied in the form of water flowing through a small opening in bottom of the pot on the day before the experiment began. Thereafter, the water supply was controlled to simulate drought stress in ID and SD, whereas IC and SC continued to be irrigated normally to keep their soil well hydrated. All treatments were examined at 7, 14, 21, 28, and 35 d after the onset of drought stress and at 7 d after rehydration or 42 d after the onset of drought stress. Healthy mature leaves were collected from the mid-canopy and transported on ice to the laboratory for the determination of physiological indices.

### 2.2 Soil relative water content

Soil relative water content was measured by the oven drying method (Centritto et al. 2011). Twenty grams of soil was collected from a depth of 20 cm from each pot and dried in an oven at 105 °C to constant weight. The soil water content on day 1 of drought stress was taken as the base 100%. All other water content values were converted into percentages.

### 2.3 Leaf relative water content

Leaf relative water content (RWC) was determined using the method described by Sharp et al. (1990). Six healthy mature

leaves were randomly collected at various treatment stages. Their fresh masses were measured using a 1/1000 electronic balance (E5500S; Sartorius, Göttingen, Germany). Turgid mass was recorded after rehydrating the samples to full saturation in the dark for 24 h in vials containing water. De-enzyming was performed at 105 °C for 30 min followed by continuous drying at 60 °C to constant mass. The samples were then weighed at approximately 20 °C. RWC was calculated according to the following formula:

$$\text{RWC} = (\text{M1} - \text{M3}) / (\text{M2} - \text{M3}) \times 100\%, \quad (1)$$

where M1, M2, and M3 are the fresh leaf mass, the mass of the leaf saturated water content, and the dried leaf mass, respectively.

## 2.4 SPAD value assay

The leaf relative chlorophyll content was determined with a portable chlorophyll content meter (SPAD-502; Konica Minolta Inc., Tokyo, Japan) Five measurements were taken per leaf (Liu et al. 2015). Because the readings were significantly related to the chlorophyll content, they were used to estimate leaf photosynthetic capacity (Kumagai et al. 2009; Li et al. 2010).

## 2.5 Photosynthetic parameters

Gas exchange parameters were monitored with a portable photosynthesis system (CIRAS-3; PP Systems, Amesbury, MA, USA). The net photosynthesis rate (Pn), transpiration rate (Tr), stomatal conductance (Gs), intercellular CO<sub>2</sub> concentration (Ci), and water use efficiency (WUE) were measured using the sixth fully expanded leaf from the apex. Measurements were taken at different treatment stages on clear days between 9h00 and 11h00 or between 8h00 and 16h00 to determine diurnal variation. The leaf temperature was 25 °C, CO<sub>2</sub> concentration was 380 ± 10 μmol mol<sup>-1</sup>, and relative humidity (RH) and light intensity were adjusted to 60% and 600 μmol m<sup>-2</sup> s<sup>-1</sup> (photosynthetic photo flux density), respectively.

## 2.6 Chlorophyll fluorescence parameters

A portable pulse-amplitude-modulated (PAM) chlorophyll fluorometer (PAM-2500; Walz, Effeltrich, Germany) was connected to a computer via a data acquisition system software (Pamwin-3; Walz) and used to measure the chlorophyll fluorescence parameters of the same leaves used to determine SPAD. Each leaf was dark-adapted for 30 min, and six measurements were taken per leaf (Guo et al. 2006). Minimal (F<sub>o</sub>) and maximal (F<sub>m</sub>) fluorescence were determined by separately activating the measuring (< 0.1 μmol m<sup>-2</sup> s<sup>-1</sup>)

and saturated pulse (> 8000 μmol m<sup>-2</sup> s<sup>-1</sup>) lights (Guo et al. 2005; Maxwell and Johnson 2000). F<sub>m</sub> yield in the light-adapted state (F<sub>m'</sub>) was determined by applying the active (538 μmol m<sup>-2</sup> s<sup>-1</sup>) and saturated pulses (> 8000 μmol m<sup>-2</sup> s<sup>-1</sup>). After steady-state photosynthesis was attained, the fluorescence was recorded, and the F<sub>o</sub> yield in the light-adapted state (F<sub>o'</sub>) was determined by activating the far-red light (van Kooten and Snel 1990). F<sub>v</sub>/F<sub>m</sub> (maximal PSII photochemical quantum efficiency), F<sub>v</sub>/F<sub>o</sub> (potential PSII activity), the photochemical quenching coefficient (qP), and non-photochemical quenching (NPQ) of the chlorophyll fluorescence were calculated according to Baker (2008). (See Table S1 for definitions of the chlorophyll fluorescence nomenclature).

## 2.7 O–J–I–P chlorophyll a fluorescence induction transient: JIP test

The rapidly induced kinetics curve was determined with the same device used to measure chlorophyll fluorescence. The leaves were dark-adapted for 30 min before measurement. Rapid changes in the fluorescence signals were recorded between 10 μs and 300 ms and reflected in the O–J–I–P curve after instantaneous red light (650 nm; 3000 μmol m<sup>-2</sup> s<sup>-1</sup>) exposure (Tan et al. 2011). The transients were subjected to JIP testing. This fast, convenient, and non-invasive technique is widely used in plant stress physiology research. It evaluates functional changes in photosystem II (PSII) under stress. The following data were used in JIP testing: fluorescence intensity at 20 μs (F<sub>o</sub>); maximal fluorescence intensity (F<sub>m</sub>); fluorescence intensity at 300 μs (K-step) (F<sub>300 μs</sub>); fluorescence intensity at 2 ms (J-step) (F<sub>J</sub>); and fluorescence intensity at 30 ms (I-step) (F<sub>I</sub>). Table S2 lists the formulae used to determine the biophysical parameters that quantify PSII energy flow based on the original data (Ceppi et al. 2012; Stirbet and Govindjee 2011).

## 2.8 Antioxidant enzyme activity and MDA content assays

Leaves were harvested, immediately frozen in liquid nitrogen, and stored at – 80 °C until post-treatment analyses were conducted. Frozen leaves (0.5 g) were crushed into fine powder under liquid nitrogen in a mortar and pestle. Soluble proteins were extracted by homogenising the powder in 5 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM ethylenediaminetetraacetic acid and 1% (w/v) polyvinylpyrrolidone. Each homogenate was centrifuged at 10,000×g and 4 °C for 20 min. The supernatants were used to determine enzyme and MDA levels (Tan et al. 2012). Superoxide dismutase (SOD) activity was assayed by the riboflavin-nitro blue tetrazolium method (Dhindsa et al. 1981). POD activity was determined by the guaiacol-colourimetric method

(Chance and Maehly 1955), and CAT activity was evaluated by the ammonium molybdate method in an ultraviolet spectrophotometer (TU-1901; Beijing Puxi General Instrument Co. Ltd, Beijing, China) (Nakano and Asada 1981). The MDA content was measured by the thiobarbituric acid method (Zhang and Kirkham 1994).

## 2.9 Physiological root analysis

The fresh weights, lengths, and volumes of the roots in the various treatments were measured. Root surface area was calculated using 20 g fresh root randomly selected from the different treatments. The roots were scanned, and their morphological parameters were assessed with the LA-S Plant Root Analyser System (Hangzhou WSeen Detection Technology Co. Ltd., Hangzhou, China).

## 2.10 Drought tolerance evaluation

The drought tolerance levels of the rootstocks were determined by membership function analysis. The parameters measured on day 35 of drought treatment were selected for the analysis and calculated as follows:

$$Z = (X_i - X_{\min}) / (X_{\max} - X_{\min}) \quad (2)$$

If the correlation was negative, the following formula was used:

$$Z = 1 - (X_i - X_{\min}) / (X_{\max} - X_{\min}), \quad (3)$$

where  $Z$  is the membership function value of the rootstocks,  $X_i$  is the measured value of the drought tolerance index for a specific rootstock, and  $X_{\max}$  and  $X_{\min}$  are the maximum and minimum values of the rootstocks, respectively (Wu et al. 2019).

## 2.11 Statistical analysis

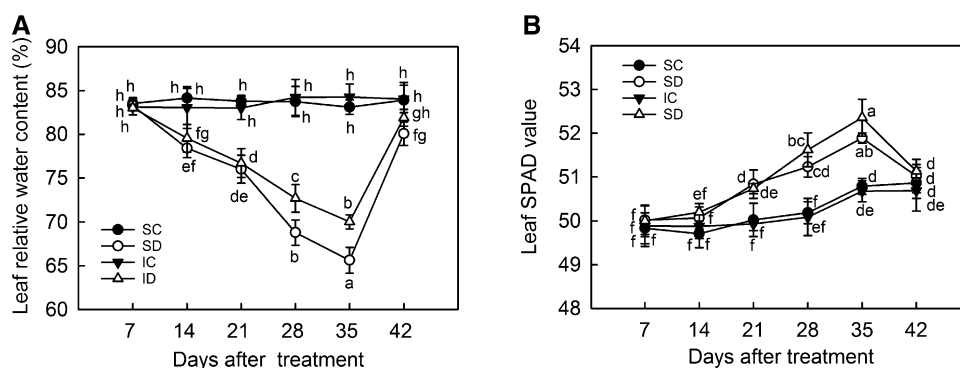
Six apple trees similar in size were used per treatment. Each treatment was performed in triplicates. All data were entered into Excel 2010 (Microsoft Corp., Redmond, WA, USA). All figures were plotted with SigmaPlot v. 14.0 (Systat Software, San Jose, CA, USA). All data were evaluated using Tukey's test ( $p < 0.05$ ) in the ANOVA program of SPSS Statistics v. 19.0 (IBM Corp., Armonk, NY, USA).

## 3 Results

### 3.1 Leaf relative water content and SPAD values

We measured the leaf relative water content of the four groups during the entire drought period and after recovery by rewatering (Fig. 1A). In the control, there was no significant difference between IC and SC in terms of leaf water content; the range was 82.44–85.99%. For the drought treatment groups, the leaf water content gradually decreased with increasing number of days of drought. After 21 d, however, it had decreased more in SD than in ID. One week after rewatering, SD and ID gradually recovered. Although ID returned to normal levels, the leaf water content of SD was significantly different from normal.

For the control, the SPAD values did not significantly change or differ between IC and SC as the experiment progressed, but they were increased with time; for the treatment groups, however, the SPAD values of SD and ID gradually increased as the drought stress intensified (Fig. 1B). After 21 d, the SPAD values were significantly higher in SD and ID than in SC and IC. Nevertheless, there was no significant difference between SD and ID in terms of SPAD value. The difference between the control and treatment groups in terms of SPAD value had reached the maximum by day 35. After



**Fig. 1** Effects of drought and rehydration on leaf relative water content (a) and SPAD values (b) of two rootstocks. Until the 35th day, the plants were under drought treatment, followed by rehydration; the measurements were taken 7 d later (the 42nd day). Different lower-

case letters near the line indicate significant differences at  $p < 0.05$ . SC: 'M.9-T337' rootstock (control); SD: 'M.9-T337' rootstock (drought treatment); IC: 'M.26' interstock (control); ID: 'M.26' interstock (drought treatment)

7 d of rehydration and recovery (day 42), the SPAD values had decreased in the treatment groups. Figure S1 shows the state of the plants after 35 d of drought.

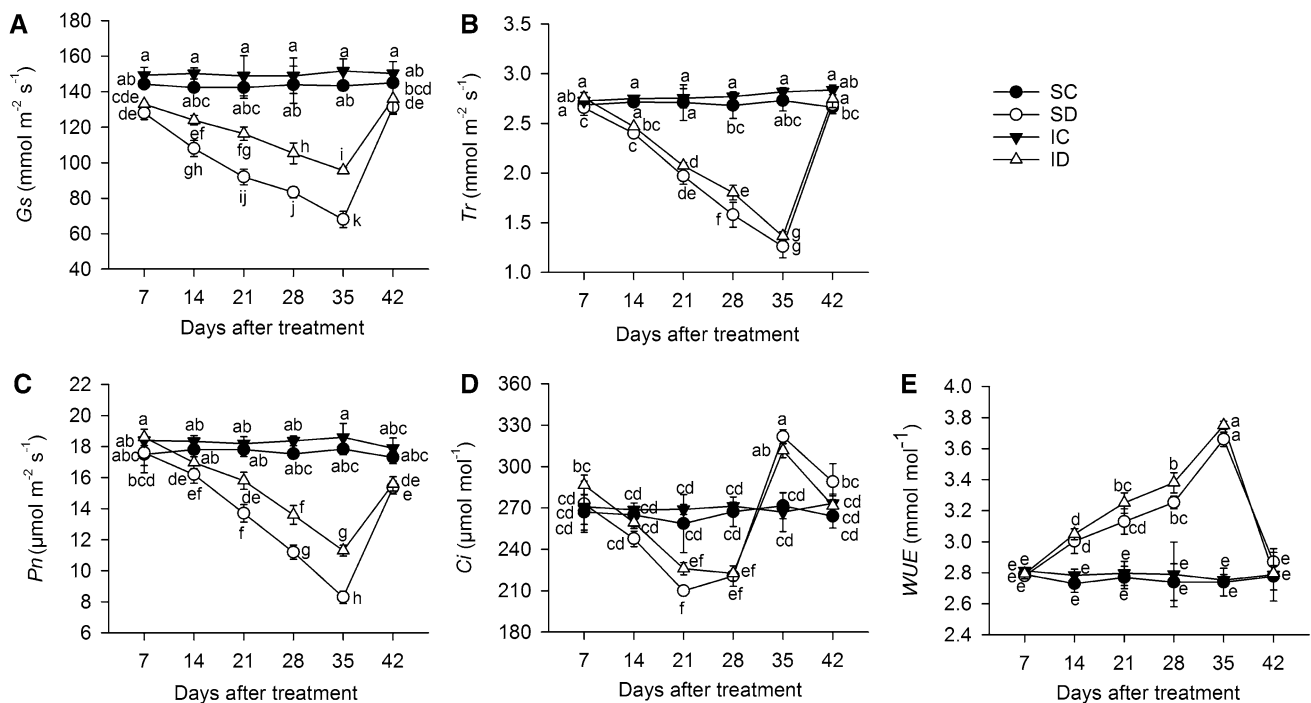
### 3.2 Changes in leaf gas exchange parameters

Foliar gas exchange parameters were measured every other week. For ID and SD, Gs gradually decreased with prolongation of drought stress, and Pn decreased in SD more than it did in ID. After rewatering, however, Gs increased in both SD and ID but to a relatively lesser extent in the latter (Fig. 2A). Tr had significantly decreased in both ID and SD after 35 d drought stress, but increased nearly to the same level as that of the control after 1 week of rehydration (Fig. 2B). The changes in Pn were similar to that in Gs (Fig. 2C). Relative to the control, Ci in the drought treatment groups initially decreased and then increased with drought stress intensification. Nevertheless, there was no significant difference between ID and SD in terms of Ci. Moreover, the values of Ci declined to normal levels after rehydration (Fig. 2D). WUE did not significantly differ between IC and SC or between ID and SD. Compared to the control groups, WUE gradually increased and was  $\sim 1.37\times$  higher in the treatment groups. After 7 d of rehydration, WUE for the treatment groups had returned to the same level as that of the control groups (Fig. 2E).

We also measured diurnal variations in foliar gas exchange parameters. Gs gradually decreased in both the control and drought treatment groups. Gs reached its lowest level at noon and slightly increased thereafter. The Gs values of the control groups were higher than those of the treatment groups at all times (Fig. 3A). Tr rapidly decreased between 10h00 and 12h00 and slowly decreased thereafter (Fig. 3B). Foliar Pn fell in the morning and rose in the afternoon (Fig. 3C). The diurnal changes in Ci were similar to those for Pn. At each observation, the Ci values were higher in the drought treatment groups than in the control groups (Fig. 3D). WUE initially decreased and increased thereafter. The WUE values were significantly higher in the drought treatment groups than in the control groups (Fig. 3E). The Gs, Tr, Pn, and Ci values were higher in the control groups than in the drought treatment groups (Fig. 3).

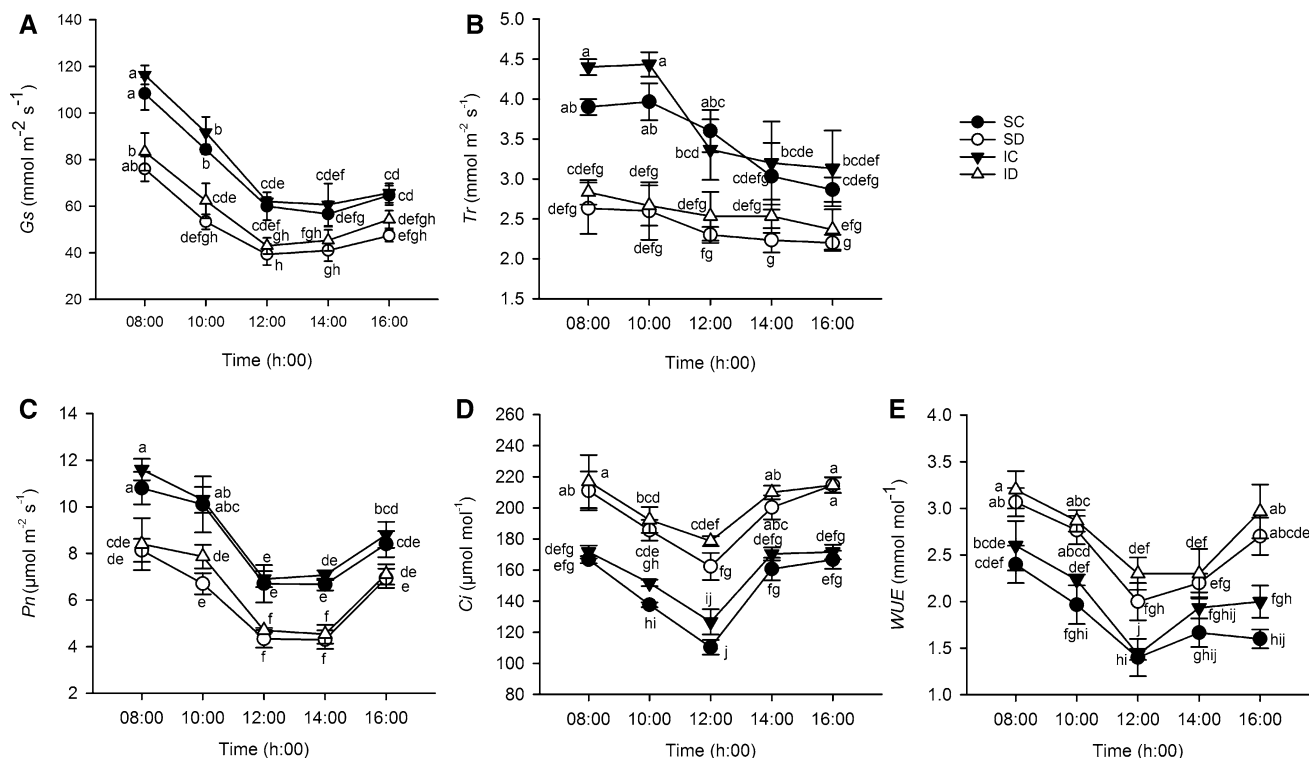
### 3.3 Effects of drought stress on chlorophyll a fluorescence parameters

The  $F_v/F_m$  and  $F_v/F_o$  ratios of the drought treatment groups gradually decreased over 35 d of drought stress but returned to normal after rewatering (day 42) (Fig. 4A, D). The qP values were also relatively lower in the drought treatment groups (Fig. 4B). In contrast, the NPQ values gradually increased (Fig. 4C). After rewatering, qP increased and NPQ



**Fig. 2** Effects of drought stress and rehydration on leaf gas exchange parameters of 'Huashuo' grafted on two rootstocks. SC: 'M.9-T337' rootstock (control); SD: 'M.9-T337' rootstock (drought treatment); IC: 'M.26' interstock (control); ID: 'M.26' interstock (drought treat-

ment); Gs: stomatal conductance; Tr: transpiration rate; Pn: net photosynthesis rate; Ci: intercellular CO<sub>2</sub> concentration; WUE: water use efficiency. Lines labeled with different lowercase letters indicate significant differences ( $p < 0.05$ )



**Fig. 3** Diurnal variation of gas exchange parameters of leaves from ‘Huashuo’ apple grafted on two rootstocks under drought stress (the 35th day). SC: ‘M.9-T337’ rootstock (control); SD: ‘M.9-T337’ rootstock (drought treatment); IC: ‘M.26’ interstock (control); ID: ‘M.26’ interstock (drought treatment). Gs: stomatal conductance; Tr: tran-

spiration rate; Pn: net photosynthesis rate; Ci: intercellular CO<sub>2</sub> concentration; WUE: water use efficiency. Lines labeled with different lowercase letters indicate significant differences between treatments ( $p < 0.05$ )

decreased (Fig. 4B, C). However, these parameters do not differ significantly between IC and ID (See Table S1 for definitions and formulae for the relevant parameters).

### 3.4 Effects of drought stress on chlorophyll a fluorescence kinetics (O–J–I–P) parameters

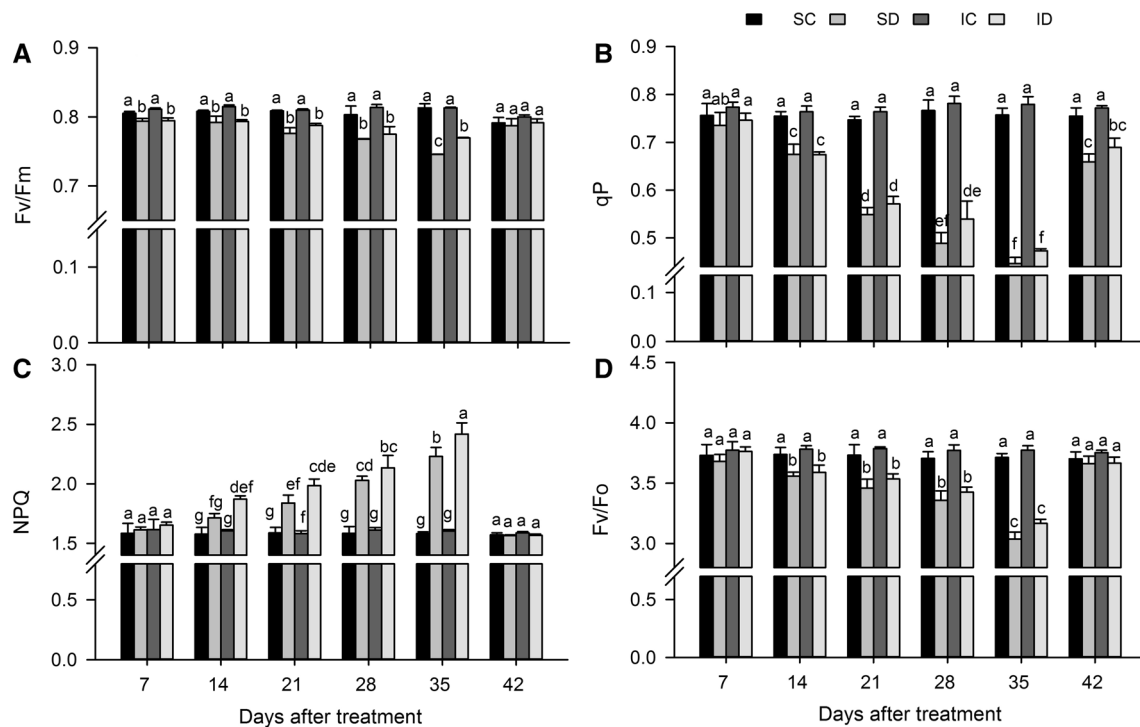
We evaluated chlorophyll fluorescence kinetics in the leaves of the apple plants at day 35 after the onset of drought stress and again at day 7 after rewatering (Fig. 5A, B). The baseline curve shapes were similar for both control groups. However, the drought and control treatments substantially differed in terms of their O–J–I–P curves. The JIP values for ID and SD decreased after 35 d of drought stress, and JIP was lower for SD than for ID. After rewatering, the foliar O–J–I–P values were higher in the drought treatment groups (ID and SD) than in the control groups (IC and SC). Moreover, the foliar O–J–I–P values were higher for ID than for SD.

The following parameters decreased in ID and SD after 35 d of drought stress: performance index on an absorption basis ( $PI_{ABS}$ ); osmotic water potential ( $\psi_o$ ); absorbed energy flux per cross section ( $ABS/CS$ ); excitation energy per cross section ( $TR_o/CS$ ); and electron transfer energy per

cross section ( $ET_o/CS$ ). However, heat dissipation per cross section ( $DI_o/CS$ ) increased in both ID and SD (Fig. 5C). The following reaction centre parameters increased in both ID and SD after 35 d of drought stress: absorbed energy flux per reaction centre ( $ABS/RC$ ); excitation energy per reaction centre ( $TR_o/RC$ ); electron transfer energy per reaction centre ( $ET_o/RC$ ); and heat dissipation per reaction centre ( $DI_o/RC$ ). However, the density of active reaction centres per unit area ( $RC/CS$ ) decreased (Fig. 5D). Definitions and formulae for all relevant parameters are listed in Table S2.

### 3.5 Effects of drought stress and rewatering on antioxidant enzyme activity and MDA content

The SOD, POD, and CAT activity levels remained relatively constant in the control (IC and SC) groups. However, their activity levels in the drought treatment (ID and SD) groups gradually increased with the increase in drought stress (0–35 d). Furthermore, the activity levels of these enzymes were higher in ID than in SD (Fig. 6A–C). Seven days after rehydration (day 42), the activity levels of all three enzymes had markedly decreased and approached the levels detected in



**Fig. 4** Effect of drought stress and rewatering treatments on the leaf fluorescence characteristics of 'Huashuo' grafted on two apple rootstocks. **a**  $F_v/F_m$ , maximal photosystem II (PSII) photochemical quantum efficiency; **b**  $qP$ , photochemical quenching coefficient; **c** NPQ, non-photochemical quenching of chlorophyll fluorescence; **d**  $F_v/F_o$ , potential PSII activity.  $F_v$ : variable fluorescence;  $F_m$ : maximal fluo-

rescence;  $F_o$ : minimal fluorescence; SC: 'M.9-T337' rootstock (control); SD: 'M.9-T337' rootstock (drought treatment); IC: 'M.26' interstock (control); ID: 'M.26' interstock (drought treatment). Columns marked with different lowercase letters indicate significant differences among treatments ( $p < 0.05$ )

the control groups. The MDA content in both ID and SD gradually increased with prolonged drought treatment and decreased after rewatering. The MDA content was higher in SD than in ID at day 21 but did not significantly differ at any other time (Fig. 6D).

### 3.6 Membership function analysis of the indices of the plants under drought stress

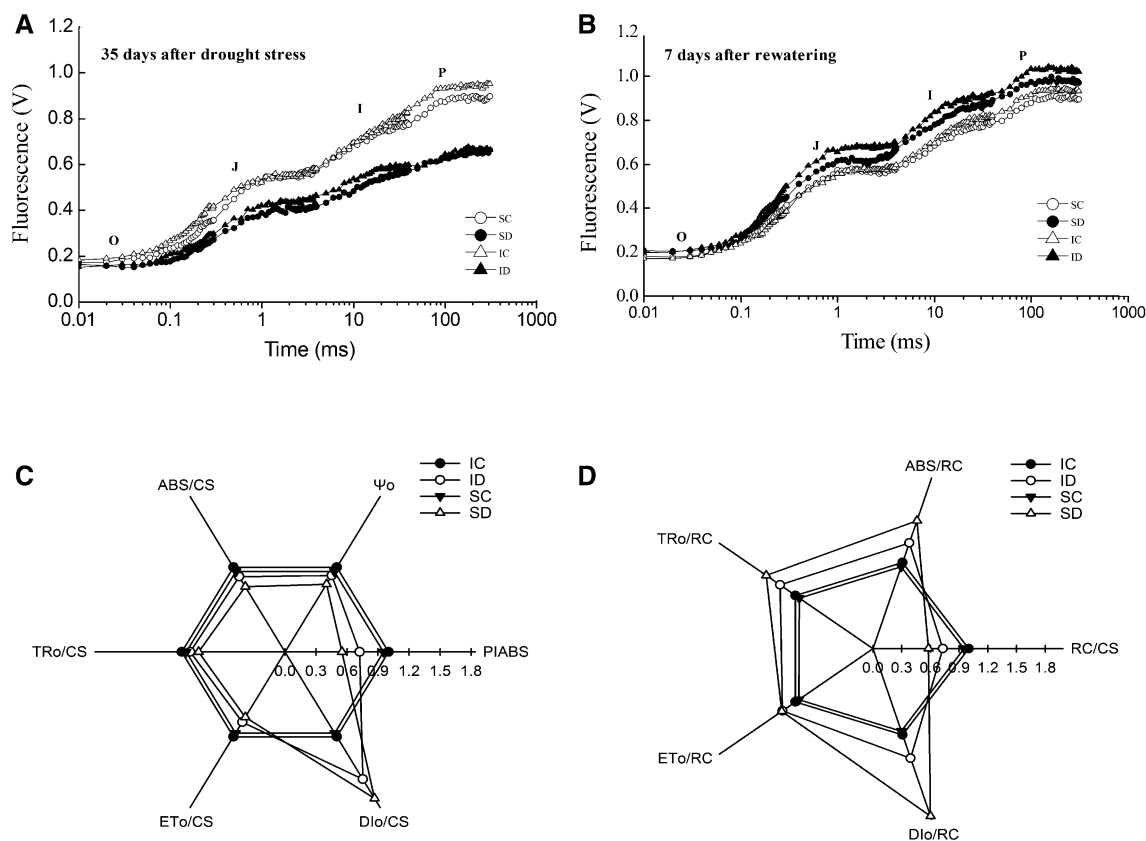
We analysed the membership function values of the various indices of the plants under drought stress. We selected data for day 35 of the drought treatment to calculate the membership function values. Table 1 lists the values for all parameters. As 'M.26' ranked first, its drought resistance was stronger than that of 'M.9-T337'.

## 4 Discussion

Leaf water content directly affects plant photosynthesis and transpiration (Hao et al. 2015; Shao et al. 2016). Drought stress can lead to a potential or direct decrease in leaf water content. Hence, this metric can indicate whether a plant requires irrigation (Cohen et al. 2005). Leaf water content

is also an index of plant drought resistance. In this study, apple leaf RWC gradually decreased after drought stress and increased after rewatering. This trend resembled that which was reported by Wang et al. (2012). RWC decreased more rapidly in 'M.9-T337' than in 'M.26' during drought stress. RWC increased more slowly in 'M.9-T337' than in 'M.26' after rewatering. The SPAD value was lower in 'M.9-T337' than in 'M.26'. Thus, the 'Huashuo' saplings with 'M.9-T337' rootstocks had lower drought resistance than those with 'M.26' rootstocks. Soil water content is the key factor determining leaf water content. In this study, we measured changes in soil water content throughout the entire drought period (Fig. S2). Oven drying is the most widely used method for determining soil water content. We evaluated changes in the roots of plants subjected to drought stress after 35 d, which showed that the root systems of the 'M.26' rootstocks were better developed than those of the 'M.9-T337' rootstocks (Fig. S3). Please see Table S3 for detailed root system data.

Drought stress may lower plant photosynthetic capacity via stomatal or non-stomatal limiting factors. When  $G_s$  and  $C_i$  changed in the same direction, the former mainly affected photosynthesis possibly by destruction of the photosynthetic organs (Niu et al. 2007; Zhou et al. 2013). In this study,



**Fig. 5** Natural variation of chlorophyll a fluorescence induction curves for ‘huahsuo’ seedlings and light use efficiency per unit area (c) or unit reaction center (d). A: Under 35 d of drought treatment; B: 7 d after rewatering. SC: ‘M.9-T337’ rootstock (control); SD: ‘M.9-T337’ (drought treatment); IC: ‘M.26’ interstock (control); ID: ‘M.26’ interstock (drought treatment). ABS/CS: absorbed energy flux

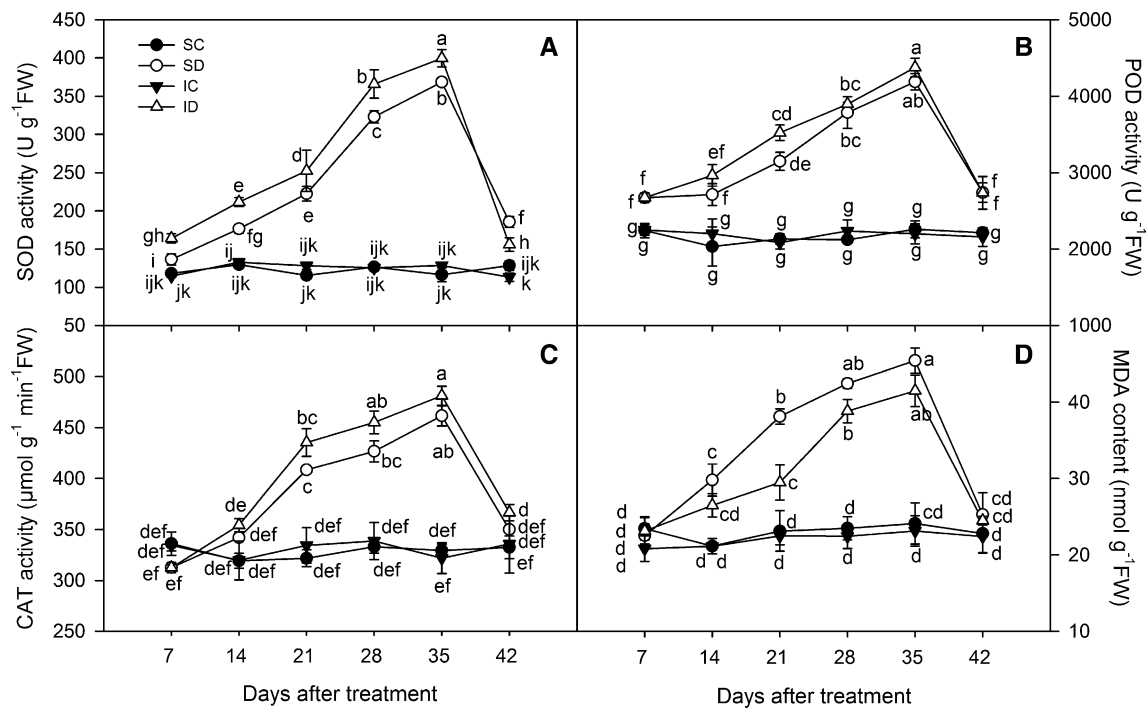
per cross section; ETo/CS: electron transfer energy per cross section; DIo/CS: heat dissipation per cross section; ABS/RC: absorbed energy flux per reaction center; TRo/RC: excitation energy per reaction center; ETo/RC: electron transfer energy per reaction center; DIo/RC: heat dissipation per reaction center

drought stress for 35 d decreased photosynthesis capacity. Gs, Pn, and Tr showed downward trends in the drought treatment group (Figs. 2A, 2B, 2C). Elevated Gs in response to the drought treatment could explain the fact that Pn was higher for ID than for SD (Figs. 2A and 2C) because the larger Gs is, the more CO<sub>2</sub> will enter the cell, indicating that the more CO<sub>2</sub> consumed in photosynthesis, the greater Pn will be. Gs reduction under drought stress is an adaptive mechanism to reduce water loss. Moreover, stomatal control lowers Tr (Figs. 2A and 2B). After 21 d of drought stress, Ci significantly differed between the drought treatment and control groups. The capacity of chloroplasts to fix CO<sub>2</sub> affects photosynthesis rather than increasing stomatal diffusive resistance. Hence, non-stomatal limitation lowers the photosynthetic rate (Fig. 2D). Diurnal variations in photosynthesis indicated that the changes in Gs and Ci followed a similar trend between 8h00 and 12h00 but diverged after 12h00. Therefore, the photosynthesis capacity is affected mainly by stomatal factors in the morning and by non-stomatal factors in the afternoon (Fig. 3A, D). A ‘napping’

phenomenon was observed at 12h00 primarily because of stomatal closure induced by photoinhibition and excessive transpiration. Gs, Tr, and Pn decreased more in SD than in ID under drought stress. Consequently, the ‘M.9-T337’ rootstock had relatively weaker drought resistance (Fig. 3A–C). WUE increased with drought stress severity. As WUE was higher in ID than in SD, drought resistance was stronger in ‘M.26’ than in ‘M.9-T337’ (Figs. 2E and 3E).

Photosynthetic energy is consumed mainly by photosynthetic electron transfer, chlorophyll fluorescence emission, and heat dissipation (Singh et al. 2017). Chlorophyll fluorescence parameters rapidly and accurately indicate the effects of drought stress on photosynthesis without damaging plant material.  $F_v/F_o$  and  $F_v/F_m$  reflect the photosynthetic potential of the PSII reaction center (Longenberger et al. 2009).  $F_v/F_m$  and  $F_v/F_o$  were inhibited in the leaves under drought stress (Fig. 4A, D). This finding was consistent with those reported by Thomas and Turner (2001). The qP reflects the redox state of the original electron acceptor Q<sub>A</sub> and the number of PSII open centres. NPQ indicates that the photosynthetic





**Fig. 6** Effects of drought stress and rewatering treatments on superoxide dismutase (SOD; **a**), peroxidase (POD; **b**), and catalase (CAT; **c**) activity, and malondialdehyde (MDA; **d**) content in the leaves of ‘Huashuo’ grafted on two apple rootstocks. SC: ‘M.9-T337’ rootstock

(control); SD: ‘M.9-T337’ rootstock (drought treatment); IC: ‘M.26’ interstock (control); ID: ‘M.26’ interstock (drought treatment). Different lowercase letters indicate significant differences between the different treatments ( $p < 0.05$ )

**Table 1** Comparison of drought resistance of rootstocks using membership function values

Rootstocks	Membership function value													Average	Order
	LRWC	SPAD	Pn	Gs	WUE	F <sub>v</sub> /F <sub>m</sub>	qP	NPQ	F <sub>v</sub> /F <sub>o</sub>	SOD	POD	CAT	MDA		
IC	1.00	0.00	1.08	1.00	0.01	1.00	1.00	0.02	1.00	0.04	0.00	0.00	1.00	0.54	2
ID	0.24	1.00	0.31	0.33	1.00	0.36	0.06	1.00	0.18	1.00	1.00	1.00	0.18	0.59	1
SC	0.94	0.15	1.00	0.90	0.00	1.00	0.91	0.00	0.92	0.00	0.03	0.05	0.96	0.53	3
SD	0.00	0.78	0.00	0.00	0.91	0.00	0.00	0.81	0.00	0.89	0.91	0.87	0.00	0.40	4

SC: ‘M.9-T337’ rootstock (control); SD: ‘M.9-T337’ rootstock (drought treatment); IC: ‘M.26’ interstock (control); ID: ‘M.26’ interstock (drought treatment). LRWC: leaf relative water content; SPAD: leaf relative chlorophyll content; Pn: net photosynthetic rate; Gs: gas exchange parameters; WUE: water use efficiency; F<sub>v</sub>/F<sub>m</sub>: maximal PSII photochemical quantum efficiency; qP: photochemical quenching coefficient; NPQ: non-photochemical quenching of chlorophyll fluorescence; F<sub>v</sub>/F<sub>o</sub>: potential PSII activity; SOD: superoxide dismutase; POD: peroxidase; CAT: catalase; MDA: malondialdehyde; Values are means ± SD of three replicates, and different lowercase letters denote significant differences between different treatments ( $p < 0.05$ )

energy absorbed by PSII antenna pigments is unavailable for photosynthetic electron transfer but is dissipated as heat instead (van Kooten and Snel 1990). We observed that F<sub>v</sub>/F<sub>m</sub>, F<sub>v</sub>/F<sub>o</sub>, and qP gradually decreased while NPQ gradually increased with prolonged drought stress (Fig. 4). Under drought stress, then, photosynthetic electron transport was blocked, the photosynthetic rate decreased, and excess light energy was captured by the leaves. However, redundant excitation energy could be eliminated through heat dissipation, which is a protective mechanism in plants (Fernandez et al. 1997; Maxwell and Johnson 2000). F<sub>v</sub>/F<sub>m</sub>, F<sub>v</sub>/F<sub>o</sub>, and qP

decreased less while NPQ increased more in ID than in SD. Relative to the ‘M.9-T337’ rootstocks, those of ‘M.26’ provided superior heat dissipation, electron transfer ability, light energy utilisation, and drought stress adaptability.

Here, the chlorophyll *a* fluorescence curves started at F<sub>o</sub> and peaked at F<sub>p</sub> when the leaves were exposed to saturating actinic light. F<sub>j</sub> and F<sub>i</sub> occurred sequentially between F<sub>o</sub> and F<sub>p</sub> (Gomes et al. 2012). Figure 5A shows the O–J–I–P curves for the two rootstocks subjected to drought stress for 35 d and reveals that the energy and electron transfer processes decreased in the photosystems. PSII activity partially

recovered after 7 d of rehydration (Fig. 5B). After 35 d of drought stress,  $PI_{ABS}$  decreased in both rootstocks. Hence, overall PSII performance also decreased (Fig. 5C). The observed reduction in  $\psi_o$  indicated that the electron transport chain was inhibited on the receptor side (Fig. 5C). A reduction in reaction centre density (RC/CS) improves the energy consumption efficiency of each active reaction centre and accounts for the observed increases in  $ABS/RC$ ,  $TRo/RC$ , and  $ET_o/RC$ . In this manner, any further damage to the PSII is mitigated. Simultaneously, light capture ( $ABS/CS$ ), excitation energy for QA ( $TRo/CS$ ), and electron transfer per unit leaf area ( $ETo/CS$ ) are reduced (Fig. 5). The observed increases in heat dissipation per unit area ( $DI_o/CS$ ) and heat dissipation per unit reaction centre ( $DI_o/RC$ ) demonstrated that the leaves had initiated the heat dissipation defence mechanism and reduced thermal damage by dissipating excess excitation energy in a timely manner (Fig. 5). These findings resembled those reported by Gao et al. (2015) for maize subjected to drought stress.

SOD, POD, and CAT scavenge active oxygen free radicals and are essential for avoiding plant injury. They work synergistically to facilitate plant drought resistance by removing ROS (Choudhury et al. 2017; Platiša et al. 2008; Ramel et al. 2009; Ren et al. 2016; Zhang et al. 2015). Ren et al. (2016) stated that drought tolerance was correlated with antioxidant enzyme activity in *Cerasus humilis* seedlings. The most drought-resistant cultivar had lower MDA content and higher SOD and ascorbate–glutathione cycle-related enzyme activity than the drought-sensitive cultivar. In this study, the observed increase in MDA content during drought stress indicated that ROS induced antioxidant enzymes (Fig. 6D), but at levels too low to prevent ROS-related cell membrane damage. Hence, lipid peroxidation-induced MDA production occurred. Similar findings were reported by Al-Ghamdi (2009). The MDA content and enzyme activity in the SC and IC rootstocks gradually increased with drought stress intensity. Moreover, the MDA content was lower in ID than in SD, while the SOD, POD, and CAT activity levels were higher in ID than in SD (Fig. 6). Thus, ID had somewhat superior drought resistance to SD. Several studies reported that CAT activity is positively correlated with drought severity (Faize et al. 2011; Mittler et al. 2011; Pinheiro and Chaves 2011; Wang et al. 2012). However, another study (Abedi and Pakniyat 2010) arrived at the opposite conclusion possibly because of species specificity. Liu et al. (2012b) proposed that ROS scavenging is induced to higher levels in apple leaves under fairly severe drought stress than in those under mild to moderate drought stress. However, ROS scavenging is induced to even higher levels in plants under severe drought stress as a consequence of enzyme damage. In this study, antioxidant enzyme activity continued to increase until day 35 d of drought stress. Hence, the enzyme system was not irreparably damaged by the drought

as it was restored after rehydration. Nevertheless, the relative soil water content was 28.77%, which indicates that the plants were experiencing severe drought. Whereas the 2-year-old plants had a certain level of drought resistance capacity, their wilting leaves indicated that the activity of their enzyme system might have soon reached a maximum critical value. The results of this study demonstrated that SOD, CAT, and POD worked synergistically to maintain the free radicals at low to normal levels, thereby protecting the plant and repairing the damage caused by drought stress (Figs. 6A–C). Rewatering recovered the antioxidant enzyme and MDA levels to a certain extent (Fig. 6). CAT activity declined to normal levels more rapidly than the other enzymes. Thus, drought promotes physiological stress resistance and adaptation in plants.

## 5 Conclusions

This study demonstrated that the performance of the PSII in ‘Huashuo’ apple leaves declined in response to drought stress. The gas exchange parameters  $G_s$ ,  $P_n$ , and  $T_r$  decreased while WUE increased under drought stress. Slow and fast chlorophyll fluorescence indicated that drought conditions enhanced leaf heat dissipation, thereby improving the efficiency of active reaction centres. Hence, chlorophyll fluorescence was a sensitive indicator of drought stress in apple leaves. Furthermore, antioxidant enzymes were induced in response to drought stress to remove excess ROS and avoid plant injury. A membership function analysis revealed that the drought resistance of ‘M.9-T337’ was inferior to that of ‘M.26’. Therefore, ‘M.26’ is a better choice for areas with serious water shortage. Nevertheless, the former could still recover in response to rehydration after prolonged drought stress. Thus, it also has good drought resistance. However, we only studied the physiological response of apple plants to drought, and the drought resistance molecular mechanism will be the direction of future related research. This will provide a theoretical reference with which to explore the molecular mechanism of the plant drought response, molecular genetic breeding, and agricultural sustainable development.

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**Author contributions** C-Y S analyzed the data, and wrote the manuscript. L L performed some of the experiment, analyzed the data and revised the manuscript. Q-L L performed the experiment. Z-F W

provided technical support. D-T G supervised the project, designed the experiments, and conceived the original idea.

## Declarations

**Conflict of interest** The authors declare that they have no conflict of interest.

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