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ORIGINAL ARTICLE

An Analysis of Physiological Index of Differences in Drought Tolerance of Tomato Rootstock Seedlings

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Abstract Drought is one of the most limiting factors for plant growth and development. In this study, experiments were carried out on five tomato rootstocks were subjected to water withdrawal and re-watering. Two RKN (root-knot nematodes)-and drought-dual resistant rootstocks were identified according to phenotype, physiological and molecular indexes such as the leaf relative water content (LRWC), electrolyte leakage (EL), water loss of the leaf, proline content, and increased activities of antioxidant enzymes, including peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR) and the transcript levels of drought stress marker genes. Further, we preliminarily investigated the mechanism underlying improved drought tolerance arising from grafting. These results will promote the application of these rootstocks in tomato production and provide new information on the mechanism of tomato grafting.

Keywords: Antioxidant enzyme, Drought tolerance, Rootstocks, Tomato (*Solanum lycopersicum*), Water stress

Introduction

Drought is a major abiotic factor that adversely affects the growth, development, metabolism and yield of higher plants (Loyola et al. 2011). Water shortages are expected to lead to global crop production losses of up to 30% by 2025 compared to current yields (Zhang 2011). The predicted increase in the number of dry days per year for many areas of the globe will further exacerbate the problem. Therefore, at present, with the aim of improving agricultural yield under the constraint of the earth's limited water resources, it is imperative to develop crops that are able to produce high yield when growing in a drought environment. Increasing crop resistance to water stress would be the most economical approach to improving productivity based on fresh water resources.

Rootstocks are widely used by horticulturists in modern agriculture. Their selection is usually based on the capacity to promote uniform, targeted vegetative growth in the scion, and adaptation to a given soil type, such as high temperature (Rivero et al. 2003). Commercial rootstocks are capable of reducing the effect of water stress on the shoot is a promising tool for enhancing drought resistance, reducing water losses during production, and improving water-use efficiency (WUE) under drought conditions (Schwarz et al. 2010). However, the potential mechanism of improved resistance or adaptation to drought resulting from grafting remains elusive.

Plants have developed a series of physiological and biochemical mechanisms to cope with drought stress. For instance, they accumulate osmolytes under drought stress, tap ground water using deep roots and close stomata to reduce water loss (Gao et al. 2009). Drought induces oxidative damage, which leads to the formation of reactive

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oxygen species (ROS) (Farooq et al. 2009a; Anjum et al. 2012). ROS include $^1O_2$, $O_2^{\bullet-}$, $H_2O^{\bullet}$, H_2O_2, $OH^{\bullet}$, $RO^{\bullet}$ organic hydroperoxide (ROOH), and excited carbonyl ($RO^{\bullet}$), etc. (Koyro et al. 2012). These species cause damage to biomolecules such as proteins, chlorophylls, membrane lipids and nucleic acids (Blokhina et al. 2003; Sánchez-Rodríguez et al. 2012a). Fortunately, plants posses complex antioxidant systems to detoxify ROS, i.e., enzymatic and non-enzymatic systems. The enzymatic system comprises superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate-glutathione (AsA-GSH) cycle enzymes ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR). The non-enzymatic system is composed of antioxidants such as ascorbic acid (AsA), vitamin E (α-tocopherol), and glutathione (GSH), etc. (Reddy et al. 2004; Genisel et al. 2012). Among the enzymatic components, SOD converts $O_2^{\bullet}$ directly into hydrogen peroxide (H_2O_2) (Koyro et al. 2012). The H_2O_2 is then further scavenged by CAT and POD into H_2O and O_2 (Farooq et al. 2009b). Additionally, CAT and APX detoxify H_2O_2 through the AsA-GSH cycle (Apel and Hirt 2004). APX converts H_2O_2 to H_2O by oxidizing ascorbate into monodehydroascorbate (MDAR), which can also be reduced to ascorbate by NAD(P)H-dependent MDHAR (Pan et al. 2006). GR is an important enzyme of the AsA-glutathione system and maintains the balance between reduced GSH and the AsA pool (Ahmad et al. 2010). GSH, which is oxidized in this process to glutathione disulfide, is recycled by GR, consuming NADPH (Ali et al. 2005). Malondialdehyde (MDA) is often considered as a reflection of membrane degradation or dysfunction (Ali et al. 2005). It has been reported that water deficit increased the quantity of MDA in wheat plants (Esfandiari et al. 2007; Sánchez-Rodríguez et al. 2012a). In this sense, low concentrations of MDA have been associated with water-stress tolerance in pea plants and wheat (Sairam et al. 2000; Sánchez-Rodríguez et al. 2012a). The maintenance of low levels of MDA has also been associated with better resistance to drought (Lima et al. 2002).

In recent years, increasing attention has been placed on understanding the antioxidative defense system in plants subjected to drought (Kaur et al. 2013). For instance, a correlation between the antioxidant capacity and drought tolerance has been found in different plant species (Dinler and Aksoy 2013). Investigations involving grass species have detected increases in the activity of CAT, GR and other peroxidases, particularly in tolerant species and varieties under water deprivation conditions (Duan et al. 2009). An enhanced ROS scavenging capacity could be a response to higher ROS production of sensitive compared with tolerant winter wheat genotypes (Simova-Stoilova et al. 2006). It has been confirmed that the activities of MDHAR, GR, and DHAR show a significant increase in rice plants subjected to a water deficit (Sánchez-Rodríguez et al. 2010b).

Tomato (*Solanum lycopersicum*) is one of the most important vegetable crops, which is also one of the most water demanding plants (Peet et al. 2005). Graft is one of the wildly used methods to reduce water loss, and also the effective way to improve WUE in vegetables. It is important to select tomato rootstocks that are resistant and productive under stress conditions, and to identify potential mechanisms of drought resistance. Based on our previous research, most of the rootstocks used in the present study are resistant against root-knot nematodes (*Meloidogyn incognita*). In tomatoes, resistance to RKN is controlled by the *Mi-1* gene (Cortada et al. 2009). *Mi-1* is a very effective nematode resistance gene and has been widely used in many commercial tomato varieties. Initially, the *Mi-1* resistance gene was introduced into cultivated tomato from *Solanum peruvianum* and this gene confers resistance to three RNK species (*M. arenaria, M. incognita* and *M. javanica*) (Smith 1944). At present, different molecular markers have been used for identification of *Mi-1* in resistance studies. Hence, the objectives of this study were to identify RKN- and drought-dual resistant rootstocks, and preliminarily analyze the underlining mechanism of drought resistance.

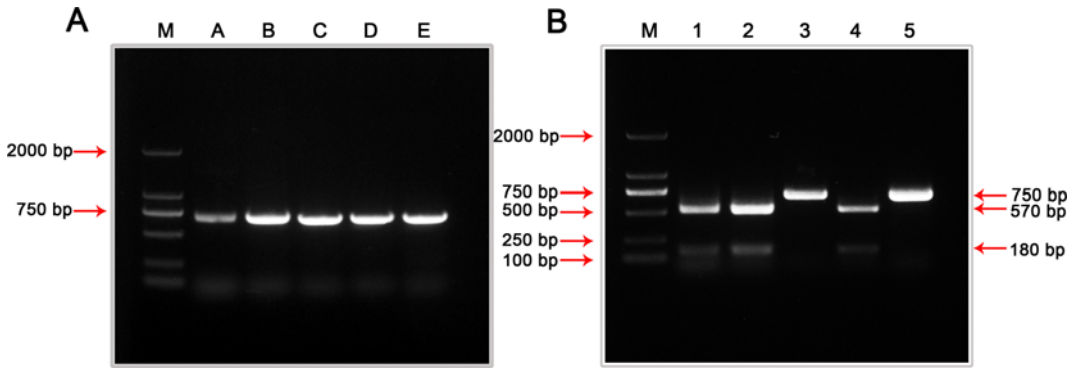

Fig. 1. Identification of the *Mi-1* gene. Products of PCR amplification (A) and PCR products after digestion with TaqI (B). M, Maker; A, 1, Beinongqiezhen; B, 2, Resistance; C, 3, Jingfan 406; D, 4, LA0655; E, 5, B1309.

Results

Identification of the *Mi-1* Gene

PCR using the REX-1 marker produced the only band of nearly 750 bp for all rootstocks (Fig. 1A). The PCR products were digested with the TaqI restriction enzymes, yielding nearly 570- and 180-bp fragments in Beinongqiezhen, Resistance and LA0655 (Fig. 1B). The results show that Beinongqiezhen (Wang et al. 2008), Resistance and LA0655 (Hu et al. 2015) are resistant against root-knot nematodes.

Phenotypes and Tolerance to Drought of the Five Rootstock Seedlings During the Drought Treatment

To test their drought tolerance, the five rootstocks were subjected to drought stress until wilting. After 9 d without watering, B1309 leaves showed chlorosis and wilted, while LA0655 and Jingfan 406 leaves wilted at 11 d and 12 d, respectively. A notable drought resistance was observed in Beinongqiezhen and Resistance, the leaves of which showed chlorosis at 14 d (Fig. 2).

Changes in the LRWC, EL, Water Loss of the Leaf and Wilting Coefficient in Candidate Rootstocks Under Drought Stress

The plant water status was estimated by measuring the LRWC. Under control conditions, all rootstock plants maintained a LRWC of ~85%. After the drought stress treatment, all species showed a reduction in LRWC. Nevertheless, from 2 DAWNL (day after water natural loss), the LRWC curves of these species started to diverge (Fig. 3A). During the first 6 DAWNL, Beinongqiezhen and Resistance exhibited a slower decrease in LRWC (Fig. 3A). At 8 DAWNL, the LRWC of Beinongqiezhen was the highest and maintained at 75.75%. When plant wilting occurred, Beinongqiezhen and Resistance exhibited a relatively higher LRWC compared with the other rootstocks (Fig. 3A). These results show the diverse water holding capacity of leaves of different rootstocks under drought stress.

EL reflects membrane damage induced by osmotic shock. All plants showed a gradual increase in EL after water natural loss, but the increase was lower in Beinongqiezhen and Resistance at 4 DAWNL (Fig. 3B). In comparison, from 2 DAWNL, the leaf EL was significantly increased in B1309 (Fig. 3B). At 8 DAWNL, B1309 EL was 21.54% versus only 16.24% and 15.70% for Beinongqiezhen and Resistance, respectively (Fig. 3B). This suggests that the degree of cell damage of Beinongqiezhen and Resistance was lower than the other rootstocks under drought stress.

The relative water loss of the detached leaves of Beinongqiezhen, Resistance, Jingfan 406, LA0655 was lower than that of the B1309 leaves throughout the drought stress experiment (Fig. 3C). After 8 DAWNL, the water loss of Beinongqiezhen and Resistance leaves was significantly affected compared with the other rootstocks. This indicated that Beinongqiezhen and Resistance maintained a low leaf water loss throughout the drought stress treatment, which ensured the normal growth of these plants.

The wilting coefficient reflects the sensitivity of plants to soil water deficit. Compared with B1309, Beinongqiezhen, Resistance, Jingfan 406 and LA0655 had lower wilting coefficients (Fig. 3D). Taken together, these results indicate

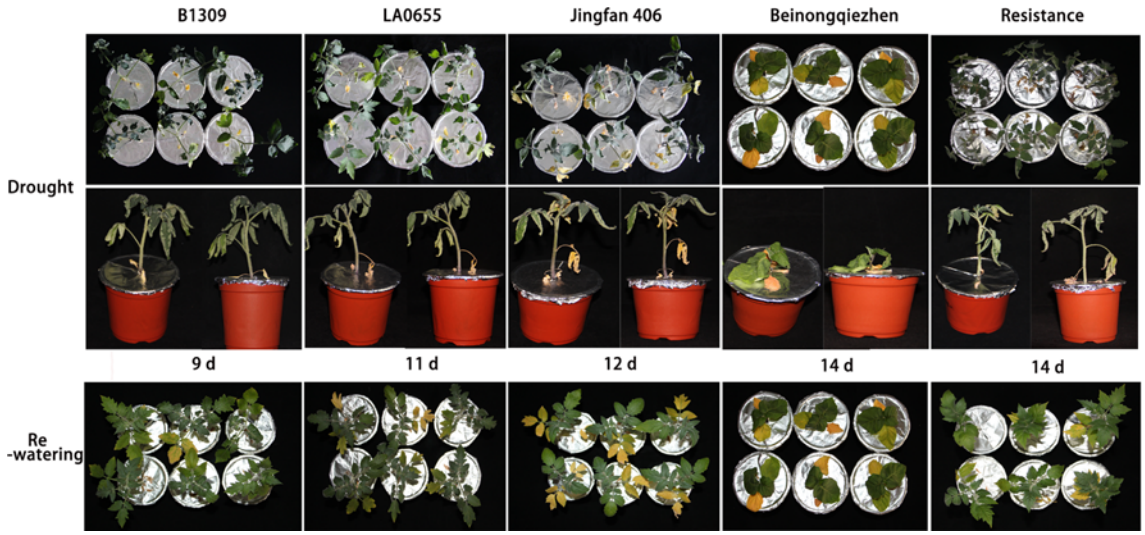

Fig. 2. Five rootstock plants after 9 d, 11 d, 12 d, 14 d and 14 d of water natural loss (wilting point), followed by a recovery period of 2 d upon re-watering. The five species are ordered from left to right according to their ability to survive under water stress.

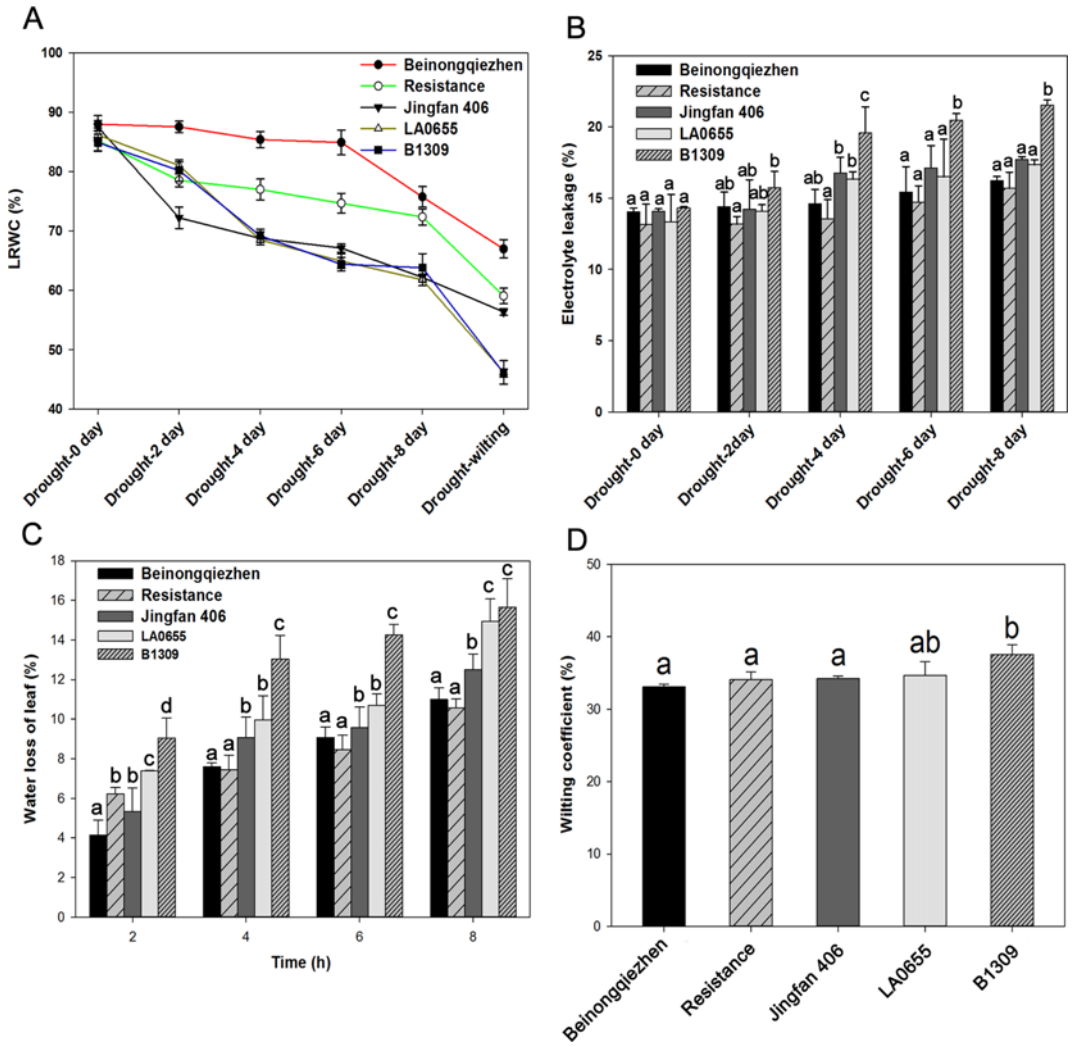

Fig. 3. Changes in LRWC (A), EL (B), water loss of the leaf (C) and wilting coefficient (D) of leaves of five rootstocks after long-term water natural loss. Measurements were made at midday on days 0, 2, 4, 6, 8 and at the time of the wilting point (in the LRWC test) during the drought treatment. Water loss is expressed as the percentage of initial fresh weight. The wilting coefficient was determined based on 3 replicates at a pot soil depth of 5 cm. Each value represents the mean of three independent measurements ± SE. Means followed by different letters indicate significant differences ($p < 0.05$).

that Beinongqiezhen and Resistance were the most drought-tolerant rootstocks among the candidates.

Analysis of Proline Content Under Drought Stress

Soluble proline is considered as an osmoprotectant and is probably associated with osmotic regulation and membrane stability under stress. Proline accumulation is a common physiological response in higher plants exposed to drought stress. During the water natural loss, a remarkable increase was observed in Resistance at the wilting point, which was approximately 20.72-fold higher than that measured under normal conditions (Fig. 4). Similarly, in Beinongqiezhen, a significant increase was observed at both the wilting point and 2 days after re-watering (Fig. 4).

Alterations in Antioxidant Enzymes under Drought Stress

Drought stress activated the antioxidant system in all rootstocks. The activities of POD, APX, GR and DHAR were gradually enhanced with as water natural loss time prolonged. The POD activity of all rootstocks peaked at 6 d after exposure to drought stress, and then declined. This increase was higher in Beinongqiezhen than in the other rootstocks. The POD activity increased significantly (by 227%) in Beinongqiezhen at 6 DAWNL compared with the control. In addition, Beinongqiezhen showed higher POD activity after re-watering, though lower activity was measured at the wilting point (Fig. 5A).

We also analyzed the enzymes in the Halliwell-Asada cycle (APX, DHAR and GR). Generally, the activities of these three enzymes were elevated during drought stress.

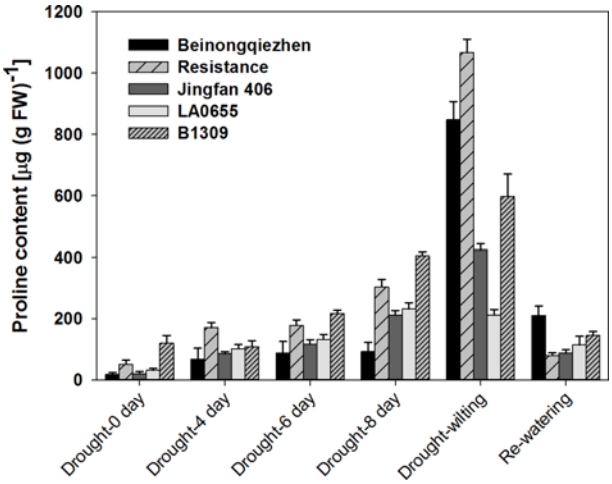

Fig. 4. Variation of free proline content in leaves under long-term water natural loss. Measurements were made on days 0, 4, 6, 8, at the time of wilting and 2 days after re-watering. Each value represents the mean of three independent measurements ± SE. Means followed by different letters indicate significant differences ($p < 0.05$).

Among the candidates, the enzyme activities of Resistance, Jingfan 406 and B1309 increased the most at the wilting point (Fig. 5). Intriguingly, we did not observe an expected significant elevation of enzyme activities in Beinongqiezhen, especially GR and DHAR (Fig. 5C, D).

Transcription Levels of Drought Stress Marker Genes in Rootstocks

The expression levels of stress-responsive genes were examined as another possible explanation for the drought tolerance of plants. The transcript levels of *LEA*, *RD29B*, *RD22*, *AREB* and *DREB1* were measured in rootstock plants (Fig. 6). Quantitative real-time PCR results showed that expression levels of all of these genes were induced by drought stress in all plants. Notably, in Resistance drought stress led to a greater increase of the transcript levels of *LEA*, *RD29B* and *AREB* where the transcript levels were increased by 3.7-, 5.6- and 4.8-fold higher than that measured under normal conditions, respectively (6A, B, D). The stress genes *AREB* and *DREB1* were also significantly upregulated in Beinongqiezhen seedlings and the mRNA levels of both were 4.2- and 10.4-fold higher than that measured under normal conditions, respectively (Fig. 6D, E).

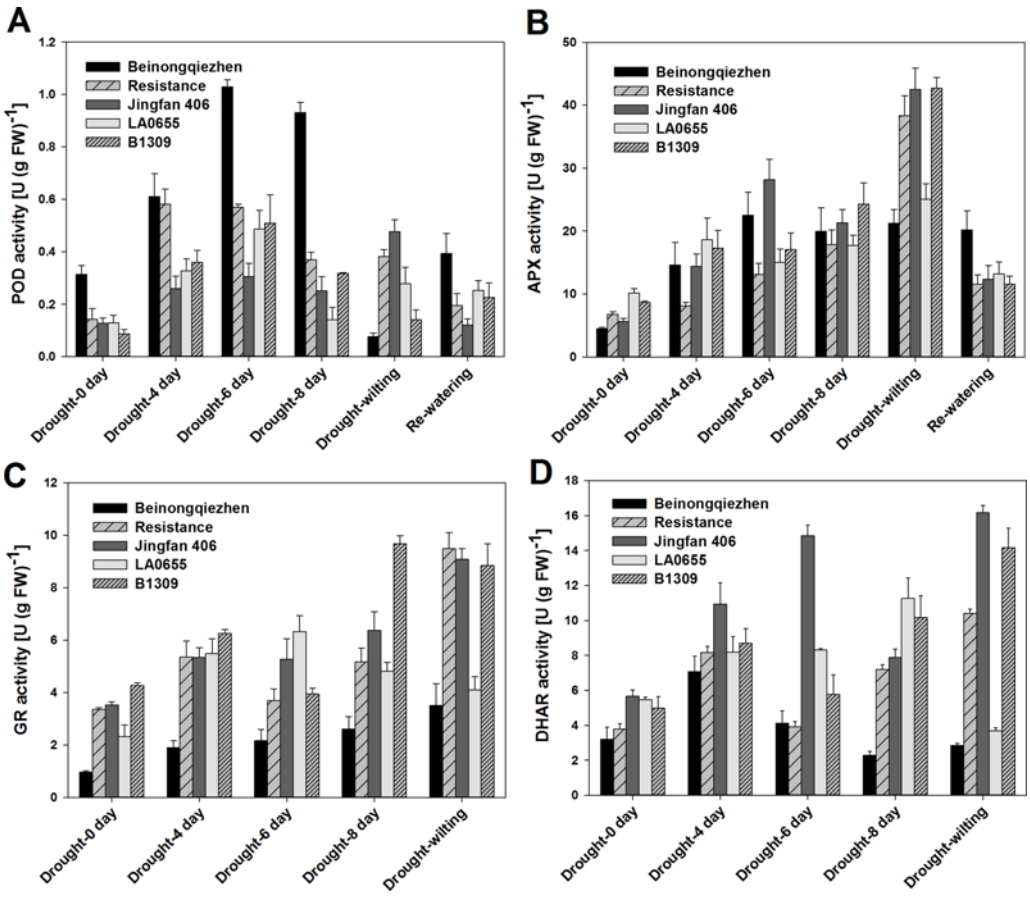

Fig. 5. Influence of long-term water natural loss on POD activity (A), APX activity (B), GR activity (C) and DHAR activity (D) in the leaves of rootstock plants. Measurements were made at midday on days 0, 4, 6, 8, at wilting and after re-watering during treatment. Each value represents the mean of three independent measurements ± SE.

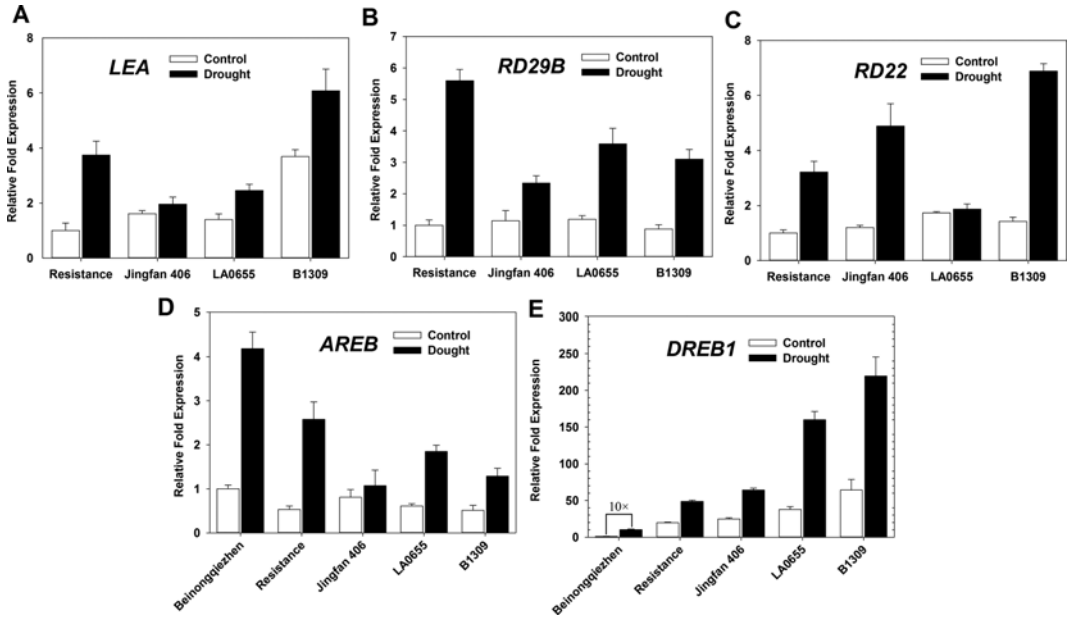

Fig. 6. The five rootstocks showed differences expression of several drought stress marker genes after DAWNL stress: *LEA* (A), *RD29B* (B), *RD22* (C), *AREB* (D) and *DREB1* (D). Transcription levels of these genes were determined by quantitative real-time PCR. Total RNA was isolated from Beinongqiezhen, Resistance, Jingfan406, LA0655 and B1309 seedlings after 2 DAWNL of water restriction. Untreated plants were used as controls. In all experiments, the expression of the constitutive *Actin* gene was used as the control. Values are means ± SE (n=3).

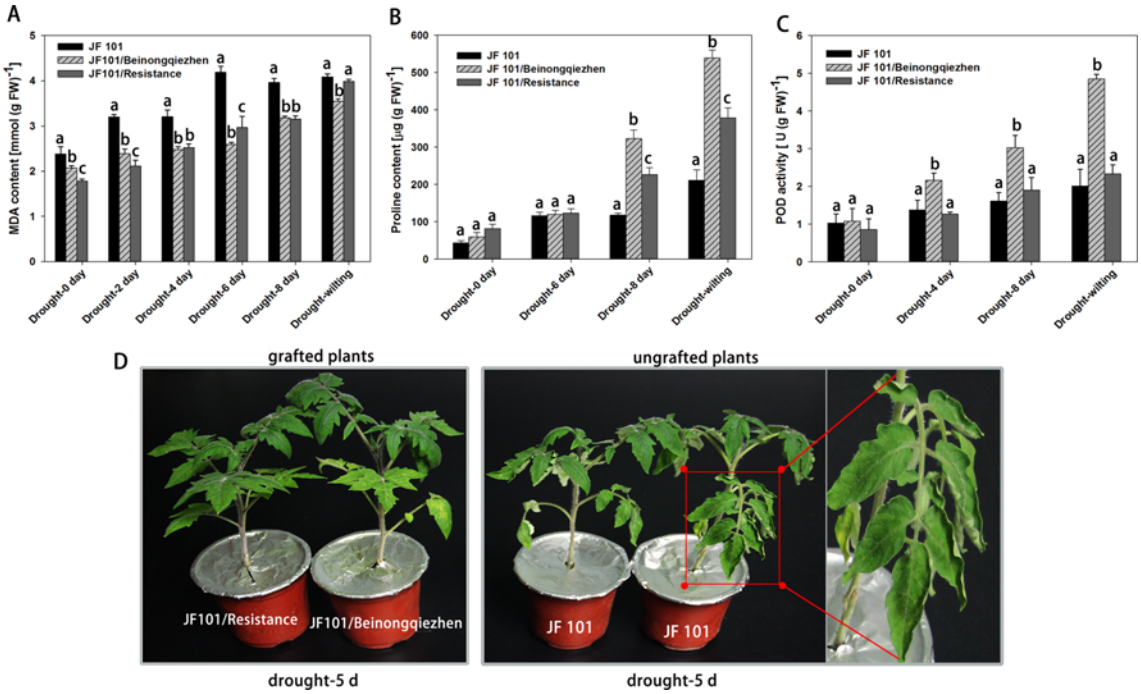

Fig. 7. MDA (A), proline content (B) and POD activity (C) in the leaf extracts of ungrafted and grafted plants under drought stress. Measurements were made at on days 0, 2, 4, 6, 8 and at wilting during treatment. Grafted (JF 101/Beinongqiezhen and JF101/Resistance) and ungrafted (JF101) plants after 5 DAWNL of water restriction (D). Each value represents the mean of three independent measurements ± SE. Means followed by different letters indicate significant differences ($p < 0.05$).

Phenotypes of Ungrafted and Grafted Seedlings During Drought Treatment

During drought stress, ROS can accumulate and cause cellular damage such as lipid peroxidation, which can be calculated by measuring the malondialdehyde (MDA) content. The maintenance of low MDA levels has been associated with increased drought stress resistance in plants (Dacosta

and Huang 2007). The MDA concentration was measured in the leaves as an indicator of lipid peroxidation in the plants. The MDA concentration was significantly increased in JF 101 at 6 DAWNL, which was approximately 1.76-fold higher than that measured on the initial day. However, the graft combinations of JF 101/Beinongqiezhen and JF 101/Resistance showed an obviously lower increase during stress, though JF 101/Resistance reached the same level as ungrafted plants at the wilting point (Fig. 7A).

During water stress, plants accumulate solutes to prevent water loss and reestablish cell turgor. These solutes include ions such as K^+, Na^+ or organic solutes such as proline, amino acids, soluble sugars and polyamines (Takashi et al. 2003). Proline is known to occur widely in higher plants and can accumulate in considerable amounts in response to water deficit and other abiotic stresses. It has been shown that stress-tolerant plants are able to accumulate proline in higher concentrations than stress-sensitive plants (Slama et al. 2008). In our study, no significant differences were observed between grafted and ungrafted plants at 6 DAWNL under water stress. Considerable differences occurred after 8 DAWNL, especially at the wilting point (Fig. 7B).

The drought treatment increased the POD activities in all plants, but the extent differed between ungrafted and grafted plants. A significant increase was observed in the JF 101/Beinongqiezhen combination under water stress at 4 d, 8 d and the wilting point, while JF 101 and JF 101/Resistance showed a weak increase throughout the drought stress experiment (Fig. 7C).

Discussion

In the present study, we screened two RKN- and drought-dual resistant rootstocks, namely Beinongqiezhen and Resistance. The significant drought-tolerant phenotypes can be observed in Fig. 2, 3 and 7. The LRWC is considered as a reliable indicator that reflects the water content relative to the maximum water content (Rosales-Serna et al. 2004; Khanna et al. 2014). A higher water-retention ability during dehydration is an important strategy for acquiring resistance and serves as a primary indicator that a drought response has been invoked (Selote and Khanna-Chopra 2006). In our study, the highest LRWC was observed in Beinongqiezhen and Resistance throughout the whole stress treatment (Fig. 3A). This result is similar to previous research conducted on wheat (Rampino et al. 2006). In addition, Beinongqiezhen and Resistance showed less water loss (Fig. 3C). Therefore, we propose that the high drought tolerance of Beinongqiezhen and Resistance is associated with a greater capacity for water retention. Electrolyte leakage (EL), an index of membrane damage, is markedly enhanced under stressful conditions (Silva et al. 2012). In our study, EL in Beinongqiezhen and Resistance showed a slower increase than the other rootstocks during the water deficit (Fig. 3B). Taken together, these results indicate at least two methods that were adopted to improve the drought tolerance ability of Beinongqiezhen and Resistance, i.e., elevated water conservation and decreased ROS-derived membrane damage.

Plants subjected to water stress tend to overproduce ROS in different plant tissues (Carla et al. 2004). The ROS generated by oxidative stress, particularly $O_2^{\bullet}$ and H_2O_2, can be toxic to cells or can be involved in a cellular signaling process that induces a number of genes and proteins involved in stress defense. Plants have evolved an entire set of scavenger systems to maintain ROS homeostasis (Selote and Khanna-Chopra 2006), which prevents ROS overproduction to resist drought stress. In the present study, the POD, GR, APX and DHAR activities changed significantly in response to drought stress (Fig. 5). The POD activities were highest in Beinongqiezhen under severe drought stress (before 8 DAWNL) and after re-watering (Fig. 5A), which may result in a lower accumulation of $O_2^{\bullet}$. However, we did not observe higher activities of GR, APX and DHAR in Beinongqiezhen (Fig. 5B, C, D), though the ascorbate/glutathione cycle constitutes an important detoxification pathway for the dissipation of H_2O_2 and other reactive oxygen radicals in chloroplasts (Sgherri et al. 2003; Sánchez-Rodríguez et al. 2012a). A fascinating phenomenon was observed: compared with Beinongqiezhen, the other varieties exhibited lower POD activities and higher GR, APX and DHAR activities. It has been reported that the tolerance of plants to progressive drought stress is associated with an increase in the activity of APX, GR and DHAR (Faize et al. 2011; Ma et al. 2011; Dinler et al. 2013; Sun et al. 2013). Therefore, we inferred that the non-enzymatic antioxidants play dominant roles in alleviating ROS accumulation during drought stress in the varieties with low POD activities. However, the functional absence of SOD, POD and CAT will lead to disastrous consequences under drought stress. In addition, we examined the expression levels of stress-responsive genes in rootstocks (Fig. 6); the results suggested that the drought tolerance ability of Beinongqiezhen and Resistance were achieved by increasing the expression levels of drought stress-induced genes.

For the grafted plants, we observed a higher proline concentration, elevated POD activity and a lower MDA content during the water stress treatment compared with JF101 (Fig. 7A, B, C). MDA is often considered as a reflection of membrane degradation or dysfunction (Ali et al. 2005). The maintenance of low levels of MDA has also been associated with better resistance to drought (Lima et al. 2002). Our results showed a significant increase in the MDA content in ungrafted JF 101, whereas in the grafting combinations, the

increase was slower under moderate drought (2-6 d) (Fig. 7A). This suggests that graft could reduce oxidative damage by decreasing the MDA content. Plants accumulate osmolytes to avoid water loss during stress (Alonso et al. 2001). Free proline is one of the most widespread accumulated osmoprotectants. This likely suggests that grafted plants accumulate proline to prevent water loss and to reestablish cell turgor. In addition, as the first line of ROS defense, enhanced POD activity dramatically improved the ROS scavenging ability, which prevented cell membrane peroxidation (measured as the MDA content). We propose that important means through which grafted rootstocks might enhance resistance to drought is through a reduction in ROS accumulation and an improvement in the capacity of their ROS-scavenging system, which is consistent with a previous study (Liu et al. 2012). Zhang et al. (2010) have reported similar observations where cucumbers grafted onto copper-tolerant rootstocks induced lower production of ROS than from those on sensitive rootstocks. In our study, we observed that the grafted plants had a positive effect on scion growth under water stress, which has been reported with apple (Cohen and Naor 2002), who has proposed that rootstocks affect scion vegetative growth mainly by influencing the status of tree water, mineral nutrition and hormones. These rootstocks can absorb water and nutrients much more efficiently as compared to nongrafted plants (Lee et al. 2010). Because the scions were the same, those differences in parameters were mainly due to a root-derived gradient and those selected for being used as rootstocks are usually much larger and more vigorous (Lee et al. 2010).

In conclusion, two drought-tolerant varieties, Beinongqiezhen and Resistance, were identified. More importantly, both rootstocks are RKN resistant. We also preliminarily analyzed the mechanism of improved drought resistance conferred by grafting. Our investigation will provide more support for tomato graft cultivation and lay the foundation for the physiology and mechanism of grafting.

Materials and Methods

Plant Material and Drought Stress Treatment

The tomato cultivar JF 101 was used as the scion and five tomato rootstocks were selected: Resistance, Beinongqiezhen (Wang et al. 2008), Jingfan 406, LA0655 (Hu et al. 2015), and B1309. The seeds of these species were germinated at 28°C and grown for 30 days in a tray filled with a 2:1 peat: vermiculite mixture in a cultivation chamber maintained under 17 h of light at 28°C and 7 h of dark at 18°C. The plantlets were fertilized with a nutrient solution once a week. The seedlings were transferred into pots filled with a 2:1 peat: vermiculite mixture of 150 g/pot when the selected seedlings had four true leaves and were maintained under the same growing conditions. The substrate amount of water in each pot was 75% field capacity (FC) and the FC was 85%. Seedlings with a uniform size were selected and divided equally into groups. The control groups for each variety remained soil moisture of 75% FC; the drought-treated groups were remained the soil moisture of 75% FC, and then allow water natural loss each day without any water supply till the day of plants wilted. During the experiment, all pots were weighed daily and maintained the desired moisture level. The surface of the pot was covered with silver paper to prevent water evaporate, the water loss is just the plants transpiration. Grafting was performed when the scion had three true leaves and the rootstock had three or four real leaves. Then, the grafted plants were maintained under controlled conditions (90-95% RH) for seven days. After the first day, during which the grafted plants remained in darkness, the photoperiod was increased over 3 days until the required conditions were achieved. Then, the grafted plants were subjected to the drought stress treatment described above. Finally, the leaves were harvested, frozen and stored at -80°C until further analysis.

Determination of Electrolyte Leakage (EL) and Leaf Relative Water Content (LRWC)

To determine the EL and LRWC, leaf samples were harvested at 0, 2, 4, 6, 8 days and at the wilting point after drought stress was imposed. Once wilting occurred, the plants were re-watering and the leaves were harvested 2 d later. The LRWC measurement was conducted following Anjum (2012). Fully developed leaves were harvested and weighed immediately to determine the fresh weight (FW) and were then immersed in double distilled water for 16 h to determine the turgid weight (TW). The leaves were then oven dried until a constant weight to determine the dry weight (DW). The LRWC was calculated as: LRWC=FW-DW/TW-DW×100. EL was assessed as described by Silva (2012). Leaf discs were placed in closed tubes containing 10^{-5} m^3 of deionized water and incubated at 25°C in a water bath for 6 h, after which the electrical conductivity of the solution (L1) was determined. The samples were then boiled at 100°C for 30 min and a second electrical conductivity (L2) reading was obtained after equilibration at 25°C. The electrolyte leakage (EL) was defined as follows: EL (%) = (L1/L2)×100.

Measurement of Water Loss from Detached Leaves and Wilting Coefficient

To measure the water loss from the detached rootstock leaves, mature leaves were detached and weighed immediately. Then, the leaves were placed in an incubator under light at 25°C. Water loss was presented as the percentage of initial fresh weight at each time point, i.e., the relative fresh weight at each time was calculated as a percentage of the initial fresh weight to indicate the rate of water loss from the leaves. The wilting coefficient is the soil relative water content at the time of plant wilting.

Determination of Proline and MDA Content

Free proline was extracted and determined as described by Gao (2009) with some modifications. A sample of leaves was homogenized in a mortar with 5 mL of a 3% (w/v) aqueous sulfosalicylic acid solution. The homogenate was centrifuged at 15,000 g for 10 min. The supernatant was treated with acid ninhydrin (2.5 g ninhydrin/100 mL of a solution containing glacial acetic acid, and 6 mol/L o-phosphoric acid at a ratio of 3:2), boiled for 40 min and cooled to room temperature. The tubes were cooled and 5 mL of benzene was added. The proline level in the sample was calculated based on absorbance at 520 nm and was expressed as ìg per g FW of sample. The free proline content was calculated based on a standard curve.

The MDA content was assayed in leaf extracts following Rao (1996) with some modifications. Leaf samples were homogenized in 5 mL of 20% (w/v) trichloroacetic acid (TCA) solution. The homogenate

was centrifuged at 10,000 g for 20 min and 3 mL of the supernatant was added to 3 ml of 0.5% (v/v) TBA in 20% TCA. The mixture was incubated in boiling water for 30 min and then quickly cooled in an ice bath. Then, the samples were centrifuged at 10,000 g for 5 min, and the absorbance of the resulting supernatant was determined at 450, 532 and 600 nm. The MDA content was expressed as nmmol MDA/g FW.

Antioxidant Enzymatic Activities in Leaf Extracts

Peroxidase (POD) activity was determined as described by Upadhyaya (1985) with some modifications. Leaf material was homogenized in liquid (50 mM PBS buffer, pH 7.8) and centrifuged at 4°C for 15 min. The reaction mixture contained 0.3% H_2O_2, 50 mM PBS buffer (pH 7.0), 0.2% guaiacol, and 100 mL of enzyme extract. An absorbance change of 0.1 at 420 nm was considered as POD activity.

The enzymes APX and glutathione reductase (GR) were assayed following Rao (1996). APX activity was determined by measuring the absorbance change of a reaction mixture containing 100 mM phosphate potassium buffer (pH 7.5), 0.5 mM AsA, 0.2 mM H_2O_2 and 0.75 mL enzyme extract at 290 nm for 3 min. GR activity was measured after monitoring the oxidation of NADPH at 340 nm for 3 min in a reaction mixture containing 100 mM Tris–HCl (pH 7.8), 2 mM Na_2-EDTA, 0.2 mM NADPH, 0.5 mM GSSG and 0.75 ml enzyme extract. Dehydroascorbate reductase activity (DHAR) was measured at 265 nm for 3 min following the change in absorbance resulting from the formation of AsA (Nakano and Asada 1980). The reaction mixture contained 25 mM phosphate sodium buffer (pH 7.0), 2.5 mM GSH, 0.4 mM DHA, and 0.1 ml enzyme extract.

Mi-1 Gene Identification

The *Mi-1* identification method followed Williamson (1994), and REX-1 was used for screening *Mi-1*. The following primer sequences were used for amplification: REX-1F (5'-TCGGAGCCTTGGTCT-GAATT-3') and REX-1R (5'-GCCAGAGATGATTCGTGAGA-3'). PCR amplification was performed in a total volume of 20 μL containing 10×PCR Buffer, 2.5 mM dNTP mixture, 0.2 mM of each primer, 1 Unit of rTaq DNA polymerase and template DNA. The PCR conditions were 5 min at 94°C; 30 cycles of 94°C for 30 sec, 55°C for 1 min, 72°C for 1 min, followed by 10 min at 72°C. The PCR products were loaded onto a 1.5% agarose gel to ascertain whether PCR amplification was successful. PCR products obtained from REX-1 were digested with TaqI following the manufacturer's instructions (Wang et al. 2009).

RNA Isolation and Real-time PCR

Total RNA was isolated from the leaf tissues of five rootstocks using Trizol reagent (Invitrogen) following the manufacturer's instructions. All RNA samples were digested with RNase-free DNase to remove genomic DNA. The quality and concentration of each of the RNA samples was determined using Namedrop 2000 spectrophotometer (Thermo Scientific). The integrity of RNA was also checked by agarose gel electrophoresis. For real-time PCR, total RNA was reverse-transcribed into cDNA using the Super-Script first-strand synthesis system (TransGen Biotech) according to the manufacturer's instructions. The cDNA was used as a template to perform real-time PCR with gene-specific primers (Supplementary Table S1) and SYBR Green Mix (TaKaRa) in a CFX96 TouchTM Real-Time PCR detection system (Bio-Rad). *Actin1* (cyclophilin) gene (Gantasala et al. 2010) was used as an internal normalization control in Beinongqiezhen. *Actin2* was also chosen as a relative quantitative reference in other rootstocks. Fold change in gene expression was calculated using ΔCt values.

Data Analysis

All experimental data were analyzed as the mean ± standard error of at least three replicates. Statistical analysis was conducted using SPSS-13.0 software. Graphical presentations of the data were prepared using Sigma Plot 12.5.

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Author's Contributions

XY performed the experiments and wrote the manuscript. WZ and SW designed the experiments and contributed to the modification of the manuscript. CL provided part of the materials. RY participated in experiment operation. FZ provided tools.

Supporting Information

Table S1. Primers used for real-time PCR expression analysis

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