Journal of Plant Physiology 226 (2018) 31–39

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Tomato plants ectopically expressing *Arabidopsis GRF9* show enhanced resistance to phosphate deficiency and improved fruit production in the field

Lili Zhang[a,b], Guangjie Li[b], Yilin Li[b], Ju Min[b], Herbert J. Kronzucker[c], Weiming Shi[b,*]

[a] University of Chinese Academy of Sciences, Beijing 100049, China
[b] State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, China
[c] School of BioSciences, The University of Melbourne, Parkville, Victoria 3010, Australia

ARTICLE INFO

Keywords:
Solanum lycopersicum
GRF9
Phosphate transporter
Proton secretion
Phosphorus acquisition
Agricultural production

ABSTRACT

Agronomic performance of transgenic tomato overexpressing functional genes has rarely been investigated in the field. In an attempt to improve low-phosphate (P) stress tolerance of tomato (*Solanum lycopersicum*) plants and promote tomato fruit production in the field, an expression vector containing cDNA to an *Arabidopsis* 14-3-3 protein, General Regulatory Factor 9 (*GRF9*), driven by a cauliflower mosaic virus 35S promoter, was transferred into tomato plants. Transgenic expression of *GRF9* was ascertained by quantitative real-time PCR analysis. The degree of low-P tolerance in transgenic plants was found to be significantly greater than that in wild-type plants, and reflected in improved root development and enhanced P content under hydroponic conditions. For transgenic tomato, roots had higher P uptake, as evidenced by tissue P content and relative expression of the genes *LePT1* and *LePT2* in both normal and low-P hydroponic solutions. GRF9 overexpressors had greatly enhanced proton extrusion from roots and heightened activity of the plasma-membrane H^+-ATPase (PM H^+-ATPase) in roots under low-P hydroponic conditions. Thus, in addition to enhanced root development, higher expression of genes coding for phosphate transporters and improved capacity for acidification in the rhizosphere emerged as key mechanisms underpinning improved P acquisition in transgenic tomato plants in soil. Subsequent field trials measuring tomato fruit production at two P levels, indicated that GRF9 can indeed improve total tomato production and may play a role in early fruit maturity. Our results suggest that the heterologous *Arabidopsis GRF9* gene can confer resistance to P deficiency in transgenic tomato plants and promote fruit production.

1. Introduction

Phosphorus is an essential macronutrient for plant growth and development, and serves various basic biological functions in the plant life cycle (Raghothama, 1999; Cordell et al., 2009). However, the availability of inorganic P in soils is very low, as it is easily bound by cations such as Fe^{3+} and Al^{3+}, or converted to organic matter via soil-microbial activity, thereby becoming immobile and difficult to utilize for plants (Raghothama, 1999; Tiessen, 2008). The P concentration in the soil, typically 10 μM or less, results in P deficiency for plant growth and compromises crop productivity on ~30–40% of arable lands worldwide (Runge-Metzger, 1995). Plants have evolved a series of adaptive strategies to overcome limited P availability in soils (Vance et al., 2003; Hoffland et al., 2006). In response to P deficiency, plants increase P uptake by altering root architecture (Ticconi et al., 2004; Osmont et al., 2007; Guo et al., 2011), by altering the expression of P-related genes (Bustos et al., 2010; Muneer and Jeong, 2015) and by changing their metabolic and developmental processes (Raghothama and Karthikeyan, 2005; Liang et al., 2013).

14-3-3 proteins, a large family of phosphoserine-binding proteins found in virtually every eukaryotic organism and tissue, play important roles in regulating plant development and stress responses in higher plants (Moore and Perez, 1967; Roberts et al., 2002; Comparot et al., 2003; Mayfield et al., 2007). Some recent studies suggest that plant 14-3-3 proteins might play a role in response to low P-deficiency by interacting with some phosphorus-deficiency response factors, such as protein kinases and phosphatases (Cao et al., 2007; Baldwin et al., 2008). In addition, 14-3-3 proteins are thought to be involved in directly regulating the plasma-membrane (PM) H^+-ATPase, which affects root growth and may enhance P absorption in phosphorus-deficient soils (Palmgren, 2001; Shen et al., 2006; He et al., 2015). In *Arabidopsis* and tomato, thirteen and twelve 14-3-3 protein isoforms have been found, respectively (Rosenquist et al., 2000; Roberts, 2003; Xu and Shi, 2006). GRF9 is one of the 14-3-3 gene family members identified in *Arabidopsis* plants (Rooney and Ferl, 1995; Roberts, 2003; Mayfield et al., 2007; Xu and Shi, 2007). *Arabidopsis GRF9* is involved in plant

* Corresponding author.
E-mail address: wmshi@issas.ac.cn (W. Shi).

https://doi.org/10.1016/j.jplph.2018.04.005
Received 15 March 2018; Received in revised form 15 April 2018; Accepted 16 April 2018
Available online 17 April 2018
0176-1617/

Table 1
Gene-specific primers used in this study.

Gene	Accession number	Primer sequence (5′ to 3′)	Size (bp)	References
Ath-GRF9	AT42590	F: TGGGTTCTGGAAAAGAGCGTGACACT R: CGAGAAGATCCTCCACGAAGCTCTCC	200	This study
LePT1	AF022873	F: GTATGCTGTTCACATTCTTGGTTCC R: TCTCTTTCTAATCCCAAATACCACA	208	Gao et al. (2010)
LePT2	AF022874	F: CATTGGACACTGGAGGCTAACC R: ATAAGAACCCATACGCTCCCA	199	Gao et al. (2010)
Le α-tubulin	TC115716	F: TGAACAACTCATAAGTGGCAAAG R: TCCAGCAGAAGTGACCCAAGAC	198	Gao et al. (2010)

root responses to water stress by participating in shoot carbon allocation, which leads to improved root growth under water stress (Comparot et al., 2003; Mayfield et al., 2012; He et al., 2015). Although *AtGRF9* has been identified to be involved in the response to P-deficiency stress, the clear function of this gene in response to P deficiency is still unknown (Cao et al., 2007).

Tomato (*Solanum lycopersicum* L.), aside from its enormous importance as a globally utilized fruit crop, is considered to be a model vegetable plant for the investigation of nutrition deficiencies and other abiotic stresses (Ivanov et al., 2012; Paolacci et al., 2014). To evaluate whether *GRF9* can perform conserved functions across species in vegetable crops, we generated transgenic tomato plants overexpressing *GRF9* derived from *Arabidopsis* (*AtGRF9*). Hydroponic experiments were carried out to elucidate the coordinated regulation of root architecture and proton exudation under differential P availability. Subsequent field experimentation was conducted to analyze tomato plant phenotypes and fruit productivity associated with P uptake. The results show that the constitutive expression of *AtGRF9* in tomato increases the degree of tolerance to P deficiency in controlled hydroponic systems and promotes fruit production in the field.

2. Materials and methods

2.1. Plant materials, growth conditions, and stress treatment

The tomato (*Solanum lycopersicum* L.) var. 'Zhongshu NO.4' was used to obtain transgenic lines. The full-length coding sequence of the *AtGRF9* gene was obtained by the polymerase chain reaction (PCR) using the primers 5′-CACCATGGGTTCTGGAAAAGAGCGTG-3′ and 5′-ATTTGATTTACCCCGAGTAAAGG-3′. The 474-bp PCR product was cloned into the pMD18-T simple vector (TaKaRa, Tokyo, Japan) and digested using *BamHI*/*SacI* (New English Biolabs, inc., America) double digestion. The DNA was cloned into pBI121 (Jefferson et al., 1987), digested by the same digestion enzymes, and designated D. *Agrobacterium* strain LBA4404, carrying the pBI121 derived binary vector D, was used in transformation experiments. The *Arabidopsis GRF9* open reading frame was driven by the cauliflower mosaic virus 35S (CaMV35S) promoter. Cotyledons were also transformed independently by co-cultivation with *Agrobacterium tumefaciens,* strain LBA4404, harboring the D vector (Gao et al., 2009). Independent T_0 transgenic plants were propagated and self-pollinated, and seeds were harvested separately. T_1 of the T_0 seeds were screened on Murashige and Skoog (MS) medium containing 50 mg l^{-1} kanamycin and identified by molecule. Finally, three transgenic lines in generation T_3 of the T_2 seeds were selected and used for following experiments. The three GRF9 overexpressing tomato lines were E2, E7, and E11, respectively.

The wild type (WT) and three lines of the *AtGRF9*-gene transgenic tomato were used as the plant materials and disinfected using 1% sodium hypochlorite (NaClO) followed by ten washes with distilled water. Seeds of tomato were germinated on moist gauze and placed in a 30 °C incubator for hydroponic experiments. After three days, germinated seeds were floated on 0.5 mM $CaCl_2$ solution until cotyledons were well developed. Then, tomato plants were transferred into black pots containing modified Hoagland solution (control: CK), which consisted of the following macronutrients: KNO_3, 1.0 mM; $Ca(NO_3)_2$, 1.0 mM; KH_2PO_4, 200 μM; $MgSO_4$, 0.4 mM; and the following micronutrients: H_3BO_3, 3.0 μM; MnCl2, 3.0 μM; $CuSO_4$, 0.5 μM; $ZnSO_4$, 1.0 μM; $NaMoO_4$, 0.1 μM. Solutions were supplied with 20 μM Fe-EDTA. For the low-P treatment, the phosphate concentration was 10 μM. The pH of solutions was adjusted to 6.0, and solutions were replaced daily. Fifteen-day-old tomato plants of uniform size were divided into two sets receiving hydroponic nutrient solution with normal 200 μM (CK) and low soluble-P (10 μM) (LP) for 6 d, respectively. The plants were grown in a plant growth chamber under fluorescent light at 200 μmol m^{-2} s^{-1} at canopy height for 16 h day^{-1} and at a temperature of 25 °C. Then, 21-day-old tomato plants were harvested for further analysis. Each independent experiment was arranged with three replicates, and each replicate contained 15 tomato plants. Furthermore, each replicate was harvested and analyzed separately.

2.2. RNA isolation, cDNA preparation, and qRT-PCR analysis

Plants were grown under CK and LP conditions for qRT-PCR. Plant tissues were collected and immediately frozen in liquid nitrogen for total RNA isolation. Total RNA was isolated from tissues using TRIzol (Invitrogen) according to the Invitrogen instructions (Zhu et al., 2016). cDNA was prepared from 1 μg of total RNA using the PrimeScript RT reagent kit (TaKaRa). For quantitative real-time PCR analysis of α-tubulin, *GRF9* transcript and phosphate transporter gene expression, 1 μL 10-fold-diluted cDNA was used for quantitative analysis, performed with SYBR Premix ExTaq (TaKaRa). The cDNA samples were used as templates to quantify target gene transcription levels using gene-specific forward and reverse primers (Table 1). Those pairs of gene-specific primers were described using Primer Premier 5 software.

Each cDNA sample was run in triplicate, because α-tubulin is a strongly and continuously expressed housekeeping gene in tomato (Wang et al., 2002). Expression data were normalized to the expression of α-TUBULIN (forward: 5′-TGAACAACTCATAAGTGGCAAAG -3′; reverse: 5′- TCCAGCAGAAGTGACCCAAGAC -3′).

Data were collected using Opticon Monitor Analysis Software version 2.02 (BioRad, America). Relative transcription levels were normalized to those of an internal control (α-tubulin) and presented as 2(-delt Ct) *102 or 2(-delt Ct) *104 or 2(-delt Ct) *106 to simplify the presentation of data (see Livak and Schmittgen, 2001; Schmittgen et al., 2004; Lin et al., 2008).

2.3. Measurement of root-architecture parameters

Root architecture parameters were measured using a root analysis instrument (WinRHIZO; Regent Instruments Inc., Quebec, ON, Canada), according to the method described by Xu and Shi (2007).

2.4. Measurement of total-plant P content

For the determination of P content, 1 g of oven-dried tissue (root and leaf) was digested in concentrated H_2SO_4 at 200–300 °C for 3 h. The

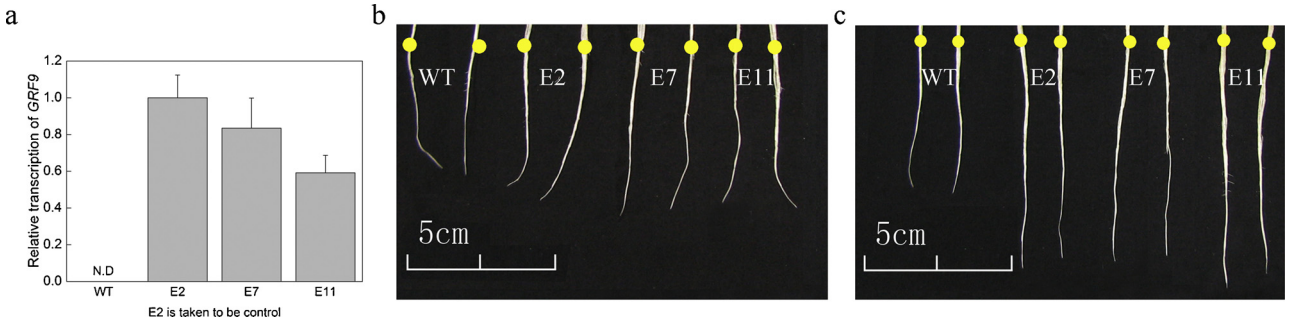

Fig. 1. Relative expression of *GRF9* and root phenotype in both wide type and three transgenic tomato plants (E2, E7, E11). Relative expression of *GRF9* in both wide type and three transgenic tomato plants (E2, E7, E11) (a) under normal P conditions, and the elongation of tap roots in both wild type and transgenic tomato plants (b and c) were measured on control and low-P media.

digested samples were filtered and diluted to a final volume of 50 mL by adding double-distilled water. The total P content in tomato plants (roots and shoots) was quantified using the Mo-Sb colorimetric method (Thomas et al., 1967).

2.5. Analysis of proton extrusion

Proton extrusion of tomato roots was analyzed following the method of Gao et al. (2010). After growth for 15 days, plants were treated with CK and LP for three days. Then, plants were transferred to 250-mL pots filled with solutions containing 200 μM or 10 μM phosphate. After 6 h, solutions were titrated with 0.01 mM NaOH to pH 6.0. The number of protons excreted by roots was calculated from the amount of NaOH used.

2.6. Enzyme measurements

5 g of fresh roots were cut and ground in ice-cold homogenization buffer with a mortar and pestle for the determination of root-PM H^+-ATPase activity, as described in Shen et al. (2006) and Zhang et al. (2011).

2.7. Field experiment

The field experiment was conducted on a crop production farm maintained by the Ministry of Agriculture (37°52′N, 102°50′E) in Wuwei, Gansu Province. This area belongs to the temperate continental arid climate, with an elevation of 1581 m. During the course of the field study, the average low temperature was 15 °C at night, daytime highs were 35 °C, and the annual average rainfall was 164 mm.

The physical and chemical characteristics of soil samples obtained from 0 to 30 cm depth were as follows: available N, P, and K was 23.55, 39.86, and 158.07 mg kg^{-1} soil, respectively.

The field experiment was carried out with and without P fertilizer. Each treatment was arranged with three replicates, on independent test areas. We set two ridges in each test area, and planted two rows of plants in each ridge with 20 tomato plants. The synthetic phosphate fertilizer (superphosphate) was applied at two rates: 0 (control: P0) and 75 (treatment: P75) kg P_2O_5 ha^{-1} $year^{-1}$, and potassium sulfate was applied at a rate of 120 kg K_2O ha^{-1} as basal fertilizer. Synthetic nitrogen fertilizer (urea) was applied at a rate of 180 kg N ha^{-1} as basal fertilizer, and 180 kg N ha^{-1} as topdressing of nitrogen fertilizer during the fruit-swelling period. Seedlings grown in a greenhouse for one month were transplanted into the experimental field.

2.8. Fruit yield and quality

Fruits were harvested at the breaker stage. The first collection of mature fruits was conducted at 65 DAT. Yield data were collected from twenty plants per repetition for each variant of the field experiment. The total, marketable, and unmarketable yields were calculated. Fruits exhibiting cracking, zippering, rotting, or blossom-end rot were excluded. Fruit production was expressed as t/ha. The following parameters were estimated: ascorbic acid and acidity content were analyzed according to Krelowska-kulas (1993) and Li et al. (2015); total soluble sugars were analyzed by the anthrone method (Yemm and Wills, 1954).

2.9. Statistical analyses

For all experiments, data were statistically analyzed using the SPSS 13.0 program (SPSS Chicago, IL, USA.). Details are as presented in figure legends. Graphs were produced using Origin 8.0. All graphs and images were prepared using Adobe Photoshop 7.0.

3. Results

3.1. Overexpression of heterologous Arabidopsis GRF9 in transgenic tomato plants

To evaluate the effect of overexpression of GRF9 in tomato plants, an *Arabidopsis GRF9* was transferred into the tomato genome using *Agrobacterium tumefaciens*-mediated transformation. After selection on kanamycin-containing medium, eleven independent kanamycin-resistant transformants (T_0) were identified by quantitative real-time PCR. From each T_0 plant, seeds (T_1) were harvested, T_2 plants were selected from kanamycin-resistant T_1 transformants, all T_2 generation plants used for experiment were analyzed by quantitative real-time PCR. AtGRF9 transcripts in the transgenic lines were detected by quantitative real-time PCR analysis of young tissues and signals were obvious in three transgenic lines, E2, E7 and E11 (Fig. 1a), whereas there was no signal in the wild-type tomato plants. These results indicated that the transgene was successfully expressed in the transgenic tomato plants. Regarding *AtGRF9*, a previous study has shown that GRF9 (14-3-3 mu) can regulate root growth in response to water stress (Mayfield et al., 2012). Therefore, root growth on normal and low-P media was further assayed. As shown in Fig. 1b and c, the average elongation of transgenic tomato tap roots was 19% and 40% higher than in the WT under normal and low-P conditions, respectively.

3.2. Changes in root system development and P accumulation in response to P deficiency

Increased root surface area is a strategy for plants to respond to P stress and to enhance P absorption. Herein, we first investigated the root system phenotypic changes of both WT and transgenic tomato plants by growing them hydroponically under control conditions or low-P conditions. Fig. 2 shows that the average increment rate of total root length, surface area, volume and diameter of the transgenic tomato plants was 20%, 14%, 73%, and 17% higher than that in the wild type under control conditions, respectively. More importantly, the increment

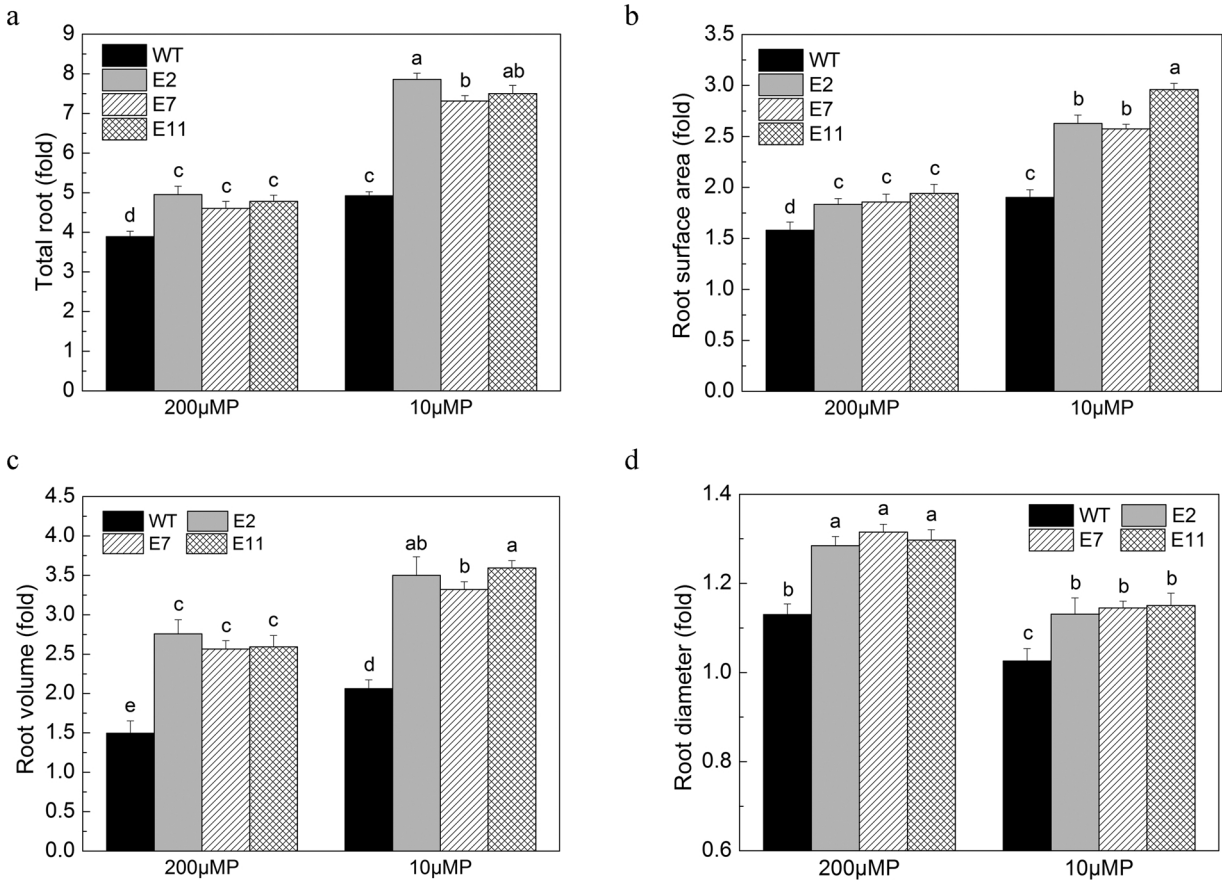

Fig. 2. The increment rate of total root length, surface area, volume and diameter of wild-type and transgenic tomato plants under both control and low-P conditions. The root architecture parameters of fifteen-day-old tomato (WT, E2, E7, and E11) were measured prior to being subjected to control or low-P conditions for 6 d in a hydroponic system. After treatment under control or low-P conditions for 6 d, root architecture parameters in twenty-one-day-old tomato (WT, E2, E7, and E11) were measured. The increment rate of total root length, surface area, volume or diameter (fold) = the root architecture parameters of twenty-one-day-old tomato/the root architecture parameters of fifteen-day-old tomato. Values are means ± standard error (SE, n = 15). Letters above the SE bars indicate whether the transgenic tomato plants had a significant influence within each P level ($P < 0.05$) as determined by analysis of variance (one-way ANOVA) followed by least-significance-difference (LSD) post-hoc tests.

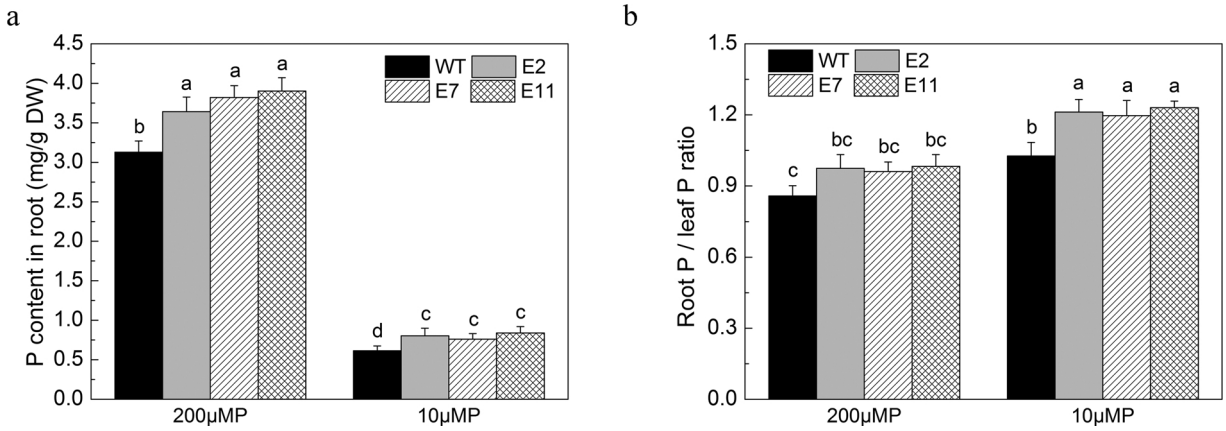

Fig. 3. The total P content in the root and the ratio of root P/leaf P of wild-type and transgenic tomato plants under both control and low-P conditions. Fifteen-day-old tomato plants (WT, E2, E7, and E11) were subjected to control or low-P conditions for 6 d in a hydroponic system. Values are means ± standard error (SE, n = 15). Letters above the SE bars indicate whether the transgenic tomato plants had a significant influence within each P level ($P < 0.05$) as determined by analysis of variance (one-way ANOVA) followed by least-significance-difference (LSD) post-hoc tests.

rate of total root length, surface area, volume and diameter of the transgenic tomato plants was also significantly (51%, 40%, 70%, and 12%, respectively) higher than wild type under low-P conditions. To remodel a lager root surface area in response to low-P stress, the increment rate of root diameter between wide type and transgenic tomato decreased. The result showed the transgenic lines exhibited enhanced root system development in response to P deficiency. Similarly, total P content in root, and the root P/leaf P ratio of the transgenic tomato plants also showed significant ($P < 0.05$) increases as compared to the WT (Fig. 3). The result showed the transgenic lines contained higher

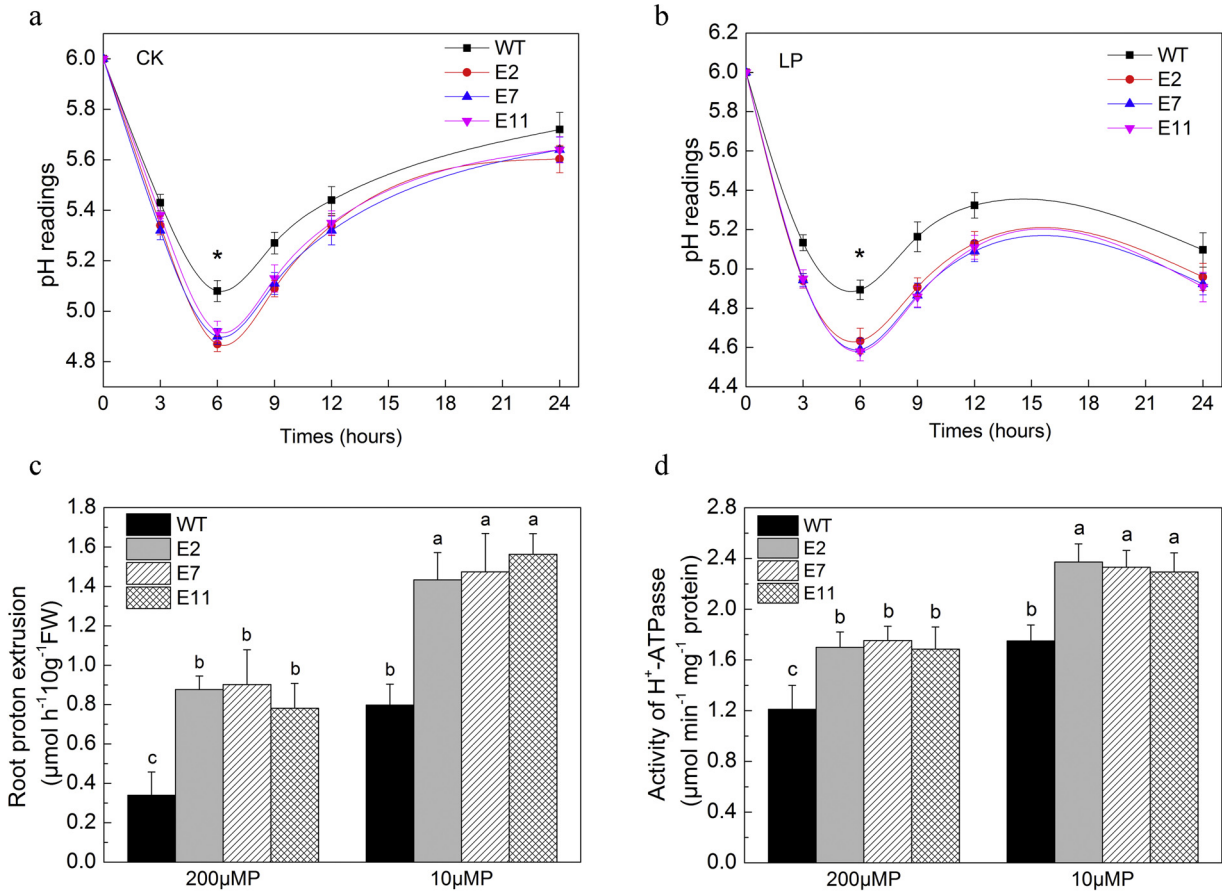

Fig. 4. Root proton excretion and activity of plasma-membrane H^+-ATPase in different P solutions. The pH in the rooting medium (a and b), root proton excretion (c), and activity of PM H^+-ATPase (d) for wild-type and transgenic tomato plants under low-P hydroponic conditions. Fifteen-day-old tomato plants (WT, E2, E7, and E11) were subjected to control or low-P conditions for 6 d in a hydroponic system. pH in growth solutions were measured in 3 h, 6 h, 9 h, 12 h, and 24 h, respectively. Values are means ± standard error (SE, n = 9). Letters above the SE bars indicate whether the transgenic tomato plants had a significant influence within each P level ($P < 0.05$) as determined by analysis of variance (one-way ANOVA) followed by least-significance-difference (LSD) post-hoc tests. Asterisks indicate significant differences between the two genotypes in the same treatment: *$P < 0.05$.

levels of P in roots than those of wild-type plants in response to P deficiency.

3.3. Root proton excretion and activity of plasma-membrane H^+-ATPase in different P solutions

Then, we investigated the hypothesis that GRF9 might mediate P uptake by decreasing rhizospheric pH. The pH in the growth medium was measured. In the hydroponic culture system, in the treatment where the initial pH was 6.0, the pH in the WT rooting medium decreased to 5.1 and 4.9 under normal-P (200 μM) and to 4.9 under low-P (10 μM) conditions, respectively. However, the pH in the transgenic lines rooting medium decreased to 4.9 and 4.6 under normal-P (200 μM) and 4.6 under low-P (10 μM) conditions, respectively (Fig. 4a and b). These results suggest that, under control and low-P conditions, GRF9 helps the root system adjust its rhizospheric pH to a value that is more suitable for P mobilization. Similarly, WT tomato roots in low-P conditions excreted 2.5 times more protons than in normal-P conditions in the hydroponic system, in agreement with previous reports (Imas et al., 1997; Gao et al., 2010). Furthermore, transgenic tomato roots excreted 2.5 and 1.9 more protons per gram fresh weight than WT roots in both normal P and low-P solutions, respectively (Fig. 4c).

Given the relationship between proton excretion and the activity of the PM H^+-ATPase, we isolated proteins and measured the activity of PM H^+-ATPase from tomato roots. As shown in Fig. 4d, we found that transgenic tomatoes exhibited an enhanced activity of PM H^+-ATPase in both normal and low-P solutions (Fig. 4d).

3.4. Relative expression of LePT1 and LePT2 in tomato roots in response to P deficiency

To examine whether the enhanced P accumulation was related to high-affinity phosphate transporter gene expression, we examined the relative expression of the genes of two high-affinity phosphate transporters, *LePT1* and *LePT2* in the both WT and transgenic tomato roots by real-time PCR. The relative expression of *LePT1* in transgenic tomato roots was significantly (33% and 27%, respectively) higher than WT under normal and low-P conditions (Fig. 5a). Consistently, the relative expression of *LePT2* in transgenic tomato roots was significantly (54%) higher than WT under low-P conditions (Fig. 5b).

3.5. Agronomic performance of GRF9 transgenic tomato plants in the field at two P levels

The agronomic performance of the *AtGRF9*-transgenic and untransformed plants was evaluated with respect to plant height, stem diameter, leaf P content, fruit quality, and production of both WT and transgenic tomato plants under field (P0 and P75) conditions. The results depicted in Fig. 6 show that the agronomic performance of the transgenic lines (E2, E7, and E11) in terms of plant height, stem diameter, and leaf P content were superior to untransformed plants under the P0 treatment in the field. A comparison of the fruit quality of

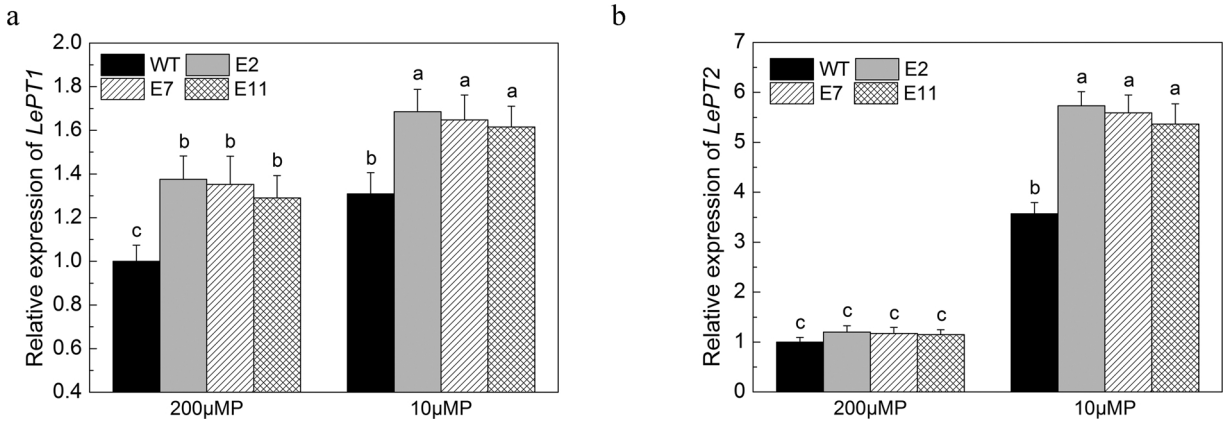

Fig. 5. The relative expression of two high-affinity phosphate transporter genes (*LePT1* and *LePT2*) in both wild-type and transgenic tomato roots by real-time PCR, under normal P and low-P conditions. Fifteen-day-old tomato plants (WT, E2, E7, and E11) were subjected to control or low-P conditions for 6 d in a hydroponic system. Values are means ± standard error (SE, n = 9). Letters above the SE bars indicate whether the transgenic tomato plants had a significant influence within each P level ($P < 0.05$) as determined by analysis of variance (one-way ANOVA) followed by least-significance-difference (LSD) post-hoc tests.

transgenic lines (E2, E7, and E11) with untransformed plants, depicted in Fig. 7a and b, showed that vitamin-C content and the sugar-acid ratio in transgenic lines were 23% and 33% higher than those of WT under P0 conditions in the field, respectively. Fruit production in transgenic plants was also measured and compared with that of WT (Fig. 7c and d). Results show the transgenic lines had 14% and 17% higher total production than wild-type plants under both P75 and P0 conditions in the field, and fruit production at the first collection stage in transgenic tomato was 68% and 32% higher than in wild-type plants under both P75 or P0 conditions.

4. Discussion

Low P availability is a major abiotic stress, affecting up to 40% of the world's arable land (Vance et al., 2003), and severely restricting crop productivity globally. A primary strategy of plants to acclimate to low-P stress is to enhance the root surface area and, thus, enhance P-uptake capacity (Yao et al., 2014; Sun et al., 2016). The molecular mechanisms of root development and P-uptake regulation during P deficiency are poorly understood. The *AtGRF9* gene has been implicated in the response to P deficiency (Cao et al., 2007). However, the clear function of this gene in response to P deficiency is still unknown. In this study, we demonstrate that the constitutive expression of *GRF9* in tomato increases the degree of tolerance to P deficiency (Figs. 2–7), an interesting and unique feature for tomato, which is generally considered to be a P-deficiency-sensitive plant like *Arabidopsis*. Results on root development, P content, and fruit production in the field imply that transgenic plants have the ability to cope with P-deficit conditions better than wild-type plants, suggesting that heterologous *GRF9* can improve environmental low-P stress resistance in agriculturally important crop plants.

Arabidopsis GRF9 mutants accumulate lower P than the WT, suggesting that *GRF9* can have functional roles in coping with fluctuations in P availability (Cao et al., 2007). We found that transgenic tomato plants with constitutive expression of *AtGRF9* accumulated P in the root and the leaf to a higher extent than the WT under both hydroponic-culture and field conditions (Figs. 3 and 6c). Therefore, it seems that *GRF9* from *Arabidopsis* performs conserved biological functions in tomato. Plants have evolved a diverse array of strategies to obtain adequate P under limiting conditions by increasing P-absorption capacity and P bio-availability, and root morphology as well as phosphate transporter activity all affect P absorption (Xu et al., 2007; Wang et al., 2008; Li et al., 2016). Previous reports showed that *Arabidopsis* 14-3-3 proteins can regulate root development in response to stress; for example, *GRF11* plays a key role in regulating root growth under Fe deficiency (Yang et al., 2013). He et al. (2015) found that GRF9-overexpressing *Arabidopsis* plants showed superior whole-plant growth and root growth than the wild type under normal and water stress conditions. In our observations, the transgenic *AtGRF9* tomato root total length, surface area, and total volume significantly increased, especially under low-P stress (Fig. 2). Improved root development is predicted to be one key factor in enhancing P-uptake capacity (Vance et al., 2003; Wang et al., 2004; Zhou et al., 2014).

Plant adaptations to low P include not only increases in root length

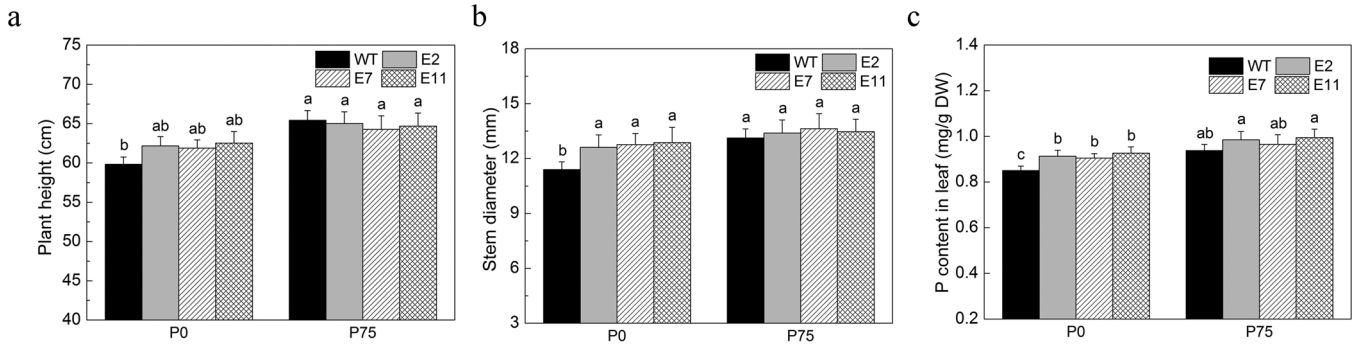

Fig. 6. Plant phenotype and leaf P content of both WT and transgenic tomato plants (E2, E7, and E11) under P0 and P75 conditions in the field. Agronomic performance of WT and transgenic tomato plants (E2, E7, and E11) in terms of plant height (a), stem diameter (b) and leaf P content (c) under P0 and P75 conditions in the field. Plant height and stem diameter were measured in the flowering period and leaf P content was investigated in the fruit ripening period.
Values are means ± standard error (SE, n = 15). Letters above the SE bars indicate whether the transgenic tomato plants had a significant influence within each P level ($P < 0.05$) as determined by analysis of variance (one-way ANOVA) followed by least-significance-difference (LSD) post-hoc tests.

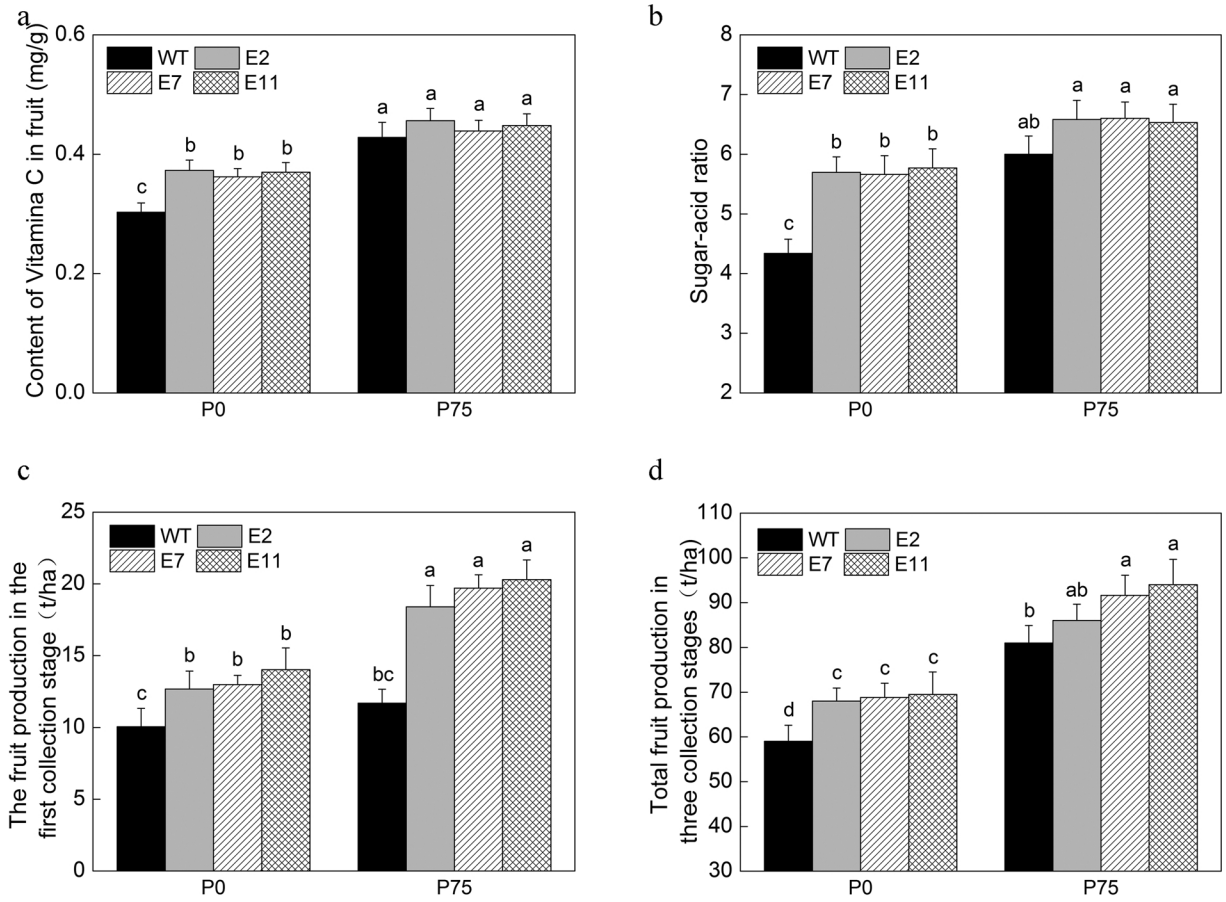

Fig. 7. Fruit quality and production of both WT and transgenic tomato plants (E2, E7, and E11) under P0 and P75 conditions in the field. The mature fruit vitamin C content (a), sugar-acid ratio (b), production (c and d) of both WT and transgenic tomato plants (E2, E7, and E11) were measured in the ripening period under P0 and P75 conditions in the field. Values are means ± standard error (SE, n = 9). Letters above the SE bars indicate whether the transgenic tomato plants had a significant influence within each P level ($P < 0.05$) as determined by analysis of variance (one-way ANOVA) followed by least-significance-difference (LSD) post-hoc tests.

for P uptake from a greater soil volume, but also rhizosphere acidification to help acquire P efficiently (Lambers et al., 2006; George et al., 2009; Ding et al., 2011; Xu et al., 2012). In higher plants, 14-3-3 proteins are known to activate plasma-membrane H^+-ATPase and proton extrusion from roots, which plays a key role in maintaining both root expansion growth and nutrient solubilization under abiotic stress; GRF9-overexpressing *Arabidopsis* plants had similar proton exudation to WT in response to water stress (Xu et al., 2012; He et al., 2015). Compared with WT tomato plants, root proton extrusion and the activity of the plasma-membrane H^+-ATPase in GRF9-overexpressing tomato plants was higher under both control and low-P conditions (Fig. 4c and d). It can be hypothesized that, depending on the environmental context, GRF9 exerts different effects on root proton extrusion. Our results suggest that GRF9 is involved in the response of transgenic tomato roots to low-P stress by activating the root plasma-membrane H^+-ATPase to release more protons, which then help maximize P uptake in response to P deficiency (Shen et al., 2006). Secretion of other organic acids, such as malate, and microbial interactions, also affect P-acquisition capacity (Li et al., 2016; Zhang et al., 2016). It will be interesting to explore in the future whether these mechanisms are also involved in the GRF9-mediated P acquisition activity.

Inorganic P is actively absorbed by plant roots using phosphate transporters, which mediate the acquisition of P across the plasma membrane following an energy-dependent proton/phosphate symporter mechanism, in which the plasma membrane H^+-ATPase is considered to be centrally involved (Song et al., 2001; Karandashov and Bucher, 2005; Wang et al., 2008). To maintain P homeostasis and meet the metabolic P demand, the expression of some high-affinity phosphate transporters (e.g. *PHT2*) appear to be diurnally induced by sucrose supply, and this provides strong evidence for the existence of an interconnection between sucrose signaling and the phosphate transporter apparatus (Lejay, 2003; Rouached et al., 2010). In *Arabidopsis*, the phosphoserine/threonine consensus binding domain of GF14μ(encoded by *GRF9*), which is involved in sucrose signaling in the root response to P deficiency, is present in several of the genes induced by P withdrawal, such as the phosphate porter *AtPHT1* (Cao et al., 2007). We infer that GRF9 may display a connected role in the phosphate transporter apparatus. In tomato, high-affinity phosphate transporters (*LePT1* and *LePT2*) are strongly expressed in the root epidermis under low-P conditions (Liu et al., 1998; Xu et al., 2007), and 14-3-3 proteins display an interaction with the phosphate transporter *PHT6* in soybean under P deficiency (Lin et al., 2017). However, there has not been a study to explore whether heterologous 14-3-3 genes can regulate endogenous *PT* gene expression in tomato. Our results in this study show that, in a P-limiting cultural system, *LePT1* and *LePT2* transcripts were significantly up-regulated in transgenic tomato roots (Fig. 5). The obvious up-regulation of the expression of phosphate transporters may be a reason why transgenic tomato with constitutive expression of *AtGRF9* display enhanced P accumulation.

Tomato is a tremendously, and globally, important fruit crop (Ivanov et al., 2012; Paolacci et al., 2014). The production of better fruits, in terms of taste and nutritional value, is an additional target in agriculture to meet consumers' sharpened demand concerning all aspects regarding food and agricultural products that satisfy both health and environmental requirements (Bona et al., 2015). Taste preference for tomato fruits has been correlated with the content of vitamin C and

the fruit sugar-acid ratio, which could be favored by P availability (Chapagain and Wiesman 2004; Copetta et al., 2011). 14-3-3 proteins could reduce the accumulation of starch by regulating the activity of SPS, and promote carbohydrate synthesis in the fruit ripening stage, to ensure a better taste of the tomato fruit (DeLille and Ferl, 2001; Wang et al., 2013). Therefore, test of the agronomic performance of low-P-tolerant tomato plants has been highly anticipated. The vitamin-C content and the sugar-acid ratio of both wild-type and transgenic tomato fruits decreased under P deficiency; however, transgenic tomato fruits were of much higher quality than WT under low-P (P0) conditions (Fig. 7a and b). Similarly, low-P-tolerant tomato plants also displayed higher final total production compared to WT under P0 field conditions (Fig. 7d). Previous studies have shown that P supplementation can improve crop productivity of grape, wheat, tomato, and soybean (Kowalska et al., 2015). One of the main benefits of P in these cases was found to be in flower bud differentiation (Bouranis et al., 2001). Transgenic tomato fruit production at the first collection stage was 68% and 32% higher compared to WT under P75 and P0 conditions, respectively (Fig. 7c). The *Arabidopsis* 14-3-3 protein μ (GRF9) has been reported to be involved in photo-periodic flower emergence by interaction with a central regulator of the photo-period pathway; thus, *Arabidopsis GRF9* mutants exhibited a delay in flowering (Mayfield et al., 2007). Other studies have reported that 14-3-3 proteins can interact with ACC synthase (isoforms ACS-6, 7 and 8 involved in ethylene synthesis), which suggests 14-3-3 proteins are involved in regulating the ethylene synthesis pathway in *Arabidopsis* or crop plants (Chang et al., 2009). Therefore, we infer that overexpressing *AtGRF9* in tomato may regulate the tomatoes in agricultural production. However, full elucidation of the mechanisms underpinning the interesting agronomic performance in *AtGRF9* transgenic tomato fruits will require further study.

The present study demonstrates that a heterologous *GRF9* gene can significantly enhance the tolerance to low-P stress in tomato plants. The tomato 14-3-3 protein gene homolog was discovered by searching the expressed sequence tag database (Xu and Shi, 2006). However, the clear function of the tomato 14-3-3 gene has remained largely unknown. We argue that it may play a role similar to the *Arabidopsis GRF9* gene in regulating a suite of low-P stress-related genes during stress acclimation. It would be of interest to know whether overexpression of the tomato *GRF9* homolog in *Arabidopsis* or tomato could yield results similar to the overexpression of *Arabidopsis GRF9* in these plants. The *AtGRF9*-transgenic tomato lines could become an important genetic resource for breeding resistance to low-P stress and may become a valuable economic crop in agriculture. We believed that a similar approach as used here may be applicable to other important crops to improve tolerance against low-P-stress conditions. Overall, the engineering of stress-tolerant crops by incorporating a master switch gene (s) like *GRF9* may be an efficient approach to minimize stress damage.

Acknowledgements

This work was supported by the National Key R&D Program of China (2017YFD0200200/2017YFD0200208), the National Natural Science Foundation of China (41171234), and the University of Melbourne.

We thank Dr. Nan Gao (Nanjing Industry University) for providing the transgenic tomato seeds. We also thank Professor Weifeng Xu for previous guidance of this work and Dr Si'en Li for his assistance in field sampling in Wuwei, Gansu, China.

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