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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

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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).

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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