RESEARCH PAPER

Involvement of 14-3-3 protein GRF9 in root growth and response under polyethylene glycol-induced water stress

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Abstract

Plant 14-3-3 proteins are phosphoserine-binding proteins that regulate a wide array of targets via direct protein–protein interactions. In this study, the role of a 14-3-3 protein, GRF9, in plant response to water stress was investigated. *Arabidopsis* wild-type, GRF9-deficient mutant (*grf9*), and GRF9-overexpressing (OE) plants were treated with polyethylene glycol (PEG) to induce mild water stress. OE plant showed better whole-plant growth and root growth than the wild type under normal or water stress conditions while the *grf9* mutant showed worse growth. In OE plants, GRF9 favours the allocation of shoot carbon to roots. In addition, GRF9 enhanced proton extrusion, mainly in the root elongation zone and root hair zone, and maintained root growth under mild water stress. Grafting among the wild type, OE, and *grf9* plants showed that when OE plants were used as the scion and GRF9 was overexpressed in the shoot, it enhanced sucrose transport into the root, and when OE plants were used as rootstock and GRF9 was overexpressed in the root, it caused more release of protons into the root surface under water stress. Taken together, the results suggest that under PEG-induced water stress, GRF9 is involved in allocating more carbon from the shoot to the root and enhancing proton secretion in the root growing zone, and this process is important for root response to mild water stress.

Key words: 14-3-3 protein, carbon allocation, GRF9, proton secretion, root growth, water stress.

Introduction

Water stress is a major limitation to crop production in agriculture. Much evidence has shown that plants have developed some adaptive strategies to cope with water stress through a complex network of biological processes (Davies and Zhang, 1991; Neumann, 2008). Among them, regulated root growth plays an important role in response to water stress (Baluska et al., 2010; Gowda et al., 2011). For example, plants may invest more carbon to roots and maintain a certain level of root growth in response to soil water stress (Sharp et al., 2004; Schachtman and Goodger, 2008; Petricka et al., 2012; Xu et al., 2013). This will help plant survival by tapping water at depth.

The 14-3-3 proteins are a large family of proteins found in virtually all eukaryotic organisms (Comparot et al.,

2003; Roberts, 2003). In higher plants, 14-3-3 proteins are phosphoserine-binding proteins that regulate the activities of a wide array of targets via direct protein–protein interactions and play indispensable roles in plant response to abiotic and biotic stresses (Ferl, 1996; Roberts *et al.*, 2002; Mayfield *et al.*, 2007). Some recent studies suggest that plant 14-3-3 proteins might play a role in response to water stress. For instance, apart from their well-established role in regulating plasma membrane (PM) H+-ATPase, which affects root growth and water acquisition (Palmgren, 2001), 14-3-3 proteins are thought to be involved in carbohydrate metabolism and transport, which also affects root adaptation to water stress (Comparot *et al.*, 2003; Gowda *et al.*, 2011). In addition, in *Arabidopsis* plants, 14-3-3 proteins can regulate root growth and chloroplast development as components of the photosensory system (Mayfield *et al.*, 2012).

In *Arabidopsis* and tomato, thirteen and twelve 14-3-3 protein isoforms have been found, respectively. GRF9 is one of the 14-3-3 gene family members identified in *Arabidopsis* plants (Roberts, 2003; Xu and Shi, 2007; Mayfield *et al.*, 2007). In this study, *Arabidopsis* GRF9 mutant (*grf9*) or GRF9-overexpressing (OE) (GRF9 overexpressed in the *grf9* mutant under the control of the 35S promoter) plants were used, and their performance in response to water stress was compared. The results show that GRF9 is involved in plant root response to water stress by participating in shoot carbon allocation and root proton secretion, which lead to more root growth under water stress.

Materials and methods

Plant materials, growth conditions, and stress treatment

Arabidopsis plants were grown hydroponically as described in Xu *et al.* (2012) with constant light. For water stress treatment, different concentrations of polyethylene glycol 8000 (PEG 8000; 5% at –0.47 MPa, 7.5% at –0.91 MPa, 10% at –1.48 MPa, 12.5% at –2.20 MPa, and 15% at –3.02 MPa) were kept in the nutrient solution (Xu *et al.*, 2013). Since PEG 8000 can induce oxygen deficiency and inhibit root growth in a hydroponic system (Verslues *et al.*, 1998), the multipoints and multidirectional aeration system (four pieces of aeration equipment were fixed along the four walls of the hydroponic pot) was adopted to increase oxygen movement or oxygen availability in this hydroponic system. After 12-day-old plants were treated uner normal growth conditions (control conditions) or water stress (PEG treatment) for 12 d, the roots and shoots of the plants were then separated, frozen, and stored at –80 °C until later analysis. Each independent experiment was arranged with three replicates and each replicate contained 10 *Arabidopsis* plants. Furthermore, each replicate was harvested separately and analysed separately. In addition, independent experiments were repeated twice at different times with the same growth conditions.

Real-time RT–PCR

Real-time reverse transcription–PCR (RT–PCR) was assayed according to the method described in Xu and Shi (2006). Total RNA was extracted from *Arabidopsis* under control conditions and water stress. Gene-specific primers for real-time RT–PCR were designed using Primer 5 software (Supplementary Table S1 available at *JXB* online). *At-ACT2* is a strongly and constitutively expressed 'housekeeping' gene in *Arabidopsis*, so the mRNA of *At-ACT2* was defined as 100 REU (relative expression units). The expression level of genes corresponds to the ratio of the copy number of cDNA of the studied gene to the copy number of the 'housekeeping' gene multiplied by 100 REU.

Subcellular localization of GRF9 and generation of genetically modified Arabidopsis plants

The full-length coding sequence of GRF9 was obtained by PCR using primers 5'-CACCATGGGTTCTGGAAAAGAGCGTG-3' and 5'-ATTTGATTTACCCCGAGTAAAGG-3', and cloned into the entry vector (pENTRTM TOPO vector) for the Gateway system (Invitrogen). After sequencing, the correct entry clone was recombined with the Gateway destination vector pGWB5 by using the LR reaction kit (Invitrogen). The formed correct p35S::GRF9-GFP (green fluorescent protein) plasmid was transformed into *Agrobacterium* GV3101 cells. The transformed *Agrobacterium* cells were then used to infiltrate the young leaves of 4-week-old tobacco plants. About 40 h after infiltration, expression of GRF9–GFP was analysed by using confocal microscopy (Olympus FV-1000 spectral type SPD mar/G/R IX81 FLUOVIEW laser confocal system) according to the method of Xu *et al.* (2013). For imaging GFP, the 488 nm line was used for excitation, and emission was detected at 520 nm. In addition, plants of the GRF9 mutant line (*grf9*) of *Arabidopsis* (Salk_004455) were obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus, OH, USA). The homozygous GRF9 mutant was identified by PCR using primers specific for the T-DNA of Salk_004455 (Supplementary Table S1 at *JXB* online). After the p35S::GRF9-GFP plasmid was transformed into *Agrobacterium* GV3101 cells, transformation of *grf9* plants was carried out according to the floral dip method (Xu *et al.* 2012). T3 homozygous *Arabidopsis* lines were used for further studies.

Measurement of dry weight, root length, and proton extrusion

Plants (roots and shoots) were separated, and then placed in an oven at 105 °C for 0.5 h to inactivate the enzymes. To obtain the dry weight, harvested plant parts were dried to a constant weight at 70 °C. The root length of plants was measured using a root analysis instrument (WinRHIZO; Regent Instruments Inc., Quebec, ON, Canada) according to the method described (Xu and Shi, 2007). The proton extrusion rate of plant roots was analysed following the method described in Xu *et al.* (2012).

Carbohydrate determination

Plant samples were extracted in 0.5 ml of 80% ethanol (v/v) for 20 min at 70 °C, and the sucrose content was determined as described (Stitt *et al.*, 1989). Starch was extracted from the residue by grinding in 0.5 ml of 50 mM sodium acetate (pH 4.8) containing α-amylase (10 U) and amyloglucosidase (6 U).

Enzyme measurements

Sucrose phosphate synthase (SPS) activity and starch synthase (SS) activity were determined as described in Sicher and Kremer (1984) and Zuk *et al.* (2005), respectively. PM H+-ATPase activity and protein concentration were determined as described in Shen *et al.* (2006) and Xu and Shi (2007), respectively.

Measurement of net H+ flux

Net fluxes of H+ in the root tip of plants were measured non-invasively using SIET (scanning ion-selective electrode technique, SIET system BIO-003A; Younger USA Science and Technology Corporation; Applicable Electronics Inc.; Science Wares Inc.; Falmouth, MA, USA). The principle of this method and instrument are detailed in Xu *et al.* (2013).

Collection and analysis of phloem exudates

Phloem exudates were measured according to Fan *et al.* (2009). After *Arabidopsis* leaves were cut, the tip of the petiole was recut in EDTA buffer (5 mM Na$_2$EDTA, pH 7.5, osmotically adjusted to 270 mosmol with sorbitol) with fresh razor blades. During phloem sap exudation, the leaves were illuminated and incubated in a chamber. The buffer solution containing phloem exudates was analysed for sucrose content.

Grafting of Arabidopsis *plants*

Grafting of 6-day-old *Arabidopsis* plants grown on sterile vertical agar plates was performed as described previously (Xu *et al.*, 2012). Seeds of *Arabidopsis* plants were surface-sterilized, and stratified at 4 °C for 3 d. The seeds were then placed onto sterile half-strength Murashige and Skoog (MS) agar (0.8%) plates. The plates were kept vertically in constant light (~120 µE) at 21 °C for 3 d in an *Arabidopsis* growth chamber, and then kept at 25 °C under an 8 h photoperiod (60 µE) for another 3 d. The seedlings were grafted using silicon tubing collars, and grafted plants were kept on identical agar plates and under the same growth conditions for another 6 d until the graft junction had healed. Successfully grafted plants (12 d old) were transferred to the hydroponic system and were treated with control conditions and water stress (10% PEG 8000) for 12 d. Then, *Arabidopsis* plants were used for experimental analysis.

Statistical analyses

Data were subjected to analysis of variance (ANOVA), and post-hoc comparisons were done with Duncan's multiple range test at the $P<0.05$ level. The statistical software program used was SPSS version 13.0. The values are the means and SD of six replicates from two independent experiments.

Results

Identification and selection of genetically modified Arabidopsis

Under PEG-induced water stress, GRF9 is 1.8-fold up-regulated (Fig. 1A) and GRF6 is 2-fold up-regulated (Supplementary Table S2 at *JXB* online). Regarding GRF9, the study by Mayfield *et al.* (2012) shows that GRF9 (14-3-3 mu) regulates root growth which is important for water use in plants, and thus it might also play important roles in plant response to water stress. With regards to GRF6, Yan *et al.* (2004) showed that overexpression of GRF6 (GF14λ) in cotton improves drought tolerance. Since the role of GRF6 in plants has thus been clarified (Yan *et al.*, 2004), the focus therefore was on GRF9 in this study. Figure 1B shows that GRF9 can be located in the nucleus, cytosol, and plasma membrane, and was induced by water stress. GRF9 transcript was not found in the homozygous GRF9 mutant line (*grf9*) of *Arabidopsis* (Supplementary Fig. S1). GRF9 was thus overexpressed in the *grf9* mutant under the control of the 35S promoter. According to the results (Supplementary Fig. S1), wild-type (Col-0), and six *Arabidopsis* transgenic lines (from OE-g1 to OE-g6) were confirmed to contain GRF9 transcript, and the GRF9 mRNA level of the overexpressing (OE) lines was significantly higher than that of the wild type (~4-fold). Further, no significant change was found in terms of the expression levels for other endogenous 14-3-3 genes between the wild-type and OE plants under control or water stress conditions (Supplementary Table S2). Thus, the wild-type Arabidopsis plants (Col-0), the homozygous GRF9 mutant (*grf9*, Salk_004455), and two GRF9 OE lines (OE-g1 and OE-g6) were selected for further study.

Growth of Arabidopsis *plants under water stress*

The growth of Col-0, *grf9*, OE-g1, and OE-g6 plants was studied for water stress tolerance by growing them hydroponically under control conditions or different concentrations of PEG 8000 (5%, 7.5%, 10%, 12.5%, and 15%). Figure 2 shows that when PEG concentrations were >10%, the dry weight of Col-0 was significantly higher than that of *grf9* but lower than that of OE-g1 or OE-g6. Figure 2 also shows that when PEG concentrations were >10%, the total root length of OE plants (OE-g1 or OE-g6) was significantly higher than that of Col-0. The root length of *grf9* was always the lowest. The relative root length inhibitory rates among the three genotypes (Col-0, *grf9*, OE-g1, and OE-g6) were also analysed. If the root length under 0% PEG is set as 100%, the relative inhibition under 10% PEG was 28.8, 35, and 37.4%, respectively, for OE, Col-0, and *grf9* plants. The statistics show that the relative inhibition by 10% PEG on OE plants was significantly less than that for Col-0, 28.8% versus 35%, although *grf9* was inhibited to almost the same extent as the wild type, 37.4% versus 35%. These results suggest that GRF9 is essential to maintain root growth under normal conditions, and overexpression of GRF9 is useful for root growth under water stress conditions.

Activities of starch synthase (SS) and sucrose phosphate synthase (SPS) under water stress

It has been suggested that SS is one of the targets of 14-3-3 proteins in carbohydrate metabolism (Sehnke *et al.*, 2001; Cao *et al.*, 2007). Under short-term water stress, plants have increased sucrose and decreased starch levels in the shoot, and the sucrose loading into phloem is also higher in the shoot. Thus, these responses can help plant to adapt to water stress. However, these adaptations are inhibited under long-term water stress, and then plant growth is impaired (Neumann, 2008). Thus, the activity of SS (Fig. 3) was investigated, and SPS was used as a control. Under control conditions or water stress, no significant difference in the SPS activity was observed between wild-type (Col-0), transgenic (OE-g1 and OE-g6), and *grf9* mutant *Arabidopsis* plants, while the activity of SS in *grf9* was significantly higher than that in Col-0 or OE plants under water stress(Fig. 3). Moreover, SS activity in Col-0 was significantly higher than that in OE plants. The sucrose/starch ratio in the leaves of OE plants was significantly higher than that in Col-0 or mutant plants, and the sucrose/starch ratio in Col-0 was significantly higher than that in *grf9* (Fig. 3; Supplementary Fig. S2 at *JXB* online).

Sucrose transport and carbohydrate content in Arabidopsis *under water stress*

The phloem sucrose transport and sucrose content were determined in Col-0, OE-g1, OE-g6, and *grf9* mutant *Arabidopsis*
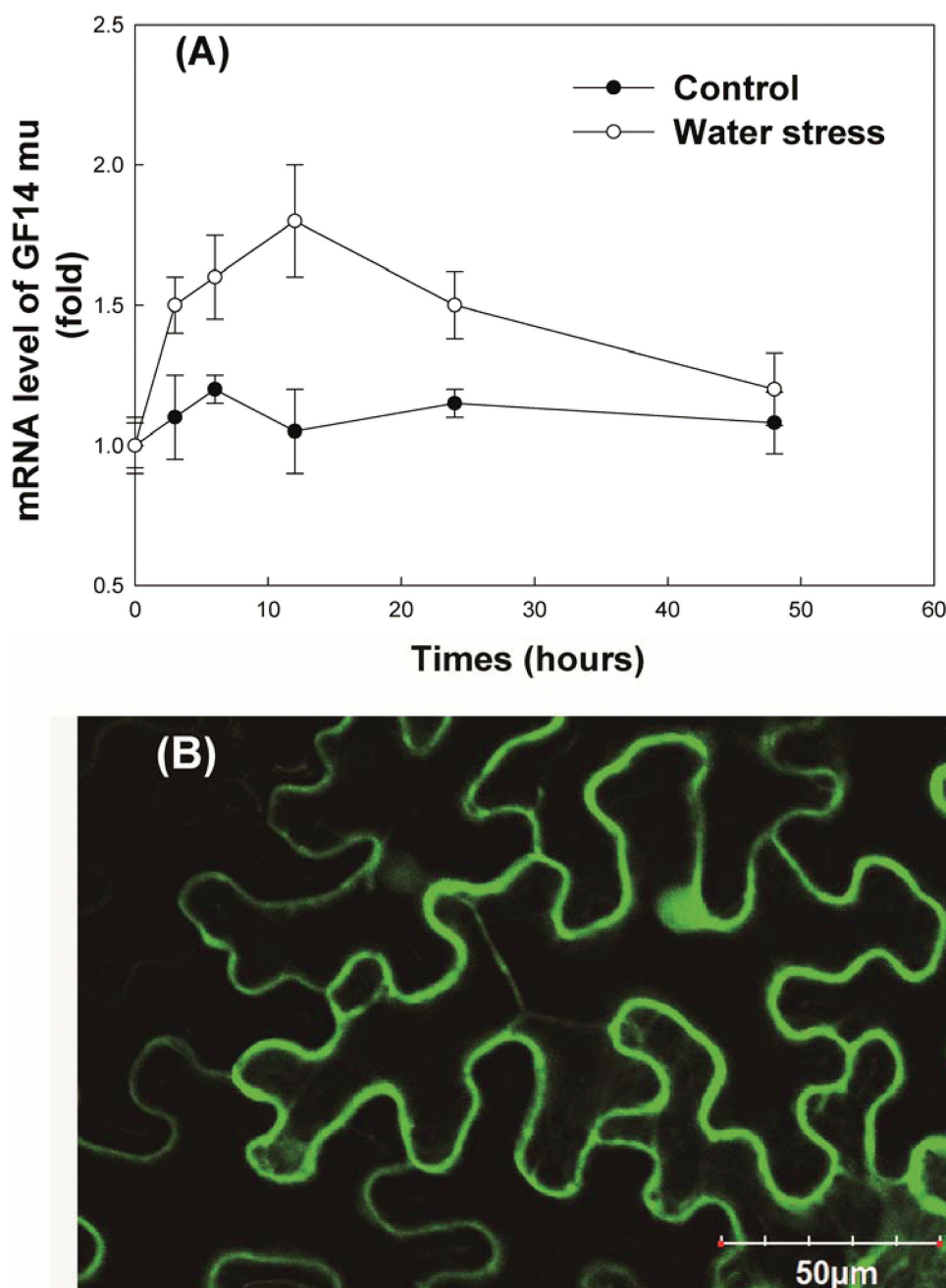
Fig. 1. The mRNA level of GRF9 in *Arabidopsis* plants under control conditions or water stress (A) and subcellular localization of GRF9 in the tobacco system (B). Twelve-day-old *Arabidopsis* plants (Col-0) were subjected to control conditions or water stress (10% PEG 8000) for 0, 3, 6, 12, 24 and 48h under a hydroponic system. The mRNA level of GRF9 in the whole plant of Col-0 at 0h was taken as 1-fold.

plants (Fig. 4). Under water stress, the phloem sucrose transport, root sucrose content, and root sucrose/shoot sucrose ratio in OE-g1 or OE-g6 were significantly higher than those in Col-0 or *grf9*. At the same time, these parameters in Col-0 were also significantly higher than in *grf9* under water stress (Fig. 4).

Activity of PM H⁺-ATPase and H⁺ flux in the root of Arabidopsis *plants under water stress*

In plants, 14-3-3 proteins can activate PM H^+-ATPase by protein–protein interactions (Palmgren, 2001). Figure 5 shows that under water stress, the PM H^+-ATPase activity and root

proton extrusion in GRF9-overexpressing *Arabidopsis* lines (OE-g1 and OE-g6) were significantly higher than those in wild-type Arabidopsis (Col-0) or GRF9 mutant Arabidopsis plants (*grf9*), and the two parameters in Col-0 were also significantly higher than those in *grf9*. Further, the proton (H^+) flux in the meristem zone (MZ), transition zone (TZ), elongation zone (EZ), and root hair zone (RHZ) was analysed in the root tip of *Arabidopsis* plants (Fig. 6). Under control conditions, no significant difference in proton flux was found in the root tip of these *Arabidopsis* plants. Under water stress, in the EZ and RHZ of the root tip, proton efflux in OE-g1 or OE-g6 was significantly higher than that in Col-0 or *grf9*, and proton efflux in Col-0 was also significantly higher than that in *grf9*.
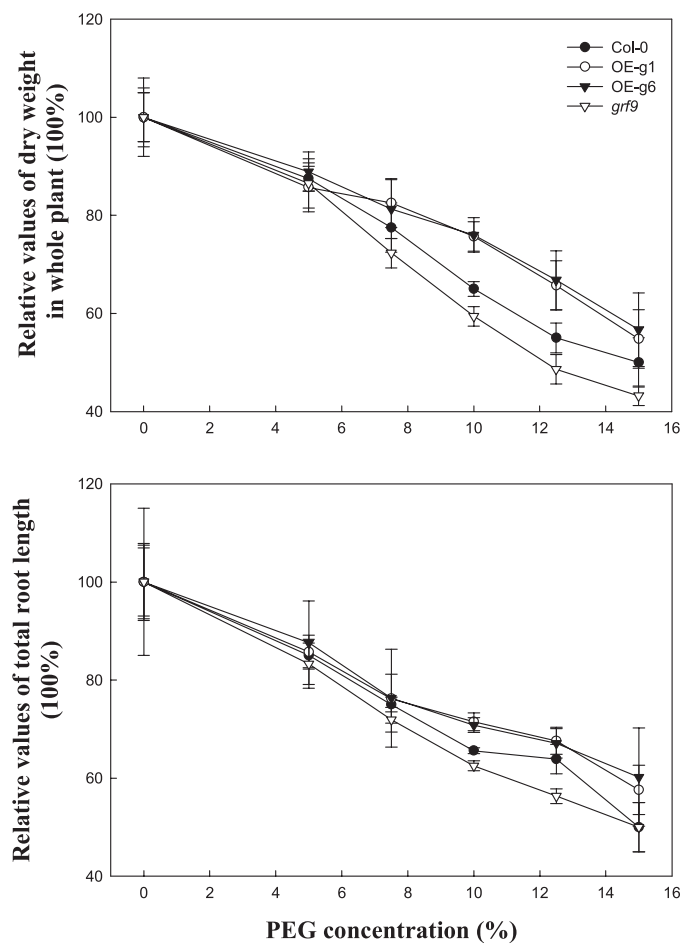
Fig. 2. Dry weight of the whole plant and the total root length in *Arabidopsis* wild-type (Col-0), 14-3-3 protein GRF9 mutant plants (*grf9*), and the two GRF9-overexpressing lines (OE-g1 and OE-g6) under different PEG concentration treatments (water stress). Twelve-day-old *Arabidopsis* plants (Col-0, *grf9*, OE-g1, and OE-g6) were subjected to control conditions or treatment with different PEG 8000 concentrations for 12 d under a hydroponic system. Absolute values of dry weight of Col-0 (2 mg), OE-g1 (2.1 mg), OE-g6 (2.08 mg), and *grf9* (1.85 mg) at 0% PEG, and root length of Col-0 (20 cm), OE-g1 (21 cm), OE-g6 (21.5 cm), and *grf9* (15.98 cm) at 0% PEG are considered as 100%, respectively, for the plant groups concerned.
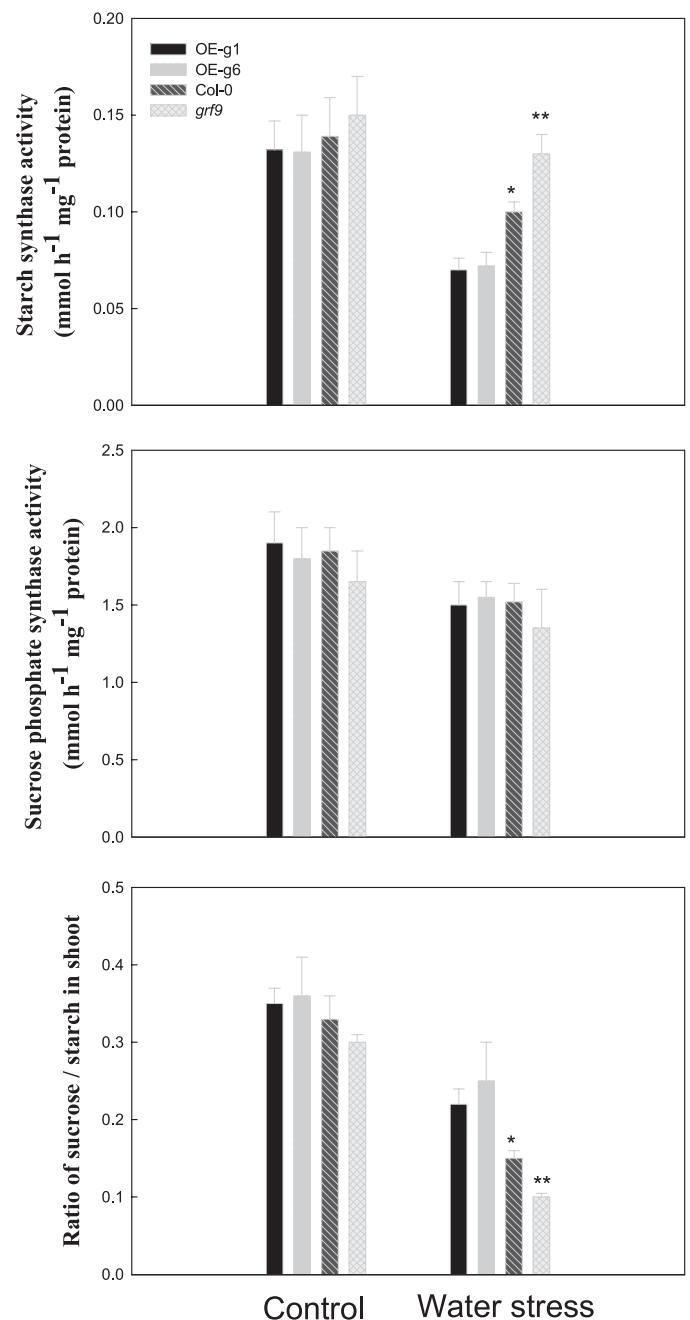

Fig. 3. The activities of starch synthase and sucrose phosphate synthase, and the sucrose/starch ratio in *Arabidopsis* wild-type (Col-0), GRF9-overexpressing (OE-g1 and OE-g6), and *grf9* mutant plants under water stress. Twelve-day-old *Arabidopsis* plants (Col-0, mutant, OE-g1, and OE-g6) were subjected to control conditions or water stress (10% PEG 8000) for 12 d under a hydroponic system.

Physiological parameters in grafted Arabidopsis *plants*

Reciprocal grafting was performed using wild-type *Arabidopsis* plants (WT), GRF9-overexpressing plants (OE), and GRF9 mutant plants (MN). Seven combinations of grafted plants were generated: overexpressing plant shoot to overexpressing plant root (designated as OES/OER), wild-type shoot to wild-type root (WTS/WTR), mutant shoot to mutant root (MNS/MNR), overexpressing plant shoot to wild-type root (OES/WTR), wild-type shoot to overexpressing plant root (WTS/OER), mutant shoot to wild-type root (MNS/WTR), and wild-type shoot to mutant root (WTS/MNR). Under control conditions or water stress, total root length, phloem exudate sucrose, and root proton extrusion were measured in these grafted plants. There was no significant difference in the total root length, phloem exudate sucrose, or root proton extrusion among these grafted plants under control conditions (Supplementary Table S3 at *JXB* online). The following effects were observed under

water stress (Fig. 7): (i) the total root length, phloem exudate sucrose, and root proton extrusion in OES/OER were significantly higher than those in WTS/WTR or MNS/MNR; (ii) the three parameters in WTS/WTR were also significantly higher than those in MNS/MNR; (iii) the total root length in OES/WTR or WTS/OER was higher than that in WTS/WTR, MNS/WTR, WTS/MNR, or MNS/MNR, but lower than that in OES/OER; and (iv) the total root length in MNS/WTR or WTS/MNR was only higher than that in MNS/MNR. Additionally, no significant difference in phloem exudate sucrose was observed between
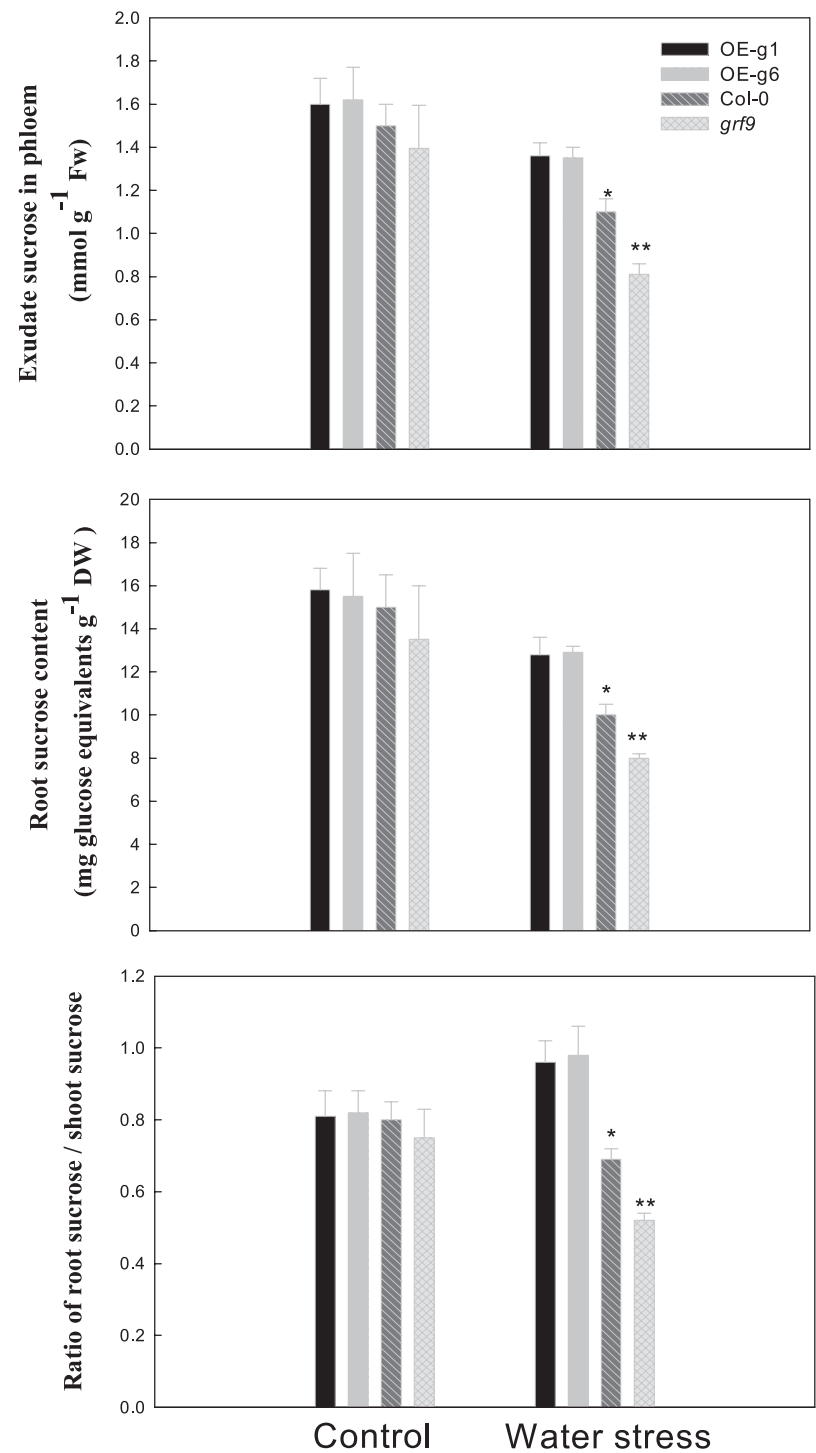
Fig. 4. Phloem exudate sucrose or root sucrose content in wild-type (Col-0), GRF9-overexpressing (OE-g1 and OE-g6), and *grf9* mutant *Arabidopsis* plants under water stress. Twelve-day-old *Arabidopsis* plants (Col-0, mutant, OE-g1, and OE-g6) were treated as described in Fig. 3.

OES/OER and OES/WTR under water stress. In addition, phloem exudate sucrose in WTS/WTR, WTS/OER, or WTS/MNR was lower than that in OES/OER or OES/WTR but higher than that in MNS/MNR or MNS/WTR. Further, no significant difference in root proton extrusion was observed between OES/OER and WTS/OER under water stress. Moreover, root proton extrusion in WTS/WTR, OES/WTR, or MNS/WTR was lower than that in OES/OER or WTS/OER but higher than that in MNS/MNR or WTS/MNR.

Furthermore, under water stress, the total root length or phloem exudate sucrose in OES/WTR was higher than that in MNS/WTR, but no significant difference in root proton extrusion was observed between OES/WTR and MNS/WTR. On the other hand, under water stress, the total root length or root proton extrusion in WTS/OER was higher than that in WTS/MNR, but no significant difference in phloem exudate sucrose was observed between WTS/OER and WTS/MNR.
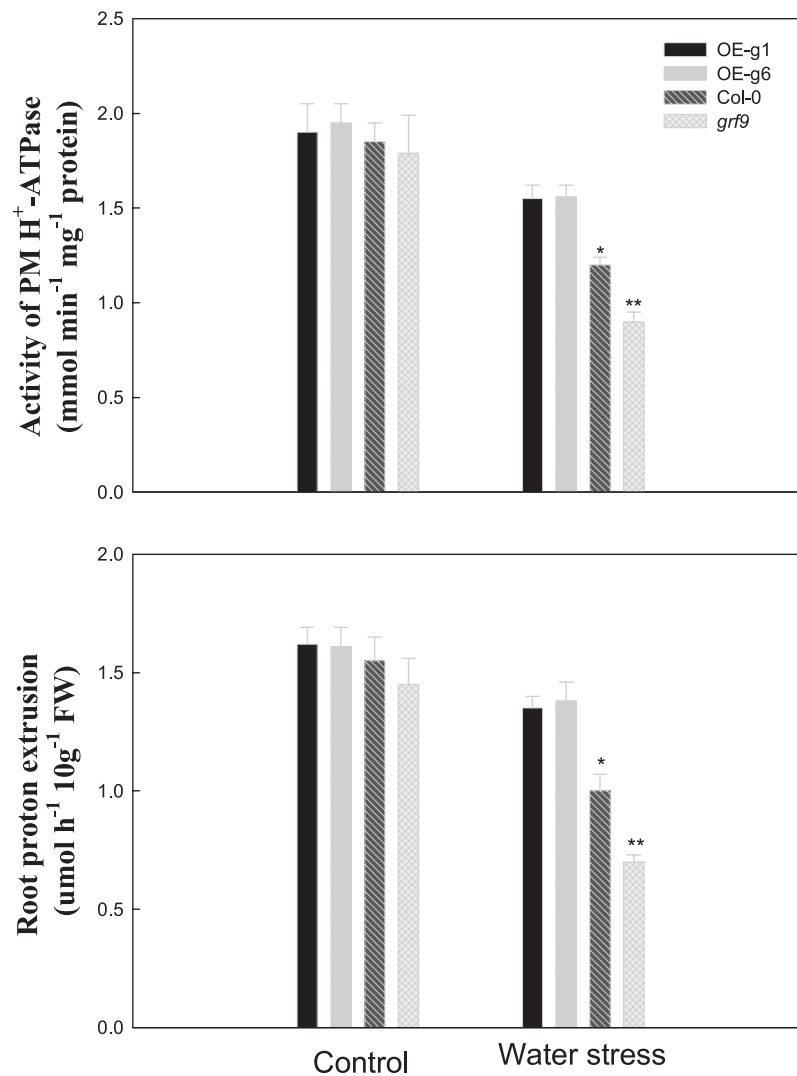
Fig. 5. PM H⁺-ATPase activity or proton extrusion in roots of wild-type (Col-0), GRF9-overexpressing (OE-g1 and OE-g6), and *grf9* mutant *Arabidopsis* plants under water stress. Twelve-day-old *Arabidopsis* plants (Col-0, mutant, OE-g1, and OE-g6) were treated as described in Fig. 3.

Discussion

Plants, sessile in their environments, have evolved to adapt to soil drying by morphological and physiological modulations. These include an enhanced ability to draw water from deep soil via an extended root system and a control of water loss from the shoot through reduced stomatal opening. It has been known for a long time that a primary adaptation of plants to drought stress is the inhibition of shoot growth and improvement of root growth (Sharp *et al.*, 2004; Gowda *et al.*, 2011). Such physiological regulation should require a change in carbon partitioning; that is, more photosynthate to be transported from growth-inhibited shoot to stress-adaptive root. The present results show that the 14-3-3 protein GRF9 may play a regulatorye role for such a response (Fig. 3). Enhanced carbon flow into the roots may also stimulate more proton secretion in the root tip to maintain root growth under water stress conditions.

Efficient sucrose transport in phloem (from shoot to root) is very important for root growth (Salerno and Curatti, 2003).

The present results suggest that *Arabidopsis* 14-3-3 protein GRF9 is involved in water stress response by regulating phloem sucrose transport to allocate more carbon from shoot to root (Fig. 4). In addition, root elongation and growth occur as a result of cell wall extension at the cellular level, during which protons are the major wall-loosening factor (Rayle and Cleland, 1992). In this study, it was found that under water stress, compared with wild-type Arabidopsis, proton secretion in GRF9-overexpressing *Arabidopsis* plants was less inhibited, while proton secretion in GRF9-deficient mutant *Arabidopsis* plants was more inhibited (Fig. 5). It was also found that the effect of GRF9 on proton secretion was not on proton influx but on proton efflux, especially in the elongation zone or root hair zone of the root tip (Fig. 6). Thus, it is proposed that the *Arabidopsis* 14-3-3 protein GRF9 is also involved in plant water stress response by enhancing proton extrusion to maintain root growth.

Recently, a study by Sun *et al.* (2014) showed that the *grf9* mutant (GF14µ is GRF9) is more tolerant to drought stress under soil drying compared with wild-type plants. However,
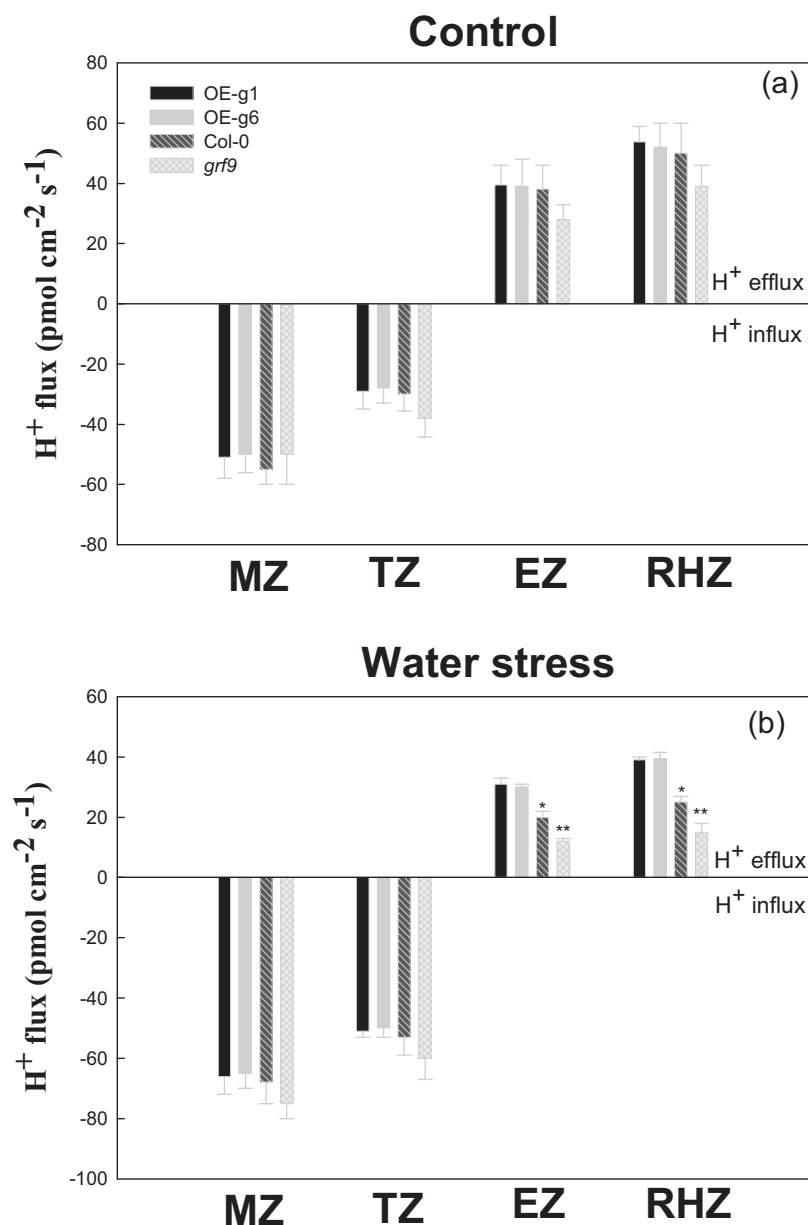
Control

Water stress

Fig. 6. Proton (H$^+$) flux in the meristem zone (MZ), transition zone (TZ), elongation zone (EZ), and root hair zone (RHZ) in the root tip of wild-type (Col-0), GRF9-overexpressing (OE-g1 and OE-g6), and *grf9* mutant *Arabidopsis* plants under water stress. Twelve-day-old *Arabidopsis* plants (Col-0, mutant, OE-g1, and OE-g6) were treated as described in Fig. 3.

the results reported here show that the total root growth of the *grf9* mutant was less than that of wild-type plants under PEG-induced water stress. Some studies have shown that PEG-induced water stress is very different from soil drying (Lilley and Ludlow, 1996; Uga *et al.*, 2013). For example, Kinandang Patong rice is more tolerant to soil drying than IR64 rice. However, under PEG-induced water stress, IR64 rice was shown to be more tolerant to osmotic stress (dehydration) than Kinandang Patong rice. It is understandable that PEG-induced water stress represents a drastic or 'immediate no-water stress', while soil drying is gradual from 'enough water' to 'no water' over time. Under PEG-induced water stress, the *grf9* mutant with shorter roots is not tolerant to osmotic stress (dehydration) compared with wild-type plants. So, just like Kinandang Patong, the *grf9* mutant can

also be more tolerant to soil drying compared with wild-type plants.

Many genes useful in plant drought responses have been identified by molecular and genetic studies using, in most cases, the model plant *Arabidopsis thaliana*. For example, one of them, *Arabidopsis* HD-START protein, can confer drought tolerance in higher plants by improving root growth (Yu *et al.*, 2008). While paying more attention to the discovery of new genes, efforts should also be focused on the regulatory genes that can fine-tune the functions of other important genes for drought response in higher plants (Umezawa *et al.*, 2004). The 14-3-3 proteins are the indispensable regulators in plant growth and development, and also play important roles in response to environmental stress (Roberts *et al.*, 2002; Yan *et al.*, 2004; Campo *et al.*, 2012). In this study, it was
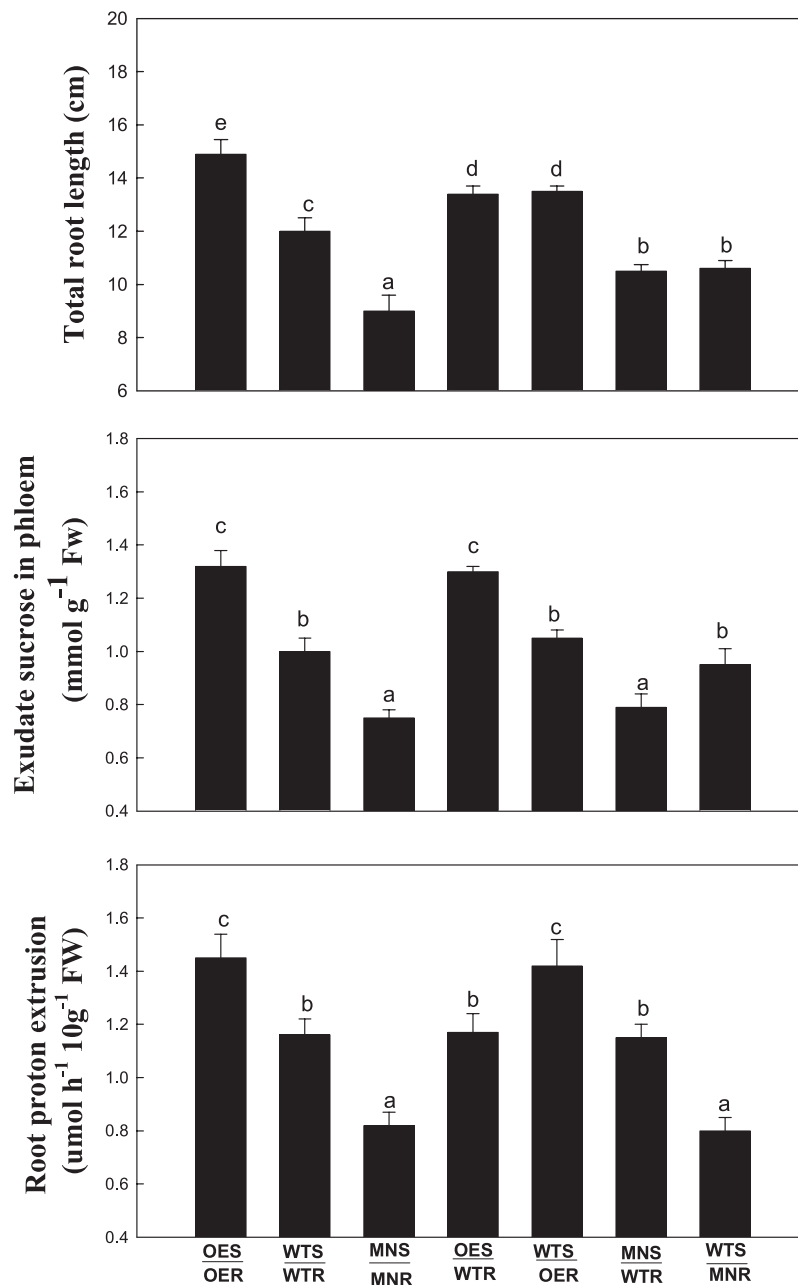
Fig. 7. Total root length, phloem exudate sucrose, and root proton extrusion of grafted plants between *Arabidopsis* wild-type, GRF9-overexpressing, and *grf9* mutant plants under water stress. Twelve-day-old grafted *Arabidopsis* plants were subjected to control conditions or water stress (10% PEG 8000) for 12 d under a hydroponic system. OES, shoot of GRF9-overexpressing plants; OER, root of GRF9-overexpressing plants; WTS, wild-type shoot; WTR, wild-type root; MNS, shoot of *grf9* mutant plants; MNR, root of *grf9* mutant plants.

found that the *Arabidopsis* 14-3-3 protein GRF9 is involved in plant adaptation to water stress by regulating shoot SS and root PM H$^+$-ATPase (Figs 2–6). It is concluded that the *Arabidopsis* 14-3-3 protein GRF9 is useful for the improvement of plant drought tolerance.

Plants have developed diverse responses to cope with water stress. These responses are possibly achieved by the co-ordination of the adaptive network comprising local and systemic responses (Yamaguchi and Sharp, 2010). The localized responses mainly occur in roots and are partially involved in root proton exudation, while the systemic responses mainly occur in shoot and are partially associated

with carbohydrate metabolic pathways. Grafting was used to generate chimeric *Arabidopsis* plants that overexpress or lack GRF9 in the shoots and in roots (Fig. 7). The results showed that compared with self-grafted wild-type plants, as long as GRF9 is overexpressed in shoots, plants show higher tolerance to water stress with higher sucrose transport in phloem, but no significant difference in root proton extrusion was found. Similarly, as long as GRF9 is overexpressed in roots, plants show higher root proton extrusion and better tolerance to water stress, but no significant difference in phloem exudate sucrose was found. Furthermore, when GRF9 was mutated in either the shoot or the root, plants

showed the same lower tolerance to water stress with lower phloem sucrose transport or lower root proton extrusion, respectively. Therefore, it is concluded that GRF9 can possibly be synchronously regulated from the local and systemic response under water stress. In shoot, GRF9 is involved in the systemic response to water stress by regulating shoot carbon allocation and increasing phloem sucrose transport to promote root growth. In root, GRF9 is directly involved in the local response to water stress by activating root PM H⁺-ATPase to release more protons.

In conclusion, using genetically modified GRF9-overexpressing or mutant *Arabidopsis* plants, it was found that GRF9, an *Arabidopsis* 14-3-3 gene family member, plays a role in response to water stress by involvement in shoot carbon allocation and root proton secretion. This process helps plant drought tolerance and explains the long observed phenomenon that plants have relatively more root growth than shoot growth when they are exposed to soil drying.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Expression of the GRF9 gene in *Arabidopsis* plants (wild-type plant, Col-0; *Arabidopsis* 14-3-3 protein GRF9 mutant, *grf9*; GRF9-overexpressing lines, from OE-g1 to OE-g6).

Figure S2. Shoot sucrose or starch content in wild-type (Col-0), GRF9-overexpressing (OE-g1 2 and OE-g6), and *grf9* mutant *Arabidopsis* plants under water stress.

Table S1. Gene-specific primers used in real-time RT–PCR for 14-3-3 genes in *Arabidopsis*.

Table S2. Expression of *Arabidopsis* endogenous 14-3-3 genes (except GRF9) in wild-type *Arabidopsis* plants and GRF9-overexpressing transgenic *Arabidopsis* plants under water stress for 3 d.

Table S3. Total root length, phloem exudate sucrose, and root proton extrusion of grafted plants in wild-type, GRF9-overexpressing, and *grf9* mutant *Arabidopsis* plants under control conditions.

Acknowledgements

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