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AtTGA4, a bZIP transcription factor, confers drought resistance by enhancing nitrate transport and assimilation in *Arabidopsis thaliana*

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ABSTRACT

To cope with environmental stress caused by global climate change and excessive nitrogen application, it is important to improve water and nitrogen use efficiencies in crop plants. It has been reported that higher nitrogen uptake could alleviate the damaging impact of drought stress. However, there is scant evidence to explain how nitrogen uptake affects drought resistance. In this study we observed that bZIP transcription factor *AtTGA4* (TGACG motif-binding factor 4) was induced by both drought and low nitrogen stresses, and that overexpression of *AtTGA4* simultaneously improved drought resistance and reduced nitrogen starvation in *Arabidopsis*. Following drought stress there were higher nitrogen and proline contents in transgenic *AtTGA4* plants than in wild type controls, and activity of the key enzyme nitrite reductase (NIR) involved in nitrate assimilation processes was also higher. Expressions of the high-affinity nitrate transporter genes *NRT2.1* and *NRT2.2* and nitrate reductase genes *NIA1* and *NIA2* in transgenic plants were all higher than in wild type indicating that higher levels of nitrate transport and assimilation activity contributed to enhanced drought resistance of *AtTGA4* transgenic plants. Thus genetic transformation with *AtTGA4* may provide a new approach to simultaneously improve crop tolerance to drought and low nitrogen stresses.

1. Introduction

Nitrogen is an essential nutrient required for plant growth and development, and nitrogen fertilizer is used worldwide to increase crop yield and quality. However, applied fertilizer nitrogen is partly taken up and used by the crop and partly lost to the environment where it causes serious environmental pollution. Therefore improved nitrogen use efficiency is an important breeding objective for crop breeders. Drought stress is one of major factors reducing crop yields, and improved water use efficiency is an important approach to increase drought resistance. It was reported that higher nitrogen uptake can relieve the negative effects of drought stress on crop plants [1–5], suggesting that nitrogen and water use efficiency interact or complement each other. Weih et al. observed that plants with higher leaf nitrogen content have better drought resistance under water stress [6], and increasing nitrogen nutrition resulted in a higher concentration of chlorophyll [4]. Moreover, the availability of nitrogen affects proline accumulation in several plants species. Proline is an inert osmolyte that protects subcellular structures and macromolecules during drought stress [7–13]. These results indicate there is a mechanism of synergistic regulation of nitrogen and water use efficiency in plants. Although the phenomenon of nitrogen uptake affecting drought resistance was observed, its molecular mechanism remains unknown.

The bZIP transcription factor family is an important group of transcription factors in plants. In *Arabidopsis* there are 75 members [14,15] involved in diverse biological processes such as pathogen defense [16–18], abiotic stress signaling [19–21], hormone signaling [22–25], light signaling [26], energy metabolism [27], and developmental processes such as flowering [28], seed maturation and germination [29,30]. Previous studies found that overexpression of bZIP transcription factors *AtTGA1* or *AtTGA4* in transgenic plants led to improved adaptation to nitrogen starvation [31]. We observed that *GmDREB3* from soybean conferred drought

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tolerance in transgenic wheat. When RNA-Seq data showed that the homologous genes of *AtTGA4* in wheat were highly induced in transgenic *GmDREB3* wheat under drought stress (unpublished data), we speculated that *AtTGA4* was involved in both drought resistance and adaptation to low nitrogen stress.

In this paper we show that overexpression *AtTGA4* improves tolerance to both drought resistance and limited nitrogen stress in *Arabidopsis*, and that drought resistance is dependent on enhanced nitrate transport and assimilation in *AtTGA4* transgenic plants. This discovery increases our understanding of the regulatory networks involved in plant responses to drought stress, and provides a new approach to improve drought resistance in crops through genetic transformation using *AtTGA4*.

2. Materials and methods

2.1. Gene cloning and production of transgenic Arabidopsis

Seven-d-old *Arabidopsis* seedlings were harvested for RNA isolation using a Plant RNA Kit (Tiangen, China). Total RNA were used to synthesize cDNA using TransScript First-Strand cDNA Synthesis SuperMix (Transgene Biotech, China). The primers used were F:TCCCCCGGATG AATACAACCTCGACAC, and R:GGACTAGTTT-CATCATCACACAGCAG. *AtTAG4* was inserted into the pBI121vector under control of the CaMV 35S promoter, and constructs were transformed into wild-type Col-0 using Agrobacterium-mediated floral dipping [32]. Four T1 generation transgenic lines were obtained, and T3 generation transgenic plants were used for analyses.

2.2. Assays of stress resistance, quantitative RT-PCR (qRT-PCR) and phenotype observations on transgenic plants

For low nitrogen stress resistance assays, seeds of wild type (Col-0, WT) and transgenic (OE) plants were surface-sterilized and sown on medium with 0.3 mM nitrogen (0.15 mM NH_4NO_3, the ionic equilibria of the medium being ensured by replacing KNO_3 with KCl). MS medium was used as the control. Materials were placed in a greenhouse with a photoperiod of 16 h light/8 h darkness. For long-term low nitrogen stress resistance assays, seven-d-old seedlings grown on MS medium were transferred to Hoagland's solution containing 0.3 mM nitrogen, and phenotypes were observed 10 days later. For analysis of drought stress resistance, seeds were sown on MS plus 8% PEG selection medium, and phenotypes were observed at 10 days post-germination. For the drying-rewetting experiment in soil in the greenhouse, seven-d-old seedlings were transferred to a 1:1 mixture of peat and vermiculite. After 10 days of growth under normal conditions water was withheld for 18 days and then rewatered for 5 days. For expression profile analysis of *AtTAG4*, WT seeds were grown in MS medium for 7 days, and then transferred to MS plus 8% PEG6000, after which samples were taken at 0.25, 0.5, 1, 3, 6, 12 and 24 h. For expression pattern analysis of *AtTGA4* under low nitrogen stress, young seedlings were transferred to the medium with 0.3 mM nitrogen, and samples were taken after 1, 2, 3, 4, 5, and 6 d. For qRT-PCR analysis, total RNA samples were isolated as described above. *AtActin* was used as the reference control; the primers are listed in Table S1. Three independent biological replicates were made.

2.3. Subcellular location of AtTGA4, enzymic activity assays of NIR (nitrite reductase) and GS (glutamine synthetase), and measurements of chlorophyll and total nitrogen contents in transgenic plants

For subcellular location assays, *AtTGA4* was inserted into the vector 16318 to fuse with green fluorescent protein (GFP) and produce the vector p16318-TAG4, which was then biologically transformed into onion epidermis cells. The empty p16318:GFP vector was used as the control. The onion epidermis was placed in a dark environment for 16–24 h before treatment with a 2 M sucrose solution. Results were observed using a confocal laser scanning microscope (ZEISS LSM 700; Germany). Enzyme activities of NIR and GS were analyzed as previously described [33,34]. Chlorophyll and total nitrogen contents of transgenic plants and controls were measured as described by He et al. [35].

3. Results

3.1. The expression pattern of AtTGA4 and subcellular localization in Arabidopsis

To investigate whether *AtTGA4* is involved in responses to drought and low nitrogen treatments, qRT-PCR was used to analyze expression patterns. Transcription of *AtTGA4* under drought treatment gradually increased with treatment time and peaked at 12 h (3.01-fold that of the control), and then decreased at 24 h (Fig. 1A). The expression level of *AtTGA4* also increased under low nitrogen stress treatment (0.3 mM nitrogen), peaked at 5 days (5.94-fold that of the control), and then gradually decreased (Fig. 1B).

The subcellular localization of protein was examined to gain evidence for its function. Fused AtTGA4-GFP protein localized to the nucleus, whereas in the control, GFP was present in the membrane, cytoplasm and nucleus (Fig. 1C). Such localization was consistent with an expected DNA binding function of AtTGA4 as a transcription factor.

3.2. Overexpression of AtTGA4 improved tolerance to limited nitrogen in transgenic Arabidopsis

Assuming that expression of *AtTGA4* is induced by nitrogen deficiency, we investigated whether *AtTGA4* was involved in adaptation to nitrogen starvation. No significant phenotypic difference was observed between transgenic and wild type plants under normal nitrogen conditions (Fig. 2A), whereas the transgenic plants had higher leaf and root surface areas, higher root diameters and longer stem and primary roots than wild type under low nitrogen conditions (0.3 mM nitrogen) (Fig. 2B–G).

To further determine whether transgenic *AtTGA4* plants also display improved tolerance to long-term nitrogen starvation, seven-d-old seedlings grown under normal conditions were dipped into Hoagland's solution containing 0.3 mM nitrogen. At 10 days after treatment transgenic plants had 1.43- to 1.72-fold higher survival rates, and 1.22- to 1.32-fold higher total nitrogen contents compared to wild type (Fig. 2H, I). These results showed that *AtTGA4* transgenic plants displayed higher levels of tolerance to nitrogen starvation.

3.3. Overexpression of AtTGA4 improved drought resistance in transgenic Arabidopsis

Drought tolerance assays showed that transgenic plants had more cotyledon greening than wild type under drought stress (MS plus 8% PEG6000) after 10 days of growth (Fig. S1A, S1B). After 17 days the phenotypes of transgenic plants were clearly different from wild-type plants, and transgenic lines had longer stems, primary root lengths, higher lateral root densities and leaf areas compared to wild type (Fig. S1C, S1D, S1E), indicating that overexpression of *AtTGA4* conferred stronger drought resistance in transgenic plants during the seedling development stage.

Drought tolerance assays in soil showed that a regime of 17-d-old plants being withheld from water for 18 days followed by rewatering for 5 days (Fig. 3A, B, C) resulted in survival rates that

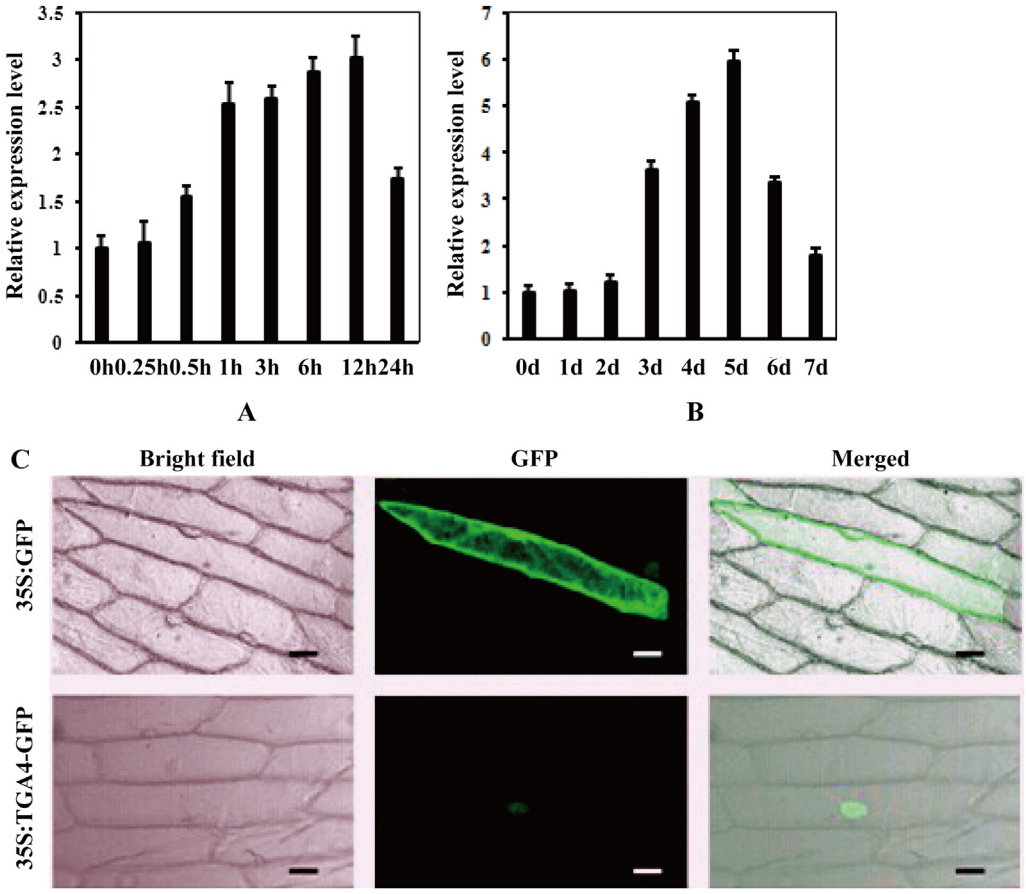

Fig. 1. Expression patterns and subcellular localization of *AtTGA4*. (A) Expression patterns of *AtTGA4* at 0.25, 0.5, 1, 3, 6, 12, 24 h following treatment with 8% PEG6000. (B) Expression patterns of *AtTGA4* at 1, 2, 3, 4, 5, 6, 7 d following treatment with low nitrogen stress (0.3 mM nitrogen). Values are means ± standard deviation (SD) (n = 3 independent experiments). (C) Visualization of 35S:AtTGA4-GFP and 35S:GFP control vectors transiently transformed into onion epidermal cells by particle bombardment by confocal microscopy. Bars = 10 μm.

were 1.5-fold higher for transgenic plants than for wild-type (Fig. 3D). Under drought stress conditions, plants often show physiological adaptation, manifested by higher POD (peroxidase) activity and proline content. There were no differences in POD activity (Fig. 3F), whereas transgenic plants showed higher proline contents than wild-type plants (Fig. 3E). Conversely, drought stress resulted in reduced chlorophyll concentrations in all plants, but under drought conditions higher chlorophyll contents were detected in transgenic plants than in wild-type (Fig. 3G). Thus overexpression of *AtTGA4* improved drought resistance in transgenic *Arabidopsis*.

3.4. Higher levels of nitrate transport and assimilation activity contribute to enhanced drought resistance in AtTGA4 transgenic plants

Assuming that overexpression of *AtTGA4* improved tolerance to both drought stress and adaptation to nitrogen starvation in transgenic plants, we speculated that nitrogen uptake might contribute to drought resistance in transgenic *AtTGA4* plants. To verify the hypothesis, we determined the total nitrogen content, enzymic activities of NIR and GS, and expression of the nitrate transporter genes in transgenic and wild type plants. Although total nitrogen content of all plants decreased after drought treatment, higher total nitrogen contents were detected in transgenic plants than in wild-type (Fig. 4A), suggesting that drought stress reduced nitrogen uptake, and that expression of *AtTGA4* led to higher nitrogen accumulation under drought stress. To further determine the effect of *AtTGA4* on nitrogen metabolism under drought stress, the activities of key enzymes NIR, which is involved in nitrate (NO^{3-}) assimilation, and GS, which is involved in ammonium (NH^{4+}) assimilation, were analyzed [36,37]. Transgenic plants had higher NIR activity than wild type plants, whereas there was no obvious difference in GS activity between transgenic and wild type plants (Fig. 4B, C). Expression of genes *NIR2.1* and *NIR2.2*, encoding nitrate reductase in transgenic plants, was also higher than in wild type plants, whereas no significant expression difference in the activity of *GLN1.4*, encoding glutamine synthetase, was observed (Fig. 4D). This implied that AtTGA4 mediated nitrate assimilation under drought stress rather than ammonium nitrogen assimilation. Expressions of dual affinity nitrate transporter *NRT1.1* and high-affinity nitrate transporters *NRT2.1*, *NRT2.2* and *NRT2.5* were also measured [38,39]. Expressions of *NRT2.1* and *NRT2.2* were higher in transgenic plants than in wild-type plants, whereas no obvious differences in expression of *NRT1.1* and *NRT2.5* were observed between transgenic and wild type plants (Fig. 4D). Thus overexpression of *AtTGA4* improved nitrate assimilation and transport processes under drought stress, and contributed to the enhanced drought resistance in transgenic plants.

4. Discussion

Drought stress inhibits plant growth by reducing water availability and restricting nitrogen uptake, transport and redistribution

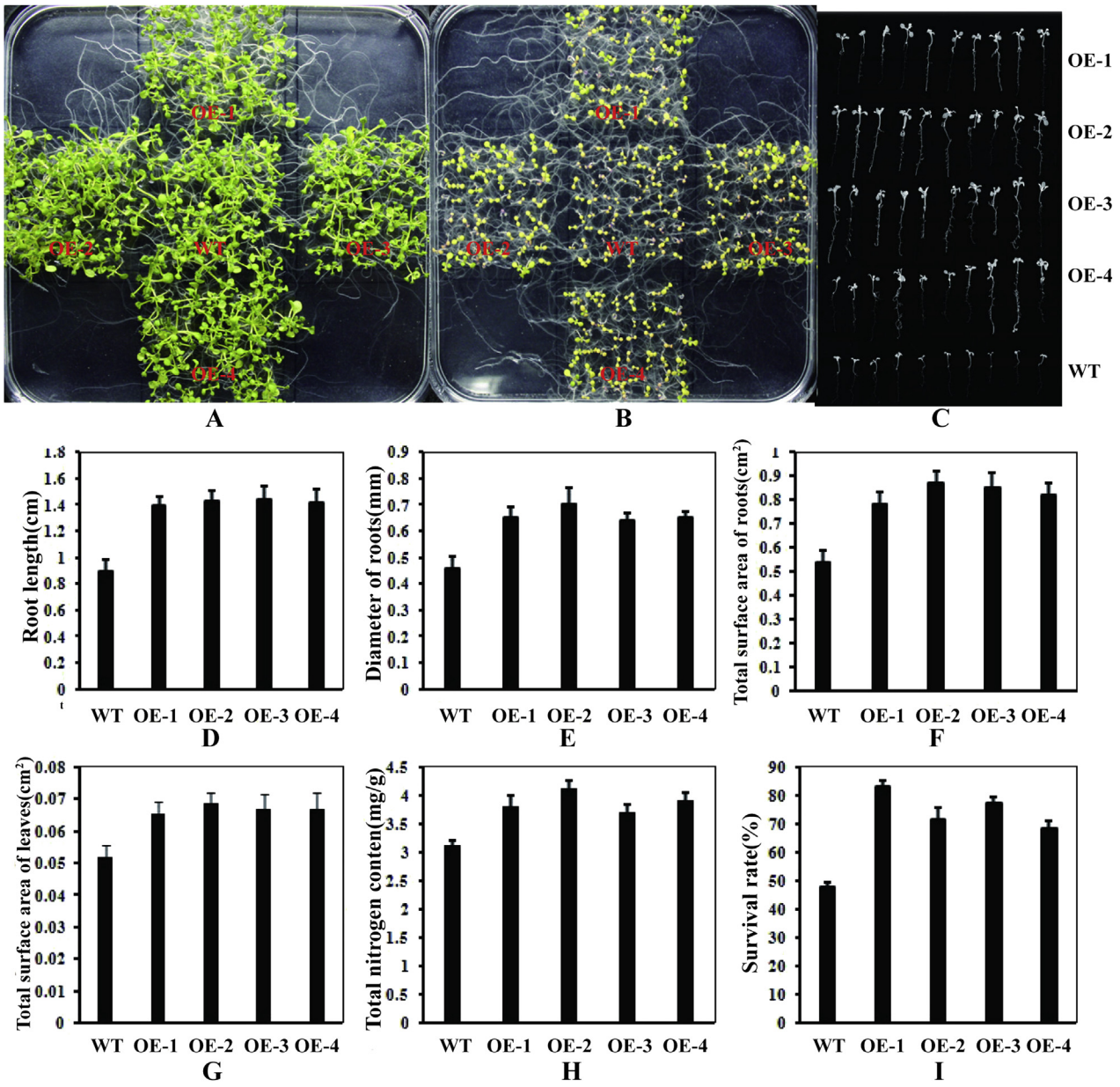

Fig. 2. Overexpression of *AtTGA4* improved adaptation to nitrogen starvation in transgenic *Arabidopsis* plants. (A) OE (overexpressing) and WT (wild-type) plants grown on MS medium. (B) OE and WT seedlings treated with low nitrogen stress (0.3 mM nitrogen). (C) Individual plant images after low nitrogen stress treatment. Statistical analysis of data for OE and WT plants are shown for root length (D), root diameter (E), total root surface area (F) and total leaf surface area (G). OE and WT plants were grown on MS medium and transferred to Hoagland's solution containing 0.3 mM nitrogen after 7 days. Results for total nitrogen content (H) and survival rate (I). Values are means ± standard deviation (SD) (n = 3 independent experiments).

[40]. Plants decrease nitrogen uptake with declining soil moisture, due to reduced nutrients coming from decomposition and mineralization [41,42], and restricted nutrient diffusion and movement in the soil [43]. Transpiration rate was also altered in response to drought, thus decreasing forces driving nutrients through the soil to the rhizosphere and though roots to shoots [44]. This suggests that drought stress is not due to water stress alone, but is accompanied by nutritional stress due to water deficit. This is consistent with previous reports suggesting that nitrogen application alleviates the negative impact of drought stress on plants. In the present research the total nitrogen content increased in transgenic *AtTGA4* plants, and expression of *NRT2.1* and *NRT2.2*, that function as transporters of nitrogen uptake from soil to roots, also increased under drought stress compared to wild type. Proline accumulation was higher in transgenic plants compared to wild type under drought stress, agreeing with previous reports that proline accumulation contributes to drought resistance. The activity of the key nitrite reductase (*NIR*) enzyme involved in nitrate assimilation was also higher in transgenic *AtTGA4* plants than that in wild type. These results increase our understanding of the regulatory networks involved in plant responses to drought stress and provide a new approach to improve crop tolerance to drought and limited nitrogen stress simultaneously through genetic transformation with *AtTGA4*.

Although the TGA subfamily belongs to the bZIP transcription factor family in *Arabidopsis*, TGA with the conserved domain "Yx_2RL [RQ]ALSS[LS]W" is distinct from other members of the bZIP family [14]. Phylogenetic analysis showed that AtTGA1 was most similar to

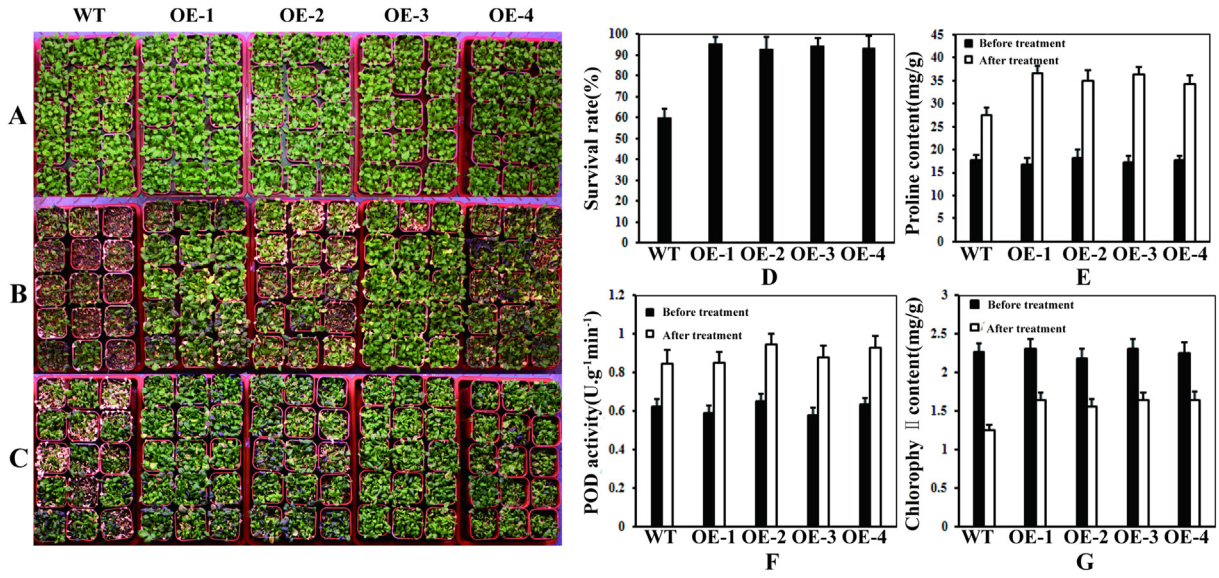

Fig. 3. Overexpression of *AtTGA4* improved drought resistance in transgenic *Arabidopsis* plants. Plants were grown on MS medium, and then seven-d-old OE and WT seedlings were placed in the greenhouse for 10 days (A); water was withheld water for 18 days (B), followed by rewatering for 5 days (C). Terminal survival rates (D), and various physiological parameters, including proline (E) and chlorophyll (G) contents, and POD activity (F) were assessed. Values are means ± standard deviation (SD) (n = 3 independent experiments).

AtTGA4 in the TGA subfamily, with an amino acid sequence similarity of 85.05% and sharing a similar conserved bZIP domain (Fig. S2). Previous work had shown that *AtTGA1* was functionally redundant in the presence of *AtTGA4* at least for phenotypes of pathogen resistance and low nitrogen stress tolerance [16,17,31]. We did not observe an obvious phenotype difference between *tga4* mutants and wild type plants under low nitrogen stress (Fig. S3), supporting the contention that *AtTGA4* and *AtTGA1* were functionally redundant at least in regard to low nitrogen stress response. *NRT2.1* and *NRT2.2* are direct downstream genes regulated by *AtTGA1* [31], but there was no evidence to indicate that these nitrate transporter genes were regulated by *AtTGA4*. In this study, expressions of *NRT2.1* and *NRT2.2* were up-regulated in transgenic *AtTGA4* plants compared with WT plants under drought

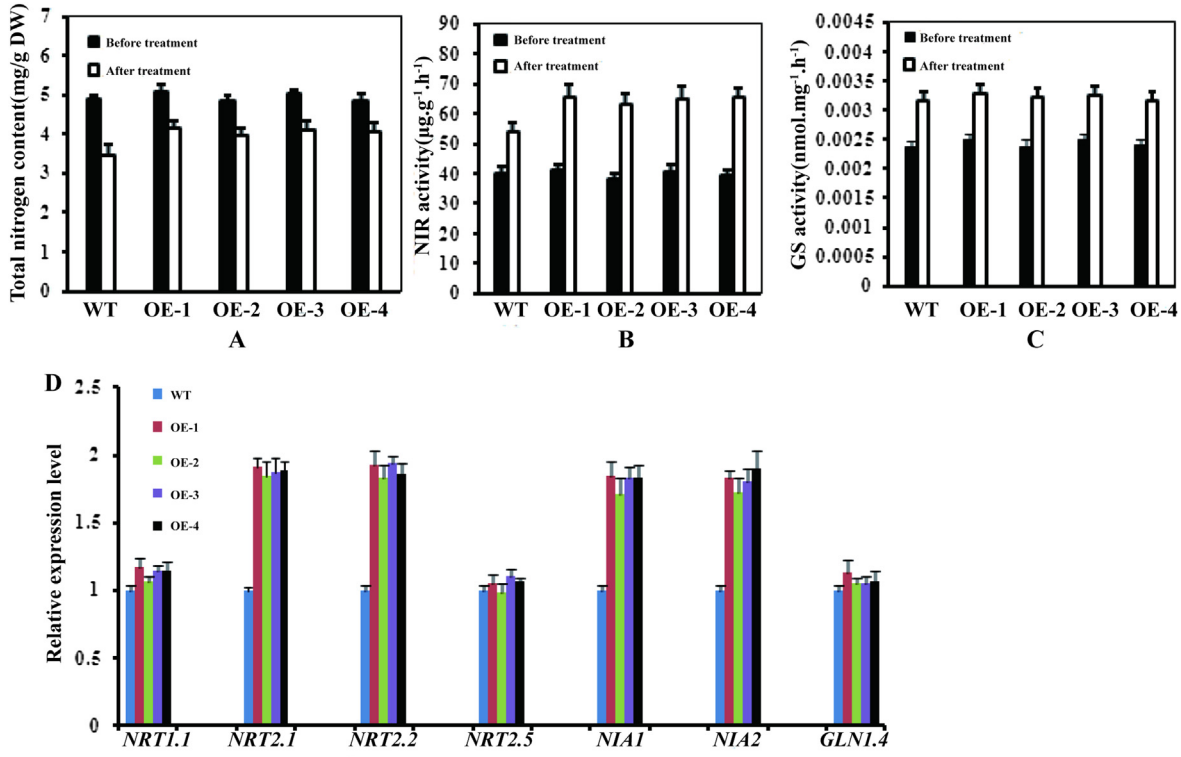

Fig. 4. Higher levels of nitrate transport and assimilation activity contribute to enhanced drought resistance of transgenic *AtTGA4* plants. Total nitrogen content (A) and related enzyme activities, including NIR (B) and GS (C) activities were measured. Expressions of nitrate transporter genes, including *NRT1.1*, *NRT2.1*, *NRT2.2*, *NRT2.5*, *NIA1*, *NIA2*, and GLN1.4 (D), were compared for OE and WT plants. Values are means ± standard deviation (SD) (n = 3 independent experiments).

stress, indicating that these genes act downstream of *AtTGA4* in *Arabidopsis*. Expression of other nitrate transporter genes, such as *NRT1.1*, was similar in transgenic and wild type plants, consistent with previous results indicating that *NRT1.1* is a sensor in nitrogen signaling [45] and suggesting that *NRT1.1* functions independently or upstream of *AtTGA4*.

Conflict of interest

None.

Acknowledgments

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Transparency document

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.009.

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