Plant Biotechnology 34, 151–158 (2017)
DOI: 10.5511/plantbiotechnology.17.0727a

Short Communication

The chimeric repressor for the GATA4 transcription factor improves tolerance to nitrogen deficiency in *Arabidopsis*

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Received July 2, 2017; accepted July 27, 2017 (Edited by T. Mizoguchi)

Abstract Nitrogen limits crop yield, but application of nitrogen fertilizer can cause environmental problems and much fertilizer is lost without being absorbed by plants. Increasing nitrogen use efficiency in plants may help overcome these problems and is, therefore, an important and active subject of agricultural research. Here, we report that the expression of the chimeric repressor for the GATA4 transcription factor (*35S:GATA4-SRDX*) improved tolerance to nitrogen deficiency in *Arabidopsis thaliana*. *35S:GATA4-SRDX* seedlings were significantly larger than wild type under nitrogen-sufficient and -deficient conditions (10 and 0.5 mM NH_4NO_3, respectively). *35S:GATA4-SRDX* plants exhibited shorter primary roots, fewer lateral roots, and higher root hair density compared with wild type. The expression levels of *NITRATE TRANSPORTER 2.1*, *ASPARAGINE SYNTHETASE* and *NITRATE REDUCTASE 1* were significantly higher in roots of *35S:GATA4-SRDX* plants than in wild type under nitrogen-sufficient conditions. Under nitrogen-deficient conditions, the expression of genes for cytosolic glutamine synthetases was upregulated in shoots of *35S:GATA4-SRDX* plants compared with wild type. This upregulation of nitrogen transporter and nitrogen assimilation-related genes might confer tolerance to nitrogen deficiency in *35S:GATA4-SRDX* plants.

Key words: *Arabidopsis*, chimeric repressor, nitrogen use efficiency, transcription factor, tolerance.

Nitrogen is an essential macronutrient for plant growth and development, and a major factor limiting agricultural production. Plants absorb and utilize ammonium (NH_4^+) and nitrate (NO_3^-) from soil (Crawford and Forde 2002; Kronzucker et al. 1997; Marschner 1995). Nitrogen levels affect crop productivity by modulating the expression of genes that affect leaf development, root architecture, senescence, flowering, and metabolite biosynthesis (Diaz et al. 2008; Rubin et al. 2009; Stitt et al. 2002; Walch-Liu et al. 2000; Wang et al. 2004; Zhang and Forde 1998). Adding nitrogen fertilizer can boost crop yields, but such inputs are costly and plants fail to use 50–70% of nitrogen provided as fertilizer. Nitrogen loss can cause soil and water pollution, and may contribute to global warming. Therefore, increasing nitrogen use efficiency (NUE) remains a crucial issue for agriculture and plant nutrient research.

Ongoing work has identified factors that regulate nitrogen uptake, translocation, and assimilation (Masclaux-Daubresse et al. 2010), including enzymes such as NITRATE TRANSPORTERs (NRTs), AMMONIUM TRANSPORTERs (ATMs), GLUTAMINE SYNTHETASEs (GLNs) and ASPARAGINE SYNTHETASEs (ASNs) (Masclaux-Daubresse et al. 2010; Vidal and Gutiérrez 2008). In addition to these enzymes, the transcription factors that regulate the expression of nitrogen-responsive genes have been analyzed. NODULE INCEPTION (NIN) functions as a key regulator of the symbiotic nitrogen fixation pathway in legumes such as *Lotus japonicus*, and NIN-Like proteins were identified as transcription factors that interact with *cis*-elements conserved in promoters of nitrate-responsive genes in *Arabidopsis thaliana* (Konishi and Yanagisawa 2013).

Several studies have manipulated transcription factor expression in attempts to enhance NUE. For example, in *Arabidopsis*, ectopic expression of *Dof1*, which regulates the expression of genes related to organic acid metabolism, resulted in increased growth under low-nitrogen conditions through the accumulation of amino acids (Kurai et al. 2011; Yanagisawa et al. 2004). In this report, we attempted to identify novel transcription factors involved in tolerance to nitrogen deficiency by screening *Arabidopsis* lines that express chimeric repressors (CRES-T) for transcription factors. Many plant transcription factors are structurally and functionally redundant and a single-gene knock-out

This article can be found at http://www.jspcmb.jp/
Published online September 16, 2017

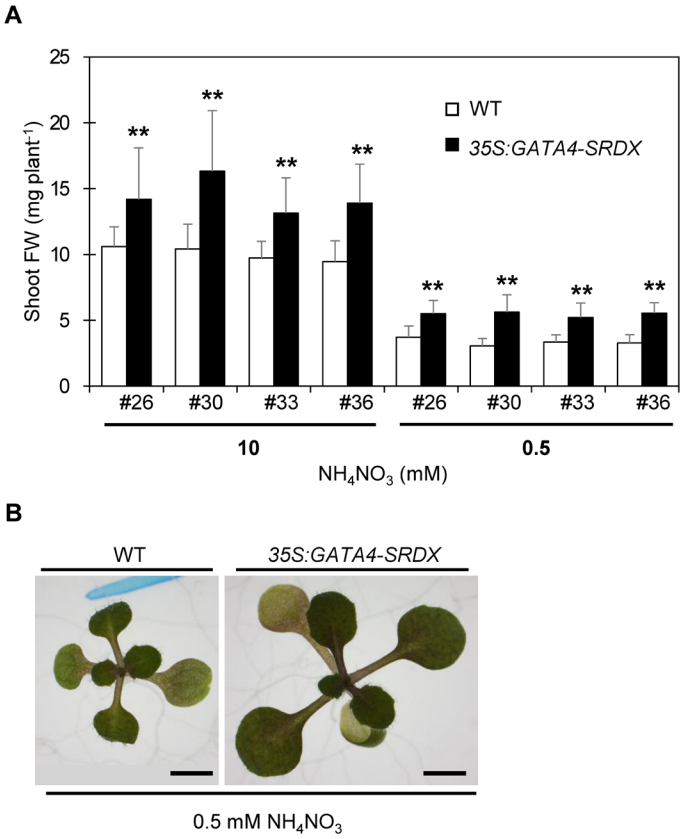

Figure 1. Phenotype at 14 days after sowing (DAS) of *35S:GATA4-SRDX* and wild type seedlings. (A) Comparison of shoot fresh weight (FW) between wild type (WT) and *35S:GATA4-SRDX* plants under nitrogen-sufficient (10 mM NH_4NO_3) and nitrogen-deficient (0.5 mM NH_4NO_3). Open and closed bars indicate wild type and *35S:GATA4-SRDX* plants, respectively. An Arabic numeral with '#' symbol under each closed bar represents an independent line of *35S:GATA4-SRDX* plants. Values are means±SD (n=16–23). Double asterisks indicate significant differences at $p<0.01$ in t-test when compared to wild type grown in each nitrogen condition. (B) Photos of 14 DAS wild type and independent line #30 of *35S:GATA4-SRDX* plants grown under nitrogen-deficient conditions. Scale bar; 2 mm.

often fails to exhibit an informative phenotype. However, in the CRES-T gene silencing system, fusion to the SRDX repression domain (SUPERMAN Repression Domain X) converts a transcription factor to a strong repressor that dominantly represses the target genes, producing phenotypes similar to loss-of-function of its redundant transcription factor genes (Hiratsu et al. 2003).

To screen our set of CRES-T lines for *Arabidopsis* transcription factors, we used Murashige-Skoog solid medium containing 0.5 mM NH_4NO_3 and 10 mM NH_4NO_3 as deficient and sufficient conditions, respectively. We screened for CRES-T lines that exhibit different sizes from wild type under nitrogen-deficient conditions. We identified a CRES-T line that produced bigger seedlings under nitrogen-deficient conditions. The fresh weight of 14-day-old seedlings of the CRES-T line was significantly higher than that of wild type under both nitrogen-sufficient and -deficient conditions (Figure 1). Genome PCR analysis revealed the transcription factor of the chimeric repressor to be GAT A4 (AT3G60530; *35S:GATA4-SRDX*). Therefore, we considered that *35S:GATA4-SRDX* plants are tolerant to nitrogen

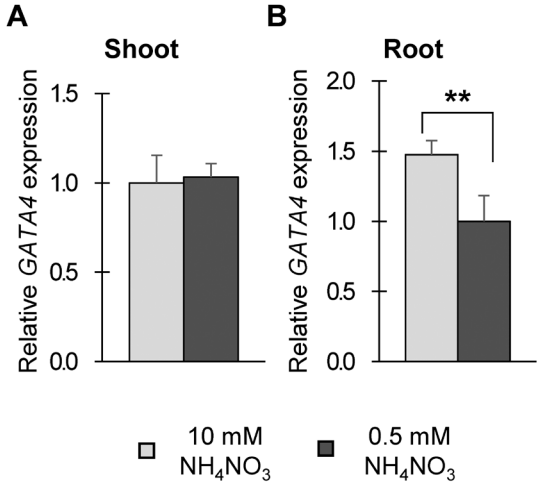

Figure 2. *GATA4* expression in response to different nitrogen conditions. Relative expression of *GATA4* in shoots (A) and roots (B) of wild type at 13 DAS grown under different nitrogen conditions analyzed by qRT-PCR, and all expression levels were normalized to that of *PP2AA3* (At1g3320), reference gene. Values are means±SD of four biological replicates. Double asterisks indicate significant difference at $p<0.01$ in t-test when compared to plants grown in nitrogen-sufficient conditions (10 mM NH_4NO_3).

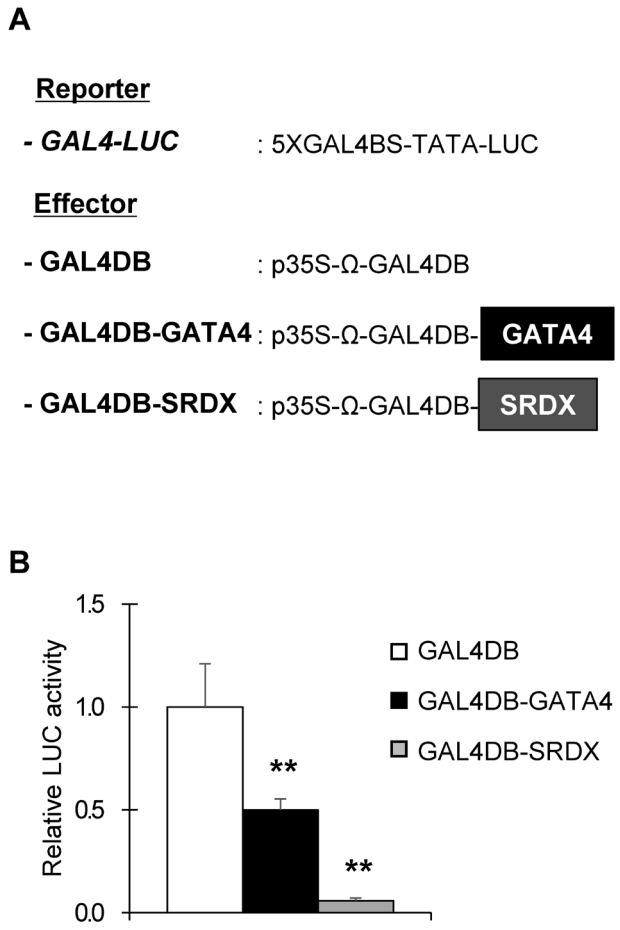

Figure 3. Transient effector–reporter analysis of GATA4 transcriptional activity. (A) Schematic representation of the constructs used in transient assay in *Arabidopsis* leaf protoplasts. The reporter construct contains firefly luciferase (*LUC*) driven by the promoter containing 5×Gal4 DNA-binding sequence (GAL4BS) and a TATA sequence. Each effector construct contains a Gal4 DNA-binding domain (GALDB) and TMV Omega leader sequence (Ω) driven by the CaMV 35 S promoter (p35S). The effector construct with the SRDX-fused Gal4 DNA-binding domain (*GALDB-SRDX*) was used as a positive control for repression. (B) Relative LUC reporter activity when each effector was co-transfected into leaf protoplasts. Values are means±SD of six technical replicates. Double asterisks indicate significant difference at $p< 0.001$ in Dunnett's test when compared with negative control (*GAL4DB*).

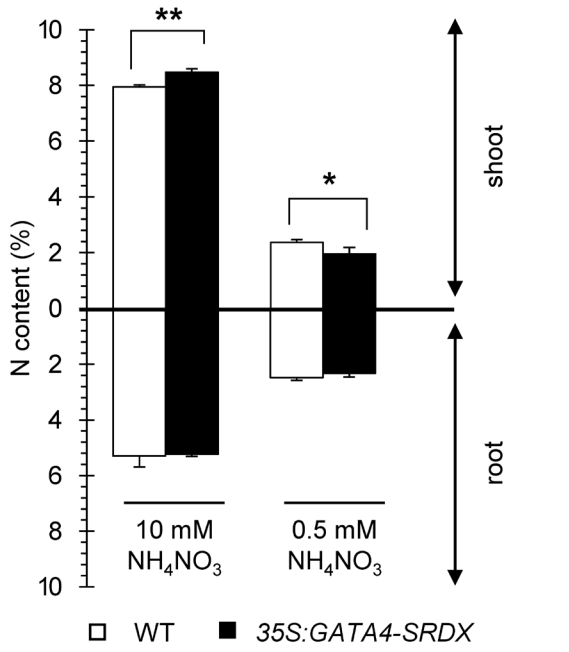

Figure 4. Nitrogen content in shoots and roots of wild type and *35S:GATA4-SRDX* plants. Plants were grown under different nitrogen conditions for 15 DAS. Values are means±SD of four independent biological replicates. Single and double asterisks indicate significant difference at $p<0.05$ and 0.01, respectively, in *t*-test when compared to wild type grown in each nitrogen condition.

deficiency.

GAT A4 is a transcription factor that belongs to the GAT A family and possesses a C_2C_2 zinc finger DNA binding domain that binds to the (A/T)GAT A(A/G) motif (Orkin 1992). The *Arabidopsis* genome has 29 genes encoding GAT A transcription factors (Manfield et al. 2006; Riechmann et al. 2000). *Arabidopsis GATA4* is expressed in all tissues including root, stem, leaf, flower, and silique at the reproductive stage and especially upregulated under darkness (Manfield et al. 2006). We analyzed whether nitrogen deficiency affects the expression level of *GATA4* by qRT-PCR and found that the expression of *GATA4* was moderately suppressed in roots under nitrogen-deficient conditions, but we observed no reduction in shoots (Figure 2).

To analyze the molecular activity of GAT A4, we performed transient expression assays using a luciferase reporter gene (*LUC*) fused to a promoter containing the Gal4 DNA binding site (*GAL4:LUC*) and an effector in which the coding region of *GATA4* was fused to the Gal4 DNA-binding domain (*GAL4DB*) driven by the CaMV 35S promoter (*35S:GAL4BD-GATA4*) in protoplasts isolated from leaf epidermal cells of *Arabidopsis*. The transient assay showed that the 35S:GAL4DB-GAT A4 effector significantly suppressed GAL4:LUC activity, compared with the control 35S:GAL4DB effector (Figure 3), suggesting that GAT A4 appears to function as a repressor.

The nitrogen content of *35S:GATA4-SRDX* shoots (about 8.5%) was significantly higher than that of wild type (about 7.9%) under nitrogen-sufficient conditions (Figure 4). By contrast, under nitrogen-deficient conditions, the nitrogen content of *35S:GATA4-SRDX* shoots (about 2.0%) was lower than that of wild type (about 2.4%, Figure 4). These results suggest that NUE is enhanced in *35S:GATA4-SRDX* plants to maintain larger shoot biomass, even under low nitrogen.

To examine the mechanisms by which *35S:GATA4-SRDX* plants tolerate nitrogen deficiency, we analyzed the expression of genes related to nitrogen transport and assimilation. *AMT1.1*, which codes for a high-

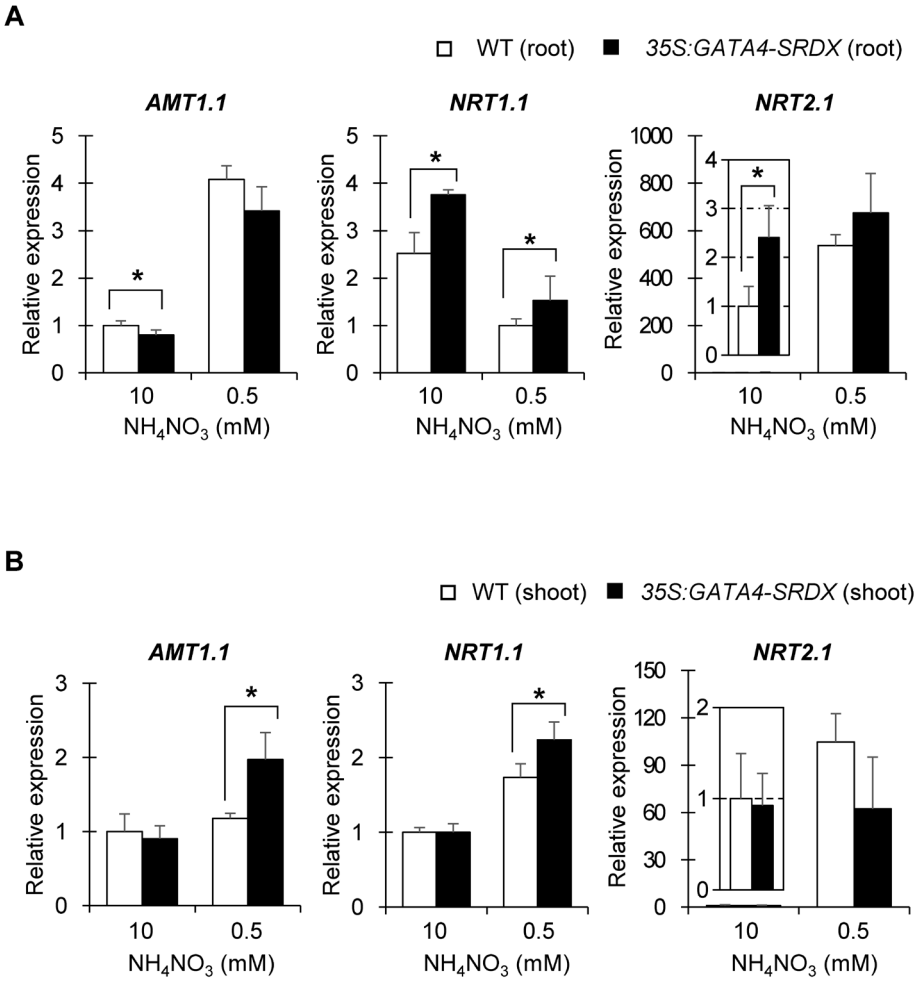

Figure 5. Expression of nitrogen transporter genes. Gene expression in roots (A) and shoots (B) of 13 DAS plants grown under different nitrogen conditions, measured by qRT-PCR, and all expression levels were normalized to that of *PP2AA3* (At1g3320), reference gene. Values are means±SD of four independent biological replicates. Single and double asterisks indicate significant differences at $p<0.05$ and 0.01, respectively, in *t*-test when compared to wild type grown in each nitrogen condition.

affinity ammonium transporter, is de-repressed in roots under nitrogen-deficient conditions, but upregulated in shoots under nitrogen-sufficient conditions (Engineer and Kranz 2007; Gazzarrini et al. 1999). Nitrate induces *NRT1.1*, which codes for a dual-affinity nitrate transporter, and *NRT2.1*, which codes for a high-affinity transporter, but they usually display opposite expression patterns under nitrogen-deficient conditions. In roots under nitrogen deficiency, *NRT1.1* is down-regulated and *NRT2.1* is dramatically induced (Forde 2000; Liu et al. 1999; Orsel et al. 2002; Wang et al. 1998). Our qRT-PCR analyses showed that *NRT1.1* and *NRT2.1* were highly expressed in roots of *35S:GATA4-SRDX* plants compared to wild type under nitrogen-sufficient conditions (Figure 5A). *NRT1.1* expression was higher in roots of *35S:GATA4-SRDX* plants than in wild type, even though its expression was suppressed in wild-type and *35S:GATA4-SRDX* roots by nitrogen deficiency (Figure 5A). *NRT2.1* expression was highly increased by nitrogen deficiency in roots of wild-type and *35S:GATA4-SRDX* plants, but did not show a significant difference between wild type and *35S:GTATA4-SRDX* (Figure 5A). We also found that the expression of *AMT1.1* was upregulated in shoots of *35S:GATA4-SRDX* plants compared to wild type even under nitrogen-deficient conditions, and *NRT1.1* was induced higher in shoots of *35S:GATA4-SRDX* plants than in wild type (Figure 5B).

Glutamine synthetases assimilate ammonium to glutamine and re-assimilate ammonia released by photorespiration or protein modification during senescence (Fuentes et al. 2001; Krapp 2015; Xu et al. 2012). The expression of genes encoding the cytosolic glutamine synthetases GLN1;1, GLN1;2, GLN1;3, and GLN1;4, was induced to higher levels in shoots of *35S:GATA4-SRDX* plants than in wild type in response to nitrogen deficiency (Figure 6A). In addition, the absorbed nitrate from soil to plant roots is reduced to nitrite by nitrate reductase, and NIA1 is one of isoforms of nitrate reductases in *Arabidopsis* (Wilkinson and Crawford 1993). The expression of *NIA1* was higher in

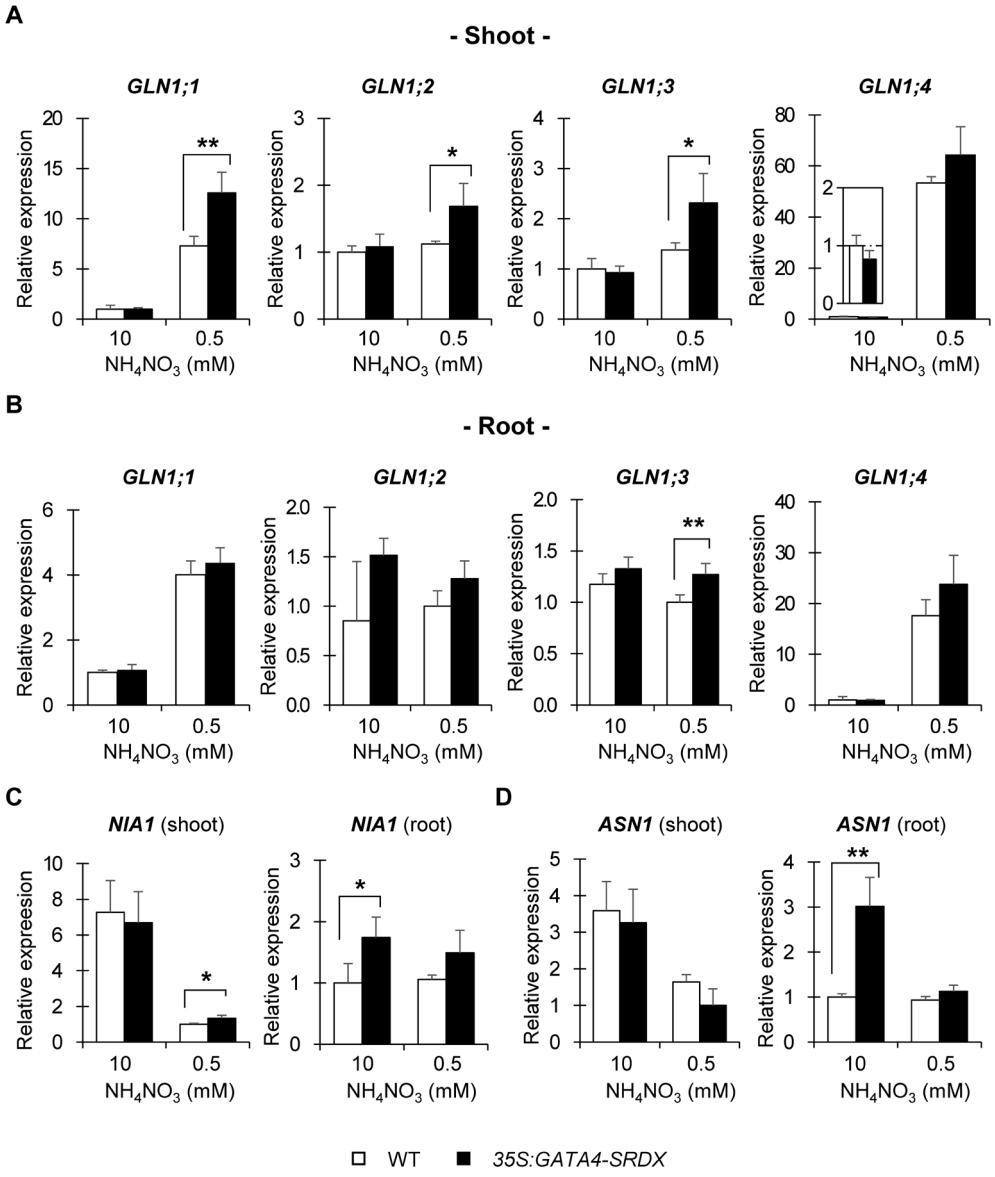

Figure 6. Expression of nitrogen assimilation-related genes. Gene expression in shoots (A) and roots (B) of 13 DAS plants grown under different nitrogen conditions, measured by qRT-PCR, and all expression levels were normalized to that of *PP2AA3* (At1g3320), reference gene. (A, B) Relative expression of *GLN1;1*, *GLN1;2, GLN1;3*, and *GLN1;4*. (C, D) Relative expression of *NIA1* (C) and *ASN1* (D) in shoots and roots. Values are means±SD of four independent biological replicates. Single and double asterisks indicate significant differences at $p < 0.05$ and 0.01, respectively, in *t*-test when compared to wild type grown in each nitrogen condition.

roots of *35S:GATA4-SRDX* plants than in wild type when the plants were grown in nitrogen-sufficient conditions (Figure 6C). This expression pattern is similar to the upregulation of *NRT1.1* and *NRT2.1*, which are induced by nitrate in roots of *35S:GATA4-SRDX* plants grown in nitrogen-sufficient conditions (Figure 5B). *ASN1*, which encodes asparagine synthetase, plays an important role in nitrogen assimilation and translocation together with glutamine synthetase (Carvalho et al. 2003; Good et al. 2004; Miflin and Lea 1976), was highly upregulated in *35S:GATA4-SRDX* roots, compared with wild type under nitrogen-sufficient conditions, but the two genotypes showed similar expression levels under nitrogen-deficient conditions (Figure 6D). These results indicate that the tolerance of *35S:GATA4-SRDX* plants to nitrogen deficiency may be due to differential upregulation of genes related to nitrogen transport and nitrogen assimilation in response to nitrogen status.

Our data base analyses showed that the *AMT1.1, NRT1.1, GLN1;1-4, NIA* and *ASN1* genes have putative GAT A binding sites in their 5' upstream regions. However, it is unlikely that those genes are the direct targets of GAT A4, because they were upregulated in *35S:GATA4-SRDX* plants. GAT A4-SRDX might repress the expression of negative regulator(s) that suppress the expression of several nitrogen metabolism-related genes.

Root architecture can be modified based on nitrogen availability. Under mild nitrogen deficiency, the growth

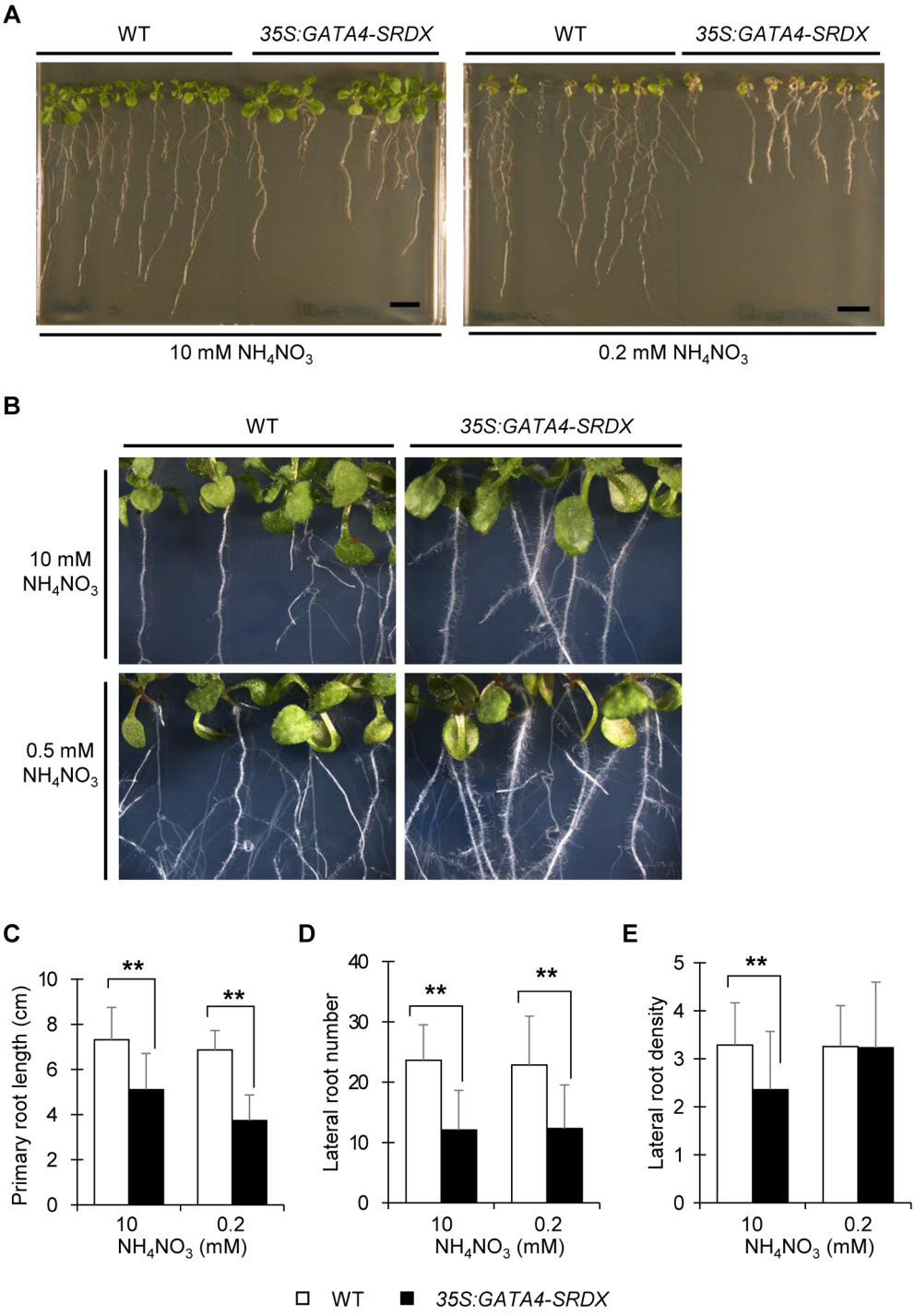

Figure 7. Root architecture of wild type and *35S:GATA4-SRDX* plants under different nitrogen conditions. (A) Photos of 15 DAS *35S:GATA4-SRDX* plants and wild type grown vertically under nitrogen-sufficient (left; 10 mM NH_4NO_3) and nitrogen-deficient (right; 0.2 mM NH_4NO_3) conditions. Scale bar; 1 cm. (B) Root hair phenotype of *35S:GATA4-SRDX* plants and wild type grown vertically under nitrogen-sufficient (top; 10 mM NH_4NO_3) and nitrogen-deficient (bottom; 0.5 mM NH_4NO_3) conditions, at 11 DAS. (C) Primary root length of 15 DAS seedlings of *35S:GATA4-SRDX* plants and wild type. (D) Number of lateral root initiations. (E) Lateral root density (number of lateral roots divided by primary root length). Values are means±SD (n=35–50). Total replicates of *35S:GATA4-SRDX* plants contain four independent lines. Double asterisks indicate significant difference at $p< 0.01$ in t-test when compared to wild type grown in each nitrogen condition.

of lateral roots is promoted to expand the root surface area for nitrogen uptake, but under severe nitrogen-deficient conditions, primary and lateral root growth are suppressed (Giehl and von Wirén 2014; Gruber et al. 2013). *35S:GATA4-SRDX* plants had shorter primary roots, fewer and shorter lateral roots, and more root hairs compared to wild type (Figure 7A, B). The growth of lateral and primary roots of *35S:GATA4-SRDX* plants was suppressed compared with wild type in nitrogen-sufficient and deficient conditions (Figure 7A, C–E). In

this experiment, we used more severe nitrogen deficient condition (0.2 mM instead of 0.5 mM), because such severe nitrogen deficiency clearly promoted the effect on root development and architecture of *35S:GATA4-SRDX* plants.

These results suggest that the GAT A4 transcription factor is involved in the regulation of root development and GAT A4-SRDX altered root architectures that affect nitrogen availability for plants. Increased root hair density may extend root surface area, thus increasing absorption of nutrients and water from soil compared to lateral roots (Gilroy and Jones 2000; Marschner 1995). As we demonstrated above, the expression of *ASN1* was highly increased in roots of *35S:GATA4-SRDX* plants (Figure 6B). Considering that *ASN1* is expressed in root hairs and in the elongation and maturation zones of the root (Brady et al. 2007; Schultz et al. 2017), the upregulation of *ASN1* in the roots of *35S:GATA4-SRDX* plants might be related to the root phenotype. Further experiments will be required to elucidate the molecular mechanisms responsible for relationship between nitrogen assimilation and root development including the roles of GAT A4 in root development and in nitrogen metabolism regardless of the alteration of root architecture.

Acknowledgements

We thank Ms. Yoshimi Sugimoto and Ms. Miyoko Yamada for their technical assistance.

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