Members of BTB Gene Family of Scaffold Proteins Suppress Nitrate Uptake and Nitrogen Use Efficiency[1]

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Development of crops with improved nitrogen use efficiency (NUE) is essential for sustainable agriculture. However, achieving this goal has proven difficult since NUE is a complex trait encompassing physiological and developmental processes. We thought to tackle this problem by taking a systems biology approach to identify candidate target genes. First, we used a supervised machine-learning algorithm to predict a NUE gene network in Arabidopsis (*Arabidopsis thaliana*). Second, we identified BT2, a member of the Bric-a-Brac/Tramtrack/Broad gene family, as the most central and connected gene in the NUE network. Third, we experimentally tested BT2 for a role in NUE. We found NUE decreased in plants overexpressing *BT2* gene compared to wild-type plants under limiting nitrate conditions. In addition, NUE increased compared to wild-type plants under low nitrate conditions in double mutant plants in *bt2* and its closely related homolog *bt1*, indicating a functional redundancy of BT1 and BT2 for NUE. Expression of the nitrate transporter genes *NRT2.1* and *NRT2.4* increased in the *bt1/bt2* double mutant compared to wild-type plants, with a concomitant 65% increase in nitrate uptake under low nitrate conditions. Similar to Arabidopsis, we found that mutation of the *BT1/BT2* ortholog gene in rice (*Oryza sativa*) *OsBT* increased NUE by 20% compared to wild-type rice plants under low nitrogen conditions. These results indicate BT gene family members act as conserved negative regulators of nitrate uptake genes and NUE in plants and highlight them as prime targets for future strategies to improve NUE in crops.

Nitrogen (N) is an essential macronutrient and a key element controlling plant growth, development, and productivity. Use of N-based fertilizers has increased more than 8-fold in the last 50 years to cope with increasing demands of agriculture and food production (Dawson and Hilton, 2011). Intensive use of N fertilizers is causing major detrimental impact on the ecosystem, including eutrophication of waters and increase of gaseous emissions of toxic N oxides and ammonia to the atmosphere (Ju et al., 2009; Lassaletta et al., 2014; Robertson and Vitousek, 2009). Moreover, excessive use of fertilizers is a major cost for farmers, which in turn affects the commercial price of vegetables and fruits. In this context, it is of paramount importance to design strategies to improve nitrogen use efficiency (NUE) for increased plant productivity in sustainable and environmentally responsible ways (Gutierrez, 2012).

NUE is a complex genetic trait and index that encompasses multiple metabolic, physiological, and developmental processes in plants exposed to a changing environment. Processes that govern NUE are broadly divided into two main categories: N uptake and N utilization efficiency, including assimilation, internal N transport, and remobilization (Gallais and Hirel, 2004; Hirel et al., 2007; Bi et al., 2009; Masclaux-Daubresse et al., 2010; Xu et al., 2012). NUE has been defined in various ways (Good et al., 2004), but yield (measured by grain, fruit, or forage depending on the crop) per unit of N available in the soil integrates all key parameters for evaluating fitness of crop cultivars and it is a common measure of NUE (Moll et al., 1982; Beatty et al., 2010; Kant et al., 2011; Gupta et al., 2012). Integrated N management strategies and overall better agricultural practices improved NUE over the last years (Jing et al., 2009). However, it is clear that improving crop genetics is key for better NUE worldwide.

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Many efforts are currently devoted toward defining target genes for generating crops with enhanced NUE (Crawford and Forde, 2002). Due to its essential role in N assimilation, Gln synthetase (GS) has been a prime target gene to improve NUE. Numerous studies reported overexpression of *GLN SYNTHETASE1* (*GS1*), the cytosolic isoform of GS, to improve NUE in different species such as tobacco (*Nicotiana tabacum*), maize (*Zea mays*), rice (*Oryza sativa*), and Arabidopsis (*Arabidopsis thaliana*; Eckes et al., 1989; Migge et al., 2000; Man et al., 2011). Overexpression of *GS1* showed positive effects on plant productivity in a few cases (Habash and Massiah, 2001; Obara et al., 2004; Martin et al., 2006). During N assimilation, GS works together with Gln oxoglutarate aminotransferase (GOGAT). Suppression of GOGAT isozymes (Fd-GOGAT and NADH-GOGAT) causes a decrease in tiller number, shoot dry weight, and yield in rice (Lu et al., 2011). Besides genes directly involved in N metabolism, overexpression of genes, including the sugar transport protein *STP13* of Arabidopsis (Schofield et al., 2009), the early nodulin gene (*OsENOD93-1*; Bi et al., 2009), and the peptide transporter/nitrate OsPTR9 (Fang et al., 2013) of rice, has been shown to positively affect production traits of the plants, as biomass, grain yield, or nitrogen content. In addition, overexpression of the transcription factor *DOF1* in Arabidopsis and rice resulted in plants with increased amino acid content, increased carbon skeleton production, and a reduction in Glc levels, suggesting a possible role for DOF1 in NUE (Yanagisawa et al., 2004). Recently, the transcription factor TaNAC2-5A was found as an interesting target to increase NUE in wheat (*Triticum aestivum*; He et al., 2015).While all these genes impact processes that are related to NUE, it is unclear whether alteration in expression of these genes leads to measurable changes in plant NUE.

We thought to find new target genes directly modulating NUE in Arabidopsis by using a systems approach. The standard systems approach comprises four steps: (1) data integration, (2) modeling, (3) hypothesis generation, and (4) experimental validation (Gutiérrez et al., 2005). We carried out the first three steps with the discriminative local subspaces (DLS) algorithm (Puelma et al., 2012). DLS generate an Arabidopsis gene network with a potential role in NUE. We used this model to predict that *BT2* is a central gene for NUE in Arabidopsis. *BT2* is a member of the Bric-a-Brac/Tramtrack/Broad (BTB) family of scaffold proteins in Arabidopsis (Gingerich et al., 2007) known to play a crucial role in both male and female gametophyte development (Robert et al., 2009). BT2 can activate telomerase expression in mature Arabidopsis leaves (Ren et al., 2007). Moreover, *BT2* gene expression is regulated by a number of signals including circadian regulation, sugar and nitrogen nutrients, hormones, cold, hydrogen peroxide, and wounding stress treatments (Mandadi et al., 2009). Based on this evidence, *BT2* has been proposed as a key component of a signaling network that integrates multiple internal and external inputs (Mandadi et al., 2009). Here, we provide evidence to support this hypothesis by demonstrating a key role for BT2 in NUE, a complex trait that results from integrating environmental and internal signals over the life cycle of the plant. Overexpression of BT2 reduces NUE, negatively affects primary root growth, and lowers plant biomass in Arabidopsis compared to wild-type plants under low nitrate conditions. In contrast, double mutant plants in *bt2* gene and its closest homolog *bt1* produced opposite phenotypes. These results indicate that BT1/BT2 are negative determinants of plant NUE and growth under low nitrate conditions. We found BT1/BT2 negatively affect nitrate uptake by down-regulating major components of the high affinity nitrate transport system in Arabidopsis. Similar results obtained in rice using a *BT1/BT2* ortholog mutant *Osbt* suggest BT proteins are part of a conserved mechanism controlling growth and NUE under N-limiting conditions in monocotyledonous and dicotyledonous plant species.

RESULTS

BT2 Is a Central Hub in Predicted NUE Gene Network of Arabidopsis

In order to identify candidate genes relevant for the control of NUE in Arabidopsis, we used the DLS algorithm (Puelma et al., 2012). DLS is a supervised machine-learning algorithm that uses available transcriptome and Gene Ontology (GO) data to infer functional gene networks (Puelma et al., 2012). DLS has been shown to outperform coexpression gene networks (Puelma et al., 2012) and is able to generate functional gene networks that integrate multiple biological processes by training on custom-made positive gene sets. The DLS output is a gene network that can be analyzed using standard network topology statistics and tools to pinpoint key genes for the regulation of the biological function of interest (Azuaje, 2014). Given that NUE is a complex process that integrates various biological processes, we defined a positive gene set using different biological processes that are known to impact or control plant NUE: nitrate assimilation (GO:0042128), nitrate transport (GO:0015706), ammonium transport (GO:0015696), ammonium response (GO:0060359), nitrogen response (GO:0019740), nitrate response (GO:0010167), regulation of seed development (GO:0080050), organ senescence (GO:0010260), endosperm development (GO:0009960), vegetative to reproductive phase transition of meristem (GO:0010228), vegetative phase change (GO:0010050), and seed maturation (GO:0010431). The union of all these GO terms resulted in a list with 220 genes that was used as the positive set for DLS. In addition, we used 2017 microarray experiments obtained from NASCArrays (ftp://uiftparabid.nottingham.ac.uk/NASCarrays/By_Experiment_ID/), including 3,911 features or experimental conditions. Using the positive set to train, DLS generated a network containing 654 genes (nodes; Supplemental Table 1) connected by functional predictions (edges; Fig. 1). DLS was able to pinpoint

overrepresented biological processes in the network that are known NUE determinants, such as senescence, response to nitrate, circadian cycle, or seed development. (Diaz et al., 2008; Masclaux-Daubresse and Chardon, 2011; Li et al., 2013). This suggests DLS can effectively predict genes involved in NUE.

Measuring node centrality and connectivity in biological networks allows identification of biologically informative genes (Azuaje, 2014, Moyano et al., 2015). DLS displays genes ranked by the normalized mean of node degree and betweenness centrality (BC). These two network statistics are frequently used to evaluate the importance of nodes in a network (Azuaje, 2014). Degree indicates the total number of nodes connected to a node, highlighting genes that are coexpressed with many other genes and hubs in the network. In contrast, BC measures the proportion of shortest paths between all pairs of nodes that pass through a specific node (Moyano et al., 2015). Nodes with high BC act as bottlenecks, which makes them essential for the flow of information and the overall connectivity and structure of the network. In the NUE network, BT2 (At3g48630) appears as the node with highest combined BC and degree, suggesting BT2 as the most important gene for the overall network structure and topology and the best candidate for future experimental validation of its role in controlling NUE (Fig. 1).

BT2 belongs to a family of BTB and TAZ DOMAIN proteins composed of five members (Robert et al., 2009) with BT1 (At5g63160) being the closest homolog with 80% sequence identity (Du and Poovaiah, 2004). Previous studies demonstrated BT1 and BT2 have functional redundancy and reciprocal transcriptional control during gametophyte development (Robert et al., 2009). Therefore, both BT1 and BT2 were selected for experimental validation as described below.

BT1 and BT2 Affect NUE in Arabidopsis Depending on External Nitrate Concentration

In order to determine the role of BT1 and BT2 in controlling NUE in Arabidopsis, we measured NUE using two different NUE indexes in wild-type, BT2 overexpressor plants (BT2OE), and *bt1/bt2* mutant plants under two contrasting nitrate concentrations: agronomic NUE (aNUE) and the nitrogen harvest index to harvest index ratio (NHI/HI). The aNUE measures seed amount per plant/N applied during the entire plant life cycle (Moll et al., 1982). NHI measures $N\%_{SEEDS}$*dry $weight_{SEEDS}$)/($N\%_{SEEDS}$ * dry $weight_{SEEDS}$ + $N\%_{BIOMASS}$ * dry $weight_{BIOMASS}$) and HI as (dry $weight_{SEEDS}$)/(dry $weight_{BIOMASS}$ + dry $weight_{SEEDS}$).

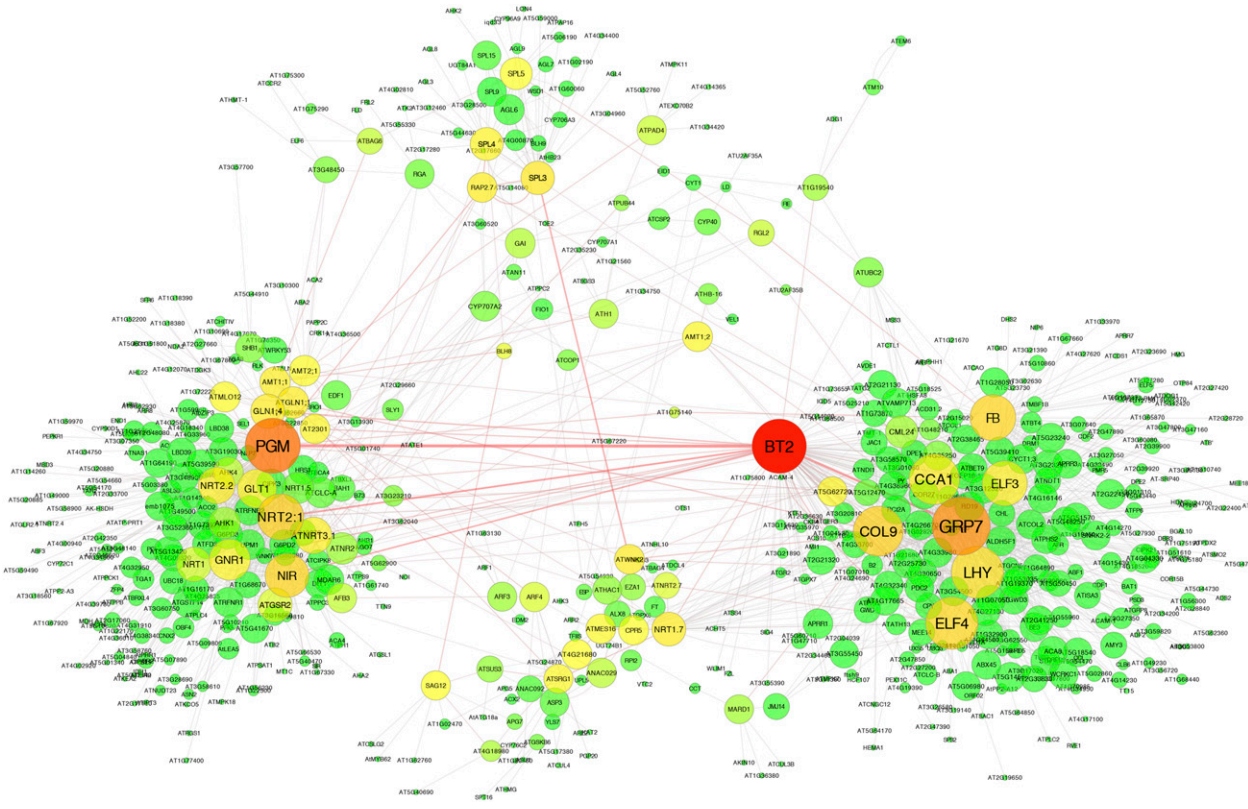

Figure 1. DLS analysis highlights BT2 as the central hub of the Arabidopsis NUE network. A NUE network of 654 genes was predicted as described in "Materials and Methods." We used Cytoscape software to visualize the resulting subnetwork in which genes are depicted as nodes and edges indicate functional interactions predicted by GENIUS. Node size is proportional to the degree of the node, and the node color indicates centrality ranging from yellow (low centrality) to red (high centrality).

This index provides a more physiological view of NUE in plants (Masclaux-Daubresse and Chardon, 2011). In a first approximation, we measured aNUE under a wide range of KNO_3 concentrations. As shown in Figure 2A, aNUE was significantly affected by external nitrate concentration in wild-type plants, with maximum aNUE obtained at 0.5 and 1 mM nitrate concentration. aNUE decreased as external nitrate concentration increased with a minimum observed at 20 and 30 mM nitrate (Fig. 2A). This contrasting effect of low or high N availability in plant development has been described previously (Lea and Azevedo, 2006; Lemaître et al., 2008; Ikram et al., 2012). In contrast to aNUE, Arabidopsis seed production was significantly ($P \leq 0.05$) increased by N concentration (Fig. 2B). Overexpression of BT2 decreased aNUE compared to wild-type plants, but only when plants were grown under low nitrate conditions (0.5 mM nitrate; Fig. 2E). No difference was observed between BT2 overexpressor and wild-type plants for NHI/HI ratio (Fig. 2F). *bt1* (Supplemental Fig. S1A) or *bt2* (Supplemental Fig. S1B) single mutants did not show differences in aNUE compared to wild-type plants, suggesting functional redundancy. In contrast, *bt1/bt2* double mutant plants exhibited higher aNUE (Fig. 2C) and NHI/HI (Fig. 2D) compared to wild-type plants under low nitrate conditions. These results indicate BT1/BT2 negatively impact NUE in Arabidopsis under limiting nitrate conditions.

In order to determine whether BT2 has a role in controlling use efficiency of other nutrients, we grew wild-type, *bt1/bt2* double mutant plants, and BT2 overexpressor lines under low (0.1 mM) and high (1 mM) concentrations of sulfate and phosphate. No significant differences were observed in *bt1/bt2* or BT2OE compared to wild-type plants for aNUE, NUE, or seed yield under contrasting phosphate or sulfate concentrations (Supplemental Fig. S2). These results indicate BT1/BT2 functions are not general for nutrient use but specific of NUE. We also found

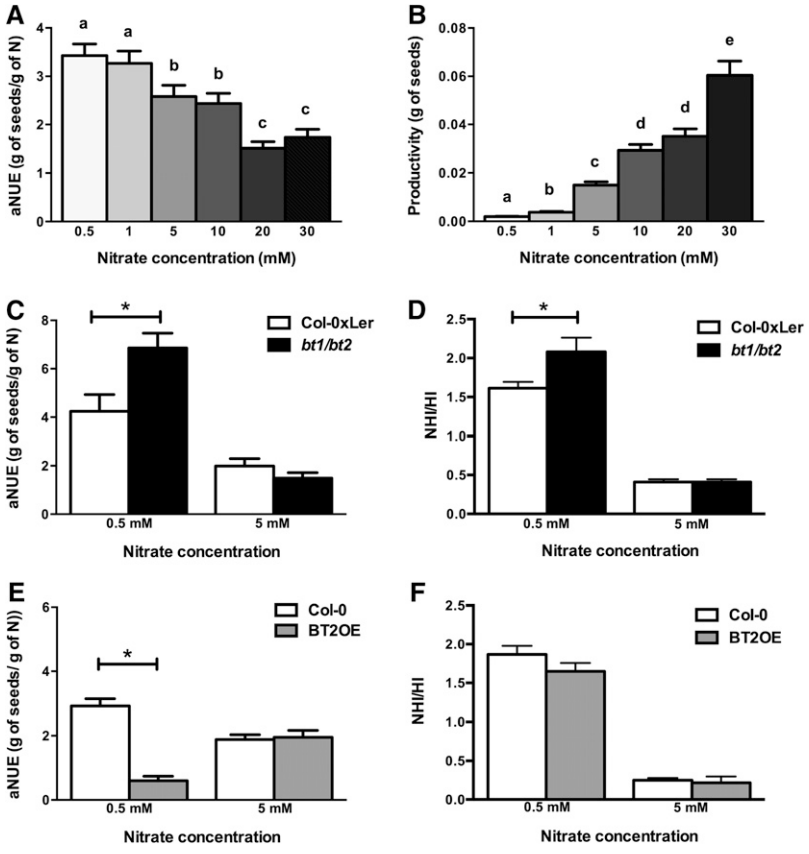

Figure 2. BT1/BT2 negatively affect NUE under low nitrate availability in Arabidopsis. Arabidopsis Col-0 ecotype was grown on an inert substrate and watered once a week with distilled water and once a week with nutrient solution without N supplemented with 0.5 mM KNO_3, 1 mM KNO_3, 5 mM KNO_3, 10 mM KNO_3, 20 mM KNO_3, or 30 mM KNO_3. All the seeds produced by each plant were collected and weighted. We show aNUE (A), calculated as grams seeds/grams of N and productivity (B), expressed as grams of seeds. Plants modified in BTB genes were grown on an inert substrate and watered once a week with distilled water and once a week with modified solution supplemented with 0.5 or 5 mM KNO_3 and two NUE indexes were measured. aNUE of wild-type and BT2 overexpressor Arabidopsis lines (C) or Col-0 × L*er* and *bt1/bt2* mutants (E) and NHI/HI in wild-type and BT2 overexpressor (D) or Col-0 × L*er* and *bt1/bt2* mutants (F). We show the mean and SE for three independent biological replicates with 12 plants each. Asterisks represent means that statistically differ ($P < 0.05$).

that changes in phosphate or sulfate availability affect phosphate use efficiency (Supplemental Fig. S2F) or sulfate use efficiency (Supplemental Fig. S2C) in wild-type plants, respectively. However, only changes in phosphate and not sulfate availability affects NUE or seed yield in wild-type plants (Supplemental Fig. S2, D and E).

BT1 and BT2 Affect Juvenile Growth of Arabidopsis under Low Nitrate Conditions

NUE has been shown to change during different developmental phases of Arabidopsis growth (Ikram et al., 2012; Masclaux-Daubresse and Chardon, 2011). In order to identify the developmental stages where BT1 or BT2 might have a more prominent impact over NUE, we monitored biomass in wild-type, BT2OE, and *bt1/bt2* mutant plants on a weekly basis during their entire life cycle. We found BT2OE plants have lower biomass compared to wild-type plants during the 2nd, 3rd, and 4th weeks after germination in the limiting condition (Fig. 3A). However, *bt1/bt2* double mutant plants exhibited higher biomass as compared to wild-type plants only during the 2nd week after germination (Fig. 3B) and only when plants were grown under low nitrate concentrations (Fig. 3C). Because of the observed timing of the phenotypes, we asked whether changes in *BT1* or *BT2* gene expression levels impacted developmental traits or transitions that occur during this period. We measured the day of cotyledon appearance and day of appearance of the first set of leaves as markers of early seedling development (Supplemental Fig. S3A), the leaf number of the first leaf with abaxial trichomes (Supplemental Fig. S3B), a morphological trait commonly used as markers of the juvenile to adult transition (Telfer et al., 1997), and the day of bolting (Supplemental Fig. S3C), as marker of reproductive phase transition (Hempel and Feldman,

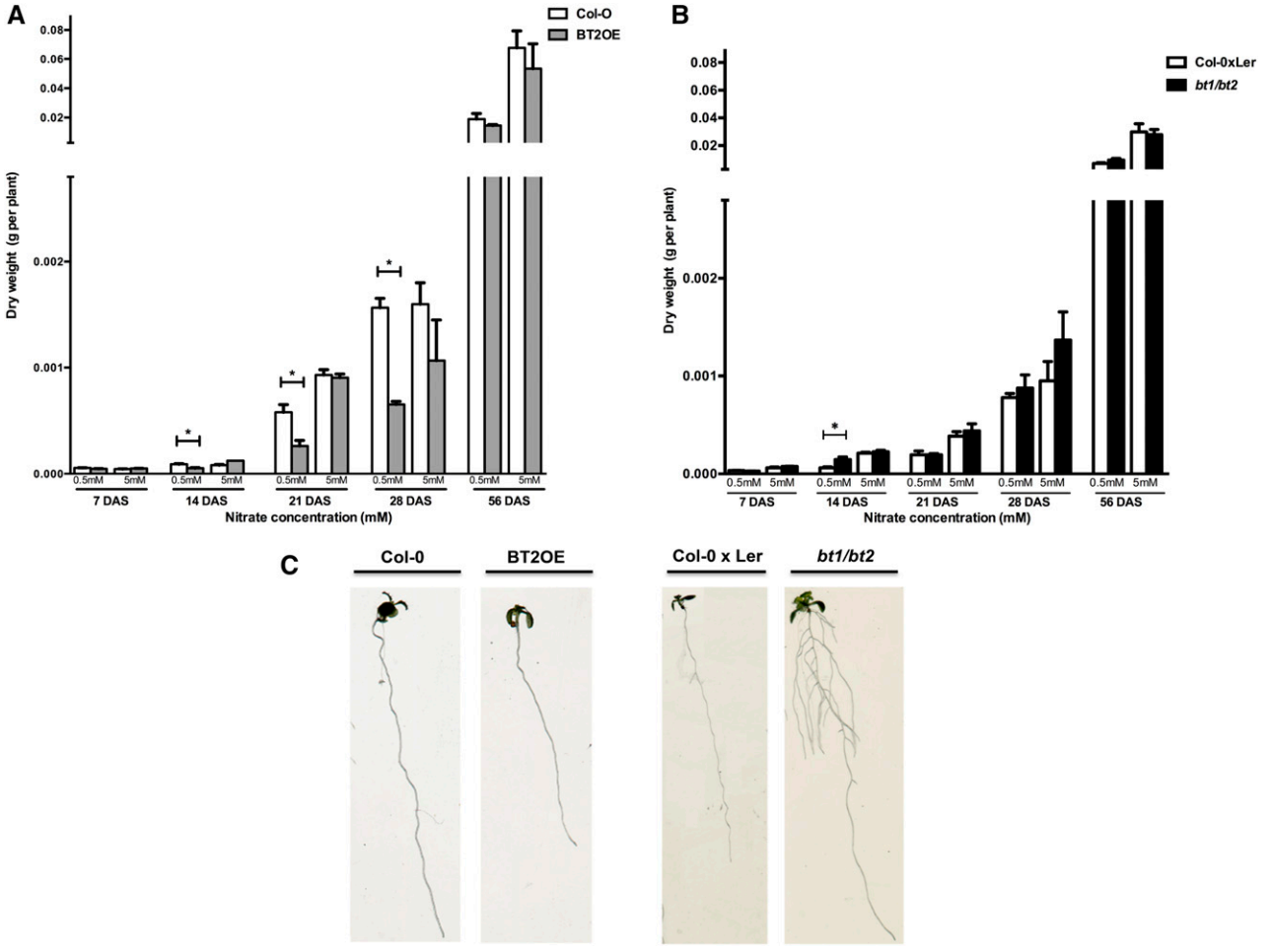

Figure 3. Altered expression of BT1/BT2 affects Arabidopsis biomass under low nitrate availability during early stages of plant development. Wild-type and BT2OE Arabidopsis plants (A) or wild-type and *bt1/bt2* plants (B) were grown on an inert substrate and watered once a week with distilled water and once a week with nutrient solution without N supplemented with 0.5 or 5 mM KNO_3 as the only nitrogen source. Biomass was measured as dry weight per plant. C, Image of 2-week-old Col-0, BT2OE, Col-0 × L*er*, and *bt1/bt2* seedlings grown under 0.5 mM KNO_3. We show the mean and SE for three independent biological replicates with at least 12 plants each. Asterisks show means that differ statistically ($P < 0.05$).

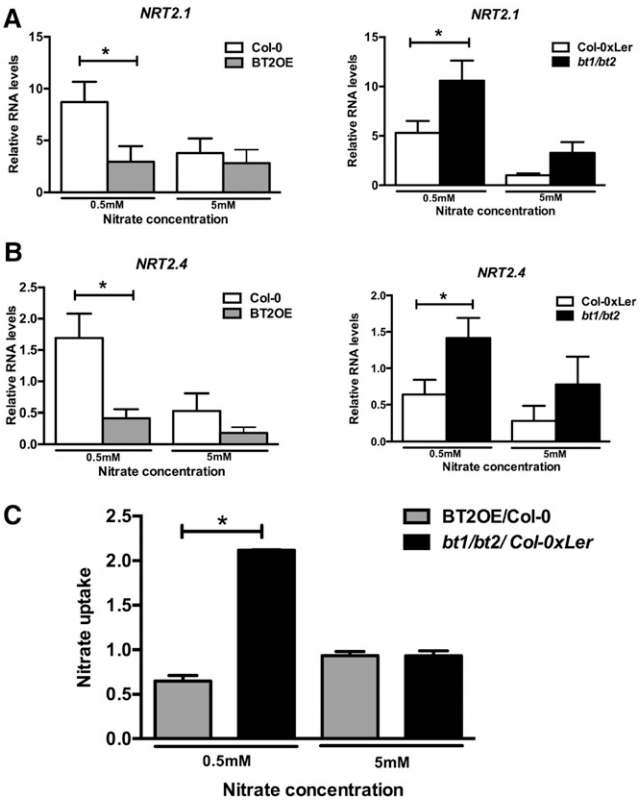

Figure 4. BT1/BT2 repressed expression of *NRT2.1* and *NRT2.4* genes and nitrate uptake under low nitrate availability. Col-0 and BT2OE or Col-0 × L*er* and *bt1/bt2* plants were grown for 2 weeks in agar plates of medium without N supplied with 0.5 or 5 mM of KNO_3. *NRT2.1* (A) and *NRT2.4* (B) transcript levels were analyzed by real-time qPCR in Arabidopsis seedlings. C, Col-0 and BT2OE and Col-0 × L*er* and *bt1bt2* plants were grown for 28 d and were treated with a nutrient solution without N supplied with 0.5 or 5 mM containing KNO_3 with 10% $^{15}NO_3$ enrichment (w/w). We show the ratio of nitrate uptake of BT2OE/Col-0 and *bt1/bt2*/Col-0 × L*er*. We show the mean and SE for three independent biological replicates with at least 12 plants each. The asterisk indicates means that significantly differ between control and treatment conditions ($P < 0.05$).

1994; Wilkinson and Haughn, 1995). We found true leaves were visible later in BT2OE and earlier in the *bt1/bt2* mutant compared to wild-type plants under low nitrate conditions (Supplemental Fig. S3A). No other developmental trait or transition was affected under the experimental conditions evaluated (Supplemental Fig. S3, B and C). The effect in the plant development is visually distinguishable in plants in the 2nd week (Fig. 3C). This indicates BT1 and BT2 have a role in the juvenile stage of plant development that can impact NUE.

BT1/BT2 Repress the Expression of High-Affinity Nitrate Transporters *NRT2.1* and *NRT2.4* and Nitrate Uptake

Our results indicate *BT1/BT2* function under low nitrate conditions to impact NUE. One of the main factors that can affect NUE in plants is the control of N uptake (Masclaux-Daubresse et al., 2010). In Arabidopsis, nitrate transporters from the NRT2 family are the main transporters involved in nitrate transport under low nitrate concentrations (Tsay et al., 1993; Huang et al., 1999; Filleur et al., 2001; Kiba et al., 2012). We evaluated the expression of the *NRT2.1*, *NRT2.2*, *NRT2.3*, *NRT2.4*, *NRT2.5*, *NRT2.6*, and *NRT2.7* genes under low and high nitrate conditions in wild-type, BT2OE, and *bt1/bt2* double mutant plants during the juvenile vegetative phase. We found *NRT2.1* (Fig. 4A) and *NRT2.4* (Fig. 4B) were differentially expressed in BT2OE and the *bt1/bt2* mutant compared with wild-type plants, specifically under low nitrate concentrations. The expression of the other NRT2 evaluated were not affected in BT2OE and in the *bt1/bt2* mutant (Supplemental Fig. S4, A–E). *NRT2.1* and *NRT2.4* have been described as the main high-affinity nitrate transporters involved in nitrate acquisition (Kiba et al., 2012). *NRT2.1* and *NRT2.4* gene expression levels were significantly lower in BT2OE compared to wild-type plants. In contrast, *NRT2.1* and *NRT2.4* gene expression was significantly higher in *bt1/bt2* than wild-type plants (Fig. 4, A and B). Moreover, we found nitrate uptake is affected in an opposite manner in the overexpressor and double mutant plants, consistent with the expression of *NRT2.1* and *NRT2.4* genes in these plants under low nitrate concentration (Fig. 4C). These results suggest the growth phenotype found for these plants might be due in part to misregulation of nitrate transport by NRT2.1 and NRT2.4 under low nitrate concentrations. These results indicate BT1 and BT2 control plant growth by controlling the expression of the NRT2.1 and NRT2.4 and, thus, nitrate transport under low nitrate concentrations.

Members of the BTB Family Play a Conserved Role in Regulating NUE in Rice Plants

To evaluate the significance of BT1 and BT2 as part of a conserved mechanism controlling NUE in plants, we examined the role of BTB orthologs in rice. We found Os01g68020 was the closest rice gene to BT1 and BT2 of Arabidopsis, and we termed it *OsBT* ("Materials and Methods"). We evaluated the phenotype in a mutant line in which *OsBT* gene is interrupted by a T-DNA insertion. The *Osbt* line (A38456, Donjing background) was isolated from the Postech collection (Jeon et al., 2000; Jeong et al., 2006) and has a T-DNA insertion in the second exon of *OsBT*. *Osbt* plants exhibit an increase in the number of tillers in the vegetative stage under limiting ammonium nitrate conditions compared to wild-type rice plants (Fig. 5A). Interestingly, we found *Osbt* mutant lines had significantly enhanced NUE under limiting N conditions compared to wild-type rice plants, measured by the two NUE indexes (Fig. 5B). When we evaluated the regulation of the N transporters (Supplemental Fig. S5, A and B), we found that three of the high affinity nitrate transporters described in rice, OsNRT2.1, OsNRT2.2, and OsNRT2.3, were induced under low ammonium nitrate conditions (Fig. 5C). These results are comparable to those obtained in Arabidopsis

for the *bt1/bt2* double mutant and indicate BTB members are part of a conserved mechanism that regulate NUE in monocotyledonous and dicotyledonous plant species.

DISCUSSION

We used a systems biology approach to identify genes involved in controlling NUE in Arabidopsis and rice. We found altering expression of BT2 alters NUE in Arabidopsis and rice plants. We also found *BT1/BT2* genes act as repressors of the *NRT2.1* and *NRT2.4* high-affinity nitrate transporter genes and nitrate transport specifically under low nitrate concentrations in Arabidopsis. *bt1/bt2* double mutants exhibited increased NUE under low nitrate conditions in Arabidopsis. Comparable NUE phenotypes obtained with *Osbt* mutants in rice indicate BTB proteins are part of a conserved mechanism that controls NUE in monocotyledonous and dicotyledonous plants.

BTBs are scaffold proteins that are characterized by their protein-protein interaction domains. Arabidopsis and rice genomes encode several BTB genes. Arabidopsis BT1 and BT2 are involved in several processes, including auxin response and telomerase activity in leaves (Ren et al., 2007), gametophyte development (Robert et al., 2009), light signals, nutrient status, hormones, and stress signaling (Mandadi et al., 2009), indicating they act as integrators of multiple cellular pathways. Two-hybrid analysis showed that BTBs are able to interact with CULLIN3 and thus might form part of E3 Ubiquitin ligase complexes (Du and Poovaiah, 2004). BTBs are also able to interact with bromodomain-containing proteins BET9 and BET10 (Du and Poovaiah, 2004). Bromodomain-containing proteins are able to interact and recognize acetylated lysines in histones, regulating transcription of target genes. However, identification of in vivo interactors of BTBs in Arabidopsis or rice in the context of NUE control are yet to be determined.

BTB proteins are predicted to respond to Ca^{2+} signals due to the presence of its calmodulin binding domain at the C terminus (Du and Poovaiah, 2004). We recently showed Ca^{2+} act as a second messenger in the nitrate signaling pathway (Riveras et al., 2015). Calcium-dependent protein kinases are key elements of nitrate signaling including CIPK8, a regulator of primary nitrate-responsive genes (Hu et al., 2009), and CIPK23, a kinase that phosphorylates the NPF6.3/NRT1.1 nitrate transceptor (Ho et al., 2009). Therefore, calcium signals triggered by nitrate availability might also serve to control BTB-mediated changes in gene expression.

Our results suggest BTBs are part of a central metabolic switch that manages offer and demand in plants. In Arabidopsis and rice, this is evident from the early vegetative stage of plant development, where BTBs might work as an early developmental brake that limits plant growth, adapting development and growth to N availability. This "brake mechanism" could be partly mediated by controlling nitrate uptake by NRT2 transporters and possibly other BTB targets. In some plants, including Arabidopsis, N uptake is repressed during the reproductive phase of development compared to early

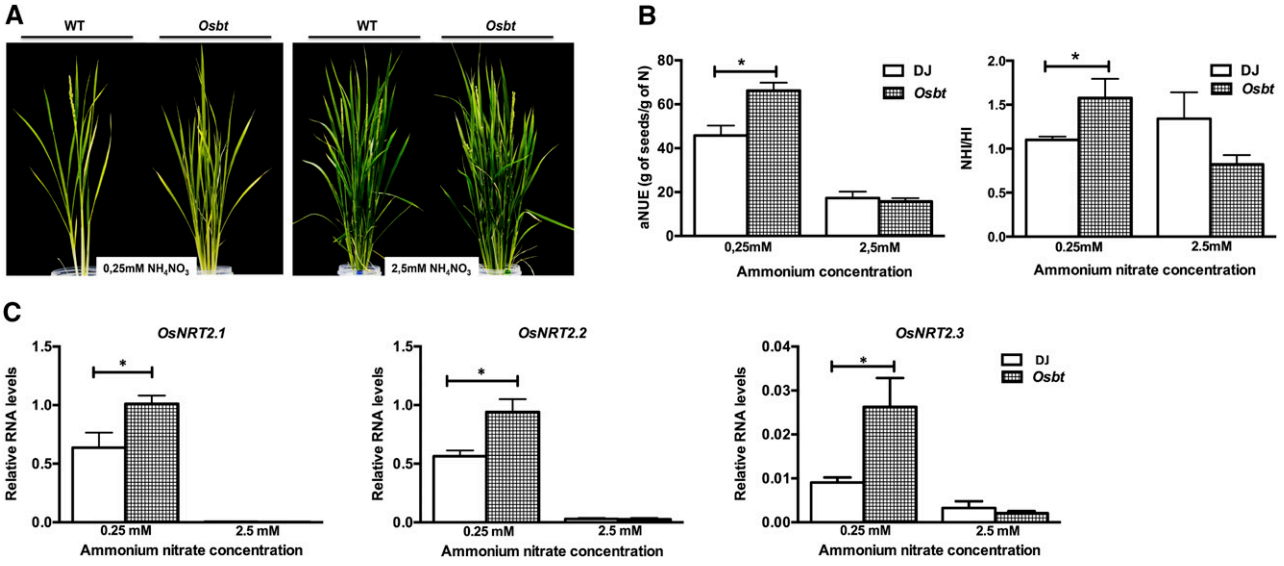

Figure 5. The *OsBT* gene represses *OsNRT2.1*, *OsNRT2.2*, and *OsNRT2.3* gene expression and NUE in rice. Wild type Donjing and *Osbt* (A3845) mutant plants were grown until seed harvest in soil and were fertilized with a nutrient solution without N supplemented with 0.25 or 2.5 mM NH_4NO_3. A, Image of 6-week-old plants. At harvest time, all the seeds and biomass per plant of wild-type and *Osbt* plants were collected weighted and analyzed to determinate the N content in seeds and biomass. B, NUE was calculated as aNUE measured by grams seeds/grams of N, or as NHI/HI. Asterisk represents means that statistically differ ($P < 0.05$). C, *OsNRT2.1*, *OsNRT2.2*, and *OsNRT2.3* transcript levels were analyzed by qRT-PCR in 1-month-old rice plants. The asterisk indicates means that significantly differ between control and treatment conditions ($P < 0.05$). We show the mean and SE for three independent replicates with at least eight plants each.

juvenile vegetative stages (Beuve et al., 2004; Malagoli et al., 2004; Masclaux-Daubresse et al., 2010). This is in accordance with BTBs role during Arabidopsis and rice early development, when nitrate uptake has a more prominent role in determining plant N status.

Previous results using different accessions of Arabidopsis suggested NUE was independent of N supply and only dependent on plant genotype (Chardon et al., 2010). However, we found aNUE and NUE measured as NHI/HI decreases as N supply increases under our experimental conditions in Arabidopsis and rice. This apparent discrepancy is due to the way NUE was measured by Chardon et al. (2010). In this work, NUE was measured as the ratio of rosette biomass to N concentration in the rosette, without normalizing by N supply (Chardon et al., 2010). Different metrics exist to determine NUE depending on the specific crop and trait studied. Since one of our goals was to find genes involved in NUE that might be used as targets for improving this trait in different cultivars, we favored using grain yield normalized per unit of N available (aNUE) as a first approximation because this is a common measure and could be extrapolated to NUE in crops (Good et al., 2004). Moreover, aNUE it is more broadly applicable to different plant species and economically important plants such as rice and other cereals. Notwithstanding, we found BT1/BT2 modulates both aNUE and NHI/HI parameters. NHI/HI index considers the plant biomass, N content in seeds and N content in biomass, including the N remobilization process. These results indicate BT1/BT2 have an important role in NUE in the plant.

Mechanisms that impact traits that are conditioned by the environment are important targets for crop productivity (Gifford et al., 2013). Growth of Arabidopsis and rice is significantly increased under low N in *bt* mutants, reaching values comparable to plants grown under sufficient N conditions. Rice is the most important food crop in the world. Half of the world's population depends on rice as their staple food; it is the cereal with the lowest NUE (Dobermann and Cassman, 2002) and a model for monocotyledonous species. Our results show NUE is increased by nearly 20% in mutant *Osbt* plants. It is estimated that a 1% increase in NUE of crops could save $1.1 billion annually (Kant et al., 2011). Our results offer a prime target for new biotechnologies to improve crop production in economically and environmentally sustainable manners.

MATERIALS AND METHODS

Plant Material and Plant Growth Conditions

Arabidopsis Plants

Arabidopsis (*Arabidopsis thaliana*) Columbia-0 (Col-0) and a crossing between Col-0 and Landsberg *erecta* (L*er*) were used as wild-type backgrounds, as indicated. *bt1-4* was obtained from the Arabidopsis Biological Resource Center mutant bank (www.arabidopsis.org), and *bt2-1* and *BT2* overexpressor line (BT2OE) were kindly donated by Dr. Thomas D. McKnight (VA State University).

Arabidopsis was grown in an inert substrate, vermiculite, under long-day (16 h light at 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$/8 h dark) conditions at 22°C in plant growth incubators (Percival Scientific). Arabidopsis plants were watered every week with 200 mL of medium containing 50 μM H_3BO_3, 1.5 mM $CaCl_2$, 50 μM $MnSO_4$, 0.08 μM $CuSO_4$, 0.05 μM Na_2MoO_4, 0.625 mM KH_2PO_4, 0.75 mM $MgSO_4$, 25 μM $ZnSO_4$, 5 μM KI, 50 μM $FeSO_4$, 50 μM Na_2EDTA, and 0.055 μM $CoCl_2$ supplemented with different amounts of nitrate in the form of KNO_3: 0.5 mM KNO_3, 1 mM KNO_3, 5 mM KNO_3, 10 mM KNO_3, 20 mM KNO_3, and 30 mM KNO_3, pH 5.7, until the plants completed their life cycle.

To evaluate gene expression in 15-d-old Arabidopsis plants, we grew plants in 50 mL of 0.8% agar vertical plates with the medium previously described supplemented with 0.5 or 5 mM KNO_3.

Rice Plants

Rice (*Oryza sativa*) japonica cv Donjing was used as wild-type ecotype. *Osbt* mutant A34852 was obtained from the POSTECH mutant bank (http://www.postech.ac.kr/life/pfg/risd/). Rice plants were grown in peat soil substrate in a growth chamber under neutral-day (12-h light/12-h dark) conditions at 28°C during day and 24°C during night and 65 to 70% humidity. The light intensity at the plant level was 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Every week, 12 rice plants were watered until flooding with the addition of 10 liters of nutrient solution until harvest. The nutrient solution contained 50 μM H_3BO_3, 1.5 mM $CaCl_2$, 50 μM $MnSO_4$, 0.08 μM $CuSO_4$, 0.05 μM Na_2MoO_4, 0.625 mM KH_2PO_4, 0.75 mM $MgSO_4$, 25 μM $ZnSO_4$, 5 μM KI, 50 μM $FeSO_4$, 50 μM Na_2EDTA, and 0.055 μM $CoCl_2$ supplemented with 0.25 or 2.5 mM ammonium nitrate (NH_4NO_3), pH 5.5.

To evaluate gene expression in 30-d-old rice plants, we grew plants hydroponically until harvest. The nutrient solution was the same described previously. The nutrient solution was replaced every week.

NUE and Biomass Measure

To evaluate NUE, we used two different methodologies. NUE agronomic index, defined as the number of seeds per plant (gr)/applied N throughout plant life cycle (gr) (Moll et al., 1982). NUE was also measured as a ratio between the Nitrogen Harvest Index (NHI) and the Harvest Index (HI), where NHI was measured as $N\%_{SEEDS} * \text{dry weight}_{SEEDS})/(N\%_{SEEDS} * \text{dry weight}_{SEEDS} + N\%_{BIOMASS} * \text{dry weight}_{BIOMASS})$ and HI as $(\text{dry weight}_{SEEDS})/(\text{dry weight}_{BIOMASS} + \text{dry weight}_{SEEDS})$. NHI/HI was measured collecting the biomass of plants at the end of their life cycle. Samples were harvested in order to determine the 15N natural abundance. After drying and weighing each biomass and seeds per plant, material was carefully weighed in tin capsules to determine the total N content and 15N abundance using a mass spectrometer coupled to an isotope ratio.

DLS Network Prediction for NUE in Arabidopsis

To identify relevant genes for NUE, a gene network was inferred using the DLS tool for MATLAB (Puelma et al., 2012). The expression data matrix and the gene annotations provided in the supplementary data of the publication were used as inputs. The expression data matrix contains 3,911 features from 2,017 Affymetrix chips. The gene annotations correspond to Gene Ontology annotations from September 7, 2010. As input to DLS, 12 biological processes were selected from Gene Ontology related to NUE, totaling 220 genes. Six of these processes are associated with N metabolism (nitrate assimilation GO:0042128, nitrate transport GO:0015706, ammonium transport GO:0015696, ammonium response GO:0060359, nitrogen response GO:0019740, and nitrate response GO:0010167), while the other six are associated with development (regulation of seed development GO:0080050, organ senescence GO:0010260, endosperm development GO:0009960, vegetative-to-reproductive phase transition of meristem GO:0010228, vegetative phase change GO:0010050, and seed maturation GO:0010431). Cytoscape (Lopes et al., 2010) was used to analyze the inferred network, and the "Network Analyzer" plug-in was applied to calculate the degree and BC of each node. A network view was constructed in which nodes have sizes that are proportional to their degrees and were colored according to their BC values. The complete network contains a total of 654 genes (Supplemental Table S1). DLS ranked the nodes with a custom score obtained by calculating the mean of the normalized values of the degree centrality (DC) and the BC of nodes:

$$\text{Score}_i = \frac{(\text{DCi}/\max(\overline{\text{DC}}) + \text{BCi}/\max(\overline{\text{BC}}))}{2}$$

The degree DCi measures the total number of nodes connected to a node i. This indicator highlight genes coexpressed with many other genes that act has central hubs. BCi measures the proportion of shortest paths between all pairs of nodes that pass through a node i.

Nitrate Uptake into the Shoot of Arabidopsis

Col-0, OEBT2, Col-0 × L*er*, and *bt1bt2* plants were grown under the same experimental conditions described above in the vermiculite substrate. Net NO_3^- uptake was measured by treating plants at dawn on day 28 when plants were still in vegetative stage. Treatment consisted of replacing the nutrient solution by an ^{15}N-containing solution that had the same nutrient composition (0.5 or 5 mM of KNO_3 and 10% enrichment in ^{15}N [w/w]). Pots were maintained in this solution for 24 h. Rosettes were cut, washed for 1 min in 0.1 mM $CaSO_4$, and then dried at 70°C for 48 h and their dry weight was determined. Total $^{15}NO_3$ content was evaluated using an ANCA-MS system (Europa Scientific), as described by Clarkson et al. (1996). Net uptake of NO_3^- for each genotype was calculated from the total ^{15}N content of plants.

RNA Isolation and qRT-PCR

RNA was isolated from whole plants or root and shoot tissues as indicated. RNA extraction was performed with the Pure Link RNA mini kit according to the manufacturer's instructions (Life Technologies). cDNA synthesis was carried out using Improm-II reverse transcriptase according to the manufacturer's instructions (Promega). qRT-PCR was carried out using the Brilliant III Ultra-Fast SYBR Green QPCR Reagents on a StepOne real-time system (Life technologies). RNA levels were normalized relative to *ADAPTOR PROTEIN-4 MU-ADAPTIN* (At4g24550) for Arabidopsis and ACTIN (Os03g50885) for rice.

Evaluation of Stage Development Changes in Vegetative Growth of Plants of Arabidopsis

BT2OEX and *bt1bt2* and their corresponding wild-type plants were grown in vermiculite and treated every week with 0.5 or 5 mM KNO_3. To evaluate developmental changes in vegetative phase change, plants were analyzed using a stereomicroscope every day after sowing. The day of appearance of cotyledons, day of appearance of first set of leaves, first set of leaves with abaxial trichomes, and the day of bolting were evaluated as reported previously by Telfer et al. (1997).

Determination of Ortholog Gene of BT1/BT2 in Rice

To examine the role of BTB orthologs in rice, we generated BTB domain alignments by the distance-based neighbor-joining method (Gingerich et al., 2007) using the following software: OrthologID (Chiu et al., 2006), Plaza 2.5 (Proost et al., 2009), and Greenphyl (Conte et al., 2008). We found a rice ortholog gene nearest to BT1 and BT2 of Arabidopsis, *OsBT* (Os01g68020).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Single mutation of *BT1* or *BT2* genes does not affect aNUE in Arabidopsis.

Supplemental Figure S2. aNUE, yield or nutrient use efficiency of *bt1/bt2* double mutant and BT2 overexpressor lines are not affected by changes in sulfate and phosphate availability.

Supplemental Figure S3. Altered expression of *BT1/BT2* genes affect early stages of Arabidopsis vegetative development.

Supplemental Figure S4. Altered expression of *BT1/BT2* genes affect the regulation of NRT2.1 and NRT2.4 but the rest of high affinity nitrate transporters in *Arabidopsis thaliana* are unaffected.

Supplemental Figure S5. *OsNRT2.4* and the ammonium transporters (OsAMTs) family are not affected by OsBT gene.

Supplemental Table S1. List of genes predicted in the NUE Network for *Arabidopsis thaliana*.

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