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Reducing expression of a nitrate-responsive bZIP transcription factor increases grain yield and N use in wheat

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Running Title: Reducing *TabZIP60* Expression Increases Wheat Yield

Key words: *Triticum aestivum*, leucine zipper transcription factor, NADH-dependent glutamate synthase, N use efficiency, grain yield

Summary

Nitrogen (N) plays critical role in plant growth, manipulating N assimilation could be a target to increase grain yield and N use. Here, we show that ABRE-binding factor (ABF)-like leucine zipper transcription factor *TabZIP60* mediates N use and growth in wheat. The expression of *TabZIP60* is repressed when the N-deprived wheat plants is exposed to nitrate. Knock down of *TabZIP60* through RNA interference (RNAi) increases NADH-dependent glutamate synthase (NADH-GOGAT) activity, lateral root branching, N uptake and spike number, and improves grain yield more than 25% under field conditions, while overexpression of *TabZIP60-6D* had the opposite effects. Further investigation shows TabZIP60 binds to ABRE-containing fragment in the promoter of *TaNADH-GOGAT-3B*, and negatively regulates its expression. Genetic analysis reveals that *TaNADH-GOGAT-3B* overexpression overcomes the spike number and yield reduction caused by overexpressing *TabZIP60-6D*. As such, TabZIP60-mediated wheat growth and N use is associated with its negative regulation on *TaNADH-GOGAT* expression. These findings indicate that *TabZIP60* and *TaNADH-GOGAT* interaction plays important roles in mediating N use and wheat growth, and provides valuable information for engineering N use efficiency and yield in wheat.

Introduction

The macronutrient nitrogen (N) is essential for plant growth, and is a primary constituent of the nucleotides and proteins. N is the most widely used fertilizer in promoting crop productivity. Plants absorb N from the soil mainly in the form of nitrate and ammonium. Once entered the cells, these inorganic N compounds are assimilated into amino acids, and

thus N assimilation represents a physiological process of the utmost importance for plant growth and development (Mokhele et al., 2012). A better understanding for the regulation of N uptake and assimilation is vital for breeding crops with improved yield and N use efficiency (Kong et al., 2013; Masclaux-Daubresse et al., 2010; Xu et al., 2012).

Plants mainly depend on nitrate transporters and ammonium transporters in the root system for N uptake from the soil. The NRT1/NPF family includes low-affinity nitrate transporters, whereas the NRT2 family encodes high-affinity nitrate transporters. NRT1/NPF and NRT2 families are involved in sensing nitrate and in regulating primary nitrate responses (Krapp et al., 2014; Xu et al., 2012). In rice, the nitrate transporters of from both NRT1/NPF and NRT2 families have been shown to increase N uptake and yield under sufficient and low N conditions (Chen et al., 2016a; Fan et al., 2016; Hu et al., 2015; Wang et al., 2018b). Quantitative trait locus (QTL) mapping has revealed the linakge between yield and N assimilation genes in wheat and maize (Hirel et al., 2001; Kichey et al., 2006; Quraishi et al., 2011). Modulating the expression of N assimilation genes has successfully improved crop yield. The transgenic expression of *AlaAT* (alanine aminotransferase) under the control of the *antiquitin* gene promoter significantly enhances yield in canola and rice (Good et al., 2007; Shrawat et al., 2008). Glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle is the first step in the assimilation of inorganic N onto carbon (C) skeletons for the production of amino acids. This has prompted geneticists and plant breeders to find ways in improving yield and N use efficiency by manipulating GS/GOGAT. Knocking out *OsGS1.1* inhibits rice growth and grain filling (Tabuchi et al., 2005), whereas *ZmGS1.3* (*Gln1-3*) overexpression in maize increases grain number (Martin et al., 2006). *TaGS2* overexpression in wheat increases N uptake, N allocation to grains and yield in wheat (Hu et al., 2018). As such, manipulating N uptake and assimilation genes can increase crop productivity.

A number of genes have been found to regulate N uptake and assimilation, and some of them have been used to engineering crops with improved yield and N use efficiency. AtNLP7 (NIN-LIKE PROTEIN 7) plays a key role in nitrate signaling and regulate the expression of many N transporters and assimilation genes in Arabidopsis (Konishi and Yanagisawa, 2013). Overexpressing *AtNLP7* in Arabidopsis increased plant biomass under both low N and high N conditions, and overexpressing *AtNLP7* in tobacco (*Nicotiana tabaccum*) also improved plant growth and N use (Yu et al., 2016). Overexpression of a

maize transcription factor (TF) *Dof1* (DNA BINDING WITH ONE FINGER) in Arabidopsis increased the expression of phosphoenolpyruvate carboxylase (PEPC) and several genes involved in the tricarboxylic acid cycle and thereby produce more carbon skeletons for the assimilation of N (Yanagisawa, 2004). And *ZmDof1* also has been shown to increase carbon flow toward N assimilation and to improve N assimilation and growth of rice under low-N conditions (Kurai et al., 2011). In wheat, the nuclear factor Y TF *TaNFYA1-6B* and NAC (NAM, ATAF1/2, and CUC2) TF *TaNAC2-5A* significantly promote root growth and enhance the expression of *NRT1* and *NRT2* families, and thus, increase N uptake and grain yield in wheat (He et al., 2015; Qu et al., 2015). *TaNAC2-5A* also has been shown to positively regulate *TaGS2* expression (He et al., 2015). The Green Revolution greatly increased crop yield, and the semi-dwarfism of green revolution varieties is conferred by mutant alleles at the *Rht* in wheat and *SD1* in rice. However, mutant *sd1* and *Rht* alleles inhibit N uptake (Li et al., 2018). A recent study in rice shows that higher expression of the *GROWTH-REGULATING FACTOR 4* (*GRF4*) TF promotes ammonium uptake and yield of Green Revolution varieties (Li et al., 2018). The rice *DEP1* encodes a G protein γ subunit and plays a key role in controlling panicle architecture (Huang et al., 2009). The dominant allele at the *DEP1* locus (*dep1-1*) is a gain-of-function mutation, and can increase transcript levels of key genes associated with ammonium uptake and assimilation (*OsAMT1.1*, *OsGS1.2* and *OsNADH-GOGAT1*), N uptake and grain yield at moderate levels of N fertilization, compared to the NIL with the *DEP1* allele (Sun et al., 2014). The rice mutant *abc1-1*, a weak mutant allele of ferredoxin-dependent GOGAT (*Fd-GOGAT*), displays a typical N-deficient syndrome (Yang et al., 2016). Moreover, the loss of function of *ARE1*, a suppressor of *abc1-1*, could partially rescue the phenotype of *abc1-1* and enhance yield (Wang et al., 2018a). Thus, identification of genes regulating N uptake and assimilation genes could help achieve higher yield and efficient N use.

The basic leucine zipper (bZIP) TFs are involved in plant development, environmental signaling and stress response (Droge-Laser et al., 2018). The Arabidopsis bZIP TFs comprises 78 members, which have been divided into 13 groups (Droge-Laser et al., 2018), and the members from Groups A, D, H and S have been reported to regulate N use. The Group S member *AtbZIP1* is a master regulator in propagating N nutrient signals (Para et al., 2014). The Group D members *AtTGA1* and *TGA4* function as important regulators of nitrate

response (Alvarez et al., 2014). The Group H members *AtHY5* and *AtHYH* regulate nitrate and ammonium transporters, and nitrate and nitrite reductase (Jonassen et al., 2008; 2009; Chen et al., 2016b; Gangappa and Botto, 2016). The Group A member *AtABI5* is involved in regulating C/N crosstalk and nitrate-induced inhibition on lateral development (Signora et al., 2001; Lu et al., 2015). Up-to-date, the roles of bZIP TFs in regulating N use has not been explored in wheat. Although a number of bZIP TFs have been used to engineer crops with improved tolerance to abiotic and biotic stresses, it has not been reported in the application of bZIP TFs in engineering wheat with improved yield and N use. The wheat bZIP TF *TabZIP60* (GenBank Accession No. KJ806560.1) was first reported in enhancing multiple abiotic stresses. Overexpression of *TabZIP60* in *Arabidopsis* confers drought and cold resistance, and increases plant sensitivity to ABA (Zhang et al., 2015). TabZIP60 shows close relation with the members in Group A of bZIP family in *Arabidopsis*, and many members of this group play critical roles in ABA signaling (Droge-Laser et al., 2018). Here, we found that *TabZIP60* is a negative regulator in wheat growth and N use. Overexpression of *TabZIP60* inhibits wheat growth, whereas reducing *TabZIP60* expression through RNAi interference improves grain yield and N use efficiency partially by upregulating *TaNADH-GOGAT* expression.

Results

Nitrate represses *TabZIP60* expression

To dissect the regulation mechanism of N assimilation, we performed RNA-seq analysis of wheat seedling roots in response to nitrate. We found that the expression of *TabZIP60* was significantly reduced 15 min after the N-deprived wheat seedlings were exposed to 2 mM nitrate. This result was confirmed by using quantitative RT-PCR. When the N-deprived wheat seedlings were exposed to 2 mM nitrate for up to 24 h, *TabZIP60* transcript abundance in roots rapidly decreased during the first half hour and then gradually decreased (Figure 1A). However, the expression of *TaNRT2.1*, which encodes a known nitrate-inducible nitrate transporter (Cai et al., 2007), significantly increased within the first hour and then decreased (Figure 1A). This result suggested that nitrate inhibited *TaBZIP60* expression. Phylogenetic analysis reveals that TabZIP60 is most closely related to the ABRE-binding factor AtABF2 followed by AtABF3 and 4 (Supplementary Figure 1), which play crucial roles in ABA signaling

(Kang et al., 2002; Uno et al., 2000). Then we examined the expression patterns of *TabZIP60* in response to ABA, and observed that it was induced by exogenous ABA in roots (Figure 1B), further confirming the previous reported ABA-induction of this gene (Zhang et al., 2015). Tissue-specific analysis revealed that the expression of *TabZIP60* was similar in the shoots and roots of seedlings (Figure 1C). Furthermore, at 14 days post-anthesis (DPA), *TabZIP60* transcripts were detected in all the investigated organs, with the highest levels observed in the older leaves (top fourth leaves), and the lowest in developing seeds (Figure 1D). These results suggested that *TabZIP60* might participate in wheat N signaling and ABA signaling simultaneously, and *TabZIP60* may function in both shoots and roots during vegetative and reproductive development.

Reducing *TabZIP60* expression increases grain yield and N uptake

To explore the roles of *TabZIP60* in mediating wheat growth and N use, we generated *TabZIP60-6D* overexpression lines and *TabZIP60* RNAi lines. Compared with the wild-type KN199 and azygous control lines (NC, negative controls), *TabZIP60* expression in the shoots and roots was significantly higher in the *TabZIP60-6D* overexpression lines (Supplementary Figure 2A), but significantly lower in the *TabZIP60* RNAi lines (Supplementary Figure2B). These results indicated that *TabZIP60-6D* was successfully overexpressed in the *TabZIP60-6D* overexpression lines and knocked down in the *TabZIP60*-RNAi lines. In field experiments, the RNAi lines showed a significant increase (25.1%–39.6%) in grain yield than the wild-type and the corresponding azygous controls, which was due to an increase (21.3%–28.6%) in spike number in 2016–2017 growing seasons (Figure 2B, C). In contrast, the overexpression lines of *TabZIP60-6D* had lower grain yield compared to the wild-type and the corresponding azygous controls in both the 2015–2016 and 2016–2017 growing seasons, primarily due to a decrease in spike number (Figure 2B, C, Supplementay Figure 3A, B). Both overexpression and knock down of *TabZIP60* did not significantly alter the 1,000-grain weight (TGW) and grain number per spike (Figure 2D, E, Supplementary Figure 3C, D). We also measured aerial N accumulation (ANA) at maturity, and found that knock down of *TabZIP60* shows a significant higher ANA compared to the wild-type, while *TabZIP60-6D* overexpression lines shows a similar ANA level with the wild-type (Figure 2F).

Reducing *TabZIP60* expression promotes root growth and N use at seedling stage

To understand the mechanism of reducing *TabZIP60* expression in enhancing N uptake, we then checked the roles of *TabZIP60* in mediating root growth. In a hydroponic culture, we observed stronger root system in the *TabZIP60* knock down lines (Figure 3A), as the knock down lines had higher root dry weight (RDW, Figure 3B), longer lateral root (LR) length (Figure 3C) than the wild-type and NC, but maximal primary root (PR) length did not significantly altered (Figure 3D). These results indicated reducing *TabZIP60* expression enhances root system by promoting lateral root growth, which may facilitate N uptake. We then detected the N concentrations in the roots of *TabZIP60* transgenic lines. The N concentration in roots of the *TabZIP60* knock down lines was significantly higher than that of KN199 and NC (Figure 3E), indicating reducing *TabZIP60* expression may improve N use. In contrast to the *TabZIP60* knock down lines, the overexpression lines had the opposite effects on RDW, LR length, and root N concentration (Figures 3A–3C and 3E). These results suggest that *TabZIP60* plays a negative role in mediating root growth and N use.

***TabZIP60* has impact on the loss of leaf N during grain filling**

To understand the role of *TabZIP60* in mediating N use, we measured N concentrations in ten aerial parts at anthesis, 14 DPA (days post-anthesis), and maturity in the 2016–2017 growing season (Supplementary Figure 4A-C). The ten aerial parts included spike, stem, and leaf blade and sheath of four leaves from the flag leaf to top fourth leaf. All the *TabZIP60-6D* overexpression lines, the *TabZIP60* RNAi lines and the wild-type KN199 exhibited a decrease in N concentrations in stem, leaf blades and sheathes with grain filling (Supplementary Figure 4B-J), indicating a N loss in these organs during grain filling. The most apparent difference between the wild-type and transgenic lines were observed for the N concentrations in leaves at 14 DPA. At anthesis, the significant differences between the wild-type and transgenic lines ware only detected in leaf blade and sheath of the top fourth leaf (Supplementary Figure 4I, J). At 14 DPA, the significant differences between the wild-type and transgenic lines were observed for the N concentrations in leaf blades and sheathes of all the four investigated leaves, and these differences disappeared at maturity (Supplementary Figure 4C-J). These results indicated that *TabZIP60* regulate leaf N level and time-course of leaf N loss during grain filling. Measurement of grain N concentration did not

find the significant difference between the wild-type and transgenic lines (Supplementary Table 1).

Reducing *TabZIP60* expression increases NADH-GOGAT activity

Previous studies have suggested that ABFs bind to ABA-responsive elements ABREs (Izawa et al. 1993, Foster et al. 1994). Since TabZIP60 is most closely related to ABFs (Supplementary Figure 1), we analyzed the promotor sequences of N-assimilation genes, and found that the *TaNADH-GOGAT* promoter contains several ABREs (Supplementary Figure 6A). Enzyme activity assay demonstrated that the NADH-GOGAT activity in roots of the overexpression lines was reduced to 57.4%–61.5% of wild-type level, whereas that of the RNAi lines was 53.2%–77.8% higher (Figure 4A). As such, *TaNADH-GOGAT* may be the target gene of TabZIP60. We then checked whether TabZIP60 could bind to the *TaNADH-GOGAT* promoter through chromatin immunoprecipitation (ChIP)-qPCR analysis. ChIP-qPCR revealed the binding enrichment of TabZIP60 to the promoter of *TaNADH-GOGAT-3B* (*GOGATpro*, Figure 4B). Furthermore, we conducted electrophoretic mobility shift assay (EMSA) to investigate whether TabZIP60 could bind to the 57-bp P1 fragment in Figure 4B, which was strongly enriched in ChIP-qPCR and contained putative ABRE elements. The results showed that TabZIP60 bound to the biotin-labeled P1 fragment (Figure 4C). In addition, the binding disappeared using the unlabeled P1 fragment as competition, and the mutated P1 probe with a mutation in the putative ABRE element was not bound by TabZIP60 (Figure 4C). These results indicated that TabZIP60 binds to *GOGATpro* in an ABRE-dependent manner. We next performed a transient expression assay to test whether TabZIP60 had any transcriptional regulatory effect on *GOGATpro*. In a luciferase (LUC) reporter assay system, firefly LUC was used as a reporter. The *LUC* gene was driven by *GOGATpro*, which was connected by five copies of the GAL4 binding element. The GAL4 DNA binding domain (BD) could bind to the GAL4 element. We fused BD with *TabZIP60*. The results showed that *GOGATpro::TabZIP60* system had lower LUC activity than control samples (*GOGATpro::GAL-BD*, *35S::bZIP60* and *35S::GAL-BD*) (Figure 4D). These results suggest that TabZIP60 directly binds to *GOGATpro* and represses the transcription of *TaNADH-GOGAT-3B*.

We also analyzed whether *TabZIP60* affected the expression of primary N-assimilation genes. Nitrate and ammonia are the major N resources for plant uptake. Nitrate is first reduced to ammonia before its incorporation into organic forms. In primary N-assimilation, ammonia is assimilated into glutamine (Gln) and Glu through the GS/GOGAT cycle, Gln and Glu can then be used to form Asp and asparagine (Asn) through the activity of aspartate aminotransferase (AAT) and asparagine synthetase (AS) (Coruzzi, 2003). In addition, GS, GOGAT, or glutamate dehydrogenase (GDH) has been implicated in N re-assimilation (Coruzzi, 2003). After analyzing gene expression in the roots of hydroponically grown wheat seedlings, we found that overexpressing *TabZIP60-6D* inhibited the expression of the genes encoding NR, cytosolic GS (GS1), Fd-GOGAT, NADH-GOGAT, GDH, and AS (Supplementary Figure 5A-H).

Overexpressing *TaNADH-GOGAT* increases grain yield

Since *TaNADH-GOGAT* acts downstream of *TabZIP60*, we further asked how *TaNADH-GOGAT* mediated wheat growth and N use. We successfully generated *TaNADH-GOGAT* transgenic lines through overexpression and RNAi approaches (Supplementary Figure1C and 1D). The field experiments showed a better performance of the agronomic traits of overexpression lines in both the 2015–2016 and 2016–2017 growing seasons (Figure 5A, Supplementary Figure 7). The grain yields of the overexpression lines were increased 16.6%-26.8% compared to the wild-type in 2015–2016 growing seasons (Supplementary Figure 7A**)**, and 18.7%-23.9% compared to their corresponding azygous controls in the next year (Figure 5B). The statistical results showed the increment primarily caused by an increase in spike number (11.2%-13.2% in 2015-2016 and 20%-25.5% in 2016-2017) (Figure 5C, Supplementary Figure 7D). In contrast, the RNAi lines showed a significant reduction in grain yield than the wild-type and the corresponding azygous controls in the two growing seasons, which was mainly due to a reduction in spike number (Figure 5C, Supplementary Figure 7D). We then measured ANA of the transgenic lines at maturity. The results shows that overexpression lines shows a significant higher grain ANA compared to the corresponding azygous controls, while RNAi lines shows a lower ANA compared to the corresponding azygous controls (Figure 5D). These results indicated that the expression level of *TaNADH-GOGAT* is positively related to grain yield and N uptake. A hydroponic culture of seedlings also revealed the positive roles of *TaNADH-GOGAT* in root growth and root N

concentrations (Figure 6A-E). Therefore, overexpression *TaNADH-GOGAT* could improve wheat growth and N use. After investigated the yield of the F_1 lines from a cross between *TabZIP60-6D* and *TaNADH-GOGAT-3B* overexpression lines, we found that the F_1 plants showed higher values for biomass, grain yield and spike number than the wild-type and *TabZIP60* overexpression lines, but were similar to that of the *TaNADH-GOGAT-3B* overexpression lines (Figure 7). These results suggest that *TabZIP60* mediates wheat growth at least partially by regulating *TaNADH-GOGAT*.

Discussion

Our current study presents evidence on the roles of *TabZIP60* in mediating wheat growth and N use. Firstly, reducing *TabZIP60* expression greatly increased lateral root branching and root N concentration of wheat seedlings, and improved grain yield and N uptake (ANA) under field conditions, while overexpressing *TabZIP60* had the opposite effects. Secondly, altering *TabZIP60* expression changed leaf N level and time-course of leaf N loss during grain filling. In the study on the mechanism underlying the regulation of *TabZIP60* on N use, we observed the inhibitory effects of *TabZIP60* on the expression of a number of genes in primary N assimilation and re-assimilation. ChIP-qPCR, EMSA, and luciferase reporter assays clearly show that TabZIP60 binds to the promoter of *TaNADH-GOGAT-3B*, possibly in an ABRE-element dependent manner, and negatively regulates *TaNADH-GOGAT-3B* expression. This negative regulation is in line with the fact that knock down of *TabZIP60* increases NADH-GOGAT activity in the roots, whereas overexpressing *TabZIP60* reduces. Applying nitrate to the N-deprived wheat seedlings inhibits *TabZIP60* expression, but induces *TaNADH-GOGAT* expression in the roots. This contrasting response of *TabZIP60* and *TaNADH-GOGAT* to nitrate can be explained, at least partially, by the negative regulatory effect of TabZIP60 on *TaNADH-GOGAT*.

GOGAT plays essential roles in primary N-assimilation and re-assimilation (Coruzzi, 2003) and presents in a small gene family in plants. Our analysis of the reference sequence from the wheat variety Chinese spring (http://plants.ensembl.org/Triticum_aestivum/Info/Index) shows that each of the three sub-genomes in wheat has one *Fd-GOGAT* on group 2 chromosomes and one *NADH-GOGAT* on group 3 chromosomes. Investigating the phenotypes of *TaNADH-GOGAT* overexpression

and RNAi lines has revealed the positive roles of these genes in root N concentration and root growth at the seedling stage, as well as in aerial N accumulation, spike number, grain number per spike, and biomass and grain yield at maturity. Similar results have been reported in rice by characterizing *OsNADH-GOGAT1* mutants. A previous study has shown that *TaNADH-GOGAT* is orthologous to *OsNADH-GOGAT1* on chromosome 3 in rice (Quraishi et al., 2011). *OsNADH-GOGAT1* is mainly expressed in the roots and is important for primary ammonium assimilation in roots, root growth, the development of active tiller number and spikelet weight (Lu et al., 2011; Tamura et al., 2010; Yamaya et al., 2002).

Our current study demonstrated that reducing the expression of *TabZIP60* increased not only grain yield but also N uptake. These increasing effects were associated with the increased NADH-GOGAT activity by reducing *TabZIP60* expression, as several phenotypes of the *TabZIP60* RNAi and overexpression lines resemble those of *TaNADH-GOGAT* overexpression and RNAi lines, respectively. Both *TabZIP60* knock down and *TaNADH-GOGAT-3B* overexpression resulted in increasing lateral root branching, root N concentration, spike number, biomass and grain yields; whereas the opposite effects were observed in both *TabZIP60-6D* overexpression lines and *TaNADH-GOGAT* knock down lines. Genetic analysis showed that overexpressing *TaNADH-GOGAT-3B* overcomes the reduced spike number, and biomass and grain yield in the overexpression lines of *TabZIP60-6D*, this result further support the claim that negative control of *TabZIP60* on wheat yield is associated with its negative control on NADH-GOGAT expression. *TabZIP60* has been reported a positive role in tolerance to multiple abiotic stresses (Zhang et al., 2015). In Arabidopsis, overexpression of *AtABF2* retards growth, but enhances tolerance to multiple abiotic stresses, whereas knockout of *AtABF2* enhances growth of seedlings (Kim et al., 2004). These results suggest that *TabZIP60*-related ABFs may mediate the balance between stress tolerance and growth. Considering the fluctuating environments in wheat growing season, further research is needed to investigate the effects of reducing *TabZIP60* on stress tolerance.

TabZIP60 is most closely related to ABFs in *Arabidopsis* (Supplementary Figure1). The expression of *TabZIP60* is induced by exogenous ABA treatment in wheat (Figure 1B), as has been shown previously in wheat (Zhang et al., 2015). Overexpression of *TabZIP60* in *Arabidopsis* increases tolerance to multiple abiotic stress and the sensitivity to ABA (Geng et

al., 2018; Zhang et al., 2015). These results indicate a role of *TabZIP60* in ABA signaling and stress tolerance. The ABA signaling pathways have been well documented, from the ABA receptors, the protein kinases, to the nuclear TFs (Raghavendra et al., 2010). In the nucleus, the TF ABI5 and related ABFs are the key targets of the protein kinases involved in ABA signaling, and the ABFs bind to the ABRE element, in concert with other transcriptional regulators, provide the ABA-responsive transcription (Droge-Laser et al., 2018; Raghavendra et al., 2010). Although ABI5 is known to control lateral root development in response to nitrate, and C/N cross talk, it is not reported to directly control the expression of N-uptake and -assimilation genes. As such, TabZIP60-related ABF(s) may connect ABA signaling with primary N assimilation. Our study also suggested the potential of manipulating ABA signaling components in increasing crop productivity. This idea is supported by the recent study that knockout of ABA receptors via CRISPR/Cas9 technology effectively promotes rice growth and increases grain yield by 31% under field conditions (Miao et al., 2018).

Experimental Procedures

Materials

Winter wheat (*Triticum aestivum*) variety Kenong 199 (KN199) was used to amplify and isolate gene sequences and generate transgenic lines. To generate overexpression lines, the cDNAs of *TabZIP60-6D* and *TaNADH-GOGAT-3B* were inserted into the *pUbi-163* vector, resulting in *pUbi::TabZIP60-6D* and *pUbi::TaNADH-GOGAT-3B* constructs. To generate knock down lines, the sequences characterized for *TabZIP60* and TaNADH-GOGAT were inserted into a *pUbi-RNAi* vector, resulting in *pUbi::TabZIP60-RNAi* and *pUbi::TaNADH-GOGAT-RNAi* constructs. The above constructs were then transformed into immature embryos of wheat variety KN199 as described elsewhere (Shan et al., 2014). The primers used for vector construction are listed in Supplementary Table 2.

Hydroponic Culture

Seedlings of the wild-type KN199 and T3 transgenic lines and their azygous control plants separated from T1 plants were used in the hydroponic cultures. Seeds were surface sterilized with 1.5% H_2O_2 for 10 h and washed five times with sterile water, and then germinated at 20 ± 1°C for seven days. Subsequently, the seedlings were transferred to plastic boxes containing 13 L of nutrient solution. The nutrient solution (normal N conditions) and growth conditions are as described by Shao et al. (2017). After growing for two weeks, the roots and shoots were harvested separately. The root morphological parameters were measured using Win-RHIZO software (Regent Instruments Canada, Inc., Ottawa, ON, Canada) as described elsewhere (Ren et al., 2012). The total N concentrations in the dried root and shoot samples were measured using a semi-automated Kjeldahl method (Kjeltec Auto 1030 Analyzer; Tecator).

Field Experiments

The wild-type KN199, transgenic lines, and their azygous control lines were used in the field experiments at the experimental station of the Institute for Cereal and Oil Crops, Hebei Academy of Agriculture and Forestry Sciences, Hebei province, China. For the *TabZIP60-6D* and *TaNADH-GOGAT-3B* overexpression lines and *TaNADH-GOGAT* RNAi lines, T3 and T4 generations were used in the 2015–2016 and 2016–2017 wheat growing seasons, respectively. For *TabZIP60* RNAi lines, the T3 generation was used in the 2016–2017 growing season. Fertilizer application (high N conditions) was as described elsewhere (Shao et al., 2017). In the 2015–2016 growing season, three replications were used. For each genotype in each replicate, 20 seeds were sown in one 2-m-long row, and the rows were spaced 23 cm apart. The yield-related traits (grain yield, spike per plant, grain number per spike, and 1,000-grain weight) of 15 representative plants in each replicate were recorded. In the 2016–2017 growing season, four biological replicates were used. For each genotype in each replicate, 40 seeds were sown in one 2-m-long row, and the rows were spaced 23 cm apart. To evaluate N distribution, the leaf, leaf sheath, stem, spike and grain samples were separately collected from 10 randomly selected culms at anthesis, 14 days post-anthesis

(DPA), and maturity. At maturity, the yield-related traits of 10 representative plants in each replicate were recorded. The total N concentrations plant samples were measured using a semi-automated Kjeldahl method (Kjeltec Auto 1030 Analyzer; Tecator).

Quantitative Real-Time PCR

Total RNA was extracted from wheat fresh samples using the Plant RNeasy Kit TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). First-strand cDNA was synthesized from 2 μg of DNase I-treated total RNA using murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). Quantitative real-time PCR analysis was performed with a LightCycler 480 engine (Roche, Mannheim, Germany) using the LightCycler480 SYBR Green I Master Mix (Roche, Mannheim, Germany). The relative expression levels were normalized to the expression of *TaACTIN* gene. The primers used for quantitative real-time PCR are detailed in Supplementary Table 2.

NADH-GOGAT Activity Assay

The roots of two-weeks-old KN199 plants cultured by hydroponic experiments were used for the analysis of NADH-GOGAT activity. Analysis of NADH-GOGAT enzyme activity was performed as described (Anderson et al., 1989) with minor modifications. A NADH-GOGAT activity kit (Comin biotechnology, Suzhou, China) were used for the detection of NADH-GOGAT activity. Briefly, plant extracts were prepared by grinding 0.1 g fresh roots in 800 μL of cold extraction buffer (buffer 1) at 4°C, and then cleared by centrifugation at 16000×*g* for 10 min at 4°C, followed by collection of the supernatants on ice. Then, we use the BCA assay as described (Bainor et al., 2011) for determination of protein concentration. To assay NADH-GOGAT activity, 20 μL plant extracts were mixed with 180 μL reaction mixture (buffer 2), and then transferred into a quartz cuvette incubated in a DU® 800 Nucleic Acid/Protein Analyzer (Beckman Coulter, US) for the Kinetics/Time Run. The NADH-GOGAT activity [nmol NADH/min/mg prot] was measured spectrophotometrically by recording the rate of NADH oxidation at 340 nm.

ChIP-qPCR

Two-week-old KN199 plants cultured hydroponically were used for the ChIP-qPCR. Anti-TabZIP60 was ordered from the Ab-Mart Company (Shanghai, China). ChIP assays were performed as described elsewhere (Bowler et al., 2004). The primers used for RT-qPCR are listed in Supplementary Table 2.

EMSA

The full-length CDS of *TabZIP60* was cloned into the *pGEX-4T-1* and transferred to *Escherichia coli* BL21 (*Transseta*) to obtain the fusion protein. EMSAs were performed using the LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific, Shanghai, China). The probes used for EMSA are listed in Supplementary Table 2.

Luciferase Reporter Assay System

The primers used for luciferase reporter assay system are listed in Supplementary Table 2. Methods from Chen lab (http://sourcedb.genetics.cas.cn/zw/zjrck/200907/t20090721_2130989.html).

Statistical Analysis

Statistical analysis was conducted using one-way ANOVA was performed with the SPSS17.0 package for Windows (SPSS, Inc., Chicago, IL, USA).

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Conflict of interest

The authors declare they have no conflict of interest.

Authors' Contributions

Y.T., J.Y., W.T., X.H., W.M., X.Z., and H.L. designed the experiments and analyzed the data. J.Y., performed most experiments, with the assistance of M.W. and M.H. Y.T., J.Y. and Y.T. wrote the paper. All authors have read and commented on the paper.

Supplementary Information

Supplementary Figure 1. Phylogenetic analysis of TabZIP60 and the bZIP members from *Arabidopsis*.

Supplementary Figure 2. Relative expression levels of *TabZIP60* and *TaNADH-GOGAT* in shoots and roots of their corresponding transgenic lines. (A) Relative expression levels of *TabZIP60* in wild-type, *TabZIP60-6D* overexpression lines and NC. (B) Relative expression levels of *TabZIP60* in wild-type, *TabZIP60* RNAi lines and NC. (C) Relative expression levels of *TaNADH-GOGAT* in wild-type, *TaNADH-GOGAT-3B* overexpression lines and NC. (D) Relative expression levels of TaNADH-GOGAT in wild-type, *TaNADH-GOGAT* RNAi lines and NC. Wheat seedlings were grown hydroponically for 14 days. KN199, wild-type; 60OE5, 60OE8 and 60OE13, *TabZIP60-6D* overexpression line; 60R2, 60R6 and 60R11, TabZIP60 RNAi line; GoOE1, GoOE2 and GoOE3, *TaNADH-GOGAT-3B* overexpression line; GoR1, GoR2 and GoR3, *TaNADH-GOGAT* RNAi line; NC in (A) to (D) indicates azygous control lines of TabZIP60-6D overexpression lines (A), TabZIP60 RNAi lines (B), *TaNADH-GOGAT-3B* overexpression lines (C), and *TaNADH-GOGAT* RNAi lines (D). The relative expression levels were normalized to

the expression of *TaACTIN.* Data are means ± SE of three replicates. * indicates the difference between KN199 and transgenic line was signifcant at $P < 0.05$.

Supplementary Figure 3. Yield-related traits of the *TabZIP60-6D* overexpression lines in the field experiment in the 2015–2016 growing season. (A) Grain yield. (B) Spike number. (C) Grain number per spike. (D) 1000-grain weight (TGW). KN199, wild-type; 60OE5, 60OE8 and 60OE13, *TabZIP60-6D* overexpression line. NC, azygous control lines. Data are means ± SE of three replicates. * indicates the difference between KN199 and transgenic line was signifcant at $P < 0.05$.

Supplementary Figure 4. N concentrations (%) in aerial organs in *TabZIP60* transgenic lines and KN199 during grain filling. (A) Spike; (B) Stem; (C) Flag leaf blade; (C) Flag leaf sheath; (E) Top 2nd leaf blade; (F) Top 2nd leaf sheath; (G) Top 3rd leaf blade; (H) Top 3rd leaf sheath; (I) Top 4th leaf blade; (I) Top 4th leaf sheath. KN199, wild type; 60OE, *TabZIP60-6D* overexpression lines; 60R, *TabZIP60* RNAi lines. DPA, days post-anthesis. Data for KN199 are mean ± SE (n = 4), Data for 60OE and 60R are mean ± SE of three transgenic lines each with four replications. * above the lines indicates that the difference between KN199 and 60OE line is significant at $P < 0.05$, while that below the lines indicates that the difference between KN199 and 60R line is significant at $P < 0.05$.

Supplementary Figure 5. Relative expression levels of genes involved in N assimilation. (A) *TaNR*; (B) *TaNADH-GOGAT*; (C) *TaFd-GOGAT*; (D) *TaGS1.1*; (E) *TaGS1.2*; (F) *TaGDH1-5*; (G) *TaGDH1-7*; (H) *TaGDH2*, (I) *TaGDH3*; (J) *TaAS*. The germinated seedlings were grown in nutrient solution for two weeks, then the roots were collected for gene expression analysis. The relative expression levels were normalized to the expression of *TaACTIN*. The data are expressed as the mean ± S.E. of three replicates. * indicates that the difference between KN199 and the transgenic line is significant at $P < 0.05$.

Supplementary Figure 6. Promotor sequence and expression analysis of *TaNADH-GOGAT.* (A) Putative ABRE cis-element in the promoter of *TaNADH-GOGAT* (PlantCARE: bioinformatics.psb.ugent.be/webtools/plantcare/html). Black boxes indicated the putative ABRE cis-element. TSS, transcription start site. (B) Expression of *TaNADH-GOGAT* and *TaNRT2.1-6B* in response to nitrate in roots at seedling stage in a hydroponic culture. (C)

Expression levels of *TaNADH-GOGAT* in shoots and roots at wheat seedling stage in a hydroponic culture. (D) Expression of *TaNADH-GOGAT* in different organs of wheat plants at 14 days post anthesis under field conditions. Data are means ± SE (n ≥ 3). Different letters in (C and D) indicate statistically significant difference at $P < 0.05$.

Supplementary Figure 7. Yield-related traits of the *TaNADH-GOGAT* transgenic in the field experiment in the 2015–2016 growing season. (A) Grain yield. (B) Grain number per spike. (C) 1000-grain weight (TGW). (D) Spike number per plant. KN199, wild-type; GoOE1, GoOE2 and GoOE3, *TaNADH-GOGAT-3B* overexpression line; GoR1, GoR2 and GoR3, *TaNADH-GOGAT* RNAi line; Control1 and 2, azygous control. Data are means ± SE, n = 3. * indicates the difference between KN199 and transgenic line was signifcant at $P < 0.05$.

Supplementary Table 1. Yield and N use-related traits of the transgenic lines.

Supplementary Table 2. Primers used in this study

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Figure Legends

Figure 1. Expression patterns of *TabZIP60.* **(A)** Responses of *TabZIP60* and *TaNRT2.1-6B* expression to nitrate in the roots. Wheat seedlings deprived of N for 2 days were exposed to a nutrient solution containing 2 mM NO_3^- for the indicated times. **(B)** Response of *TabZIP60* to exogenous ABA in roots. HN, 2 mM NO_3^-; HN + ABA, 2 mM NO_3^- + 50 μM ABA. **(C)** Expression levels of *TabZIP60* in shoots and roots at the seedling stage. **(D)** Expression of *TabZIP60* in different organs of wheat plants at 14 days after flowering under field conditions. The relative expression levels were normalized to the expression of *TaACTIN*. Data are expressed as the mean ± SE of three replicates.

Figure 2. Agronomic traits of *TabZIP60* transgenic lines in the 2016-2017 growing season. **(A)** Images of transgenic lines and KN199. Bar = 10 cm. **(B)** Grain yield per 10 plants. **(C)** Spike number per plant. **(D)** 1000-grain weight (TGW). **(E)** Grain number per spike. **(F)** Aerial N accumulation per 10 plants (ANA). 60OE5, 60OE8, and 60OE13indicate overexpression lines; 60OE5-, 60OE8- and 60OE13- are the corresponding azygous control lines. 60R2, 60R6, and 60R11 indicate the knockdown lines, 60R2-, 60R6-, and 60R11-indicate the corresponding azygous control lines. The data are expressed as the mean ± SE of four replicates. * indicates that the difference between the transgenic line and its corresponding azygous line is signifcant at $P < 0.05$.

Figure 3. *TabZIP60* affects root growth of wheat seedlings. The seven-day-old germinated seedlings of wild-type (KN199), *TabZIP60-6D* overexpression lines (60OE5, 8, 13), *TabZIP60* RNAi lines (60R2, 6, 11), and azygous control lines (NC) separated from T1 plants were grown for 14 days in nutrient solutions that contained 2 mM NO_3^-. (A) Root images of *TabZIP60* transgenic lines. Bar = 20 mm. (B) Root dry weight. (C) Lateral root (LR) length. (D) Maximal primary root (PR) length. (E) Root N concentration. The data are expressed as the mean ± SE, $n \geq 3$. The data of NC are presented as the mean of all azygous control lines. Different letters in (B) to (D) indicate statistically significant differences at $P < 0.05$.

Figure 4. TabZIP60 regulates the expression of *TaNADH-GOGAT-3B.* (A) TaNADH-GOGAT activity in roots of the wild-type and *TabZIP60* transgenic lines. KN199, wild-type; 60OE5 and 8, *TabZIP60-6D* overexpression line; 60R2 and 60R6, *TabZIP60* RNAi line. Different letters above the column indicate statistically significant differences at $P < 0.05$. (B) ChIP-qPCR assay of TabZIP60 binding to *TaNADH-GOGAT-3B* promoter *in vivo*. TSS, transcription start site. (C) EMSA of TabZIP60 binding to the P1 fragment from (B) *in vitro*. (D) TabZIP60-6D represses the promoter activity of *TaNADH-GOGAT-3B* in a transient expression assay using *Arabidopsis* leaves. The data are expressed as the mean ± SE ($n \geq 3$). * indicates statistically significant differences at $P < 0.05$.

Figure 5. Yield and N use-related traits of the *TaNADH-GOGAT* transgenic lines in the field experiment in the 2016–2017 growing season. **(A)** Biomass per 10 plants. **(B)** Grain yield per 10 plants. **(C)** Spike number per 10 plants. **(D)** Aerial N accumulation per 10 plants (ANA). GoOE1, GoOE2 and GoOE3 indicated positive overexpression lines, GoOE1-, GoOE2- and GoOE3- indicated the azygous lines of GoOE1, GoOE2 and GoOE3, respectively. GoR1, GoR2 and GoR3 indicated positive RNAi lines, GoR1-, GoR2- and GoR3- indicated the azygous lines of GoR1, GoR2 and GoR3, respectively. Data are means ± SE of three replicates. * indicates the difference between the positive transgenic line and its corresponding azygous line was significant at $P < 0.05$.

Figure 6. *TaNADH-GOGAT* affects root growth and N use in wheat seedlings. The 7-day old germinated seedlings of wild-type (KN199), *TaNADH-GOGAT-3B* overexpression lines (GoOE1, GoOE2 and GoOE3), *TaNADH-GOGAT* RNAi lines (GoR1, GoR2 and GoR3), and azygous control lines (NC) were grown for 14 days in nutrient solutions that contained 2 mM NO_3^-. **(A)** Root images of *TaNADH-GOGAT* transgenic lines. Bar = 20 mm. **(B)** Root dry weight. **(C)** Lateral root (LR) length. **(D)** Maximal primary root (PR) length. **(E)** Root N concentration. Data are means ± SE, $n \geq 3$. Data of NC are presented as mean value of all the azygous control lines. Different letters in **(B)** to **(D)** indicate statistically significant differences at $P < 0.05$.

Figure 7. *TaNADH-GOGAT-3B* overexpression overcomes the yield reduction by overexpressing *TabZIP60-6D* overexpression. **(A)** Biomass per plant. **(B)** Grain yield per plant. **(C)** Spike number per plant. KN199, wild-type; 60OE, *TabZIP60-6D* overexpression lines; GoOE, *TaNADH-GOGAT-3B* overexpression lines; 60OE × GoOE (F_1), F_1 between 60OE and GoOE cross. Seven F_1 lines were developed using three 60OE lines (60OE5, 8, and 13) and three GoOE lines (GoOE1, 2 and 3). The data are expressed as the mean ± S.E. (n = 7). Different letters above the columns indicate statistically significant differences at $P < 0.05$.