The Nitrate-Inducible NAC Transcription Factor TaNAC2-5A Controls Nitrate Response and Increases Wheat Yield[OPEN]

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Nitrate is a major nitrogen resource for cereal crops; thus, understanding nitrate signaling in cereal crops is valuable for engineering crops with improved nitrogen use efficiency. Although several regulators have been identified in nitrate sensing and signaling in Arabidopsis (Arabidopsis thaliana), the equivalent information in cereals is missing. Here, we isolated a nitrate-inducible and cereal-specific NAM, ATAF, and CUC (NAC) transcription factor, TaNAC2-5A, from wheat (Triticum aestivum). A chromatin immunoprecipitation assay showed that TaNAC2-5A could directly bind to the promoter regions of the genes encoding nitrate transporter and glutamine synthetase. Overexpression of TaNAC2-5A in wheat enhanced root growth and nitrate influx rate and, hence, increased the root’s ability to acquire nitrogen. Furthermore, we found that TaNAC2-5A-overexpressing transgenic wheat lines had higher grain yield and higher nitrogen accumulation in aerial parts and allocated more nitrogen in grains in a field experiment. These results suggest that TaNAC2-5A is involved in nitrate signaling and show that it is an exciting gene resource for breeding crops with more efficient use of fertilizer.

Nitrogen (N) is one of the primary nutrients that limit plant productivity, and low N frequently represents a major constraint to crop yield (Jones et al., 2013). The dramatic increases in global crop productivity during the past five decades have occurred alongside 20-fold increases in the use of N fertilizer (Glass, 2003); N fertilizer use is expected to increase by at least 3-fold by 2050 (Good et al., 2004). High N fertilizer input has resulted in low nitrogen use efficiency (NUE) in cropping systems and has caused a series of environmental and economic problems (Hirel et al., 2007; Galloway et al., 2008; Canfield et al., 2010; West et al., 2014). Therefore, breeding crops with improved productivity in low-N environments represents an effective approach for increasing NUE in crop production (Rengel and Marschner, 2005).

Overall NUE in plants is calculated as the product of nitrogen uptake efficiency (NpE) and N utilization efficiency (Good et al., 2004; Hirel et al., 2007). These processes are governed by complicated gene networks that mediate the uptake, assimilation, remobilization, and storage of N (Good et al., 2004; Masclaux-Daubresse et al., 2010; McAllister et al., 2012; Xu et al., 2012). Differences in grain yield in wheat (Triticum aestivum) have been found to result largely from variations in NpE (Van Sanford and Mackown, 1986; Dhugga and Waines, 1989; Mohammadi et al., 2006), indicating the importance of understanding the genetic control of NpE for efforts to breed wheat varieties with improved NUE. A deep and large root system that responds strongly to N availability is considered to be the ideal root architecture for efficient N acquisition (Garrett et al., 2009; Mi et al., 2010; Trachsel et al., 2013). Numerous reported positive associations of quantitative trait loci (QTLs) for N uptake and QTLs for root architecture traits support the importance of root system traits in efforts to develop crop varieties with more efficient N acquisition (Mohammadi et al., 2006).

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the NAC superfamily is one of the largest plant TF families and is found only in plants. Proteins of this family are characterized by a highly conserved DNA-binding domain and variable C-terminal domains (Ooka et al., 2003). More than 100 members of this family have been identified in both Arabidopsis and rice; phylogenetic analysis indicates that these can be divided into six major groups (Nakashima et al., 2012). A number of transporter has the opposite effects (Fang et al., 2013). These studies clearly demonstrate that efficient root-to-shoot transport and remobilization of N are required to support plant growth and achieve high yields. In major cereal crops, the N assimilation genes NITRATE REDUCTASE, GLUTAMINE SYNTHETASE (GS), and GLUTAMATE SYNTHASE are known to colocalize with QTLs affecting grain yield and NUE (Hirel et al., 2001; Obara et al., 2001, 2004; Habash et al., 2007; Coque et al., 2008; Zhang et al., 2010; Li et al., 2011; Quraishi et al., 2011). Furthermore, a number of studies have shown that ectopic expression of the genes involved in primary N metabolism and amino acid biosynthesis can increase N uptake and assimilation and/or promote plant growth and yield (McAllister et al., 2012). Therefore, the selection of optimal alleles of N assimilation genes or the modulation of the expression of such genes via biotechnological methods are promising strategies in efforts to breed crops with improved NUE and yield. Considering that carbon skeletons synthesized in photosynthesis are required for N-efficient crops, carbon flow is important in promoting N assimilation. The plant-specific DNA BINDING WITH ONE FINGER (Dof) TF Dofl from maize (Zea mays) can increase the expression of PHOSPHOENOLPYRUVATE CARBOXYLASE and several genes involved in the tricarboxylic acid cycle and thereby produce more carbon skeletons for the assimilation of N in Arabidopsis (Arabidopsis thaliana; Yanagisawa et al., 2004). This TF has been used successfully to increase the net photosynthesis rate and to increase carbon flow toward N assimilation and, consequently, to improve N assimilation and growth of rice under low-N conditions (Kurai et al., 2011). While positionally cloning Gpc-B1, a wheat QTL for grain protein concentration on chromosome 6B, Uauy et al. (2006b) found that this QTL encodes a NAC (for NAM, ATAF, and CUC) TF (NAM-B1) that accelerates leaf senescence and increases nutrient remobilization from leaves to developing grains. In rice, the NAC-like, activated by apetala3/pistillata (OsNAP) was also found to positively regulate leaf senescence and the accumulation of N and other mineral nutrients in grains. Reduced OsNAP expression led to delayed leaf senescence and an extended grain-filling period, resulting in an increase in grain yield (Liang et al., 2014). Detailed analysis showed that the accumulation of OsNAP transcripts up-regulated several amino acids and peptide transporters, yielding clues to understanding the mechanism of N remobilization from senescing leaves to grains (Liang et al., 2014). These studies shed light on the critical role of NAC TFs in regulating N use in major cereal crops.
NACs have been found to play broad roles in the regulation of root and aerial development, leaf senescence, hormone responses, and tolerance to multiple biotic and abiotic stresses, including drought, salt, and low-temperature stresses (Nakashima et al., 2012; Nuruzzaman et al., 2013). In wheat, several NAC TFs have been characterized for their response to abiotic and biotic stresses; some of these have been shown to play roles in adaptation to stresses such as drought, salt, and low temperature (Xia et al., 2010; Xue et al., 2011; Mao et al., 2012, 2014; Tang et al., 2012a; Chen et al., 2013; Feng et al., 2014). For example, the expression of TaNAC2 on chromosome 5A (hereafter referred to as TaNAC2-5A) is induced by drought, salt, cold, and abscisic acid treatment, and it slightly promotes primary root growth and enhances tolerance to drought, salt, and freezing stresses when overexpressed in Arabidopsis (Mao et al., 2012). Recent studies have shown that the expression of several NACs is responsive to nutrient availability (de Zélicourt et al., 2012; Woo et al., 2012; Vidal et al., 2013). AtNAC4, a target of the auxin receptor AUXIN SIGNALING F-BOX3 (AtAFB3) in Arabidopsis, has been shown to be a key regulatory element controlling a nitrate-responsive network. Knockout of AtNAC4 alters lateral root growth in response to nitrate (Vidal et al., 2013). A more recent study showed that the response of AtAFB3 and AtNAC4 to nitrate treatment depends on the nitrate-transport function of NRT1/PTR FAMILY6.3 (AtNPF6.3, also known as NRT1.1; Vidal et al., 2013). Moreover, AtNAC4 itself is necessary for the nitrate response of the zinc finger TF OBF BINDING PROTEIN4 (AtOBP4; Vidal et al., 2013).

Here, we report that TaNAC2-5A is nitrate inducible. When overexpressed in wheat, TaNAC2-5A increased root growth and increased the rate of nitrate uptake under hydromonic conditions. In a field experiment, overexpression of TaNAC2-5A increased grain yield, grain N concentration, and N harvest index under various N supply levels. We also found that TaNAC2-5A had the ability to bind the promoter region of the genes functioning in nitrate transport and N assimilation. These results suggest that TaNAC2-5A plays an essential role in nitrate metabolism and that this gene is a valuable resource for efforts to develop crops with more efficient N use and higher yields.

RESULTS

TaNAC2-5A Is Nitrate Inducible

A microarray analysis revealed that the probe corresponding to TaNAC2-5A (accession no. AY625683) was differentially expressed in the roots of wheat plants grown under various nitrate supply levels. To further evaluate the response patterns of TaNAC2-5A expression to nitrate, we analyzed the expression of TaNAC2-5A in wheat roots using quantitative real-time reverse transcription (RT)-PCR. When plants that had been deprived of N for 2 d were exposed to 2 mM nitrate for time periods ranging from 0 to 48 h, the mRNA levels of TaNAC2-5A increased gradually but significantly, reaching their highest expression level at 12 h and decreasing thereafter (Fig. 1A). This expression pattern was similar to that observed for TaNRT2.1-6B (Fig. 1A), which is known to be nitrate inducible (Cai et al., 2007). This result indicated that TaNAC2-5A was nitrate inducible. We then analyzed the expression of TaNAC2-5A in different organs. At the seedling stage, shoots and roots had similar TaNAC2-5A expression levels under high-N conditions. However, under low-N conditions, shoots had higher TaNAC2-5A expression levels than did roots (Fig. 1B). In addition, we observed that the expression of TaNAC2-5A increased following long-term (14-d) low-N treatment in both shoots and roots, as compared with the high-N treatment (Fig. 1B). At the grain-filling stage (14 d after flowering), TaNAC2-5A was expressed in all of the organs that we tested, with the highest expression levels ranging from 0 to 48 h, the mRNA levels of TaNAC2-5A increased gradually but significantly, reaching their highest expression level at 12 h and decreasing thereafter (Fig. 1A). This expression pattern was similar to that observed for TaNRT2.1-6B (Fig. 1A), which is known to be nitrate inducible (Cai et al., 2007). This result indicated that TaNAC2-5A was nitrate inducible. We then analyzed the expression of TaNAC2-5A in different organs.
observed in old leaves and flag leaves and the lowest expression level observed in young spikes (Fig. 1C).

Overexpression of TaNAC2-5A Promotes Root and Shoot Growth and Increases Shoot N Accumulation in Wheat Seedlings

To test the function of TaNAC2-5A in mediating plant growth and N use, we generated TaNAC2-5A overexpression transgenic wheat lines. The three selected transgenic lines had higher TaNAC2-5A expression levels in shoots and roots than did the wild type in hydroponic culture (Fig. 2), indicating that TaNAC2-5A was overexpressed in these transgenic lines. We then compared the performance of the wild type and the transgenic lines in a soil pot trial at the seedling stage (Fig. 3A). For the wild-type plants, both nil-N and low-N treatments reduced tiller number (Fig. 3B), shoot dry weight (Fig. 3C), shoot N concentration (Fig. 3E), and shoot N accumulation (Fig. 3F), as compared with the high-N treatment. Compared with the wild type, two or all of the three transgenic lines had significantly higher tiller numbers under the nil-N and low-N conditions (Fig. 3B), significantly higher shoot dry weights (Fig. 3C) and shoot N accumulation (Fig. 3F) under all three N conditions, and significantly higher root dry weights under the nil-N treatment (Fig. 3D). Although the root dry weight of the wild type did not respond to N treatment, that of the transgenic lines exhibited positive responses to both the nil-N and the low-N treatments, as compared with the high-N treatment (Fig. 3D). The transgenic lines had lower shoot N concentrations than did the wild type under the nil-N treatment. In contrast, the transgenic lines had higher shoot N concentrations than did the wild type under low-N conditions and similar shoot N concentrations to the wild type under high-N conditions (Fig. 3E). Considering that the transgenic plants had higher shoot dry weights but lower shoot N concentrations than did the wild type plants under the nil-N conditions, these results indicate that the transgenic lines used N more efficiently to produce shoot dry matter than did the wild type under conditions of severe N deficiency.

Overexpression of TaNAC2-5A Increases Grain Yield, Aerial N Accumulation, and N Harvest Index

We further compared the grain yield and the N use of the wild type and the transgenic lines (OE1 and OE2) under field conditions (Table I). OE1 had 10% \( P < 0.05 \) and 17.1% \( P < 0.05 \) higher grain yields than did the wild type under low-N and high-N conditions, respectively. OE2 had 6.7% \( P < 0.05 \) and 5% (not significant) higher grain yield than did the wild type under low-N and high-N conditions, respectively. We evaluated the yield components (spike number, grain number per spike, and 1,000-grain weight) in these lines and found that the increases in spike number and 1,000-grain weight in the transgenic lines likely contributed to the observed increases in grain yield. The two transgenic lines also had higher grain N concentrations, grain N accumulation, aerial N accumulation (total N accumulation in aerial parts), and N harvest indexes than did the wild-type plants, under both low-N and high-N conditions. In contrast, the transgenic lines tended to have slightly lower straw N concentrations and lower N accumulation in straw than did the wild type. These results indicate that TaNAC2-5A increased aerial N accumulation and promoted N allocation to grains. Additionally, the transgenic lines had higher grain zinc concentrations than did the wild type, under both low-N and high-N conditions.

Overexpression of TaNAC2-5A Up-Regulates the Expression of Nitrate Transporters and GS and Increases the Root Nitrate Influx Rate in Wheat

Since TaNAC-5A had positive effects on NUE, we wondered whether overexpressing TaNAC-5A would up-regulate the expression of the genes involved in N transport and assimilation. We analyzed the effects of overexpressing TaNAC2-5A on the expression of nitrate transporter genes. Of the investigated nitrate transporter genes, TaNRT2.1-6B and TaNRT2.2-6D are known to be nitrate inducible (Cai et al., 2007). Phylogenetic analysis showed that TaNPF7.1-6D and TaNPF7.2-6B are closely related to AtNPF7.2/NRT1.8 and AtNPF7.3/NRT1.5, which are known to be required for xylem loading and unloading of nitrate (Buchner and Hawkesford, 2014). In roots, the two transgenic lines had higher TaNRT2.1-6B, TaNRT2.1-6D, TaNPF7.1-6D, and TaNPF7.2-6B expression than did the wild type. In shoots, the two transgenic lines had higher TaNRT2.1-6B and TaNPF7.1-6D expression than did the wild type (Fig. 4, A–D). GS plays an essential role in the assimilation of ammonium into amino acids (Miflin and Habash, 2002). We then investigated

\[ \text{Relative expression level} = \frac{\text{Expression level of transgenic line}}{\text{Expression level of wild type}} \times 100\% \]

\[ \text{Shoot} \]

\[ \text{Root} \]

Figure 2. Expression levels of TaNAC2-5A in shoots and roots in wild-type (LC) and transgenic (OE1, OE2, and OE3) wheat. Wheat seedlings were grown for 14 d in a nutrient solution that contained 2 mM nitrate. The relative expression levels of TaNAC2-5A were normalized to the expression of \( \beta\)-TUBULIN. Data are means ± se of three replicates. Asterisks indicate that the difference between the means of the wild type and the transgenic lines was significant at the \( P < 0.05 \) (*) and \( P < 0.01 \) (**) levels.
the expression of TaGS2 encoding plastid-located GS (Li et al., 2011). Overexpression of TaNAC2-5A caused the up-regulation of TaGS2 (representing the summed expression of TaGS2-2A, TaGS2-2B, and TaGS2-2D) in both shoots and roots (Fig. 4E). These results suggest that the overexpression of TaNAC2-5A up-regulated the expression of genes involved in nitrate transport and N assimilation. We further measured the nitrate fluxes at the root surface by using a scanning ion-selective electrode technique. Wheat seedlings pretreated for 7 d with 1 mM nitrate and 0.2 mM nitrate were used to measure nitrate fluxes in measuring solutions that contained 1 mM nitrate (high-nitrate treatment) or 0.2 mM nitrate (low-nitrate treatment). The results showed that both of the transgenic lines had higher nitrate influx rates than did the wild type, under both high-nitrate and low-nitrate treatments (Fig. 4F).

Figure 3. Plant growth and N accumulation of the wild type and the transgenic lines in the soil pot trial. A, Seedlings of the wild type (LC) and the transgenic lines (OE1, OE2, and OE3) grown under low-N and high-N conditions. B, Tiller number per plant. C, Shoot dry weight per plant. D, Root dry weight per plant. E, Shoot N concentration. F, Shoot N accumulation per plant. The wild type and the transgenic lines were grown for 30 d in soil supplied with 0 mg N kg\(^{-1}\) soil (nil-N treatment), 10 mg N kg\(^{-1}\) soil (low-N treatment), or 100 mg N kg\(^{-1}\) soil (high-N treatment). Data are means ± se of four replications. Asterisks indicate that the difference between the means of the wild type and the transgenic lines was significant at the \(P < 0.05\) (*) and \(P < 0.01\) (**) levels.

TaNAC2-5A Has Transcriptional Activity and Is Capable of Binding the Promoter Regions of TaNRT2.1-6B, TaNP7.1-6D, and TaGS2-2A

Polyclonal antibodies for TaNAC2 were prepared and used to visualize the subcellular localization of TaNAC2 in wheat root cells with an immunofluorescence labeling technique. TaNAC2-5A and its homologous alleles TaNAC2-5B and TaNAC2-5D shared high similarity, and the peptides that were selected to raise antibodies did not show differences among these three TaNAC2 proteins (Supplemental Fig. S1); therefore, the antibodies we used may have recognized all three TaNAC2 proteins. TaNAC2 was localized in the nucleus (Fig. 5A). We also observed that TaNAC2-5A conferred protein transcription activation activity in yeast (Saccharomyces cerevisiae) cells (Fig. 5B, right).
NAC proteins have a highly conserved DNA-binding domain near their N-terminal regions and variable C-terminal domains (Ooka et al., 2003). It is already known that the C-terminal end of NAC proteins contains the transcription regulatory regions that can either activate or repress transcription (Puranik et al., 2012). We examined the function of the DNA-binding domain and the C-terminal domain in the transcription activation activity of TaNAC2-5A. Figure 5B shows that yeast cells transfected with an N-terminal domain deletion mutant of TaNAC2-5A (pBD-TaNAC2-5AΔC) could not grow. This result indicates that the transcription activation activity of TaNAC2-5A depends on the function of its C-terminal domain.

As we now knew that TaNAC2-5A had transcription activation activity and that the overexpression of TaNAC2-5A up-regulated the expression of TaNRT2.1, TaNPF7.1-6D, TaNPF7.2-6B, and TaGS2, we wondered whether TaNAC2-5A could directly bind the promoter region of these genes. To address this, a chromatin immunoprecipitation (ChiP) experiment was performed to detect these gene promoter fragments in...
the DNA-TaNAC2-5A complexes of wheat plants. By analyzing the available sequences of TaNRT2.1-6B, TaNRT2.1-6D, TaNPF7.1-6D, TaNPF7.2-6B, and TaGS2, we predicted several putative motifs in their promoter regions (Fig. 6A) that were likely recognizable by NAC TFs (Simpson et al., 2003; Ernst et al., 2004; Tran et al., 2004). ChIP enrichment analysis showed that TaNAC2-5A had the ability to bind the fragments from the promoter regions of TaNRT2.1-6B, TaNPF7.1-6D, and TaGS2-2A (Fig. 6B), possibly by recognizing both the CACG and CGT(G/A) motifs.

Overexpressing TaNAC2-5A Increases Nitrate Influx and the Expression of Nitrate Transporter Genes in Arabidopsis

To further dissect the molecular mechanism of increased nitrate uptake rate observed in wheat when overexpressing TaNAC2-5A, we introduced a TaNAC2-5A overexpression construct into Arabidopsis (Supplemental Fig. S2), a plant for which a number of nitrate transporters have already been functionally characterized. We found that the TaNAC2-5A overexpression Arabidopsis plants had longer primary roots and more lateral roots than did the wild type, under both low-N and high-N conditions (Fig. 7, A–C). As has been shown previously (Ma et al., 2014), low-N treatment increased primary root length and lateral root number as compared with high-N treatment (Fig. 7, B and C); these increases in the transgenic plants were more apparent than those in the wild-type plants. These results indicate that overexpressing TaNAC2-5A enhanced primary root length and lateral root number in response to low N availability.

The roots of the transgenic plants also had higher rates of nitrate influx than did wild-type roots (Fig. 7D). We then analyzed the expression of AtNPF6.3/NRT1.2, AtNPF4.6/NRT1.2, and AtNRT2.1, which are known to mediate nitrate transport from the environment into root cells (Huang et al., 1996, 1999; Wirth et al., 2007). We also evaluated the expression of AtNPF7.3/NRT1.5 and AtNPF7.2/NRT1.8, which function in the long-distance transport of nitrate from roots to shoots (Lin et al., 2008; Li et al., 2010). The transgenic lines had higher expression levels for AtNPF4.6/NRT1.2, AtNPF7.3/NRT1.5, and AtNRT2.1 than did the wild type. The expression of AtNPF6.3/NRT1.1 and AtNPF7.2/NRT1.8 transcripts was similar in the transgenic and wild-type plants (Fig. 7E). These results indicate that overexpression of TaNAC2-5A in Arabidopsis enhanced nitrate uptake rate, possibly by up-regulating the expression of nitrate transporters. ChIP enrichment analysis showed that TaNAC2-5A strongly bound to the P2 fragment of AtNRT2.1, which contains a putative CGT(G/A) motif (Fig. 7G).

Figure 6. Subcellular localization and transcriptional activation activity of TaNAC2-5A. A, Nuclear localization of TaNAC2-5A in root tips of wheat seedlings. Seedlings 7 d after germination were used to stain TaNAC2 protein with immunofluorescence labeling. The primary antibody was a polyclonal antibody to TaNAC2, and the secondary antibody was a goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated antibody. Intra-cellular fluorescence was observed using a confocal microscope equipped with an argon laser (488 nm). Bar = 10 μm. B, Transactivation activity of TaNAC2-5A, TaNAC2-5AΔN, and TaNAC2-5AΔC in yeast cells. The TaNAC2-5A open reading frame, or the fragment of TaNAC2-5A encoding the N-terminal (TaNAC2-5AΔN) or C-terminal (TaNAC2-5AΔC) domain of TaNAC2-5A, was fused in frame with the GAL4 DNA-binding domain (BD) in the pGBKT7 vector. Empty pGBK7 was used as a negative control. Ade, Adenine; SD, synthetic dropout.

Figure 5. Subcellular localization and transcriptional activation activity of TaNAC2-5A. A, Nuclear localization of TaNAC2-5A in root tips of wheat seedlings. Seedlings 7 d after germination were used to stain TaNAC2 protein with immunofluorescence labeling. The primary antibody was a polyclonal antibody to TaNAC2, and the secondary antibody was a goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated antibody. Intra-cellular fluorescence was observed using a confocal microscope equipped with an argon laser (488 nm). Bar = 10 μm. B, Transactivation activity of TaNAC2-5A, TaNAC2-5AΔN, and TaNAC2-5AΔC in yeast cells. The TaNAC2-5A open reading frame, or the fragment of TaNAC2-5A encoding the N-terminal (TaNAC2-5AΔN) or C-terminal (TaNAC2-5AΔC) domain of TaNAC2-5A, was fused in frame with the GAL4 DNA-binding domain (BD) in the pGBKT7 vector. Empty pGBK7 was used as a negative control. Ade, Adenine; SD, synthetic dropout.
GmNAC32 and GmNAC2 (soybean; Fig. 8, F and G) but repressed the expression of TaNAC6 (wheat; Fig. 8H) and AtNAC32 and AtNAC102 (Arabidopsis; Fig. 8, I and J). These results indicate that the NAC TFs in clades I and II differed in their responses to nitrate and that the clade I NAC TFs may be important in controlling nitrate responses in cereal crops.

**DISCUSSION**

**TaNAC2-5A Is Involved in Nitrate Signaling and Regulates N Transport and Assimilation Genes**

Nitrate is both a key nutrient and a signaling molecule for plants. A number of molecular components involved in nitrate signaling have been identified to date in Arabidopsis. These include the nitrate sensor AtNPF6.3/NRT1.1 (Ho et al., 2009), the CBL-INTERACTING PROTEIN KINASEs AtCIPK8 and AtCIPK23 (Ho et al., 2009; Hu et al., 2009), the RWP-RK TF NIN LIKE PROTEIN7 (AtNLP7; Castaings et al., 2009; Marchive et al., 2013), the LATERAL ORGAN BOUNDARY DOMAIN (LBD) gene family of TFs (AtLBD37, AtLBD38, and AtLBD39; Rubin et al., 2009), the auxin receptor AtAFB3 and its target AtNAC4 (Vidal et al., 2013), the TF TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR1-20 (Guan et al., 2014), and the TGACG motif-binding factors AtTGA1 and AtTGA4 (Alvarez et al., 2014). However, nitrate signaling is not understood well in cereal crops such as wheat, rice, and maize, which are the most important food crops in the world. This study suggests that TaNAC2-5A is an important regulatory element in a nitrate-responsive network that may directly or indirectly activate the expression of downstream genes. TaNAC2-5A expression was rapidly (within 15 min after nitrate treatment) and strongly induced after exposure to nitrate whenever the wheat plants were pretreated with N-deprived solution (Fig. 1A) or ammonium as sole N source (Fig. 8A). Besides TaNAC2-5A, the rice OsNAC19 and the maize ZmNAC67 from the cereal-specific clade I of NAC TFs were also found to be nitrate inducible (Fig. 8, B and C), suggesting the importance of this clade of NAC TFs in nitrate signaling in cereal crops. TaNAC2-5A expression also appears to be controlled by internal N status, as TaNAC2-5A was upregulated in both roots and shoots by long-term low-N treatment (Fig. 1B).

Overexpression of TaNAC2-5A in wheat increased nitrate influx and up-regulated the expression of genes encoding nitrate transporter and GS. The putative high-affinity nitrate transporter TaNRT2.1-6B was predominantly expressed in roots and had much higher expression than did TaNRT2.1-6D (Fig. 4, A and B).
indicating its possible role in nitrate uptake. A previous study reported that TaNPF7.1-6D expression had the highest root transcript level of all NPF/NRT1 genes analyzed (Buchner and Hawkesford, 2014). Our results also indicated that TaNPF7.1-6D was predominantly expressed in roots and was expressed at a much higher level than was TaNPF7.2-6B (Fig. 4, C and D). Therefore, the larger net nitrate influx of the transgenic lines may have resulted from the increased expression of TaNRT2.1-6B and TaNPF7.1-6D in the TaNAC2-5A lines. GS plays an essential role in the metabolism of N by catalyzing the condensation of Glu and ammonium.
to form Gln (Miflin and Habash, 2002). Our previous study showed that TaGS2 haplotypes are associated with N-use traits and yield-related traits in wheat (Li et al., 2011). In wheat leaves, GS activity was strongly correlated to soluble protein and N content (Kichey et al., 2006; Habash et al., 2007). Furthermore, QTLs for leaf GS activity colocalized with those for leaf protein and N content and with yield components in wheat (Habash et al., 2007). Therefore, the increased TaGS2 expression may contribute to the improved NUE observed following the overexpression of TaNAC2-5A.

We observed that TaNAC2-5A has transcription activation activity and can directly bind to the promoter regions of TaNRT2.1-B1, TaNPF7.1-D1, and TaGS2-2A (Figs. 5 and 6). Thus, TaNAC2-5A directly regulates genes putatively involved in nitrate transport and N assimilation. A previous study in Arabidopsis reported that AtNAC4 controls a nitrate-responsive network; however, AtNAC4 seems not to act on the AtNPF6.3/NRT1.1 and AtNRT2.1 nitrate transporters and does not act on N metabolic genes (Vidal et al., 2013). Therefore, it seems that TaNAC2-5A and AtNAC4 may be involved in different pathways in controlling gene expression in plant responses to nitrate. Although TaNAC2-5A belongs to a cereal-specific clade of NAC proteins, overexpression of TaNAC2-5A in Arabidopsis up-regulated the expression of several nitrate transporters and increased nitrate influx, and TaNAC2-5A had the ability to bind to the promoter of AtNRT2.1 (Fig. 7, D–G). These findings suggest that TaNAC2-5A has great potential for use in improving NUE in both monocots and eudicots.

Overexpressing TaNAC2-5A Increases the Root’s Ability to Acquire N

Plants are able to adapt to low N availability by increasing primary root length and lateral root branching to efficiently explore soil N resources (Giehl and von Wirén, 2014). Here, we showed that overexpression of TaNAC2-5A increased root dry weight in wheat, stimulated primary and lateral root growth in Arabidopsis, and increased root growth responses to low N availability in both wheat and Arabidopsis (Figs. 3D and 7, B and C). TaNAC2-5A can thus be considered an attractive gene resource for use in engineering deep and large root systems that are highly responsive to N availability; these are root architecture traits that are considered ideal for efficient N acquisition (Garnett et al., 2009; Mi et al., 2010; Trachsel et al., 2013). The increased root size combined with the increased nitrate influx rates (compare with Fig. 4F) provided a strong basis for the TaNAC2-5A-transgenic wheat lines to efficiently acquire soil N resources, as has been shown at the seedling stage in the pot experiment (Fig. 3F) and throughout the entire plant life cycle in the field experiment (Table I).

Overexpressing TaNAC2-5A Increases Aerial N Accumulation and Grain Yield

The field experiment showed that overexpressing TaNAC2-5A increased grain yield, N accumulation in aerial parts (aerial N accumulation), and N allocation to grains (Table I). In the pot trial (compare with Fig. 3F) and the field experiment (compare with Table I), the transgenic line OE1 had higher N accumulation in aerial parts and grains.
parts than did OE2 under various N supply levels; this was possibly due to the fact that OE1 had higher expression of nitrate transporters analyzed and nitrate influx rate than did OE2 (Fig. 4). Although overexpressing TaNAC2-5A increased N allocation to grains, we did not observe early leaf senescence in the transgenic lines during grain filling. This was possibly because overexpressing TaNAC2-5A increased the root’s ability to acquire N. The NAC TF NAM-B1 has been shown to increase grain protein, zinc, and iron content in wheat (Uauy et al., 2006b), but it accelerates senescence and thus has adverse effects on total grain yield (Uauy et al., 2006a; Brevis and Dubcovsky, 2010). A similar result was reported for the rice NAC TF OsNAP (Liang et al., 2014).

At the grain-filling stage, TaNAC2-5A was mainly expressed in old leaves and flag leaves (Fig. 1C), indicating that TaNAC2-5A may have an important role in regulating N transport and metabolism in leaves during grain filling. This idea is in line with the fact that TaNAC2-5A directly regulates TaNPF7.1-6D and TaGS2-2A. A previous study suggested that TaNPF7.1-6D is involved in N loading and unloading (Buchner and Hawkesford, 2014). TaGS2 is known to be important in the assimilation or recycling of ammonium in leaves during grain development and filling (Bernard et al., 2008). Additionally, the increased N harvest index following the overexpression of TaNAC2-5A (compare with Table I) in this study suggests that TaNAC2-5A may control N transport from leaves to grains. TaNAC2-5A may also be useful for increasing the micronutrient nutritional values of grain, as the transgenic lines had higher grain zinc concentrations than did the wild type, under both low-N and high-N conditions (Table I). The increased grain zinc concentration is especially valuable for wheat growers in developing countries, where plant-derived foods are major contributors to the micronutrient requirements for most people. In light of all of this information, TaNAC2-5A promises to be a valuable resource for developing wheat varieties with improved NUE and grain yield.

In summary, TaNAC2-5A is nitrate inducible and regulates the root’s ability to acquire N, N allocation to grains, and grain yield. Although TaNAC2-5A was shown to bind to the promoter regions of genes involved in nitrate transport and N assimilation, further research will be needed to dissect the gene networks operating downstream of TaNAC2-5A. Such studies will not only improve our understanding of nitrate signaling but will also provide new knowledge and gene resources that can be used in the development of wheat varieties with improved NUE and grain yield.
NAC Gene Expression in Response to Nitrate in Different Plant Species

To measure the responses in the expression of NAC genes to nitrate, seedlings (7 d after germination) were grown for 14 d in a nutrient solution that contained ammonium as the sole N source. The plants were then transferred to a nutrient solution that contained nitrate as the sole N source for nitrate induction. After the plants were treated with nitrate for different durations, the roots were collected for gene expression analysis. The N sources used before nitrate treatment were 2 mM NH₄Cl for wheat and rice (Oryza sativa) and 5 mM NH₄Cl for maize (Zea mays), soybean (Glycine max), and Arabidopsis, and those used after nitrate treatment were 2 mM KNO₃ for wheat and rice and 5 mM KNO₃ for maize, soybean, and Arabidopsis. The concentrations of other nutrients in the nutrient solution used for the wheat plants were described previously (Ren et al., 2012); those used for rice were modified from a Yoshida nutrient solution that was described previously (Cai et al., 2008), and those used for maize, soybean, and Arabidopsis were the same as those in Hoagland solution (Hoagland and Arnon, 1950). KCl was used to balance the potassium concentrations before and after the nitrate treatments. The plants were grown in plastic boxes that contained 10 L of nutrient solution; this solution was refreshed every 3 d. The plants were grown in a growth chamber with a 16/8-h light/dark cycle; the temperature was set at 20°C for wheat, 22°C for Arabidopsis, and 28°C/23°C for rice, maize, and soybean.

Measurement of Total N Concentration and Zinc Concentration in Plant Samples

Plant samples were dried at 80°C prior to measuring dry weight. The dried samples were milled and subsequently digested with concentrated H₂SO₄ for the determination of total N, using a semiautomated Kjeldahl method (TecatorKjeltec Auto 1030 Analyzer; Tecator). For zinc, grain samples were ground to a fine powder with a mortar and pestle and zinc concentrations were measured using an Agilent 7500A inductively coupled plasma mass spectrometer (Agilent Technologies) after the samples had been digested with HNO₃ and H₂O₂.

Measurements of Nitrate Flux at the Root Surface

Seedlings (7 d after germination) were grown for 7 d in a nutrient solution that contained 1 mM nitrate (high-nitrate treatment) or 0.2 mM nitrate (low-nitrate treatment). The roots of these plants were then transferred to a measuring solution and allowed to balance for 10 min. The net nitrate fluxes were measured using a scanning ion-selective electrode technique (systemBIO-003A; Younger USA Science and Technology; Applicable Electronics; Science Wares) at Xuyue Science and Technology, using methods that have been described previously (Luo et al., 2013; Zheng et al., 2013). For measuring nitrate flux of the high-nitrate-treated roots, the measuring solution contained 1 mM KNO₃, 0.1 mM CaCl₂, and 0.3 mM MES (pH 6); for measuring nitrate flux of the low-nitrate-treated roots, the measuring solution contained 0.2 mM KNO₃, 0.1 mM CaCl₂, and 0.3 mM MES (pH 6). To measure nitrate flux rate at the root surface of Arabidopsis plants, seedlings (4 d after germination) were grown for 4 d on a solid medium that contained 6 mM KNO₃, 1% (w/v) agar, and 1% (w/v) Suc. The nitrate flux rate was measured in measuring solution containing 1 mM KNO₃, 0.1 mM CaCl₂, and 0.3 mM MES (pH 6). The maximum nitrate flux rates along the root were recorded.

Quantitative Real-Time PCR

Total RNA from plant tissues was extracted with the Plant RNeasy Kit (Qiagen). First-strand complementary DNA was synthesized from 2 µg of DNase I-treated total RNA using the PrimeScript RT Reagent Kit (TaKaRa) according to the manufacturer’s instructions. Quantitative real-time PCR analysis was performed with a LightCycler 480 engine (Roche) using LightCycler 480 SYBR Green I Master Mix (Roche). β-TUBULIN (in common wheat) or ACT2 (in Arabidopsis) was used as an internal control. The specific primers used for quantitative real-time PCR are detailed in Supplemental Table S1.

Transcriptional Activation Analysis of TaNAC2-5A

Transactivation activity assays were based on the GAL4-based Matchmaker Two-Hybrid System 3 (Clontech). The TaNAC2-5A open reading frame, or the fragment of TaNAC2-5A encoding the N-terminal (TaNAC2-5A-N) or C-terminal (TaNAC2-5A-C) domain of TaNAC2-5A, was fused in-frame with the GAL4 DNA-binding domain in the pGBKT7 vector. Empty pGBKT7 was used as a negative control. These different constructs were transformed into yeast (Saccharomyces cerevisiae) strain AH109. The yeast liquid cultures were diluted to an optical density absorbance of 0.5 at 600 nm, and a 1-µl drop of culture was added to plates that contained Trp-, His-, and adenine-negative yeast synthetic dropout agar medium. The plates were grown for 3 d at 30°C for the transcriptional activation assays. The relevant PCR primer sequences are given in Supplemental Table S1.

Subcellular Localization of TaNAC2

The subcellular localization of TaNAC2 was investigated using immuno-fluorescence labeling methods, as described previously (He et al., 2006). The primary antibody was a polyclonal antibody to TaNAC2, and fluorescein isothiocyanate-conjugated goat anti-rabbit antibody was used as the secondary antibody (Sigma; diluted 1:100 in phosphate-buffered saline). Intracellular fluorescence was observed using a confocal microscope (Zeiss LSM 710 NLO) equipped with an argon laser (488 nm).

ChiP Assay

The TaNAC2-5A-overexpressing wheat and Arabidopsis transgenic lines were used for the ChiP assays; these assays were performed according to methods described previously (Bowler et al., 2004). Anti-TaNAC2 antibody was used to precipitate the DNA-TaNAC2 complexes. The DNA was released with proteinase K and then purified for further PCR analysis. The enrichment of DNA fragments was determined using quantitative real-time PCR, with the primers detailed in Supplemental Table S1.

Statistical Analysis of Data

One-way ANOVA was performed using SAS statistical software (SAS Institute).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Sequence alignment of TaNAC2.

Supplemental Figure S2. Relative expression levels of TaNAC2-5A in the seedlings of the wild-type Arabidopsis and transgenic lines.

Supplemental Figure S3. Phylogenetic tree of NAC proteins.

Supplemental Table S1. The primers used in this study.

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