

A Wheat CCAAT Box-Binding Transcription Factor Increases the Grain Yield of Wheat with Less Fertilizer Input¹

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Increasing fertilizer consumption has led to low fertilizer use efficiency and environmental problems. Identifying nutrient-efficient genes will facilitate the breeding of crops with improved fertilizer use efficiency. This research performed a genome-wide sequence analysis of the A (NFYA), B (NFYB), and C (NFYC) subunits of Nuclear Factor Y (NF-Y) in wheat (*Triticum aestivum*) and further investigated their responses to nitrogen and phosphorus availability in wheat seedlings. Sequence mining together with gene cloning identified 18 NFYAs, 34 NFYBs, and 28 NFYCs. The expression of most NFYAs positively responded to low nitrogen and phosphorus availability. In contrast, microRNA169 negatively responded to low nitrogen and phosphorus availability and degraded NFYAs. Overexpressing *TaNfya-B1*, a low-nitrogen- and low-phosphorus-inducible NFYA transcript factor on chromosome 6B, significantly increased both nitrogen and phosphorus uptake and grain yield under differing nitrogen and phosphorus supply levels in a field experiment. The increased nitrogen and phosphorus uptake may have resulted from the fact that that overexpressing *TaNfya-B1* stimulated root development and up-regulated the expression of both nitrate and phosphate transporters in roots. Our results suggest that *TaNfya-B1* plays essential roles in root development and in nitrogen and phosphorus usage in wheat. Furthermore, our results provide new knowledge and valuable gene resources that should be useful in efforts to breed crops targeting high yield with less fertilizer input.

Nitrogen (N) and phosphorus (P) fertilizers have made a significant contribution to the improvement of crop yields (Godfray et al., 2010; Gregory and George, 2011). To meet the food demands for the estimated nine billion humans on Earth, the consumption of N and P fertilizers will likely more than double by 2050 (Tilman et al., 2001). However, the overuse of fertilizers has resulted in low fertilizer use efficiency, resource waste, and environmental problems (Tilman et al., 2001; Liu et al., 2013). As such, systematic approaches to efficiently utilize N and P fertilizers, such as the development of N- and P-efficient crop varieties, should be made. As N and P fertilizers are applied together in many agriculture systems, a promising approach would be to breed crops

that can efficiently use both N and P; this strategy could lead to high yields with lower economic and environmental costs.

Conventional plant breeding has made remarkable improvements to both N and P use efficiency. For instance, modern wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) varieties significantly out-yield the old varieties at all N levels tested (Sylvester-Bradley and Kindred, 2009; Cormier et al., 2013). The International Maize and Wheat Improvement Centre Wheat Program has selected wheat varieties that can efficiently acquire P under low-P conditions but also respond to P application (Manske et al., 2000). In addition to conventional breeding, molecular breeding that employs marker-assisted selection and transgenic technologies has also improved N and P use efficiencies in crops. Marker-assisted selection for *PHOSPHORUS UPTAKE1*, a major rice (*Oryza sativa*) quantitative trait locus that confers tolerance to P deficiency in soils, significantly increases rice grain yields in P-deficient soils (Chin et al., 2010). In recent years, transgenic technology has been widely used to engineer crops with improved N use efficiency by manipulating amino acid biosynthesis, N translocation and remobilization, and N signaling (McAllister et al., 2012; Xu et al., 2012). Such methods have also been used to develop crops with efficient P use by enhancing P acquisition capacities (Tian et al., 2012; Zhang et al., 2014). However, to date, most of these transgenic studies have targeted the efficient

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use of a single nutrient, not the efficient use of both N and P.

Complicated gene networks control both N and P use in plants. A variety of transcription factors in N and P signaling networks have been identified recently; these are known to modulate the expression of genes involved in root development and genes involved in the uptake, assimilation, remobilization, and storage of N and P (Chiou and Lin, 2011; Alvarez et al., 2012; Gutiérrez, 2012; Zhang et al., 2014). Some of these transcription factors have been shown to be of value in the engineering of crops for improved N or P use efficiency. For example, the plant-specific transcription factor *Dof zinc finger protein1* from maize (*Zea mays*) can increase the expression of phosphoenolpyruvate carboxylase and several genes involved in the tricarboxylic acid cycle, which results in the production of more carbon skeletons for the assimilation of N in *Arabidopsis thaliana* (Yanagisawa et al., 2004). This transcription factor has been used successfully to improve N use efficiency and enhance growth in rice under low-N conditions by increasing carbon flow toward N assimilation (Kurai et al., 2011). The MYB-CC-type transcription factor *PHOSPHATE STARVATION RESPONSE1* (*PHR1*) plays a central role in the signaling network for sensing P availability (Rubio et al., 2001; Chiou and Lin, 2011). *PHR1* and its homologs in *Arabidopsis* and rice regulate root development and a set of inorganic phosphate (Pi) starvation-inducible genes, including those involved in Pi uptake and redistribution (Rubio et al., 2001; Nilsson et al., 2007; Zhou et al., 2008; Bustos et al., 2010). Recently, a *PHR1* homolog in wheat (*TaPHR1*) was shown to stimulate lateral branching, increase P uptake and grain yield, and up-regulate a subset of Pi starvation response genes in wheat (Wang et al., 2013). In addition to transcription factors, microRNAs also play vital roles in N and P signaling (Chiou et al., 2006; Pant et al., 2009; Fischer et al., 2013; Zeng et al., 2014), and some microRNAs are regulated by both N and P supply levels (Pant et al., 2009; Kant et al., 2011). The E3 ligase NITROGEN LIMITATION ADAPTATION (NLA) was initially shown to function as a positive regulator of the adaptability of *Arabidopsis* to N limitation (Peng et al., 2007). More recently, NLA was found to regulate Pi signaling/homeostasis through its interaction with the E2 conjugase PHOSPHATE2 (*PHO2*), a key negative regulator in the Pi response pathway (Park et al., 2014). *NLA* and *PHO2* are, respectively, the targets of two Pi starvation-induced microRNAs, miR827 and miR399 (Bari et al., 2006; Lin et al., 2013). These two microRNAs were also recently found to be regulated by N starvation (Fischer et al., 2013). These results indicate that NLA, *PHO2*, miR827, and miR399 function in regulating the cross-talk between the N and P signaling pathways. Modulation of these factors may facilitate the development of crops with more efficient N and P use.

The microRNA169 (miR169) family is known to be regulated by both N and P starvation (Pant et al., 2009; Lundmark et al., 2010; Xu et al., 2011, 2013; Zhao et al.,

2011; Liang et al., 2012). Different members of this microRNA family show distinct response patterns to N or P starvation (Pant et al., 2009; Liang et al., 2012; Xu et al., 2013). As such, miR169 and its targets may be valuable resources for engineering plants with altered N and/or P use efficiencies. For example, under N starvation treatment in *Arabidopsis*, miR169 is down-regulated while its potential targets *NFYA2*, *NFYA3*, *NFYA5*, and *NFYA8* are up-regulated (Zhao et al., 2011). Overexpression of miR169a inhibited the expression of several of *NFYAs* and accumulated less N in transgenic *Arabidopsis* (Zhao et al., 2011).

CCAAT box-binding transcription factors are conserved among all eukaryotes; they are named Nuclear Factor Y (NF-Y) in plants, CCAAT-binding factor in mammals, and Heme Activator Protein (HAP) in yeast (*Saccharomyces cerevisiae*; Mantovani, 1999). NF-Y transcription factors are heterotrimers composed of three subunits: NF-YA, NF-YB, and NF-YC (hereafter, NF-YA, NF-YB, and NF-YC are referred as *NFYA*, *NFYB*, and *NFYC*, respectively). Several studies suggested that members of the NF-Y family play essential roles in the control of flowering (Kumimoto et al., 2008, 2010; Wei et al., 2010; Li et al., 2011; Yan et al., 2011), seed development (Yamamoto et al., 2009), photosynthesis (Stephenson et al., 2010, 2011), and nodule development (Comber et al., 2006, 2008; Zanetti et al., 2010). Other NF-Y family members are known to enhance tolerance to abiotic stresses such as drought (Nelson et al., 2007; Stephenson et al., 2007; Li et al., 2008; Ni et al., 2013), salinity (Zhao et al., 2009; Leyva-González et al., 2012), and cold (Leyva-González et al., 2012; Shi et al., 2014). A relationship between NF-Ys and N utilization was first reported in yeast: the HAP complex regulates the expression of *Glucose dehydrogenase1* and an N-starvation-specific gene, *vacuolar serine protease (isp6⁺)* (Dang et al., 1996; Nakashima et al., 2002; Riego et al., 2002). Although miR169 has been reported to potentially function in N and P signaling, the functions of its targets (*NFYAs*) in mediating N and P use are not well understood in crops such as wheat.

Here, we show that both N and P starvation cause the down-regulation of miR169 but the up-regulation of the *NFYAs* in wheat. Overexpressing *TaNFYA-B1* in wheat stimulated lateral branching, up-regulated the expression of nitrate and phosphate transporters, increased N and P uptake, and increased grain yields under control, low-N, and low-P conditions. Thus, *NFYAs* represent valuable genetic resources for efforts to breed crops that utilize both N and P more efficiently.

RESULTS

Cloning and Phylogenetic Analysis of NF-Y Transcription Factors

In a microarray experiment to identify N and P starvation-responsive genes in wheat, we found that the probes corresponding to NF-Y transcription factors

were induced by both low-N and low-P treatments. In order to more comprehensively understand the responses of different NF-Y transcription factors to N and P supply levels, we first isolated NF-Y genes using both complementary DNA (cDNA) cloning and sequence mining from public databases, including the National Center for Biotechnology Information dbEST, the genome sequences of the wheat cv Chinese Spring (International Wheat Genome Sequencing Consortium, 2014), and the two grasses thought to be the A genome and D genome donors of wheat (*Triticum urartu* and *Aegilops tauschii*; Jia et al., 2013; Ling et al., 2013). We identified a total of 80 unique NF-Y genes in the wheat genome. These included 18 NFYAs, 34 NFYBs, and 28 NFYCs (Supplemental Table S1), which encode the A, B, and C subunits of NF-Y, respectively. The chromosomal locations of 76 NF-Y genes were determined according to the draft sequence of the 21 individual chromosomes of cv Chinese Spring (Supplemental Table S1), while the putative chromosomal locations of the remaining four genes (e.g. *TaNFYB-B2*, *TaNFYB-D3*, *TaNFYB-A13*, and *TaNFYB-D13*), which were isolated by cDNA cloning, were assigned according to their similarities with NFYBs from cv Chinese Spring, *T. urartu*, and *A. tauschii*. Of the 18 NFYAs, six each were from the A, B, and D genomes; of the 34 NFYBs, 11, 12, and 11 were from the A, B, and D genomes, respectively; of the 28 NFYCs, seven, nine, and 12 were from the A, B, and D genomes, respectively (Supplemental Table S1).

Phylogenetic trees for NFYA (Supplemental Fig. S1), NFYB (Supplemental Fig. S2), and NFYC (Supplemental Fig. S3) were created after alignment of the putative NF-Y protein sequences from wheat, *T. urartu*, *A. tauschii*, barley, maize, rice, *Brachypodium distachyon*, *Arabidopsis*, and yeast. The phylogenetic trees clearly showed that the NF-Y proteins of wheat were most closely related to the homologs from *T. urartu* and *A. tauschii*, followed by those of barley. The phylogenetic tree of NFYAs was divided into three clades, and each clade contained NFYAs from all the plant species that were used to create this phylogenetic tree (Supplemental Fig. S1). For the 18 NFYAs from wheat, *TaNFYA5* (*TaNFYA-A5*, *TaNFYA-B5*, and *TaNFYA-D5*) fell into clade I; *TaNFYA1* (*TaNFYA-A1*, *TaNFYA-B1*, and *TaNFYA-D1*), *TaNFYA3* (*TaNFYA-A3*, *TaNFYA-B3*, and *TaNFYA-D3*), and *TaNFYA4* (*TaNFYA-A4*, *TaNFYA-B4*, and *TaNFYA-D4*) were in clade II; and *TaNFYA2* (*TaNFYA-A2*, *TaNFYA-B2*, and *TaNFYA-D2*) and *TaNFYA6* (*TaNFYA-A6*, *TaNFYA-B6*, and *TaNFYA-D6*) were in clade III (Supplemental Fig. S1).

Expression of *TaNFYAs* Is Induced by N and P Starvation

Quantitative real-time reverse transcription (RT)-PCR was used to evaluate the mRNA expression levels of the NF-Y genes in the roots and shoots of wheat seedlings grown under different N and P supply levels. Primers were designed to amplify the homologous alleles at a

particular locus; for example, the relative expression level of *TaNFYA1* might represent that of all three homologous alleles of *TaNFYA1* (*TaNFYA-A1*, *TaNFYA-B1*, and *TaNFYA-D1*). Compared with the control treatment, low-N treatment significantly increased the expression levels of *TaNFYA1* to *TaNFYA4* in shoots (Fig. 1A), and both low-N and low-P treatments significantly increased the expression levels of *TaNFYA1* to *TaNFYA4* in roots (Fig. 1B). However, neither low-N nor low-P treatment significantly affected the expression of *TaNFYA5* or *TaNFYA6* in shoots or roots (Fig. 1). We also investigated the responses of the *TaNFYB* and *TaNFYC* genes to N and P supply levels. The expression of *TaNFYB1* in shoots was up-regulated by low-P treatment. The expression of *TaNFYB3* in shoots and the expression of *TaNFYB2* to *TaNFYB5* in roots were repressed by low-N and low-P treatments (Supplemental Fig. S4). Most of the tested *TaNFYC* genes did not respond significantly to alternation of N or P supply levels. The exceptions to this were that *TaNFYC3* was up-regulated in shoots by low-N

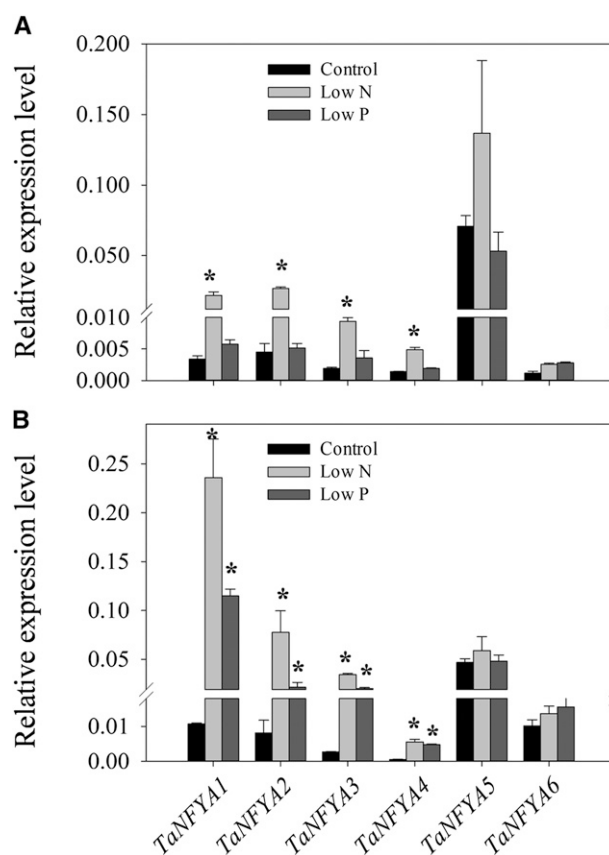


Figure 1. The responses of *TaNFYA* genes to N and P starvation. Wheat seedlings 7 d after germination were grown for 20 d in nutrient solutions that contained 2 mM N + 0.2 mM P (control), 0.2 mM N + 0.2 mM P (low N), and 2 mM N + 0.02 mM P (low P). The shoots (A) and roots (B) were then collected for gene expression analysis. cDNA was standardized by reference to an actin (*TaACTIN*) standard. Data represent means \pm SE of three independent biological replicates. Asterisks indicate that differences between the control and low N or low P were significant at the 0.05 level.

treatment and *TaNFYC1* was down-regulated in roots by low-N treatment (Supplemental Fig. S5). These results indicated that TaNF-Y genes responded differentially to N and P availability and that low-N and low-P treatments mainly induced the expression of TaNFYA genes.

Expression of *T. aestivum* (tae)-miR169 Is Reduced by N and P Starvation

It has been reported that miR169 can regulate the expression of NFYAs by cleaving the mRNA of NFYAs (Li et al., 2008; Zhao et al., 2011). To determine whether tae-miR169 responds to N and P starvation, small RNAs were extracted from the roots and shoots of Xiaoyan 54 plants grown under control, low-N, and low-P conditions and sequenced. The clean sequence reads for individual small RNA samples varied between 6,024,563 and 12,328,574. Bioinformatics analysis of the sequence data identified nine tae-miR169 members and revealed that the expression of tae-miR169a, tae-miR169b, tae-miR169e, tae-miR169f, tae-miR169h, and tae-miR169n was inhibited in both shoots and roots by both low-N and low-P treatments. The other three members (tae-miR169d, tae-miR169m, and tae-miR169v) were present only in very low numbers of transcripts; thus, whether their expression was regulated by N and P supply levels will require further investigation (Table I). To confirm the results of the small RNA sequencing, microRNA northern blotting was used to detect the expression of tae-miR169 by using tae-miR169b as a probe. The results showed that the expression of tae-miR169 in shoots was repressed by low-N treatment and that in roots was repressed by both low-N and low-P treatments (Fig. 2). This repression was stronger in roots than in shoots and more dramatic with low-N treatment than with low-P treatment (Supplemental Fig. S6). Our subsequent analysis showed that all nine tae-miR169 members nearly perfectly matched with their putative target sites in the 3' untranslated region of *TaNFYA1* to *TaNFYA4*

and *TaNFYA6* but not in *TaNFYA5* (Fig. 3). *TaNFYA-B1* was degraded by tae-miR169f (Supplemental Fig. S7).

The Molecular Cloning and Characterization of *TaNFYA-B1*

To further determine the roles of TaNFYA genes in mediating N and P use in wheat, *TaNFYA1* was chosen as the representative subunit for further study. *TaNFYA1* was selected because it had the strongest responses to N and P supply levels among the investigated TaNFYA genes (Fig. 1). The genome sequences of *TaNFYA-A1* and *TaNFYA-D1* both have five exons and four introns in the same arrangement, but the second intron is absent in *TaNFYA-B1* (Supplemental Fig. S8). By using the length polymorphisms of the first intron, we successfully located *TaNFYA-A1*, *TaNFYA-B1*, and *TaNFYA-D1* on the long arms of chromosomes 6A, 6B, and 6D, respectively (Supplemental Fig. S9), confirming the results of the cv Chinese Spring draft genome sequence (Supplemental Table S1). After sequencing the cDNAs corresponding to the homologous *TaNFYA1* alleles, we found that *TaNFYA-B1* accounted for about 60% of the cDNA sequences (data not shown), suggesting that *TaNFYA-B1* had higher expression levels than did *TaNFYA-A1* or *TaNFYA-D1*. By overexpressing a 35S::*TaNFYA-B1*::GFP fusion in Arabidopsis, we observed that TaNFYA-B1 was localized to the nucleus (Supplemental Fig. S10A). A transcriptional activity assay in yeast cells showed that TaNFYA-B1 had transcriptional activation activity (Supplemental Fig. S10B).

Overexpressing *TaNFYA-B1* in Wheat Increases Grain Yield under Different N and P Supply Levels in a Field Experiment

In order to investigate the functions of TaNFYA genes, we developed *TaNFYA-B1* transgenic wheat plants

Table I. Abundance of the miR169 family in wheat

Xiaoyan 54 seedlings were grown for 7 d in nutrient solutions that contained 2 mM N + 0.2 mM P (control), 0 mM N + 0.2 mM P (low N), and 2 mM N + 0 mM P (low P). Total RNA was isolated from roots and shoots separately for small RNA sequencing. Transcript abundance is presented as transcripts per million.

Seedling	Transcript Abundance					
	Shoot			Root		
	Control	Low P	Low N	Control	Low P	Low N
tae-miR169a	406.92	133.62	133.23	74.54	45.59	36.26
tae-miR169b	1,399.30	573.98	488.27	180.80	69.00	42.36
tae-miR169d	0.00	0.00	0.00	0.32	0.16	0.12
tae-miR169e	39.86	22.74	13.45	49.97	20.78	4.46
tae-miR169f	28.28	9.30	6.58	13.71	0.99	0.82
tae-miR169h	163.06	51.46	51.52	52.16	10.60	2.70
tae-miR169m	0.40	0.50	0.14	0.00	0.00	0.00
tae-miR169n	14.00	4.65	3.86	1.30	0.25	0.12
tae-miR169v	0.13	0.00	0.14	0.16	0.08	0.00

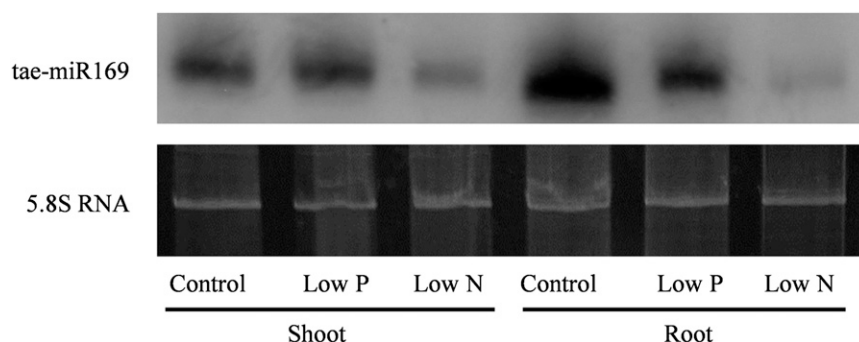


Figure 2. Northern blot of tae-miR169b from the shoots and roots of wheat plants grown under different N and P supply levels. Wheat seedlings were grown for 20 d in the control, low-P, and low-N nutrient solutions, and then total RNA from the shoots and roots was separated by PAGE; RNA blots were probed with [γ - 32 P]ATP-labeled oligonucleotides. The 5.8S RNA bands were visualized by ethidium bromide staining of polyacrylamide gels and served as loading controls.

using a maize ubiquitin promoter-controlled construct. Two homozygous transgenic lines (OE1 and OE2) were obtained. *TaNFYA-B1* expression in these lines was severalfold higher than in the wild-type Longchun 23 plants (Fig. 4C). The performance of the wild-type and transgenic lines were evaluated under control, low-N, and low-P conditions in a field experiment. Both of the transgenic lines had significantly higher grain yield and spike numbers than the wild type in all three of the treatments (Fig. 4, A, D, and E). However, the transgenic lines had similar or lower 1,000-grain weights and spike grain numbers than the wild type (Fig. 4, B, F, and G). These results indicated that overexpression of *TaNFYA-B1* increased grain yield by increasing spike number.

We also evaluated N and P use in the transgenic lines. The two transgenic lines had higher grain N concentration than the wild type under control conditions, and OE1 had higher grain N concentration than the wild type under low-P conditions (Fig. 4H). OE1 also had higher grain P concentration than the wild type in all three treatments (Fig. 4I). Overexpressing *TaNFYA-B1* did not consistently affect straw N concentration or straw P concentration (Fig. 4, J and K). The two transgenic lines had higher N and P uptake than the wild type under all three treatments (with the exception of P uptake of OE2 under low-P conditions; Fig. 4, L and M). For all three treatments, the transgenic lines and the wild type had similar N and P utilization efficiencies (Supplemental Fig. S11); the

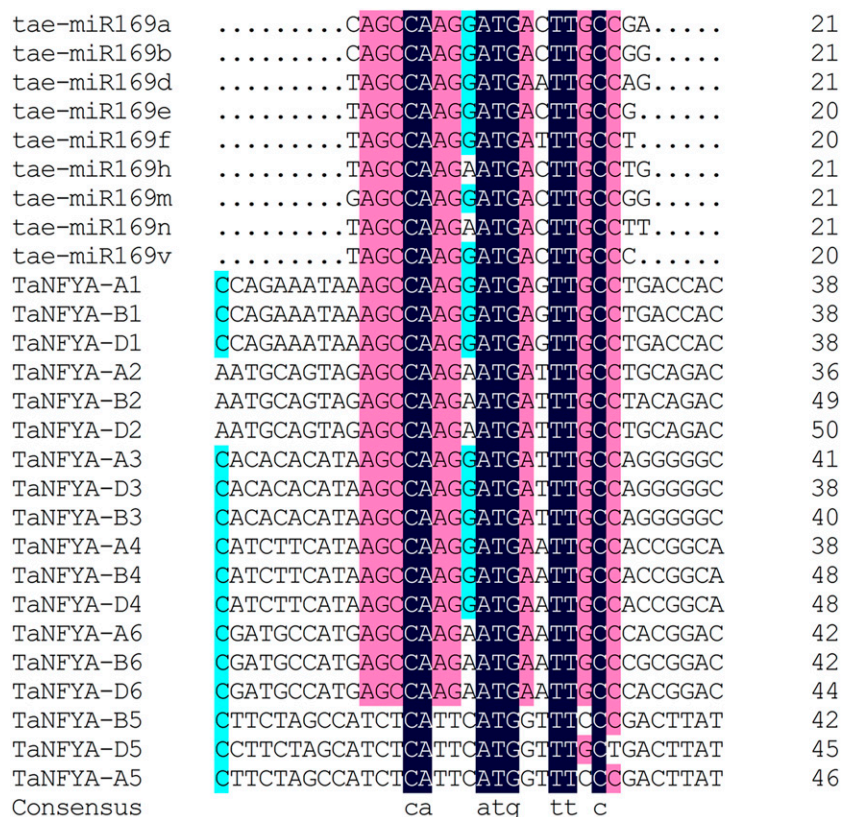
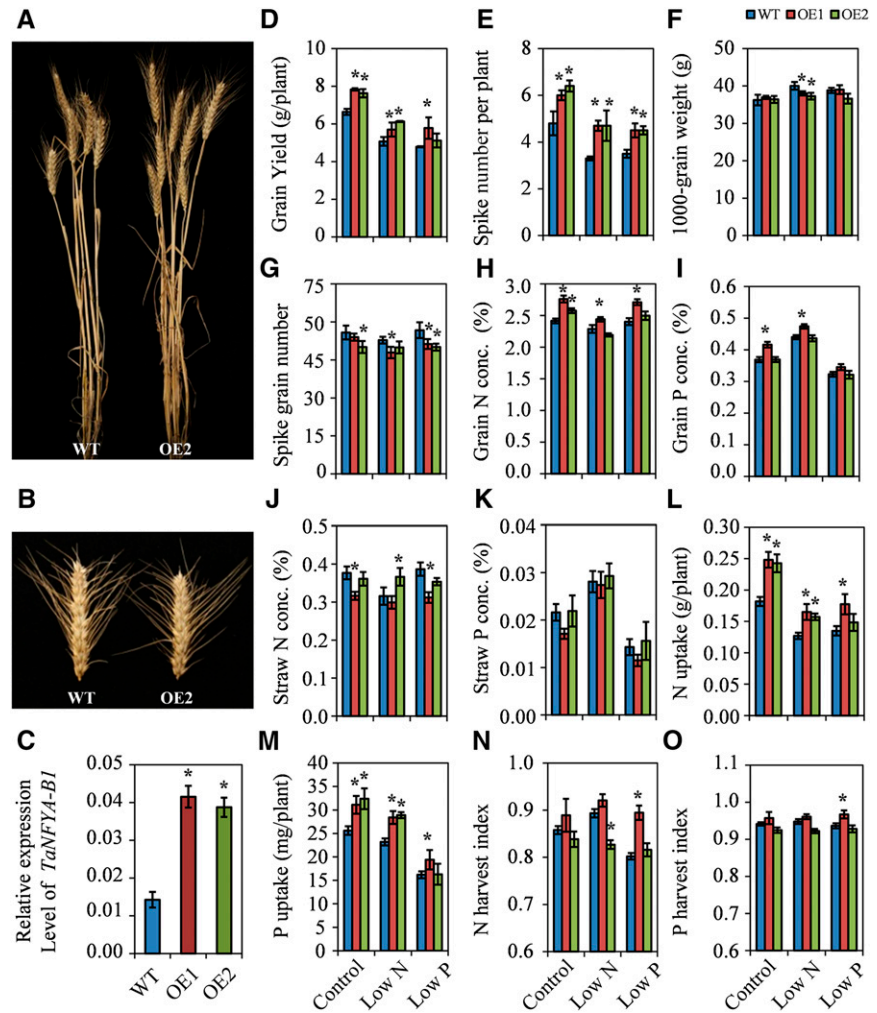


Figure 3. Putative tae-miR169 binding sites in the 3' untranslated region of *TaNFYA* genes. The target sites of *TaNFYA* genes were aligned with miR169 mature sequences with DNAMAN6.0. The mature sequences of tae-miR169 were obtained through sequencing small RNA libraries of wheat seedlings with Illumina Genome Analyzer Iix.

Figure 4. Agronomic traits and N and P use of the wild type and *TaNFYA-B1* transgenic lines in the field experiment. The wild type (WT) and the transgenic lines (OE1 and OE2) were grown in soils that were treated with 18 g N m⁻² + 13.5 g P m⁻² (control), 0 N + 13.5 g P m⁻² (low-N treatment), and 18 g N m⁻² + 0 P (low-P treatment). A, Representative plants of the wild type and OE2 under control conditions. B, Primary spikes of the plants in A. C, Expression levels of *TaNFYA-B1* in the seedlings grown under control conditions. D to O, Grain yield per plant (D), spike number per plant (E), 1,000-grain weight (F), grain number per spike (G), grain N concentration (H), grain P concentration (I), straw N concentration (J), straw P concentration (K), N uptake (L), P uptake (M), N harvest index (N), and P harvest index (O). Data represent means ± SE of three replicates. Asterisks indicate that differences between the wild type and the transgenic lines were significant at the 0.05 level.



transgenic lines and the wild type also had similar N and P harvest indexes (Fig. 4, N and O). These results suggested that overexpression of *TaNFYA-B1* increased N and P uptake under different N and P supply levels but did not apparently affect N and P utilization efficiency.

Overexpressing *TaNFYA-B1* Promotes Lateral Root Growth

To understand the mechanisms by which *TaNFYA-B1* increases N and P uptake, we first investigated how *TaNFYA-B1* affected root growth. In a hydroponic culture system with plants at the seedling stage, we observed that the two transgenic lines had higher lateral root length than the wild type (Fig. 5, A and C). The transgenic plants had similar primary root length to the wild type (Fig. 5B). In a soil-pot experiment, we also observed that overexpressing *TaNFYA-B1* increased N uptake and P uptake under both low-N and high-N conditions and root dry weight under low-N conditions (Supplemental Fig. S12). Therefore, both the hydroponic culture and soil-pot experiments demonstrated that overexpressing *TaNFYA-B1* enhanced

root development, possibly by increasing lateral root growth.

To analyze the mechanism by which overexpression of *TaNFYA-B1* enhanced root development, we compared the expression of auxin biosynthesis genes in the wild-type and transgenic lines. It has been reported that overexpressing *TRYPTOPHAN AMINOTRANSFERASE RELATED2* (*AtTAR2*), an auxin biosynthetic gene, promoted lateral growth in *Arabidopsis* (Ma et al., 2014). We analyzed the effect of overexpressing *TaNFYA-B1* on the expression of *TAR2* homologs in wheat. We determined the expression of the three homologous alleles of *TaTAR2* located on chromosomes 5A, 5B, and 5D and found that overexpressing *TaNFYA-B1* up-regulated the expression of *TaTAR2-A1*, *TaTAR2-B1*, and *TaTAR2-D1* in roots (Fig. 5D).

Overexpressing *TaNFYA-B1* Increases the Expression of Nitrate and Phosphate Transporter Genes

Nitrate transporters of the NRT1 and NRT2 families and phosphate transporters of the PHT1 family are the key membrane proteins that function in nitrate and

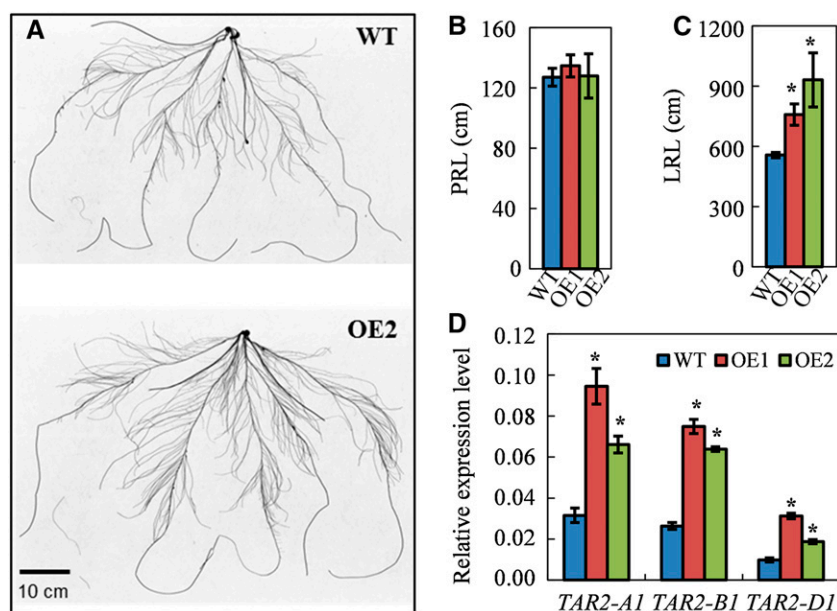


Figure 5. Root morphological parameters of the wild type (WT) and the transgenic lines (OE1 and OE2) hydroponically grown under control nutrient conditions. Wheat seedlings were grown for 20 d in the control nutrient solution, and then the roots were scanned with an STD1600 scanner and analyzed for the root morphological parameters with WinRHIZO software (Regent Instruments). A, Root images of the wild type and OE2. B, Primary root length (PRL). C, Total lateral root length (LRL). D, Expression levels of *TaTAR2* in roots. Data represent means \pm SE of at least five replicates. Asterisks indicate that differences between the wild type and the transgenic lines were significant at the 0.05 level.

phosphate uptake from soil, respectively (Schachtman et al., 1998; Xu et al., 2012). As OE1 and OE2 plants absorbed more N and P than did the wild-type plants, and there is at least one putative CCAAT box in the promoters of *NRT* and *PHT* genes (Supplemental Fig. S13), we evaluated the expression of these genes in the roots of plants that were grown under control conditions. The results showed that both of the transgenic lines had higher mRNA expression levels of *NRT1.1*, *NRT2.1*, *PHT1.1/1.9*, *PHT1.2*, *PHT1.3/1.4*, *PHT1.5*, *PHT1.6*, *PHT1.7*, and *PHT1.10* than did the wild type (Fig. 6, A–I).

Overexpressing *TaNFYA-B1* Increases Root Nitrate Influx

As overexpression of *TaNFYA-B1* increased the expression of nitrate transporters in roots, we measured the net nitrate flux rates using the scanning ion-selective electrode technique (SIET; Luo et al., 2013; Zheng et al., 2013). The roots of wild-type and transgenic lines (OE1 and OE2) grown for 20 d in the control nutrient solution (2 mM nitrate) were used to measure net nitrate flux rates in measuring solution containing 0.2 mM nitrate. At the root tip (0 mm from the root tip), wild-type and transgenic line roots all exhibited net nitrate efflux; at sites 3, 6, and 9 mm from the root tip, the roots of the wild type exhibited net nitrate efflux, while the roots of the transgenic lines exhibited net nitrate influx (Fig. 6j). These results indicated that overexpressing *TaNFYA-B1* enhanced root nitrate influx.

DISCUSSION

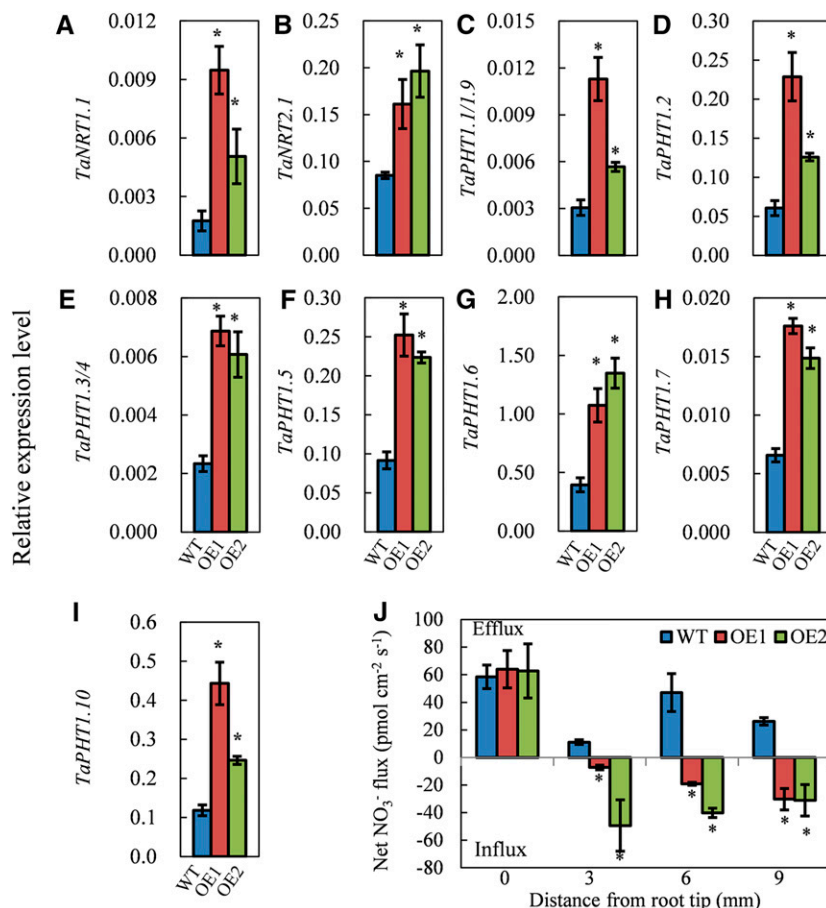
The NF-Y Family in Wheat

The CCAAT box is one of the most ubiquitous elements existing upstream of eukaryotic promoters. The

three subunits of NF-Y transcript factors form a heterotrimer that binds to these CCAAT boxes and thus act as a transcriptional activator or repressor (Laloum et al., 2013). Unlike the situation in animals, each NF-Y subunit is encoded by a single gene; a large expansion in the members of the NF-Y family has occurred in plants (Laloum et al., 2013). For example, there are nine *NFYAs*, 10 *NFYBs*, and 10 *NFYCs* in Arabidopsis (Gusmaroli et al., 2001, 2002). In a previous study in wheat, 10 *NFYAs*, 11 *NFYBs*, and 14 *NFYCs* were identified (Stephenson et al., 2007). In this study, a total of 80 wheat NF-Y genes were identified (Supplemental Table S1). By analyzing the draft genome of the wheat A genome progenitor *T. urartu* (Ling et al., 2013), we identified six *NFYAs*, 12 *NFYBs*, and eight *NFYCs* (Supplemental Table S1). In a previous study of *A. tauschii*, the D genome donor of common wheat, six *NFYAs*, 12 *NFYBs*, and 11 *NFYCs* were identified (Jia et al., 2013). After comparing the copy numbers of NF-Y genes in wheat, *T. urartu*, and *A. tauschii*, we concluded that there are six *NFYAs*, and theoretically as many as 12 *NFYBs* and 15 *NFYCs*, in each of the three genomes of wheat. In the sequenced cereals rice, maize, and *B. distachyon*, the copy numbers of the NF-Y genes vary between seven and 13 for *NFYA* genes, 11 and 17 for *NFYB* genes, and seven and 12 for *NFYC* genes (Supplemental Table S1). Thus, the copy numbers of NF-Y genes in each wheat genome fell into the range of that known for other cereals.

The members of the NF-Y family in plants have undergone functional diversification (Laloum et al., 2013). It has been reported that NF-Ys play roles in regulating photosynthesis, flowering, seed development, nodule development, and adaptations to abiotic stresses such as drought, salinity, and nutrient deficiency (for references, see the introduction). However,

Figure 6. Expression levels of nitrate and phosphate transporter genes in the wild type (WT) and the transgenic lines (OE1 and OE2). Wheat seedlings were grown for 20 d in the control nutrient solution, and then total RNA was isolated from the roots and used for gene expression analysis. A to I, Expressions levels of *TaNRT1.1* (A), *TaNRT2.1* (B), *TaPHT1.1/1.9* (C), *TaPHT1.2* (D), *TaPHT1.3/4* (E), *TaPHT1.5* (F), *TaPHT1.6* (G), *TaPHT1.7* (H), and *TaPHT1.10* (I). J, Net NO_3^- flux rate. NO_3^- fluxes were measured by SIET with 0.2 mM NO_3^- in the measuring solution. Positive and negative flux values indicate efflux and influx, respectively. Data represent means \pm SE of at least three replicates. Asterisks indicate that differences between the wild type and the transgenic lines were significant at the 0.05 level.



the functions of NF-Ys in wheat are still largely unknown, mainly due to the complex and large genome of wheat. The information regarding the chromosomal locations of the NF-Y genes from wheat (Supplemental Table S1) and phylogenetic trees of the families of the three NF-Y subunits (Supplemental Figs. S1–S3) will help in analyzing the collinear relationships for the NF-Y genes between wheat and model cereals such as rice. The information about NF-Y functions obtained in studies of model cereals promises to facilitate the study in wheat.

NF-Y Genes in Wheat Respond Variously to N and P Starvation

In plants, a subset of miR169 members are repressed by N and P starvation (Pant et al., 2009; Liang et al., 2012; Xu et al., 2013), and several miR169 NFYA targets are induced in such conditions (Pant et al., 2009; Zhao et al., 2011). We observed similar results in this study in wheat: low-N and low-P treatments decreased *tae-miR169* abundance (Fig. 2; Table I) and increased the expression of *TaNFYA1* to *TaNFYA4* (Fig. 1). In correlation with the converse responses of *tae-miR169* and *TaNFYA1* to *TaNFYA4* to low-N and low-P treatments, *tae-miR169f* was able to degrade its target

TaNFYA-B1 (Supplemental Fig. S7). The lack of a miR169 target in *TaNFYA5* was a possible reason for the lack of any response to low-N or low-P treatments for *TaNFYA5* (Fig. 3). Although there was a putative miR169 target site in the three *TaNFYA6* genes (Fig. 3), we did not detect any response to low-N and low-P treatment for these genes (Fig. 1). In contrast with the up-regulation of *TaNFYA* genes in low-N and low-P treatments, the majority of *TaNFYB* and *TaNFYC* genes did not positively respond to these same treatments (Supplemental Figs. S4 and S5). Previously, various *TaNf-Y* genes have been shown to differentially respond to drought stress in wheat (Stephenson et al., 2007). Therefore, *TaNf-Y* genes could play different roles in the adaptation to abiotic stresses.

Overexpression of *TaNFYA-B1* Promotes Wheat Growth and Grain Yield

NF-Y transcription factors have been shown to improve drought tolerance in *Arabidopsis* (Li et al., 2008), maize (Nelson et al., 2007), and soybean (*Glycine max*; Ni et al., 2013) and to reduce N starvation-induced senescence in *Arabidopsis* (Leyva-González et al., 2012). Here, we found that overexpressing *TaNFYA-B1* improved N uptake, P uptake, and grain yield in wheat

under different N and P supply conditions in a field experiment (Fig. 4, D, L, and M). The increased N and P uptake in the transgenic lines appears to favor an increase in the number of tillers (Supplemental Fig. S12) and spikes (Fig. 4E) and ultimately in grain yield. In a recent 10-year period (2004–2013), the average wheat grain yield in China varied from 4,251.9 to 5,050.6 kg ha⁻¹ (<http://faostat.fao.org/>). Under control conditions in the field experiment, the theoretical grain yield per square meter (the product of grain yield per plant and plant density per square meter) of the wild-type and transgenic lines fell into the range of the average wheat grain yield in China in that recent 10-year period (Supplemental Fig. S14A). As such, *TaNFYA-B1* showed potential in engineering wheat with improved yield and fertilizer use efficiency under the growth conditions of average yield. The negative effect of overexpressing *TaNFYA-B1* on spike grain number may limit the use of this gene in breeding high-yield wheat, as spike grain number greatly contributes to grain yield under the growth conditions of high yield. Although overexpressing *AtNFYA2*, *AtNFYA3*, *AtNFYA7*, and *AtNFYA10* delayed N starvation-induced senescence, such overexpression of these genes impaired cell elongation and caused a dwarf phenotype in Arabidopsis (Leyva-González et al., 2012). Moreover, the expression of these four genes is induced by N and/or P limitation (Zhao et al., 2011; Leyva-González et al., 2012). Thus, it seems that the NFYA genes are positively responsive to N and P starvation but do not essentially promote plant growth. As such, more research is needed to evaluate the functions of the different NFYA genes in engineering wheat varieties with improved grain yield under different N and P availabilities.

Overexpression of *TaNFYA-B1* Increases N and P Uptake, Possibly by Enhancing Root Growth and Up-Regulating the Expression of Nitrate and Phosphate Transporters

Breeding wheat with a large root system is an important strategy to improve N and P use efficiency (Manske et al., 2000; Liao et al., 2004; An et al., 2006; Ehdaie et al., 2010). Here, we showed that overexpressing *TaNFYA-B1* enhanced root development by stimulating lateral root growth (Fig. 5, A–C; Supplemental Fig. S12D) and that *TaNFYA-B1* overexpression also increased N and P uptake. It has been demonstrated that miR169/NFYA modules are important regulators of primary and lateral root development. For instance, expressing a miR169-resistant version of *AtNFYA2* in Arabidopsis indirectly increased lateral root initiation and lateral root density but did not affect primary root growth (Sorin et al., 2014). However, the molecular mechanisms through which miR169/NFYA modules regulate root development are still not understood well. Our study shows that *TaNFYA-B1* possibly acts on auxin biosynthesis to promote lateral root development. TAR2 functions in the indole-3-pyruvic acid biosynthetic pathway, the main route for the de novo synthesis of

auxin in plants (Mashiguchi et al., 2011). In Arabidopsis, overexpressing *AtTAR2* increases auxin accumulation in both shoots and roots and increases lateral root growth under both high-N and low-N conditions (Ma et al., 2014). The more abundant *TaTAR2* transcripts in the roots of *TaNFYA-B1*-overexpressing plants (Fig. 5D) might favor auxin biosynthesis and, hence, lateral root development.

The up-regulated expression of nitrate and phosphate transporters in the *TaNFYA-B1*-overexpressing plants also might contribute to improved N and P uptake. Transporters of the NRT1 and NRT2 families mediate the uptake of nitrate (Xu et al., 2012), which is the main N source taken up by wheat. Thus, the elevated expression of *TaNRT1.1* and *TaNRT2.1* (Fig. 6, A and B) and the increased nitrate influx rate in roots (Fig. 6J) should facilitate N acquisition by the *TaNFYA-B1*-overexpressing plants. When the wheat plants prepared in 2 mM nitrate solution were used to measure nitrate flux in 0.2 mM nitrate solution by the SIET method, the wild type exhibited net nitrate efflux while the transgenic lines showed net nitrate influx at sites 3, 6, and 9 mm from the root tip (Fig. 6J). It has been reported that net nitrate efflux can occur when a plant grown in a high-nitrate solution is transferred to a low-nitrate solution (Zheng et al., 2013). The larger net nitrate influx of the transgenic lines was possibly due to the increased expression of *TaNRT1.1* and *TaNRT2.1* in the *TaNFYA-B1* lines (Fig. 6, A and B). In line with the positive regulation of nitrate transporters by *TaNFYA-B1*, overexpression of miR169a down-regulated the expression of *AtNRT1.1* and *AtNRT2.1* (Zhao et al., 2011). To cope with P deficiency, plants increase the expression of PHT1 transporters in roots and thus increase the ability of their root systems to utilize P from soils (Zhang et al., 2014). Increasing the expression of PHT1 transporters through transgenic approaches has been shown to be successful in engineering crops with improved P uptake (Tian et al., 2012). As such, the increased mRNA levels of a number of PHT1 transporters (Fig. 6, C–I) in roots should account for the improved P uptake by the plants overexpressing *TaNFYA-B1*. It is already known that it is the NFYA subunit of the NF-Y complexes that binds to the CCAAT box (Laloum et al., 2013). Since the promoters of the investigated NRT and PHT1 transporters contain putative CCAAT boxes (Supplemental Fig. S13), it is worthy to check if *TaNFYA-B1* directly binds to the promoters and thus activates the expression of these transporters.

In summary, we performed a genome-wide identification of miR169 and NF-Y transcription factors and evaluated their responses to N and P availability. *TaNFYA-B1* was shown to be a valuable genetic resource/target for the engineering of wheat varieties with more efficient use of N and P and higher grain yields under different N and P availability conditions. The up-regulation of auxin biosynthetic genes and nutrient transporters by overexpressing *TaNFYA-B1* suggested that *TaNFYA-B1* may control a complex

gene network. Further dissecting the gene network controlled by TaNFYA-B1 and other NF-Y members will facilitate our understanding of the roles of NF-Y transcription factors in mediating nutrient transport from soils to grains and in regulating root and aerial development and yield formation.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The wheat (*Triticum aestivum*) varieties Xiaoyan 54 and Longchun 23 were used in this study. Xiaoyan 54 was used to isolate the sequences and evaluate the expression of the TaNF-Y genes, and Longchun 23 was used to generate the transgenic lines.

Hydroponic Culture

The Xiaoyan 54 plants used for the TaNF-Y gene expression analysis and the transgenic lines that were phenotyped at the seedling stage were grown hydroponically. Seven days after germination, wheat seedlings were grown for 20 d in plastic pots containing 1 L of nutrient solution; this solution was refreshed every 2 d. The hydroponic culture was carried out in a growth chamber with the following conditions: 20°C ± 1°C, 50% to 70% relative humidity, a photon fluence rate of 300 μmol photons m⁻² s⁻¹, and a 16-h-day/8-h-night cycle. To analyze the expression of the TaNF-Y genes in Xiaoyan 54 plants, three treatments (control, low N, and low P) were used. The nutrient solution of the control treatment was described previously (Ren et al., 2012). The N and P supply levels of the control, low-N, and low-P treatments were set as 2 mM N [Ca(NO₃)₂] + 0.2 mM P (KH₂PO₄), 0.2 mM N + 0.2 mM P, and 2 mM N + 0.02 mM P, respectively. CaCl₂ and KCl were used to balance the calcium and potassium concentrations of the different treatments. The roots and shoots of three plants were collected separately for gene expression analysis. To analyze the phenotypes of the transgenic lines overexpressing TaNFYA-B1, seedlings of the wild-type Longchun 23 and T4 transgenic lines OE1 and OE2 were grown in the control nutrient solution. The roots were used to measure root morphology, the expression of nitrate and phosphate transporter genes, and net nitrate flux rates. The root morphological parameters were measured as described by Ren et al. (2012). Nitrate influx rate was measured as described by Zheng et al. (2013).

Field Experiment

The field experiment was conducted in the experimental station of the Institute of Genetics and Developmental Biology in Beijing. The experiment contained three treatments, which each had three replications. The control treatment had 18 g m⁻² N in the form of urea with 12 g m⁻² applied prior to sowing and 6 g m⁻² applied at the stem elongation stage, and 13.5 g m⁻² P (calcium superphosphate) applied prior to sowing. The low-N treatment had no N application but 13.5 g m⁻² P. The low-P treatment had no P application but 18 g m⁻² N. For each genotype in each replicate, 90 seeds were sown in three 1.5-m-long rows, and the rows were spaced 23 cm apart (87 seeds m⁻²). The plant density was thinned to 22 plants per row (63.8 plants m⁻²). The grain yield per plant, spike number per plant, and grain number of the primary spike were recorded on 30 representative plants for each sample group. The 1,000-grain weight was determined according to the dry weight of 500 dried grains. Total N concentrations in straw and grain were measured using a semiautomated Kjeldahl method (Tecator Kjeltac Auto 1030 Analyzer; Tecator). To measure total P concentration, the straw and grain samples were digested with concentrated H₂SO₄ and H₂O₂ and then the P concentrations of these digestions were determined using a Molybdate Blue colorimetric method (Murphy and Riley, 1962).

Gene Cloning and Phylogenetic Analysis

The sequences of the NF-Y genes from wheat, *Aegilops tauschii*, rice (*Oryza sativa*), *Brachypodium distachyon*, and Arabidopsis (*Arabidopsis thaliana*; Stephenson et al., 2007; Petroni et al., 2012; Jia et al., 2013) were used as queries

to search the EMBL-European Bioinformatics Institute GenBank to identify the NF-Y genes of wheat, *Triticum urartu*, barley (*Hordeum vulgare*), maize (*Zea mays*), and *B. distachyon* (<http://www.ebi.ac.uk>). In addition to sequence mining from public databases, cDNA cloning was also employed to isolate NF-Y genes by using the primers listed in Supplemental Table S2. After removing redundancies, the NFYA, NFYB, and NFYC subunits from all of the plants investigated were aligned using ClustalX 2.0 (Thompson et al., 2002). We used the neighbor-joining method to generate a phylogenetic tree, and the phylogenetic tree was drawn using MEGA 5.0 (Tamura et al., 2011).

To identify microRNA169 members, Xiaoyan 54 seedlings were grown for 7 d in nutrient solutions that contained 2 mM N + 0.2 mM P (control), 0 mM N + 0.2 mM P (low N), and 2 mM N + 0 mM P (low P). Total RNA was isolated from roots and shoots separately using TRIzol reagent (Thermo). The total samples were sent to the Beijing Genomics Institute for small RNA processing and sequencing. The small RNAs with lengths between 18 and 30 nucleotides were enriched and then reverse transcribed to cDNA as described previously (Liang et al., 2014). Total cDNA was sequenced by Illumina Genome Analyzer Ix. Raw reads were filtered to get clean reads. During this process, the following reads were removed: all low-quality reads including 3' adapter reads and 5' adapter contaminants, reads with poly(A), and reads larger than 30 nucleotides and smaller than 18 nucleotides.

Quantitative Real-Time PCR and MicroRNA Northern Blot

Total RNA from plant tissues was extracted with TRIzol reagent (Thermo). The first-strand cDNA was synthesized from 2 μg of DNase I-treated total RNA using murine leukemia virus reverse transcriptase (Promega). Quantitative real-time RT-PCR analysis was performed with a LightCycler 480 engine (Roche) using the LightCycler480 SYBR Green I Master Mix (Roche). *ACTIN2* mRNA was used as an internal control. The primers for real-time RT-PCR are detailed in Supplemental Table S2.

For microRNA gel-blot analysis, 20 μg of RNA was separated by denaturing (7 M urea containing) 15% PAGE and then blotted onto a nylon membrane (Hybond NX; GE Healthcare); the RNA blot was cross-linked by exposing to UV light for 1 min. miR169b was used as the probe and was prepared by end labeling with [³²P]ATP using T4 polynucleotide kinase (Thermo Scientific). RNA gel blots were hybridized with the miR169b probe. Nonsaturated signals were quantified on a Molecular Dynamics Storm 840 PhosphorImager (Molecular Dynamics).

Vector Construction and Transformation

In order to generate the vector for wheat transformation, the maize ubiquitin promoter (pUbi) was subcloned into the *Pst*I site of the pAHC25 vector (Christensen et al., 1992), resulting in a vector named pUbi-pAHC25. Then, the TaNFYA-B1 cDNA was inserted into the *Bam*HI/*Kpn*I site of pUbi-pAHC25, resulting in the construct pUbi::TaNFYA-B1-pAHC25. The construct was transformed into immature embryos of wheat variety Longchun 23 using the method described by Wang et al. (2013).

Measurement of Net Nitrate Flux Using a Noninvasive Microtest Technique

Wild-type and TaNFYA-B1 transgenic line plants grown in the control solution for 20 d were used to measure net nitrate fluxes. For measuring net nitrate fluxes, the roots were transferred to measuring solution (0.2 mM KNO₃, 0.1 mM CaCl₂, and 0.3 mM MES, pH 6) and allowed to balance for 10 min, and then the net nitrate fluxes were measured by using a SIET (systemBIO-003A; Younger USA Science and Technology) at Xuyue Science and Technology in Beijing, as described (Luo et al., 2013; Zheng et al., 2013).

Statistical Analysis

One-way ANOVA was performed with SPSS11.5 for Windows (SPSS).

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: TaNRT1.1, AY587265; TaNRT2.1, AF288688; TaPHT1.1-A1, KJ170110; TaPHT1.1-D1, KJ170111; TaPHT1.2-A1, KJ170112; TaPHT1.2-B1, KJ170113; TaPHT1.2-D1, KJ170114; TaPHT1.3-D1, KJ170115; TaPHT1.4-D1, KJ170116; TaPHT1.4-D2, KJ170117; TaPHT1.5-A1, KJ170118; TaPHT1.5-B1, KJ170119; TaPHT1.5-D1, KJ170120;

TaPHT1.6-B1, KJ170121; TaPHT1.6-D1, KJ170122; TaPHT1.7-D1, KJ170123; TaPHT1.8-B1, KJ170124; TaPHT1.8-D1, KJ170125; TaPHT1.9-D1, KJ170126; TaPHT1.10-D1, KJ170127; TaPHT1.10-D2, KJ170128; TaTAR2-A1, KM078759; TaTAR2-B1, KM078760; and TaTAR2-D1, KM078761. Sequence data of the NF-Y genes in this study can be found in the GenBank/EMBL data libraries under the accession numbers listed in Supplemental Table S1.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Phylogenetic analysis of NFYAs.

Supplemental Figure S2. Phylogenetic analysis of NFYBs.

Supplemental Figure S3. Phylogenetic analysis of NFYCs.

Supplemental Figure S4. The responses of TaNFYB genes to N and P starvation.

Supplemental Figure S5. The responses of TaNFYC genes to N and P starvation.

Supplemental Figure S6. The relative expression level of ta-miR169 under different N and P supply levels.

Supplemental Figure S7. The regulation of ta-miR169f on TaNFYA-B1 and the cleavage site mapping.

Supplemental Figure S8. The gene structures of TaNFYA1 genes.

Supplemental Figure S9. Chromosome localizations of TaNFYA1.

Supplemental Figure S10. Subcellular localization and transcription activation of TaNFYA-B1.

Supplemental Figure S11. The N and P utilization efficiency of the wild type and transgenic lines grown in the field experiment.

Supplemental Figure S12. The performance of the wild-type and transgenic lines grown in a pot experiment.

Supplemental Figure S13. The putative CCAAT-box in the promoter of nitrate and phosphate transporters.

Supplemental Figure S14. Theoretical grain yield, spike number, and the N and P uptake per square meter of the wild-type and transgenic lines grown in the field experiment.

Supplemental Table S1. The identified NF-Y genes in wheat.

Supplemental Table S2. The primers used in this study.

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