**RESEARCH ARTICLE**

**TaNBP1**, a guanine nucleotide-binding subunit gene of wheat, is essential in the regulation of N starvation adaptation via modulating N acquisition and ROS homeostasis

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**Abstract**

**Background:** Nitrate (NO$_3^-$) is the major source of nitrogen (N) for higher plants aside from its function in transducing the N signaling. Improving N use efficiency of crops has been an effective strategy for promotion of the sustainable agriculture worldwide. The regulatory pathways associated with N uptake and the corresponding biochemical processes impact largely on plant N starvation tolerance. Thus, exploration of the molecular mechanism underlying nitrogen use efficiency (NUE) and the gene wealth will pave a way for molecular breeding of N starvation-tolerant crop cultivars.

**Results:** In the current study, we characterized the function of **TaNBP1**, a guanine nucleotide-binding protein subunit beta gene of wheat (*T. aestivum*), in mediating the plant N starvation response. TaNBP1 protein harbors a conserved W40 domain and the TaNBP1-GFP (green fluorescence protein) signals concentrate at positions of cytoplasm membrane and cytosol. **TaNBP1** transcripts are induced in roots and leaves upon N starvation stress and that this upregulated expression is recovered by N recovery treatment. **TaNBP1** overexpression confers improved phenotype, enlarged root system architecture (RSA), and increased biomass for plants upon N deprivation relative to the wild type, associating with its role in enhancing N accumulation and improving reactive oxygen species (ROS) homeostasis. Nitrate transporter (NRT) gene *NtNRT2.2* and antioxidant enzyme genes *NtSOD1*, *NtSOD2*, and *NtCAT1* are transcriptionally regulated under **TaNBP1** and contribute to the improved N acquisition and the increased AE activities of plants.

**Conclusions:** Altogether, **TaNBP1** is transcriptional response to N starvation stress. Overexpression of this gene enhances plant N starvation adaptation via improvement of N uptake and cellular ROS homeostasis by modifying transcription of NRT gene *NtNRT2.2* and antioxidant enzyme genes *NtSOD1*, *NtSOD2*, and *NtCAT1*, respectively. Our research helps to understand the mechanism underlying plant N starvation response and benefits to genetically engineer crop cultivars with improved NUE under the N-saving cultivation conditions.

**Keywords:** Wheat (*Triticum aestivum* L.), Guanine nucleotide-binding protein subunit beta, G-protein, Gene expression, N starvation stress, Functional characterization

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Background

Nitrogen (N) is an essential macronutrient for the plant growth and development. For a long time, increased input of the N fertilizers has contributed greatly to the improvement of the crop productivity. However, overdose of N input during crop production has also caused serious environment problem in addition to the increased investment [1]. Improvement of N use efficiency (NUE) of crops under the N-saving cultivation conditions has thus been an effective strategy for sustainable agriculture given that it can alleviate the N-associated environmental pollution.

Plants have evolved multifaceted strategies in response to external N availabilities [2, 3]. Upon N deficiency, plants respond to the N-starvation signaling via initiation of response pathways involving transcriptional modulation of a suite of N-starvation responsive genes, whose synergic action contributes to the plant N starvation adaptation. Of which, distinct transcription factors (TFs) and nitrate transporters (NRTs) have been confirmed to be involved in the N starvation response. For example, the TF encoding genes, including Arabidopsis nitrate regulated 1 gene (ANR1) and nodule inception-like protein 7 gene (NL7P) as well as maize DNA finger binding protein gene (ZmD0F1), act as crucial mediators in the regulation of N starvation tolerance via mediating external nitrate response and internal N assimilation [4–6]. The Arabidopsis NRT genes (i.e., CHL1 and NRT2.1) play important roles in sensing nitrate signal, taking up external nitrate, and translocating internal N across tissues [7, 8]. It is now well documented that plants are able to sense external nitrate availability. Therefore, nitrate acts as a signal molecule in plants involving regulation of many biological processes associated with N intake, metabolism, and related gene expression [9]. However, although a large set of investigations performed focuses on understanding of the N starvation responses and adaptation pathways, detailed mechanisms as to how plants perceive and transduce the N signaling still remain largely unknown.

GTP-binding proteins (G-proteins) are heterotrimeric consisting of three subunits, Ga, Gβ, and Gγ. In plant species as well as other eukaryotes, proteins in this functional class play essential roles in transducing the signals initiated by internal cues as well as environmental stressors [9, 10]. The subunits constituting G-protein execute distinct functions across diverse biological processes. For example, Arabidopsis dimer Gβγ1 and Gβγ2 involves plant defensiveness to a variety of fungal pathogen infection [11]; it acts as an essential mediator in preventing plant infection and injury from P. syringae, a kind of bacterial pathogen, via regulating programmed cell death (PCD) and reactive oxygen species (ROS) homeostasis [10]. Likewise, subunit Ga of the G-protein endows plants enhanced defensiveness against P. syringae infection and injury through adjustment of stomata movement [12]. Several Ga-like subunits have been validated to be involved in establishing extra-large G proteins referred to as XLGs with subunits Gβ and Gγ, conferring plants increased GTP hydrolysis capacity by which to promote the substrate degradation and modulate the flowering characterization of plants [13, 14].

Subunit Gβ involves the constitution of the G-protein and exerts similar biological functions to Ga and Gγ, two other G-protein subunits. It is also involved in plant pathogen defensiveness [15]. In addition, this subunit has also been an essential mediator in multiple physiological processes, including root gravitropic response [16], tissue differentiation [17], and channel-mediated ion transportation [18]. These findings suggest the diverse roles of subunit Gβ in regulating plant growth, development, and abiotic stress responses.

Wheat (T. aestivum L.) is one of the important cereal crops cultivated around the world. Thus far, a large set of investigations has been performed focusing on understanding of the physiological and biochemical mechanisms underlying plant N starvation response [19]. However, the molecular networks associating with N starvation signaling perceiving and transducing still remain largely unknown in the T. aestivum species. Previously, based on microarray analyses, we identified a guanine nucleotide-binding protein (NBP) encoding TaNBP1 gene (GenBank accession No. AK332651), a beta subunit (Gβ) gene for G-protein of wheat, to be upregulated in expression under N starvation condition. In this study, we functionally characterized TaNBP1, a subunit Gβ encoding gene of wheat, in regulating the N starvation stress tolerance. Our results indicate that TaNBP1 is N-starvation inducible and acts as an essential modulator in plant N deficiency tolerance through regulating N acquisition and cellular ROS homeostasis.

Methods

Characterization analysis of TaNBP1

Sequence similarities of TaNBP1 and its homologous proteins in plant species were determined using MEGA7 software (https://www.mega.com). Conserved domain shared by the NBP proteins including TaNBP1 and its plant counterparts was specified as previously described [20]. Phylogenetic relationship among TaNBP1 and its homologous genes was established using the DNAStar software (https://www.dnastar.com).

3-D structure prediction and subcellular localization analysis on TaNBP1

An online tool referred to as SWISS-MODEL algorithm (https://swissmodel.expasy.org/interactive) that simulates protein three-dimensional structure was adopted to predict the 3-D structure of TaNBP1. To define the
subcellular localization of TaNBPI sorted from endoplasmic reticulum (ER), we generated an expression cassette harboring a TaNBPI-GFP (green florescence protein encoding gene) fusion gene as previously described [21], in which, the coding sequence (CDS) of TaNBPI was amplified by RT-PCR using specific primer pairs (Additional file 1: Table S1) and integrated in frame with GFP under control of the constitutive CaMV35S promoter. GFP signals derived from the fusion in transformed tobacco epidermis cells were detected as described by Guo et al. (2013) [21].

Expression analysis of TaNBPI
Shiyou 20, an elite high yielding wheat cultivar in North China used in our microarray analyses to identify genes differentially expressed upon N starvation stress, was selected to investigate the expression patterns of TaNBPI at two N supplies. With this aim, the seeds were germinated under 25 °C in darkness. After germination, the young seedlings were cultured hydroponically in a growth chamber. To this end, roots of the young seedlings were immersed into standard Murashige and Skoog (MS) solution (16 mM N) through holes of plastic foam that floated on the nutrient solution, which was renewed twice one week and air-circulated by a mini pump. Growth conditions for the seedlings were as follows: photoperiod of 16 h/8 h (light/dark), temperature of 22 °C, light intensity of 230 μmol m⁻² s⁻¹ during light phase, and air humidity of 65 to 75%. At the third leaf-expansion stage, wheat seedling were subjected to N starvation treatment by transferring into a modified MS solution that contained reduced N (0.02 mM N), which was established by reducing NH₄NO₃ and KNO₃ amounts and supplementing KCl in the meantime to sustain unaltered K content in the solution. Additionally, a followed N recovery treatment was initiated by retransferring the 27 h N-deprived seedlings again to a standard MS solution. At 0 h (before N starvation), 1, 3, 9, and 27 h after N starvation and N recovery treatments, root and leaf tissues were collected. Transcripts of TaNBPI1 in both tissues examined were determined based on qRT-PCR. Briefly, total RNA in the tissues was extracted by TRIzol reagents (Invitrogen, USA). After treatment with RNase-free DNase (TaKaRa, Dalian, China) to avoid genomic DNA contamination, the total RNA (2 μg) was subjected to first-strand cDNA synthesis using RT-AMV transcriptase (TaKaRa, Dalian, China) in 20 μL reaction volume using oligo (dT)18 at 42 °C for 30 min, according to the manufacturer’s instructions. qRT-PCR analysis was performed in a total volume of 25 μL containing 12.5 μL of SYBR Premix ExTaqTM (TaKaRa, Dalian, China), 0.5 μL of forward and reverse primers, 1 μL cDNA and 10.5 μL nuclease-free water. TaNBPI transcripts in tissues examined were calculated based on the 2-ΔΔCT method using wheat Tatubulin as an internal control. The gene-specific primers used for qRT-PCR analysis are shown in Additional file 1: Table S1.

Generation of transgenic N. benthamian lines with TaNBPI overexpression
Transgenic N. benthamian lines with TaNBPI overexpression were generated to characterize the gene function in mediating N starvation tolerance. With this aim, an expression cassette harboring the TaNBPI CDS was constructed using conventional approach. Briefly, the TaNBPI CDS was amplified by RT-PCR with specific primers (Additional file 1: Table S1), then inserted into the Ncol/BstEI restriction sites in binary vector pCAMBIA3301 at position downstream the CaMV35S promoter. Genetic transformation of the expression cassette into A. tumefaciens stain EHA105 and further generation of the N. benthamian lines with TaNBPI overexpression were performed as described previously [22].

Assays of phenotypes and biomass of transgenic lines under different N treatments
Two lines with higher TaNBPI expression levels (Lines 2 and 3, Additional file 1: Figure S1A) were selected to address the gene function in mediating the plant N starvation response. To this end, seeds of Lines 2 and 3 at T3 generation were germinated in darkness. Ten days after germination, uniform seedlings of the transgenic and wild type (WT) were hydroponically cultured in standard MS solution for normal growth (16 mM N) or subjected to N starvation treatment by growing under modified MS solution containing reduced N (0.06 mM N); this modified deficient N solution was prepared similarly to that for N-deprived wheat seedlings. The transgenic and WT seedlings under contrasting N conditions were cultured under same growth conditions as wheat seedlings. During culture process, nutrient solutions were air-circulated using a mini pump and renewed twice within each week. Six weeks later, phenotypes of the transgenic and WT plants were recorded using a digital camera and the biomass were obtained after drying the plant samples in an oven at 80 °C for 48 h.

Assays of N concentrations and NRT gene expression patterns in N-deprived transgenic lines
N concentrations and the nitrate transporter (NRT) gene expression patterns in the transgenic lines (Lines 2 and 3) and WT were evaluated after the N starvation treatment. Of which, the N concentrations were assessed as described previously (Guo et al. 2011) [23]. Plant N accumulative amounts in transgenic lines and WT were calculated by multiplying N concentration and plant biomass. To characterize the NRT genes that putatively involve the
mediation of N uptake and internal N translocation across tissues, a set of NRT encoding genes of tobacco, including NtNRT1.1-s, NtNRT1.1-t, NtNRT1.2-s, NtNRT1.2–t, NtNRT2.1, and NtNRT2.2, were subjected to expression evaluation using the N-deprived TaNBPI overexpression lines and WT as samples, based on semiquantitative RT-PCR or qRT-PCR [21]. Tobacco constitutive gene referred to as Nttubulin was used for normalization of the NRT gene transcripts using specific primers (Additional file 1: Table S1).

Assays of growth and N-associated traits in NtNRT2;2 overexpression lines

NtNRT2.2 was shown to be significantly upregulated in expression in the N-deprived TaNBPI overexpression lines (Lines 2 and 3) compared with wild type, suggesting its involvement in the TaNBPI-mediated N starvation response. To evaluate the function of this NRT gene in mediating plant N uptake, transgenic N. benthamian lines with NtNRT2.2 overexpression were generated using similar procedure for establishment of TaNBPI overexpression lines. Primers for amplification of Nt NRT2.2 CDS are shown in Additional file 1: Table S1. To define the NtNRT2.2-mediated N acquisition character, NtNRT2.2–1 and NtNRT2.2–2, two lines with high expression of this NRT gene (Additional file 1: Figure S1B), were subjected to cultivation under N starvation treatment. With this purpose, seeds of NtNRT2.2–1 and NtNRT2.2–2 at T3 generation were germinated in darkness. Ten days after germination, uniform seedlings of the transgenic and wild type (WT) were hydroponically cultured in standard MS solution for normal growth (16 mM N) or subjected to N starvation treatment by growing under modified MS solution containing reduced N (0.06 mM N), which was established to be same for N-deprived WT and lines with TaNBPI overexpression as aforementioned. Likewise, nutrient solutions were air-circulated using a mini pump and renewed twice within each week during the culture process. Phenotypes, biomass and N concentrations were assessed six weeks later followed the aforementioned description.

Assessments of ROS parameters and expression patterns of antioxidant related genes in TaNBPI overexpression lines

Given the close relation between cellular reactive oxygen species (ROS) homeostasis and plant N starvation response, we assessed the activities of a set of antioxidant enzymes (AEs), including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), and the contents of malondialdehyde (MDA), hydrogen peroxide (H2O2), and superoxide anion in the N-deprived TaNBPI overexpression lines (Lines 2 and 3) and wild type. The parameters described above were determined as reported by Huang et al. 2010 [24]. To define genes possibly involving regulation of the TaNBPI-mediated SOD, CAT, and POD activities, a suite of AE genes of tobacco, including five encoding SOD proteins (i.e., NtSOD1, NtSOD2, NtSOD3, NtMnSOD1, and NtMnSOD2), six coding for CAT proteins (i.e., NtCAT, NtCAT1, NtCAT3, NtCAT1;1, NtCAT1;2, and NtCAT1;3), and eleven encoding POD proteins (i.e., NtPOD1:1 to NtPOD1:7, NtPOD2:1, NtPOD2:2, NtPOD4, and NtPOD9), were subjected to expression evaluation using the N-deprived TaNBPI overexpression lines based on RT-PCR or qRT-PCR. Accession numbers and primer pairs used for these AE genes are shown in Additional file 1: Table S1.

Functional analysis of differential AE genes in modulating the AE activities

Expression analysis on the AE encoding genes revealed that three of which, including NtSOD1, NtSOD2, and NtCAT1, were shown to be differentially expressed in the N-deprived TaNBPI overexpression lines. To characterize their function in mediating plant AE activities under N starvation conditions, transgenic lines overexpressing these AE genes were generated. With this aim, CDS of the AE genes was PCR amplified and separately inserted into the binary vector pCAMBIA3301 at the Ncol/BstEII restriction sites under control of the CaMV35S promoter. Constructing expression cassettes harboring these AE genes and genetically transforming them into tobacco were performed to be similar in generating the TaNBPI2 overexpression lines as described above. Primer pairs used for amplifying the AE genes are shown in Additional file 1: Table S1.

Two representative lines for each AE gene were selected to define the gene-mediated AE activities under both N normal and N starvation treatments. With this purpose, seeds of WT and transgenic lines at T3 generation with overexpression of the AE genes, including NtSOD1–1 and NtSOD1–2 for NtSOD1, NtSOD2–1 and NtSOD2–3 for NtSOD2, and for NtCAT1–2 and NtCAT1–3 NtCAT1, were germinated in darkness. Ten days after germination, uniform transgenic and WT seedlings were vertically cultured onto agar media containing 1/2 MS salts (8 mM N) or modified 1/2 MS salts with reduced N (0.06 mM N) which was established similarly to that for N-deprived wheat seedlings as aforementioned. In addition, same growth conditions were adopted for culturing these transgenic and WT seedlings. Three weeks after treatments, the transgenic and WT seedlings were subjected to assay of AE activities performed similarly to those for TaNBPI overexpression lines.

Statistical analysis

The mean values of the plant biomass, N concentration, N amount, activities of SOD, CAT, and POD, content of
MDA, and qRT-PCR data in WT and transgenic lines under sufficient or deficient N conditions were derived from results of four replicates. Standard errors of the mean values and the significant differences among the mean values were analyzed using the Statistical Analysis System software (SAS Corporation).

Results

The characterization of TaNBP1

TaNBP1 cDNA is 1273 bp-long that encodes a 335 aa polypeptide (Additional file 1: Figure S1); the predicted molecular mass and an isoelectric point (pI) of TaNBP1 are 36.28 kD and 6.32, respectively. At amino acid level, TaNBP1 shares high similarities to its counterparts from B. distachyon, D. oligosanthes, O. brachyantha, S. italica, S. bicolor, T. urartu, and Z. mays; all of them harbor the conserved WD40 domain involving the constitution of seven blade units (Fig. 1). At nucleic acid level, TaNBP1 shows high identities to the homologous genes in diverse plant species, with highest similarities to those from H. vulgare (AK359815), B. distachyon (XM_003567896), O. sativa (CT833917), and S. italica (XM_004961250) (Additional file 1: Figure S2). These results suggest that TaNBP1 shared similarly evolved pathway to its plant counterparts.

Based on three dimensional structure (3-D) prediction analysis, it was revealed that TaNBP1 harbors a typical β-propeller feature domain initiated by conserved WD40 repeats (repeats I to VII) (Fig. 2a). Based on distribution of the fusion TaNBP1-GFP detected in transformed tobacco epidermal cells, TaNBP1 was suggested to be located onto positions of cytoplasm membrane and cytosol, given that the GFP signals derived from the fusion were concentrated on these cellular locations (Fig. 2b).

The expression of TaNBP1 in response to external N levels

The expression patterns of TaNBP1 in tissues of roots and leaves were detected under the varied external N levels. Under N normal condition (16 mM N), the TaNBP1 transcripts were shown to be low in both roots and leaves. Upon N starvation stress (0.02 mM N), the TaNBP1 expression in both tissues was gradually upregulated over a 27 h treatment regime, reaching a peak at 27 h after the treatment (Fig. 3). Moreover, the upregulated transcripts of TaNBP1 in N-deprived tissues were gradually restored upon an N normal recovery treatment, showing that its induced expression was gradually lowered in both tissues along with the N recovery progression (Fig. 3). These results indicate that TaNBP1 is temporal response to the external N levels.

TaNBP1 endows plants improved growth and N acquisition upon N-starvation stress

Transgene analysis was performed to characterize the TaNBP1 function in mediating N starvation response. Line 2 and Line 3, two T3 lines possessing more target transcripts (Additional file 1: Figure S3A), were selected and subjected to the N normal and N starvation stress treatments. Under N normal condition, Lines 2 and 3 exhibited comparable phenotypes and biomass with the wild type (WT) (Fig. 4a and c). Under N starvation treatment, however, Lines 2 and 3 both showed enlarged phenotypes, improved root system architecture (RSA), and increased biomass with respect to WT (Fig. 4b and c) (with increase of 54.69 and 59.90% in roots and 83.44 and 122.09% in aerial tissues in Line 2 and Line 3, respectively). These results indicate that TaNBP1 is crucial in mediating plant N starvation tolerance.

N concentrations in the transgenic lines (Lines 2 and 3) and WT were assessed after the N starvation treatment. No obvious variations in both N concentration and N accumulative amount were observed in transgenic and WT plants under N normal condition (Fig. 4d). In contrast, Lines 2 and 3 exhibited increased accumulative N amounts relative to wild type under N starvation treatment (Fig. 4e, with increase of 63.54 and 79.02% in Line 2 and Line 3, respectively), although the lines displayed comparable N concentrations with WT under the N-deficient condition (Fig. 4d). These results suggest that the TaNBP1 is involved in enhanced N accumulation that contributes to the plant N starvation tolerance.

NtNRT2.2 shows upregulated expression in TaNBP1 overexpression lines and confers plants enhanced N acquisition upon N starvation stress

The expression of a suite of tobacco NRT encoding genes, including NtNRT1.1-s, NtNRT1.1-t, NtNRT1.2-s, NtNRT1.2-t, NtNRT2.1, and NtNRT2.2, in the N-deprived transgenic lines (Lines 2 and 3) and WT plants. Among them, the NtNRT2.2 transcripts were drastically upregulated in lines 2 and 3 with respect to those in WT plants (Fig. 5a, b), contrasting to unaltered expression patterns in other NRT genes examined (i.e., NtNRT1.1-s, NtNRT1.1-t, NtNRT1.2-s, NtNRT1.2-t, and NtNRT2.1) among the transgenic and WT plants. These results suggest that the transcription of NtNRT2.2 is under control of TaNBP1 and the upregulated expression of this NRT gene possibly contributes to increased N accumulation in transgenic lines.

The function of NtNRT2.2 in improving N accumulation under N starvation stress was evaluated based on transgene analysis. NtNRT2.2–1 and NtNRT2.2–2, two NtNRT2.2 overexpression lines possessing more target transcripts than others (Additional file 1: Figure S3B), were selected and subjected to N normal and N
starvation treatments. Under N normal condition, NtNRT2.2–1 and NtNRT2.2–2 exhibited comparable phenotypes (Fig. 5c), biomass (Fig. 5d), N concentrations (Fig. 5e), and N accumulation (Fig. 5f) with wild type. Under N starvation treatments, however, the transgenic lines displayed improved phenotype (Fig. 5c), increased biomass (Fig. 5d, increase from 29.15 to 32.60%), and elevated N accumulation (Fig. 5f, increase from 25.00 to 36.32%) relative to WT. The increased N amounts in transgenic lines were caused by improved biomass, given that similar N concentrations were observed in transgenic and WT seedlings (Fig. 5e). These results together indicate that NtNRT2.2 is crucial in mediating plant N acquisition when plants are challenged by N starvation stress.

**TaNBP1 overexpression improves cellular ROS homeostasis under N starvation conditions**

A set of ROS-associated parameters, including the activities of SOD, CAT, POD, contents of MDA, and the amounts of H$_2$O$_2$ and superoxide anion in the TaNBP1 overexpression lines and wild type was assessed after the N normal and N starvation treatments. Under N normal...
condition, the transgenic lines (Lines 2 and 3) exhibited comparable SOD, CAT, and POD activities and MDA, H$_2$O$_2$ and superoxide anion contents with WT plants (Fig. 6a to f). Under N starvation treatment, Lines 2 and 3 displayed increased activities of SOD (Fig. 6a, increase from 44.48 to 52.32%), CAT (Fig. 6b, increase from 48.13 to 52.06%), and POD (Fig. 6c, increase from 55.00 to 65.53%), lowered contents of MDA (Fig. 6d, decrease from 18.69 to 27.88%), and reduced H$_2$O$_2$ and superoxide anion amounts compared with WT (Fig. 6e and f). Therefore, TaNBPI confers increased AE activities and improved cellular ROS homeostasis for plants treated by N starvation stress, which contributes to the TaNBPI-mediated N starvation tolerance.

**AE gene expression patterns in the N-deprived TaNBPI overexpression lines**

A suite of tobacco AE encoding genes, including five for SOD (i.e., NtSOD1, NtSOD2, NtSOD3, NtMnSOD1, and NtMnSOD2), six for CAT (i.e., NtCAT, NtCAT1, NtCAT1;1, NtCAT1;2, NtCAT1;3, and NtCAT3), and eleven for POD (i.e., NtPOD1;1, NtPOD1;2, NtPOD1;3, NtPOD1;4, NtPOD1;5, NtPOD1;6, NtPOD1;7, NtPOD2;1, NtPOD2;2, NtPOD4, and NtPOD9), was subjected to expression evaluation in the N-deprived TaNBPI overexpression lines. Among them, two SOD genes (i.e., NtSOD1 and NtSOD2) and one CAT gene (i.e., NtCAT1) exhibited significantly upregulated in expression in Line 2 and 3, contrasting to other AE genes examined that showed unaltered transcripts abundance among the transgenic and WT plants (Fig. 7a to d). Therefore, the expression of these differential AE genes is possibly controlled under TaNBPI and the upregulated transcription
of them possibly impacts on the modified AE activities and cellular ROS homeostasis of the N-deprived TaNBP1 overexpression lines.

The differential SOD and POD genes mediate drastically cellular AE activities

The functions of NtSOD1, NtSOD2, and NCAT1 in mediating cellular AE activities were characterized based on transgene analysis. Based on target transcripts abundance detection, two lines with strong target gene expression, including SOD1–1 and SOD1–2 for NtSOD1, SOD2–1 and SOD2–3 for NtSOD2, and CAT1–2 and CAT1–3 for NtCAT1 (Additional file 1: Figures S4A to S4E), were selected and subjected to the N starvation treatment. Under both N normal and N starvation treatments, all of the transgenic lines showed much higher corresponding AE activities than wild type (Fig. 8a-c), namely, SOD1–1 and SOD1–2 as well as SOD2–1 and SOD2–3 showed enhanced SOD activities (Fig. 8a and b) whereas CAT1–2 and CAT1–3 displayed increased CAT activities (Fig. 8c) with respect to WT. These results indicate that the differential AE encoding genes involve the regulation of SOD and CAT activities and contribute to the improved cellular ROS homeostasis in the N-deprived TaNBP overexpression lines.

Discussion

The subunits Gα, Gβ and Gγ involved in assembly of G-proteins are conserved in all eukaryotes. The members in family constituting these subunits are generally limited in plant species. For example, only a subunit Gα encoding gene (GPA1) [25], a subunit Gβ encoding gene
(AGBI) and a set of genes encoding subunit Gβ-like [26], and three subunit Gγs genes (AGG1, AGG2 and AGG3) that encode Gγ1, Gγ2 and Gγ3, respectively [27], have been identified in Arabidopsis. Characterization on the G-protein subunits has validated their critical roles in the modulation of diverse biological processes associated with plant growth, development and stress responses [9, 10].

Subunit Gβ of G-proteins is involved in regulation of various physiological processes in plants, including to modulate plant flowering initiation [14], regulate seed germination, ABA-mediated stomatal opening and inward K⁺-channel activation [29], and modulate plant phenotype and silique development [30, 31]. In this study, characterization on the wheat Gβ subunit TaNBP1 indicated that it bears conserved WD40 repeats, which are involved in the 3-D structure establishment of the NBP1 family proteins. Our analysis on detecting the GFP distribution at subcellular level from TaNBP1-GFP fusion revealed that the signals were concentrated onto cytoplasm membrane and cytosol, suggesting that TaNBP1...
targets to these positions after sorted from endoplasmic reticulum, where it established the heterotrimeric G-protein with subunits Gα and Gγ.

Distinct N signaling is elicited upon N starvation stress, which initiates transcriptional alteration on a large set of the regulatory and the functional genes [32]. A suite of genes involving plant N starvation stress response, including those coding for NRT proteins [32], ammonia transporters [33], Dof1 transcription factor [5], glutamine synthetase [34], and auxin receptor (AFB3) [35], shows modified transcription patterns upon the varied external N levels. In this study, we found that the expression patterns of TaNBP1 exhibited variation under modified N input conditions, showing that the TaNBP1 transcripts were gradually increased in roots and leaves over a 27 h regime under N starvation treatment. Moreover, the upregulated expression of this wheat gene was gradually downregulated once the N-deprived plants were resubjected to a N normal recovery treatment. These expression patterns suggest that TaNBP1 is involved in plant N starvation response. Previously, a cis-acting regulatory element referred to as NRE was shown to be critical in transcriptional regulation of a suite of N-responsive genes, including those encoding nitrite reductase (NIR) and NRT proteins in Arabidopsis, such as NRT2.1 and NRT2.2 [32]. Further characterization of the cis-regulatory elements underlying N starvation response of TaNBP1 can help understand its transcriptional mechanism upon low-N stress.

Functional characterization on subunit Gβ has revealed its various biological roles, including modulation of hypocotyl cell division [30], seed germination [31], seedling establishment and root elongation [31, 36], stomatal opening and inward K⁺-channel responses in the guard cells [18]. In this study, the observation on N deprivation response prompted us to characterize the
function of TaNBPI in regulating plant N starvation tolerance. Under N starvation treatment, the lines with TaNBPI overexpression exhibited improved phenotypes, increased N accumulative amounts, and higher plant biomass with respect to the wild type (Fig. 4a-e). The significant improvement on N uptake and plant dry matter production under low-N stress in transgenic lines suggests that TaNBPI is essential in modulating the plant adaptation to N starvation stress. It is also valuable in crop genetically engineering with high NUE under N-deficient conditions.

Low-affinity transport system (LATS) and high-affinity transport system (HATS) are the two major N uptake systems in plant species and other eukaryotes, which play crucial roles in the mediation of plant N acquisition and internal N translocation across tissues upon various N input conditions [37]. Of which, HATS is constisted
of the high-affinity nitrate transporters encoded by NRT2 family genes, which are functional in plant N uptake under N starvation stress [38]. Previously, based on mutants analysis, the functions of a set of NRT2 family members in mediating N acquisition under low-N stress were characterized. For example, mutants with knockout of NRT2.1, NRT2.2, NRT2.4, and NRT2.7, the Arabidopsis NRT2 family genes, show drastic reduction on root nitrate absorption capacity [39–41]. In this study, we analyzed the expression patterns of a suite of NRT encoding genes to address whether they were involved in the TaNBP1-mediated improvement on N uptake under N starvation stress. Our results indicated in contrast to other NRT genes examined that showed unaltered transcription in the N-deprived TaNBP1 overexpression lines and wild type, one NRT family member referred to as NtNRT2.2 showed upregulated in expression in transgenic lines relative to that of wild type. Transgene analysis on NtNRT2.2 indicated that the lines with overexpression of this NRT gene conferred plants improved phenotype and increased N accumulative amounts (Fig. 5c, d, f). These findings suggested that NtNRT2.2 contributes to plant N uptake under the low-N stress. Therefore, the TaNBP1–mediated N starvation tolerance is associated with the upregulated expression of NtNRT2.2, whose transcription under TaNBP1 control impacts on the plant N accumulation, phenotype behavior, and biomass production once plants are challenged by the N starvation stress.

Root system architecture (RSA) impacts largely on the water acquisition and the inorganic nutrient uptake from growth media. In addition to the NRT protein mediated N uptake, the RSA modulated by low-N stress also drastically impacts on the plant N uptake, whose establishment has been reported to be associated with the involvement of distinct NRT genes. For example, the Arabidopsis NRT2.1 involves the physiological processes of initiation and extension of the primary and secondary roots, aside from its role in regulating NO₃⁻ uptake [32]. In this study, our analysis on RSA characterization in lines with overexpression of TaNBP1 or NtNRT2.2 revealed that they all displayed enlarged RSA system under N starvation treatment compared with wild type (Figs. 4c and 5c). This finding suggested the involvement of TaNBP1 in mediating RSA establishment, which was possibly accomplished through its transcriptional regulation on NtNRT2.2. Previously, the RSA behaviors modulated upon altered N levels was shown to be associated with auxin, a type of the phytohormones. The corresponding evidence was provided in maize (Z. mays), whose root growth is inhibited under high nitrate conditions and the inhibition intensity is correlated with the reduction on auxin concentration in root tips. Alleviation of transduction of the auxin signaling from shoot to root then involves modulation of the RSA behavior [42]. Moreover, several investigations have indicated the internal connections between the G-proteins, NRT proteins, and the cellular auxin transportation characterization. For example, the Arabidopsis Gβ (also designated as AGB1) mediates acropetal transportation of auxin within the central cylinder, affecting the action of auxin in epidermis and/or cortex and further modulating RSA establishment [43]; Arabidopsis NRT1.1 regulates negatively cellular auxin transportation and modulates positively the auxin-mediated RSA establishment under N starvation conditions [38]. These findings together suggest the synergic action mechanism underlying G-protein, NRT protein, and auxin in regulating the RSA behavior. Further characterization on RSA behaviors regulated by TaNBP1 and NtNRT2.2 can help understanding of the root phenotype formation of plants.

Cellular ROS overproduction upon diverse stresses results in damage of proteins, lipids, and nucleic acid, leading ultimately to cellular injury or cell death [44]. On the other hand, plants have evolved a suite of corresponding protection strategies (i.e., enzymatic and non-enzymatic antioxidative systems) to protect themselves from the oxidative damage [45]. Antioxidant enzymes (AE) such as SOD, CAT, and POD are critical mediators in detoxification of ROS initiated by environmental stressors. Recently, it has been validated that the transcription of distinct AE genes is regulated under control of the G-protein subunits. For example, the transcripts of the CAT gene in rice (O. sativa) are regulated by RGG1, a γ subunit of G-protein; overexpression of RGG1 confers plants enhanced CAT activities and improved salt tolerance [46]. This finding suggests the putative connection between the subunits of G-protein and cellular ROS homeostasis of plants. In this study, based on assessment of the ROS-associated parameters in N-deprived TaNBP1 overexpression lines, we revealed the function of TaNBP1 in mediating cellular ROS homeostasis under N deprivation. Our results indicated that the TaNBP1 overexpression lines exhibited increased SOD, CAT, and POD activities, lowered MDA contents, and reduced H₂O₂ and superoxide anion accumulative amounts (Fig. 6a to f) with respect to those of wild type. Further transgene analysis on a set of differential AE genes, including NtSOD1, NtSOD2, and NiCAT1, three AE genes showing significantly upregulated in expression in lines with TaNBP1 overexpression, confirmed the roles of them in modulating AE activities. These results suggest that the TaNBP1-mediated N starvation tolerance was also associated with the gene function in detoxification of cellular ROS. Transcriptional mechanisms of the differential AE genes underlying TaNBP1 regulation are needed to be further characterized.
**Conclusion**
Our investigation indicates that TaNBPI is transcriptional response to external N levels. Overexpression of TaNBPI confers plants improved phenotype, N accumulative amount, and biomass production under N starvation conditions, indicating that TaNBPI acts as one of essential regulators in plant adaptation to the N-starvation stress. TaNBPI–mediated plant N-starvation tolerance is closely associated with the gene functions in improving N acquisition, RSA establishment, and cellular ROS homeostasis through transcriptional regulation of NRT gene NtNRT2.2 and distinctive AE genes such as NtSOD1, NtSOD2, and NtCAT1. Overexpression of these differential genes significantly regulates plant N uptake, root architecture establishment, and AE activities. TaNBPI can be used as one of valuable target genes in crop genetically engineering with high NUE under the N-saving cultivation conditions.

**Additional file**

**Additional file 1:** Table S1. PCR primers used in this study. Figure S1. The full length cDNA of TaNBPI and the corresponding translated amino acids. The start codon ATG and the termination codon TAG of TaNBPI are labeled by red background. Seven conserved WD40 repeat domains (I to VII) consisting of a sevenfold β-propeller in TaNBPI are highlighted by blue background. Figure S2. Phylogenetic relations between TaNBPI and its homologous genes from various plant species. Figure S3. Target gene transcripts in lines overexpressing TaNBPI and NtNRT2.2. a. TaNBPI transcripts in transgenic lines; b. NtNRT2.2 transcripts in transgenic lines. WT, wild type. Line 1 to Line 7, independent transgenic lines with TaNBPI overexpression. NtNRT2.2–1 to NtNRT2.2–6, independent lines with NtNRT2.2 overexpression. In a. TaNBPI expression levels in transgenic lines are normalized by the constitutive TaTubulin transcripts. In b. NtNRT2.2 expression levels in transgenic lines are normalized by the constitutive Nttubulin transcripts. Internal standard reference genes are set an expression level of 1. Figure S4. Target gene transcripts in lines overexpressing differential AE genes. a. NtSOD1 transcripts in transgenic lines; b. NtSOD2 transcripts in transgenic lines; c. NtCAT1 transcripts in transgenic lines; WT, wild type. Expression levels of the AE genes in transgenic lines are normalized by the constitutive Nttubulin transcripts whose expression level is set as 1. (DOC 162 kb)

**Abbreviations**
AE: Antioxidant enzyme; CAT: Catalase; MS: Murashige and skoog; NRT: Nitrate transporter; ORF: Open reading frame; POD: Peroxidase; ROS: Reactive oxygen species; WT: Wild type

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**Availability of data and materials**
All data generated or analyzed during this study are included in this published article and its supplemental data files.

**Author’s contributions**
KX designed the research and wrote the paper. ZL, YZ, XW, MY, and CG conducted the research, analyzed the data, and performed the statistical analysis. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**
Not applicable.

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Not applicable.

**Competing interests**
The authors declare that they have no competing interests.

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**References**
1. Lu XT, Xing GX, Chen XP, Zhang SL, Zhang LJ, Liu XL, Cui ZL, Yin B, Christie P, Zhu ZL, et al. Reducing environmental risk by improving N management in intensive Chinese agricultural systems. Proc Natl Acad Sci U S A. 2005;102(63):10441–6.
2. Toh M, Muller C, Mo M, Gibon Y, Carillo P, Morcuende R, Scheible WR, Krapp A. Steps towards an integrated view of nitrogen metabolism. J Exp Bot. 2002;53:959–70.
3. Vidal E, Gutiérrez RA. A systems view of nitrogen nutrient and metabolite responses in Arabidopsis. Curr Opin Plant Biol. 2008;11:521–9.
4. Zhang H, Forde BG. An Arabidopsis MADS box gene that controls nutrient-induced changes in root architecture. Science. 1998;280:407–9.
5. Yanagisawa S, Akiyama A, Kikumasa S, Uchimiyama H, Miwa T. Metabolic engineering with DoF transcription factor in plants: improved nitrogen assimilation and growth under low-nitrogen conditions. Proc Natl Acad Sci U S A. 2004;101:7833–8.
6. Castaños L, Camargo A, Pocholle D, Gaudon V, Texier Y, Boulet-Mercy S, Taconnat L, Renou JP, Daniel-Verde F, Fernandez E, et al. The nodulin-like protein 7 modulates nitrate sensing and metabolism in Arabidopsis. J Exp Bot. 2005;56:326–35.
7. Ho CH, Lin SH, Hu HC, Tsay YF. CHL1 function as a nitrate sensor in plants. Cell. 2009;138:1184–94.
8. Ho CH, Tsay YF. Nitrate, ammonium, and potassium signaling and sensing. Curr Opin Plant Biol. 2010;13:604–10.
9. Krapp A, David LC, Chardin C, Girin T, Marmagne A, Leprince AS, Chassou S, Ferrari-Méri S, Meyer C, Daniel-Verde F. Nitrate transport and signalling in Arabidopsis. J Exp Bot. 2014;65(3):789–98.
10. Delgado-Cerezo M, Sánchez-Rodríguez C, Escudero V, Miedes E, Fernández PV, Jordá L, Hernández-Blanco C, Sánchez-Vallet A, Bednarek P, Schulze-Lefert P, et al. Arabidopsis heterotrimERIC G-protein regulates cell wall defense and resistance to necrotrophic fungi. Mol Plant. 2012;5:119–128.
11. Trusov Y, Sewelam N, Rooke JE, Kunkel M, Novak E, Schenk PM, Botella JR. Heterotrimeric G proteins-mediated resistance to necrotrophic pathogens includes mechanisms independent of salicylic acid-, jasmonic acid/ethylene- and abscisic acid-mediated defense signaling. Plant J. 2009;58:69–81.
12. Zeng W, He SY. A prominent role of the flagellin receptor FLAGELLIN-SENSING2 in mediating stomatal response to Pseudomonas syringae pv tomato DC3000 in Arabidopsis. Plant Physiol. 2010;153:1188–98.
13. Ding L, Pandey S, Assmann SM. Arabidopsis extra-large G-proteins (XLGs) regulate root morphogenesis. Plant J. 2008;53:248–63.
14. Heo JBL, Sung S, Assmann SM. Ca2+–dependent GTPase, extra-large G protein 2 (XLG2), promotes activation of DNA-binding protein related to vernalization 1 (RTV1), leading to activation of floral integrator genes and early flowering in Arabidopsis. J Biol Chem. 2012;287:8242–53.
15. Zhu H, Li G, Ding L, Cui X, Berg H, Assmann SM, Xia Y. Arabidopsis extra-large G-protein 2 (XLG2) interacts with the GB subunit of heterotrimeric G protein and functions in disease resistance. Mol Plant. 2009;25:13–25.
16. Pandey S, Monshausen GB, Ding L, Assmann SM. Regulation of root-wave response by extra large and conventional G proteins in Arabidopsis thaliana. Plant J. 2008;55:311–22.
17. Ullah H, Chen JG, Temple B, Boyes DC, Alonso JM, Davis KR, Ecker JR, Jones AM. The β-subunit of the Arabidopsis G protein negatively regulates auxin-induced cell division and affects multiple developmental processes. Plant Cell. 2003;15:395–409.

18. Fan LM, Zhang W, Chen JG, Taylor JP, Jones AM, Assmann SM. Abscisic acid regulation of guard-cell K+ and anion channels in GP- and RGK-deficient Arabidopsis lines. Proc Natl Acad Sci U S A. 2008;105:8976–81.

19. Zhou B, Serret MD, Elazab A, Bort Pie J, Araus JL, Aranjuelo I, Sanz-Sázáñ E. Wheat ear carbon assimilation and nitrogen remobilization contribute significantly to grain yield. J Integr Plant Biol. 2016;58:914–26.

20. Urano D, Chen J, Botella JR, Jones AM. Heterotrimeric G protein signalling in the plant kingdom. Open Biol. 2013;3:120–86.

21. Guo C, Zhao X, Liu X, Zhang L, Gu J, Li X, Lu W, Xiao K. Function of wheat phosphate transporter gene TaPT12.1 in pi translocation and plant growth regulation under replete and limited pi supply conditions. Planta. 2013;237:1163–78.

22. Sun Z, Ding C, Li X, Xiao K. Molecular characterization and expression analysis of TaZTP15, a C2H2-type zinc finger transcription factor gene in wheat (Triticum asemum L). J Integr Agric. 2012;11:31–42.

23. Guo C, Li J, Chang W, Zhang L, Cui X, Xiao K. Effects of chromosome substitution on the utilization efficiency of nitrogen, phosphorus, and potassium in wheat. Front Agric China. 2011;5:253–61.

24. Huang XS, Liu JH, Chen XJ. Overexpression of PtrABF gene, a bZIP transcription factor isolated from Pseudomonas infolifata, enhances dehydration and drought tolerance in tobacco via scavenging ROS and modulating expression of stress-responsive genes. BMC Plant Biol. 2010;10:230.

25. Ma H, Yanofsky MF, Meyerowitz EM. Molecular cloning and characterization of GPA1, a G protein alpha subunit gene from Arabidopsis thaliana. Proc Natl Acad Sci U S A. 1990;87:3821–5.

26. Weiss CA, Gamaat CW, Mukai K, Hu Y, Ma H. Isolation of cDNAs encoding guanine nucleotide-binding protein beta-subunit homologues from maize (Zea mays) and Arabidopsis (Arabidopsis). Proc Natl Acad Sci U S A. 1994;91:5504–8.

27. Thung L, Trusov Y, Chakravorty D, Botella JR. Gammam1+ Gammam2+ Gammam3=Gbeta: the search for heterotrimeric G-protein gamma subunits in Arabidopsis is over. J Plant Physiol. 2012;169:542–5.

28. Lee S, Rojas CM, Ishiga Y, Pandey S, Mysore KS. Arabidopsis heterotrimeric G-proteins play a critical role in host and nonhost resistance against Pseudomonas syringae pathogens. PLoS One. 2013;8:e82445.

29. Mishra G, Zhang W, Deng F, Zhao J, Wang X. A bifurcating pathway directs abscisic acid effects on stomatal closure and opening in Arabidopsis. Science. 2006;312:264–6.

30. Ullah H, Chen JG, Young JC, Boyes DC, Alonso JM, Davis KR, Ecker JR, Jones AM. The β-subunit of the Arabidopsis G protein negatively regulates auxin-induced cell division and affects multiple developmental processes. Plant Cell. 2003;15:395–409.

31. Ullah H, Chen JG, Wang S, Jones AM. Role of a heterotrimeric G protein in regulation of Arabidopsis seed germination. Plant Physiol. 2002;129:897–907.

32. Remans T, Nacy P, Peverent M, Grin T, Tillard P, Lepetit M, et al. A central role for the nitrate transporter NRT1.2 in the integrated morphological and physiological responses of the root system to nitrogen limitation in Arabidopsis. Plant Physiol. 2006;140:909–21.

33. Engineer CB, Kranz KG. Reciprocal leaf and root expression of AtAmt1.1 and root architectural changes in response to nitrogen starvation. Plant Physiol. 2007;143:236–50.

34. Fuentes SJ, Allen DJ, Ortiz-Lopez A, Hernandez G. Over-expression of cytosolic glutamine synthetase increases photosynthesis and growth at low nitrogen concentrations. J Exp Bot. 2001;52:1071–81.

35. Vidal EA, Araus V, Lu C, Parry G, Green PJ, Conzatt GM, Gutierrez RA. Nitrate-responsive miR839/AFB1 regulatory module controls root system architecture in Arabidopsis thaliana. Proc Natl Acad Sci U S A. 2010;107:4477–82.

36. Pandey S, Chen JG, Jones AM, Assmann SM. G-protein complex mutants are hypersensitive to abscisic acid regulation of germination and postgermination development. Plant Physiol. 2006;141:243–56.

37. Ludewig U, Neuhäuser B, Dynowski M. Molecular mechanisms of ammonium transport and accumulation in plants. FEBS Lett. 2007;581:2301–8.

38. Krouk G, Tillard P, Gojon A. Regulation of the high-affinity NO3− uptake system by NRT1.1-mediated NO3− demand signaling in Arabidopsis. Plant Physiol. 2006;142:1075–86.

39. Little DY, Rao H, Oliveira F, Daniel-Devele F, Krapf A, et al. The putative high-affinity nitrate transporter NRT2.1 represses lateral root initiation in response to nutritional cues. Proc Natl Acad Sci U S A. 2005;102:13693–8.

40. Chopin F, Orsel M, Dorbe MF, Chardon F, Truong HN, et al. The Arabidopsis ATNRT2.7 nitrate transporter controls nitrate content in seeds. Plant Cell. 2007;19:1590–602.

41. Kiba T, Feria-Bourellier A-B, Lafouge F, Letzhema V, Bouter-Mercey S, et al. The Arabidopsis nitrate transporter NRT2.4 plays a double role in roots and shoots of nitrogen-starved plants. Plant Cell. 2012;24:245–58.

42. Tian Q, Chen F, Liu J, Zhang F, Mi G. Inhibition of maize root growth by high nitrate supply is correlated with reduced IAA levels in roots. J Plant Physiol. 2008;165:942–51.

43. Kushwah S, Jones AM, Laemi A. Cytokinin interplay with ethylene, auxin, and glucose signaling controls Arabidopsis seedling root directional growth. Plant Physiol. 2011;156:1851–66.

44. Gill SS, Tuteja N. Reactive oxygen species and antioxidative machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem. 2010;48:909–30.

45. You J, Chan Z. ROS regulation during abiotic stress responses in crop plants. Front Plant Sci. 2015;6:1092.

46. Swain DM, Sahoo RK, Srivastava VK, Tripathy BC, Tuteja R, Tuteja N. Function of heterotrimeric G-protein γ subunit RGG1 in providing salinity stress tolerance in rice by elevating detoxification of ROS. Plants. 2016; https://doi.org/10.1007/s00425-016-2614-3.