TaLBD1, a LOB transcription factor gene in T. aestivum, confers plant adaptation to low-N stress via modulating N acquisition-associated processes

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Abstract

Distinct members of the transcription factor (TF) families act as the critical mediators in plant low-N stress response by regulating transcription of the stress defensive-associated genes. In this study, we characterized *TaLBD1*, a member of the Lateral Organ Boundary (LOB) TF family in *T. aestivum*, in modulating plant adaptation to the N deprivation condition. The TaLBD1 protein harbors conserved domains specified by the plant LOB TF proteins, which targeted onto nucleus after sorted from endoplasmic reticulum (ER). The transcripts of *TaLBD1* in roots and aerial tissue were shown to be response to modified external N levels, showing in the tissues with a pattern to be gradually upregulated following a 27-h N starvation treatment. Transgene characterization on *TaLBD1* in *N. tabacum* indicated that it confers plants improved phenotype, root system architecture (RSA) establishment, dry mass production, and N accumulation under N starvation (NS) condition. Analyses on expression patterns of the genes in nitrate transporter (NRT) and those in PIN-FORMED (PIN) families revealed that the NRT gene *NtRNT2.4* and PIN gene *NtPIN6* displayed significantly upregulated expression in the NS-challenged lines. Further knockdown expression of them indicated that *NtNRT2.4* regulates N uptake whereas *NtPIN6* modulates RSA behavior. These results suggested that the *TaLBD1*-mediated plant NS adaptation is associated with its role in transcriptionally regulating distinct NRT and PIN members that improve N uptake and RSA establishment of plants upon low-N stress. High throughput RNA-seq analyses indicated that a quantity of genes modified expression upon NS underlying *TaLBD1* regulation. These differentially expressed (DE) genes were enriched into the gene ontology (GO) terms associated with signal transduction, transcription, protein biosynthesis, primary or secondary metabolism, and stress defensiveness. Our results together suggested that *TaLBD1* acts as one of the crucial regulators in plant adaptation to low-N stress. It can be used as a valuable index for evaluating plant NS response and a potential target for molecular breeding of high N use efficiency (NUE) crop cultivars.

Key Message

The member of the LOB transcription factor family *TaLBD1* in *T. aestivum* confers plant tolerance to N starvation stress via the role in modulating N uptake and RSA establishment.

Introduction

Nitrogen (N) acts as of one the indispensible inorganic nutrients for all of living organisms. The supplies of N in arable field impact largely on plant growth, development, and the crop production worldwide. In past of several decades, the chemical N fertilizers applied in agrosystem contributed greatly to the promotion of crop productivity and the sustainable agriculture development around the world. However, the N use efficiency (NUE) of crop plants is frequently lowered following with the increased application of N fertilizers given the N nutrition to be prone to leaching and evaporation, which results in intensified pollution of the agrosystem environment aside from elevation of the production investment. Therefore, it has been an essential issue in the modern agriculture production by improving the crop NUE through which to promote the sustainable agriculture development (Wang et al. 2019; Laurens et al. 2021).

The N acquisition in roots and its internal translocation across plant tissues are mediated by diverse physiological and biochemical processes. Under N starvation (NS) condition, a large set of genes encoding signal receptors, kinases, transcription factors (TF), and the stress-defensive proteins in N signaling pathway, modified transcription efficiencies upon low-N stress (Kant et al. 2011; Lang et al. 2014; Antal et al. 2016; Wang et al. 2019). The components consisting of the signaling pathway function cooperatively each other to impact on the NS defensiveness-associated biological processes and to define plant response to the low-N stress.

Acting as one of the largest families of transcription factor, the Lateral Organ Boundary (LOB) Domain (LBD) TF proteins exist specifically in plant species, which are evolved from same progenitor with the charophyte algae (Coudert et al. 2012; Chanderbali et al. 2015). Investigations indicated that the members in LOB TF family are typified by a domain referred to as LOB (also designated as AS2) that consists of a set of conserved motifs: C-motif, Gly–Ala–Ser (GAS) block, and leucine-zipper-like coiled-coil motif (Ohashi et al. 2018). Among these, C-motif is functional in binding the *cis*-acting regulatory elements situated in the downstream gene promoters; GAS block and the coiled-coil motif are involved in protein-protein interactions between the LBD protein and its interacting counterparts. To date, a subset of the LOB members have been functionally characterized. They have been documented to be functional in various physiological processes associated with growth, development, and plant abiotic stress responses, such as leaf primordium differentiation (Ohashi-Ito et al. 2018; Zhang et al. 2020), cellular secondary metabolism (Zhang et al. 2020), lateral root formation (Okushima et al. 2007), microspore cell division (Yang et al. 2021), and transduction of the N signaling in plants after perception of the modified environmental N availability (Kimura et al. 2017). These results together suggest that the LOB TF members extensively involve the modulation of diverse biological processes in plants.

Root system architecture (RSA) impacts largely on plant acquisitions for water and inorganic nutrients, acting as one of the essential factors in determining plant adaptation to the N starvation (NS) conditions (Yusefi-Tanha et al. 2020). Thus far, a subset of members in nitrate transporter (NRT) family has been documented to positively regulate plant N uptake and NS adaptation, given their functions for improvement of N taken up under low-N stress conditions (Yusefi-Tanha et al. 2020). On the other hand, characterization on RSA upon stressors has revealed that a suite of factors associated with auxin signaling, including cellular localization, concentration and translocation of this phytohormone, affecting largely root growth and RSA feature due to its the synergistic effects in modulating root primordium differential and lateral root elongation processes (Brunetti et al. 2018; Doyle et al. 2019). As to this issue, members of the PIN-FORMED (PIN) family have been indicated to mediate polar auxin transportation across tissues, impacting on the physiological processes associated with initiation and elongation of the primary and lateral roots and contributing to plant NS tolerance by enlargement of root system (Ye et al. 2001; Baetsen et al. 2021). However, although a large set of the physiological processes associated with plant NS responses were characterized, the molecular mechanisms underlying NRT-mediated plant N uptake and PIN-modified RSA establishment mediated by LOB TF members are needed to be further characterized.

Wheat (*T. aestivum*) acts as one of the most important cereals and is cultivated extensively worldwide. The productivity of wheat impacts greatly on the grain supply capacity and food security for humankind around the world. To date, although studies were conducted focusing on the identification and characterization analyses of the LBD TF family members in plant species, such as the model plant *A. thaliana* and *O. sativa* (Evans et al. 2007; Soyano et al. 2008; Matsumura et al. 2009), functions of the LBD transcription factor family members in mediating growth and plant stress response as well as the underlying mechanisms are still largely unknown in *T. aestivum*. In this study, we characterized *TaLBD1*, a gene of the LBB family of transcription factor in wheat, in regulating plant response to the low-N stress. Our findings indicated that *TaLBD1* sensitively responds to low-N stress at the transcriptional level and confers plants improved NS adaptation through its role in improving N uptake and RSA establishment.

Materials And Methods

Characterization analysis on *TaLBD1*

Our previous RNA-seq analysis revealed that *TaLBD1* (GenBank accession No. AK330221), a member of the LBD transcription factor family in *T. aestivum*, was significantly upregulated in transcription in wheat (cv. Shinong 086) roots upon low-N stress (unpublished data). This finding prompted us to further investigate the function of this TF gene in mediating plant NS response in more detail. The homologous genes of *TaLBD1* across various plant species were obtained based on a BLASTn analysis by searching against with the GenBank database in NCBI (https://www.ncbi.nlm.nih.gov) using the target gene as a query. Phylogenetic relations among *TaLBD1* and its homologous genes distributed diverse plant species were established using the MegAlign algorithm that is supplemented in the DNAStar software.

Subcellular location analysis of the TaLBD1 protein

The subcellular localization of the TaLBD1 protein after endoplasmic reticulum (ER) assortment was firstly predicted based on an online tool referred to as NL Stradamus. Moreover, an additional experiment using transgene analysis was conducted to validate the online prediction results of the target subcellular localization. With this purpose, the open reading frame (ORF) of *TaLBD1* was amplified based on RT-PCR using gene specific primers (Table S1). It was then integrated in the binary vector pCAMBIA3300 to be fused with the reporter gene (i.e., green fluorescent protein encoding gene, *GFP*) in frame after sequencing confirmation. The expression cassette was then subjected to genetic transformation onto the epidermal cells of *N. tabacum* (cv. Wisconsin 35) using an *A. Tumefaciens*-mediated transformation approach as described previously (Keen et al. 2020). The GFP signals initiated from fusion TaLBD1-GFP in the transformed epidermal cells were detected under fluorescent microscope to define the location of the target protein at subcelluar level.

Expression analysis of *TaLBD1*

The expression patterns of *TaLBD1* under low-N stress conditions were evaluated using the root and leaf tissues of *T. aestivum* (cv. Shinong 086) treated with varied external N input levels. With this purpose,

young seedlings of wheat after regular germination in a growth chamber were cultured in a standard Murashige and Skoog (MS) solution (affluent N, 16 mM) to the third-leaf stage as previously described (Jiang et al. 2006). At that growth stage, the seedlings were subjected to NS treatment by transferring into a MS solution with supply of lowered N level (0.06 mM N). At the time points of 0 h (prior to treatment), and 1 h, 3 h, 9 h, and 27 h during NS treatment, the tissues of root and leaf were separately collected. In addition, the recovery effects of the NS-mediated *TaLBD1* expression were investigated. To this end, aliquots of the seedlings after 27 h NS treatment were re-transferred in a standard MS solution for recovered normal growth. At 1 h, 3 h, 9 h, and 27 h following the N recovery treatment, tissues of root and leaf were sampled at the time points mentioned and subjected to assessment of the target transcripts. The transcripts of *TaLBD1* in all of the collected samples were evaluated based on qRT-PCR that was performed similarly as described previously (Guo et al. 2013) using gene specific primers (Table S1). *Tatubulin*, a constitutive gene shown in *T. aestivum*, was used as an internal reference to normalize the target transcripts (Table S1).

Assays of the growth traits and photosynthetic parameters in *TaLBD1* transgenic lines

Transgenic tobacco lines with overexpression of *TaLBD1* were generated to define the gene function in mediating plant response to NS. With this purpose, the ORF of *TaLBD1* was amplified based on RT-PCR using the gene specific primer pairs (Table S1). After sequencing confirmation, the ORF was inserted into the restriction sites *Bgl*II/*Bst*EII in binary vector pCAMBIA3301 under the control of the CaMV35S promoter. Procedure for the generation of the transgenic lines of target gene was similar to that as reported previously (Sun et al. 2012).

We selected Line 2 and Line 3, two lines at T3 generation with high expression level of *TaLBD1*, to define the target gene-mediated NS response in plants. With this aim, these two transgenic lines together with wild type (WT, as a control) plants were subjected to cultivation under two N input treatments, including affluent N (AN) in which the plants of transgenic lines and WT were cultured in standard MS solution with affluent N (16 mM N) and NS treatment in which those of transgenic lines and WT were grown in MS solution with supply of lowered N level (0.3 mM N). During the N input treatments, the growth conditions provided for plants were as follows: a photoperiod of 14 h/10 h (day/night) with the light intensity of 400 $\mu E/m^2s$ during light phase, a temperature range of 26°C/22°C (day/night), and a relative air humidity range changed from 60% to 75%. During the process of N input treatments, the solutions applied in AN and NS treatments were air-circulated using a mini pump and renewed twice each week. Six weeks after the treatments, we assessed the plant growth traits in transgenic and WT plants, including the phenotypes, plant biomass, root fresh weights and root volumes. Among these, the phenotypes of whole plant and root tissue were recorded as images taken by a digital camera; the plant biomass and root biomass were obtained from three of representative plants after conventional oven-drying; the root fresh weights and root volumes were determined according to the conventional approach. In addition, several of photosynthetic parameters, including photosynthetic rate (Pn), photosystem II photochemical efficiency (ΨPSII), and non-photochemical quenching coefficient (NPQ), were measured in the transgenic and WT plants after the two N level treatments. Of which, Pn, gs, and Ci in representative leaves (i.e., the

third leaves with fully expansion) were measured using the photosynthesis assay system (LiCOR-6200) following the manufacturer's suggestion; and parameters ΨPSII and NPQ were assessed to be similar as those reported previously (Guo et al. 2013).

Assays of the N contents and expression patterns of the NRT family genes

The N-associated traits in the transgenic lines after N input treatments were assessed to address the gene function in mediating plant N uptake upon the NS condition. Of which, the N concentrations in plants were measured as described previously (Guo et al. 2013); the accumulative N amounts in plants were calculated by multiplying the N concentrations and the plant biomass. To understand the molecular processes underlying *TaPLD1*-mediated N taken up and the expression patterns of nitrate transporter (NRT) family members, a set of NRT genes in *N. tabacum*, including *NtNRT1.1-s*, *NtNRT1.1-t*, *NtNRT2.5*, and *NtNRT2.6*, were subjected to transcripts evaluation in the N-deprived transgenic lines. The transcripts abundance of the NRT family genes examined in the transgenic and WT plants were evaluated based on qRT-PCR using the gene-specific primers (Table S1). During which, a constitutive gene referred to as *Nttubulin* in *N. tabacum* was used as an internal reference to normalize the transcripts of the NRT genes.

Assay of the expression patterns of the PIN-FORMED family genes

The root system architecture (RSA) establishment in plants is largely determined by the PIN-FORMED (PIN) protein-mediated auxin concentration and translocation at cellular level, which impacts on the plant uptake capacity for water and inorganic nutrients in growth media under abiotic stress conditions (Gray et al. 2001; Reed et al. 2001; Brunetti et al. 2018; Doyle et al. 2019). To determine the PIN protein-mediated RSA establishment underlying *TaLBD1* regulation, several genes in the PIN family in *N. tabacum*, namely, *NtPIN1*, *NtPIN1b*, *NtPIN6*, and *NtPIN9*, were subjected to evaluation of expression levels in the *TaLBD1* transgenic lines after NS treatment. The transcripts of these PIN family genes in roots were analyzed based on qRT-PCR performed to be similar to that as mentioned above. Gene specific primer pairs used for the amplification of the PIN genes are shown in Table S1. Likewise, *Nttubulin* was used as an internal reference to normalize the target transcripts.

Transgene analysis on distinct genes in NRT and PIN families

Two genes in the NRT and PIN families, including *NtNRT2.4* and *NtPIN6* that displayed significantly upregulated in expression in the N-deprived *TaLBD1* lines (i.e., Line 2 and Line 3), were further subjected to transgene analysis given their modified transcription and putative involvement of plant NS adaptation. With this purpose, the ORFs of *NtNRT2.4* and *NtPIN6* were amplified in anti-sense orientation based on RT-PCR using the gene specific primers (Table S1). They were then inserted into the restriction sites *Nco*I/*Bst*EII in binary vector pCAMBIA3301 under the control of the CaMV35S promoter using the aforementioned approach. Among the transgenic lines generated, we selected five lines including three ones with drastic knockdown expression of *NtNRT2.4* (i.e., NtNRT2.4-1, NtNRT2.4-3 and NtNRT2.4-4) and two ones with significant depressed expression of *NtPIN6* (i.e., AnPIN6-1 and AnPIN6-2) to be subjected to NS treatment as mentioned above. Six weeks after treatments, the phenotypes, plant biomass, N

concentrations and plant N accumulative amounts in the *NtNRT2.4* lines and the phenotypes, plant biomass, root fresh weights, and root volumes in the *NtPIN6* lines were assessed. Of which, the N-associated traits and root growth traits in the lines were assessed similarly to those performed in the *TaLBD1* transgenic lines mentioned above.

Transcriptome analysis

The high-throughput RNA-seq analyses were performed to characterize the transcriptiome profile mediated by *TaLBD1* upon NS condition. With this purpose, the transgenic line overexpressing *TaLBD1* (i.e., Line 2) together with WT were cultured regularly in standard MS solution as aforementioned to the fifth leaf stage. At that time, the transgenic and WT plants were separately subjected to the NS treatment (0.06 mM N) as aforementioned for one week. Total RNA in the roots of Line 2 and WT plants was extracted using TRIzol reagent (Invitrogen) and subjected to construction of the RNA-seq libraries in triplicates after confirmation of RNA quality, following the procedure as described previously (Zhong et al. 2011). Primary transcripts generated in the RNA-seq libraries were sequenced using the Illumina HiSeq 2500 platform. The transcripts in libraries generated with high quality levels in the N-deprived transgenic line and WT were obtained after the removal for adaptors in the reads, the reads with sequence length less than 40 bp, and those with low quality based on analysis using the software Trimmomatic (Bolger et al. 2014). The clean reads with high quanlity in libraries were further subjected to the alignment analysis against the transcripts database of the reference genome (*N. tabacum*, Novogene Co, LTd, Beijing). We defined the genes as differentially expressed (DE) when they exhibited transcripts variation over 2-fold between the transgenic line and WT (Robinson et al. 2010), using a default parameter of false discovery rate (FDR) to be less than 0.05 (Benjamini and Hochberg 1995). The Gene Ontology (GO) terms of the DE genes identified were functionally categorized using the online tool referred to as Plant MetGenMap (http://bioinfo.bti.cornell.edu/cgi-bin/MetGenMAP/home.cgi), in which we adopted a CPAN pearl module to define them as described previously (Boyle et al. 2004). The biological roles of the DE genes identified in the *TaLBD1* transgenic lines were determined based on categories of their functional annotations.

Expression analysis on randomly selected DE genes identified from RNAseq analysis

Ten of the DE genes identified from above RNA-seq analyses, including five with pattern of upregulated and five with that of downregulated, were subjected to transcripts evaluation based on qRT-PCR using the gene specific primer pairs (Table S1), by which to validate results in the RNA-seq analyses. Among the genes examined, the five genes with a pattern of upregulated expression included: *mitogen-activated protein kinase kinase* (*MAPKK*), *leucine zipper*, *ribosomal protein L3A*, *malate dehydrogenase*, and *peroxidase*; the five ones with a pattern of downregulated expression included: *cytokinin-regulated kinase*, *WRPK*, *phosphoglyceromutase*, *metal transporter*, and *chitinase*. The transcripts of above DE genes in the N-deprived transgenic lines (Sen 2) and WT were evaluated based on qRT-PCR performed as mentioned above using the gene specific primers. Of which, the cDNA samples derived from Line 2 and WT treated by NS were used as the templates in PCR reactions. Similarly, the constitutive *Nttubulin* was used as an internal reference to normalize target transcripts.

Statistical analysis

Averages of the plant and root biomass, N concentration, N accumulative amount, root fresh weight, root volume and the expression levels were all derived from the triplicate results. Standard errors of the averages and significant differences among the averages were analyzed using the Statistical Analysis System software.

Results

The characterization of *TaLBD1*

TaLBD1 has a full length cDNA of 1267 bp that encodes a 303-aa polypeptide (Fig. S1). Similar to its counterparts of the LOB TF family in plant species, the TaLBD1 protein harbors a conserved domain referred to as C-motif (CX2CX6CX3C) (5 aa-19 aa) at the N terminus (Fig. S2). Phylogenetic relation analysis revealed that *TaLBD1* shares high similarities to the homologous genes across diverse plant species at nucleic acid level, such as the LOB family genes in *H.vulgare*, *P.edulis*, *O. sativa*, *Z. mays*, *I. triloba*, *B. nivea* and *A. thaliana* (with sequence identities from 76.5 to 95.8%, Fig. 1). Based on online prediction analysis, the TaLBD1 protein is suggested to be targeted onto nucleus after endoplasmic reticulum (ER) assortment. It was in consistent with the results in detecting the cellular localization of fusion TaLBD1-GFP in the *N. tabacum* epidermal cells. The GFP signals derived from the cells mentioned harboring *TaLBD1-GFP* were shown to be confined in nucleus under observation of a fluorescent microscope (Fig. 2A). The TaLBD1 localization analysis results indicating that it targets onto nucleus is in accordance with the nature of transcription factor in nucleus to exert roles in regulating transcription of the downstream genes.

TaLBD1 expression is sensitive in response to NS condition

The transcripts of *TaLBD1* were assessed in wheat plant tissues upon modified N input levels to understand the transcriptional response of the target gene upon NS condition. Results indicated that the expression levels of *TaLBD1* were low in both root and aerial tissues under affluent N (AN) condition. However, the *TaLBD1* transcripts in the tissues examined were shown to be increased dramatically upon NS, showing a pattern to be gradually elevated along with progression of a 27 h NS regime and reaching peak levels at the end of treatment (Fig. 2B). Moreover, the *TaLBD1* expression displays a recovery effect upon a normal restoration growth condition; the upregulated transcripts of *TaLBD1* in both tissues upon NS were detected to be restored steadily in the tissues following a 27 h of N recovery treatment (Fig. 2B). These results together suggested that the *TaLBD1* expression is sensitive in response to external N availability and is possibly involved in mediating plant low-N stress response given its induced transcription upon NS condition.

TaLBD1 confers plants improved tolerance to low-N stress

Two of transgenic tobacco lines at T3 generation, namely, Line 2 and Line 3 with strong expression levels of *TaLBD1* (Fig. S3), were selected to be subjected to NS treatment to characterize the gene function in mediating plant NS response. Under AF condition, these two lines (Line 2 and Line 3) were comparable on the growth traits, such as phenotypes, plant biomass, and root biomass with the WT plants (Figs. 3A-3C). Under NS treatment, however, both of the transgenic lines displayed significantly improved phenotype (Fig. 3A), RSA feature (Fig. 3B), and biomass in whole plant and root tissues (Fig. 3C) with respect to WT. In parallel with the above growth traits, compared with WT, both of the transgenic lines were enhanced on photosynthetic function, elevating the photosynthetic rate (Pn), enhancing photosystem II biochemical efficiency (ΨPSII), and reducing the nonphotochemical quenching (NPQ) in the upper expanded leaves of plants (Figs. 3D-3F). These results of growth- and photosynthetic-associated traits shown in NS-challenged transgenic lines suggested that *TaLBD1* plays an important role in enhancing the plant tolerance to low-N stress.

TaLBD1 overexpression improves N accumulation capacity of plants

The N-associated traits were assessed in the transgenic lines (i.e., Lines 2 and 3) and WT after the NS treatments. Similar to the growth traits mentioned above, the N concentrations and N accumulative amounts in the plants of Line 2 and Line 3 were comparable with those shown in WT under AN condition (Figs. 4A-4B). Under NS treatment, both of the transgenic lines (i.e., Lines 2 and 3) and WT were shown to be decreased on the N concentrations and the N accumulative amounts in plants compared with those shown under AN condition (Figs. 4A-4B), suggesting the negative effects of NS on plant N uptake. However, compared with WT, Line 2 and Line 3 showed increased N concentrations and elevated N accumulative amounts in plants compared to those shown in the WT ones under the NS condition (Figs. 4A-4B). These results indicated the function of *TaLBD1* in positively regulating the plant N acquisition capacity under low-N stress conditions. That the transgenic lines displayed improved N-associated traits, namely, increased N concentration and N accumulation in plants under NS condition, suggested that the *TaLBD1*-improved plant adaptation to NS to be largely attributed to the gene function in positively regulating plant N uptake that further improves plant growth- and photosynthetic-associated traits.

Expression and function characterizations of the NRT genes

The expression patterns of a suite of NRT family genes involving N uptake and internal N translocation across tissues were subjected to transcripts evaluation in NS-treated transgenic lines and WT plants to understand the molecular processes underlying *TaLBD1*-mediated N accumulation. Among nine genes of the NRT family in *N. tabacum* examined, *NtNRT2.4* was shown to be significantly upregulated in expression in the transgenic lines (i.e., Line 2 and Line 3) with respect to WT (Fig. 5A). The modification on expression levels of *NtNRT2.4* between the transgenic lines and WT under NS condition was in contrast to other genes in NRT family examined that were unaltered on transcription r in the transgenic and WT plants (Fig. 5A). Therefore, *NtNRT2.4* is suggested to be regulated underlying *TaLBD1* at transcription level and to possibly contribute to NS adaptation of the *TaLBD1* transgenic lines.

Three lines with significant knockdown expression of *NtNRT2.4* (NRT2.4-1, NtNRT2.4-3 and NRT2.4-4) (Fig. S4) were selected and subjected to experimental characterization for the gene function in regulating plant N uptake. Under NS treatment, all of the transgenic lines, namely, NRT2.4-1, NtNRT2.4-3 and NRT2.4-4, were shown to be significantly alleviated on plant phenotypes (Fig. 5B), biomass, N concentrations, and the accumulative N amounts compared with the WT plants (Figs. 5C-5E). The results that repressed expression of *NtNRT2.4* led to drastic alleviation on plant N accumulation and growth behavior suggested that the *TaLBD1*-mediated plant improved NS adaptation is largely ascribed to its role in enhancing plant N uptake due to transcriptional regulation of distinct NRT family genes.

Expression and functions of the PIN-FORMED family genes

The transcripts of ten genes in the PIN-FORMED (PIN) family in *N. tabacum* were evaluated in the N-deprived transgenic lines (Lines 2 and 3) and WT plants to deepen understanding the putative PIN genes involving RSA establishment underlying the *TaLBD1* regulation. Among the PIN genes examined, *NtPIN6* was shown to be significantly upregulated in expression in the transgenic lines with *TaLBD1* overexpression (i.e., Line 2 and Line 3) relative to the WT plants after NS treatment (Fig. 6A). Other PIN family genes aside from *NtPIN6* were in contrast in expression levels, showing unchanged transcription in the NS-treated transgenic and WT plants (Fig. 6A). These expression analysis results on PIN family genes suggested that distinct members, such as *NtPIN6*, is transcriptionally regulated underlying *TaLBD1* and is possibly involved in improving RSA establishment in transgenic lines challenged by NS condition.

Transgene analysis on *NtPIN6* was conducted to characterize the gene function in regulating plant RSA establishment upon low-N stress conditions. NtPIN6-1 and NtPIN6-2, two lines with *NtPIN6* knockdown expression (Fig. S5), were selected and subjected to NS treatment. Results indicated that the *NtPIN6* knockdown expression lines (i.e., NtPIN6-1 and NtPIN6-2) were shown to be significantly alleviated on growth of root tissue (Fig. 6B), reduced on plant and root biomass, fresh weights, and decreased on root volumes (Figs. 6C-6E) compared to those shown in the WT plants after NS treatment. These transgene analysis results on *NtPIN6* together suggested that *TaLBD1*-mediated plant NS tolerance is also attributed largely to its role in transcriptionally regulating the PIN-FORMER family genes, such as *NtPIN6*, that positively impact on RSA establishment of plants challenged by NS condition.

Differentially expressed (DE) genes underlying the regulation of *TaLBD1*

The differentially expressed (DE) genes mediated by *TaLBD1* were identified under N starvation condition based on the high-throughput RNA-seq analyses. A total of 1971 genes were shown to be categorized in group of DE in the N-deprived transgenic line (i.e., Line 2) with respect to WT. Among these DE genes, 962 were upregulated and 1009 downregulated (Fig. 7A, Dataset 1-Dataset 2). To verify the transcripts results derived from the RNA-seq analyses, ten DE genes were randomly selected from the transcripts datasets derived from above transcriptome assay, including five genes of upregulated and another five ones of downregulated that were shown in the N-deprived transgenic plants. As a results, all of the DE genes displayed similar expression levels, with comparable variation-folds in transcription as shown in the transcriptome analyses. These results validated the reproducibility of transcripts characterized in the

RNA-seq analysis (Fig. 8). Quantities of the DE genes indentified in the *TaLBD1* overexpression lines suggested that the target gene acts as one of the important regulators in plant N starvation signaling transduction via modulating gene transcription at a large level.

The DE genes identified in transcriptome analyses were shown that only a small ratio haa been functionally annotated thus far (including known functions of 130 upregulated and 161 downregulated DE genes) (Dataset 1-Dataset 2). The DEGs with a large ratio to be functional unknown is possibly ascribed to the relatively slow progress of the gene annotation research programs in *N. tabacum* to date. Based on the annotated biological functions, the DE genes of upregulated are categorized into the following functional groups: signal transduction (with numbers of 16),transcriptional regulation (15), protein metabolism (14), chromosome remodeling (5), primary metabolism (26), secondary metabolism (11), transport (2), oxidative stress defense (4), phytohormone response (6), abiotic stress response (7), biotic stress response (4), cellular structure (10), cell cycling (4), development (3), and miscellaneous (3) (Fig.7B; Dataset 3). Likewise, the DE genes with downregulated pattern are classified into the following functional groups: signaling transduction (20), transcriptional regulation (8), protein metabolism (9), chromosome remodeling (6), primary metabolism (29), secondary metabolism (19), transport (7), phytohormone response (6), abiotic stress response (5), biotic stress response (12), cellular structure (14), cell cycling (2), development (2), and miscellaneous (22) (Fig. 7C; Dataset 4). The results on transcriptome analyses in the *TaLBD1* transgenic lines after NS treatment suggested that this LOB TF gene exerts roles in extensively regulating the gene transcription, which impacts on the modulation of the physiological processes associated with plant tolerance to low-N stress.

Discussion

The transcription factor members of the LOB family are involved in the modulation of diverse physiological processes associated with plant growth, development, and plant stress responses, playing critical roles in mediating leaf polarity establishment (Zhu et al. 2020; Busche et al. 2021), tracheary element development (Soyano et al. 2008), boundary delimitation (Ohashi et al. 2015; Ohashi et al. 2018), cytokinin signaling, inflorescence branch formation (Guo et al. 2020), female gametophyte development (Evans et al. 2007), and KNOX gene regulation (Semiarti et al. 2001; Chalfun et al. 2005; Long et al. 2014; Long et al. 2015), and plant responses to low-N stress (Rubin et al. 2009). In this study, our characterization analysis on *TaLBD1*, a gene of the LOB TF family in *T. aestivum*, revealed that it shares high similarities with its homologous genes across various plant species. The TaLBD1 protein harbors the same conserved domains its plant counterparts, with a subcellular localization in nucleus after ER assortment. These characterizations of *TaLBD1* indicates that it acts as one of the transcription factors of the LOB family in *T. aestivum*, exerting the TF functions in nucleus to regulate downstream genes at transcriptional level.

The plant response to NS signaling is defined cooperatively by the modified transcription of the N deprivation-responsive genes. Distinct *cis*-acting regulatory elements, such as the nitrate-responsive elements (NRE) identified in the promoter regions of the N uptake- and assimilation-associated genes,

regulate the transcription efficiencies of the NS-responsive genes upon low-N stress (Jian et al. 2018). For instance, *NRT2.1* and *NRT2.2*, two genes of NRT family in *A. thaliana*, display induced expression levels upon the low-N stress, given the DNA-protein interaction occurred between the NRE motif in their promoters with the DNA binding domain situated in distinct TFs (Jian et al. 2018). In this study, our expression analysis on *TaLBD1* indicated that it is sensitive in response to the low-N stress in both root and aerial tissues, suggesting that *TaLBD1* is involved in mediating plant NS response due to its modified expression patterns upon NS conditions. Further characterization on the *cis*-acting regulatory elements situated in the *TaLBD1* promoter that involve NS response can deepen the understanding of the transcriptional mechanism of LOB family members in *T. aestivum* upon low-N stress.

The function of the LOB members in mediating transduction of the N deprivation signaling has been documented (Rubin et al. 2009). However, the physiological mechanisms underlying LOB TF-mediated plant NS adaptation are still largely unknown. In this study, we generated the tobacco transgenic lines with *TaLBD1* overexpression to characterize the biological roles of target gene in mediating plant response to low-N stress. Our transgene analysis revealed that *TaLBD1* exerts significant roles in mediating plant NS adaptation. Compared with wild type, the transgenic lines (i.e., Line 2 and Line 3) showed improved behavior on growth traits, showing enlarged stature, increased biomass and fresh weights of whole plant and root tissue, and elevated root volumes after the NS treatment. The transgene analysis results of target gene on growth traits mentioned were in closely accordance with the photosynthetic parameters in tragsnenic lines that exhibited elevated Pn, enhanced photosystem II biochemical efficiency, and lowered nonphotochemical quenching (NPQ) with respect to WT under NS. These results together indicate that *TaLBD1* plays an important role in improving plant low-N stress tolerance given its improvement of photosynthetic function in the NS-challenged plants..

Enhancement of N uptake acts as one of the effective strategies for plant NS response. The members of the nitrate transporter (NRT) family involve the constitution of the high-affinity transport (HAT) system for N acquisition, contributing largely to the improved N uptake capacity in the N-deprived plants (Remans et al. 2006; Da et al. 2019). Previous characterization on the HAT system has revealed the critical roles of the NRT2 group in positively regulating plant N acquisition processes under the low-N stress conditions (Wang et al. 2020). For example, the mutants nrt2.1 with knockout of *AtNRT2.1*, a member of the NRT2 family in Arabidopsis, leads to the reduced HATS activity and N accumulative amount upon NS relative to the wild type (Li et al. 2007). In this study, we assessed the N accumulation property in the *TaLBD1* transgenic lines (i.e., Lines 2 and 3) under NS treatment to understand the N uptake-associated physiological processes underlying *TaLBD1* regulation. Both Line 2 and Line 3 were elevated on the N concentration and N accumulative amounts after NS treatment with respect to the WT plants. These findings on transgenic lines suggested that *TaLBD1* positively regulates the N uptake enhancement by which to improve the plant photosynthetic function, growth behavior, RSA establishment, and finally the plant low-N stress tolerance. Based on expression and transgene analysis, we indentified one of the NRT family members, namely, *NtNRT2.4* that was upregulated in expression in the N-deprived transgenic lines, exerting huge roles in the *TaLBD1*-mediated plant NS adaptation. Compared with WT, the lines with knockdown expression of *NtNRT2.4* dramatically alleviated the growth traits and lowered the N

accumulative amounts plants under NS treatment. The results on NRT gene expression and functional analyses suggested that distinct genes in NRT family, such as *NtNRT2.4*, involve the constitution of action modules LBD1-NRT2.4, which contributes to improved N uptake and low-N stress tolerance of plants underlying the *TaLBD1* regulation.

The phytohormone auxin acts as one of the critical growth regulators, involving modulation of diverse biological processes associated with plant growth and development, such as initiation and formation of the lateral roots that affect capacities of nutrient and water acquisition in plants (Brunetti et al. 2018). The auxin signaling pathways involving the modulation of various physiological processes are integrated through the synergistic effects covering diverse physiological and biochemical processes (Gray et al. 2001; Doyle et al. 2019). Thus far, the members of the PIN-FORMED (PIN) family have been documented to act as the essential regulators in modulating RSA establishment through their roles in controlling the levels and translocation properties of auxin in cells (Rogers et al. 2016; Lee et al. 2021). In this study, our characterization analysis on RSA feature in the transgenic lines overexpressing *TaLBD1* revealed that the effect of target gene in enlarging RSA under NS treatment, suggesting that the *TaLBD1*-mediated plant NS tolerance is also ascribed to the gene function in promoting RSA establishment. To provide insight into the PIN protein-mediated RSA behavior upon NS, we analyzed the transcripts of the PIN-FORMED family genes in the N-deprived lines with *TaLBD1* overexpression. We found that in contrast to other PIN family genes were unaltered on transcripts between the transgenic and WT plants, *NtPIN6* displayed significantly upregulated pattern in expression in the N-deprived *TaLBD1* lines compared to WT. Further transgene analysis on *NtPIN6* validated the PIN gene function in positively regulating RSA establishment of plants challenged by NS condition. These findings together suggested the existence of a putative pathway LBD1-PIN6-RSA underlying distinct LOB TF modulation, which contributes to plant low-N stress tolerance through improving root growth. Recently, distinct members of the LOB TF family have been recorded to be involved in the transduction pathways of the auxin signaling, exerting roles in the mediation of lateral root formation (Filleur et al. 2001). Further characterizing the PIN protein - modulated RSA establishment underlying the LOB TF regulation can provide insights into the mechanisms of plant low-N stress tolerance.

High throughput RNA-seq analysis approach has provided a power tool in elucidating the molecular mechanisms underlying plant stress response and defensiveness (Nemhauser et al. 2004; Lang et al. 2014; Keen et al. 2020). In this study, we conducted the RNA-seq analyses with the purpose to disect the molecular processes of plant NS adaptation mediated by *TaLBD1*. Among the transcripts detected in the N-deprived transgenic line with *TaLBD1* overexpression (Line 2) and the WT plants treated by NS, a total of 1971 genes were shown to be differentially expressed (DE) in Line 2. Of which, 962 were upregulated and 1009 downregulated. Gene Ontology (GO) analysis on the DE genes was performed, which indicated the GO terms of these DE genes to be enriched into various functional groups, including signal transduction, transcriptional regulation, protein biosynthesis and degradation, primary and secondary metabolism, stress response, and phytohormone response, etc. These results on transcriptiome analyses suggested that *TaLBD1* regulates the transcription of a large set of genes specifically, by which to impact on plant tolerance to the low-N stress given the modified physiological processes associated with N

uptake (i.e., via function module LBD1-NRT2.4), RSA establishment (via biochemical pathway LBD1-PIN6-RSA formation), and other biological functions, such as photosynthesis. It can be valuable to characterize the DE genes which function as the essential regulators in plant stress response, by which to deepen understanding of the NS adaptation mechanisms in crop plants.

Conclusion

The TaLBD1 protein harbors the conserved domains as the members of its plant LOB TF counterparts, targeting onto the nucleus after endoplasmic reticulum (ER) assortment. *TaLBD1* expression is sensitive in response to NS treatment, showing induced transcripts in root and aerial tissues upon N starvation. Overexpression of *TaLBD1* confers plants improved growth traits under low-N stress treatment; the transgenic tobacco lines overexpressing this wheat LOB TF gene were improved on RSA establishment, plant and root biomass production, photosynthetic function, and the N accumulation capacity in plants. Distinct genes in the NRT family referred to as *NtNRT2.4* and the PIN-FORMED family gene *NtPIN6* were both significantly upregulated in expression in the N-deprived lines overexpressing *TaLBD1*, which exerted critical roles in enhancing the N uptake capacity and in improving RSA establishment of the plants under NS condition, respectively. RNA-seq analysis identified a large set of genes with significantly modified transcription underlying the regulation of *TaLBD1*, which are enriched into the functional groups associated with diverse biological processes, including signal transduction, transcription, protein biosynthesis, primary or secondary metabolism, and stress defensiveness. *TaLBD1* acts as a valuable target gene in evaluating plant NS adaptation across wheat cultivars and in genetically engineering of the high NUE crop cultivars suitably cultivated under the N-saving conditions.

Declarations

Author contribution statement

Kai Xiao designed the research. Yanyang Zhang, Chenyang Ni, Tianjiao Li, Le Han, and Pingping Du conducted the experiment and performed data analysis. Kai Xiao wrote the paper. All authors contributed to the paper and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Figures

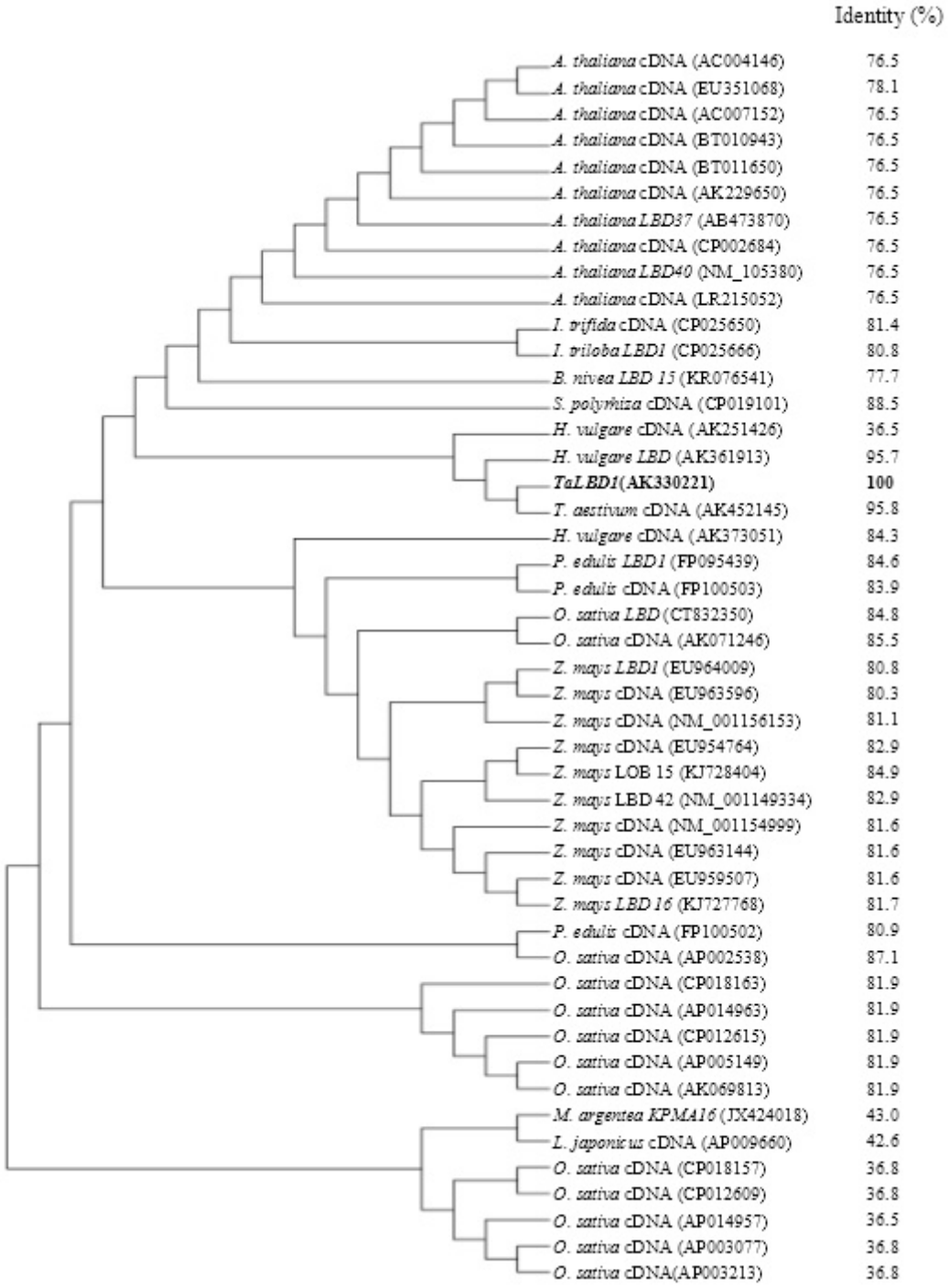

Figure 1

Phylogenetic relations among *TaLBD1* and its homologous genes from various plant species

A. thaliana-Arabidopsis; *I. trifida*-petunias; *I. triloba*-sweet potato; *B. nivea*-ramie; *S. polyrhiza*-duckweed; *H. vulgare*-barley; *T. aestivum*-wheat; *P. edulis*-kudzu; *O. sativa*-rice; *Z. mays*-maize; *M. argentea*- marcus silver; *L. japonicus*- lotus japonicus.

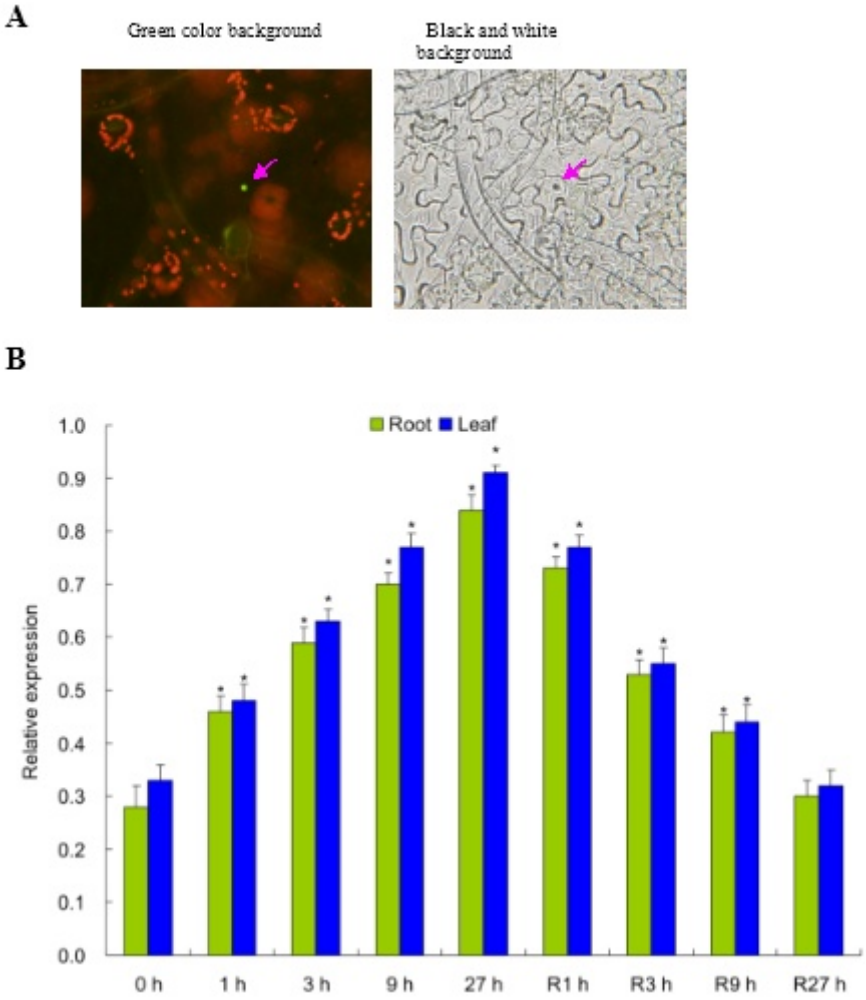

Figure 2

Subcellular localization of TaLBD1 and the expression patterns of *TaLBD1* following the external N treatments

A, The TaLBD1-GFP fusion detected under green microscope. **B**, expression patterns of *TaLBD1* upon the N starvation treatment. In **A**, the arrows point to cell nucleus. In **B**, 0 h, time point prior to N starvation stress. 1 h, 3 h, 9 h, and 27 h, time points after N starvation treatment. R3 h, R9 h, and R27 h, time points after N normal recovery treatment. Error bars represent standard errors and symbol * indicates significant differences between transgenic lines and WT calculated by one-way ANOVA with significance level of 0.05.

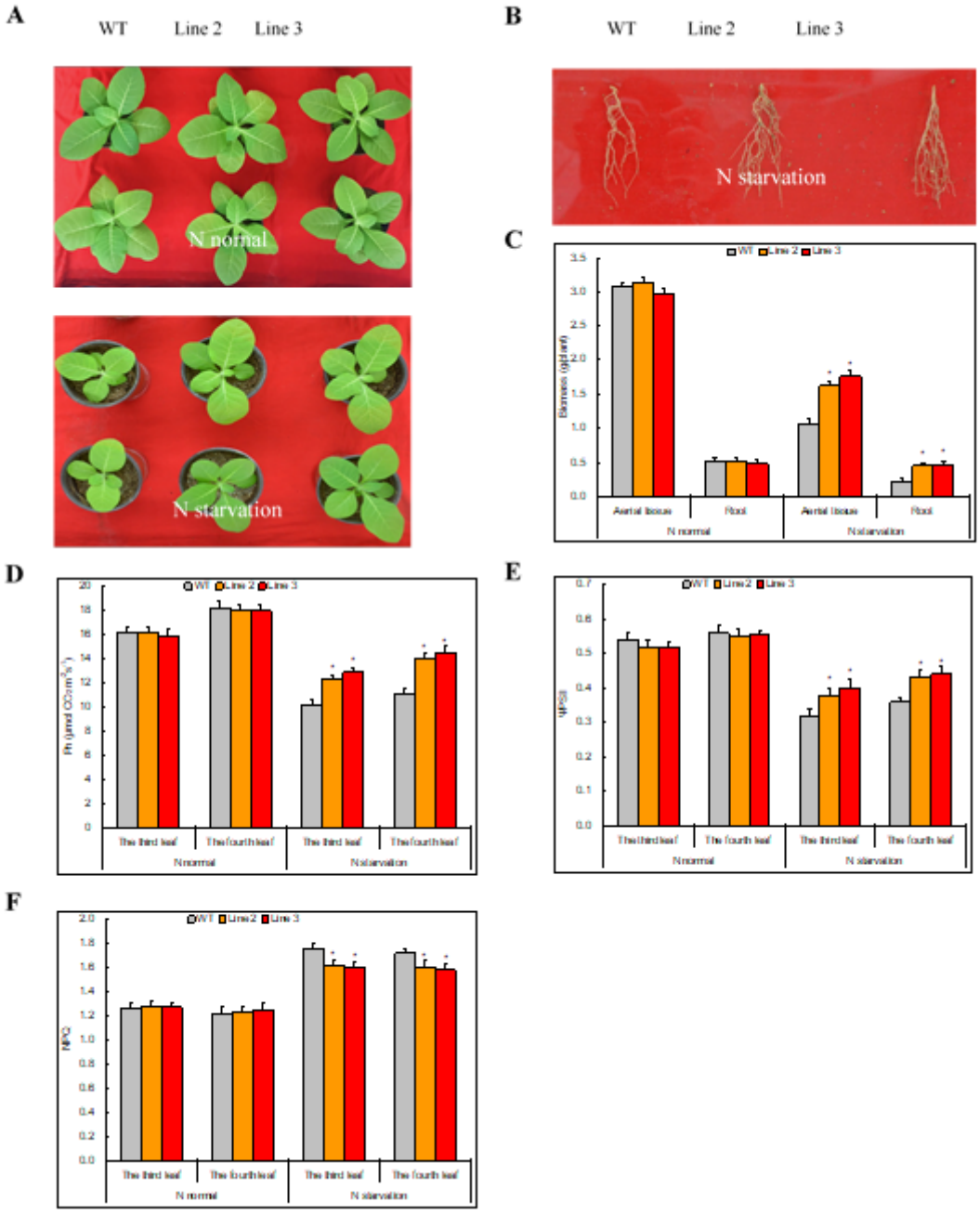

Figure 3

Phenotypes, biomass, and photosynthetic parameters in *TaLBD1* transgenic lines under N starvation treatment

A, phenotypes of plants. **B**, phenotypes of roots under N starvation. **C**, biomass in aerial tissues and roots. **D**, photosynthetic rate (Pn). **E**, photosystem II efficiency (ΨPSII), **F**, nonphotochemical quenching (NPQ). WT, wild type. Line 2 and Line 3, two lines with *TaLBD1* overexpression. In **C** to **F**, the average values are derived from the triplicate results. Error bars represent standard errors and symbol * indicates significant differences between transgenic lines and WT under same N treatment calculated by one-way ANOVA with significance level of 0.05.

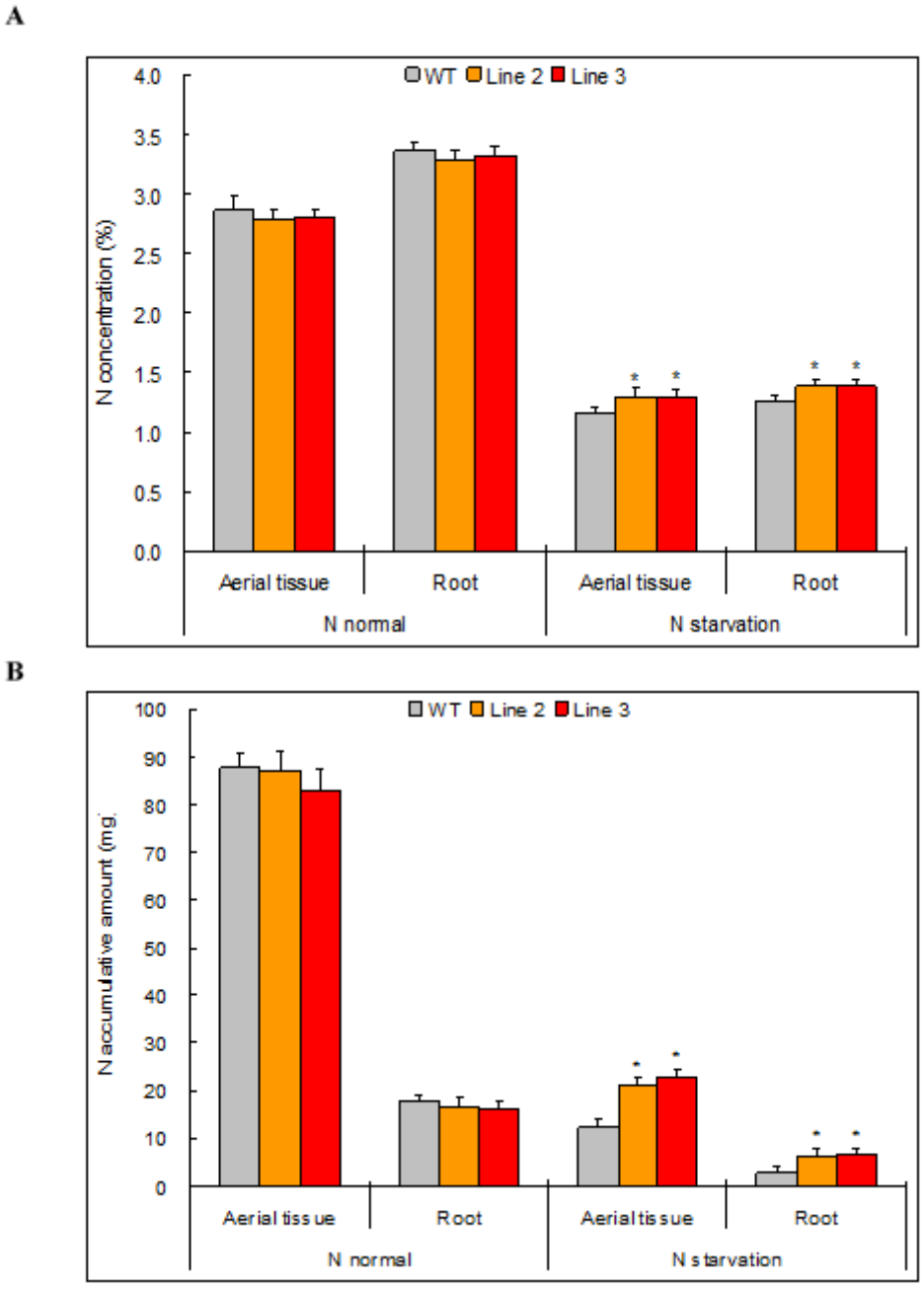

Figure 4

The N-associated traits in *TaLBD1* transgenic lines under the N starvation treatment

A, N concentrations in aerial tissues and roots. **B**, N accumulative amounts in aerial tissues and roots. WT, wild type. Line 2 and Line 3, lines with *TaLBD1* overexpression. The average values are derived from the triplicate results. Error bars represent standard errors and symbol * indicates significant differences between transgenic lines and wild type under same N treatment calculated by one-way ANOVA with significance level of 0.05.

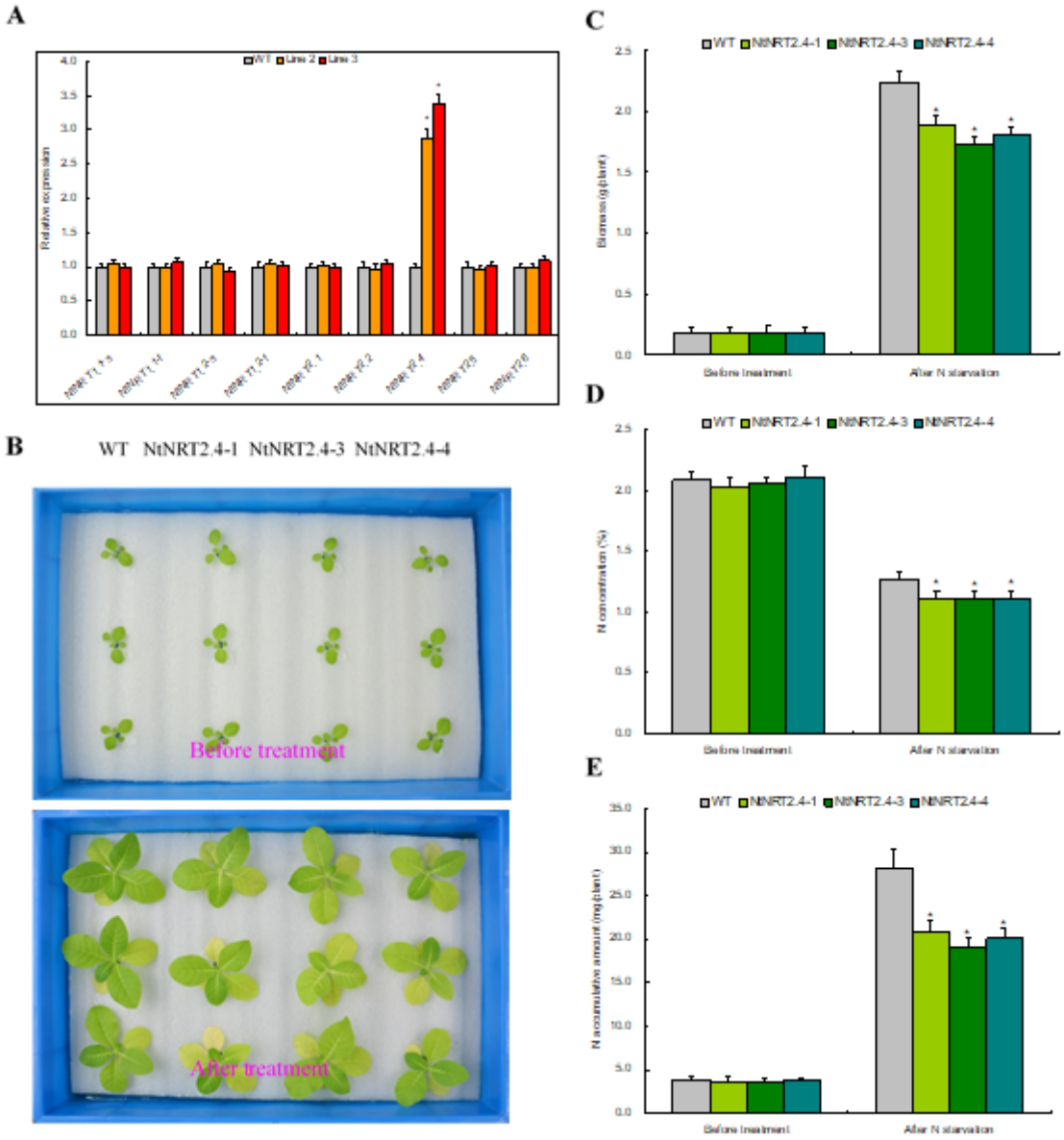

Figure 5

Expression patterns of the NRT family genes and functional analysis on distinct differential NRT gene under N starvation treatment

A, Expression patterns of the NRT family genes. **B**, phenotypes on transgenic lines with *NtNRT2.4* knockdown. **C**, biomass on lines with *NtNRT2.4* knockdown. **D**, N concentrations on lines with *NtNRT2.4* knockdown. **E**, N accumulative amounts on lines with *NtNRT2.4* knockdown. In **A** and **C** to **E**, the average values are derived from the triplicate results. WT, wild type. NtNRT2.4-1, NtNRt2.4-3 and NtNRT2.4-4, three lines with *NtNRT2.4* knockdown. Error bars represent standard errors and symbol * indicates significant differences between transgenic lines and WT calculated by one-way ANOVA with significance level of 0.05.

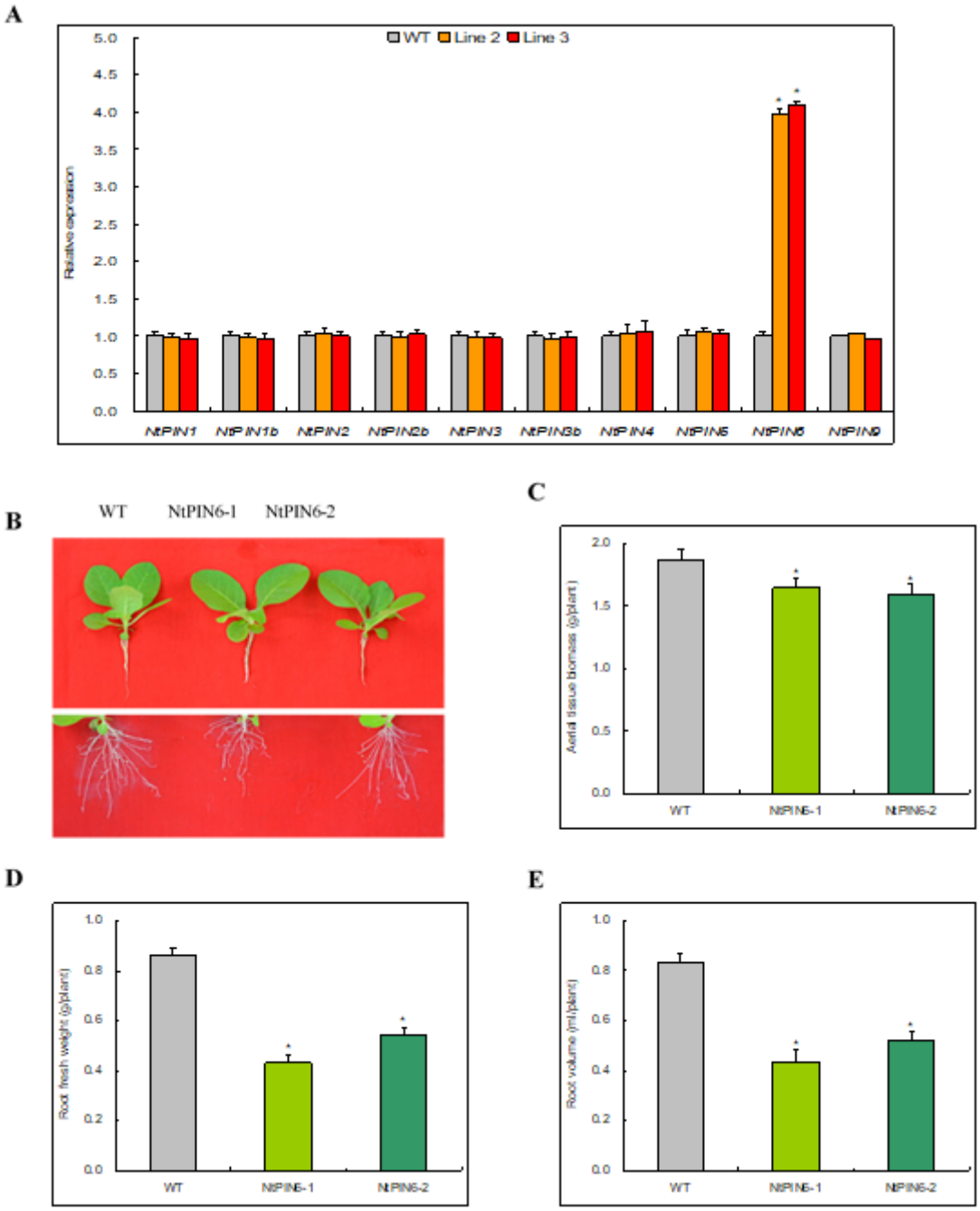

Figure 6

Expression patterns of the PIN-FORMED family genes and functional analysis on distinct differential PIN gene under N starvation treatment

A, expression patterns of the PIN-FORMED family genes. **B**, plant and root phenotypes on transgenic lines with *NtPIN6* knockdown. **C**, biomass on lines with *NtPIN6* knockdown. **D**, Root fresh weights on lines with *NtPIN6* knockdown. **E**, root volumes on lines with *NtPIN6* knockdown. In **A** and **C** to **E**, the average values are derived from the triplicate results. WT, wild type. NtPIN6-1 and NtPIN6-2, two lines with *NtPIN6* knockdown. Error bars represent standard errors and symbol * indicates significant differences between transgenic lines and WT calculated by one-way ANOVA with significance level of 0.05.

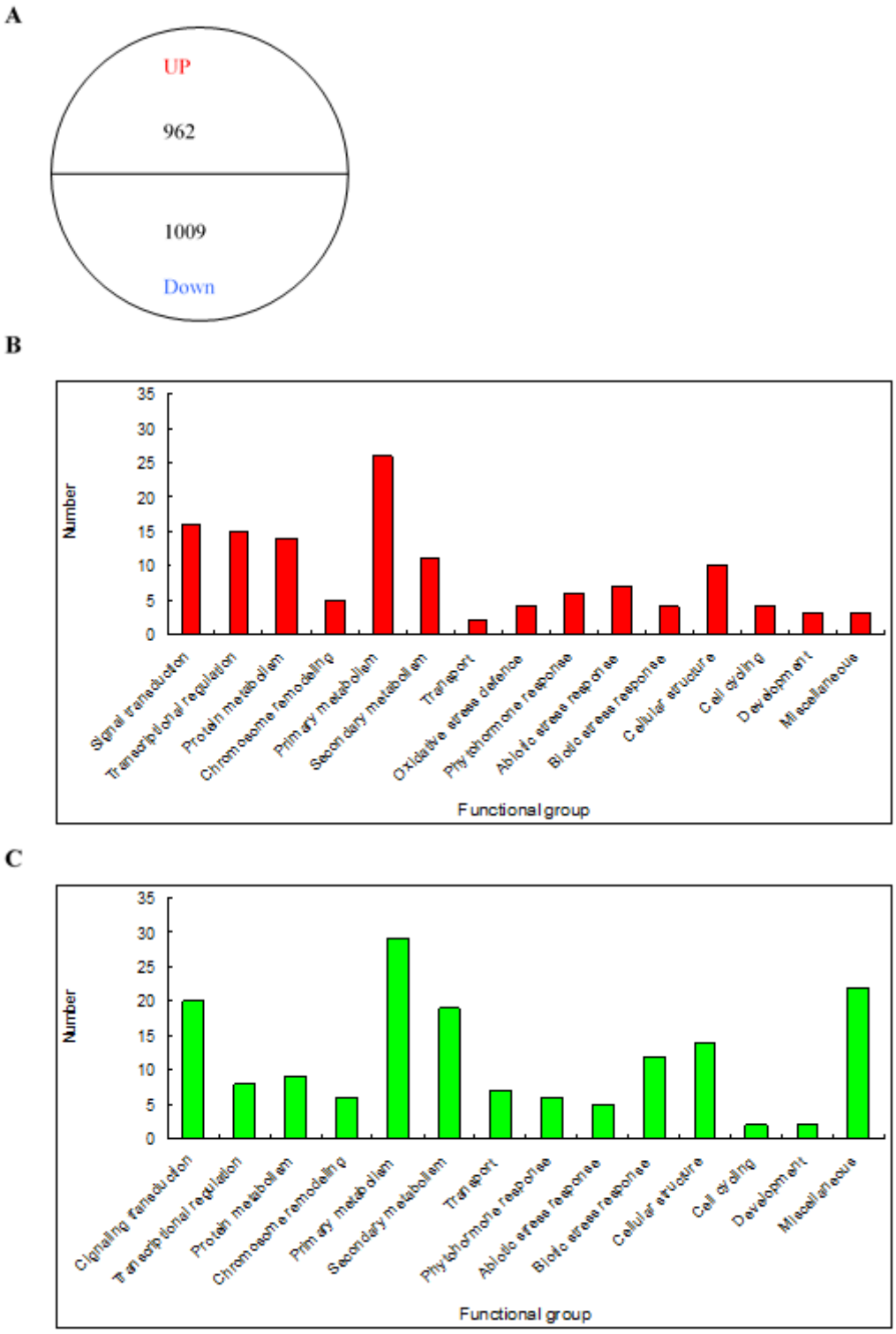

Figure 7

Functional groups of the DEGs with upregulated and downregulated expression patterns in lines with *TaLBD1* overexpression treated by N starvation stress

A, Venn diagram showing the numbers of the DEGS with upregulated and downregulated expression patterns. **B**, Functional groups of the upregulated DEGs. **C**, Functional groups of the downregulated DEGs.

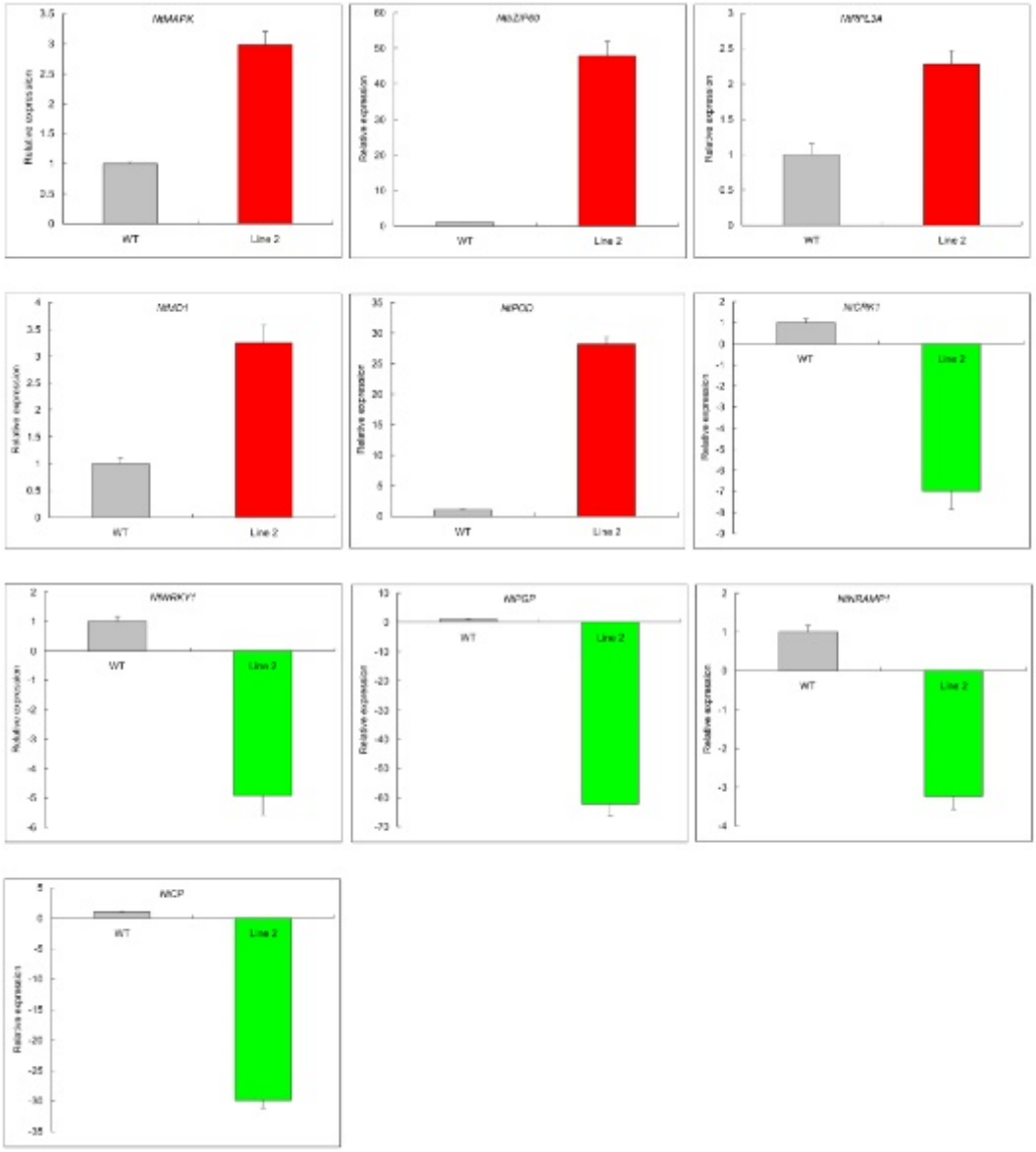

Figure 8

Expression patterns of ten randomly selected DEGs identified in lines with *TaLBD1* overexpression after N starvation treatment

NtMAPKK (Genbank accession No. BP530009), *NtbZIP60* (AB281271), *NtRPL3A* (AY395738), *NtMD1* (AJ299256), and *NtPOD* (AB044153), five DEGs with upregulated expression pattern in transcriptome analysis. *NtCRK1* (AF302082), *NtWRKY1* (AB022693), *NtPGP* (X70651), *NtNRAMP1* (AB505625), and *NtCP* (S44869), five DEGs with upregulated expression pattern in transcriptome analysis. Data are normalized by internal standard gene *Nttubulin* and shown by average plus standard error.

Supplementary Files

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