TaLBD1, a LOB Transcription Factor Gene In *T. Aestivum*, Improves Plant N Starvation Adaptation Via Modulating N Acquisition-Associated Processes

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Abstract

Members of transcription factor (TF) families contribute largely to plant N starvation tolerance by regulating downstream stress defensive genes. In this study, we characterized *TaLBD1*, a Lateral Organ Boundary (LOB) TF gene in *T. aestivum*, in regulating plant low-N stress adaptation. TaLBD1 harbors the conserved domains specified by plant LOB proteins, targeting onto nucleus after endoplasmic reticulum (ER) assortment. The *TaLBD1* transcripts were response sensitively to N starvation (NS) signaling, showing to be gradually upregulated in aerial and root tissues over a 27-h NS condition. The *N. tabacum* lines overexpressing *TaLBD1* improved phenotype, root system architecture (RSA) establishment, biomass, and N contents of plants under NS treatment. The nitrate transporter gene *NtNRT2.4* and PIN-FORMED gene *NtPIN6* significantly upregulated in expression in NS-challenged lines; knockdown expression of *NtNRT2.4* decreased N uptake and that of *NtPIN6* alleviated RSA establishment relative to WT. These results validate the function of NRT and PIN genes in regulating plant N uptake and RSA behavior. RNA-seq analyses revealed that a quantity of genes modify expression in N-deprived lines overexpressing *TaLBD1*, which enriched into functional groups of signal transduction, transcription, protein biosynthesis, primary or secondary metabolism, and stress defensiveness. These findings suggested that the *TaLBD1*-improved NS adaptation attributes largely to its role in transcriptionally regulating NRT and PIN genes as well as in modulating those functional in various biological processes. *TaLBD1* is a crucial regulator in plant N starvation tolerance and valuable target for molecular breeding high N use efficiency (NUE) crop cultivars.

Key Message

The LOB TF member *TaLBD1* in *T. aestivum* confers plants N starvation tolerance via modulation of N uptake and RSA establishment.

Introduction

Nitrogen (N) acts as of one the indispensible inorganic nutrients for all of the living organisms. The conditions of N supply impact largely on growth, development, and productivity potential of the plants. In several of past decades, application of N fertilizers contributed greatly to the sustainable crop production development around the world; however, lowered N use efficiency (NUE) in crop plants is accompanied with the overdosed N fertilizers, which failed utilization efficiently due to the nature of N nutrition being prone to be leaching and evaporation, leading to intensified pollution of environment aside from the elevated production investment. Therefore, improving the NUE for crop plants has been an essential issue for promotion of the sustainable agriculture worldwide (Wang et al. 2019; Laurens et al. 2021).

The acquisition of N and the internal translocation of it across various tissues in plants are accomplished underlying mediation of diverse physiological and biochemical processes. Under the N starvation (NS) condition, a large set of genes functional in various categories including those encoding N signaling receptors, kinases, transcription factors (TF), and stress-defensive proteins, display modified efficiencies

on transcription or translation (Kant et al. 2011; Lang et al. 2014; Antal et al. 2016; Wang et al. 2019). These components operate synergistically in mediating the plant NS response through modulating the NS defensiveness-associated biological processes.

Acted as one of the large families of TF, the members of Lateral Organ Boundary (LOB) Domain (LBD) family are plant specific that have been evolved from the same progenitor with charophyte algae (Coudert et al. 2012; Chanderbali et al. 2015). The proteins of the LOB family are specified by a LOB domain (also designated as AS2) consisting of following conserved domains: a C-motif, a Gly–Ala–Ser (GAS) block, and a leucine-zipper-like coiled-coil motif (Ohashi et al. 2018). Among these, the C-motif functions in binding the *cis*-acting regulatory elements situated in the downstream gene promoters whereas the coiled-coil motif involves the interaction processes between LBD TF and other types of proteins. Previously, extensive investigations have suggested that the LOB members play essential roles in regulating various physiological processes associated with growth, development, and abiotic stress responses of plants, including 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 N signaling transduction once plant perception of the modifying environmental N availability (Kimura et al. 2017). These results together suggest that the LOB TF members function as essential modulators in mediating various biological processes in plants.

The property of N uptake and characterization of root system architecture (RSA) are two determinants in plant adaptation to N starvation (NS) conditions (Yusefi-Tanha et al. 2020). Thus far, a subset of nitrate transporter (NRT) members that contribute to plant tolerance to low-N stress has been reported (Yusefi-Tanha et al. 2020). In addition, the RSA feature has been documented to be regulated synergistically by a set of external factors, including the localization, concentration and translocation of phytohormone auxin at cellular level (Brunetti et al. 2018; Doyle et al. 2019). To this issue, distinct members of PIN-FORMED (PIN) family were recorded to be involved in polar transportation of the cellular auxin, playing critical roles in modulation of physiological processes associated with initiation and elongation of primary and lateral roots, by which to contribute to plant NS tolerance (Ye et al. 2001; Baetsen et al. 2021). However, the molecular processes as to how the NRT members regulate N uptake and weather the PIN proteins mediate RSA acclimation to low-N stress underlying LOB TF modulation are needed to be further characterized.

Wheat (*T. aestivum*) is one of the important cereal crops to provide huge food source for humanity around the world. Todate, although the LBD TF family members have been identified and subjected to functional characterization in some plant species, such as *A. thaliana* and *O. sativa* (Evans et al. 2007; Soyano et al. 2008; Matsumura et al. 2009), the members of this TF family in *T. aestivum* remain largely to be investigated. In this study, we characterized *TaLBD1*, a gene of the LOB TF family in wheat, in mediating plant adaptation to NS stress. Our findings suggest that *TaLBD1* sensitively responds to external N starvation condition at transcriptional level and confers improved plant NS tolerance by improving N uptake and RSA establishment of plants challenged with low-N stress.

Materials And Methods

Characterization analysis on *TaLBD1*

Our previous RNA-seq analyses aimed at elucidating profiles of transcriptome in *T. aestivum* (cv. Shinong 086) upon low-N stress identified *TaLBD1*, a member of the LBB transcription factor family (GenBank accession No. AK330221), displayed significantly upregulated transcription (unpublished data). This finding encouraged us to further investigate its molecular characterization given the potential function of TF in mediating stress response in plants. The homologous genes of *TaLBD1* distributed in various plant species were obtained based on BLASTn search against with the GenBank database in NCBI (https://www.ncbi.nlm.nih.gov). The phylogenetic relations among *TaLBD1* and its plant counterparts were established using the MegAlign algorithm supplemented in the DNAStar software.

Subcellular location analysis of the TaLBD1 protein

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

Expression analysis of *TaLBD1*

The roots and leaves of *T. aestivum* (cv. Shinong 086) treated with varied N levels were subjected to evaluation of *TaLBD1* transcripts. To this end, wheat seedlings were cultured in a standard Murashige and Skoog (MS) solution (affluent N, 16 mM) as previously described by (Jiang et al. 2006). At the third-leaf growth stage, the NS treatment was set up for the seedlings by culturing them in a modified MS solution supplemented with lowered N supply (NS, 0.06 mM N). Tissues of roots and leaves were sampled at 0 h (prior to treatment), and 1 h, 3 h, 9 h, and 27 h after the NS treatment. In addition, an N recovery treatment was established to address the target gene response to recovered normal N condition. For that, an aliquot of the seedlings after 27 h of NS were re-cultured in a standard MS solution. The roots and leaves were collected after 1 h, 3 h, 9 h, and 27 h during the N recovery condition. The *TaLBD1* transcripts in collected tissues were evaluated based on qPCR performed to be similarly to previously described by Guo et al. (2013), using gene specific primers (Table S1). *Tatubulin*, a constitutive gene 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 tobacco lines overexpressing *TaLBD1*

TaLBD1 was overexpressed in an ectopic species (i.e., *N. tabacum*) to define its function in mediating plant NS response. With this purpose, the ORF of *TaLBD1* was amplified using RT-PCR together with the gene specific primers (Table S1). It was then inserted into the restriction sites *Bgl*II/*Bst*EII in vector pCAMBIA3301 under the control of the CaMV35S promoter. The procedure for generating transgenic lines was similar to that reported previously (Sun et al. 2012).

Line 2 and Line 3, two lines at T3 generation with high expression level of *TaLBD1*, were selected to define the gene function in regulating NS response of the plants. With this purpose, the transgenic lines and wild type (WT) plants were subjected to two N level treatments, including affluent N (AN) by culturing in a standard MS solution (16 mM N) and NS treatment by growing in a modified MS solution supplemented with lowered N (0.3 mM N). The growth conditions for plants in two N level treatments were as follows: a photoperiod of 14 h/10 h (light/dark) with light intensity of 400 μE/m^2s during light phase, temperature of 26°C/22°C (light/dark), and relative air humidity from 60% to 75%. During culture process, the solutions were air-circulated using a mini pump and renewed twice for each week. Six weeks after treatments, the plant growth traits of transgenic lines and WT, including phenotype, biomass, fresh weights and volumes of roots were assessed. Among these, phenotypes of plants and root tissues were recorded based on a digital camera; biomass of plants and root tissues was obtained from three representative plants after conventional oven-drying; fresh weights and root volumes of root tissues were determined according to the conventional approach. In addition, a set of photosynthetic parameters, including photosynthetic rate (Pn), photosystem II photochemical efficiency (ΨPSII), and non-photochemical quenching coefficient (NPQ), were measured in the transgenic lines and WT plant after N level treatments. Of which, Pn, gs, and Ci were measured using the photosynthesis system (LiCOR-6200) following the manufacturer's suggestion; ΨPSII and NPQ were assessed as reported previously (Guo et al. 2013).

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

The N contents and the expression patterns of a suite of nitrate transporter (NRT) family genes involving N uptake were analyzed in transgenic lines after N level treatments, by which to address the gene function in mediating plant N nutrition under NS. Of which, N concentrations were measured as described by Guo et al. (2013). Accumulative N amounts in plants were calculated by multiplying the N concentrations and plant biomass. The NRT family genes in *N. tabacum* that were subjected to the expression analysis included *NtNRT1.1-s*, *NtNRT1.1-t*, *NtNRT2.5*, and *NtNRT2.6*. Transcripts of the NRT family genes in transgenic and WT plants were evaluated using qRT-PCR together with corresponding gene-specific primers (Table S1). *Nttubulin*, a constitutive gene in *N. tabacum*, was used as an internal reference to normalize the target transcripts.

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

Cellular auxin level controls largely on the root system architecture (RSA) establishment of plants, playing an important role in mediating water and nutrient acquisition of root tissues under abiotic stress

conditions (Brunetti et al. 2018; Doyle et al. 2019). Members of the PIN-FORMED family act as the critical mediator in regulating internal auxin translocation and the cellular auxin level, by which to affect RSA establishment (Gray et al. 2001; Reed et al. 2001). To understand the putative PIN family members that contributed to modified RSA feature underlying *TaLBD1* regulation, we indentified the genes of PIN-FORMED family genes in *N. tabacum*, namely, *NtPIN1*, *NtPIN1b*, *NtPIN6*, and *NtPIN9*, and evaluated expression levels of them in the transgenic lines under NS treatment. To this end, qRT-PCR was performed to assess the transcripts of these PIN family genes. Gene specific primers used for amplification of them are listed in Table S1. *Nttubulin* was used as internal reference to normalize the target transcripts.

Transgene analysis on distinct NRT and PIN-FORMED family genes

The NRT family gene *NtNRT2.4* and the PIN-FORMED family gene *NtPIN6* displayed significantly upregulated expression in N-deprived transgenic lines (i.e., Line2 and 3), suggesting their putative involvement in mediating plant N uptake and RSA establishment. Therefore, we performed transgene analysis on them to characterize their functions in mediating N uptake and RSA establishment, respectively. With this purpose, the ORF in anti-sense orientation of *NtNRT2.4* and *NtPIN6* were separately amplified based on RT-PCR using gene specific primers (Table S1). They were then inserted into restriction sites *Nco*I/*BstE*II in vector pCAMBIA3301 under the control of the CaMV35S promoter as aforementioned. The lines with significant knockdown expression of target genes were established to be similarly for generating the *TaLBD1* overexpression lines. Three transgenic lines designated as NtNRT2.4-1, NtNRT2.4-3 and NtNRT2.4-4 for *NtNRT2.4* and two lines AnPIN6-1 and AnPIN6-2 for *NtPIN6*, were selected and subjected to two N level treatments as mentioned above (i.e., AN with 16 mM N and NS with 0.3 mM N). Six weeks after treatments, the phenotypes, biomass, N concentrations and N accumulative amounts in *NtNRT2.4* lines were assessed. Likewise, the phenotypes of plants and root tissues, plant biomass, and fresh weights and volumes of root tissues were evaluated in *NtPIN6* lines. The N-associated traits and root growth traits were assessed to be similarly to those performed in the *TaLBD1* overexpression lines mentioned above.

Transcriptome analysis

High-throughput RNA-seq analyses were performed to characterize the transcriptiome profile underlying modulation of *TaLBD1* under the NS condition. To this end, Line 2, the transgenic line overexpressing *TaLBD1* together with WT were cultured regularly in a standard MS solution as aforementioned. At the fifth leaf stage, they were subjected to NS treatment for another one week. Total RNA in Line 2 and WT plants was extracted using TRIzol reagent (Invitrogen) and subjected to construction of RNA-seq libraries following the procedure as described previously (Zhong et al. 2011). Transcripts generated in the RNA-seq libraries were sequenced using the Illumina HiSeq 2500 system. Valuable transcripts in libraries generated from the N-deprived transgenic lines and WT were obtained by removing the adaptors in reads, the reads with sequence length less than 40 bp, and those being low quality based on the software Trimmomatic (Bolger et al. 2014). These clean reads were then subjected to alignment analysis against the database for transcripts of the reference genome (*N. tabacum*, Novogene Co, LTd, Beijing). We defined

the genes to be differentially expressed (DE) when they exhibited 2-fold variation on transcripts across the transgenic and WT plants (Robinson et al. 2010), using a false discovery rate (FDR) less than 0.05 (Benjamini and Hochberg 1995). The DE genes were categorized into distinct GO terms using the online tool referred to as Plant MetGenMap (http://bioinfo.bti.cornell.edu/cgi-bin/MetGenMAP/home.cgi), in which a CPAN pearl module was applied as described previously (Boyle et al. 2004). Functional groups of the DE genes identified in transgenic lines were determined based on gene GO annotations.

Expression analysis on randomly selected DE genes in RNAseq analysis

Ten of DE genes identified in the RNA-seq analyses, including five to be upregulated and five downregulated, were subjected to evaluation of transcripts based on qPCR using gene specific primers (Table S1), to validate the RNA-seq analysis results. The five genes with upregulated expression pattern included those encoding mitogen-activated protein kinase kinase, leucine zipper protein, ribosomal protein L3A, malate dehydrogenase, and peroxidase; the five ones with downregulated expression pattern were those coding for cytokinin-regulated kinase, WARK protein, phosphoglyceromutase, metal transporter, and chitinase. cDNA samples derived from Line 2 and WT after NS treatment were used as the templates in qRT-PCR. Likewise, the constitutive *Nttubulin* was used as an internal reference to normalize target transcripts.

Statistical analysis

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

Results

The characterization of TaLBD1

The full length cDNA of *TaLBD1* is 1267 bp that encodes a 303-aa polypeptide (Fig. S1). Similar to the members of LOB TF family in plant species, the TaLBD1 protein harbors a conserved C-motif (CX2CX6CX3C) (5 aa-19 aa) at N terminus (Fig. S2). Based on phylogenetic relation analysis, it was revealed that *TaLBD1* shares high similarities to the homologous genes distributed in diverse plant species at nucleic acid level, including those from *H.vulgare*, *P.edulis*, *O. sativa*, *Z. mays*, *I. triloba*, *B. nivea* and *A. thaliana* (with identities changing from 76.5 to 95.8%, Fig. 1). TaLBD1 targets onto nucleus after endoplasmic reticulum (ER) assortment based on online prediction analysis, which is consistent with result of the experiment indicating that the GFP signals derived from fusion TaLBD1-GFP were confined in nucleus of *N. tabacum* epidermal cells (Fig. 2A). Subcellular localization of the TaLBD1 protein onto nucleus is in agreement with its TF nature exerting roles in regulating transcription of the downstream genes.

TaLBD1 expression is response sensitively to the NS treatment

The expression levels of *TaLBD1* were drastically altered in both roots and leaves once challenged with NS condition. Under affluent N condition (AN), the transcripts of *TaLBD1* in tissues examined were low. In contrast, they were increased dramatically under NS treatment, being gradually elevated over a 27 h NS regime treatment and reaching a peak at end of the treatment (Fig. 2B). Moreover, the upregulated transcripts of *TaLBD1* upon NS were restored steadily in tissues following a 27 h ofN recovery treatment (Fig. 2B). These results together suggested that *TaLBD1* is sensitive in response to Nsignaling condition and possibly involves the NS signaling transduction in plants.

TaLBD1 confers plants significantly improved tolerance to the low-N stress

Two tobacco lines at T3 generation, namely, Line 2 and Line 3 with strong expression of *TaLBD1* (Fig. S3), were subjected to two treatments with contrasting N levels. Under AF condition, comparable growth traits, including phenotypes and biomass in plants and roots were observed in both the transgenic lines and WT (Figs. 3A). Under NS treatment, however, the transgenic lines significantly improved phenotype (Fig. 3A), RSA establishment (Fig. 3B), and biomass in both plants and roots (Fig. 3C) compared with WT. In line with above growth traits, the transgenic lines displayed improved photosynthetic parameters, such as elevated photosynthetic rate (Pn), enhanced photosystem II biochemical efficiency (ΨPSII), and reduced nonphotochemical quenching (NPQ) with respect to WT (Figs. 3D-3F). These results suggested that *TaLBD1* acts as a crucial regulator in enhancing plant tolerance to the low-N stress.

Overexpression of TaLBD1 confers plant improved N accumulation capacity

N-associated traits in the transgenic lines (i.e., Lines 2 and 3) and WT were assessed after the N level treatments. Under AN condition, similar to the growth traits mentioned above, the transgenic lines were comparable on N concentrations and N accumulative amounts with WT (Figs. 4A-4B). Under NS treatment, all of the transgenic lines (Lines 2 and 3) and WT were lower on N concentrations and the N accumulative amounts than shown under the N normal condition (Figs. 4A-4B). However, Lines 2 and 3 increased N concentrations and N accumulative amounts compared to WT under NS treatment (Figs. 4A-4B). Behaviors of these N-associated traits in transgenic lines are consistent with the growth traits and photosynthetic parameters shown under contrasting N level treatments, suggesting that the *TaLBD1*-improved plant N tolerance is largely attributed to the gene function in positively regulating N uptake of plants challenged with low-N stress.

Expression patterns of NRT genes and their function in mediating plant NS adaptation

Expression patterns of a suite of NRT family genes were analyzed in the N-deprived transgenic and WT plants to understand the molecular processes underlying N uptake mediated by *TaLBD1*. Among nine genes of NRT genes in *N. tabacum* examined, *NtNRT2.4* was significantly upregulated in expression in the transgenic lines with respect to WT (Fig. 5A). Its modified expression pattern upon N starvation was in contrast to other NRT genes whose transcripts were similar in both transgenic and WT plants (Fig. 5A).

Therefore, *NtNRT2.4* is a gene to be putatively regulated underlying *TaLBD1* regulation at transcription level and contributes to the NS adaptation of the N-deprived transgenic lines.

Three typical lines with *NtNRT2.4* knockdown expression (NRT2.4-1, NtNRT2.4-3 and NRT2.4-4) (Fig. S4) were subjected to characterization of gene function in regulating N uptake. Under NS treatment, these lines alleviated significantly on phenotypes (Fig. 5B), biomass, N concentrations, and accumulated N amounts in plants compared with WT (Figs. 5C-5E). These results suggested that *NtNRT2.4* functions effectively in mediating the N uptake by impacting on N accumulation and growth-associated processes of the plants treated by low-N stress.

Expression patterns of PIN-FORMED family genes and their roles in mediating root RSA establishment

Transcripts of ten genes in PIN-FORMED family in *N. tabacum*, were evaluated in the N-deprived transgenic lines (Lines 2 and 3) and WT to understand weather any of them involves RSA establishment underlying *TaLBD1* regulation. In contrast to other PIN genes whose transcripts were similar each other in transgenic lines and WT plants, *NtPIN6* displayed significantly upregulated expression in Lines 2 and 3 relative to WT (Fig. 6A). These results suggested that *NtPIN6* is transcriptionally regulated by *TaLBD1* and contributes to the improved RSA establishment in transgenic lines challenged with NS condition.

Transgene analysis on *NtPIN6* was performed to characterize its function in regulating RSA establishment. Under NS treatment, NtPIN6-1 and NtPIN6-2, two typical lines with *NtPIN6* knockdown expression (Fig. S5) significantly alleviated root growth (Fig. 6B), reduced biomass, decreased root fresh weights, and lowered root volumes of the plants compared to WT plants (Figs. 6C-6E). These results together suggested that distinct genes in PIN-FORMER family, such as *NtPIN6*, positively impact on RSA establishment in plants overexpressing *TaLBD1* treated by NS condition.

The differentially expressed (DE) genes underlying regulation of TaLBD1

The DE genes in lines overexpressing *TaLBD1* were identified under N starvation condition based on a high-throughput RNA-seq analysis. A total of 1971 genes were differentially expressed in transgenic line (i.e., Line 2) under NS treatment with respect to WT. Among these, 962 were categorized into the expression pattern of upregulated and 1009 of downregulated (Fig. 7A, Dataset 1-Dataset 2). To verify the results derived from the transcriptome datasets, we analyzed the transcripts of ten DEGs randomly selected from the datasets, including five upregulated and another five downregulated shown in the N-deprived transgenic lines. All of the DE genes displayed expression levels with comparable variation-folds shown in the transcriptome analyses. These results validated the nature of reproducibility in our transcriptome analysis (Fig. 8). Therefore, *TaLBD1* acts as an important regulator in plant N starvation signaling transduction system which modulates transcription of downstream genes at global level.

Among the DE genes identified in transcriptome analyses, only small ratio of them has been annotated to known biological functions thus far (including a total of 130 upregulated and 161 downregulated) (Dataset 1-Dataset 2). That the DEGs with a large ratio identified to be function unknown is suggested

due to the limitation of the gene annotation programs in *N. tabacum* species. Based on the annotated biological functions, the upregulated DEGs are categorized into 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 downregulated DEGs with annotated functions are classified into 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 N-deprived transgenic plants suggested that *TaLBD1* exerts roles in regulating transcription of numerous genes, which are involved in various biological functions by synergistically acting in physiological processes associated with plant N starvation tolerance.

Discussion

The members of the LOB transcription factor family play critical roles in mediating physiological processes associated with plant growth, development, and stress responses, involving modulation of 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), KNOX gene regulation (Semiarti et al. 2001; Chalfun et al. 2005; Long et al. 2014; Long et al. 2015), and N deprivation adaptation (Rubin et al. 2009). In this study, our characterization on *TaLBD1*, a gene of the LOB TF family in *T. aestivum*, revealed its high similarities at cDNA level with the homologous genes across diverse plant species. The conserved domains situated in TaLBD1 and the subcellular localization onto nucleus of the protein are consistent with members of the LOB TF family, which exert putative biological functions in nucleus through regulation of the downstream genes at transcriptional level.

Plant response to NS is closely associated with transcriptional modulation of the N deprivation-responsive genes. Previously, a set of *cis*-acting regulatory elements such as nitrate-responsive elements (NRE), was identified to be situated in promoters of the N uptake- and assimilation-associated genes, contributing to modified transcription efficiency of NS-responsive genes (Jian et al. 2018). For instance, *NRT2.1* and *NRT2.2*, two genes of NRT family in *A. thaliana*, displayed induction on expression upon low-N stress, which is closely associated with interaction of the NRE motifs in them with distinct TFs (Jian et al. 2018). In this study, our expression analysis on *TaLBD1* indicated that its transcripts are response to low-N stress in aerial and root tissues, which suggests its involvement in mediating NS response of the plants. Further characterization on *cis*-acting regulatory elements situated in the promoter of this *LOB* gene can deepen understanding the transcriptional mechanism of it upon NS condition in *T. aestivum*.

The function of LOB members in mediating transduction of N deprivation signaling has been recorded (Rubin et al. 2009). In this study, we selected *N. tabacum* as an ectopic expression system to generate transgenic lines with *TaLBD1* overexpression, by which to characterize putative role of target gene in mediating plant NS response. Results indicated that the transgenic lines (i.e., Line 2 and Line 3) significantly improved low-N stress tolerance with respect to control, the wild type (WT) without subjected to genetic transformation of target gene. The transgenic lines showed much better on phenotype, biomass of aerial tissues and roots, fresh weight and volume of root tissues under NS treatment compared with WT. These results are in agreement with behavior of the improved photosynthetic parameters, such as elevated Pn, enhanced photosystem II biochemical efficiency, and reduced nonphotochemical quenching (NPQ) in the N-deprived lines overexpressing *TaLBD1*. Together, our findings suggest that *TaLBD1* acts as an essential regulator in mediating the plant N starvation response..

Plant low-N stress tolerance is largely dependent on the capacity of N uptake of plants challenged with NS condition. It has been clearly documented that nitrate transporter (NRT) system consisting of members of the high-affinity transport (HAT) exerts crucial role in regulating N accumulation in N-deprived plants (Remans et al. 2006; Da et al. 2019). Characterization on HAT system has revealed that members of the RT2 group act as critical components, which contribute to the N acquisition capacity of plants under low-N conditions (Wang et al. 2020). For example, the mutants nrt2.1 with knockout of *AtNRT2.1*, a gene of NRT2 family in Arabidopsis, reduces HATS activity and decreases N content relative to wild type (Li et al. 2007). In this study, we assessed the N accumulation property in transgenic lines (i.e., Lines 2 and 3) under NS treatment to understand the physiological processes associated with *TaLBD1*-mediated low-N stress tolerance improvement. Higher N concentration and more accumulative amounts of N were observed in N-deprived transgenic plants than WT. These findings suggest that *TaLBD1* confers plant enhanced N uptake by which to improve plant NS tolerance. Our expression analysis on NRT family genes in *N. tabcucm* revealed that *NtNRT2.4* enhances expression levels significantly in N-deprived transgenic lines relative to WT. Moreover, using transgenic lines with *NtNRT2.4* knockdown expression, we experimentally verifieded the NRT gene function in mediating plant NS response. Compared with WT, the lines with knockdown expression of *NtNRT2.4* dramatically alleviated growth traits and lowered N accumulative amounts of the plants under NS treatment. These results suggest that distinct NRT genes underlying control of *TaLBD1* to constitute putative action modules, such as LBD1-NRT2.4, which contribute to plants improved N uptake and tolerance to low-N stress.

Auxin acts as one of critical members of phytohormones and involves multiple biological processes associated with plant growth and development, including initiation and formation of lateral roots to impact on nutrient and water acquisition of plants (Brunetti et al. 2018). The auxin signaling involving modulation of various physiological processes is transduced through synergistic actions of diverse biochemical pathways (Gray et al. 2001; Doyle et al. 2019). Thus far, it has been documented that members of the PIN-FORMED family play essential roles in controlling the auxin levels and distribution patterns in cells, acting as an important determinant in modulating establishment of RSA (Rogers et al. 2016; Lee et al. 2021). In this study, our characterization on RSA in transgenic lines overexpressing

TaLBD1 revealed that the target gene confers plants enlarged RSA feature under NS treatment, suggesting that the *TaLBD1*-mediated plant NS tolerance is also ascribed to its regulation on RSA establishment. In addition, based on expression analysis, the members of PIN-FORMED family with modified transcription in N-deprived lines were identified. In contrast to other genes with unaltered transcripts in transgenic and WT plants, *NtPIN6* displayed significantly upregulated expression in N-deprived lines with *TaLBD1* overexpression compared to WT; transgene analysis on this PIN gene validated its function in significantly improving RSA establishment of plants challenged with the NS condition. These findings together suggest that the putative pathway LBD1-PIN6-RSA acts as another mechanism for low-N stress tolerance in plants underlying regulation of *TaLBD1*. Recently, it has been documented that distinct members of the LOB TF family are involved in auxin signaling transduction pathways, which exert biological roles in mediating formation of lateral root tissues (Filleur et al. 2001). Further investigation on mechanisms as to how distinct PIN-FORMED family members modulate RSA establishment mediated by LOB TF members can provide insights into molecular processes associated with plant root development and low-N stress adaptation.

High throughput transcriptome analysis provides effective approach to elucidate molecular processes underlying the plant stress response (Nemhauser et al. 2004; Lang et al. 2014; Keen et al. 2020). In this study, based on RNA-seq analysis, we investigated the genes with modified transcription in N-deprived transgenic lines overexpressing *TaLBD1*. As a result, a total of 1971 genes, including 962 to be upregulated and 1009 downregulated, were identified. These differentially expressed (DE) genes are categorized 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 suggested that *TaLBD1* mediates plant low-N stress tolerance to be also associated with its function in modulating the biological processes mentioned above, aside from its regulation on N uptake (i.e., via function module LBD1-NRT2.4) and improvement on RSA establishment (via biochemical pathway LBD1-PIN6-RSA formation). Further characterization on the DE genes playing critical regulatory roles in plant stress response underlying regulation of *TaLBD1* can benefit to elucidate the biochemical pathways contributing to plant N starvation adaptation in crop plants.

Conclusion

TaLBD1 harbors the conserved domains shared by members of the plant LOB TF family, targeting onto nucleus after endoplasmic reticulum (ER) assortment. *TaLBD1* is sensitive in response to low-N stress treatment, showing induced transcripts upon N starvation in aerial and root tissues and whose induced expression under NS was recovered following N recovery treatment. *TaLBD1* confers plants improved growth traits treated by low-N stress, displaying to be better on RSA, biomass production, photosynthetic function, and N accumulation for plants. Distinct genes in NRT family referred to as *NtNRT2.4* and PIN-FORMED family *NtPIN6* significantly upregulated in expression in N-deprived lines overexpressing *TaLBD1*, exerting roles in the *TaLBD1*-mediated improvement of plant low-N stress tolerance by enhancing N uptake and RSA establishment. RNA-seq analysis identified large sets of genes with

significantly modification on transcription underlying regulation of *TaLBD1*, which are categorized into functional groups associated with signal transduction, transcription, protein biosynthesis, primary or secondary metabolism, and stress defensiveness, etc. *TaLBD1* is a valuable target gene for genetic engineering of high NUE crop cultivars that are 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

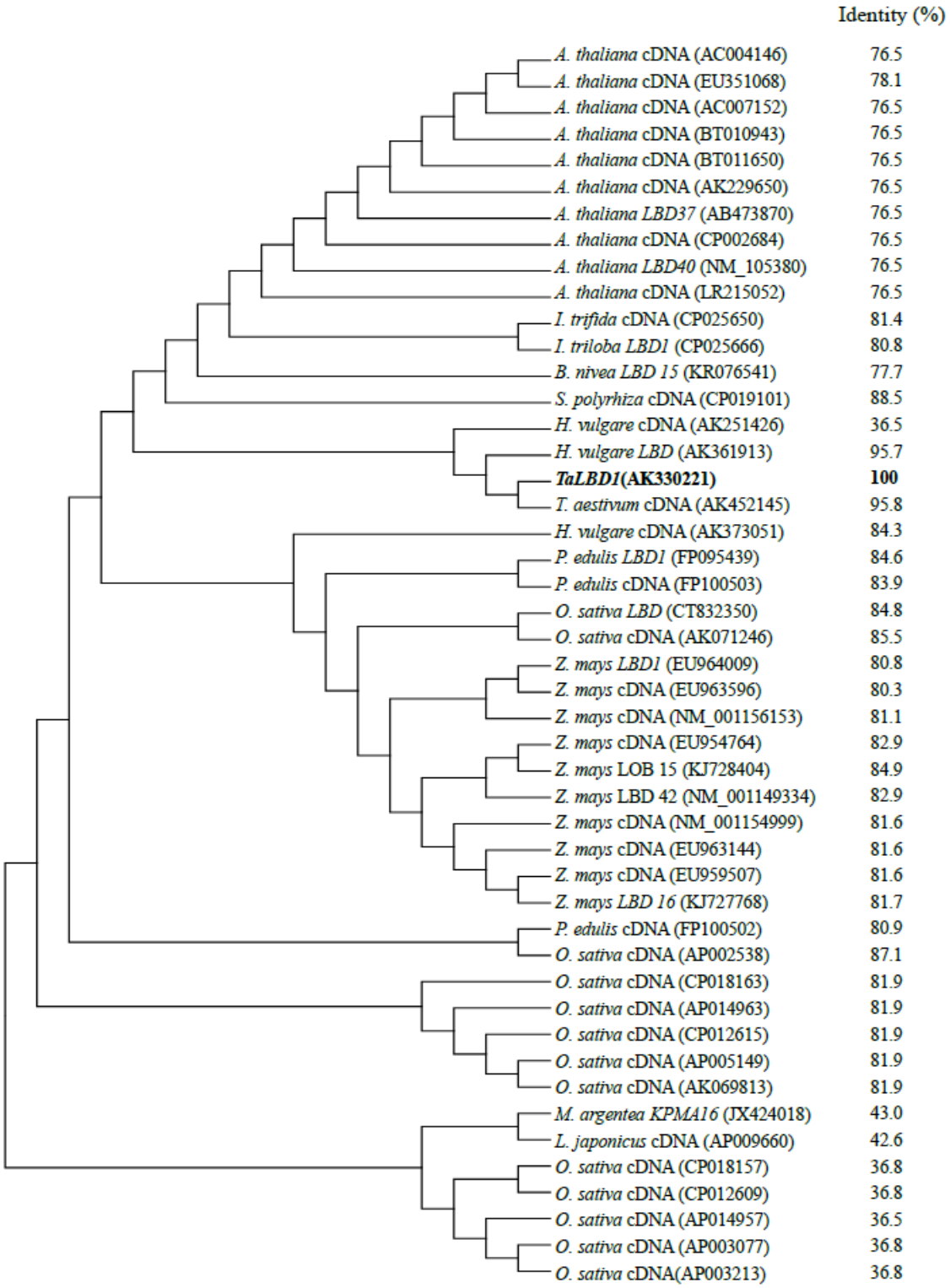

Figure 1

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.

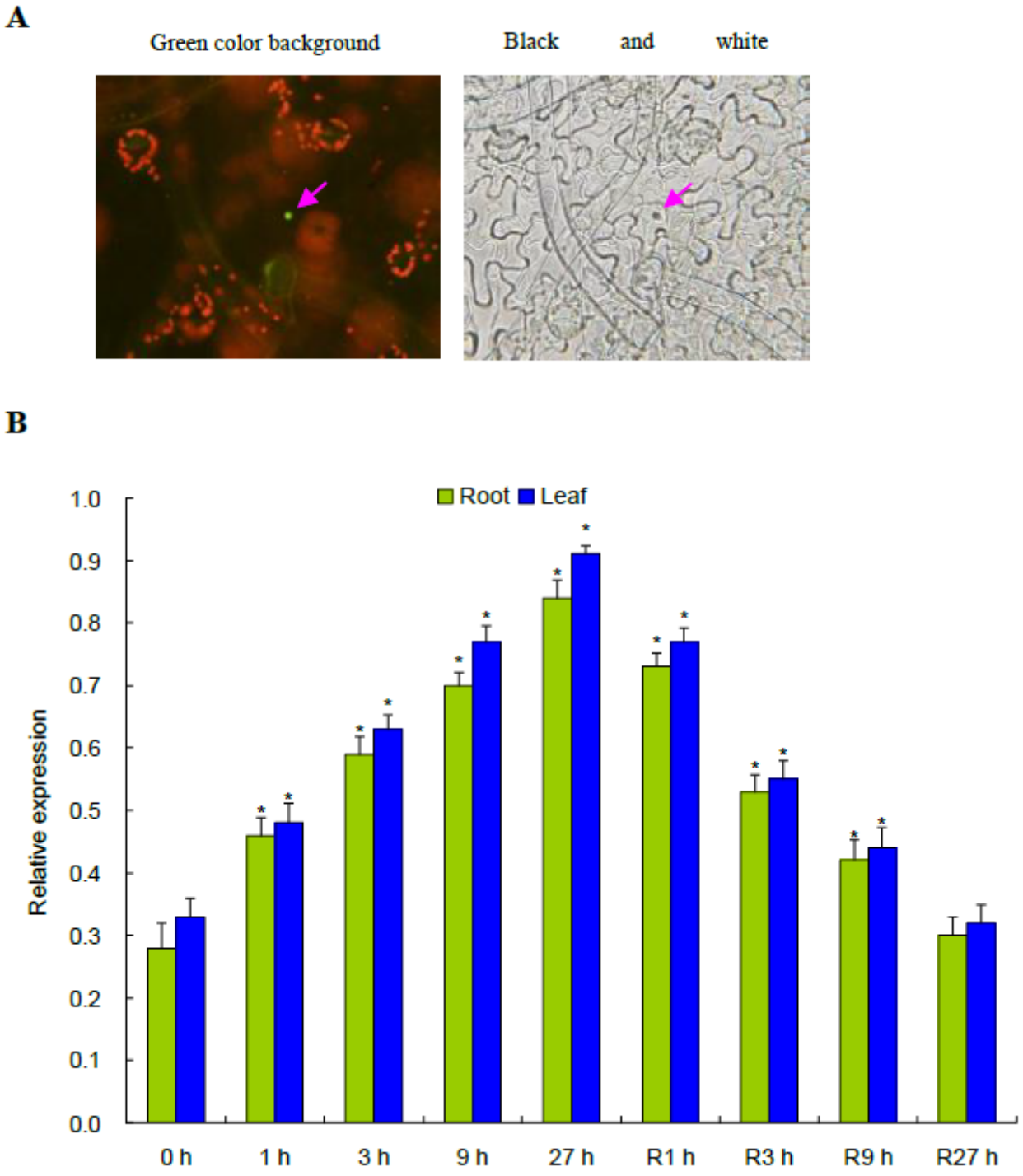

Figure 2

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.

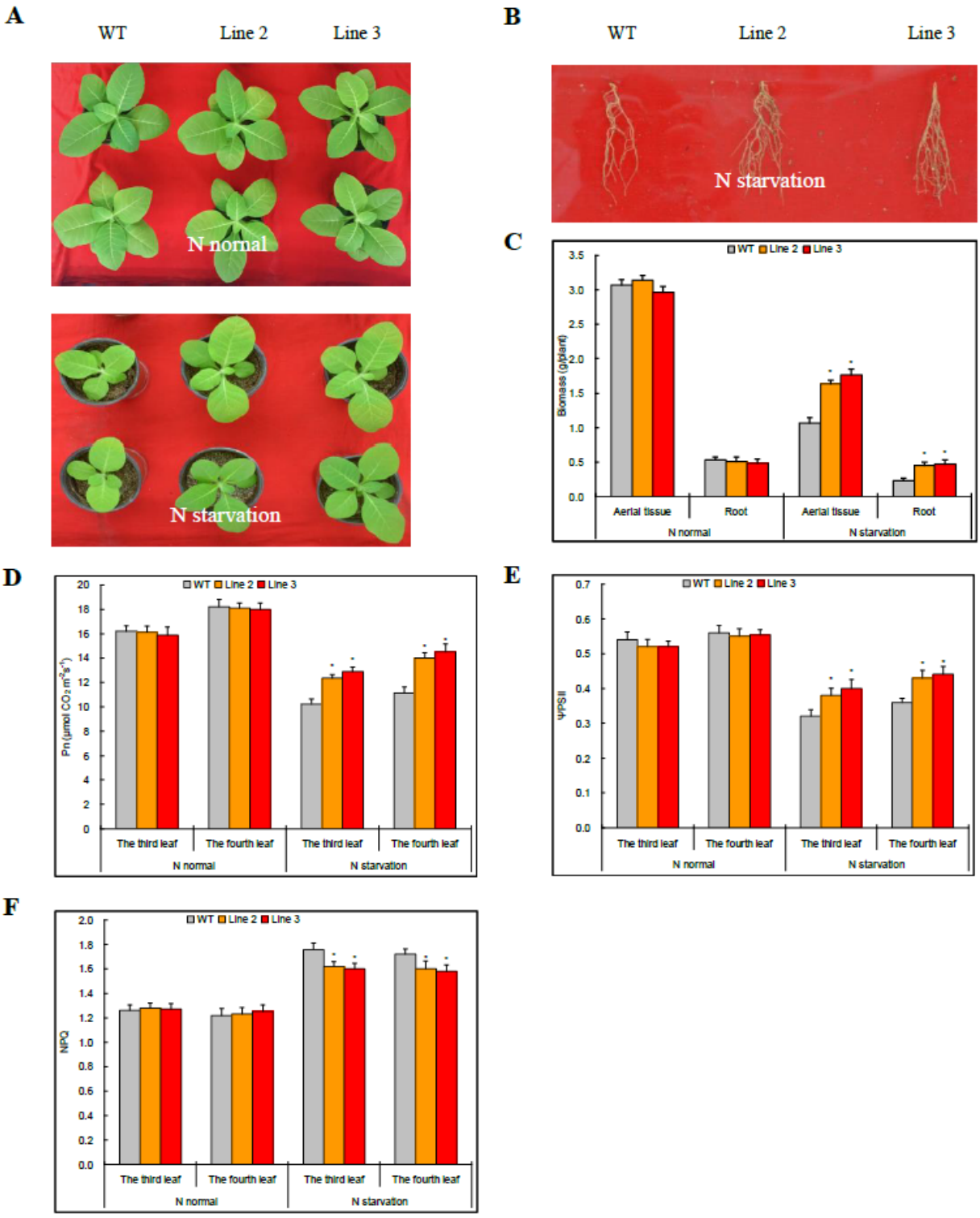

Figure 3

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.

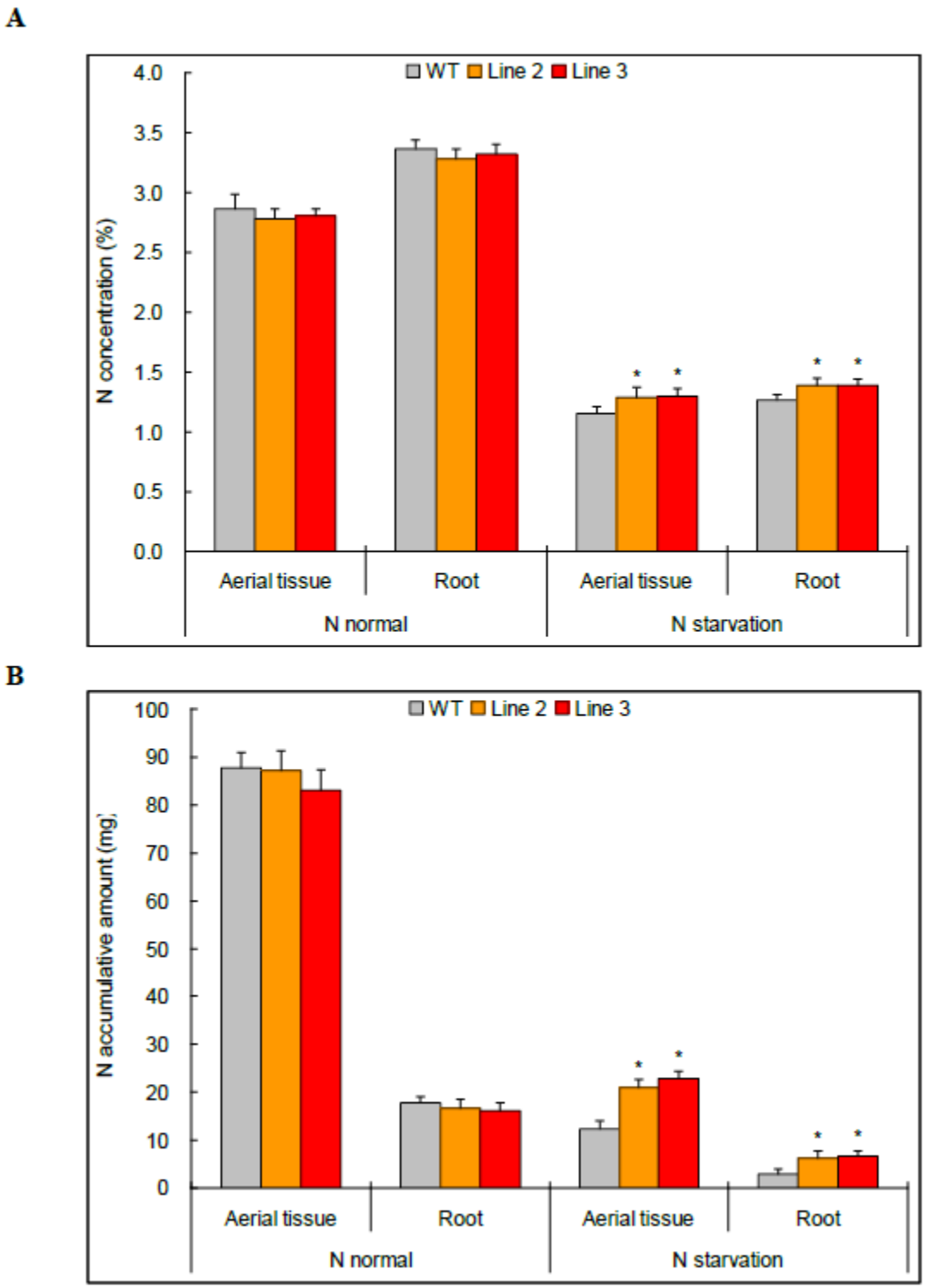

Figure 4

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.

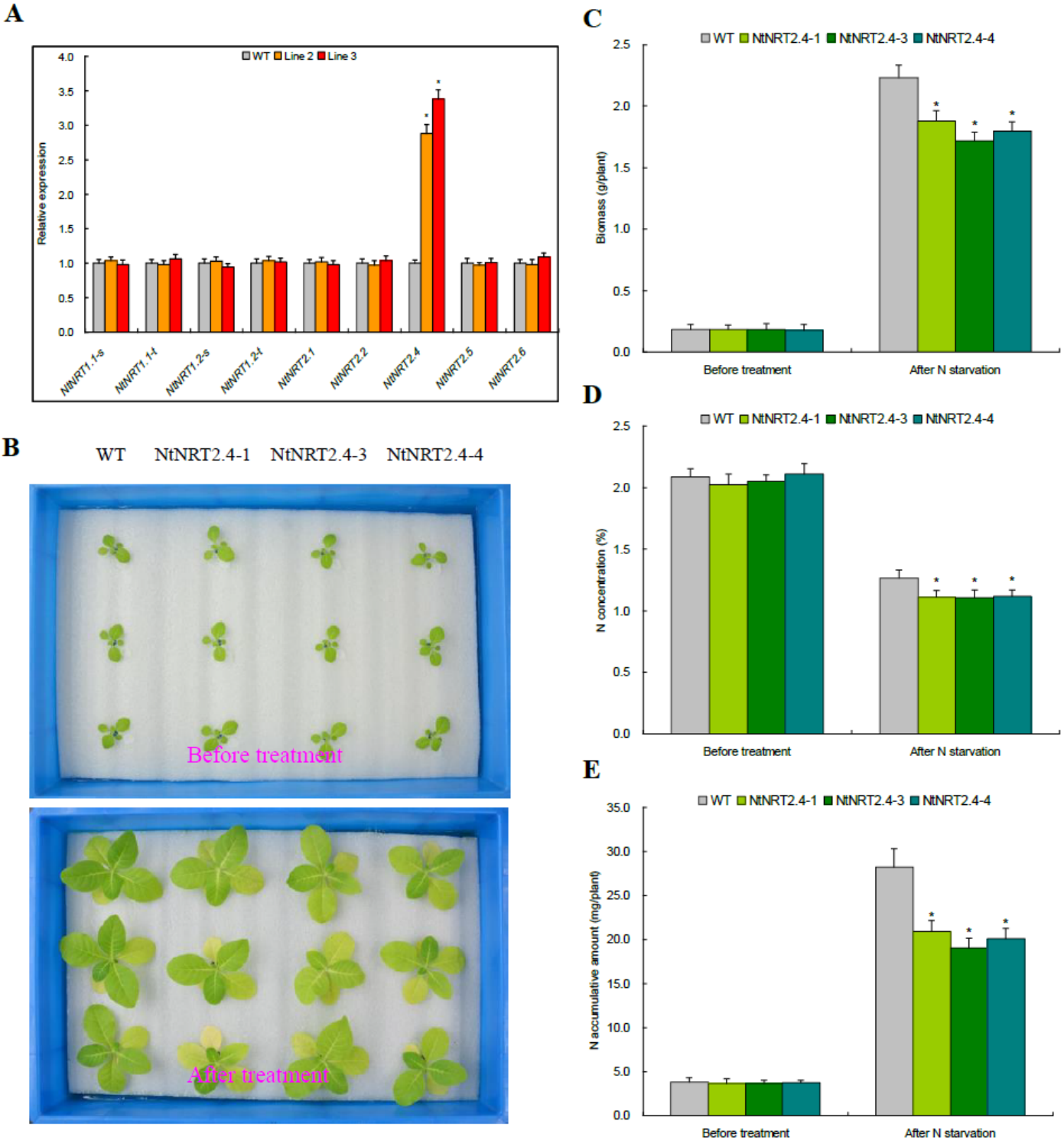

Figure 5

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.

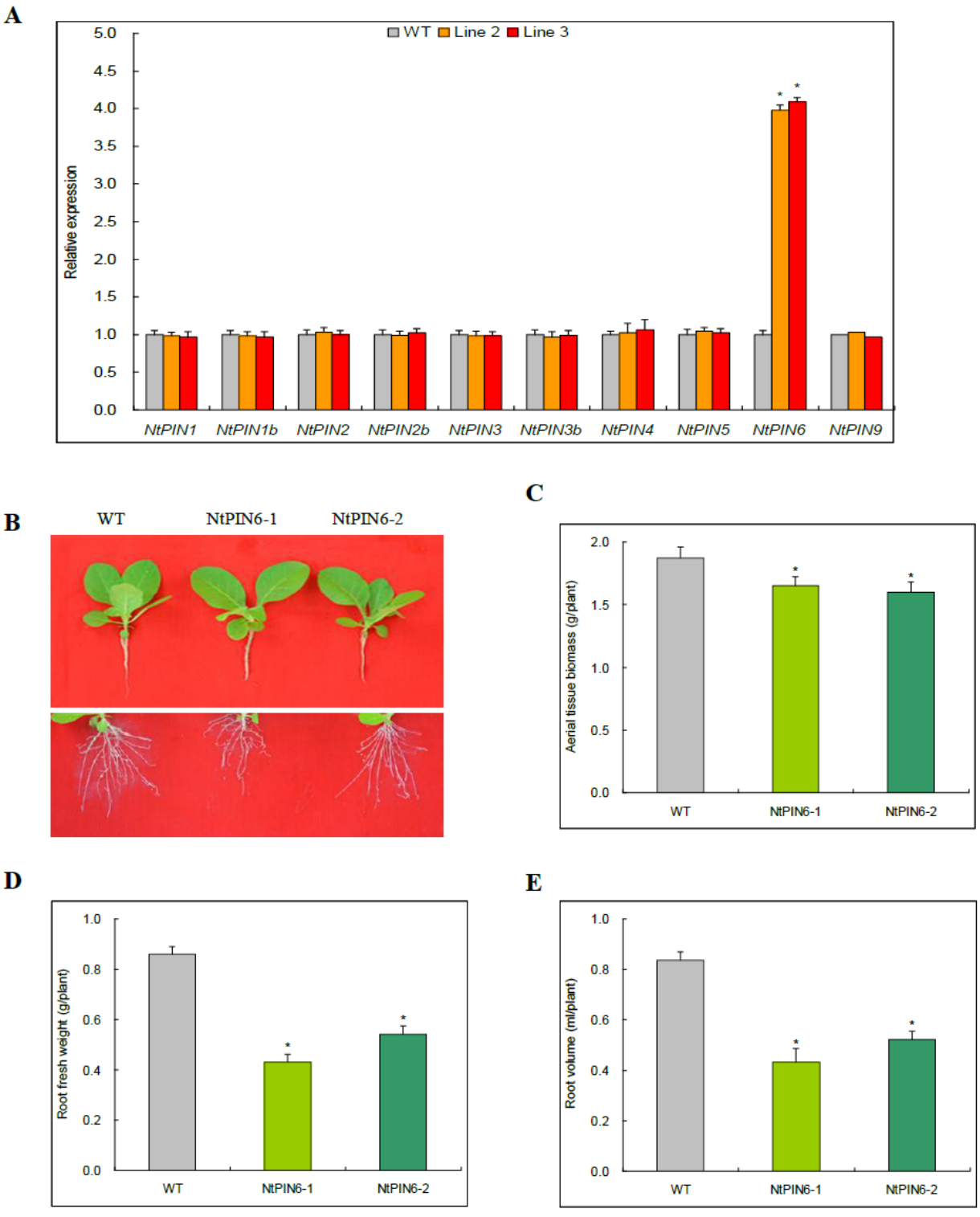

Figure 6

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.

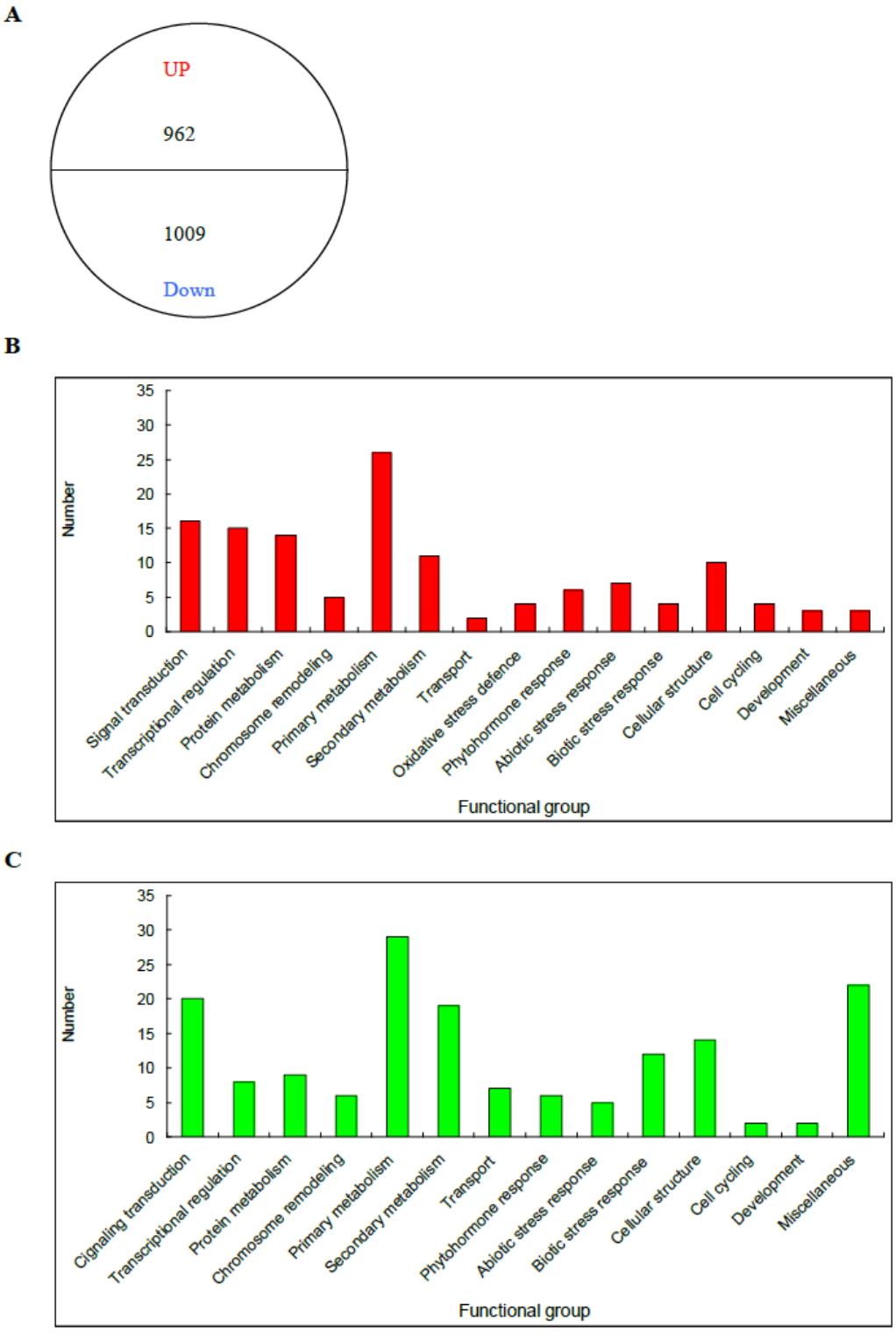

Figure 7

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.

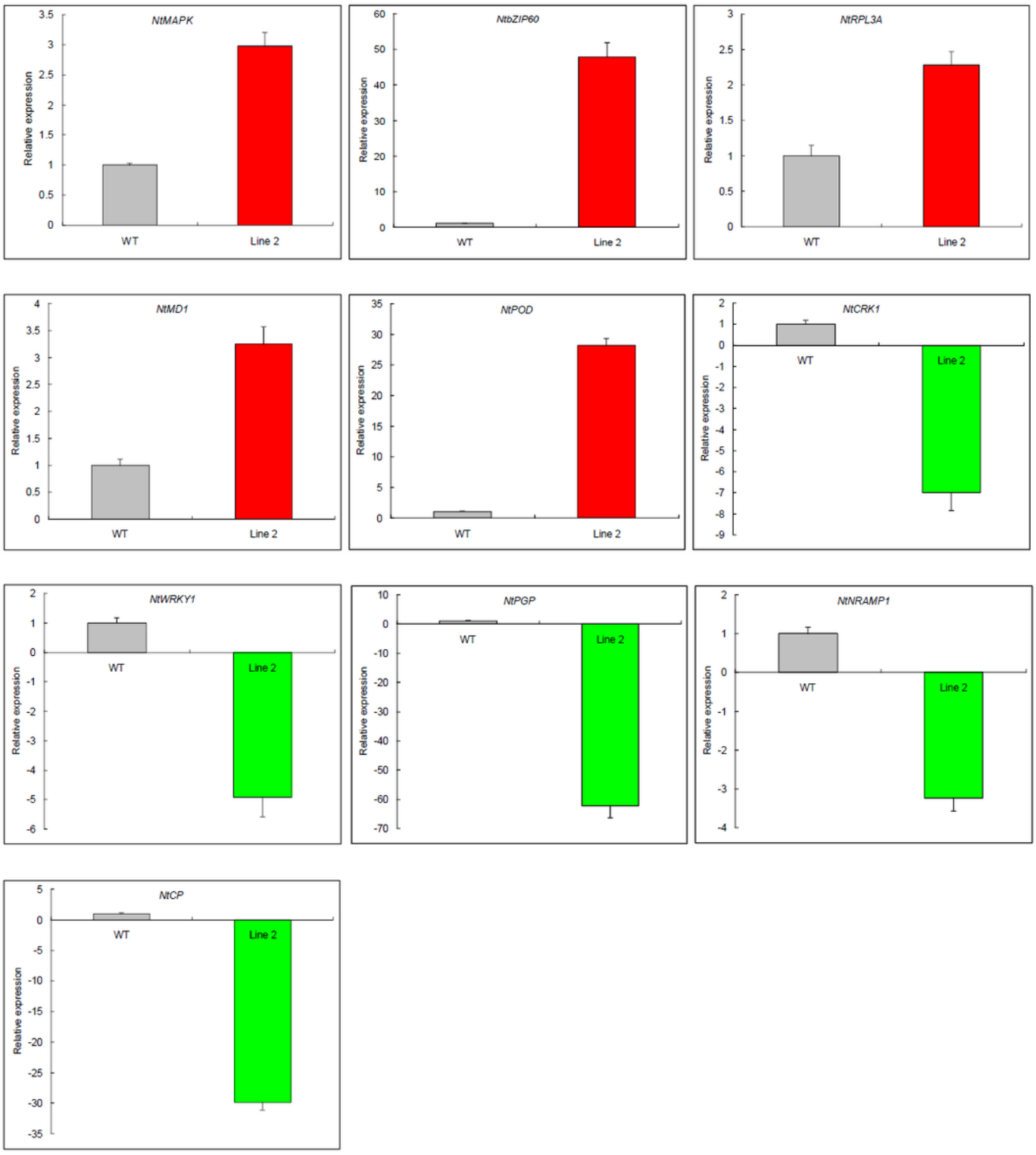

Figure 8

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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