

# Wheat microRNA Member TaMIR444a Is Nitrogen Deprivation-Responsive and Involves Plant Adaptation to the Nitrogen-Starvation Stress

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**Abstract** microRNAs (miRNAs) are involved in regulating various plant developmental processes and mediating plant-adaptive responses to nutrient deprivation. In this study, the characterization of a wheat miRNA member TaMIR444a and the role of this miRNA in mediating plant tolerance to the N-starvation stress were investigated. Results indicated that the expression levels of TaMIR444a and NtMIR444a, the homologue of TaMIR444a in tobacco, were upregulated in roots and leaves under N deprivation, whereas the transcription of their target genes showed reverse expression patterns in above tissues. These results suggest that miR444a is conserved across plant species of dicots and monocots and can possibly establish the miRNA/target modules for mediating plant response to N deficiency. Overexpression of TaMIR444a in tobacco improved the plant growth feature, biomass, N content, photosynthetic parameters, and antioxidant enzymatic activities under N deprivation. Based on microarray analyses, a large number of genes were identified to be differentially expressed in the TaMIR444a-overexpressing plants; these differential genes are categorized into functional groups of signal perception and transduction, transcription regulation, primary

and secondary metabolism, phytohormone response, cellular protection and defensive responsiveness, etc. qPCR analyses revealed that the nitrate transporter (NRT) genes *NtNRT1.1-s*, *NtNET1.1-t*, and *NtNRT2.1* and the antioxidant enzyme genes (AEEs) *NtCAT1;1*, *NtPOD1;3*, and *NtPOD4* were significantly upregulated by TaMIR444a, suggesting that the altered transcription of these NRT and AEE genes is associated with the improvement of the N acquisition and the cellular ROS detoxification in the N-deprived transgenic plants. Together, our findings demonstrate that miR444a acts as one critical regulator in mediating plant tolerance to the N-starvation stress through modulation of the regulatory networks associated with N acquisition, cellular ROS homeostasis, and carbon assimilation. Our findings have provided insights into the mechanisms of plant tolerance to N deficiency mediated by the distinct miRNA pathways.

**Keywords** Wheat (*Triticum aestivum* L.) · microRNA · N deprivation · Expression · Genetic transformation · Differential gene · Growth feature

Si Gao and Chengjin Guo contributed equally to this work.

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## Introduction

Nitrogen (N) is an essential macronutrient for plant growth and development and acts as one of the major factors in impacting crop productivity (Frink et al. 1999). Over the past several decades, N fertilizers applied in arable land have been dramatically increased worldwide aiming at acquiring high yields. However, the large input and the low use efficiency of the applied N fertilizers have been resulting in negative impacts on the environment except the increased cost (Ju et al. 2009). Improving N use efficiency (NUE) in crop production thus has been used as one of the important practices in sustainable agriculture (Hirel et al. 2007).

Plants have evolved multiple strategies to respond to the variation of N availability. At molecular level, based on genome-wide microarray analyses, a large quantity of genes functioning in diverse biological processes, including signal transduction, transcription regulation, primary and secondary metabolism, protein synthesis, stress response, cellular growth processes, and stress defense, have been identified to show altered transcripts under the N-limited conditions (Wang et al. 2003). Recently, a set of genes encoding the regulatory proteins and nitrate transporters (NRTs) has been demonstrated to be involved in plant responses to the varied external N conditions. For instance, *Arabidopsis Nitrate Regulated 1 (ANR1)* and *nodule inception-like protein 7 (NLP7)*, two transcription factor (TF) genes in *Arabidopsis*, and *ZmDOF1*, a DOF-type TF gene in maize, act as critical signaling components in sensing and transducing the external N levels (Yanagisawa et al. 2004; Castaings et al. 2009). The *Arabidopsis* NRT genes *AtNRT1.1* and *AtNRT2.1* play an important role in mediating the N signaling transduction and act as critical regulators in improving N assimilation and acquisition (Ho et al. 2009; Ho and Tsay 2010). These findings suggest that plants cope with the varied external N levels through modifying a variety of biological processes, which are mediated by genes with diverse biological roles (Stitt et al. 2002; Vidal and Gutiérrez 2008; Wang et al. 2013; Cai et al. 2013; Jin et al. 2015).

Acting as important regulatory molecules, microRNAs (miRNAs) involve various plant developmental processes, including flowering, leaf and root growth, embryo development, and auxin signaling (Sunkar and Zhu 2004; Jones-Rhoades et al. 2006; Mica et al. 2006; Wong et al. 2011; Asha et al. 2013). In addition, recent studies have revealed that miRNAs also regulate the plant responses to various nutrient deprivations (Jones-Rhoades and Bartel 2004; Fujii et al. 2005; Gifford et al. 2008; Pant et al. 2009). For example, the *Arabidopsis* miR169 is significantly downregulated by N deprivation and it then transcriptionally regulates the target genes *AtNRT1.1* and *AtNRT2.1* (Zhao et al. 2011). miR167 in *Arabidopsis* mediates the lateral root initiation and the plant growth under the N-deficient conditions, through targeting a set of N deprivation-defensive genes (Gifford et al. 2008). Several miRNAs in wheat, such as TaMIR1118, TaMIR1129, and TaMIR1136, respond to N deficiency and can possibly establish distinct miRNA/target modules to mediate the plant response to N deprivation (Zhao et al. 2015). Additionally, some miRNA members are also associated with the plant adaptation to other inorganic nutrients, of which *Arabidopsis* miR399 regulates plant phosphate homeostasis under Pi deprivation through posttranscriptional cleavage of transcripts of the ubiquitin-conjugating E2 enzyme (PHO2)-encoding gene (Fujii et al. 2005; Chiou et al. 2006; Bari et al. 2006). After response to external sulfide variations, miR395 transcriptionally regulates the ATP sulfurylase genes *AP51*, *AP53*, and *AP54*

and a low-affinity sulfur transporter gene *AtST*, establishing the miR395/target module to affect the plant sulfur assimilation (Jones-Rhoades and Bartel 2004). miR398 mediates the cellular copper homeostasis under copper deprivation via regulating a copper/zinc superoxide dismutase (SOD) gene (Yamasaki et al. 2007). These results collectively indicate that the miRNA/target pathways act as critical regulators for mediating the plant responses to external N and other inorganic nutrients.

Up to date, although a subset of N deprivation-responsive miRNAs has been characterized in plants, the mechanisms underlying the plant adaptation to N deprivation mediated by the miRNA/target modules are still largely to be determined. In this study, we reported the characterization of TaMIR444a and its function in mediating plant adaptation to the N-starvation stress. TaMIR444a was drastically upregulated by N deprivation and demonstrated to play critical roles in mediating plant tolerance to N deficiency through regulating diverse physiological and biochemical processes associated with N acquisition, carbon assimilation, and reactive oxygen species (ROS) detoxification.

## Materials and Methods

### Expression Analysis of TaMIR444a and Its Tobacco Homologue as well as Their Target Genes

Seedlings of wheat (cv. Shixin 828) and tobacco (cv. Wisconsin 38) were cultured hydroponically with MS solution as described by Sun et al. (2012). At the three-leaf expansion stage, the wheat and tobacco seedlings were subjected to the N-starvation treatments by transferring the seedlings into a modified MS solution with reduced N (20  $\mu$ mol/L N) from the normal N condition (MS, 6 mmol/L N). Roots and leaves of the wheat and tobacco were sampled at the time points of 0 h (before treatment), 12, 24, and 48 h, and 3, 7, and 14 d after the N-deficient treatment to evaluate the responsiveness of the miRNAs to the N-starvation stress. In addition, to address the issue of how these miRNA members respond to the N recovery treatment, an aliquot of wheat and tobacco seedling treated by 48 h of N deprivation was retransferred into MS solution for the N recovery treatments. Roots and leaves of the seedlings were sampled at 12, 24, and 48 h after recovery to assess the expression patterns of the miRNAs under the restored N condition. Total RNA in samples was extracted using TRIzol reagent (Invitrogen) and used to synthesize cDNAs using M-MLV transcriptase (TaKaRa) according to the manufacturer's instructions. The expression levels of TaMIR444a and NtMIR444a were detected based on semiquantitative RT-PCR and qPCR analyses performed as described by Liu et al. (2013), using the specific primers of TaMIR444a (Table S1). *Tatubulin* and *Nttubulin*, two constitutive genes in wheat and tobacco, respectively, were used as internal

references in the RT-PCR and qPCR analyses with specific primers (Table S1). Expression levels of these miRNAs in qPCR were quantified according to the  $2^{-\Delta\Delta CT}$  method (Guo et al. 2013).

The target genes of TaMIR444a and NtMIR444a were predicted by an online tool referred to as psRNATarget (<http://plantgrn.noble.org/psRNATarget/>). The expression levels of the target genes in roots and leaves under various external N conditions were assessed based on qPCR with specific primers (Table S1) performed as aforementioned for detection of the miRNA expression.

### Generation and Molecular Characterization Analysis of the Transgenic Plants

The TaMIR444a precursor sequence was amplified by RT-PCR and integrated into the binary vector pCAMBIA3301 at a position downstream of the CaMV35S promoter. Given that the genetic transformation of wheat is difficult currently, we transformed the expression cassette into tobacco (cv. Wisconsin 38) based on an *Agrobacterium*-mediated approach as described by Sun et al. (2012). To validate the insertion of TaMIR444a in the transgenic lines, PCR analysis was performed to amplify the transcripts of *bar*, a selection gene in expression cassette conferring resistance to phosphinothricin (PPT), with specific primers (Table S1). A small RNA blot analysis was performed to evaluate the expression levels of TaMIR444a in the transgenic T3 lines as described by Zhao et al. (2015), using the roots treated by 48 h of N deprivation as samples. The tobacco U6 RNA was used as an internal standard for normalization of the TaMIR444a transcripts. Southern blot analysis was performed to characterize the genome insertion pattern of TaMIR444a in transgenic lines of lines 3 and 5 following the procedure described by Li et al. (2011), using the *bar* gene labeled with alkaline phosphatase as probe.

### Analyses of the Growth Feature, Dry Mass, and N Concentration of the Transgenic Plants Under the N-Sufficient and N-Deficient Conditions

Lines 3 and 5, two T3 lines with stronger expression of TaMIR444a together with wild type (WT), were selected to determine the function of TaMIR444a in mediating the plant growth under N-sufficient and N-deficient conditions. The filter paper-based and the hydroponic culture approaches were used to assess the growth features of the transgenic lines and WT. To this end, the transgenic and WT seedlings were cultured onto filter papers soaked with MS solution (6 mmol/L N) and the modified MS solution with reduced N (60  $\mu$ mol/L) for 2 weeks or cultured in the above MS solutions for 5 weeks. After treatments, the growth features of the transgenic lines and WT were recorded by a digital camera. The dry mass of

the samples was obtained after drying for 48 h at 85 °C. The N concentration in roots and aboveground tissues was assayed as described by Guo et al. (2011). The N content in above tissues was calculated by multiplying the dry mass with N concentration.

### Measurement of Photosynthetic Parameters and Antioxidant Enzymatic Activities of the Transgenic Plants Under N-Sufficient and N-Deficient Conditions

The hydroponically cultured transgenic and WT plants treated by sufficient and deficient N were further subjected to measurement of the photosynthetic and ROS-associated parameters. The photosynthetic parameters assayed included photosynthetic rate ( $P_n$ ), PSII efficiency ( $\Phi_{PSII}$ ), and nonphotochemical quenching (NPQ), which were measured as described by Guo et al. (2013). The ROS-associated parameters evaluated included activities of SOD, catalase (CAT), and peroxidase (POD) and contents of malondialdehyde (MDA),  $H_2O_2$ , and superoxide anion, which were determined as described by Liang et al. (2003).

### Transcriptome Analysis of the Transgenic and WT Plants Under N Deprivation

Total RNA in root tips of the transgenic (lines 3 and 5) and WT seedlings after 48 h of the N-deficient treatment was extracted using TRIzol Reagent (Invitrogen) then cleaned and concentrated with RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA). Seven hundred nanograms of cleaned total RNA was then used to synthesize the Cy3-labeled cDNAs (Agilent's RNA Spike-in Kit) and then subjected to hybridization analysis using an Agilent's 4 $\times$ 44K tobacco chip following manufacturer's suggestions. After hybridization, the chips were washed using the ready-made solutions and treated with Agilent's Stabilization and Drying solutions (product number 5190-0423). The signals on chips were scanned (Agilent Technologies Scanner, model G2565CA) and generated the numeric data using the software (Agilent Feature Extraction software, version 10.5.1.1). The tool of R Project for Statistical Computing was used to analyze the raw numerical data set. Three biological replicates were conducted for each sample to yield reproducible results. An associative analysis approach was used to define the differential genes as described by Dozmorov and Centola (2003), in which a false discovery rate (FDR) cutoff of 0.01 was used in defining the differential genes, whose transcripts showed absolute values over 2.0 on fold change between the transgenic and WT plants. Gene biological functions were specified according to roles in GO biological processes of their homologs in *Arabidopsis*.

### Expression Analysis of the Randomly Selected Differential Genes in the Microarray Analysis

Twenty-two of differential genes identified in the microarray analyses, including 12 upregulated and 10 downregulated, were subjected to expression analysis based on qPCR to verify the microarray results. The qPCR was performed as aforementioned using the cDNAs in microarray analysis as templates and specific primer (Table S2).

### Expression Analysis of AEE and NRT Genes of the Transgenic and WT Plants

Five, 6, and 11 genes encoding SOD, CAT, and POD, respectively, together with 6 genes encoding NRTs in tobacco, were subjected to detection of transcripts to determine whether any of them are involved in improving the antioxidant enzymatic activities and the N acquisition mediated by TaMIR444a. The SOD genes analyzed included *NtMnSOD1* (X14482), *NtSOD1* (KJ874395), *NtFeSOD* (KF724056), *NtSOD2* (EU123521), and *NtMnSOD2* (AB093097); the CAT genes examined included *NtCAT* (EF532799), *NtCAT1* (NTU07627), *NtCAT1;1* (NTU93244), *NtCAT1;2* (HF564632), *NtCAT1;3* (HF564631), and *NtCAT3* (HF564633); and the POD genes evaluated included *NtPOD1;1* (L02124), *NtPOD1;2* (AB044154), *NtPOD1;3* (AB044153), *NtPOD1;4* (D11396), *NtPOD1;5* (AB178953), *NtPOD1;6* (AB027753), *NtPOD1;7* (AB027752), *NtPOD2;1* (AB178954), *NtPOD2;2* (KF701483), *NtPOD4* (AY032675), and *NtPOD9* (AY032674). The NRT genes examined included *NtNRT1.1-s* (AB102805), *NtNRT1.1-t* (AB102806), *NtNRT1.2-s* (AB102807), *NtNRT1.2-t* (AB102808), *NtNRT2.1* (AJ557583), and *NtNRT2.2* (AJ557584). qPCR analyses were performed as mentioned above to assess the transcripts of these genes in transgenic (lines 3 and 5) and WT seedlings treated by 48 h of N deprivation, with specific primers (Table S3).

### Statistical analysis

Averages of gene expression levels in qPCR analysis, plant dry mass, concentrations and contents of N, photosynthetic parameters, antioxidant enzymatic activities, and MDA contents were derived from the results of four replicates. Standard errors of the averages and the significant differences among averages were analyzed by using the Statistical Analysis System software (SAS Corporation, Cory, NC, USA).

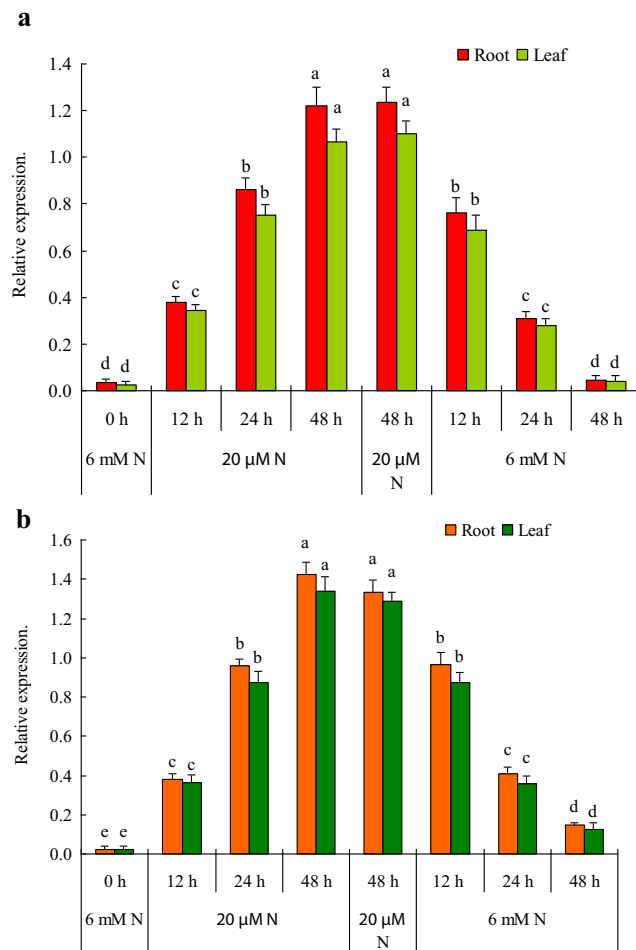
## Results

### TaMIR444a and NtMIR444a Are Induced Whereas the Target Genes Are Downregulated by N Deprivation

TaMIR444a and NtMIR444a share an identical precursor sequence based on PCR analysis, although the latter has not been released in the miRNA database (Release 21, [www.mirbase.org](http://www.mirbase.org)). The precursor sequences of TaMIR444a and NtMIR444a are shown in Fig. S1. This finding indicates that miR444a acts as a conserved miRNA member across plant species of dicots and monocots.

TaMIR444a and NtMIR444a showed similar expression patterns under various external N conditions. Under N sufficiency, they exhibited low expression levels in roots and leaves. Upon exposure to the N-starvation stress, the transcripts of these miRNAs were significantly induced in roots and leaves, showing a pattern to be gradually increased over a 48-h treatment (Fig. 1a, b; Fig. S2). Once treated by a N recovery treatment, the transcripts of the miRNAs in roots and leaves were swiftly downregulated and restored gradually to the prestimulus level after a 48-h treatment (Fig. 1a, b; Fig. S2). In addition, the miRNA members kept high expression levels under longer N starvation conditions (3, 7, and 14 d), which were comparable to those treated by 48 h of N deprivation (Fig. S3). This finding suggests that miR444a is transcriptionally upregulated by N deprivation with an expression pattern of typical concentration- and temporal-dependent manner.

TaMIR444a was predicted to target eight genes including those encoding MIKC-type MADS-box TF WM32B (*TaWM32*, TC368997), maturase K (*TaMK*, TC410100), MGC80202 protein (*TaMGC*, CV775588), expressed protein (unknown gene 1, TC450645), MIKC-type MADS-box TF WM30 (*TaWM30*, TC370370), one encoding unannotated protein (unknown gene 2, CA648881), MIKC-type MADS-box TF (*TaMADS*, TC424265), and GRAS family TF (*TaGRAS*, CA624957); NtMIR444a was shown to target four genes that encode DSK2 (*NtDSK2*, TC133521), elongation factor 1 (*NtEF1*, AM833250), pyridoxal kinase (*NtPK*, AM819983), and olfactory receptor (*NtOR*, BP131636), respectively (Table 1). A subset of target genes of the miRNAs encodes TFs categorized into MIKC-types and GRAS-type, kinase, and receptor, suggesting the involvement of miR444a in mediating biological processes via modulation of the target genes encoding signaling components. The expression patterns of the target genes exhibited to be contrasting to the miRNAs; namely, they were downregulated by N deprivation, and the reduced expression of them under the N-starvation stress was restored by the N recovery treatment (Fig. 2a–d). Therefore, the responses of these target genes to external N levels are mediated by miR444a possibly through



**Fig. 1** Expression patterns of TaMIR444 and NtMIR444a in roots and leaves of wheat and tobacco under the N-sufficient and N-deficient conditions **a** qPCR results of TaMIR444a; **b** qPCR results of NtMIR444a. In **a** and **b**, the expression level is listed in relative value to show a ratio on transcript abundance of TaMIR444a or NtMIR444a to the internal standard tubulin. Data derived from three biological replicates were used for significant analysis followed by Student's *t* test. Error bars indicate SE, and different lowercase letters stand for to be significant ( $P < 0.05$ )

the mechanism of posttranscriptional cleavage. As an exception, a gene encoding an unknown protein (TC450645) targeted by TaMIR444a and a gene encoding an elongation factor (*NtEF1*, AM833250) targeted by NtMIR444a kept stable transcripts under various N treatments, suggesting that these two targets are regulated by miR444a possibly via the mechanism of translation repression.

### Overexpression of TaMIR444a in Tobacco Improves Plant Growth and Dry Mass Production Under N Deprivation

An expression cassette harboring the TaMIR444a precursor sequence was constructed, whose schematic diagram is shown in Fig. 3a. Seven independent transgenic tobacco lines (lines 1 to 7) were generated. The TaMIR444a insertion was detected

in the transgenic T3 lines by PCR analysis. Results showed that the selection gene *bar* was detected in all of the transgenic lines rather than in WT (Fig. 3b), indicating the integration of TaMIR444a into genome of the transgenic lines. Small RNA blot analysis revealed that TaMIR444a expressed in most of transgenic lines (Fig. S4a).

Lines 3 and 5, two transgenic lines with stronger expression of TaMIR444a and different insertion pattern in genome (Fig. S4a and S4b), and WT were subjected to evaluation of the growth feature and dry mass under N sufficiency (6 mM/L N) and N deficiency (60 μmol/L). Under the N-sufficient condition, the transgenic lines showed similar phenotype and dry mass to WT. Under the N-deficient condition, however, the transgenic lines exhibited improved growth feature and dry mass compared with WT (Figs. 4a–d). These results indicate that TaMIR444a acts as a critical regulator in mediating the adaptation to the N-starvation stress.

### Overexpression of TaMIR444a Increases Plant N Concentration, N Content, and Photosynthetic and ROS-associated Parameters Under N Deprivation

Similar to the behaviors of phenotype and dry mass, lines 3 and 5 exhibited similar N concentration and N content in roots and aboveground tissues to WT under the N-sufficient condition (Fig. 5a, b); they displayed higher N concentration and more N content in above tissues than WT under the N-deficient condition (Fig. 5a, b). The increased N concentration and N content in transgenic plants under N deprivation indicated that these traits contribute to the TaMIR444a-improved plant growth and dry mass production through improvement of the N acquisition process.

The photosynthetic parameters such as  $P_n$ ,  $\Phi_{PSII}$ , and NPQ impact largely on plant dry mass accumulation via regulating carbon assimilation capacity, in which  $\Phi_{PSII}$  reflects the actual potential efficiency of the excitation energy captured by PSII centers and is calculated by  $(F'_m - F_s)/F'_m$  ( $F'_m$  and  $F_s$  define the maximum fluorescence in light and actual fluorescence level, respectively); NPQ shows the level of fluorescence quenching due to the slowly relaxing high-energy status of the thylakoids, i.e., directly proportional to the rate constant for the energy dissipation, and is defined by  $(F_m - F'_m) - 1$  ( $F_m$  means the maximum chlorophyll fluorescence). In this study, investigation on the photosynthetic parameters revealed that the transgenic plants also showed comparable photosynthetic parameters, such as  $P_n$ ,  $\Phi_{PSII}$ , and NPQ (Fig. 5c–e), antioxidant enzymatic activities of SOD, CAT, and POD (Fig. 6a–c), content of MDA, and amount of  $H_2O_2$  and superoxide anion, with WT plants under the N-sufficient condition (Fig. 6d–f). Under the N-deficient condition, the transgenic plants exhibited higher  $P_n$  and  $\Phi_{PSII}$  and lower NPQ than WT (Fig. 5c–e), higher activities of SOD, CAT, and POD (Fig. 6a–c), lower content of MDA, and less amount of  $H_2O_2$  and superoxide anion than WT plants

**Table 1** Information of the target genes interacted by TaMIR444 and NtMIR444a

Plant species	Gene name	Target accession No.	Alignment between MIR444a and target	Target interacting region	Biological function
Wheat	<i>TaWM32</i>	TC368997	CGUCGUUCGAACUCCGUCGUU :::~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~ GCAGCAAGCUUGAGGCAGCAA	376-396	MIKC-type MADS-box transcription factor WM32B
	<i>TaMK</i>	TC410100	GUCGUUCGAACUCCGUCGUU .:~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~ UAGCUAACUUGAGGCAGCAA	469-488	Maturase K
	<i>TaMGC</i>	CV775588	CGUCGUUCGAACUCCGUCGUU .:~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~ GCAGAAAGCUUCGGCAGUAA	463-483	MGC80202 protein
	unknown gene 1	CA648881	GUCGUUCGAACUCCGUCGUU .:~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~ UGGCAAGUUUGGGCGGUAA	334-353	Annotation not available
	<i>TaWM30</i>	TC370370	CGUCGUUCGAACUCCGUCGUU .:~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~ GCAGCAAGCUUGAGACAACAA	401-421	MIKC-type MADS-box transcription factor WM30
	unknown gene 2	TC450645	CGUCGUUCGAACUCCGUCGUU .:~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~ GCAGCAAGCUUGAAGGCAGCAU	299-319	Expressed protein
	<i>TaMADS</i>	TC424265	CGUCGUUCGAACUCCGUCGUU .:~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~ GCAGCAAGCUUGAGACAACAA	371-391	MIKC-type MADS-box transcription factor
	<i>TaGARS</i>	CA624957	GUCGUUCGAACUCCGUCGUU .:~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~ GAGCAGGCUUGAAGCAGCAG	226-245	GRAS family transcription factor
Tobacco	<i>NtDSK2</i>	TC133521	GUCGUUCGAACUCCGUCGUU .:~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~ CAGAAUGCUUGAGGCAGCAG	1343-1362	DSK2
	<i>NtEF1</i>	AM833250	CGUCGUUCGAAC-UCCGUCGUU .:~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~ GCAGCAAGCUUCUAGGCAGCAA	320-341	Elongation factor 1
	<i>NtPK</i>	AM819983	GUCGUUCGAACUCCGUCGUU .:~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~ CUGUUAAGCUUGAGACAGCAA	191-210	Pyridoxal kinase
	<i>NtOR</i>	BP131636	GUCGUUCGAACUCCGUCGUU .:~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~~::~ CUGCAAGCUUCAGGUAGCAG	89-108	Olfactory receptor

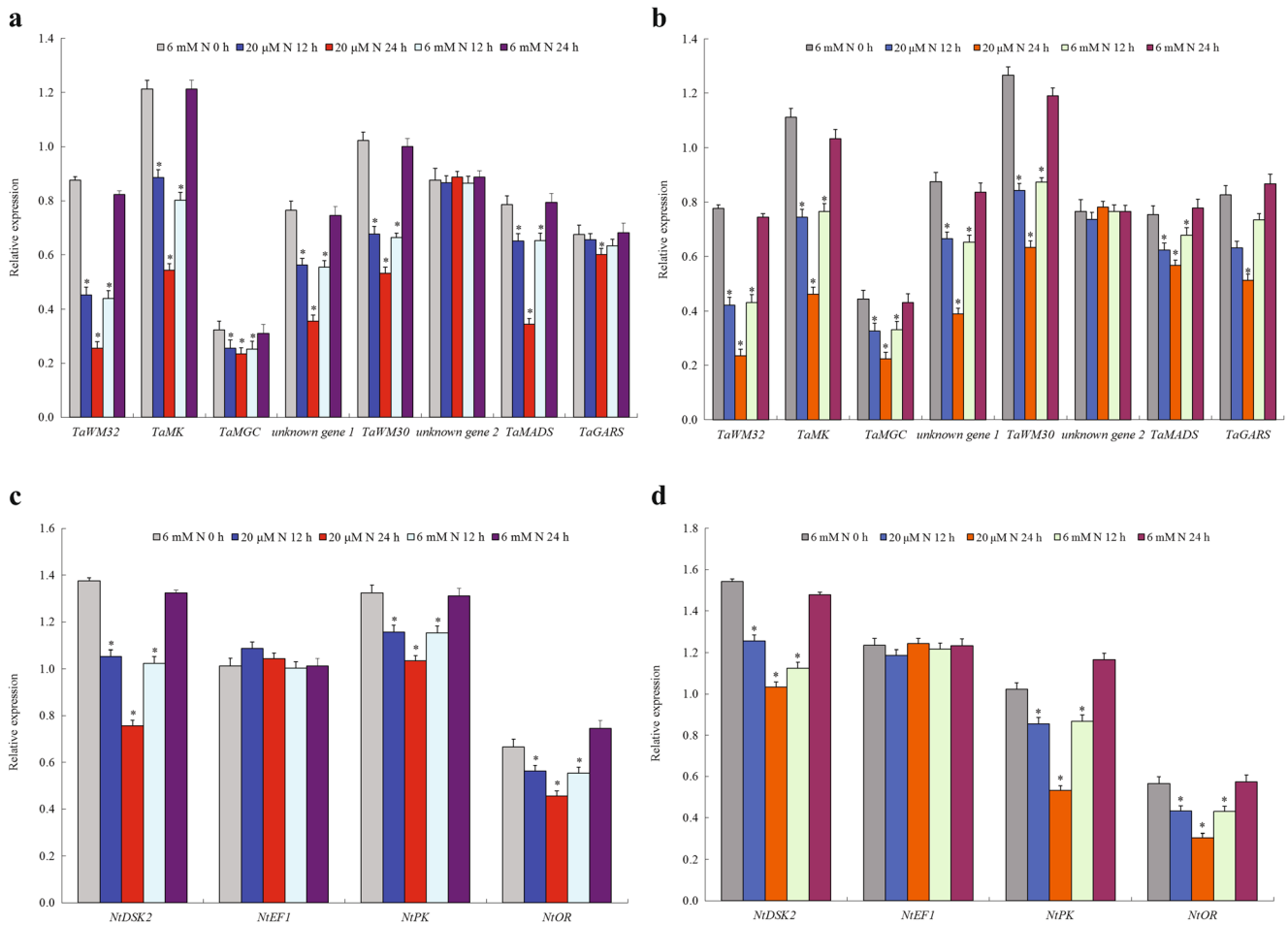
(Fig. 6d–f). These results suggested that the TaMIR444a-mediated tolerance to N deprivation is closely associated with the improvement of cellular ROS detoxification that further impacts on N acquisition, photosynthesis behavior, growth feature, and dry mass production of the plants under the N-starvation stress.

### Overexpression of TaMIR444a Modifies Largely the Transcriptome of Plants Under N Deprivation

Based on microarray analyses, totally 1733 genes were identified to be differential in the TaMIR444a-overexpressing plants treated by N deprivation, in which 921 were upregulated and

812 were downregulated (Fig. 7a, Tables S4 and S5). qPCR analysis revealed that 22 of the randomly selected differential genes, including 12 upregulated and 10 downregulated, showed comparable expression patterns to those evaluated by microarray analyses (Fig. S5). These results indicate that the differential genes identified in microarray analyses are reproducible. A large number of differential genes were identified in transgenic plants, suggesting that TaMIR444a regulates the transcription of the target genes, whose altered expression can further transcriptionally regulate the downstream genes underlying the TaMIR444a/target modules.

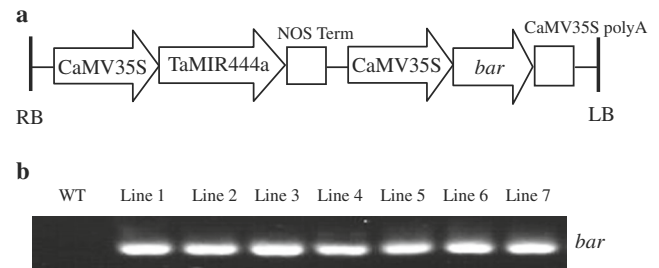
A small number of the differential genes identified in the microarray analyses, including 124 of upregulated and 119 of



**Fig. 2** Expression patterns of the target genes of TaMIR444a and NtMIR444a under varied external N levels **a** TaMIR444a in roots. **b** TaMIR444a in leaves. **c** NtMIR444a in roots. **d** NtMIR444a in leaves. In **a** and **b**, *TaWM32* (TC368997), *TaMK* (TC410100), *TaMGC* (CV775588), unknown gene 1 (A648881), *TaWM30* (TC370370), unknown gene 2 (TC450645), *TaMADS* (TC424265), and *TaGARS* (CA624957). In **c** and **d**, *NiDSK2* (TC133521), *NiEF1* (AM833250),

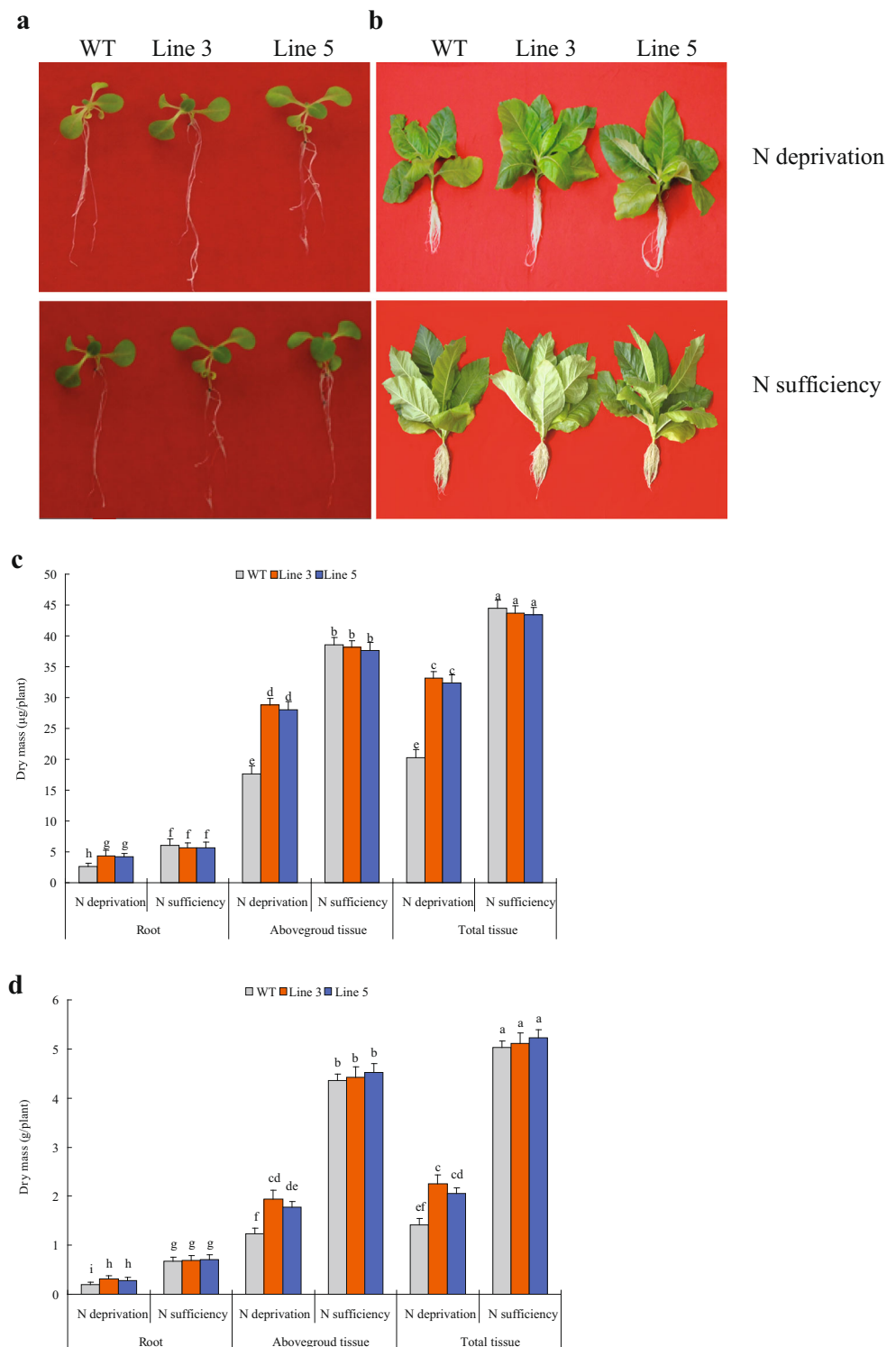
*NiPK* (AM819983), and *NiOR* (BP131636). The expression level is shown in relative transcript abundance of the target gene to the internal standard. Three biological replicates for each biological replicate were used for data analysis followed by Student’s t test (\*<0.05). Asterisks stand for to be significant compared with normal growth (6 mM N, 0 h). Constitutive expressed gene tubulin was used as the endogenous control. Error bars indicate SE

downregulated, have been annotated to biological roles (Tables S6 and S7). A large quantity of the differential genes is functionally unknown, which is possibly ascribed to the relatively slow progress on tobacco genome sequencing and the gene annotation programs. Based on the biological roles, the upregulated differential genes were grouped into the following categories: signaling perception (with numbers of 2), signal transduction (18), transcription (12), protein metabolism (8), chromosome remodeling (3), primary metabolism (21), secondary metabolism (11), transport (5), oxidative stress defensiveness (6), phytohormone response (1), abiotic stress response (7), biotic stress response (7), cellular structure (8), cell cycling (4), development (2), and miscellaneous (8) (Fig. 7b); the downregulated genes were grouped into the following categories: signaling transduction (with numbers of 16), transcription (11), protein metabolism (4), chromosome remodeling (2), primary metabolism (27), secondary



**Fig. 3** Molecular characterization of the transgenic plants overexpressing TaMIR444a **a** Schematic diagram of the binary expression cassette harboring TaMIR444a, in which TaMIR444a and a selecting gene bar are under the control of a CaMV35S promoter. The gene bar confers the plant PPT resistance. *LB* left border, *RB* right border. **b** PCR analysis of bar to confirm the TaMIR444a insertion in the transgenic plants. *WT* wild type; *line 1* to *line 7*, seven independent transgenic tobacco plant lines integrating the TaMIR444a precursor sequence

**Fig. 4** Growth feature and dry mass of the TaMIR444a-overexpressing and WT plants under N-sufficient and N-deficient conditions **a** Phenotype of the seedlings cultured onto filters soaked by N-deprived and N-sufficient solutions for 2 weeks. **b** Phenotype of the plants cultured hydroponically by N-deprived and N-sufficient solutions for 5 weeks. **c** Dry mass of the seedlings of **a**. **d** Dry mass of the plants of **b**. Nine representative seedlings or six plants of the transgenic lines and WT in each treatment were selected. *WT* wild type. *Line 3* and *line 5*, two transgenic lines with strong expression of TaMIR444. In **c** and **d**, error bars indicate SE and different lowercase letters stand for to be significant ( $P < 0.05$ )

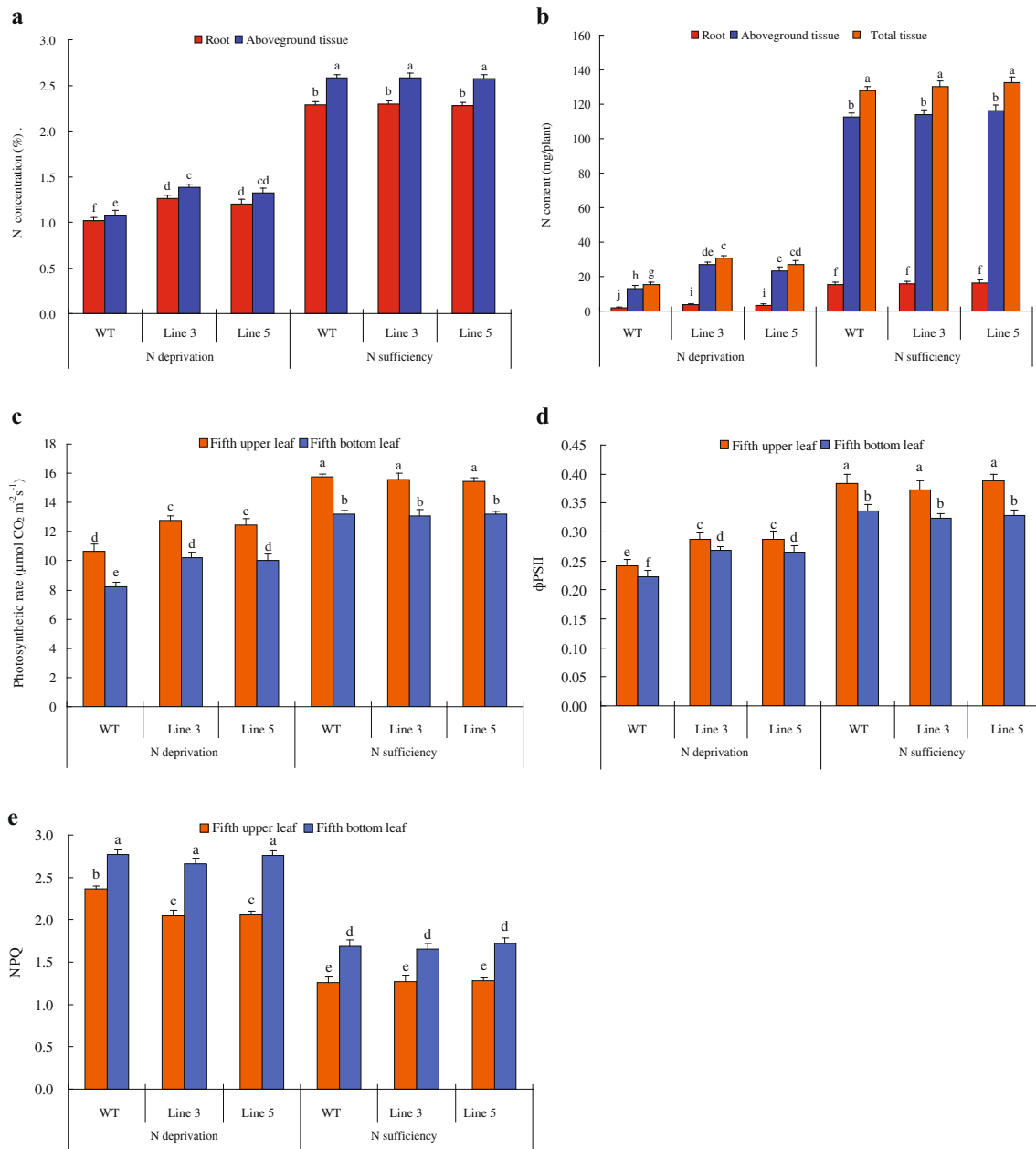


metabolism (16), transport (6), oxidative stress defense (2), phytohormone response (4), abiotic stress response (7), biotic stress response (9), cellular structure (5), cell cycling (1), development (1), and miscellaneous (7) (Fig. 7c). The upregulated and downregulated functional genes, the corresponding group category, probe set in tobacco chip, and expression

variation fold under contrasting N levels are listed in Tables S6 and S7.

Together, our investigation indicates that TaMIR444a drastically modifies the transcriptome. The differentially expressed genes mediated by TaMIR444a are possibly associated with the improved adaptation to N deprivation by





**Fig. 5** N concentration, N content, and photosynthetic parameters of the TaMIR444a-overexpressing and WT plants under N-sufficient and -deficient conditions 5 **a** N concentrations. **b** N contents. **c** Pn. **d** Fv/Fm. **e** NPQ. WT wild type. Line 3 and line 5, two lines of overexpressing

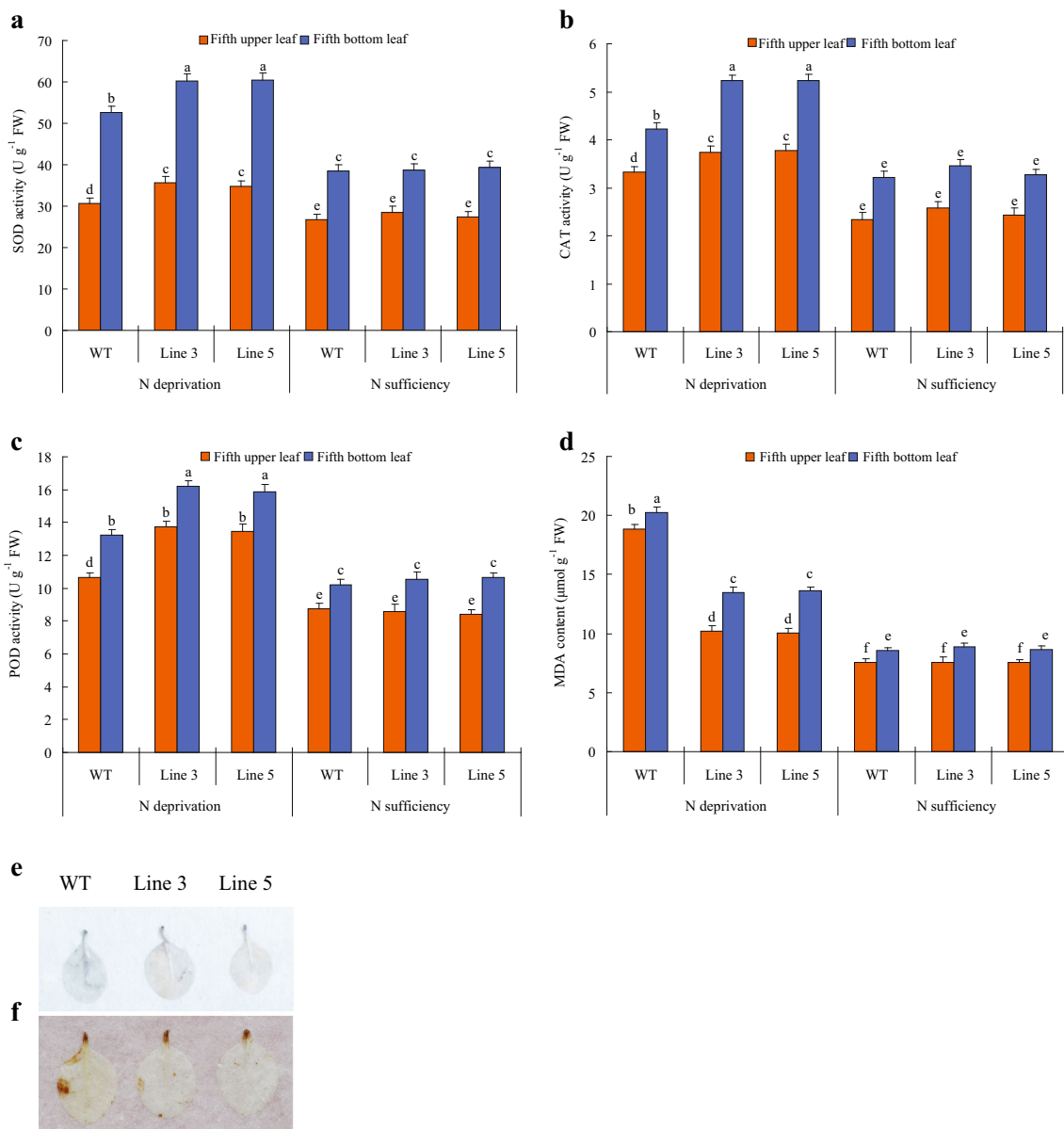
TaMIR444. The upper fifth and the bottom fifth leaves in six representative transgenic and WT plants in each treatment were selected to evaluate the parameters. Error bars indicate SE, and different lowercase letters stand for to be significant ( $P < 0.05$ )

regulating various biological processes involving N acquisition and internal translocation, ROS homeostasis, carbon assimilation, and dry mass production.

### A Set of AEE and NRT Genes Shows Varied Expressions in Transgenic Plants Under N Deprivation

The expression levels of antioxidant enzyme-encoding (AEE) genes and NRT genes were investigated in the N-deprived transgenic and WT plants. Compared with WT, most of the

AEE and NRT genes exhibited unchanged expression in the transgenic lines. However, *NtCAT1;1*, *NtPOD4*, and *NtPOD1;3* that encode antioxidant enzymes and *NtNRT1.1-s*, *NtNRT1.1-t*, and *NtNRT2.1* that encode NRTs displayed higher expression levels in the transgenic plants than in WT plants (Fig. 8a–c; Fig. 9), suggesting that these AEE and NRT genes are regulated by TaMIR444a and possibly contribute to the increased antioxidant enzymatic activities and N acquisition in the TaMIR444a-overexpressing plants under the N-deficient conditions.



**Fig. 6** Antioxidant enzymatic activities and histochemical staining of the reactive oxygen species in the TaMIR444-overexpressing and WT plants under N deprivation **a** SOD activities; **b** CAT activities; **c** POD activities; **d** MDA contents; **e** DAB histochemical staining to show the H<sub>2</sub>O<sub>2</sub> contents under the N-deficient condition; **f** NBT histochemical staining

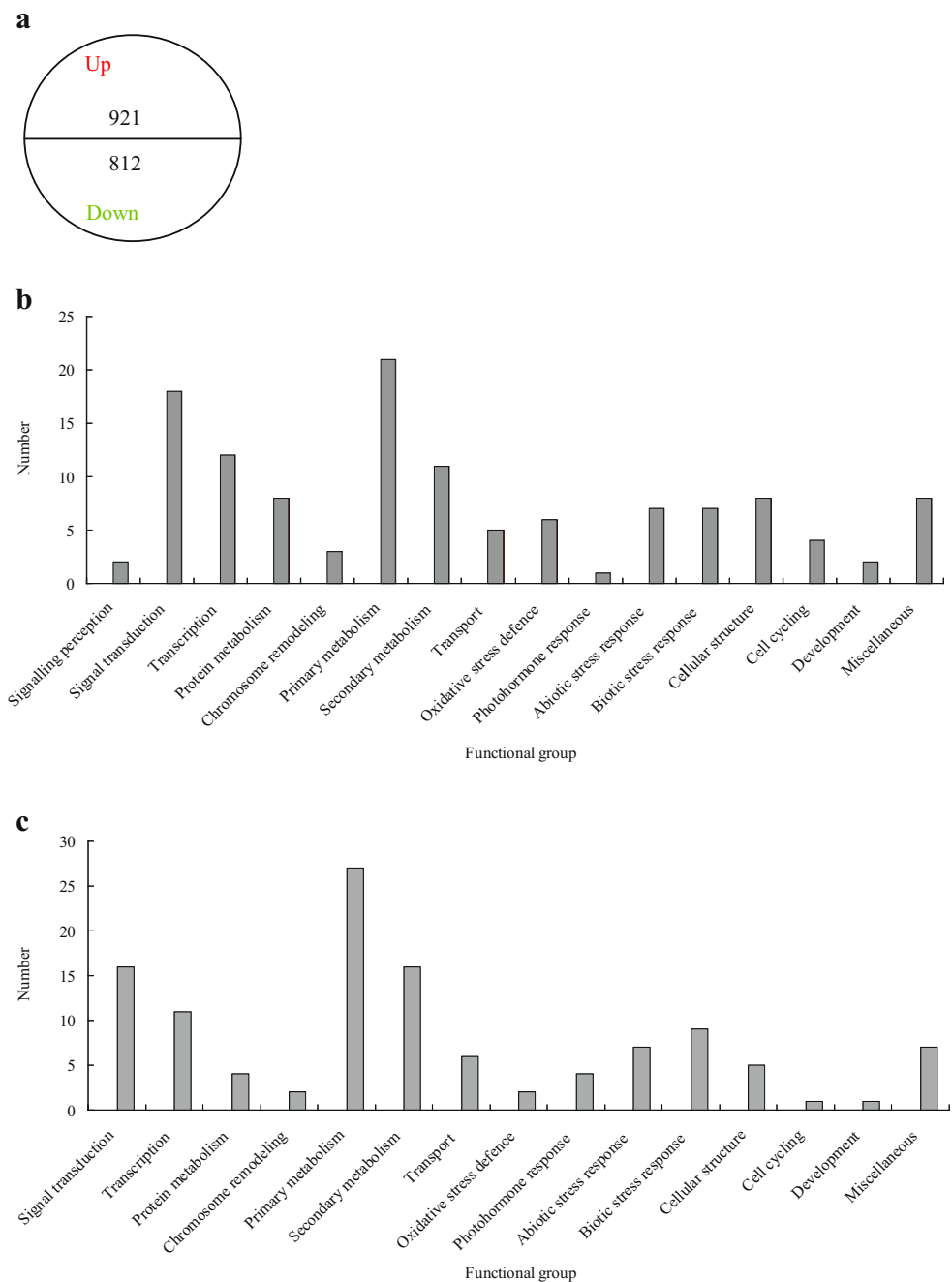
to show the superoxide anion contents. **a–d** The upper fifth and the bottom fifth leaves of six representative transgenic and WT plants in each treatment were selected to evaluate the parameters. In **a–d**, error bars indicate SE and different lowercase letters stand for to be significant ( $P < 0.05$ )

## Discussion

Through the mechanisms of posttranscriptional cleavage and translation repression, miRNAs in plant species are involved in the regulation of diverse plant biological processes, such as the development and phytohormone response (Jones-Rhoades et al. 2006) as well as the stress adaptations (Fujii et al. 2005; Jones-Rhoades et al. 2004, 2006; Sunkar et al. 2006; Zhao et al. 2013; Zhao et al. 2014; Zhao et al. 2015). In this study, characterization analysis of TaMIR444a and

NtMIR444a, the homologue of TaMIR444a in tobacco, revealed that they sensitively respond to the external N levels, whose transcript abundance was upregulated by N deprivation, and the induced expression levels under the N-starvation stress can be restored to a low level after N recovery treatments. In contrast to the miRNAs, most of the target genes exhibited an opposite expression pattern in responding to the external N conditions. These findings indicate that miR444a can establish the miRNA/target module for mediating plant responses to the N-starvation stress.

**Fig. 7** Functional classifications of the upregulated and downexpressed genes regulated by TaMIR444a **a** Venn diagram to show numbers of the up- and down-expressed genes regulated by TaMIR444a. **b** Functional groups of the upregulated genes and the corresponding number in each group. **c** Functional groups of the downregulated genes and the corresponding number in each group

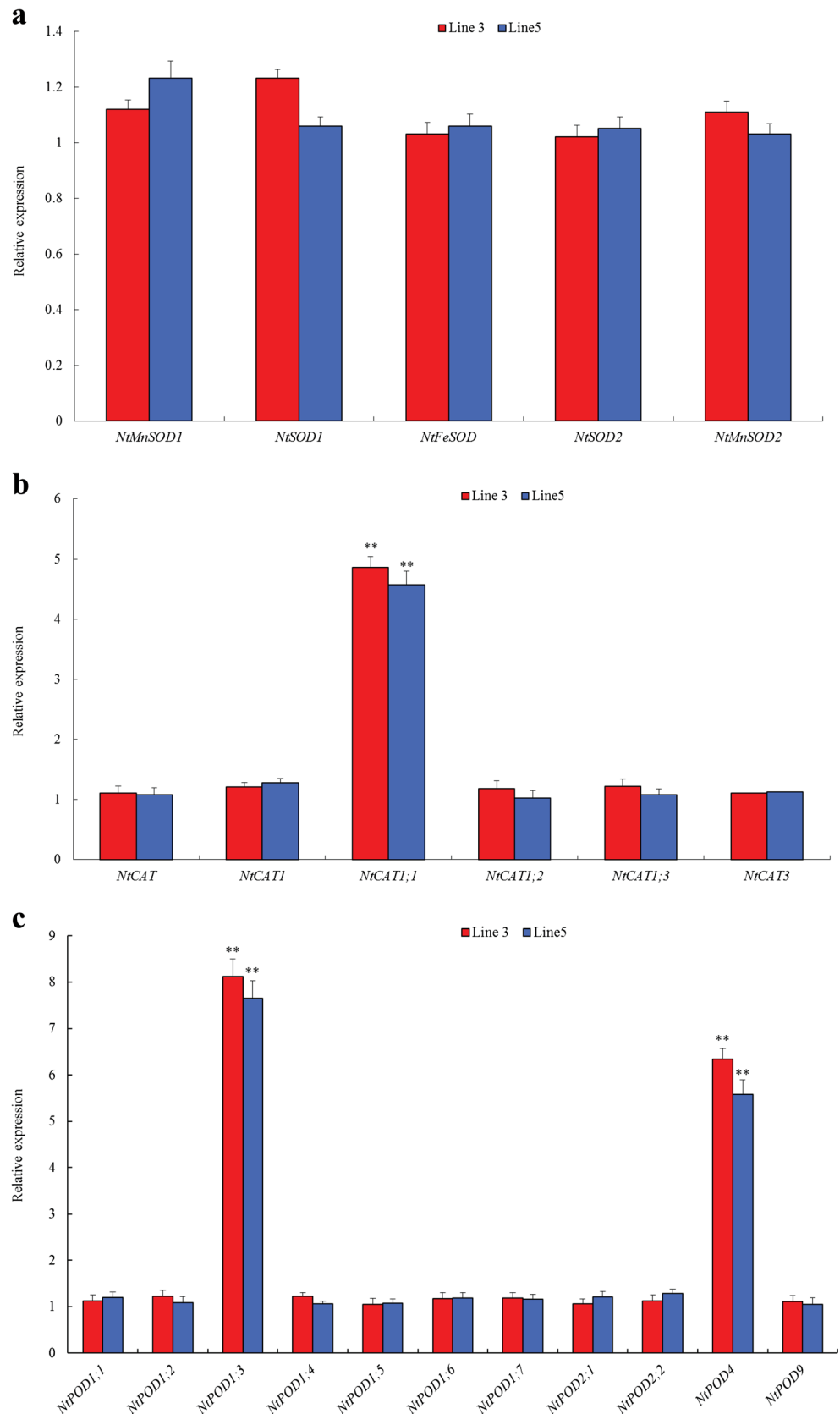


Several studies have reported that an array of plant miRNAs involves plant response to N deprivation. In *Arabidopsis*, miR169 mediates plant N acquisition under N deprivation through targeting two NRT genes, *AtNRT1.1* and *AtNRT2.1* (Zhao et al. 2011). miR167 regulates plant adaptation to N deficiency by modifying the lateral root initiation (Gifford et al. 2008). Several of wheat miRNAs respond to N deficiency and possibly establish distinct miRNA/target networks to mediate plant tolerance to the N-starvation stress (Zhao et al. 2015). In this study, overexpression of TaMIR444a in tobacco

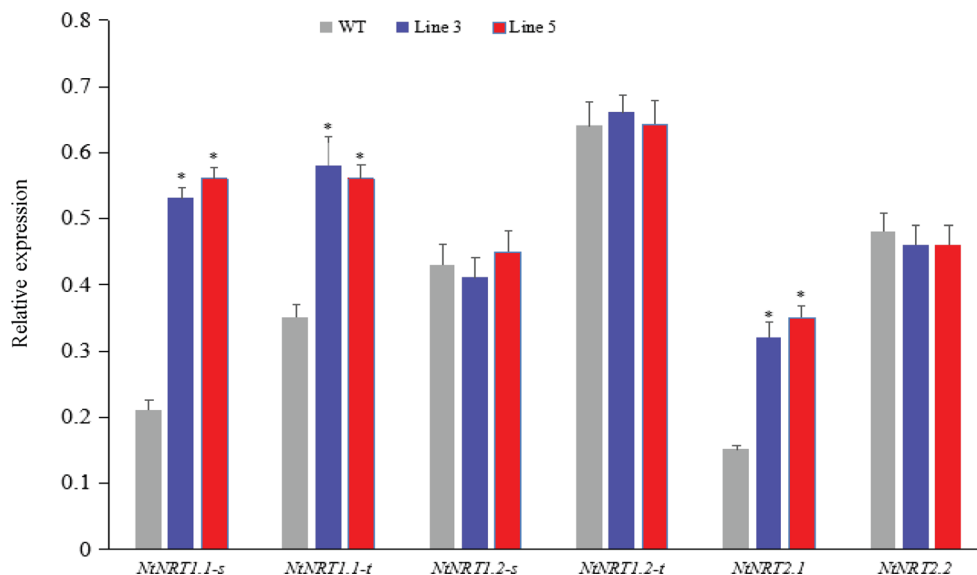
drastically improved a set of growth traits, including growth feature, dry mass production, N concentration and content, photosynthetic parameters, and antioxidant enzymatic activities under the N-deficient conditions. Therefore, TaMIR444a acts as one critical regulator in mediating plant tolerance to the N-starvation stress.

N taken up and internal translocation in plants are associated closely with NRTs (Crawford and Glass 1998). Two nitrate uptake systems evolved in plants, namely, the low-affinity transport and the high-affinity transport, function effectively in mediating plant  $\text{NO}_3^-$  acquisition from

**Fig. 8** Expression patterns of the genes encoding SOD, CAT, and POD in the N-deprived TaMIR444-overexpressing and WT plants under N deprivation **a** SOD genes. *NtMnSOD1* (X14482), *NtSOD1* (KJ874395), *NtFeSOD* (KF724056), *NtSOD2* (EU123521), and *NtMnSOD2* (AB093097). **b** CAT genes. *NtCAT* (EF532799), *NtCAT-1* (NTU07627), *NtCAT1;1* (NTU93244), *NtCAT1;2* (HF564632), *NtCAT1;3* (HF564631), and *NtCAT3* (HF564633). **c** POD genes. *NtPOD1;1* (L02124), *NtPOD1;2* (AB044154), *NtPOD1;3* (AB044153), *NtPOD1;4* (D11396), *NtPOD1;5* (AB178953), *NtPOD1;6* (AB027753), *NtPOD1;7* (AB027752), *NtPOD2;1* (AB178954), *NtPOD2;2* (KF701483), *NtPOD4* (AY032675), and *NtPOD9* (AY032674). The expression level is shown a relative transcript abundance of the antioxidant enzyme encoding gene to the internal standard. Error bars indicate SE, and double asterisks represent to be significant at 0.01 % level in comparison with WT



**Fig. 9** Expression patterns of the tobacco nitrate transporter (NRT) genes in the TaMIR444a-overexpressing and WT plants under N deprivation



soil and internal N translocation under various N conditions (Wang et al. 1998; Liu et al. 1999; Orsel et al. 2002; Li et al. 2007; Kiba et al. 2012). In this study, *NtNRT1.1;1*, *NtNRT1.1;2*, and *NtNRT2.1*, three tobacco NRT genes, were upregulated in the N-deprived plants overexpressing TaMIR444a. These results suggest that the NRT genes are possibly involved in improving the N acquisition in the transgenic plants through mediating N taken up. Previously, miR169a was shown to comprise a module with *AtNRT1.1* and *AtNRT2.1*, two NRT genes in *Arabidopsis* and involved in mediating the long-distance N signaling transduction under N deprivation (Zhao et al. 2011). The issue of how miR444a regulates the transcription of these differential NRT genes is needed to be further addressed.

In this study, the improved growth feature and dry mass production of the transgenic plants were closely consistent with the increased photosynthetic parameters, such as  $P_n$  and  $\Phi_{PSII}$ . Based on microarray analyses, a subset of differential genes associated with photosynthesis metabolism, such as those encoding FtsZ-like chloroplast protein (AJ133453), chloroplast pigment-binding protein CP29 (DQ676843), glucose-6-phosphate 1-dehydrogenase (DW003514), photosystem I psaH protein (X61665), sucrose-phosphate synthase isoform B (DQ213015), chloroplast oxygen-evolving protein 16-kDa subunit (TC126580), and chloroplast carbonic anhydrase (M94135), was identified (Tables S6 and S7). These findings provide a link between the TaMIR444a-mediated carbon assimilation and these differential photosynthesis-associated genes. Further investigation of these genes can shed light on the putative pathway covering miR444a, a carbon assimilation gene, and the photosynthesis behavior in plants under N deprivation.

Cellular ROS are induced under stress conditions including nutrient deficiency (Miller et al. 2008). High levels of antioxidant enzymatic activities can endow plants with improved resistance to the oxidative stress initiated by stress conditions and alleviate the ROS toxicity to plants (Shalata et al. 2001). In this study, overexpression of TaMIR444a endowed plants with enhanced activities of SOD, CAT, and POD and reduced amount of MDA,  $H_2O_2$ , and superoxide anion under N deprivation. These findings indicate that the TaMIR444a-mediated improvement of cellular ROS homeostasis possibly acts as a central regulator in regulating growth feature, photosynthetic parameters, and N acquisition of plants through detoxification of the induced ROS by N deprivation. Three AEE genes *NtCAT1;1*, *NtPOD4*, and *NtPOD1;3* are possibly involved in improving the antioxidant activities of the TaMIR444a-overexpressing plants through upregulated transcription. Functional analysis of the AEE genes can be helpful to elucidate the mechanism underlying the TaMIR444a-mediated antioxidant activities and these upregulated AEE genes.

Genechip analysis has been widely used in the investigation of the plant transcriptome in responding to internal and external cues (Wang et al. 2000, 2003). Based on a microarray analysis, totally 3008 genes were identified to be regulated by OsmiR156, a conserved miRNA member in rice (Xie et al. 2012). Overexpression of OsmiR156b in switchgrass significantly modified the expression of 2346 genes in plants, in which 1020 were upregulated and 1326 were downregulated (Fu et al. 2012). These findings indicate that distinct plant miRNA can dramatically modify the plant transcriptome, albeit that the miRNA member targets limited genes (Zhao et al. 2013, 2015). In this study, based on microarray analyses, we

identified 1733 genes to show differential expression in the TaMIR444a-overexpressing plants, including 921 up-regulated and 812 downregulated (Tables S4 and S5). These results indicate that miR444a can transcriptionally regulate a large number of genes, through it directly regulates the target genes as well as indirectly modulates the genes downstream of the miR444a/target pathways. Functional characterization of the key differential genes through gene overexpression or gene knockout technologies can provide new insights into the miR444a-mediated plant response to the N-starvation stress.

miRNA-mediated plant development and response to stress stimuli are closely associated with the target genes encoding TFs (Zhang and Forde 1998; Williams et al. 2005; Singh et al. 2002). miR159/319, miR160, miR167, and miR396 regulate plant development mainly by post-transcriptional regulation of the target genes encoding TF families of MYBs/TCPs, ARFs, and GRF (Reyes and Chua 2007; Mallory et al. 2005; Liu et al. 2007; Wu et al. 2006). In this study, based on microarray analyses, we identified 22 differential genes in the transgenic plants that encode TFs of MYB, bZIP, WRKY, and ZFP-type members, with 12 upregulated and 10 downregulated. Previously, an *Arabidopsis* MADS-box gene *ANR1* was identified to regulate plant response to the N availability through modulating lateral root development (Gan et al. 2005; Remans et al. 2006). The putative modules comprising miR444a and these differential TF genes are needed to be further defined.

In conclusion, stress-responsive miRNAs mediate plant response to abiotic stresses through reprogramming intricate physiological processes and biochemical metabolisms. In this study, we revealed that miR444a is N deprivation-responsive and acts as one critical regulator in mediating plant tolerance to the N-starvation stress. The microarray analyses revealed that TaMIR444a transcriptionally regulates a large number of genes, which are grouped into various functional categories, such as signaling perception and transduction, transcription regulation, protein metabolism, chromosome remodeling, primary and secondary metabolism, transport, oxidative stress defense, phytohormone response, abiotic and biotic stress responses, cellular structure and cycling, development, miscellaneous, etc. Our findings indicate that the TaMIR444a-mediated plant tolerance to N deprivation involves complicated gene networks that modify various physiological and biochemical processes associated with N acquisition, ROS detoxification, carbon assimilation, and plant dry mass accumulation. Further establishment of the regulatory networks covering miR444a and differential genes can provide novel insights into understanding the molecular mechanisms underlying plant response to the external N signaling mediated by miRNA/target pathways.

## Compliance with Ethical Standards

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

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