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ORIGINAL ARTICLE

# TaMIR1139: a wheat miRNA responsive to Pi-starvation, acts a critical mediator in modulating plant tolerance to Pi deprivation

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## Abstract

***Key message*** **Wheat miRNA member TaMIR1139 targets genes functional in various families and plays crucial roles in regulating plant Pi starvation tolerance.**

**Abstract** Through regulating target genes at posttranscriptional or translational level, plant miRNAs are involved in mediating diverse biological processes associated with growth, development, and responses to adverse stresses. In this study, we characterized the expression pattern and function of TaMIR1139, a miRNA member of wheat (*T. aestivum*) under Pi deprivation. TaMIR1139 precursor is also present in *N. tabucum*, suggesting the conserved nature of miR1139 across monocots and eudicots. TaMIR1139 targets seven genes within different families. The transcripts abundance of TaMIR1139 was induced upon Pi deprivation and the upregulated expression under Pi starvation was downregulated by the Pi recovery treatment, In contrast, the genes targeted by TaMIR1139 exhibited reduced transcripts upon Pi starvation and their downregulated expression was recovered by Pi-recovery condition, suggesting the regulation of them under TaMIR1139 through a cleavage mechanism. TaMIR1139 overexpression conferred the Pi-deprived plants improved phenotype, biomass, photosynthesis, and Pi acquisition. Transcriptome analysis identified numerous genes involving biological process, cellular components, and molecular function were differentially expressed in the TaMIR1139 overexpression lines, which suggests the TaMIR1139-mediated plant Pi starvation tolerance to be associated with the role of miRNA in extensively modulating the transcript profiling. A phosphate transporter (PT) gene *NtPT* showed significantly upregulated expression in TaMIR1139 overexpression lines; overexpression of it conferred plants improved Pi acquisition upon Pi starvation, suggesting its contribution to the TaMIR1139-mediated plant low-Pi stress resistance. Our investigation indicates that TaMIR1139 is critical in plant Pi starvation tolerance through transcriptionally regulating the target genes and modulating the Pi stress-defensiveness processes.

**Keywords** Wheat (*Triticum aestivum* L.) · MiRNA member · Pi deprivation · Expression · Plant growth · Photosynthetic traits · Trancriptome profiling · Pi acquisition

## Introduction

As one of the essential inorganic nutrients, phosphorus (P) is present in all organisms and plays an important role in plant growth and development (Raghothama 1999). Plants acquire P by the root system from growth media in the form of inorganic phosphate (Pi), whose concentration in the arable soils is frequently limited (Hinsinger 2001). It has been indicated that approximately 70% of the global crop field is suffered from phosphate deficiency (López-Arredondo et al. 2014). Therefore, application of phosphate fertilizer acts as an effective strategy in crop production, which provides adequate amount of available Pi for plant normal growth, development, and yield formation (Poirier and Bucher 2002).

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Zhipeng Liu and Xiaoying Wang contribute equally to this work.

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To cope with low-Pi stress, plants have evolved a variety of adaptive strategies, including enhancement of Pi uptake through modulating corresponding physiological and biochemical processes, such as alteration of root architecture, increase of Pi taken up activity, and secretion of organic acids and phosphatases (Raghothama 1999). These responsive processes are largely resulted from the transcriptional variation of a large set of Pi-responsive or -defensive genes (Raghothama 1999; Poirier and Bucher 2002; Yuan and Liu 2008). Additionally, phytohormones such as abscisic acid (ABA), ethylene, auxin, and cytokinin play synergistic roles in the regulation of Pi homeostasis when plants are challenged by Pi stress through modulating the Pi signaling-associated pathways (Hillwig et al. 2008; Devaiah et al. 2009; Lei et al. 2011).

MicroRNAs (miRNA) are a group of the non-coding small RNA family members, which play critical roles in mediating plant growth, development, and in regulating plant abiotic stress adaptation via modifying target genes at posttranscriptional or translational level (Voinnet 2009; Jones-Rhoades et al. 2006). During past decade, a suite of the miRNA family members that are involved in transducing nutrient signaling, including starvation of nitrogen (N) (Paul et al. 2015; Gao et al. 2016), Pi (Jones-Rhoades and Bartel 2006; Fujii et al. 2005; Chiou et al. 2006), sulfur (Jeong et al. 2011; Jagadeeswaran et al. 2010), and copper (Beauclair et al. 2010; Jin et al. 2015), has been documented. For example, in the model plants Arabidopsis (*A. thaliana*), miRA156, miR399, miR778, miR827, and miR2111 display induced transcripts abundance upon the Pi-starvation stress (Fujii et al. 2005; Hsieh et al. 2009; Pant et al. 2009). Of which, the action mode that miR399 mediates plant internal Pi homeostasis has been characterized in more detail; this miRNA member promotes Pi uptake and Pi internal transport via activating expression of two PT genes referred to as *Pht1;8* and *Pht1;9*, whose regulation is controlled by *PHO2* (*UBC24*, a ubiquitin-conjugating E2 enzyme gene), a target gene of miR399 (Fujii et al. 2005; Aung et al. 2006; Bari et al. 2006; Chiou et al. 2006; Pant et al. 2009). Target gene characterization analysis on miR827 and miR2111 indicates that these two miRNA members interact with the genes functional in different families, including those encoding Kelch repeat-containing F-box protein (Hsieh et al. 2009), primary metabolism-associated protein, and ubiquitin E3 ligase (Hsieh et al. 2009; Pant et al. 2009). These findings suggest that distinct miRNA family members contribute largely to the plant Pi starvation adaptation based on their roles in transcriptionally or translationally regulating target genes that are involved in diverse biological pathways.

Wheat is one of the important cereals and widely cultivated around the world. Recently, the miRNAs and isomiRs in cereals to be stress response have been investigated (Budak et al. 2015). Moreover, a suite of investigations on establishing wheat abiotic stress miRNome (Alptekin et al. 2017), dissecting miRNA members in wheat such as genome D, 4A, 5D and 1AL (Kantar et al. 2012; Lucas and Budak 2012; Kurtoglu et al. 2013; Sun et al. 2014; Akpinar and Budak 2016), and identifying wheat miRNA ancestors (Burcu and Budak 2017) and novel miRNAs using next-generation sequencing (Kurtoglu et al. 2014; Budak et al. 2014), has been performed. In addition, the miRNA members being responsible to distinct abiotic stresses such as those of Pi deprivation (Zhao et al. 2013), nitrogen starvation (Sinha et al. 2015; Zuluaga et al. 2017), drought (Kantar et al. 2010; Akpinar et al. 2015; Zhao et al. 2015; Bakhshi et al. 2017), and high salinity (Wang et al. 2014a, b), have been investigated in wheat and barley plants. Based on transgene analysis, several wheat miRNA members, including miR399 in mediating plant Pi-starvation adaptation (Ouyang et al. 2016) and TaMIR444a in regulating plant low-N stress tolerance (Gao et al. 2016), have been functionally characterized. However, given the intricate nature of plant abiotic stress responses at molecular level, the mechanisms underlying the miRNA-mediated plant growth, development, and abiotic stress response are still largely unknown, especially in wheat and other cereal species. In this study, we characterized the function of TaMIR1139, a wheat Pi starvation-inducible miRNA member (Zhao et al. 2013), in mediating the plant Pi deprivation adaptation. TaMIR1139 and the target genes are Pi starvation-responsiveness and this miRNA member contributes largely to plant growth, Pi acquisition, biomass production under the low-Pi stress. These finding suggest that TaMIR1139 is critical in regulating plant resistance to the Pi deprivation condition.

## Materials and methods

### Characterization of TaMIR1139

Previously, our expression analyses on a suite of wheat miRNA family members revealed that TaMIR1139 (accession number MI0006021 in the website www. mirbase.org) is low-Pi stress responsive (Zhao et al. 2013). To characterize whether this miRNA member is genetically conserved, the TaMIR1139 homolog of tobacco (*N. tabacum*), an endicot model plant species, was subjected to identification based on a reverse transcriptase-polymerase chain reaction (RT-PCR). With this aim, the tobacco (cv. Wisconsin 35) roots cultured in standard MS solution were used to extract total RNA using TRIzol reagent (Invitrogen). cDNA synthesis from the total RNA and RT-PCR analysis were conducted as previously described (Sun et al. 2012) using TaMIR1139 precursor specific primers (Table S1).

### Identification of the TaMIR1139 target genes

To characterize the target genes interacted with TaMIR1139, an online tool referred to as psRNATarget (Plant microRNA Potential Target Finder; http://plantgrn.noble.org/psRNATarget/) was run to scan against two of wheat cDNA databases using the mature TaMIR1139 sequence as query, one of which is *Triticum aestivum* (bread wheat), transcript, cDNA library, TGACv1 and another of which is *T. aestivum* (wheat), DFCI Gene Index (TAG), version 12, released 2010-04-18. The target gene functions were defined based on BLASTn search results in NCBI (https://www.ncbi.nlm.nih.gov/).

### Expression analysis of the miRNA members and target genes upon varied P input levels

qRT-PCR was performed to characterize the expression patterns of TaMIR1139, its tobacco homolog NtMIR1139, and the target genes upon varied Pi levels. With this aim, wheat (cv. Shiyou 20) and tobacco (cv. Wisconsin 35) seedlings were cultured under standard MS solution (sufficient-Pi, 1.2 mM Pi) for 2 weeks, then transferred separately in the modified MS solution with reduced Pi (0.012 mM) for 48 h for Pi starvation treatment, and followed for further 48 h for Pi recovery treatment initiated by transferring the 48 h Pi-deprived seedlings again in standard MS solution. At time points before Pi starvation (0 h), 6, 12, 24, and 48 h after Pi starvation, and 6, 12, 24, and 48 h after Pi recovery, root tissues in wheat were collected and subjected to transcripts abundance evaluation for TaMIR1139 and the target genes whereas those in tobacco were to expression assessment for NtMIR1139 based on qRT-PCR using specific primers for miRNA members and the target genes (Table S1). In brief, total RNA from roots was extracted by TRIzol reagents (Invitrogen, USA), then subjected to synthesis of first-strand cDNA with RT-AMV transcriptase (TaKaRa, Dalian, China) after RNase-free DNase (TaKaRA, Dalian, China) treatment for avoidance of genomic DNA contamination. qRT-PCR was performed in a total volume of 25 µL containing 12.5 µL of SYBR Premix ExTaqTM (TaKaRa, Dalian, China), 0.5 µL each of forward and reverse primers, 1 µL cDNA and 10.5 µL nuclease-free water. Transcripts of TaMIR1139, NtMIR1139 and the TaMIR1139 target genes were calculated based on $2^{-\Delta\Delta CT}$ method after normalization against wheat *Tatubulin* and tobacco *Nttubulin* with specific primers (Table S1).

### Characterization of the target cleavage nature

To validate the target cleavage nature under control of TaMIR1139, two target genes including *TaPK2* (serine/threonine-protein kinase encoding gene, GenBank accession number XM_020325918) and *TaTT* (tyrosine-specific transporter gene, GenBank accession number XM_020292847) were selected to subjected to analysis for RNA ligase-mediated 5′ rapid amplification of cDNA ends (RLM-RACE) as previously described (Song et al. 2012). To this end, total RNA derived from wheat (cv. Shiyou 20) root tissues cultured at normal growth (0 h), 48 h after Pi-starvation, and 48 h after Pi-recovery was polyadenylated at 37 °C for 60 min in a 50 µl reaction mixture [containing 5 µg of total RNA, 1 mM ATP, 2.5 mM $MgCl_2$, and 8 U poly (A) polymerase] (Ambion, USA). RLM-RACE was performed using the GeneRacer kit (Invitrogen) according to the manufacturer's suggestion.

### Generation of tobacco TaMIR1139 overexpression lines

The role of TaMIR1139 in mediating plant Pi-starvation adaption was investigated in the miRNA overexpression lines of tobacco, an eudicot model plant species containing identical of the TaMIR1139 precursor, due to whose convenient genetic transformation. To this end, RT-PCR was performed to amplify the TaMIR1139 precursor in wheat roots using specific primers (Table S1), then which was inserted into restriction sites *Nco*I/*Bst*EII in the binary vector pCAMBIA3301 under the control of CaMV35S promoter. The established expression cassette was further integrated into EHA105, an *A. tumefaciens* strain using a conventional heat shock approach. Transgenic tobacco lines with TaMIR1139 overexpression were generated following the procedure as described by Sun et al. (2012). Target miRNA expression levels in transgenic lines were evaluated based on qRT-PCR performed to be similar in assessing this miRNA expression pattern as aforementioned.

### Assays of growth features, biomass, photosynthesis traits, and P-associated traits

Sen 1 and Sen 2, two lines with much more TaMIR1139 transcripts (Fig. S1), were selected and subjected to the characterization of miRNA in mediating plant Pi starvation response. With this aim, evenly 7-day-old transgenic seedlings at T3 generation together with wild type (WT) were vertically cultured on agar MS media supplemented with sufficient-Pi (1.2 mM) or deficient-Pi (0.05 mM) as previously described (Ding et al. 2016). After 21 days of treatments, phenotypes of the transgenic and WT plants were recorded using a digital camera. To validate the results obtained from agar MS media culture, Sen 1 and Sen 2 as well as WT were also hydroponically cultured in MS solutions containing contrasting Pi levels for a longer duration. To this end, 10-day-old transgenic and WT seedling were cultured in standard MS solution (sufficient-Pi, 1.2 mM) or

modified MS solution containing lowered Pi (0.02 mM) for 6 weeks. The solutions for culture were regularly renewed during the treatments (twice per week) and air circulated using a mini pump. After treatments, phenotypes of the transgenic and WT plants were recorded using digital camera. Moreover, biomass of the transgenic and WT plants was obtained after drying the samples for 48 h at 85 °C. Photosynthetic parameters, including photosynthetic rate (Pn), PSII efficiency (ΦPSII), and nonphotochemical quenching (NPQ), were assayed in upper expanded leaves as described by Guo et al. (2013). Plant P concentrations were assessed following the approach reported by Guo et al. (2011) and P accumulative amounts were obtained by multiplying plant biomass and P concentrations.

## Characterization of expression profile specifically regulated by TaMIR1139 upon Pi starvation

The miRNA/target modules exert various biological functions via modulating gene transcription at global level (Xie et al. 2012; Fu et al. 2012). To define the systematic expression profiling mediated by TaMIR1139 in Pi starvation-challenged plants, high-throughput transcriptome sequencing analyses were performed in the transgenic line and WT after low-Pi stress. For this, Sen 1 seedlings and wild type were cultured in MS solution for 2 weeks and then subjected to Pi-starvation treatment (0.02 mM Pi) for another 1 week. Root tissues of the transgenic and WT seedlings were then collected for high-throughput sequencing analysis. Total RNA extraction, mRNAs isolation, and library construction for the transgenic WT samples were conducted by Huanuo, a biotechnology company in China (Beijing, China). Briefly, raw sequences were filtered by removal of the adapter sequences using SeqPrep (https://github.com/jstjohn/SeqPrep). Deteriorate quality bases at the end were trimmed using sickle (https://github.com/najoshi/sickle). Reads of less than 20 bp after processing were discarded. All clean reads were aligned to the reference sequences using Tophat (http://tophat.cbcb.umd.edu/) with default parameters. Transcript abundance was estimated using the fragments per kilobase of exon per million mapped reads (FPKM) values within a 95% confidence interval. Cuffdiff software (http://cufflinks.cbcb.umd.edu/) was used to calculate and analyze differentially expressed genes (DEGs). The *P* value denotes expression difference between two samples Pi-deprived TaMIR1139 overexpression line and Pi-deprived wild type, whereas the false discovery rate (FDR) determines the threshold of *P* value. FDR $\leq 0.05$ and log2 ratio $\geq 1$ were considered statistically significant (Thimm et al. 2004). To validate the results obtained in transcriptome analysis, six genes with significantly upregulated in expression in Pi-deprived TaMIR1139 overexpression line (Sen 1) were subjected to further expression evaluation based on qPCR. These genes included one encoding pyruvate kinase (*NtPK*, ID # LOC107760370 with GenBank accession no. XM_016578407.1), one encoding WD repeat-containing protein SCD1 (*NtSCD1*, ID#LOC107772888 with GenBank accession no. XM_016592362.1), one encoding WRKY transcription factor (*NtWRKY*, ID# LOC107780258 with GenBank accession no. XM_016600787.1), one encoding cyclin-dependent kinase (*NtCDK*, ID# LOC107817252 with GenBank accession no. XM_016643050.1), one encoding superoxide dismutase (*NtSOD*, ID# LOC107774639 with GenBank accession NO. XM_016594232.1), and one encoding zinc finger protein (*NtZFP*, ID# LOC107777165 with GenBank accession no. XM_016597150.1). Assessment of the transcripts abundance of these genes in Pi-deprived TaMIR1139 overexpression line (Sen 1) and wild type was performed by adopting similar approach using gene specific primers (Table S1) that defines the TaMIR1139 target genes as aforementioned.

## Expression pattern analysis of the tobacco phosphate transporter (PT) genes

Pi acquisition and internal Pi translocation in plants are mediated by phosphate transporters (PTs) (Mudge et al. 2002; Shin et al. 2004). To characterize whether TaMIR1139 modifies the PT genes transcriptionally that further contributes to the TaMIR1139-mediated plant Pi starvation response, totally six PT encoding genes of *N. tabacum* identified in the NCBI Genbank database, including *NtPT* (DI040486), and *NtPT1* to *NtPT5* (AB020061, AF156696, AB042950, AB042951 and AB042956), were subjected to transcripts abundance evaluation in the Pi-deprived transgenic and WT plants. Transcripts of these PT genes were evaluated by qRT-PCR using the approach to be similar in assessing the transcripts abundance of TaMIR1139 target genes as mentioned above with these PT gene specific primers (Table S1).

RT-PCR was performed to amplify the open reading frame (ORF) of *NtPT* using gene specific primers (Table S1), then which was inserted into *Nco*I/*Bst*EII restriction sites in the binary vector pCAMBIA3301 at position downstream the CaMV35S promoter. Integration of the expression cassette into *A. tumefacien* strain EHA105, transformation of *NtPT* into tobacco, and generation of transgenic lines with this PT gene overexpression were performed to be similar in establishing TaMIR1139 overexpression lines as described above. Line 2 and line 4, two lines sharing more *NtPT* transcripts at T3 generation based on qRT-PCR together with WT, were subjected to functional characterization of the PT gene in mediating Pi acquisition under contrasting Pi-supply conditions (sufficient-Pi, 1.2 mM and deficient-Pi, 0.02 mM) based an agar media culture. After 2 weeks of treatment,

phenotypes, biomass, and P-associated traits of plants were recorded or assessed to be similar as aforementioned.

### Statistics analysis

Averages of gene expression levels in qRT-PCR analysis, plant biomass, photosynthesis parameters, P concentrations and P accumulative amounts in transgenic lines and WT were derived from the results of four replicates. Differential genes identified from the line overexpressing TaMIR1139 were validated by triplicates. Standard errors of averages and significant differences were analyzed using Statistical Analysis System software (SAS Corporation, Cory, NC, USA).

## Results

### The eudicot species tobacco harbors wheat miR1139 homolog

The precursor sequence of TaMIR1139 is 216 nt in length, which harbors a 22 nt-long in length of mature sequence (5′-AGAGUAACAUACACUAGUAACA-3′). A secondary stem-loop structure initiated by the TaMIR1139 precursor is shown in Fig. S1. Based on RT-PCR analysis, the tobacco homolog of TaMIR1139 (designated as NtMIR1139 hereafter) was identified to be identical to TaMIR1139 (Fig. 1a). This result indicates that the miR1139 members are conserved across monocots and eudicots.

### TaMIR1139 targets seven genes that are classified into various functional categories

Online predication results revealed that TaMIR1139 interacts with seven genes. Among these putative target genes, five are derived from a cDNA database of wheat referred to as *T. aestivum* (bread wheat), transcript, including: (1) TRIAE_CS42_1BS_TGACv1_050305_AA0170400, a receptor-like serine/threonine-protein kinase encoding gene (*TaPK1*, XM_020329410); (2) TRIAE_CS42_4BL_TGACv1_321691_AA1064200, a serine/threonine-protein kinase encoding gene (*TaPK2*, XM_020325918); (3) TRIAE_CS42_1DL_TGACv1_061103_AA0185340, a cytokinin oxidase 2 (CKX2) encoding gene (*TaCKX2*, HM195293); (4) TRIAE_CS42_4BL_TGACv1_322074_AA1068310, a tyrosine-specific transport gene (*TaTT*, XM_020292847); and (5) TRIAE_CS42_6AL_TGACv1_475510_AA1536580, a 3-ketoacyl CoA synthetase encoding gene (*TaCS*, KU737579). Another two target genes were derived from an expressed sequence tag (EST) library of wheat referred to as DFCI Gene Index (TAG), version 12, including: (1) a glycine cleavage system H protein encoding gene (*TaGCS*, CJ636238), and (2) a functionally uncharacterized gene (TC449025). Interaction characterization between the mature sequence of TaMIR1139 with the target genes are shown in Fig. 1b. These results indicate that TaMIR1139 interacts with a set of genes that are categorized into different function families, associating with protein phosphorylation (*TaPK1* and *TaPK2*), cytokinin metabolism (*TaCKX2*), amino transportation (*TaTT*), protein metabolism (*TaGCS*), and secondary metabolism (*TaCS*). These target genes within various families exert distinct biological roles under the regulation of TaMIR1139.

### miRNA members and the target genes are responsive to the varied external Pi input levels

qRT-PCR was performed to understand the expression patterns of TaMIR1139, NtMIR1139, and the target genes upon varied external Pi conditions. As previously indicated (Zhao et al. 2013), the expression levels of TaMIR1139 was drastically induced upon the Pi-starvation stress, showing a pattern to be gradually elevated along with progression of a 48-h Pi-starvation regime (Fig. 2). Upon Pi recovery condition, the transcripts of TaMIR1139 under 48-h Pi deprivation were gradually reduced over a 48-h Pi recovery regime, restoring finally to the levels similar to before Pi-starvation stress (Fig. 2). NtMIR1139 exhibited similar expression patterns to TaMIR1139, whose expression levels were shown to be induced by Pi deficiency and down-regulated by the re-supplied sufficient-Pi condition (Fig. 2). These results indicated that miR1139 family is Pi-starvation responsible, whose transcripts abundance is sensitively regulated by external Pi levels. All of the seven target genes of TaMIR1139 exhibited modified expression patterns in root tissues upon varied Pi levels, which are converse to miR1139 members; their transcripts are gradually decreased over a 48-h Pi starvation regime and the Pi-starvation reduced expression is gradually recovered along with the progression in a 48-h Pi recovery regime, although the variation on expression levels is different among them (Fig. 2). To validate the cleavage characterization of the target genes, two of the them including *TaPK2* and *TaTT* were further subjected to cleavage product evaluation upon varied external P supplies based on 5′ RACE analysis. Results confirmed the cleavage nature of these target genes (Fig. S2). The expression patterns of the target genes upon external Pi conditions suggest that they are regulated by the miRNA member through a cleavage mechanism.

### TaMIR1139 overexpression modifies plant growth and photosynthesis parameters upon Pi-starvation stress

Sen 1 and Sen 2, two T3 generation lines showing much more TaMIR1139 transcripts than other four lines (Fig. S3), together with WT were subjected to functional evaluation of

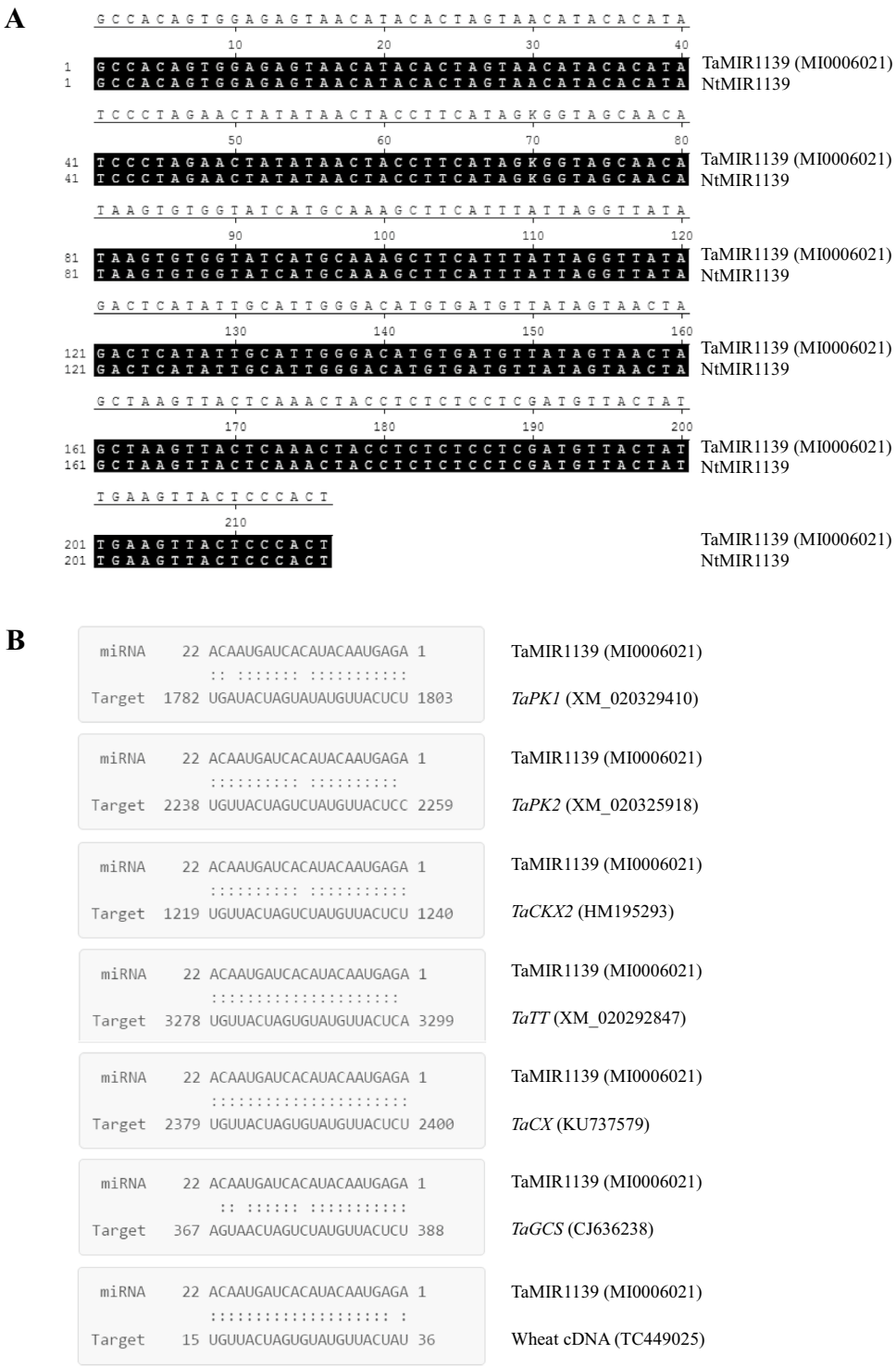



**Fig. 1** Characterization of TaMIR1139, NtMIR1139, and the target genes. **a** Alignment result between TaMIR1139 and NtMIR1139. **b** Interaction characterization between TaMIR1139 and the target genes. In **b**, *TaPK1, TaPK2, TaCKX2, TaTT, TaCX, TaGCS*, and wheat cDNA, seven TaMIR1139 target genes

the miRNA in mediating plant Pi-starvation stress response. After 2 weeks of agar media culture under the sufficient-Pi condition (1.2 mM Pi), Sen 1 and Sen 2 seedlings exhibited comparable growth features with WT seedlings (Fig. 3a); however, the lines displayed drastically improved phenotypes relative to WT after 3 weeks of Pi-starvation stress (0.02 mM Pi) (Fig. 3a). To validate above results, the transgenic and WT plants were cultured in MS solutions with sufficient- and deficient-Pi conditions for a longer period. Similar to results from agar media culture as mentioned above, Sen 1 and Sen 2 displayed similar growth features to WT after sufficient-Pi treatment and behaved improved

phenotypes relative to WT after the Pi-starvation stress (Fig. 3b). Biomass in transgenic and WT seedlings or plants are consistent with the growth features; showing to be comparable among the transgenic lines and WT under the sufficient-Pi condition and to be drastically increased in transgenic lines with respect to WT under Pi starvation (Fig. 4a, b). These results together suggest that TaMIR1139 is a critical mediator in improving Pi deprivation tolerance.

Photosynthesis behaviors are closely associated with biomass production and plant growth given that the photosynthetic assimilates contribute largely to the tissue differentiation and the organ establishment (Gu et al. 2017; Slattery et al. 2017). To characterize if TaMIR1139 regulates photosynthesis that affects the plant growth under Pi starvation stress, photosynthetic rate (Pn), PSII efficiency (ΦPSII), and nonphotochemical quenching (NPQ) in upper transgenic and WT levels were assessed under the sufficient-Pi and deficient-Pi treatments. As expected, Pn, ΦPSII, and NPQ were comparable among the transgenic lines and WT under the sufficient-Pi treatment. Under Pi starvation, the transgenic lines showed higher Pn and ΦPSII and lower NPQ than wild type (Fig. 5a–c), suggesting that TaMIR1139 confers P-deprived plants increased photosynthetic efficiency via regulation of the nonphotochemical quench capacity of chlorophyll within PSII. These results indicate that the TaMIR1139-mediated improvement on growth and biomass production under Pi starvation is associated with the miRNA function in positively regulating photosynthesis parameters.

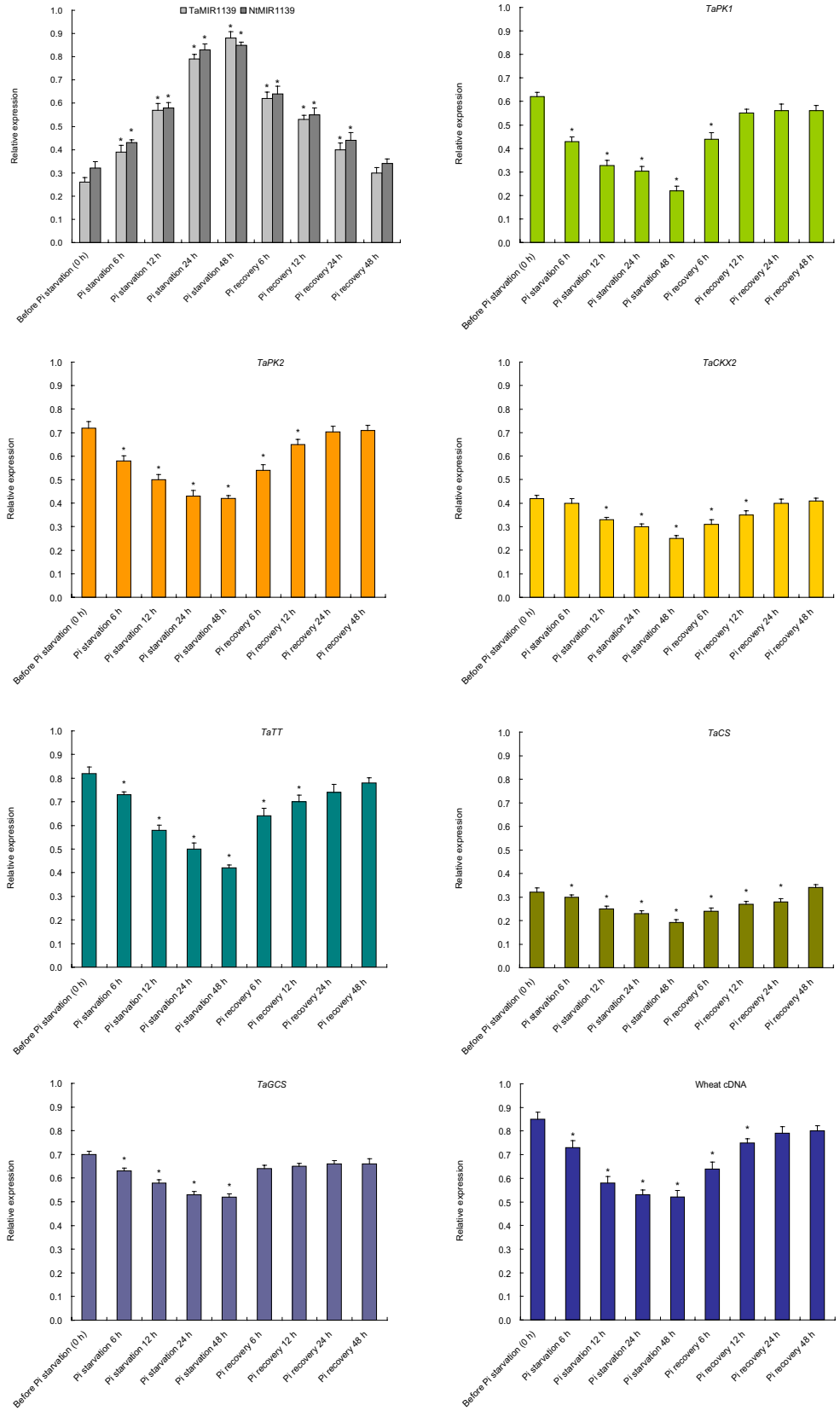
## TaMIR1139 overexpression improves Pi acquisition of plants upon Pi starvation treatment

P concentrations and accumulative amounts in the transgenic lines and WT were assessed after the sufficient-Pi and Pi starvation treatments. Under the sufficient-Pi treatment, the transgenic seedlings or plants (Sen 1 and Sen 2) exhibited comparable P concentrations and P accumulative amounts with WT (Fig. 6a–d). Under the Pi starvation treatment, however, the transgenic lines showed similar P concentrations (Fig. 6a, b) and dramatically increased P accumulative amounts (Fig. 6c, d) relative to WT. These results suggest that the TaMIR1139-mediated improvement under low-Pi stress on plant growth, biomass, and photosynthesis parameters is correlated with the miRNA modulated increase of the Pi taken up activity.

## Transcript profiles regulated by TaMIR1139 under the Pi-starvation stress

The genes targeted by TaMIR1139 share different biological functions, including a set to act as signaling regulators (i.e., serine/threonine protein kinase encoding genes *TaPK1* and *TaPK2*). It, therefore, could be speculated that the miRNA-mediated plant Pi starvation response is closely associated with the role of miRNA in extensively modulating the expression profile, via posttranscriptionally regulating target genes. To systematically elucidate the gene network regulated by TaMIR1139, a high throughput transcriptome sequencing analysis was performed to globally characterize the differential genes in Pi-deprived TaMIR1139 overexpression line (Sen 1). Totally 1343 genes were identified to be differential in the transgenic line, including 418 to be upregulated and 925 downregulated (Fig. 7a, b). To validate the results obtained in transcriptome analysis, six genes with significantly upregulated in expression in Pi-deprived TaMIR1139 overexpression lines, including *NtPK, NtSCD1, NtWRKY, NtCDK, NtSOD*, and *NtZFP* were subjected to transcripts abundance evaluation. Expression analysis revealed that all of these genes show comparable transcript levels with RNA-Seq analysis (Fig. S4). Therefore, the results derived from the transcriptome data are reproducible and credible.

Functional characterization on the upregulated genes categorized them into three large classes, including biological process, cellular component, and molecular function. Among them, the biological process is further classified into follow sub-classes, including metabolic process, protein phosphorylation, oxidation–reduction process, proteolysis, carbohydrate metabolic process, and transcription regulation; the cellular component is further divided into follow sub-classes, including constitution of membrane, integral to membrane, nucleus, intracellular, ribosome, and cytoplasm; the molecular function is further classified into sub-classes, including protein binding, zinc ion binding, sequence-specific DNA binding protein, and DNA binding (Fig. 7c). For example, a large set of genes involving various biological pathways have been identified to be drastically upregulated in the Pi-deprived TaMIR1139 overexpression line (Sen 1), including those associated with signaling transduction [i.e., serine/threonine kinase encoding genes (ID# LOC107759098 and ID# MSTRG.8890.1), WD40 repeat encoding gene (ID# LOC107772888), CYTH-like domain encoding gene (ID# MSTRG.30973.2)]; transcription regulation [i.e., *NtWRKY* (ID# LOC107780258), *NtZFP* (ID# LOC107777165), RNA polymerase gene (ID# LOC107795733), helicase gene (ID# LOC10778076), BTB/POZ domain encoding gene (ID# LOC107805205), RNA recognition motif encoding genes (ID# LOC107767634, ID# LOC107799110 and MSTRG.4029.4)]; transportation [i.e., major facilitator superfamily gene (ID#; LOC107832780), potassium transporter gene (ID# LOC107825304), auxin efflux carrier encoding gene (ID# MSTRG.20500.1), nucleotide-diphospho-sugar transferase gene (ID# LOC107773227), SulP transporter gene (ID# LOC10781169), oligopeptide transporter gene (ID# MSTRG.32789.3)]; translation regulation [i.e., translation initiation factor gene (ID# LOC107791261)]; and biochemical metabolism [i.e., Glycoside hydrolase genes


TaMIR1139
NtMIR1139
TaPK1
TaPK2
TaCKX2
TaTT
TaCS
TaGCS
Wheat cDNA
Relative expression
Before Pi starvation (0 h)
Pi starvation 6 h
Pi starvation 12 h
Pi starvation 24 h
Pi starvation 48 h
Pi recovery 6 h
Pi recovery 12 h
Pi recovery 24 h
Pi recovery 48 h

◂**Fig. 2** Expression patterns of TaMIR1139, its tobacco homolog NtMIR1139, and the target genes. Data are normalized by internal standards and shown by average plus standard error. Asterisk indicates to be statistically significant compared with the time point before Pi starvation (0 h) ($P < 0.05$)

(ID# LOC107816298 and ID# LOC107823482), tRNA-dihydrouridine synthase genes (ID# MSTRG.44583.2 and ID# MSTRG.44583.3)] (Supplemental file 1). These discoveries indicate that TaMIR1139 regulates transcription of global genes functional in diverse biological processes, which impact on growth, biomass production, photosynthesis behavior, and Pi acquisition of plants upon exposure to the low-Pi stress.

### Transcription of PT genes mediated by TaMIR1139 and the role in mediating plant Pi starvation response

The TaMIR1139 overexpression lines (Sen 1 and Sen 2) displayed drastically increased P accumulation under Pi starvation treatment, which suggests that the connection between the miRNA and distinct PT genes contributes to Pi acquisition. To define whether the PT genes are mediated by TaMIR1139, expression patterns of six PT encoding genes in *N. tabacum* (i.e., *NtPT*, and *NtPT1* to *NtPT5*) were analyzed in Pi-deprived transgenic lines and WT. Among the PT genes examined, contrasting to others that showed unaltered transcripts among transgenic and WT plants, *NtPT* exhibited drastically upregulated expression in Sen 1 and Sen 2 relative to WT (Fig. 8a). This result suggests that this PT gene is transcriptionally regulated under TaMIR1139 via a possibly independent-manner, which contributes to improved Pi taken up activity in the transgenic lines. To experimentally validate the PT gene role in regulating Pi acquisition, two T3 lines (line 2 and line 4) with *NtPT* overexpression (Fig. S5) together with WT were subjected to sufficient-Pi and Pi starvation treatments. The transgenic lines exhibited improved phenotypes (Fig. 8b), biomass (Fig. 8c), and P accumulative amounts (Fig. 8e) relative to WT under Pi-starvation treatment, albeit comparable above traits observed among them under the sufficient-Pi condition (Fig. 8b–e). These results confirm that *NtPT* acts as an important regulator in mediating Pi taken up in the Pi starvation-challenged plants and contributes to the TaMIR1139-mediated P accumulation. The molecular connection between TaMIR1139 and the *NtPT* transcription needs to be further characterized.

## Discussion

Pi starvation-responsive miRNA members act as critical mediators in plant adaptation to Pi deprivation. Arabidopsis miR399 and its homologs in other plant species, such rice (Hu et al. 2015), wheat (Ouyang et al. 2016), and barley (Hackenberg et al. 2013) play important roles in modulating internal Pi homeostasis (Bari et al. 2006; Chiou et al. 2006; Pant et al. 2009). Transgene analysis on this miRNA has indicated that it confers plants an ability of Pi overaccumulation (Chiou et al. 2006) through posttranscriptional regulation of *PHO2*, a miR399 target gene (Aung et al. 2006; Bari et al. 2006) that is functional in an ubiquitin-dependent protein degradation pathway (Sunkar and Zhu 2004) and via modified expression of distinct PT genes (i.e., Arabidopsis *Pht1;8* and *Pht1;9*) (Lin et al. 2008). Other miRNA family members such as miR827 and miR2111 are also functional as crucial regulators in plant Pi starvation response through transcriptional response to low-Pi signaling, by which to further regulate the target genes at posttranscriptional level (Hackenberg et al. 2013; Paul et al. 2015). In this study, expression analysis on TaMIR1139 and its tobacco homolog revealed that both of them are sensitively response to external Pi supplies. The Pi starvation responsive patterns together with its identical precursor in *N. tabacum* (NtMIR1139) suggest the conserved characterization of miR1139 across diverse plant species. *cis*-acting regulatory elements are essential in controlling gene transcription after specific action with distinct transcription factors (Kaur and Pati 2016; Fasani et al. 2017). Previously, characterization on the Pi starvation-responsive regulatory elements revealed that a set of them, including PIBS and PIBS-like situating in promoter regions, acts as the *cis*-acting regulatory motifs in transcriptionally regulating genes in Pi starvation response (Schu¨nmann et al. 2004; Glassop et al. 2005). Enrichment of these motifs promotes gene transcription efficiency in response to Pi starvation whereas base mutation of the motifs causes significant impairment of genes in Pi starvation response (Schu¨nmann et al. 2004). Further characterization of the *cis*-acting regulatory elements in TaMIR1139 that impact on its Pi starvation response as well as its target genes can provide novel insights into gene transcriptional regulation upon Pi deprivation.

miRNA members involve the mediation of diverse biological processes through regulation of the target genes, large sets of which are categorized into transcription factor (TF) families that play essential roles in modulating plant growth, development, organ identity, metabolism, and biotic or abiotic stress responses (Palatnik et al. 2003; Aukerman and Sakai 2003). Compared with other family genes, the TF family ones are frequently acted as preferential targets interacted by the miRNA members (Liu et al. 2017). Besides the TF encoding genes, genes categorized into other functional families are also targeted by distinct miRNAs and play critical roles in various physiological processes. For example, Arbidopsis miR399 mediates plant Pi homeostasis largely through regulation of a PHO2 gene that encodes an E3 ligase, which involves protein degradation

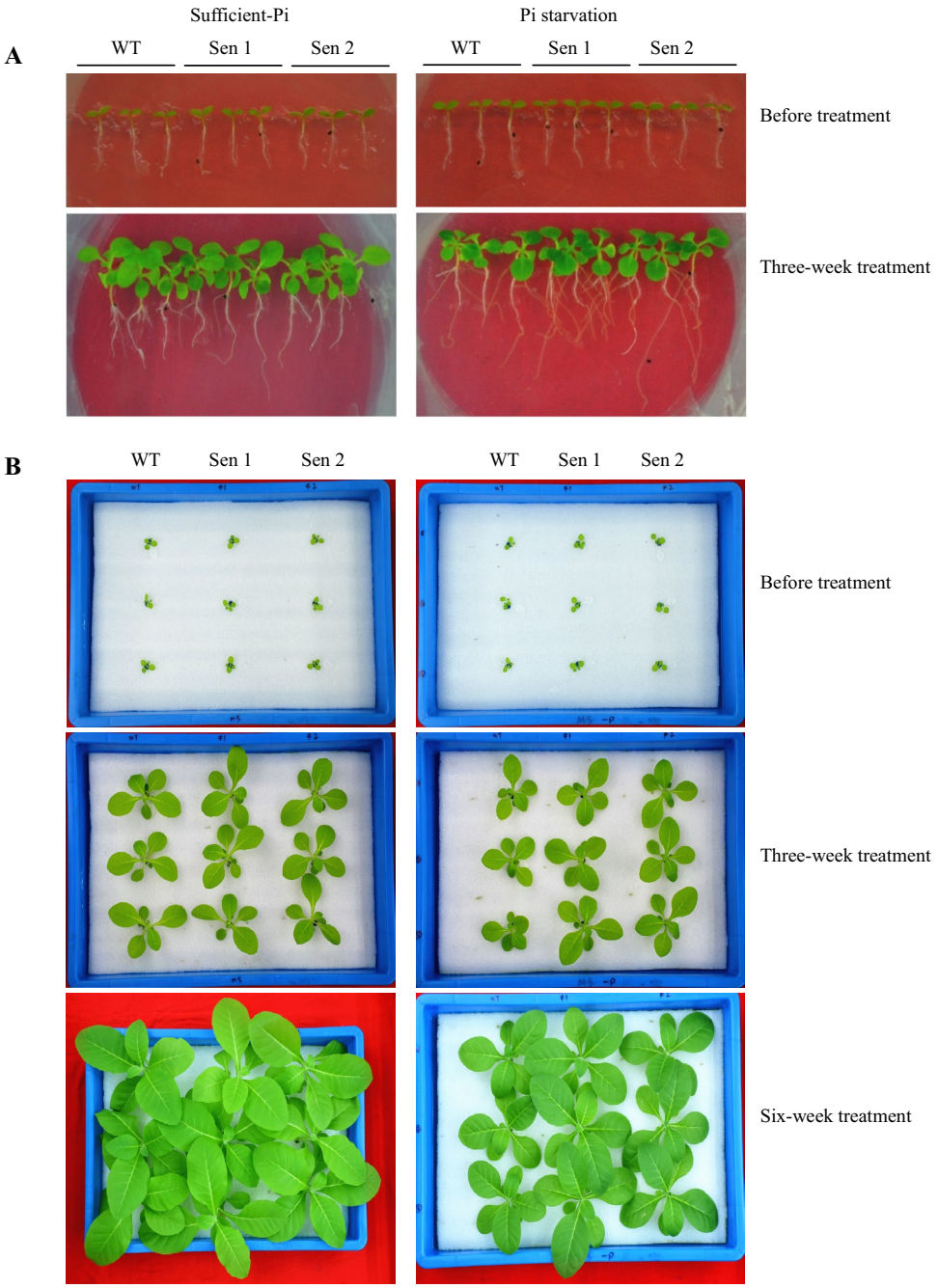



**Fig. 3** Phenotypes of the transgenic lines and WT under sufficient-Pi and Pi starvation treatments. **a** Phenotypes of seedlings cultured on agar media containing standard Pi (sufficient-Pi, 1.2 mM) or lowered Pi (Pi starvation, 0.05 mM Pi). **b** Phenotypes of plants hydroponically cultured in the standard MS solution (sufficient-Pi, 1.2 mM) or the modified MS solution containing reduced Pi (Pi starvation, 0.05 mM Pi). *Sen 1 and Sen 2* two transgenic lines, *WT* wild type

via the ubiqutin-associated pathway (Aung et al. 2006). TaMIR444a, an N-starvation inducible miRNA member of wheat, mediates plant low-N stress adaptation through regulation of the target genes encoding receptor-like proteins via a cleavage mechanism (Gao et al. 2016). In this study, totally seven genes were predicted to be targeted by TaMIR1139. Functional characterization on these target genes revealed their biological families, including two encoding receptor-like serine/threonine protein kinase (*TaPK1* and *TaPK2*), one cytokinin oxidase 2 (*TaCKX2*), one glycine cleavage system H protein (*TaGCS*), one tyrosine-specific transport (*TaTT*), one 3-ketoacyl CoA synthase encoding gene (*TaCS*), and one to be functional uncharacterized (TC449025). Expression analysis indicated that all of target genes displayed reverse expression patterns to TaMIR1139. Moreover, 5′-RACE analysis on two target genes (i.e., *NtPK2* and *NtTT*) provided the cleavage evidence that these target genes are regulated under miRNA at the posttranscriptional level. Thus, the action modules established by this miRNA and the target genes are involved in plant Pi starvation response through the target genes functional in diverse biological processes.

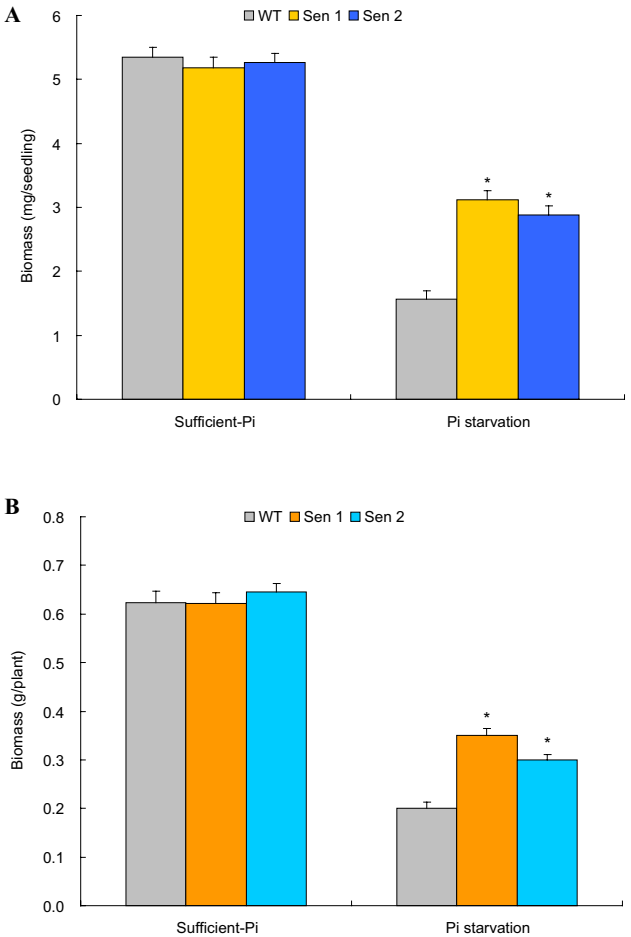



**Fig. 4** Biomass of transgenic lines and WT under sufficient-Pi and Pi starvation treatments. **a** Biomass of seedlings cultured on agar media. **b** Biomass of plants cultured in MS solutions. In **a**, 3-week-old seedlings after Pi treatments were subjected to assessment of the traits. In **b**, 6-week-old plants after Pi treatments were subjected to assessment of the traits. Data are shown by average plus standard error and asterisk indicates to be statistically significant compared with WT ($P < 0.05$). *Sen 1 and Sen 2* two transgenic lines, *WT* wild type

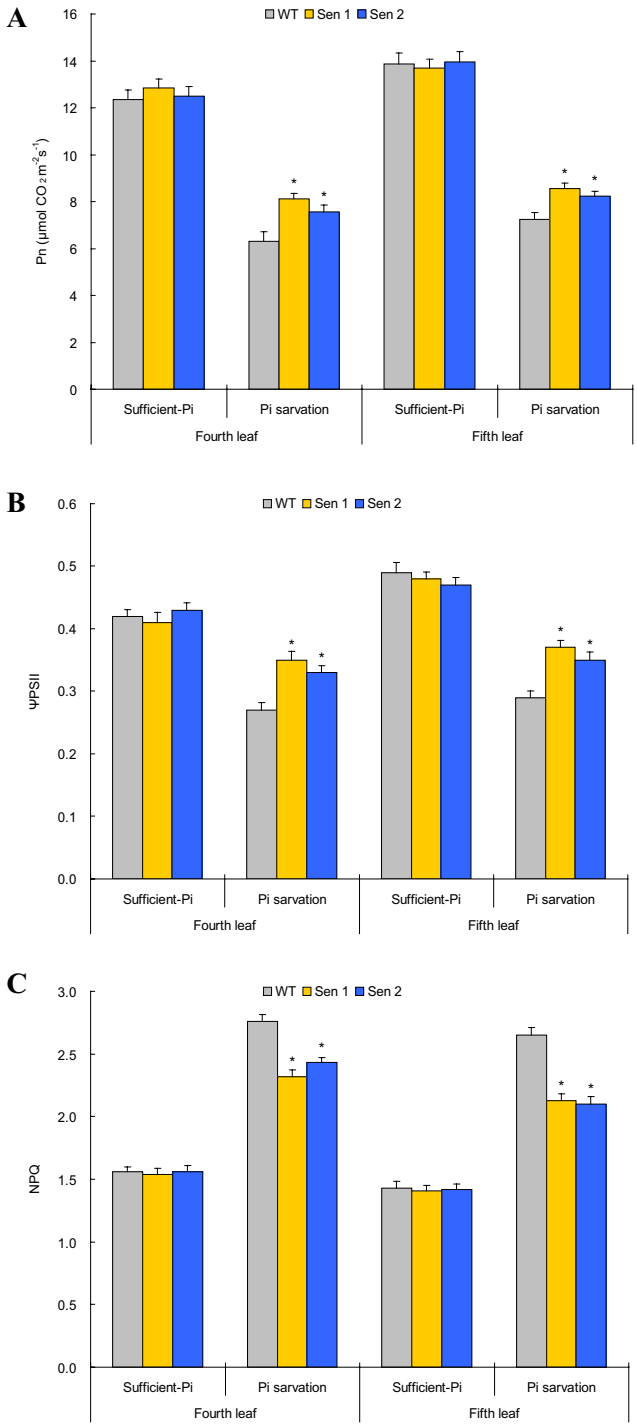



**Fig. 5** Photosynthesis parameters of the transgenic lines and WT under sufficient-Pi and Pi starvation treatments. **a** Pn. **b** ΨPSII. **c** NPQ. In **a**–**c**, 6-week-old plants after Pi treatments were subjected to assessment of the parameters. Data are shown by average plus standard error and asterisk indicates to be statistically significant compared with WT ($P < 0.05$). *Sen 1 and Sen 2* two transgenic lines, *WT* wild type

Although the target genes interacted by distinct miRNA members are limited, a large quantity of genes are shown to be modified in transcription under regulation of the miRNA members (Jones-Rhoades et al. 2006; Samad et al. 2017). For example, global profiling analysis in lines overexpressing OsMIR156, a conserved miRNA family member in rice mediating drastically on plant growth and development, revealed that totally 3008 genes involved in diverse biological processes are differentially expressed (Xie et al. 2012). Similarly, a total of 2346 genes, including 1020 to be upregulated and 1326 downregulated, are shown to be differential ones in switchgrass lines with OsMIR156b overexpression, synergistically contributing to the OsMIR156b-mediated plant growth and phenotype behavior (Fu et al. 2012). Recently, TaMIR444a has been confirmed to modify

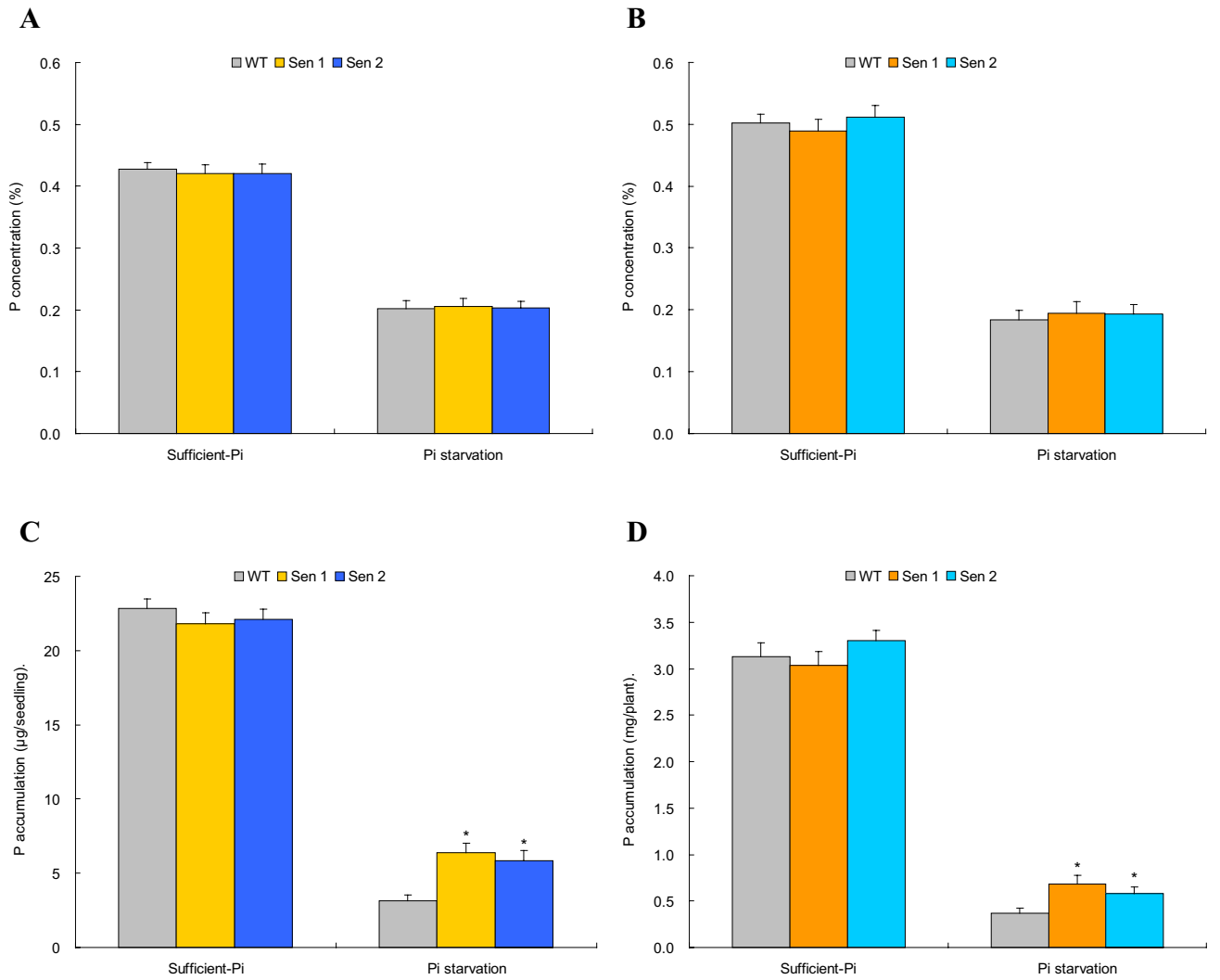



**Fig. 6** P-associated traits of the transgenic lines and WT under sufficient-Pi and Pi starvation treatments. **a** P concentration of seedlings cultured on agar media. **b** P concentration of plants cultured in varied Pi solutions. **c** P accumulation of seedlings cultured on agar media. **d** P accumulation of plants cultured in varied Pi solutions. In **a** and **c**, 3-week-old seedlings after Pi treatments were subjected to assessment of the traits. In **b**–**d**, 6-week-old plants after Pi treatments were subjected to assessment of the traits. Data are shown by average plus standard error and asterisk indicates to be statistically significant compared with WT ($P < 0.05$). *Sen 1 and Sen 2* two transgenic lines, *WT* wild type

the transcription of thousands of genes under N starvation treatment (Gao et al. 2016). In this study, characterization of the transcriptional profiling indicated that totally 1343 genes are differentially expressed under regulation of TaMIR1139, including 418 to be upregulated and 925 downregulated upon Pi starvation, which are categorized into functional classes of biological process, cellular component, and molecular function. This finding suggests that TaMIR1139 mediates plant Pi starvation adaptation to be associated with its role in extensively modulating the gene network impacting on plant growth, photosynthesis behaviors, and the P-associated traits. The transcription of these differential genes modulated by TaMIR1139 is suggested to be realized possibly via an indirect manner, given that the target genes of this miRNA are limited. It can be speculated that the modified transcription of the differential genes is derived from the diverse functions of the target genes. For example, the target genes *TaPK1* and *TaPK2* that encode receptor-like serine/threonine protein kinases act as critical components in Pi starvation signaling pathways (Fei et al. 2017; Zhao et al. 2017) that modulate the transcription of suites of corresponding downstream genes. The target gene *TaCKX2* encoding a cytokinin oxidase impacts on cytokinin biosynthesis metabolism and the cytokinin signaling transduction, causing extensively transcriptional modification of the genes underlying the miRNA regulation (Werner et al.

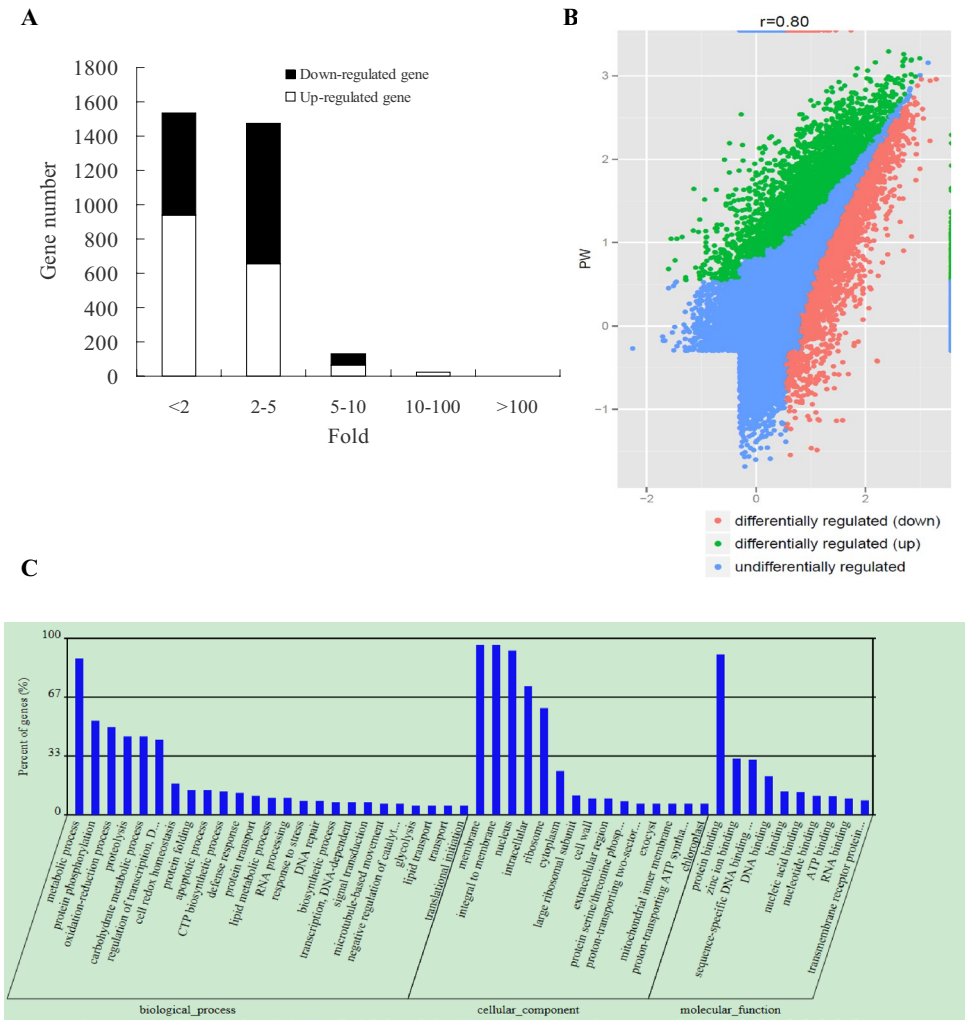



**Fig. 7** Transcriptome profile of the TaMIR1139 overexpression lines upon Pi starvation. **a** Statistical fold change results of the differentially expressed genes (DEG) showing upregulated or downregulated expression pattern; **b** dot distribution pattern of DEG; **c** GO categories of the upregulated DEG

2001; Macková et al. 2013). Therefore, the genes *TaPK1, TaPK2*, and *TaCKX2* targeted by TaMIR1139 synergistically act with other target genes, such as *TaGCS, TaTT*, and *TaCS*, to coordinately regulate the expression profiling in the TaMIR1139 overexpression lines under the Pi starvation treatment. Further characterizing the biological function of the target genes can establish the action modules TaMIR1139/target gene and help understand the plant Pi starvation adaptation mechanism underlying the miRNA member.

Pi is sole form of phosphorus to be taken up by plant root systems (Chiou and Lin 2011; López-Arredondo et al. 2014). Pi acquisition from media into plants is mediated by the PT proteins encoded by a gene family referred to as PHT1. Distinct PHT1 family members are Pi-starvation inducible and play an important role in mediating Pi taken up under Pi starvation conditions (Mudge et al. 2002; Muchhal et al. 1996; Karthikeyan et al. 2002; Shin et al. 2004). It has been shown that the modified transcription of PT genes upon Pi starvation is regulated by upstream mediators, such as transcription factors PHOSPHATE STARVATION RESPONSE1 (PHR1; Rubio et al. 2001), WRKY75 (Devaiah et al. 2007), and WRKY45 (Wang et al. 2014a, b). In this study, the tobacco lines overexpressing TaMIR1139 exhibited improved P accumulative amount under Pi starvation, suggesting that distinct PT genes contribute to Pi taken up under the deficient-Pi condition. Based on expression analysis on six PT encoding genes in *N. tabacum, NtPT* exhibited an upregulated expression pattern in the transgenic lines with TaMIR1139 overexpression; overexpression of this PT gene confers plants improved growth, biomass production, and P-associated traits, confirming its role in mediating the Pi-starvation adaption to the TaMIR1139 overexpression lines. Previously, analysis on Arabidopsis miR399 revealed that this miRNA member regulates Pi homeostasis to be closely associated with the miRNA role in indirectly regulating *Pht1;8* and *Pht1;9*, two PHT1 members of the PT family through a PHO2-mediated ubiquitin pathway (Fujii et al. 2005; Aung et al. 2006; Bari et al. 2006; Chiou et al. 2006; Pant et al. 2009). The transcription mechanism of

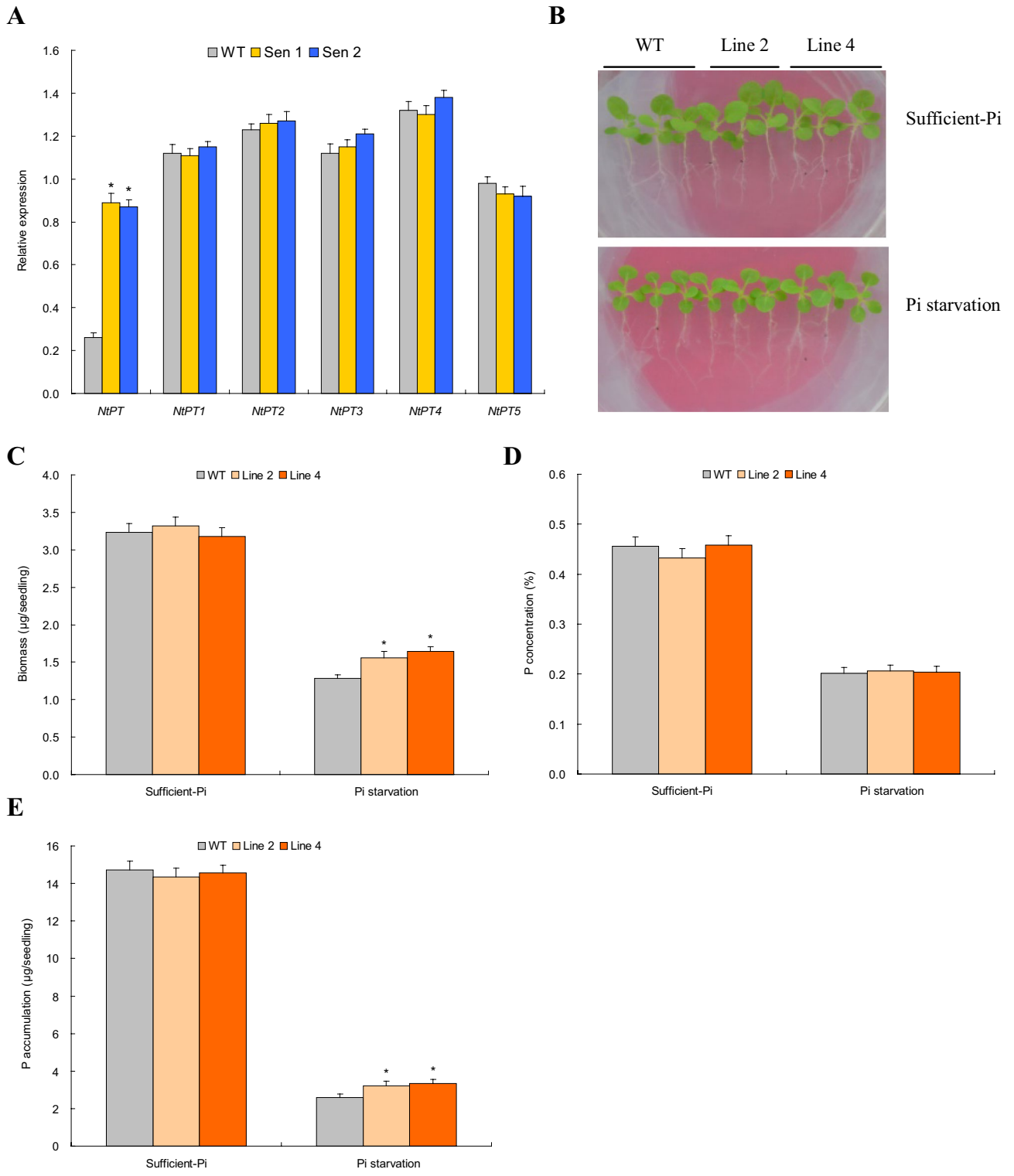



**Fig. 8** Characterization of the expression pattern and function of tobacco PT genes. **a** Expression patterns of tobacco PT genes in Pi-deprived WT and transgenic lines overexpressing TaMIR1139. **b** Phenotypes of *NtPT* overexpression lines and WT; **c** biomass of *NtPT* overexpression lines and WT; **d** P concentrations of *NtPT* overexpression lines and WT; **e** P accumulative amounts of *NtPT* overexpression lines and WT. Two weeks of transgenic and WT seedling treated by sufficient-Pi (1.2 mM Pi) and Pi starvation (0.05 mM Pi) were shown in **b** and subjected to assessment of biomass, P concentration, and P accumulation in **c**–**e**. In **a**, Sen 1 and Sen 2, two lines with TaMIR1139 overexpression; in **b**–**e**, line 2 and line 4, two lines with *NtPT* overexpression. *WT* wild type. In **c**–**e**, data are shown by average plus standard error and asterisk indicates to be statistically significant compared with WT ($P < 0.05$)

*NtPT* and its wheat homolog underlying miR1139 needs to be further characterized.

In past decade, functional characterization on plant miRNA members suggests that distinct ones of them can be acted as potential targets in breeding crop cultivar with improved abiotic stress tolerance (Kumar et al. 2010; Buiatti et al. 2013). In this study, the TaMIR1139 overexpression lines exhibited drastic improvement on plant growth, photosynthesis parameters, and P-associated traits upon Pi-starvation stress through regulating target genes, indicating that TaMIT1139 might be valuable in crop genetic improvement for high P use efficiency under the Pi-starvation stress condition.

**Author contribution statement** KX designed the research. ZL, XW, XC, GS, and QB conducted the experiment and performed data analysis. KX wrote the paper.

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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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