Plant Cell Rep (2016) 35:1371–1383
DOI 10.1007/s00299-016-1970-6

ORIGINAL ARTICLE

Characterization and primary functional analysis of a bamboo NAC gene targeted by miR164b

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Received: 2 February 2016 / Accepted: 21 March 2016 / Published online: 28 March 2016

Abstract

Key message ***PeSNAC1*****, a stress-related NAC1 from *Phyllostachys edulis*, was characterized. Ectopic expression in Arabidopsis indicated that *PeSNAC1* together with *ped*-miR164b participated in the regulation of organ boundaries and stress tolerance.**

Abstract NAC (NAM, ATAF1/2 and CUC2) participates in many different processes regulating plant growth, development, and stress response. A total of 125 NAC genes have been predicted in moso bamboo (*Phyllostachys edulis*), but their roles are poorly understood. *PeSNAC1* targeted by *ped*-miR164b was focused for further study. The cleavage of *PeSNAC1* mRNA guided by *ped*-miR164b was validated using RLM-5′ RACE. Tissue-specific expression analysis demonstrated that *ped*-miR164b had a declining trend from root, sheath, leaf, to that of stem, which was opposite to that of *PeSNAC1*. Transgenic Arabidopsis plants overexpressing either *PeSNAC1* (OX-PeSNAC1) or, *ped*-miR164b (OX-ped-miR164b) driven by the *CaMV35S* promoter were generated. OX-ped-miR164b plants showed similar phenotype of *cuc2* mutants whose growth was seriously suppressed. Compared with Col-0, sense OX-PeSNAC1 plants grew rapidly and flowered earlier, whereas antisense plants grew slowly and exhibited delayed flowering. Sense OX-PeSNAC1 plants had the greatest number of lateral roots, while antisense OX-PeSNAC1 and OX-ped-miR164b plants had fewer lateral roots than Col-0. Under NaCl and PEG6000 stresses, survival rates were higher and F_v/F_m values declined more slowly in sense OX-PeSNAC1 plants than in Col-0, with lower survival rates and a more rapid decrease in F_v/F_m values conversely observed in antisense OX-PeSNAC1 and OX-ped-miR164b plants. These findings indicated that *ped*-miR164b-targeted *PeSNAC1* may play key roles in plant development and tolerance to salinity and drought stresses.

Keywords Bamboo · NAC · miR164b · RLM-5′ RACE · qRT-PCR · Lateral root · NaCl and drought stresses · F_v/F_m

Abbreviations

ATAF	*Arabidopsis* transcription activation factor
BLAST	Basic local alignment search tool
CaMV35S	Cauliflower mosaic virus *35S*
cDNA	Complementary DNA
F_v/F_m	Maximal quantum yield of PSII
ORF	Open reading frame
CUC	Cup-shaped cotyledon (CUC), a NAC protein
miRNA	MicroRNA, small regulatory RNAs
NAC	Abbreviation of NAM, ATAF1/2 and CUC2, a plant-specific TF family

Communicated by B. Li.

Electronic supplementary material The online version of this article (doi:10.1007/s00299-016-1970-6) contains supplementary material, which is available to authorized users.

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NAM	No apical meristem (NAM), a NAC protein
PCR	Polymerase chain reaction
qPCR	Real time quantitative PCR
RACE	Rapid-amplification of cDNA ends
RLM-5′ RACE	RNA ligase-mediated 5′ RACE
TF	Transcription factor

Introduction

Plant biomass and agricultural productivity are seriously affected by abiotic stress due to the environmental deterioration globally (Ahuja et al. 2010). To ensure survival, plants have developed numerous effective molecular mechanisms over the long course of evolution to protect cellular activities and maintain plant integrity under adverse conditions (Pastori and Foyer 2002). Transcription factors (TFs) play important roles in plant growth and development by regulating the expression of stress-related genes to control plant biological processes (Meshi and Iwabuchi 1995). NAC (NAM, ATAF1/2, CUC2) TFs constitute a large plant-specific gene family involved in the regulation of plant developmental processes and response to environmental stresses (Zhu et al. 2015). The expression of some NAC TFs is negatively regulated by microRNAs (miRNAs) at the post-transcriptional level in a wide variety of eukaryotic organisms (Jeong et al. 2011). In Arabidopsis, miR164 targets five NAC domain-encoding mRNAs: *NAC1*, *CUC1*, *CUC2*, *At5g07680*, and *At5g61430* mRNAs (Mallory et al. 2004). *NAC1* plays an important role in the transmission of auxin signals that promote lateral root emergence. *AtNAC1* has been characterized as an intermediary in the auxin-signaling pathway that activates genes encoding molecules involved in the specification of lateral root initiation (Xie et al. 2000). In addition, transgenic *ZmNAC1*-overexpressing Arabidopsis plants have an increased number of lateral roots compared with the wild type (Li et al. 2012), while *TaNAC29*-overexpressing plants have enhanced tolerance to high salinity and dehydration (Huang et al. 2015).

Bamboos, which are woody grasses with rhizomes and underground roots, are found exclusively in forest habitats. The most economically important bamboo, moso bamboo (*Phyllostachys edulis*), possesses desirable features such as a fast growth rate, extensive usability, high yield, and strong regeneration capacity. Much attention has been paid to the regulatory mechanism promoting rapid growth in bamboo. One such study involved histological investigations and protein expression analyses using bamboo culm materials from different developmental stages and internodes (Cui et al. 2012). The results of that study revealed that culm development is initially dominated by cell division followed by cell elongation in middle and late stages, with the expressed proteins involved in many physiological and metabolic processes constituting a complex network to regulate culm development. Following the publication of the draft genome of moso bamboo (Peng et al. 2013), a genome-wide analysis of TFs identified 1768 TFs classified into 54 families (http://planttfdb.cbi.pku.edu.cn/). Among them, 125 NAC TFs were clustered into 17 groups and their gene structures and conserved domains were subsequently predicted by Li et al. (2015). In another study, miRNA expression profiling of bamboo was performed using next-generation sequencing (He et al. 2013). In addition, 84 conserved miRNAs belonging to 17 families as well as 81 novel miRNAs have been identified in ma bamboo (*Dendrocalamus latiflorus*) (Zhao et al. 2013), while 75 miRNAs and 24 miRNA* variants of 22 families have been found in moso bamboo (Xu et al. 2014). Finally, comparative profiling of miRNAs between sympodial and monopodial bamboos has been conducted using two representatives: moso bamboo and ma bamboo, respectively (Zhao et al. 2014b). Despite all these data, little information is available regarding how miRNAs regulate TFs in bamboo.

In this study, we used a bioinformatics approach to identify the NAC genes targeted by miR164b and isolated sequences of the *ped*-miR164b precursor, the *ped*-miR164b promoter, and the target gene *PeSNAC1* from moso bamboo. We then validated the *ped*-miR164b target site in *PeSNAC1* using RNA ligase-mediated (RLM) rapid amplification of cDNA ends (RACE). To understand the function of *ped*-miR164b and *PeSNAC1*, we further analyzed the expressions of *ped*-miR164b and *PeSNAC1* in moso bamboo and characterized the phenotypes of transgenic Arabidopsis plants expressing either *ped*-miR164b or *PeSNAC1*. We found that the expressions of *PeSNAC1* and *ped*-miR164b in different tissues exhibited opposite trends. In addition, *PeSNAC1*-over-expressing plants grew faster and had more lateral roots than the Columbia-0 (Col-0) wild type, while antisense (*PeSNAC1*) plants grew more slowly and had fewer lateral roots, some lines in the latter group even had phenotypes similar to those of transgenic plants expressing the *ped*-miR164b precursor. Further analysis of plants under salinity and drought stresses revealed that sense *PeSNAC1*-overexpressing plants had higher survival rates and F_v/F_m values than those of Col-0, while those of antisense and *ped*-miR164b expressing plants were lower. These findings indicate that *PeSNAC1* expression is negatively regulated by *ped*-miR164b, which in turn may influence tolerance to salinity and drought stresses through regulation of root development. Taken together, our results shed some light on the function of

PeSNAC1 targeted by *ped*-miR164b and can serve as a basis for the study of miRNA target genes in bamboo.

Materials and methods

Plant materials and growth conditions

Seedlings of moso bamboo and wild-type *Arabidopsis thaliana* (ecotype Col-0) were planted in nutritional bowls (basal diameter/mouth diameter/height = 6.5/10/8 cm) containing vermiculite presoaked with plant nutrient solution (one-third strength B5 medium). The seedlings were grown at 22 ± 1 °C under a 16-h light/8-h dark photoperiod with a light intensity of 200 μmol m^{-2} s^{-1}.

Sequence retrieval and analysis

Homologous sequences of NAC genes and miR164 in moso bamboo were retrieved from BambooGDB (http://www.bamboogdb.org) (Zhao et al. 2014a). Open reading frames (ORFs) of *NAC* genes were determined with DNASTAR, BLASTN and BLASTX, and a multiple alignment of NAC proteins was conducted using DNAMAN to find the conserved domains. The GSDS gene structure display server (http://gsds.cbi.pku.edu.cn) was used to uncover the number and the positions of introns (Hu et al. 2015). The secondary stem-loop structure of *ped*-miR164b was predicted using the RNAfold WebServer (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) (Gruber et al. 2008), *cis*-elements in the *ped*-miR164b promoter region were analyzed with PlantCARE (http://bioinformatics.psb.ugent.be/Webtools/plantcare/html/) (Lescot et al. 2002). The target site of *ped*-miR164b was predicted using psRNATarget (http://plantgrn.noble.org/psRNATarget/?function=3#) (Dai and Zhao 2011).

RNA/DNA extraction and gene isolation

Roots, stems, leaves and sheaths were collected from 1-year-old moso bamboo seedlings and quickly frozen in liquid nitrogen. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacture's protocol and treated with RNase-free DNase I (Tiandz, Beijing, China). First-strand cDNA was synthesized from 1 μg total RNA using a Reverse Transcription System (Promega, Beijing, China). cDNA was synthesized from miRNAs using miRNA-specific stem-loop primers according to Unver and Budak (2009). Genomic DNA was extracted from leaves using the CTAB method (Gao et al. 2006).

The primers listed in Table 1 were designed according to the results of the bioinformatics analyses. Leaf cDNA and genomic DNA were used as templates for PCR amplifications performed in 20 μL reaction volumes containing PrimeSTAR DNA polymerase (Takara, Dalian, China). Primer pairs used for the amplifications were as follows: F-SNAC and R-SNAC (for *PeSNAC1*), F-pre164b and R-pre164b (for the *ped*-miR164b precursor), and F-pro164b and R-pro164b (for the *ped*-miR164b promoter) (Table 1). The PCR amplification products were inserted into a pGEM-T easy vector (Promega) and subsequently confirmed by sequencing on an ABI 3730 DNA Analyzer (Applied Biosystems, USA).

Tissue-specific expression analysis

Quantitative real-time PCR (qRT-PCR) and stem-loop qRT-PCR were used in this study to, respectively, detect expression levels of *PeSNAC1* and *ped*-miR164b in different tissues of moso bamboo. Primers specific to individual miRNAs were designed using a method described by Chen et al. (2005). U6 snRNA and *PeNTB* were, respectively, used as internal controls for miRNA (Ding et al. 2011) and *PeSNAC1* (Fan et al. 2013) analyses. Detailed information on primers used in the quantitative analysis is listed in Table 1. qRT-PCR amplifications were carried out using a SYBR Green I Master kit (Roche, Germany) on a QTOWER 2.2 real-time PCR system (Analytick Jena). Each amplification was performed in a final reaction volume of 10 μL containing 5.0 μL 2× SYBR Premix Ex *Taq*, 0.2 μL of each primer (10 μM), 0.8 μL cDNA and 3.8 μL nuclease-free water. All reactions were repeated three times. Stem-loop qRT-PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, and 62 °C for 10 s. The procedure used for traditional qRT-PCR consisted of 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 10 s. The qRT-PCR experiments were performed as biological triplicates. Expression levels relative to internal reference genes were calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Rlm-5′ race

Outer and inner primers (listed in Table 1) were designed for RLM-5′ RACE on the basis of miRNA target site prediction. Nested-RACE PCRs were conducted by RLM reverse transcription without CIP and TAP treatments in conjunction with a Full RACE Kit with TAP (Takara). The cDNA for RLM-RACE was synthesized according to the manufacturer's instructions. Outer PCR was carried out in 20 μL volumes consisting of 2 μL of 10× GC Buffer II, 8 μL 1× cDNA Dilution Buffer II, 2 μL cDNA template, 1 μL miR164b outer primer (10 μM), 1 μL 5′RACE outer

Table 1 Primer sequences for gene cloning and expression analysis

Name	Sequences (5′ → 3′)	Application
Stem-loop primer	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAG	Reverse transcription
F-miR164b	AGCAGCATTGGAGAAGCAGGG	ped-miR164b isolation and quantitative analysis
R-miR164b	CTCAACTGGTGTCGTGGAGTC	
F-pre164b	CGTGTACTACTTTTGCCGCCTATAT	ped-miR164b precursor isolation
R-pre164b	AGTCAATTGGAATTCGAGCCCCAT	
F-SNAC	ATGGTGGAGGCGAGGCTGC	*PeSNAC1* isolation
R-SNAC	TCAATCGAGTGAGTTCCACATCTGTGA	
F-pro164b	AGCAGGGTAGCTATGCTATCTGCACC	ped-miR164b promoter isolation
R-pro164b	GCTCGCCCCTCCTCATACACCT	
miR164b outer primer	CACACACCCAAGTCTTCTTTAAGAGTTC	RLM-5′ RACE outer PCR reaction
5′RACE outer primer	CATGGCTACATGCTGACAGCCTA	
miR164b inner primer	GCCTTCAAACCTGGTGAACTGGCTCAGC	RLM-5′ RACE inner PCR reaction
5′RACE inner primer	CGCGGATCCACAGCCTACTGATGATCAGTCGATG	
F-U6	GGACATCCGATAAAATTGGAACGATACAG	Internal control of ped-miR164b in bamboo
R-U6	AATTTGGACCATTTCTCGATTTATGCGTGT	
F-NTB	TCTTGTTTGACACCGAAGAGGAG	Internal control of *PeSNAC1*
R-NTB	AATAGCTGTCCCTGGAGGAGTTT	
F-PeSNAC	CCTCCCCTCATTGACAACTTCATT	*PeSNAC1* quantitative analysis
R-PeSNAC	CCTGAGCAAAATTACTCTGAGGCG	
F-miR	CGGGATCCCGTGTACTACTTTTGCCGCCTATAT	ped-miR164b expression vector construction and transgenic plants testing
R-miR	GCTCTAGAAGTCAATTGGAATTCGAGCCCCATC	
F- Actin	CATCAGGAAGGACTTGTACGG	Internal control of ped-miR164b in *Arabidopsis*
R- Actin	GATGGACCTGACTCGTCATAC	
F-CUC1	TGCATGAGTATCGCCTTGAC	*CUC1* expression testing
R-CUC1	AACGCCACGCCATCACCGAC	
F-CUC2	TGAAGGCAAATTCTCTTACC	*CUC2* expression testing
R-CUC2	AGGCCGTAGTAGTAGTAGGG	
F-NAC1	CGAGGCCGTAAAACCGAT	*NAC1* expression testing
R-NAC1	GGGTTAGGGTTCTTGCATGG	
F-S	CCATCGATATGGTGGAGGCGAGGCT	Sense *PeSNAC1* expression vector construction and transgenic plants testing
R-S	CGGGATCCTCAATCGAGTGAGTTCCACAT	
F-A	CGGGATCCATGGTGGAGGCGA	Antisense *PeSNAC1* expression vector construction and transgenic plants testing
R-A	CCCAAGCTTTCAATCGAGTGAGTTCCACAT	
F-GUS	ATGTTACGTCCTGTAGAAACCC	ped-miR164b promoter transgenic plants testing
R-GUS	TCATTGTTTGCCTCCCTGCT	
F-AtUbiquitin	ATGGCTGAAGAGGATATCCAGC	Internal control of *PeSNAC1* and ped-miR164b promoter
R-AtUbiquitin	GAAACACTTCATATGGACGATGG	

primer (10 μM), 0.25 μL LA *Taq* (5 U μL^{-1}) and 5.75 μL double-distilled water (ddH_2O). PCR cycling conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 20 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension of 72 °C for 10 min. Inner PCR was then performed in 20 μL volume consisting of 4 μL of 5× PrimeSTAR Buffer (Mg^{2+} plus), 1.6 μL dNTP mix (2.5 mM each), 1 μL outer PCR amplification product, 1 μL miR164b inner primer (10 μM), 1 μL 5′RACE inter primer (10 μM), 0.2 μL PrimeSTAR HS DNA polymerase (2.5 U μL^{-1}) and 11.2 μL ddH_2O. The inner PCR protocol involved an initial denaturation of 98 °C for 3 min, followed by 20 cycles of 98 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a

final extension of 72 °C for 10 min. The generated PCR fragments were cloned into pGEM-T easy vectors and sequenced.

Construction of plant expression vectors and transformation

The ORF sequence of *PeSNAC1* was amplified with primers designed to introduce *Bam*H I or *Cla* I sites. The primers used to generate the sense fragment were F-S and R-S, while those for the antisense fragment were F-A and R-A—all based on the nucleotide sequence of *PeSNAC1* (Table 1). *PeSNAC1* overexpression vectors were constructed by inserting the fragments into modified pCAMBIA1301 vectors driven by the *CaMV35S* promoter. The sense and antisense constructions were designated as pC1301-PeSNAC1-S and pC1301-PeSNAC1-A, respectively. The precursor of *ped*-miR164b was also incorporated into a modified pCAMBIA1301 vector under the control of the *CaMV35S* promoter by using the precursor fragment with *Bam*H I and *Xba* I sites at 5′ and 3′ ends, respectively. The promoter fragment was cloned into the *Sal* I-*Xba* I site of the binary vector pBI101 to obtain a transcriptional fusion of the *ped*-miR164b promoter and the GUS coding sequence. The constructs were then separately introduced into *Agrobacterium tumefaciens* (strain GV3101) by electroporation. Transformations of Arabidopsis plants were conducted using the floral dip method (Clough and Bent 1998). Putative transgenic plants harboring *PeSNAC1* and *ped*-miR164b were selected on medium supplemented with 50 mg L^{-1} hygromycin, while those containing the *ped*-miR164b promoter were selected using 50 mg L^{-1} kanamycin. Phenotypes of resistant plants were observed and photographed. Resistant seedlings showing no evidence of segregation were selected for further analysis.

Verification of transgenic plants

Transgenic plants of *ped*-miR164b were verified by qRT-PCR using F-miR/R-miR primer pair, with the actin gene used as an internal control (Song et al. 2013). In addition, expression analysis of endogenous genes such as *CUC1*, *CUC2* and *NAC1* was carried out using primer pairs designed according to their respective sequences (AB049069, AB002560, and NM_104479) (Table 1).

Transgenic plants of the *ped*-miR164b promoter and *PeSNAC1* were validated by RT-PCR using F-GUS/R-GUS, F-S/R-S, and F-A/R-A primer pairs (Table 1). *AtUbiquitin* (NM180850) was used as an internal control under the same RT-PCR conditions with primers F-AtUbiquitin and R-AtUbiquitin (Yang et al. 2015). Vertical plate culture was used for root growth observations, and the number of lateral root was counted using 20 seedlings of each line. Student's *t* test was used for the statistical analysis of the unit lateral root numbers.

Salinity and drought stress treatments

Seeds of transgenic Arabidopsis plants were sown on half-strength Murashige–Skoog (MS) solid medium containing hygromycin, while those of the wild type (Col-0) were cultivated on half-strength MS solid medium alone. Following incubation at 4 °C for 2 days, the seedlings were cultivated under normal conditions. One week after germination, both transgenic and Col-0 plants were transferred to half-strength MS solid medium containing NaCl (200 mM). At the same time, another set of transgenic and wild type plants were transferred to solid medium with incorporated PEG6000 (30 %) to simulate drought stress. Each treatment included 100 seedlings of each line. The survival rate of each NaCl-treated transgenic line was counted after 10 days, while that of each line treated with PEG6000 was counted after 20 days. During this period, chlorophyll fluorescence parameters were measured using an IMAGING-PAM fluorometer (Waltz, Germany) and used to calculate the maximum quantum yield of photosystem II, F_v/F_m, as $(F_m - F_0)/F_m$ (Demmig-Adams and Adams 1996), where F_0 is the minimum fluorescence in the dark-adapted state and F_m is the dark-adapted maximum fluorescence upon illumination with a pulse (0.6 s) of saturating light. F_0 and F_m were determined after 20 min dark adaptation. Parameters were measured for 10 replicates per treatment per line. To determine significant differences ($p < 0.05$), all data were statistically analyzed by one-way analysis of variance in SPSS.

GUS activity assay

GUS activity was examined in T_3 seedlings harboring the *ped*-miR164b promoter fused with the *GUS* reporter gene. The transgenic plants were stained in a 0.1 % X-Gluc solution according to the method of Jefferson (1987). After incubation at 37 °C for a period of 3 h to overnight, tissues were destained in 70 % ethanol until chlorophyll pigments were completely bleached. Finally, photos were taken using a camera (SONY DSC-HX50).

Results

Identification and isolation of *PeSNAC1* and *ped*-miR164b

Six of the 125 NAC domain-encoding genes in the bamboo genome database (Zhao et al. 2014a) were identified as

miR164b-targeted genes (S1): PH01001309G0120, PH01000110G0680, PH01000183G1320, PH01000093G0340, PH01000483G1000, and PH01000501G0450. These genes belonged to the stress-related NAC (SNAC) subgroup (Zhu et al. 2015) and were accordingly named *PeSNAC1–6*. *PeSNAC1* was isolated from moso bamboo leaves using RT-PCR with primers N-F and N-R. The open reading frame (ORF) was 867 bp, and the genomic sequence corresponding to ORF was 3675 bp including three exons and two introns. A multiple alignment revealed that PeSNAC1 shared a highly conserved N terminal DNA binding domain (>94.52 % homology) and a highly variable C-terminal transcriptional regulatory domain compared with NACs of other plants such as *Oryza sativa, Zea mays*, *Triticum aestivum*, and *Aarbidopsis thaliana* (S2), this finding indicates that *NAC*s have not been conserved during evolution. In addition, a putative miR164b-binding region was located downstream of the NAC domain in the *PeSNAC1* coding region (626–646 bp).

Two candidates belonging to the miR164 family (*ped*-miR164a and *ped*-miR164b) were identified in the bamboo genome database. The mature sequence of *ped*-miR164b, which was 21-bp long (5′-UGGAGAAGCAGGGCACGUGCU-3′), had a different nucleotide at its 3′ end compared with the standard mature sequence of miR164 (5′-UGGAGAAGCAGGGCACGUGCA-3′). This result indicates that the miR164 family sequence is indeed highly conserved (Koyama et al. 2010). The precursor of *ped*-miR164b was isolated by PCR using specific primers (F-pre164b and R-pre164b) designed based on the PH01000543 sequence. The 82-bp precursor sequence could form a stable stem-loop structure with the generated mature sequence on the 5′ end of the arm, consistent with miR164a and miR164b in Arabidopsis (Mallory et al. 2004). The promoter region sequence upstream of the *ped*-miR164b precursor, obtained using primers F-pro164b and F-pro164b, comprised 1397 bp containing essential promoter elements, such as the TATA-box and the CAAT-box. Some putative *cis*-acting regulatory elements, such as ABRE, MBS, and ARE (S3), were also identified, thus indicating that *ped*-miR164b may be involved in abiotic stress regulation.

Identification of the *ped*-miR164b-mediated cleavage site

According to PsRNATarget prediction, a strongly matching target site for miR164b was identified in the *PeSNAC1* ORF at 626–646 bp. To verify that *PeSNAC1* is the direct target of *ped*-miR164b, RLM-5′RACE was used to confirm the *ped*-miR164b-mediated cleavage site. A cleavage product generated by the processing of the *PeSNAC1* mRNA fragment by *ped*-miR164b was successfully detected (Fig. 1a); as confirmed by sequencing, this product was 119 bp long. Sequencing also revealed that 9 of 10 cDNA clones possessed this cleavage site, which was located in the middle of the *ped*-miR164b-*PeSNAC1* base-pairing interaction region and corresponded to the 11th nucleotide position of the mature *ped*-miR164b sequence (Fig. 1b). This result confirmed that transcripts of *PeSNAC1* are targeted for degradation by *ped*-miR164b at miR164-directed cleavage positions identical to those previously identified in the target mRNA in Arabidopsis (Guo et al. 2005) and *Z. mays* (Li et al. 2012).

Tissue-specific expression analysis of *ped*-miR164b and *PeSNAC1*

Because the expression patterns of miRNAs can provide clues to their functions (Yao et al. 2007), qRT-PCR was used to detect expression levels of *ped*-miR164b and *PeSNAC1* in different tissues of moso bamboo. According to the qRT-PCR analysis, both *PeSNAC1* and *ped*-miR164b were expressed in the examined tissues. The expression of *ped*-miR164b exhibited a declining trend from roots, sheaths, leaves, and stems, with the highest level in roots, the lowest in stems, and similar levels in leaves and sheaths (Fig. 2a). The expression of *PeSNAC1* showed an opposite trend to that of *ped*-miR164b, with the lowest level in roots and the highest in stems (Fig. 2b). This result suggests that *ped*-miR164b has a regulatory effect on the instant amount mRNA of *PeSNAC1*.

Ectopic expression of *ped*-miR164b in Arabidopsis

Six *ped*-miR164b overexpressing (OX-ped-miR164b) lines were obtained through resistance screening. These

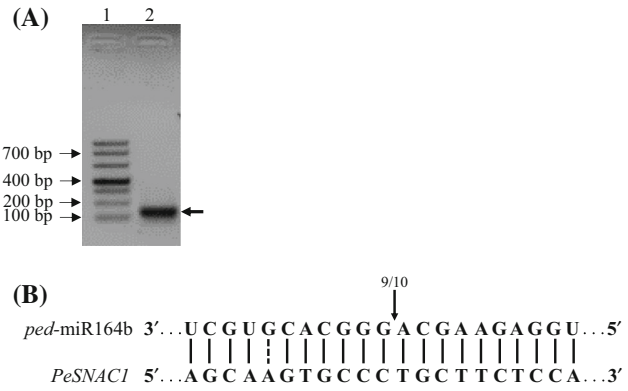

Fig. 1 Analysis of target site for *ped*-miR164b in *PeSNAC1* transcript. **a** The resulting agarose gel showed the nested PCR product that we cloned and sequenced, and the product was expected to be approximately 120 bp. *1* DNA ladder; *2* PCR product. **b** The frequency of 5′ RACE clones corresponding to the cleavage site (indicated by *arrow*) was shown as a fraction, with the number of clones matching the target message in the denominator

OX-ped-miR164b lines exhibited seriously suppressed growth and varying degrees of phenotypic changes. For instance, roots were shorter and lateral roots were fewer in the overexpressing lines than in Col-0 (S4), and some cotyledons and petioles were fused to different degrees, such as cup-shaped, small or dissymmetric leaves and fused petioles, resulting in failed seed production and even death (Fig. 3A). These phenotypes are similar to those of Arabidopsis *cuc2* enhancer mutants (Hibara et al. 2006), whose *CUC2* transcript is targeted for degradation by miR164 (Raman et al. 2008). The only two OX-ped-miR164b lines (miR-2 and miR-6) that were able to produce seeds without resistance segregation in the T_3 generation were further selected for gene expression analysis. According to the qRT-PCR results, *ped*-miR164b was much more highly expressed in the two transgenic lines than in Col-0 ($p < 0.01$) (Fig. 3B). In addition, expression analysis of endogenous genes such as *CUC1*, *CUC2*, and *NAC1* in miR-6 showed that they were all much more weakly expressed than in Col-0 ($p < 0.01$) (Fig. 3C). This result indicates that *ped*-miR164b can negatively regulate the expression of *CUC1*, *CUC2*, and *NAC1*.

We also generated transgenic Arabidopsis plants harboring the *ped*-miR164b promoter fused with *GUS*. GUS activity was detected in young roots, leaves, hypocotyls, and the shoot apical meristem of these plants (Fig. 4). The higher promoter activity of the gene in roots might indicate

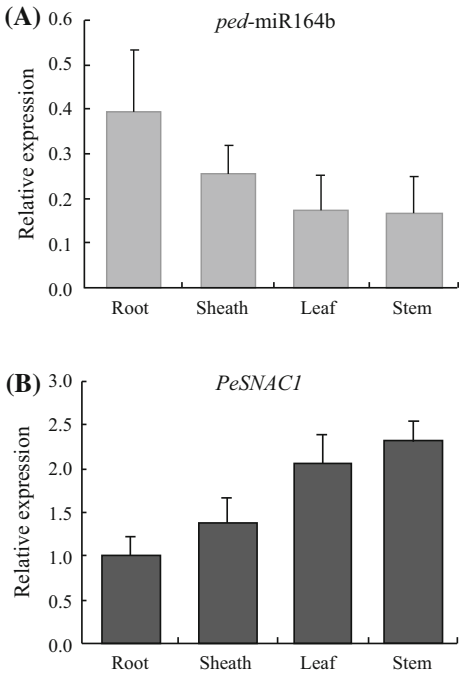

Fig. 2 Expression analysis of *ped*-miR164b and *PeSNAC1* in different tissues. **a** *ped*-miR164. **b** *PeSNAC1*. *Vertical bars* represent standard deviation of three independent biological replicates

that *ped*-miR164b participates in the regulation of root development.

Ectopic expression of *PeSNAC1* in Arabidopsis

Nine sense *PeSNAC1*-overexpressing (OX-PeSNAC1) lines and nine antisense OX-PeSNAC1 lines were acquired by hygromycin selection. Morphological observation revealed that the seedlings of sense OX-PeSNAC1 lines grew more rapidly and flowered earlier than Col-0, with growth and flowering of antisense OX-PeSNAC1 lines showing the opposite trend compared with Col-0. Although a significant difference in aboveground phenotypes was not observed between sense OX-PeSNAC1 lines and Col-0, OX-ped-miR164b lines and antisense OX-PeSNAC1 line A-1 had similar phenotypes, including small, dissymmetric or absent leaves that may have attributed to their failure to produce seeds (S5). Four sense OX-PeSNAC1 T_3 transgenic lines (S-1, S-6, S-8 and S-9) and four antisense OX-PeSNAC1 lines (A-1, A-2, A-8 and A-9) showing no evidence of segregation were the focus of further analysis. The results of semi-quantitative PCR analysis indicated that *PeSNAC1* was transcribed in both sense (Fig. 5a) and antisense (Fig. 5b) OX-PeSNAC1 transgenic plants but not in Col-0.

Root morphology of transgenic plants

To investigate the effects of OX-PeSNAC1 and OX-ped-miR164b on Arabidopsis roots, seedlings of S-1, A-2 and miR-6 lines were randomly selected for observation and measurement of lateral roots on vertical plates. In 7-day-old seedlings, no significant difference was observed in taproot lengths of transgenics and Col-0. At this time, no lateral root initials were apparent in Col-0, A-2 or miR-6, whereas short lateral roots and two to four lateral root initials were found in some S-1 transgenic lines (data not shown).

At 14 days after germination, taproot lengths of S-1 and A-1 were, respectively, slightly longer or slightly shorter than those of Col-0; however, these differences were not significant. In contrast, miR-2 taproot lengths were significantly shorter than those of Col-0 ($n = 20$, $p < 0.01$). The average number of lateral roots per centimeter of taproots of S-1 (3.52 ± 0.37) was higher than that of Col-0 (2.3 ± 0.55) ($p < 0.01$); this observation is consistent with findings in *ZmNAC1*-overexpressing plants (Li et al. 2012). On the other hand, average numbers of lateral roots per centimeter in primary roots of A-2 and miR-6 were 0.98 ± 0.28 and 1.83 ± 0.47, respectively, significantly less ($p < 0.01$) than the number in Col-0 (Fig. 6). These results indicated that both *ped*-miR164b and *PeSNAC1* are involved in root development, especially with respect to the number of lateral roots.

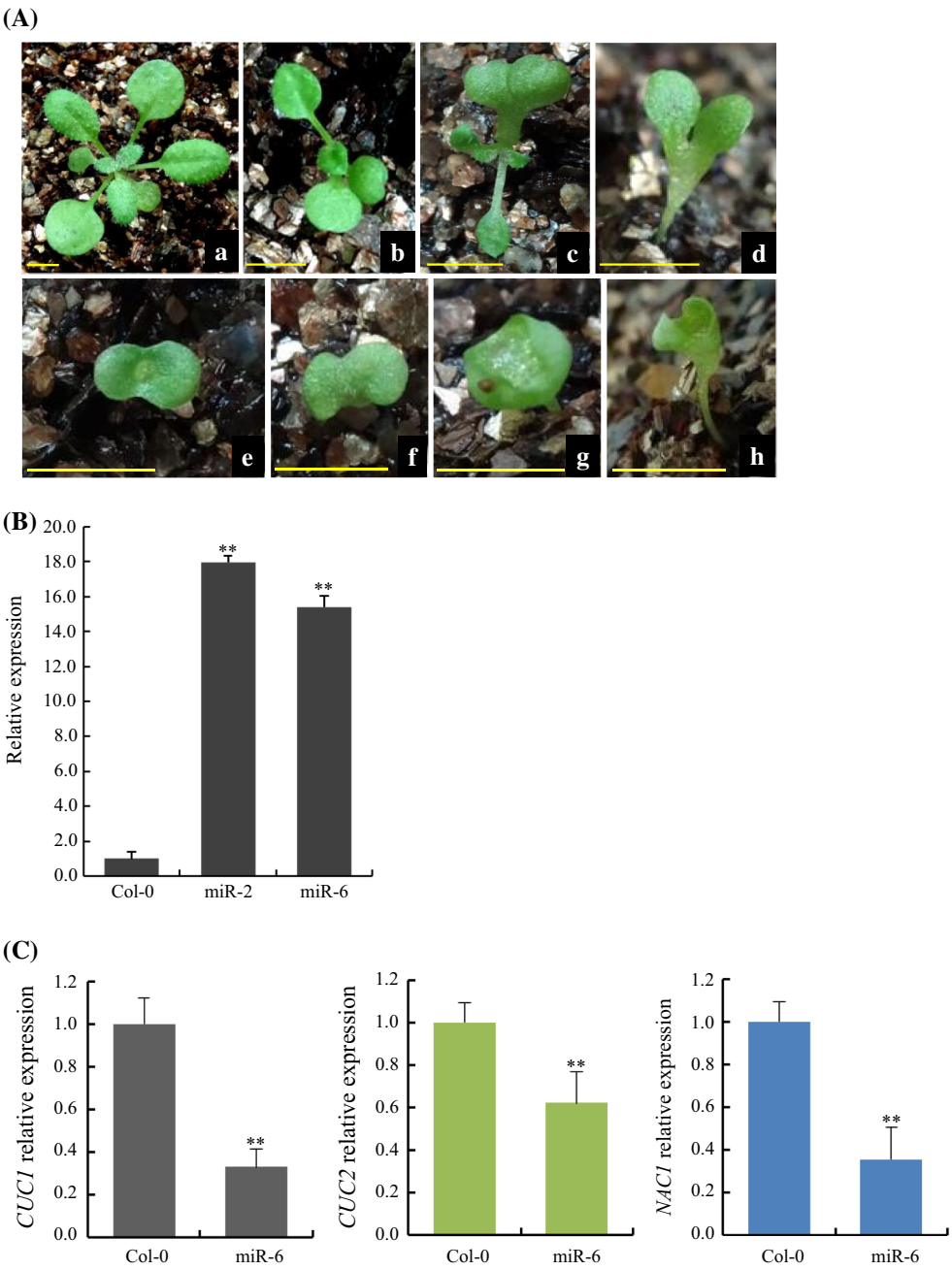

Fig. 3 Ectopic expression of *ped*-miR164b in *A. thaliana*. **A** Phenotype of *ped*-miR164b transgenic plants. *a* Wild type (Col-0); *b–h ped*-miR164b transgenic plants (*b* dissymmetric leaf; *c*, *d* fused petiole; *e*, *f* fused cotyledon; *g*, *h* cup shaped cotyledon). *Bar* 0.5 cm. **B** Expression analysis of miR164b in transgenic plants using qRT-PCR. **C** Expression analysis of *CUC1*, *CUC2* and *NAC1* in miR-6 and Col-0 using qRT-PCR. *Vertical bars* represent standard deviation of three independent biological replicates. *Asterisks* indicate the strongly significant difference between the transgenic lines and Col-0 ($p < 0.01$)

Analysis of transgenic plant resistance to stresses

To examine whether the transgenic plants exhibited tolerance to salinity and drought stresses, a comparative analysis was conducted using transgenic lines S-1, A-2 and miR-6 treated with NaCl and PEG6000. After 7 days NaCl treatment, A-2 and miR-6 seedlings exhibited severely inhibited phenotypes compared with Col-0, with leaf rolling and even death observed. In contrast, S-1 seedlings showed more tolerance than those of Col-0 (S6). The measurement of chlorophyll fluorescence kinetics parameters can be used as a quick, nonintrusive probe in studies of plants under stress. For example, the maximal photochemical efficiency (F_v/F_m) is an index to estimate the degree of photoinhibition (Maxwell and Johnson 2000). F_v/F_m values of Col-0 and all the transgenic lines displayed a downward trend during NaCl treatment. The only obvious difference among lines was observed after 4 days NaCl treatment. Compared with Col-0, F_v/F_m values of A-2 and miR-6 decreased rapidly, and that of S-1 declined slowly (S7). After 7 days treatment, F_v/F_m was maintained at a relatively high level in S-1; in contrast, the F_v/F_m value of A-2 was similar to that of miR-6, which remained at a lower level than that of Col-0 (Fig. 7a). No significant

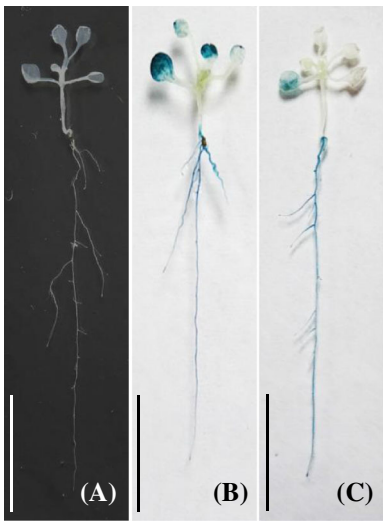

Fig. 4 Expression pattern of ped-miR164b promoter fused with *GUS* in *A. thaliana*. **a** Col-0; **b**, **c** different transgenic lines. *Bar* 1 cm

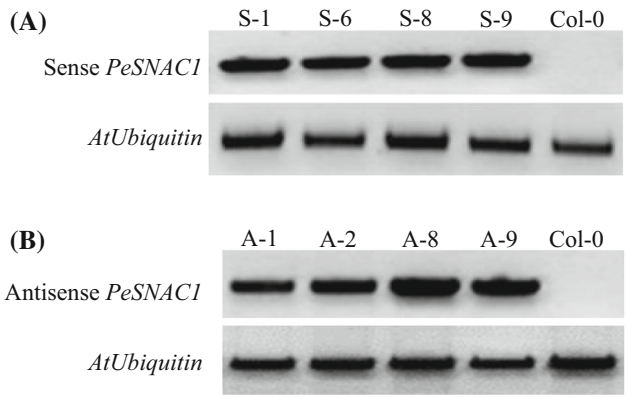

Fig. 5 Expression analysis of *PeSANC1* transgenic plants by semi-quantitative PCR. Col-0 was used as control. **a** Sense lines: S-1, S-6, S-8, and S-9. **b** Antisense *lines*: A-1, A-2, A-8, and A-9

difference in F_v/F_m was observed between transgenic plants and Col-0, but a significant difference ($p < 0.05$) was found between S-1 and miR-6. According to calculated statistics, the survival rate of S-1 (~90 %) was higher and those of miR-6 (~20 %) and A-2 (~30 %) were lower than that of Col-0 (~50 %) after 10 days NaCl treatment (Fig. 7b).

During drought stress, A-2 and miR-6 seedlings showed similar responses—yellowing and even dying. Compared with Col-0 (S8), seedlings of S-1 were the last to turn into yellow. Similar to the trend observed under salinity stress, F_v/F_m values in Col-0 and transgenic lines declined over the course of the drought stress treatment. During the first 6 days of drought treatment, there was no obvious difference in F_v/F_m values between transgenic plants and Col-0. At 9 days of treatment, F_v/F_m was obviously decreased, especially in miR-6 and A-2, with S-1 (S9) exhibiting the smallest decline. After 14 days treatment, F_v/F_m of miR-6 was significantly lower and that of S-1 was significantly higher than that of Col-0 ($p < 0.05$). Although the value of A-2 was lower than that of Col-0, the difference was not significant (Fig. 8a). After 20 days treatment with PEG6000, the survival rate of S-1 (~85 %) was higher and survival rates of miR-6 (~5 %) and A-2 (~2 %) were lower than that of Col-0 (~50 %) (Fig. 8b). These results indicate that *PeSNAC1* can confer abiotic stress tolerance on transgenic Arabidopsis.

Discussion

Following the description of the first identified NAC gene, *NAM* of petunia (Souer et al. 1996), multiple studies have expanded our knowledge of NAC TFs. Many NACs have been identified, including 138 NACs in Arabidopsis (http://planttfdb.cbi.pku.edu.cn/), 151 in rice (*O. sativa*; Nuruzzaman et al. 2010), 152 in soybean (*Glycine max*; Le et al. 2011), 79 in grape (*Vitis vinifera*; Wang et al. 2013), and 118 in *Brachypodium distachyon* (Zhu et al. 2015). The NAC family, one of the largest TF families in plants, is divided into 14 subfamilies. Although the exact functions of CUC (development-related NACs), VND (secondary wall-synthesis NACs), TIP (membrane-associated NACs), TERN (tobacco elicitor-responsive NAC) and SNAC subfamilies are unknown, they may have similar regulatory roles (Shen et al. 2009; Zhu et al. 2015). Because NAC TFs have multiple functions related to the regulation of plant growth and development, much attention has focused on their functional identification. The availability of the draft genome of moso bamboo (Peng et al. 2013) and BambooGDB database (http://www.bamboogdb.org/) have facilitated further analysis of bamboo TFs and miRNAs. All 125 NAC TFs and 93 conserved miRNAs have been functionally annotated in BambooGDB. Bioinformatics analysis of NACs in moso bamboo allowed us to verify the function of the *ped*-miR164b target gene *PeSNAC1* in this study.

miRNAs play vital regulatory roles in both animals and plants, mainly by promoting cleavage or translation inhibition of targeted mRNAs (Bartel 2004). The biological functions of miRNAs are intimately relevant to the functions of their target genes. Identification of potential target genes is therefore an effective and essential approach for in-depth investigation of complex miRNA-mediated regulatory mechanisms. In plants, miR164 was first predicted in Arabidopsis, with its mature sequence found to be fairly conserved (Rhoades et al. 2002); this finding has been confirmed by the identification of miR164 key recognition sites in *OMTN* genes of 158 rice varieties (Fang et al. 2014). Truncation of *PeSNAC1* mRNA by *ped*-miR164b

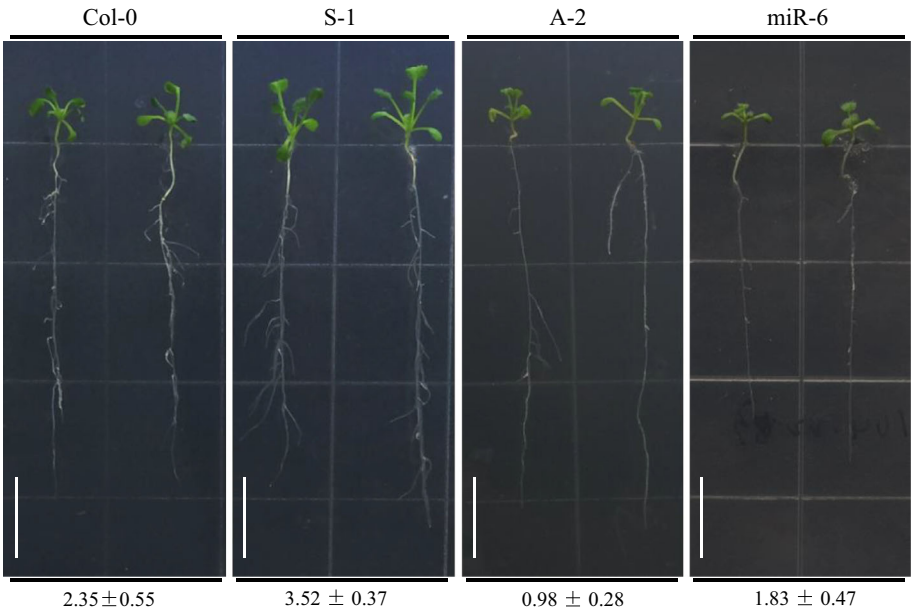

Fig. 6 Effects of overexpression of *PeSNAC1* and *ped-miR*164b on lateral root development. Col-0 and transgenic seedlings of S-1, A-2, and miR-6 were grown on MS medium with 2 % sucrose. Seedlings were photographed 14 days after germination. Lateral root numbers were counted with a binocular microscope directly. Numbers of lateral roots per centimeter of primary root of Col-0, S-1, A-2, and miR-6 were presented at *bottom* ($n = 20$). *Bar* 1 cm

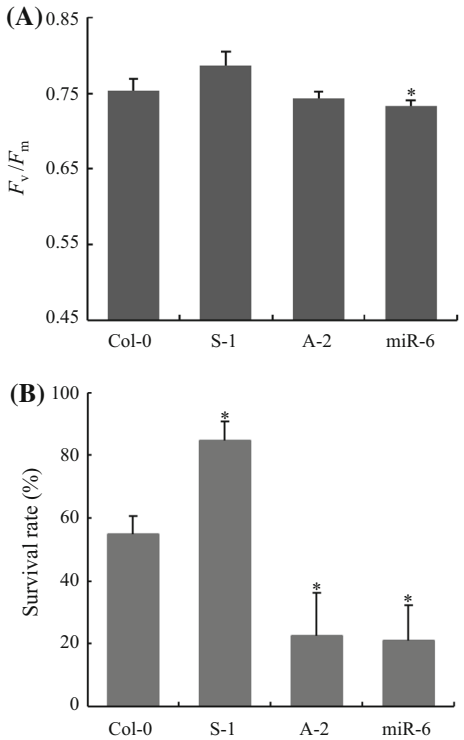

Fig. 7 Response of Col-0 and transgenic plants to salinity stress. **a** Comparison analysis of F_v/F_m between transgenic plants and Col-0 after 7 days NaCl treatment. *Vertical bars* represent standard deviation of ten replicates. *Asterisks* indicated significant differences between S-1 plants and miR6 ($p < 0.05$). **b** Survival rate analysis of transgenic plants and Col-0 after 10 days NaCl treatment. Cotyledon greening were determined as survival. *Vertical bars* represent standard deviation of the mean ($n = 3$, treatments with 60 seedlings). *Asterisks* indicate the significant difference between the transgenic lines and Col-0 ($p < 0.05$)

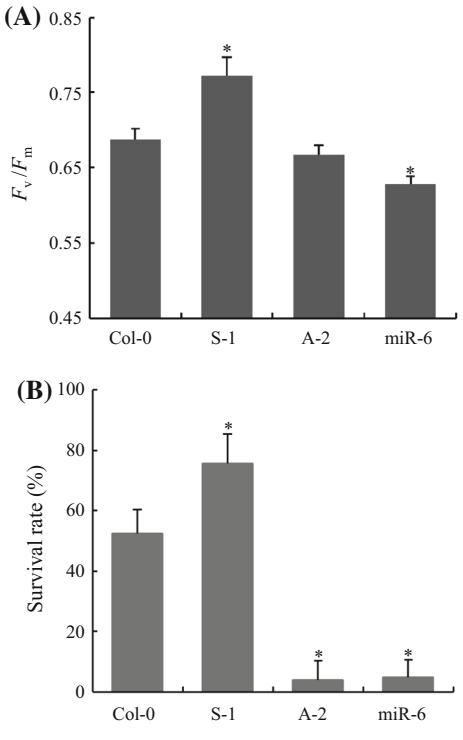

Fig. 8 Response of Col-0 and transgenic plants to drought stress. **a** Comparison analysis of F_v/F_m between transgenic plants and Col-0 after 14 days PEG6000 treatment. *Vertical bars* represent standard deviation of ten replicates. *Asterisks* indicated significant differences between transgenic plants and Col-0 ($p < 0.05$). **b** Survival rate analysis of transgenic plants and Col-0 after 20 days PEG6000 treatment. Cotyledon greening were determined as survival. *Vertical bars* represent standard deviation of the mean ($n = 3$, treatments with 60 seedlings). *Asterisks* indicate the significant difference between the transgenic lines and Col-0 ($p < 0.05$)

revealed that the cleavage site was between nucleotides 11 and 12 of the target site, consistent with findings in rice (Fang et al. 2014) and maize (Li et al. 2012). This result is consistent with the principle that miRNAs recognize their targets based on near-perfect sequence complementarity (Rhoades et al. 2002). The evolutionary conservation of miR164 suggests that the target sites may have important functions.

Different miR164 family members are present in plants, such as three members (ath-miR164a/b/c) in Arabidopsis (Moldovan et al. 2010), six (osa-miR164a/b/c/d/e/f) in rice (Sunkar et al. 2008), and two (*ped*-miR164a/b) in moso bamboo. Previous studies have demonstrated that miR164 can target five NAC domain-encoding mRNAs, namely, *NAC1*, *CUC1*, *CUC2*, *At5g07680*, and *At5g61430* mRNAs. In Arabidopsis, boundary establishment and maintenance is controlled by three partially redundant genes, *CUC1*, *CUC2*, and *CUC3* (Rhoades et al. 2002). miR164 is reported to target *CUC1* and *CUC2* mRNAs, but not that of *CUC3* for degradation in plants (Laufs et al. 2004). In addition, a single mutation of either *CUC* gene has been found to have no major effect on boundary formation, whereas double mutants have fused cotyledons that reflect abnormal boundary specification during embryo development (Raman et al. 2008). In our study, the overexpression of *ped*-miR164b in Arabidopsis resulted in phenotypes similar to those of *cuc2* mutants. We thus hypothesized that *ped*-miR164b constrains the boundary formation by randomly reducing the expression of some related targets including *CUC1* and *CUC2*. This prediction was confirmed by the down-regulation of *CUC1* and *CUC2* in OX-ped-miR164b plants.

Proteins with similar domains may have identical or similar biological functions (Lin et al. 2007). miR164-targeted NAC genes have been reported to participate in the regulation of growth and development in Arabidopsis (Mallory et al. 2004; Guo et al. 2005; Li et al. 2012). Our alignment indicated that PeSNAC1 share a highly conserved domain with NACs in other plants, which implies that *PeSNAC1* may also function in growth and developmental processes. Small-leaf, dissymmetric-leaf and afoliate phenotypes were observed in antisense OX-PeSNAC1 plants, very similar to OX-ped-miR164b plant phenotypes. Antisense OX-PeSNAC1 plants had fewer lateral roots than Col-0 plants, while sense OX-PeSNAC1 plants had more. On the basis of these observations, we speculate that *PeSNAC1* together with *ped*-miR164b participates in the regulation of organ boundary maintenance and normal development in bamboo—an idea that requires further confirmation.

To fully understand whether ectopic expression of *ped*-miR164b or *PeSNAC1* alters the seedling tolerance, we analyzed survival rates under salinity and drought stresses. Sense OX-PeSNAC1 plants exhibited a higher survival rate than Col-0 on NaCl and PEG6000 media, while rates of OX-ped-miR164b and antisense OX-PeSNAC1 plants were relatively lower. Chlorophyll fluorescence measurement is an effective internal probe for the study of plant photosynthesis under stress. Measurement of F_v/F_m under drought stress indicated that the intrinsic efficiency of photosystem II in OX-ped-miR164b and antisense OX-PeSNAC1 plants fell more rapidly, while that of sense OX-PeSNAC1 plants fell more slowly compared with Col-0, indicating that sense OX-PeSNAC1 plants are more stress tolerant than Col-0. However, further researches are required to identify the detailed function of TFs and miRNAs in bamboo.

Author contribution statement Conceived and designed the experiments: ZMG. Performed the experiments: LLW, HSZ, DLC. Analyzed the data: HYS, LCL, LLW. Contributed reagents/materials/analysis tools: YFL, ZMG. Contributed to the writing of the manuscript: LLW, ZMG.

Acknowledgments This work received financial support from the Special Fund for Forest Scientific Research in the Public Welfare from State Forestry Administration of China (No. 201504106) and the Sub-Project of National Science and Technology Support Plan of the Twelfth Five-Year in China (No. 2015BAD04B01 and No. 2015BAD04B03).

Compliance with ethical standards

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

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