MiR164-*TaNAC14* module regulates root development and abiotic-stress tolerance of wheat seedlings

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Abstract Previous studies have uncovered the miR164 family and the miR164-targeted NAC transcription factor genes in rice (*Oryza sativa*) and *Arabidopsis* that play versatile roles in developmental processes and stress responses. In wheat (*Triticum aestivum* L.) we find nine genetic loci of tae-miR164 (*tae-MIR164a* to *i*) producing two mature sequences that down-regulate the expression of newly identified target genes of *TaNACs* (*TaNAC1*, *TaNAC11*, and *TaNAC14*) by the cleavage of the respective mRNA. Overexpression of tae-miR164 or one of its target genes *TaNAC14* demonstrated that miR164-*TaNAC14* module greatly affected root growth and development and stress (drought and salinity) tolerance of wheat seedlings, *TaNAC14* promotes root growth and development of wheat seedlings and enhanced drought tolerance, while miR164 inhibits root development and reduces drought- and salinity-tolerance by down-regulating the expression of *TaNAC14*. Our findings identify the miR164-*TaNAC14* module as well as other tae-miR164 regulated genes to generate new genetic resources for stress-resistance wheat breeding.

Keywords: *Triticum aestivum*, tae-miR164, miR164-targeted *TaNACs*, miR164-*TaNAC14* module, growth and development, abiotic-stress tolerance[1]

1. Introduction

Wheat (*Triticum aestivum* L.) is one of the most important staple crops worldwide, and its yield is directly related to human food security. The data from FAO (UN Food and Agriculture Organization) shows that the total wheat production worldwide in 2018/2019 year is about 731 million metric tons, but the total consumption is about 733 million metric tons, a gap existing between wheat supply and demand. With the increase in world population and the decrease in arable land area brought by rural urbanization, the food security problem becomes more and more prominent. Due to the frequent occurrence of extreme conditions worldwide over recent years, potential global wheat production was declined by more than 5% relative to what would achieved in case it didn't happen (Lobell *et al*. 2011). Therefore, high yield and tolerance to adverse environmental conditions are always the main targets in wheat breeding program.

MicroRNAs (miRNAs) are a class of small endogenous single-strand noncoding RNAs of 21 to 24 nucleotides, which are widely distributed in animals and plants (Lee *et al*. 2004; Chen 2009). In plants, miRNAs exhibit near-perfect complementarity to their targeted mRNAs and negatively regulate gene expression at post-transcriptional levels through the cleavage and/or translational suppression of the target mRNAs (Chen 2004; Bartel 2009). The miRNAs are diverse in sequence, expression and

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function (Cuperus *et al*. 2011), with some miRNAs being conserved at an interspecies level, while others being species-specific or tissue-specific.

As powerful post-transcriptional regulators in plants, many miRNAs participate in modulating plant growth and development (Chen 2009; Wang *et al*. 2011; Zhang *et al*. 2018), the transition from vegetative to productive phase (Lauter *et al*. 2005; Wang *et al*. 2015; Jian *et al*. 2017) and seed development (Zhu *et al*. 2008; Curaba *et al*. 2012; Han *et al*. 2014). For example, miR156 and miR529 can finely regulate the expression level of *OsSPL* family members in rice, which regulate miR172/*AP2* and *PAP2*/*RCN1* pathways integrating other regulatory factors to form optimal spike types (Wang *et al*. 2015). The miR396-*OsGRF6* module regulates rice spike grain number and spike branch development related transcription factors by regulating auxin synthesis and signal transduction (Li *et al*. 2016). In *Arabidopsis*, miR159 is essential for the correct timing of vegetative development, miR159 targets an R2R3 MYB domain transcription factor *MYB33*, and miR159/*MYB33* module functions as modifiers of vegetative phase change (Guo *et al*. 2017). More recently, it was elucidated in rice that miR156, miR529 and miR535 can target the *OsSPL*s family of controlling factors to regulate tillering number, spike branching number, grain size and quality by down-regulating target gene expression (Peng *et al*. 2019).

Some other miRNAs play vital regulatory function in plant responses to biotic and abiotic stress (Xin *et al*. 2010; Kantar *et al*. 2011). For example, miR408, one of the most conserved plant miRNAs, participates in responses to abiotic stress, including salinity stress, osmotic and drought stresses by down-regulation of its target genes Plantacyanin (At2G02850), Cupredoxin (At1G72230), Laccase (At2G30210), and Uclacyanin (At2G44790) in *Arabidopsis*, emphasizing the central function of miR408 in plant survival (Ma *et al*. 2015). Overexpression of miR408 in *Arabidopsis*, rice, and tobacco (*Nicotiana tabacum* L.) showed that higher expression enhances photosynthesis, growth, and seed yield in diverse plants (Pan *et al*. 2018). *TaMIR1119*, a miRNA family member of wheat, plays critical roles in regulating plant drought tolerance through transcriptionally regulating the target genes that modulate osmolyte accumulation, photosynthetic function, and improve cellular ROS homeostasis of plants (Shi *et al*. 2018).

The miR164 family is one of the most conserved miRNA in plants. Previous studies in *Arabidopsis* have elucidated that the miR164 family consists of three members (ath-miR164a/b/c) that perform the cleavage of the mRNAs of five NAC (NAM, ATAF1/2, and CUC2) transcription factor genes (*NAC1*/At1g56010, At5g61430, At5g07680, *CUC1*/At3g15170, and *CUC2*/At5g53950) to regulate developmental processes (Mallory *et al*. 2004; Guo *et al*. 2005). In rice, the miR164 family is composed of six members (osa-miR164a-f) that target nine genes, of which, six (*OMT1*–*OMT6*) are from the NAC family. The conserved miR164-targeted NAC genes (*OMTN2*, *OMTN3*, *OMTN4*, and *OMTN6*) negatively regulate drought tolerance as well as development process (Fang *et al*. 2014). In wheat, it was demonstrated that miR164 family target a group of highly conserved *NAC*s and a new class of less conserved phytosulfokine (PSK) precursor gene by miRNAome and degradome sequencing of developing wheat grains (Li *et al*. 2015; Geng *et al*. 2020). However, compared to *Arabidopsis* and rice, our knowledge of the miR164 family and miR164-target NAC genes in wheat is very limited to date, even though wheat is the most important staple crop for human life worldwide.

In the current study, we conducted genome-wide identification of the miR164 family and the miR164-targeted NAC genes in wheat based on newly published wheat genome reference sequence database IWGSC RefSeq v2.0 (http://www.wheatgenome.org/), performed expression profiling of tae-miR164 and the miR164-targeted *NACs* across different wheat tissues/organs or in diverse abiotic-stress conditions. In addition, we explored the structural characteristics of tae-miR164 and the miR164-targeted *NAC* and their functions in wheat development and responses to abiotic stresses.

The main target of this study was to uncover the biological function of miR164-*NACs* modules in wheat and to provide candidate genes for high yield or stress-resistant wheat breeding.

2. Materials and methods

2.1. Plant materials and growth conditions

Winter wheat (*T. aestivum* L.) cultivar Xiaoyan 6 was used in this experiment for analyzing expression patterns of tae-miR164 and its target gene *TaNACs*. Wheat plants were grown under natural conditions in the experimental fields of Northwest A & F University, Yangling, China (108°E, 34°15'N) in the growth seasons in 2017 to 2019. Ten wheat tissues/organs, which included root (R), leaf (L) and stem (S) of wheat seedlings at five-leaf stage, flag leaf (FL) from wheat plant at booting and heading stages, young spike 5 cm long (YS5) from wheat plants at booting stage and spike 10 cm long (YS15) from wheat plant at heading stage, grains 5, 10, 15, and 20 days post-fertilization (GR5, GR10, GR15, and GR20, respectively), were collected and immediately frozen in liquid nitrogen and kept at -80℃ for further usage, three independent biological replicates for individual tissues/organs being included.

Wheat cultivar Chinese spring was used for cloning of tae-MIR164 and its target genes *TaNACs*. Wheat seeds were germinated for one week at room temperature, and then transplanted into pots with ten plants in each. The plants were grown in a growth chamber with 16/8 h light/dark cycle, temperature regime of 20-25/15-18℃, relative humidity of 50-60% and watered as needed. The leaf samples were collected from wheat plants at three-leaf stage and immediately frozen in liquid nitrogen and kept at -80℃ for further usage.

To explore the expression profiles of tae-miR164 and the miR164-targeted *NACs* under various abiotic stresses and phytohormone treatments, the seedlings of wheat *cv*. Xiaoyan 6 were grown in growth chamber as described above for 2 weeks, and then stress- or phytohormone-treatment was applied. For drought stress, two-week-old seedlings were washed gently with water to remove soil around root, and then transferred into Hogland solution with 15% PEG-6000 (Hoagland and Arnon 1937). For high salinity treatment, the two-week-old seedlings were transferred to 0.2 mol L^{-1} NaCl solution. For cold stress, the seedlings were transferred to a growth chamber at temperature at 4℃. Commonly used plant growth regulators, including plant growth promoter indole-3-acetic acid (IAA) and kinetin (KT) and plant growth retarder abscisic acid (ABA), were used for phytohormone treatments, i.e., 0.1 mmol L^{-1} of ABA, 0.1 mmol L^{-1} of IAA, or 0.1 mmol L^{-1} of KT were sprayed into the leaves of the wheat seedlings. Leaf samples were collected at different time-points (0, 1, 3, 6, 12, and 24 h) under various treatments and immediately frozen in liquid nitrogen and kept at -80℃ for total RNA isolation.

2.2. Genome-wide identification of *TaMIR164* in wheat

One mature sequence of tae-miR164 and its precursor sequence (tae-MIR164) are registered in miRBase database/*Triticum aestivum* (http://www.mirbase.org/textsearch.shtml?q=Triticum+aestivum), and six sequences are annotated as miR164 precursors in newly published wheat genome reference sequence database IWGSC RefSeq v2.0 (http://www.wheatgenome.org/). The seven precursor sequences were compared with each other and blasted against the IWGSC RefSeq v2.0 in the present study, in order to define all the members of *tae-MIR164* family in wheat. The recovered potential precursor sequences of tae-miR164 in wheat genome were used to conduct structure prediction through the RNA Folding Form (http://unafold.rna.albany.edu/?q=mfold/ RNA-Folding-Form) in order to confirm that the precursors can correctly fold into hairpin secondary structures. The potential tae-miR164s predicted from the stem-loop MIR164s ~~should~~ were expected to meet the common criteria described previously (Yan *et al*. 2012).

2.3. Genome-wide prediction of miR164 target gene *NACs* in wheat

All the *NAC* transcript sequences retrieved from IWGSC RefSeq v2.0 (http://www.wheatgenome.org/) and the mature sequences of tae-miR164 in wheat were used to predict miR164-targeted *TaNAC*s through online software psRNA Target (http://plantgrn.noble.org/v1_psRNATarget/), based on the near-perfect complementary principle (with no more than four mismatches) and the criteria reported previously (Rhoades *et al*. 2002).

2.4. Total RNA isolation and cDNA synthesis

Wheat tissue/organ samples collected above were used for RNA isolation. Total RNA was isolated using Total RNA Rapid Extraction Kit for Polysaccharides Polyphenol Plant (Biotech, Beijing, China) according to the manufacturer's instructions. First-strand cDNA was synthesized using TaKaRa cDNA synthesis kit (TaKaRa, Dalian, China).

2.5. Cloning of *tae-MIR164* and the cDNA of putative miR164- targeted *TaNACs* in wheat

The sequence of tae-MIR164 was retrieved from the miRBase (http://mirbase.org/index.shtml), and then searched against wheat genome sequence for the genome sequence of *TaMIR164*. The region covered the precursor sequence was selected to design primers. The gDNA of Chinese spring isolated with cetyltrimethylammonium bromide (CTAB) method (Porebski *et al*. 1997) was used as amplification template to clone tae-MIR164. The primer sequences are shown in Appendix A.

To clone the cDNA of putative tae-miR164 target genes *TaNACs*, primers were designed based on the sequences of putative target genes *TaNACs* retrieved from IWGSC Ref v 2.0, and the coding sequences (CDS) of *TaNACs* were amplified using the cDNA synthesized from the mixed total RNA of roots, leaves and developmental grains of Chinese spring. The primer sequences used in this experiment are shown in Appendix A. More than ten clones were sequenced and compared to obtain the full-length cDNA of individual *TaNACs*.

2.6. Transient co-expression assay of tae-MIR164 and its putative target *TaNACs* in the leaves of *Nicotiana benthamiana*

To confirm whether the putative target *TaNAC*s can be targeted and their expression can be down-regulated by tae-miR164, we developed the expression vectors of tae-MIR164a (Pro35S::MIR164) and the CDS of individual *TaNAC*s fused with reporter gene β-glucuronidase (*GUS*) (Pro35S::TaNACs -GUS) (the protocols of the expression vector construction were described in the following paragraph). We also generated tae-miR164 resistant version *TaNAC* mutants (*TaNACsm* for short) by synonymous substitutions of nucleotides at the tae-miR164 binding sites in *TaNACs* according to the protocol of site overlap extension-PCR-mutagenesis (Ho *et al*. 1989) using mutant forward primer TaNACsm F and reverse primer TaNACsm R shown in Appendix A, in order to develop fusion expression vectors Pro35S::TaNACsm-GUS that were used as positive controls.

To develop above constructs, we used plasmid pCAMBIA3301 as the expression vector, the CDS of the protein coding genes (*TaNACs* and *TaNACsm*) with restriction sites in 5'- and 3'-end (XbaI and BglII) were subcloned into pCAMBIA3301 between the *CaMV35S* promoter and the *GUS* gene, generating a fusion protein gene TaNACs-GUS driven by the *CaMV35S* promoter, and the precursor sequences of

miR164 with restriction sites in 5'- and 3'-end (Nco I and Nhe I) were subcloned into pCAMBIA3301 between the *CaMV35S* promoter and the *NOS* terminator, generating miR164 overexpression vector. The resulted constructs Pro35S::MIR164, Pro35S::TaNACs-GUS, and Pro35S::TaNACsm-GUS were first confirmed by sequencing, and then introduced into *Agrobacterium* strain *GV3101* by freeze-thaw method. Transformation of *N. benthamiana* leaves were conducted according to the method previously described by Liu and Axtell (2015). The *Agrobacterium* strain (OD_{600}=0.5) harboring certain construct above that suspended in infiltration media (88.85 mL water, 1 mL of 1 mol L^{-1} $MgCl_2$ stock, 10 mL of 100 mmol L^{-1} MES stock, and 150 μL of 100 mmol L^{-1} acetosyringone stock) were transformed into the leaves of *N. benthamiana* one-month old plants with 3-5 young leaves. The *Agrobacterium* strain harboring expression vectors Pro35S::GUS was used as controls. After injection, the leaves were incubated at 25℃ for 3 days. Histochemical staining of GUS was assayed as described by Jefferson *et al*. (1987).

2.7. Quantitative real time reverse transcriptase-PCR (qRT-PCR)

For quantification of tae-miR164 abundance in wheat samples, the cDNA of each wheat sample was used as a template, *U6* was used as an internal control, and a stem-loop qRT-PCR method was performed as described previously (Chen *et al*. 2005) .

For determining the expression levels of miR164-targeted *TaNACs* in wheat samples, the cDNA of each wheat sample was used as a template, *Actin* (GenBank: MF405765.1) was used as an internal control, and qRT-PCR was performed as reported previously (Chi *et al*. 2019). The sequences of the primers used for qRT-PCR were shown in Appendix A. The qRT-PCR was conducted on CFX96 real-time PCR (Bio-Rad) with three replicates for each sample using GoTaq® qPCR Master Mix (Promega). CT (the threshold cycle above background) values were calculated using Bio-Rad Cycler software. The relative expression levels of the tested genes were analyzed according to the comparative $2^{-\Delta\Delta CT}$ method reported previously (Livak and Schmittgen 2001).

2.8. Subcellular localization of TaNAC14

To determine the subcellular localization of TaNAC14, the full-length coding sequence of *TaNAC14* from B genome was amplified and fused upstream of GFP in the p16318-GFP expression vector driven by CaMV35S promoter to generate the construct p16318-TaCYP78A5-GFP. The control vector p16318-GFP and The p16318-TaCYP78A5-GFP and control vector p16318-GFP were separately transferred into wheat mesophyll protoplasts by polyethylene glycol (PEG)-mediated transient expression system followed by incubation in the dark at 25℃ for 15h (Yoo *et al*. 2007), with the expression vector of the NUCLEAR LOCALIZATION SEQUENCE short peptide (NLS)-mCherry that is nuclear localization as a marker. Transformed cells were observed with a fluorescence microscope (DMi8, Leica).

2.9. Transcriptional activity assay of TaNAC14 in yeast

To test the transcriptional activity of TaNAC14, the experiment was carried out following the manual of Matchmaker™ Gold Yeast Two-Hybrid System in the yeast system. The coding sequence of *TaNAC14* with restriction sites (Nde I and EcoRI in 5'- and 3'-end, respectively) was constructed into yeast expression plasmid pGBKT7 vector to obtain pGBKT7-TaNAC14, the primer pair used to amplify the full-length coding region of *TaNAC14* being listed in Appendix A. The recombinant construct was

confirmed by sequencing and then transformed into the strain of Y_2H Gold using PEG-LiCl method (Gietz and Schiestl 2007), with empty pGBKT7 as a negative control. The transcriptional activity of TaNAC14 was verified on the synthetic dropout medium.

2.10. Construction of *TaNAC14* overexpression vector and development of transgenic wheat lines

The coding sequence of *TaNAC14* with restriction sites (XbaI and Nhe I in 5'- and 3'-end, respectively) was amplified and subcloned into pCAMBIA3301 between the *CaMV35S* promoter and the *NOS* terminator, generating *TaNAC14* overexpression vector Pro35S::TaNAC14. The resulted construct was first confirmed by sequencing and then transformed into *Agrobacterium tumefaciens* strain *EHA105*.

The embryogenic calli of the immature embryo 15 days post-anthesis of spring wheat line JW1 was used as recipient materials, and the *Agrobacterium tumefaciens* strain *EHA105* harboring the construct Pro35S::TaNAC14 was used as medium to conduct wheat transformation according to the protocol described previously (Ishida *et al*. 2015). The positive transgenic plants were identified by applying 0.2% glufosinate (BASTA) to leaves, and the copy number of the transgene *TaNAC14* in the transgenic plants of T_0 generation was determined by *TaqMan* probe real-time quantitative PCR method as described previously (Zhang *et al*. 2020). Transgenic lines were obtained by continuous self-crossing of single-locus transgenic plants and screening by BASTA.

2.11. Phenotypic determination and salinity tolerance identification of wheat seedlings

The selected full seeds of different transgenic lines of T_3 generation and wild type wheat JW1 were sterilized with 1% sodium hypochlorite solution for 15 min and then washed with sterile water three times. These sterile seeds were immersed in sterile water at room temperature for 24 hours and germinated in petri dishes for 4 days. Seedlings of the same size were selected and transferred to tubes with Hoagland's nutrient solution and cultivated for 14 days, renewing the nutrient solution ever three days. The principal root length and root number of wheat seedling were measured at one-leaf stage (7 days) and two-leaf stage (14 d), at least 20 plants of each transgenic line and wild type wheat were tested.

Two-leaf seedlings of each genotype wheat line were classified into two groups. One group was subjected to salinity-stress by cultivating in Hoagland nutrient solution with increasing concentrations of NaCl for 22 days, i.e., the initial concentration of NaCl was 100 mmol L^{-1} and increase by 50 mmol L^{-1} every 3 days until the concentration reached 300 mmol L^{-1}, and maintain this concentration for 10 days. The other group as control was cultivated in Hoagland nutrient solution for the same time. Fresh weight for each plant of different genotype line was recorded immediately before and after salinity-treatment, and fresh weight for each plant was also recorded in the control group. The increase of biomass per plant of different genotype wheat lines and wild type wheat JW1 in salinity-treatment group or control group undergoing 22-day-cultivation was determined. At least 15 plants of independent transgenic lines and wild type wheat were tested for a replicate, and three independent biological replicates were included. The survival rates of the different plant lines were recorded. More than 50 plants from individual transgenic lines and wild type wheat were used to conduct the experiment, and three independent biological replicates were included.

2.12. Drought tolerance evaluation of wheat seedlings

The plump seeds of transgenic wheat T3 lines and wild type wheat JW1 were planted in pots (six plants for each pot) with 2:1 soil:vermiculite in greenhouse (with 16/8 h light/dark cycle, temperature range of 20-25/15-18℃, and humidity of 60%) and watered as needed. Two-leaf wheat seedlings grown in soil were subjected to drought-stress treatment by withholding water for 16 days till soil heavy-drought (the soil water capacity reaches 30% of the maximum soil water capacity) and followed by rewatering seven days for recovery, and then the survival rates of the different plant lines were recorded. More than 50 plants from individual transgenic lines and wild type wheat were used to conduct the experiment, and five independent biological replicates were included. Leaf relative water content (RWC) of different genotype wheat plants was determined by using the second leaves fully expanded from three-leaf stage seedlings before drought-stress and after drought treatment by withholding water for 6 and 13 days, respectively, according to protocol as described previously (Lu *et al*. 2009)

The water loss rate (WLR) of the leaves from different genotype wheat plants was evaluated according to the protocol reported previously (Hu *et al*. 2020) with some modification. The second leaves fully expanded from three-leaf stage seedlings of individual transgenic lines were cut and their fresh weights were recorded immediately. The leaves were placed on dry dishes in a growth chamber, which posses a temperature of 22−25°C and a relative humidity of 50−60%, and were weighed every 10 min within the first hour, and then weighed at 3, 6, 12, and 24 h, and recorded as $W_{10\ min}$, $W_{20\ min}$, $W_{30\ min}$, $W_{40\ min}$, $W_{50\ min}$, $W_{60\ min}$, $W_{3\ h}$ to $W_{24\ h}$, respectively. The WLR for each time points was determined by the formula: WLR%=[(FW-Wt)/FW]×100, whereas, Wt refers to $W_{10\ min}$ to $W_{24\ h}$. More than five leaves from three-leaf stage seedlings of individual lines were used for the analysis and three biological experiments were conducted.

2.13. Statistical analysis

Statistical analysis was performed using SPSS software (http://www.spss.com/spss) and statistical contrasts were conduct by *t*-test.

3. Results

3.1. Genome-wide identification of tae-miR164 family members and their sequence in wheat

Previous studies have elucidated the miR164 family and the miR164-targeted *NAC* genes in *Arabidopsis* and rice, however, little is known about the miR164 family and its target genes in wheat. Only one mature sequence of tae-miR164 and its precursor tae-MIR164, which was named as tae-MIR164a in this study, is published in miRBase (http://www.mirbase.org, Release 22.1, Oct. 2018). In order to identify all the miR164 family in wheat, the sequence of the tae-MIR164a was retrieved from the miRBase and blasted against wheat genome reference sequence (IWGSC RefSeq v2.0). Consequently, nine genetic loci (*tae-MIR164 a* to *i*) were identified to encode eight precursors of tae-miR164 that generate two mature sequences of tae-miR164, the loci *tae-MIR164d* and *tae-MIR164f* generating the same precursor and the same mature sequence of tae-miR164 (Appendix B, Appendix C and D). The loci *tae-MIR164a/b/c* and *tae-MIR164d/e/f* locating in chromosome 1A, 1B, and 1D, respectively, produce mature sequence (5'-TGGAGAAGCAGGGCACGTGCA-3'); while the loci *tae-MIR164g/h/i* locating in chromosome 6A, 6B, and 6D, respectively, produce mature sequence (5'-TGGAGAAGGAGCGCACGTGCT-3') (Appendix D and Fig. 1-A).

The phylogenetic analysis of the miR164 family in wheat and the miR164 family from three related species, including *Arabidopsis*, *Oryza sativa*, and *Brachypodium distachyon*, exhibited that *tae-MIR164a/b/c* are the orthologous genes of *osa-MIR164b* and *bdi-MIR164f*, *tae-MIR164d/e/f* are the

orthologous genes of *osa-MIR164e* and *bdi-MIR164e*, while *tae-MIR164g/h/i* are the orthologous genes of *osa-MIR164d* and *bdi-MIR164c* (Fig. 1-B).

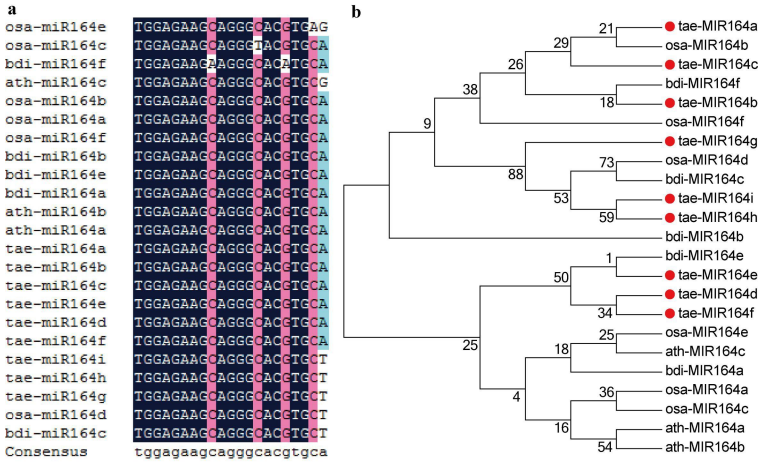

Fig. 1 Conservation of miR164 mature sequences and phylogenetic analysis of tae-MIR164 family in wheat. A, the conservation of miR164 mature sequences derived from nine genetic loci of *Tae-MIR164* in wheat genome. B, the phylogenetic relationships of the tae-MIR164 family in wheat and three related species. The sequences of tae-MIR164s in wheat were retrieved from wheat genome sequence IWGSC Ref v 2.0; the sequences of MIR164s in *Brachypodium distachyon* (bdi-MIR164s), *Arabidopsis* (ath-MIR164s), and *Oryza sativa* (osa-MIR164s) were obtained from miRBase. The red circles denote tae-MIR164s in wheat. Phylogenetic tree was generated by MEGA 6.0 using the Maximum likelihood (ML) method with 500 bootstrap replicates.

3.2. Prediction and validation of miR164-targeted *NAC* genes in wheat

In *Arabidopsis*, all the five miR164-targeted genes identified are from *NAC* family. While in rice, nine miR164-targeted genes were identified, of which six belong to *NAC* genes, two phytanoyl-CoA dioxygenase genes, and one phytosulphokine precursor gene (Fang *et al*. 2014). In wheat, five *NAC* genes, including *TaNAC7*, *TaNAC21/22*, *TaNAC23*, *TaNAC92*, and *TaCUC1*, were previously predicted to be miR164 targets (Geng *et al*. 2020). In the presently study, to identify all the miR164-targeted *NAC* genes in wheat, all the *NAC* transcripts from wheat reference genome sequence IWGSC RefSeq v2.0 and the mature sequences of tae-miR164 were used to prediction miR164 targets through online software psRNA Target (http://plantgrn.noble.org/v1_psRNATarget/), based on the near-perfect complementary principle (with no more than four mismatches) and the criteria reported previously (Rhoades *et al*. 2002). Consequently, six *NAC* genes (*TaNAC1*, *TaNAC7*, *TaNAC11*, *TaNAC14*, *TaNAC21/22*, and *TaNAC92*) containing tae-miR164 complementary sites were identified as putative tae-miR164 targets, each with three homoeologs in A, B and D subgenome (Appendix E-a, Appendix F). The miR164 binding sites (miR164BS) of these miR164-targeted *TaNAC*s located downstream of the NAC domain in the coding regions (Appendix E-b). Of the six miR164-targeted *TaNACs*, *TaNAC7*, *TaNAC21/22*, and *TaNAC92* were previously documented as a miR164 target genes (Feng *et al*. 2014; Geng *et al*. 2020), the other three (*TaNAC1*, *TaNAC11*, and *TaNAC14*) are newly identified miR164-targeted *NAC* genes in wheat in the present study, even though the functions of the *TaNAC1* has been reported previously (Meng *et al*. 2013). The biological role of the miR164-targeted three *TaNACs* (*TaNAC11*, *TaNAC14*, and *TaNAC92*) remains unclear.

To verify the accuracy of the tae-miR164 target prediction above, we first introduced mutations into miR164BS of the newly identified miR164 targets *TaNACs* (*TaNAC1*, *TaNAC11*, and *TaNAC14*) to

generate mutant genes *TaNACms* which were deregulated by miR164, previously identified miR164 target *TaNAC7* also included (Appendix E-c). Then, we conducted transient co-expression of tae-MIR164a and the fusion expression vectors of *TaNACs* (Pro35S::TaNACs-GUS) or miR164-resistant version *TaNACms* (Pro35S::TaNACms-GUS) in *Nicotiana benthamiana* leaves by *Agrobacterium*-mediated co-transformation. It can be seen that the sections co-expressing tae-MIR164a and fused gene *TaNACs-GUS* (labeled as MIR164 and TaNAC for simplicity) showed a lower expression level of GUS than those of the controls where only empty vector Pro35S::GUS (labeled as Vector for simplicity) was expressed, and the positive controls where only *TaNACs-GUS* (labeled as TaNAC for simplicity) was expressed (the left pane in Appendix E-d and -e). However, the sections co-expressing tae-MIR164a and *TaNACms-GUS* (labeled as TaNACm and MIR164 for simplicity) exhibited a similar expression level of GUS as those of the control and the positive control (the right pane in Appendix E-d and -e). While the negative control where only tae-MIR164a (labeled as MIR164 for simplicity) was expressed showed no expression of GUS (Appendix E-d and -e). These suggested that the tae-miR164 can down-regulate the expression of the putative targets *TaNAC1*, *TaNAC7*, *TaNAC11* and *TaNAC14*.

Taken together, *TaNAC1*, *TaNAC11*, and *TaNAC14* are newly identified tae-miR164 target genes in wheat, and the biological function of miR164-targeted *TaNAC11* and *TaNAC14* remains unknown.

3.3. Expression profiles of tae-miR164 and its target genes *TaNACs* in wheat

Ten wheat tissues/orgains, including R, S, L, FL, YS5, YS15, GR5, GR10, GR15, and GR20, were used for quantification of tae-miR164 abundance by qRT-PCR. The results indicated that tae-miR164 was ubiquitously expressed in all of the ten tissues/organs with most abundant in root (Fig. 2-A). The expression profiles of miR164-targeted *TaNACs* across various wheat tissues/organs were explored based our RNA-Seq data reported previously (Chi *et al*. 2019), and heatmap of *TaNACs* expressions was created by TBtools (Chen *et al*. 2020) based on the transformed data of $\log_2$ (FPKM) values of *TaNACs*, whereas FPKM means fragments per kilobase of transcript per million mapped reads. The heatmaps showed that the miR164-targeted *TaNACs* exhibited different expression levels and diverse expression patterns across the tissues/organs tested (Fig. 2-B). Notably, the three homoeologs of *TaNAC14* showed ubiquitously high expression levels in all of the ten tissues/organs, with particularly high abundance in reproductive tissues (YS5, YS15, GR5-GR20) (Fig. 2-B). These implied that the miR164-targeted *TaNACs* may have diverse biological functions during various developmental stages of wheat, and miR164-*TaNAC14* module might play crucial role during growth and development of wheat.

The expression patterns of tae-miR164 and the miR164-targeted *TaNACs* were also determined under abiotic stress (drought, cold, and high salinity) and phytohormone (ABA, IAA, and KT) treatment. The results showed that the expression of tae-miR64 was inhibited to various degrees under abiotic stresses and ABA treatment (Fig. 2-C). With regards to the miR164-targeted *TaNACs*, under drought and cold stress, the expression of all the miR164-targeted *TaNACs* was greatly induced with exception of *TaNAC1* that was decreased (Fig. 2-D). While under high salinity stress, the expression of all the five miR164-targeted *TaNACs* was largely induced (Fig. 3-D). The expression patterns of the *TaNACs* were contrary to that of tae-miR64 under diverse abiotic stress conditions (Fig. 2-C and D), implying the *TaNACs* regulated by tae-miR164. As for phytohormone (ABA, IAA, and KT) treatments, the expression of these *TaNACs* was induced at various degrees (Fig. 2-D). In general, tae-miR164 and its targeted *TaNACs* exhibited opposite transcript abundance during wheat development and under various abiotic stresses.

Considering the expression profiles of the *TaNACs* during wheat development and under abiotic

stress described above, miR164-*TaNAC14* module was selected for subsequent functional studies.

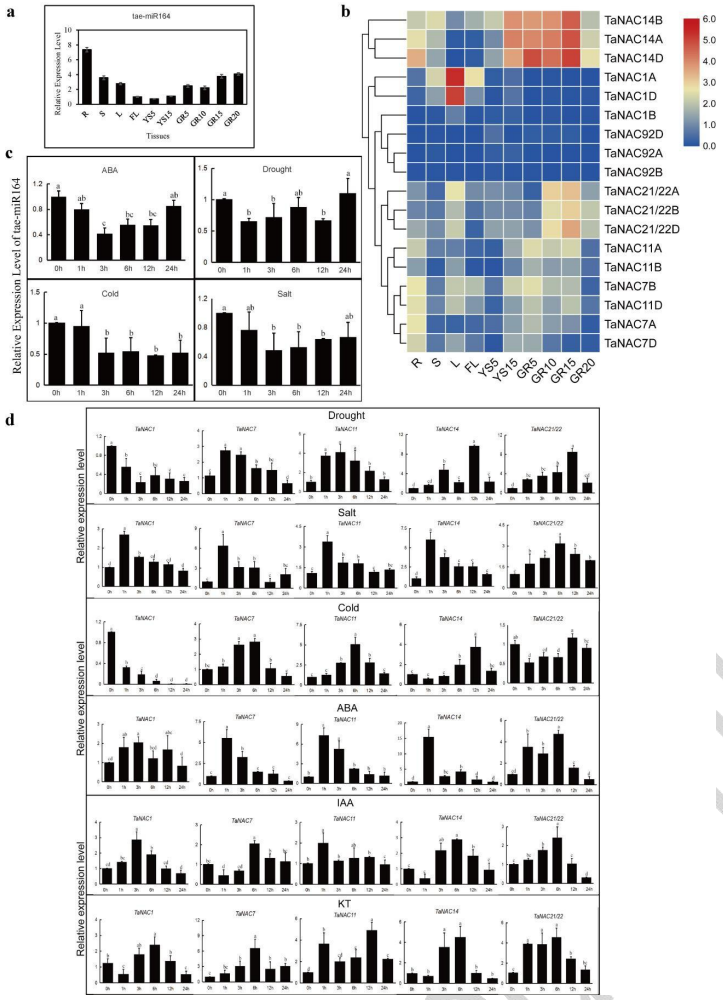

Fig. 2 Expression patterns of tae-miR164 and its putative target gene *TaNACs* across different wheat tissues and under various abiotic stresses. A, the spatio-temporal expression patterns of tae-miR164 in wheat. B, the heatmap of *TaNACs* expression in different wheat tissues/organs. Log_2- expression values of *TaNACs* that was normalized as fragments per kilobase of transcript per million mapped reads (FPKM) were used to create the hotmap. R, S, and L represent root, stem and leaf of five-leaf-stage seedlings, respectively; FL, flag leaf of wheat plants at booting and heading stages; YS5 and YS15 indicate young spikes from wheat plant at booting and heading stages, respectively; GR5, GR10, GR15, and GR20 represent grains at 5, 10, 15, and 20 days post fertilization, respectively. C, expression profiles of tae-miR164 under various abiotic stresses and phytohormone treatments. D, expression of *TaNACs* under various abiotic stresses and phytohormone treatments. Drought,15%PEG-6000 treatment; salt, 0.2 mol L^{-1} NaCl treatment; cold, low temperature (4℃) treatment; ABA, 100 μmol L^{-1} ABA treatment; KT, 100 μmol L^{-1} KT treatment; IAA, 100 μmol L^{-1} IAA treatment. The error bars on the histograms represent the standard deviation of three biological replicates. The horizontal axis present 10 wheat tissues or time point under various abiotic stresses; the left vertical axes show the relative expression levels of the gene tested by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR), the minimum gene expression levels or the expression levels of leaves without treatments being defined as one.

3.4. TaNAC14 locates in nucleus and possesses transcriptional activity

TaNAC14, as one of the plant specific NAC transcript factors, presumably locates in the nucleus. Based on the full-length coding sequence of *TaNAC14-2B* we determined the subcellular location of

TaNAC14-2B in wheat protoplasts by transient expression. GFP signals was observed in the whole cells of the controls, whereas TaNAC14-2B-GFP (TaNAC14-GFP for simplicity) fusion protein was co-localized in the nucleus with the nuclear localization marker NLS-mCherry (Appendix G-a)

Transcription activation activity of TaNAC14 was verified in yeast using TaNAC14-2B. The recombinant construct pGBKT7-TaNAC14-2B (pGBKT7-TaNAC14 for simplicity) was introduced into the yeast Y_2Hgold and screened on the yeast nonselective (SD/-Trp) or selective (SD/-Trp-His-Ade) media. As expected, yeast colonies with pGBKT7-TaNAC14 could grow on the SD/-Trp and SD/-Trp-His-Ade media, but those with pGBKT7 only can grow on SD/-Trp, suggesting that TaNAC14-2B possesses transcription activity (Appendix G-b).

3.5. miR164-*TaNAC14* module regulates root growth and development of wheat seedlings

To explore the functions of the miR164-*TaNAC14* module on wheat growth and development, we generated more than fifteen transgenic wheat lines overexpressing tae-miR164 (named as miR164-OE lines) or overexpressing *TaNAC14-2B* (designed as NAC14-OE lines) under the control of the 35S promoter of Cauliflower Mosaic virus (CaMV). We compared the phenotypes of wild-type plants (JW1) and transgenic wheat seedlings under normal growth condition and found that miR164-OE lines had fewer primary roots (3), shorter principal roots (11.0-11.3 cm), and fewer root hairs than the wild-type plants (4 and 13.4 cm, respectively) at two-leaf stage, conversely, the NAC14-OE lines had more primary roots (5-6), and quite longer principal roots (14.8-16.6 cm), and more root hairs compared with the wild-type plants, with miR164-OE-3 and -10 as representatives of miR164-OE lines and NAC14-OE-3, -12, -9 as representatives of NAC14-OE lines shown in Fig. 3-A-C. Quantitative analysis of *TaNAC14* expression level showed that the NAC14-OE lines had the most abundance, followed by the wild type plants, and the miR164-OE lines showed the lowest level (Fig. 3-D). These suggested that the miR164-*TaNAC14* module regulates wheat root growth and development, *TaNAC14* enhancing root growth and development whereas tae-miR164 inhibiting growth and development by negatively regulating the expression of *TaNAC14*. A recent study demonstrated that miR164-overexpressing rice lines exhibited significantly reduced root lengths compared with the WT, while a new type miR164-target TaPSK5-overexpressing rice lines showed a significantly greater primary root lengths than the WT control, suggesting that TaPSK5 encodes a positive regulator of root growth, and its regulatory effect is restricted by miR164 (Geng *et al*. 2020). This is in line with our result.

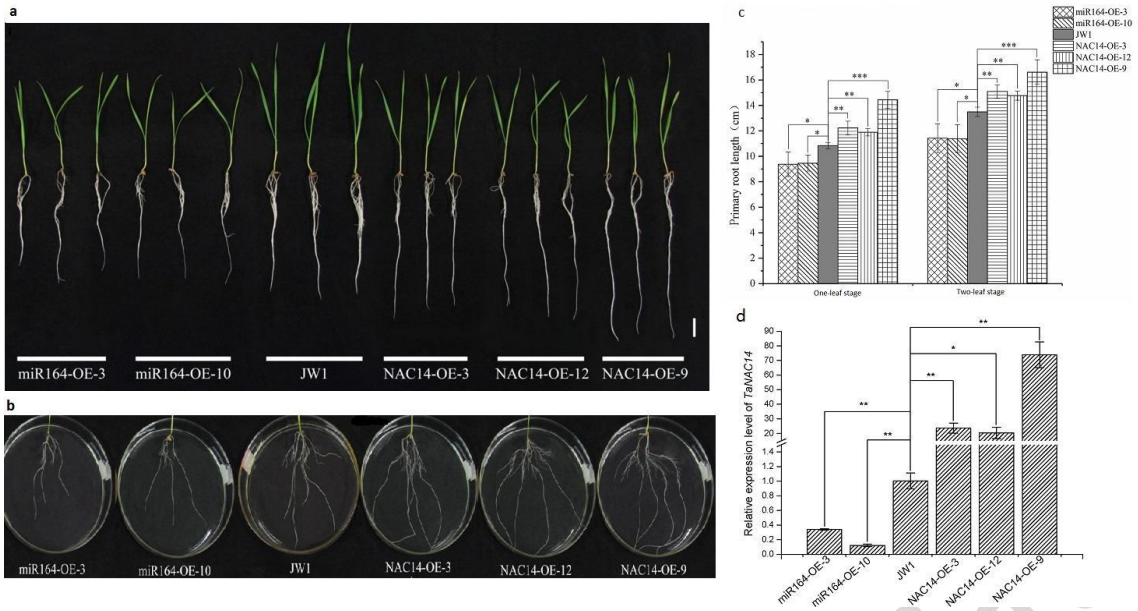

Fig. 3 Phenotypes of transgenic wheat lines and wild type plants at seedling stage. A, phenotypes of whole plants of transgenic wheat lines and wild type wheat plants at two-leaf stage. B, phenotypes of primary roots and root hairs of transgenic wheat lines and wild type plants at two-leaf stage. C, primary root length of transgenic wheat lines and wild type plants at one-leaf and two-leaf stages. Data are mean±SD (*n*=20 plants) from three independent experiments. D, relative expression levels of *TaNAC14* in transgenic wheat lines and wild type plants. Values are means±SD of three biological replicates. Statistical contrasts were performed by t-test, and significant differences are marked with asterisk (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$). miR164-OE, transgenic wheat lines overexpressing tae-miR164; NAC14-OE, transgenic wheat lines overexpressing *TaNAC14*; JW1, wild type wheat.

3.6. miR164-*TaNAC14* module regulates drought and salinity tolerance of wheat seedlings

The abiostress-responsive expression patterns of miR164 and *TaNAC14* encourages us to investigate the effect of the miR164-*TaNAC14* module on wheat plant stress tolerance. For drought tolerance test, the transgenic lines and wild-type plants at two-leaf-stage were withheld watering for 16 days till soil heavy-drought (the soil water capacity reaches 30% of the maximum soil water capacity) and then rewatered 7 days for recovering. It can be seen that the miR164-OE lines are quite sensitive to drought treatment, whereas the NAC14-OE lines exhibited much drought tolerance, compared to the wild type wheat JW1 (Fig. 4-A). Before drought treatment, there was no difference in RWC between transgenic lines and wild-type JW1. However, after 13 days of drought stress, the NAC14-OE lines had much high RWC (58.6-87.4%), while miR164-OE lines had much lower RWC (32.6-21.2%), compared with the wild type wheat JW1 (39.4%) (Fig. 4-B). Furthermore, after drought and rewatering, the NAC14-OE lines exhibited the highest survival rates (89-95%), followed by the wild type wheat JW1 (about 67%), whereas the miR164-OE lines showed the lowest survival rates (37-59%) (Fig. 4-C). These imply that the miR164-*TaNAC14* module regulates plant drought tolerance and overexpression of *TaNAC14* enhanced drought tolerance in Wheat. In addition, the NAC14-OE lines showed much lower water loss rate than the wild type JW1, whereas the miR164-OE lines were the opposite (Fig. 4-D), further indicating the role of *TaNAC14* in enhancing wheat drought tolerance, and its regulatory effect being restricted by miR164.

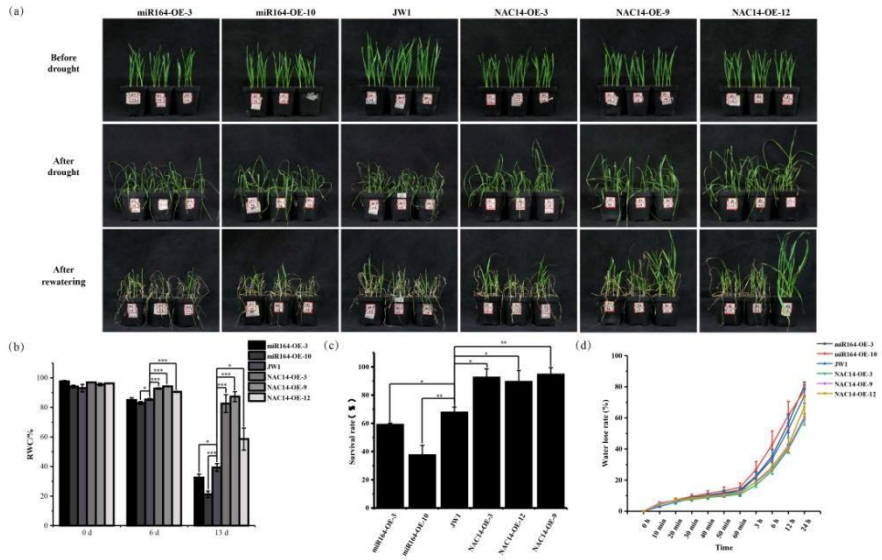

Fig. 4 Drought tolerance of transgenic wheat lines and wild-type wheat. A, phenotypes of the different genotype wheat lines before drought treatment (top panel), after withholding water for 16 days (middle panel), and after rewatering for 7 days (bottom pane). Two-leaf stage plants were used for drought-stress treatment. B, comparison of leaf relative water content (RWC) among different wheat genotypes under drought stress. 0 d, before drought treatment; 6 and 13 d, withholding water for 6 and 13 days, respectively. Values are mean±SD of five independent experiments. Statistical contrasts were performed by t-test, and significant differences are marked with asterisk (*, P<0.05; ***, P<0.001). C, survival rates of the different genotype plant lines undergoing drought and rewatering. Values are mean±SD (n=50) from five independent experiments. Statistical contrasts were performed by t-test, and significant differences are marked with asterisk (*, P<0.05; **, P<0.01). D, water loss rates of the leaves from different genotype plant lines. The second leaves fully expanded from three-leaf stage seedlings of individual transgenic lines were used for the analysis and three biological experiments were conducted. miR164-OE-, transgenic wheat lines overexpressing tae-miR164; NAC14-OE-, transgenic wheat lines overexpressing *TaNAC14*; JW1, wild type wheat.

With regard to high salinity tolerance test, all transgenic lines and wild-type plants at two-leaf-stage were divided into two groups, one as treatment was cultivated in Hoagland nutrient solution with increasing concentrations of NaCl for 22 days as described in Materials and methods, the other as control was cultivated in Hoagland nutrient solution for the same time. As can be seen, the miR164-OE lines showed increased sensitive to salinity-stress, compared with the wild type plants JW1, while the NAC14-OE lines exhibit similar salinity-tolerance with JW1 (Fig. 5-A). In the control group, the increase of biomass per plant of individual transgenic lines was no difference with that of the wild type wheat JW1 after 22 days of cultivation (Fig. 5-B). However, in the salinity treatment group, the increase of biomass per plant of the miR164-OE lines was significantly lower than that of JW1, whereas the increase of biomass per plant of the NAC14-OE lines was no significant difference with that of JW1 (Fig. 5-B). Furthermore, the survival rates of the miR164-OE lines after salinity treatment was much lower than that of JW1, where there was no difference in the survival rates between the NAC14-OE lines and wild type plants JW1, this result is consistent with that in rice reported previously (Fang *et al*. 2014). These data indicated that miR164-*TaNAC14* module affects salinity-tolerance at seedling stage, tae-miR164 decreasing salinity-tolerance of wheat seedlings.

Taken together，the miR164-*TaNAC14* module affects drought- and salinity-stress tolerance of wheat seedlings, and tae-miR164 decreases drought- and salinity-stress tolerance, where *TaNAC14* confers drought-tolerance.

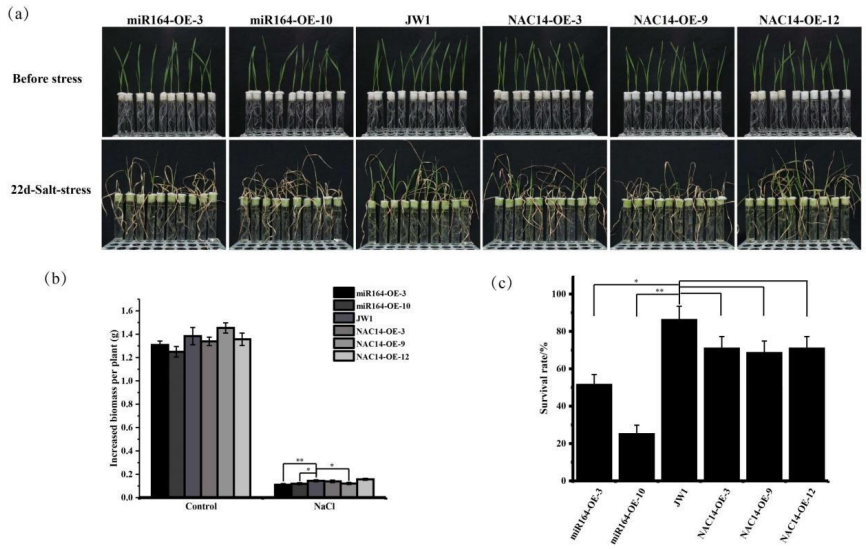

Fig. 5 Salinity tolerance of transgenic wheat lines and wild-type wheat. A, phenotypes of the different genotype wheat lines before salinity treatment (upper panes) and after high salinity treatment for 22 days (lower panes). Two-leaf stage plants were used for salinity-stress treatment by cultivating in Hoagland nutrient solution with increasing concentrations of NaCl for 22 days, i.e., the initial concentration of NaCl was 100 mmol L^{-1} and increase by 50 mmol L^{-1} every 3 days until the concentration reached 300 mmol L^{-1}, and maintain this concentration for 10 days. B, the increase of biomass per plant of different genotype wheat undergoing 22 days of cultivation in Hoagland solution (control group) or in Hoagland nutrient solution with increasing concentrations of NaCl (NaCl treatment group). Values are mean±SD (n=15 plants) from three independent experiments. Statistical contrasts were performed by t-test, and significant differences are marked with asterisk (*, P<0.05; **, P<0.01). C, survival rates of the different genotype wheat lines undergoing high salinity treatment. Values are mean±SD (n=50) from three independent experiments. Statistical contrasts were performed by t-test, and significant differences are marked with asterisk (*, P<0.05; **, P<0.01).

4. Discussion

4.1. The tae-miR164 family and its target genes in wheat

Similar to plant transcription factors, one miRNA can regulate multiple target genes, and multiple miRNAs can also regulate the same target gene (Peng *et al*. 2019). Previous studies have demonstrated that miR164 targets plant-specific NAC transcription factors (Laufs *et al*. 2004; Mallory *et al*. 2004; Guo *et al*. 2005; Kim *et al*. 2009; Fang *et al*. 2014). In *Arabidopsis*, three members of the miR164 family (ath-miR164a/b/c) target the mRNAs of five *NAC* genes (At1g56010, At5g61430, At5g07680, At3g15170, and At5g53950) to regulate developmental processes (Mallory *et al*. 2004; Guo *et al*. 2005). In rice, six members of the osa-miR164 family (osa-miR164a to f) target nine genes, of which, six (*OMT1-OMT6*) are from the NAC family (Fang *et al*. 2014). In wheat, only one mature tae-miR164 and its precursor sequence tae-MIR164 are registered in miRBase (http://www.mirbase.org/textsearch.shtml?q=Triticum+aestivum). It was demonstrated that tae-miR164 can target both conserved plant transcription factors *TaNACs* and less conserved *TaPSK5* in developmental grains of wheat (Li *et al*. 2015; Geng *et al*. 2020). According to plant TFDB (Plant Transcription Factor Database, http://planttfdb.gao-lab.org/), the wheat NAC transcription factor family include 263 members registered which are divided into eight subfamilies, members of d subfamily were related to growth and development process of plants (Shen *et al*. 2009; Borrill *et al*. 2017). A previous

study reported that *TaNAC21/22*, a NAC transcription factor from the NAM subfamily, is a target gene of tae-miR164 that negatively regulates resistance of wheat to stripe rust (Feng *et al*. 2014). More recently, several *NACs*, including *TaNAC7*, *TaNAC23*, *TaNAC92*, and *TaCUC1*, were predicted to be the targets of miR164 in wheat (Geng *et al*. 2020). Due to the large and complex genome sequence of wheat, our knowledge of the miR164 family and the miR164-targeted *NAC* genes in wheat is still very limited to date.

In the present study, nine genetic loci (*tae-MIR164a* to *i*) were identified to produce eight precursors of tae-miR164 that generate two mature miR164 sequences in wheat (Fig. 1, Appendix B, C and D). Based on bioinformatics analysis, three more *TaNAC* genes (*TaNAC1*, *TaNAC11*, and *TaNAC14*) were newly identified as tae-miR164 targets in this study besides the previously identified three *TaNACs* (*TaNAC7*, *TaNAC21/22*, *and TaNAC92*), each of these miR164-targeted *TaNACs* having three homoeologs (Appendix F and Appendix E-b). Furthermore, these putative novel tae-miR164 target genes (*TaNAC1*, *TaNAC11*, and *TaNAC14*) were confirmed by transient co-expression analysis of tae-miR164 and the putative target gene *TaNACs* or miR164-resistant *TaNACms* in leaves of *N. benthamiana* (Appendix E-d and -e).

In rice, three other genes (two genes encoding phytanoyl-CoA dioxygenases; the other one encoding phytosulphokine precursor) that are not from the NAC family were predicted to be osa-miR164 target besides the six *NAC* genes (*OMTN1*-*OMTN6*) (Li *et al*. 2010; Zhou *et al*. 2010; Fang *et al*. 2014). Like the case in rice, tae-miR164 in wheat can also target other genes which are not from the NAC family. For examples, mitogen-activated protein kinase gene *TaMAPK4* was identified as a tae-miR164 target gene that play an important role in signaling during the wheat-*Puccinia striiformis* interaction (Wang *et al*. 2018), and *TaPSK5* is also identified as a less conserved target of miR164 in wheat that regulates root growth and yield traits (Geng *et al*. 2020).

4.2. MiR164-*TaNAC14* module regulates root growth and development in wheat

In *Arabidopsis*, miR164-targeted *NAC* genes participate in the control of plant growth and development (Mallory *et al*. 2004; Guo *et al*. 2005). In rice, transgenic RNAi lines of miR164-targeted *OMTNs* exhibited twisted leaves and fusion organs, these phenotypes were similar as those presented by the miR164-OE plants (Fang *et al*. 2014). The rice transcript factor OsNAC6 mediated root structural adaptations, including increased root number and root diameter (Lee *et al*. 2017). A recent study showed that overexpression of miR164 in rice plants resulted in a significant decrease in miR164-targeted *OsPSK5* transcript levels and reduced shoot length and root lengths by more than 25% and compared with the WT, while overexpression of miR164 target gene *TaPSK5- D* promoted root growth but did not alter shoot growth in rice, indicating that miR164 plays a widespread role in plant development, possibly by regulating the expression of multiple targets including *PSK5* and *NACs* (Geng *et al*. 2020). In the current study, the spatio-temporal expression profiles of tae-miR164 and miR164-targeted *TaNAC14* exhibited that tae-miR164 is ubiquitously expressed across ten tissues/organs tested with most abundant in root, while *TaNAC14* has extremely high expression level in reproductive organs of wheat (Fig. 2-A and B), suggesting that miR164-*TaNAC14* module might play important roles in regulating vegetative and reproductive growth of wheat. Considering tae-miR164 negatively regulating expression of *TaNAC14*, the transgenic wheat lines overexpressing tae-miR164 or *TaNAC14* were generated to explore the effects of miR164-*TaNAC14* module on growth and development of wheat in the present study. The transgenic wheat lines overexpressing *TaNAC14* had much longer principal roots, more primary roots and root hairs than the wild type plants at seedling stages, while the miR164-OE lines that had reduced *TaNAC14* expression levels possessed shorter

principal roots, fewer primary roots and root hairs than the wild type plants (Fig. 3). These results indicate that *TaNAC14* can promote root growth and development of wheat at seedling stage and tae-miR164 plays negatively role in root growth and development by down-regulating *TaNAC14* expression. Our findings enriched the results in transgenic rice that *TaPSK5* encodes a positive regulator of root growth and its regulatory effect is restricted by miR164 (Geng *et al*. 2020).

4.3. MiR164-*TaNAC14* module regulates drought and salinity tolerance of wheat seedlings

A previous study demonstrated that the expression level of osa-miR164 and *OsNAC2* were up-regulated by drought- and salinity-stress in the roots of rice at the seedling stage, and *OsNAC2* also significantly up-regulated to cold stress (Fang *et al*. 2014). In the present study, expression level of tae-miR164 was down-regulated under abiotic stress, such as drought-, salinity- and cold-stress (Fig. 2-C), conversely, the expressions of the miR164-targeted *TaNACs* (*TaNAC7*, *TaNAC11*, *TaNAC14*) were induced at various degree by abiotic stress (Fig. 2-D). *TaNAC1* presented decreased expression levels under drought- and cold-stress but increased expression levels under high-salinity stress (Fig. 2-D), while *TaNAC21/22* showed increased expression levels under drought- and salinity-stress but declined expression levels under low temperature condition (Fig. 2-D). Our results suggest that these miR164-targeted *TaNAC*s in wheat might play diverse functions in abiotic- stress conditions.

Furthermore, the drought and salinity tolerance of the transgenic wheat lines overexpressing tae-miR164 or *TaNAC14* were tested in the present study. Our results showed that the transgenic wheat lines overexpressing tae-miR164 exhibited decreased drought tolerance, but the transgenic wheat lines overexpressing *TaNAC14* showed increased drought tolerance compared to the wild type plants at seedling stage (Fig. 4). These suggest that miR164-*TaNAC14* module affects drought tolerance of wheat seedlings, *TaNAC14* enhances drought tolerance, while tae-miR164 negatively regulating drought tolerance. This is consistent with the functions of miR164-*TaNAC14* module in root growth and development of wheat seedlings. Thus, we speculate that *TaNAC14* enhances drought tolerance of wheat seedlings mainly through promoting root growth and development. Previous studies in rice demonstrated that *OsNAC5* and *OsNAC6* promoted root number and root diameter, which enhanced drought tolerance (Jeong *et al*. 2013; Lee *et al*. 2017). These results in rice are in line with our findings in wheat. However, in rice, the transgenic lines overexpressing miR164-targeted NAC genes *OMTN2*, *OMTN3*, *OMTN4*, and *OMTN6* showed increased drought sensitivity to varying degrees at the reproductive stage but not alter drought tolerance at other developmental stages, this suggests that the roles of the *OMTN* genes in regulating drought resistance may be associated with reproductive development (Fang *et al*. 2014). The result in rice is inconsistent with our results in wheat at vegetative developmental stage, which may suggest that miR164-*OMTNs* module in rice and miR164-*TaNAC14* module in wheat have different functions in drought tolerance in this developmental stage. How do the transgenic wheat lines overexpressing miR164 or *TaNAC14* affect drought tolerance at reproductive stage is under investigation. As for salinity tolerance test in the present study, the transgenic wheat lines overexpressing tae-miR164 exhibited decreased salinity tolerance, while the transgenic wheat lines overexpressing *TaNAC14* showed no significant difference in salinity tolerance, compared to the wild type plants at seedling stage (Fig. 5). This result is consistent with that in rice, where the transgenic rice lines overexpressing miR164-targeted *NAC* genes *OMTN2*, *OMTN3*, *OMTN4*, and *OMTN6* and the wild type plants showed no significant difference in their tolerance to salinity-stresses (Fang *et al*. 2014).

Notably, recent studies in rice and wheat demonstrated that overexpression of *OsNAC6* and *OsNAC10* in rice and *TasNAC8-6A and TaNAC071-A* in wheat significantly enhanced drought tolerance

and increased grain yield (Lee *et al*. 2017; Mao *et al*. 2020, 2021). Here we found that overexpression of *TaNAC14* can promote root growth and development and enhance drought tolerance of wheat at seedling stage. Our findings provides with new potential genetic resources for stress-resistance wheat breeding. However, whether overexpression of *TaNAC14* can also confer drought tolerance of wheat plants at the reproductive stage and increase grain yield is under investigation.

5. Conclusion

Nine genetic loci of tae-miR164 (*tae-MIR164a to i*) in wheat were identified to generate two mature sequences that down-regulated the expression of newly identified target genes (*TaNAC1*, *TaNAC11*, and *TaNAC14*) by the cleavage of the respective mRNA. Overexpression of tae-miR164 or one of its target genes *TaNAC14* in wheat demonstrated that miR164-*TaNAC14* module greatly affect root development and stress (drought and salinity) tolerance at seedling stage. *TaNAC14* promotes root growth and development of wheat seedlings and enhanced drought tolerance, while miR164 inhibited root development and reduced drought and salinity tolerance by down-regulating the expression of *TaNAC14*. Our findings identify the miR164-*TaNAC14* module as well as other tae-miR164 regulated genes to generate new genetic resources for stress-resistance wheat breeding.

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Conflict of interest

Authors claim there is no conflict of interest.

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