miR399-*UBC24* module enhances freezing tolerance through regulating CBF signaling pathway and starch degradation

Kankan Peng[1], Yu Tian[1], Xianze Sun[1], Chunhua Song[1], Zhipeng Ren[1], Yuzhuo Bao[1], Jinpu Xing[1], Yuanshan Li[1], Qinghua Xu[1], Jing Yu[1], Da Zhang[1], and Jing Cang[1]

[1]Northeast Agricultural University

May 16, 2021

Abstract

Although the regulation in Pi homeostasis of miR399 have been studied in various plants, its molecular mechanisms in response to freezing stress are still elusive. In this work, we found that the expression of tae-miR399 and its target gene *TaUBC24* in tillering nodes of strong cold resistance winter wheat cultivar Dongnongdongmai1 (Dn1) was significantly altered subjected to severe winter. tae-miR399 and its target gene TaUBC24 were also responsive to short-term freezing stress in tillering nodes of Dn1 seedlings. TaUBC24 physically interacted with TaICE1. Ehanced freezing tolerance was observed in overexpressing tae-miR399 Arabidopsis lines. Under freezing stress, overexpressing tae-miR399 decreased the expression of *AtUBC24* to increase the expression of genes in CBF signaling pathway, Pi translocation pathway and starch metablism, including *AtCBFs*, *AtCOR47*, *AtCOR413IM*, *AtPHT1;4*, *AtAPLs* and *AtBAMs*, inhibit the degradation of AtICE1 and AtPHO1, and promote the activities of SOD, POD and CAT. These findings indicated that the increased freezing tolerance was dependent upon elevating CBF signaling pathway, phosphorus utilization efficiency, starch degradation, accumulation of soluble sugar and ability of ROS scavenge. These results will aid our understanding of molecular mechanism of how miR399-*UBC24* module plays a cardinal role in regulating plant freezing stress tolerance through mediating the downstream pathways.

Introduction

Low temperature stress is the major abiotic stress that is not conducive to plant growth and yield. Basing the different effects of cold stress in plants, it can be classified chilling stress and freezing stress (Jan, Mahboob ul, & Andrabi, 2009). Chilling stress disturbs membrane processes including opening of ion channels, membrane associated electron transfer reactions (Ruelland, Vaultier, Zachowski, & Hurry, 2009). The freezing temperature has more negative effects in plants. The extracellular freezing which means cellular water migrates to this extracellular ice causing cell dehydration and shrinkage could occur in freezing stress (Dowgert & Steponkus, 1984). Ultimately, ice can penetrate the symplast (Gusta & L., 2004), causing a deterioration of the intracellular structures and death of tissues. In addition, ROS is accumulated continuously, no matter under chilling stress or freezing stress, which leads to the degradation of various proteins and protein complexes in plants and destroys the process of plant biogenesis (Ruelland et al., 2009). Unraveling the mechanisms of how cold resistant plant varieties adapt to extremely cold environments could provide valuable information for enhancing the performance of conventional crops under freezing stress, via genetic engineering. Plants have evolved sophisticated mechanisms that limit cold-induced damage. A series of comprehensive physiological and biochemical events take place during plants withstanding cold stress (Y. Ding, Shi, & Yang, 2019). At the physiological level, many substances or protective proteins are synthesized in plants, such as soluble sugars, proline, and cold-resistance proteins, of which are involved in osmotic potential, ice crystal formation, membrane stability and ROS scavenging in plants. These physiological and biochemical changes in plants are regulated by different signaling and metabolic pathways (Asif et al., 2014), such as CBF signaling pathway, sugar metabolism and ROS scavenge system. Exploring upstream regula-

tion mechanism of these pathways is of great significance for revealing the molecular basis of plant freezing tolerance.

CBF signal pathway endows plants cold tolerance by regulating the expression of downstream cold-resistance proteins (Chinnusamy et al., 2003a). CBF/DREB1 (C-REPEAT BINDING FACTOR/DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN1) genes are involved in cold acclimation of plants and rapidly induced under cold stress (Q. Liu et al., 1998; Stockinger, Gilmour, & Thomashow, 1997). Subsequently, COR genes, a class genes encoding osmolyte and cryprotective proteins, are induced by CBFs to protect plant from freezing injury (Shi, Ding, & Yang, 2018; Yamaguchi-Shinozaki & Shinozaki, 1994). Inducer of CBF expression transcription factor (ICE) is a MYC type bHLH transcription factor, which is the most characteristic transcription activator of CBF gene (Chinnusamy et al., 2003b). The ICE1 has been proved to play an important role in response to low temperature stress in varied plants, such as rice (Dianjun, Yu, & Kuide, 2007), Arabidopsis (Chinnusamy et al., 2003b) and tomato (Juan et al., 2015). Not only CBFs gene, ICE1 can also bind CORs promoter to activate CORs gene expression (Tang et al., 2020). Due to the importance of ICE1 in CBF signaling pathway, its upstream regulation mechanism has also been widely studied. Ding et al. report that ubiquitination, sumo acylation and phosphorylation are important for ICE1 in Arabidopsis cold tolerance (Yanglin Ding et al., 2019). ICE1 is ubiquitinated and degraded by E3 ubiquitin ligase HOS1 (high expression of osmotically responsive gene 1), while sumo E3 ligase SIZ1 (SAP and Miz) mediates sumo acylation of ICE1 and stabilizes ICE1 under low temperature stress (Miura et al., 2007). In addition, protein kinase OST1 (open stomata 1) mediates ICE1 phosphorylation and cold tolerance in Arabidopsis. OST1 is cold activated and is negatively regulated by protein phosphatase EGR2 (clade e growth regulating 2) and ABI1 (abscisic acid insensitive 1), independent of ABA (Y. Ding et al., 2019; Y. L. Ding et al., 2015). Cold activated OST1 phosphorylates ICE1 to enhance its stability by destroying its interaction with HOS1 (Y. L. Ding et al., 2015). Other study also finds that the protein stability and transcriptional activity of OsICE1 are positively regulated by OsMPK3 (MAP kinase 3) in rice (Z. Zhang et al., 2017). However, the upstream regulation mechanism of ICE1 in plant freezing tolerance is still elusive.

Starch degradation is involved in cold-induced sugar accumulation (Ruelland et al., 2009). When suffering cold stress, amylases activity in plants increases. Subsequently, starch is degraded to glucose and fructose, which is the substrate for the accumulation of soluble sugar (Kaplan et al., 2007; Ruelland et al., 2009). The *AtBAM3*(At4g17090) encoding β-amylase, is induced by low temperature stress (Kaplan & Guy, 2005; Lundmark, Cavaco, Trevanion, & Hurry, 2006). The*bam3* Arabidopsis mutant is sensitive to cold, and the accumulation of soluble sugar is significantly reduced(Kaplan & Guy, 2005; Yano & R., 2005). Overexpressing *PbrBAM3* in tobacco (*Nicotiana tabacum*) and pear (*P.ussuriensis*) can increase β-amylase activity, promote starch degradation under low temperature stress, and enhance its cold tolerance (Zhao et al., 2019). Except*BAM3* , another gene encoding β-amylase, *BAM1* , also plays an important role in starch degradation under chilling and freezing stress (T. Peng, Zhu, Duan, & Liu, 2014). Moreover, α-glucan hydration dikinase (GWD) can phosphorylate C-3 and C-6 of α-glucan, this starch phosphorylation may promote starch degradation by increasing the water-insoluble glucan to become hydrophilic ensuring better access to starch degrading enzymes(Hejazi, Fettke, Haebel, Edner, & Ritte, 2010). And its coding genes *SEX1* and *SEX4* are induced by low temperature stress (Berrocal-Lobo et al., 2011; Hejazi et al., 2010). Based on the importance of starch metabolism in plant response to low temperature stress, its upstream regulatory mechanism is still to be explored.

miR399 is an essential regulator of *UBC24* (*PHO2*) expression during plant growth and response to stress. The miR399-*UBC24* (*PHO2*) regulation module is conserved in plants including Arabidopsis (Bari, Datt Pant, Stitt, & Scheible, 2006), rice (*Oryza sativa*) (Hu et al., 2011), wheat (*Triticum aestivum*) (J. Wang, Sun, et al., 2013) and maize (*Zea mays*) (Du, Wang, Zou, Xu, & Li, 2018). The miR399-*UBC24* model was first found to play a role in plant response to Pi deficiency stress (Fujii, Chiou, Lin, Aung, & Zhu, 2005). When Pi is sufficient, UBC24 can degrade the Pi transporter PHOSPHATE TRANSPORTER 1 (PHT1) by ubiquitination (Huang et al., 2013). Under Pi-deficient condition, miR399 is strongly induced to downregulate the expression of *UBC24* to increase the level of PHT1 (Aung et al., 2006; Hu et al., 2011). miR399 has been shown to regulate plant reproductive development. The miR399-*UBC24* module

regulates SEPALLATA MADS box transcription factor genes (SEPs) and ICE1 protein level to effect floral organ development in Citrus (R. Wang et al., 2020). The miR399-*UBC24* module is also involved in plant other process such as sugar metabolism (Y. Wang et al., 2017), nutrienrt starvation responses (Hu et al., 2015), salt, drought and ABA signaling (Baek et al., 2016). In addition, the results of multi plant species miRNA sequencing showed that miR399 was induced by low temperature (R. Hu et al., 2019; Koc, Filiz, & Tombuloglu, 2015). Overexpressing miR399 can improve plant growth in low temperature (Gao, Qiang, Zhai, Min, & Shi, 2015). However, the mechanism of miR399 in response to freezing stress is unknown.

Wheat (*Triticum aestivum*) is one of the main food crops for the global population (Rizwan et al., 2019). Freezing stress is one of the main factors damaging the yield and quality of economic crops such as wheat. To avoid winterkill it is very important to obtain the wheat freezing resistance gene (Babben et al., 2018). Dongnongdongmai1 (Dn1) is the first winter wheat cultivar that can overwinter safely in alpine region of Heilongjiang province (The minimum temperature in this area can reach -30) and the rate of returning green is greater than 85% (K. K. Peng et al., 2021; Tian et al., 2021). Winter wheat's resistance to cold includes microRNAs (miRNAs)-short, single-stranded, non-coding RNAs that regulate the posttranscriptional gene expression by targeting mRNAs for cleavage or repressing translation (Lu, Xu, et al., 2020). Therefore, Dn1 is valuable germplasm resources for mining freezing resistance genes for crop variety improvement.

In this work, we systematically studied the interaction of tae-miR399-*TaUBC24* and the expression of tae-miR399-*TaUBC24* in winter wheat using bioinformatics and research methods in molecular biology, physiology and biochemistry. Then we determined the physical and genetic interaction of TaUBC24 and a inducer of CBF expression transcription factor protein, TaICE1, and reported that in addition to its role in Pi homeostasis and starch degradation, the mechanism of how tae-miR399-*UBC24* module and ICE1 synergistically regulated plant tolerance to freezing stress, which providing a broader view on the novel upstream regulating mechanism of both CBF singnaling pathway and starch degradation response to freezing stress.

Materials and Method

Plants materials and growth conditions

Winter wheat (*Triticum aestivum*) cultivar Dongnongdongmai1 (Dn1) was obtained from the Wheat Breeding Institue of Northeast Agricultural University, Harbin, China. The field trial referred to previous study (Tian et al., 2021),was carried out in autumn, September 8th, 2018, at Northeast Agricultural University Experimental Site (45°7'N, 126°6'E) in Harbin, China. Then the tillering nodes were collected at 5 (October 15th, 2018), 0 (November 2nd, 2018), -10 (November 23rd, 2018) and -25 (January 14th, 2019) (average of minimum temperature for ten consecutive days) and store at -80degC. The indoor trail was carried out as follow: Dn1 seeds were sowed in plastic pots (5 plants/ pot) containing soil and vermiculite (1:1, v/v) and grown in green house for 15 days at 22 in a 16 h photoperiod under 160-180 μmol m^{-2} s^{-1} light. Then seedlings were transferred to 4 for cold acclimation. After 28 d, the Dn1 seedlings were frozen at -10 for 2 h. the tillering nodes were pooled from each seedlings and the tissue immediately frozen in liquid nitrogen and stored at -80

Arabidopsis thaliana accession Columbia (Col-0) was used for gene transformation and as a phenotypic control. The stratification and planting conditions of Arabidopsis referred to previous study (J. Wang, Ma, Kojima, Sakakibara, & Hou, 2013). Its stratification condition was 4°C for 3 d in darkness and culturing condition was as follow: 24°C, 16/8 photoperiod, light intensity of 120-150 μmol $m^{-2}s^{-1}$, and 60% relative humidity. During freezing treatment, the soil culture one-month old Arabidopsis seedlings were treated in low-temperature incubator at 4 for 3 d as cold acclimation and then the temperature dropped to -10 for 2 h. After freezing treatment, the plants were recovered for 7 d under normal condition. The Arabidopsis survival rate was counted after the recovery phase. Plants that turned yellow and could not be regenerated were defined as death. Survival rates were calculated from the results of three repeated experiments, and the amount of each plant was approximately 80 in one experiment.

Real-time quantitative PCR assay

Plants total RNA was isolated by Trizol (Nakayama et al. 2017) and cDNA from mRNA and miRNA synthese were performed by HiScript III 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China) and Mir-X miRNA First-Strand Synthesis Kit (Takara, USA). qRT-PCR analyses were performed by using the ChamQ^{TM} Universal SYBR qPCR Master MIX (Vazyme). The qPCR primers of wheat and *Arabidopsis thaliana* genes were listed in Table S1. Each reaction was performed in a final volume of 20 μl in a Stratagene MX3000P^{TM} System (Genetimes, Shanghai, China). The program referred to instruction of qPCR reagent and data were quantified using the comparative $2^{-\Delta\Delta^{\sim}T}$method after the PCR program. All the gene expression experiments were three biological and three technical repeats. Subsequently, the mean of the technical replicates for each biological replicate were calculated, which values were used for statistical analysis.

Cloning and bioinformatics analysis of tae-miR399 and *TaUBC24*

The precursor and mature sequences of tae-miR399 were obtained according to the miRNA database of Dn1 and the wheat miRNA data of miRbase (*http://www.mirbase.org/*). According to the precursor and mature sequence of tae-miR399, the chromosome position of tae-miR399 was obtained in wheat open genome library wheat URGI (*https://wheat-urgi.versailles.inra.fr/*). The base conservation analysis of mature miRNA sequences was analyzed with WebLOGO (*http://weblogo.berkeley.edu/logo.cgi*). The online software RNAfoldWebSever was used to predict the stem-loop structure of miR399 precursors. The 2 kb promoter sequence upstream of the genes were downloaded from wheat genome (*https://urgi.versailles.inra.fr/download/iwgsc/IWGSC_RefSeq_Assemblies/v1.0/*) and analysed with PlantCARE software (*http://bioinformatics. psb.ugent.be/webtools/plantcare/html/*) for the active elements in its promoter. The potential target genes of tae-miR399 were predicted by psRNAtarget (*http://plantgrn.noble.org/psRNATarget/analysis*). The complete gene and protein sequences of TaUBC24 (Gene ID:TRIAE_CS42_1DL_TGACv1_062114_AA0208970.2) were obtained from wheat open genome library wheat URGI (*https://wheat-urgi.versailles.inra.fr/*). The physicochemical properties of TaUBC24 protein sequence was predicted by using ProtParam (*https://web.expasy.org/protparam/*). Plant UBC24 protein sequences were obtained from UniProt (https://www.uniprot.org/). Multiple sequence alignments were analyzed using DNAMAN. A phylogenetic tree was generated using neighbor joining (NJ) method with a 1000 bootstrap value by software MEGA 6.0. Subcellular localization prediction was performed by the online tool CELLO v.2.5 (http://cello.life.nctu.edu.tw). To obtain the full-length precursor sequences of tae-miR399 and CDS of *TaUBC24* , total RNA was isolated from tillering nodes under -25°C treatment. The full-length precursor sequences of tae-miR399 was amplified using primers*pre-tae-miR399* -F: 5'-TCGTGTGTGAATCACAGGG-3' and primers*pre-tae-miR399* -R: 5'-GGGCAATTCTCCTTTGGCA-3', and Taq DNA Polymerase (CWBIO, Beijing, China). And the full-length CDS of*TaUBC24* was amplified using primers *TaUBC24* -F: 5'-GCTTGGAGAGATGGATCTGTTTG-3' and primers *TaUBC24* -R: 5'-GTCACTGGCCACAGGCGA-3', and Taq DNA Polymerase (CWBIO).

tae-miR399-mediated cleavage of *TaUBC24*

To validate tae-miR399 mediated cleavage of *TaUBC24* , 5' RLM-RACE analysis was conducted using FirstChoice® RLM-RACE Kit (Thermofisher, USA). 3' gene specific primers for *TaUBC24* were listed in Table S2. Interactions between tae-miR399 and *TaUBC24*on potential target genes were demonstrated using transient co-expression assays in *N. benthamiana* leaves. The precursor sequence of tae-miR399 was cloned from Dn1 genomic DNA and then cloned into pBI121 using Homologous recombination technology. The full-length of partial *TaUBC24* sequence containing MREs was amplified using primers *pTaUBC24* -F: 5'-CTGAACAGTCGTTGGTTGCTCT-3' and primers*pTaUBC24* -R: 5'-CTAAAGAACCGTCTCTGAACAGC-3', and Taq DNA Polymerase (CWBIO). The PCR product was cloned into pBI121, which drives expression using the cauliflower mosaic virus 35S promoter. To co-express tae-miR399 and MREs, *A. tumefaciens* (EHA105) strains containing these plasmids were coinfiltrated into 7-week-old *N. benthamiana*leaves. β-Glucuronidase (GUS) staining were conducted 2 d after infiltration. All primers of 5' RLM-RACE were designed using the software Primer 6.0 and listed Table S2.

Plant expression vector construction and transformation

The full-length precursor sequence of tae-miR399 was cloned into Pcambia230035Su plant expression vector with control of CaMV35S promoter to generate 35S::tae-miR399 constructs using USER^{TM}cloning technology (Nour-Eldin, Hansen, Nørholm, Jensen, & Halkier, 2006). The above recombinant constructs were then transformed into*A. tumefaciens* strain EHA105. The 35S:: tae-miR399 constructs was transformed into Arabidopsis wild-type (Col-0) plants using floral-dip method to obtain tae-miR399 overexpressing plants (Clough & Bent, 1998). Transgenic plants were selected by 1/2 MS medium containing 50 mg L^{-1} kanamycin.

Determination of physiological indices

The MDA (malondialdehyde) content in leaves of Arabidopsis plants were performed as described previously (Breuil & Saddler, 1985; Shabala, Shabala, Martynenko, Babourina, & Newman, 1998; Sofo, Dichio, Xiloyannis, & Masia, 2004). Relative electrolyte leakage (%) in leaves of Arabidopsis plants was determined according to the (Dionisio-Sese & Tobita, 1998) method. The total phosphorus content was determined by using Total Phosphorus Assay Kit (Yoyobio, Shanghai, China). The inorganic phosphorus content was determined by using Inorganic Phosphorus Assay Kit. The content of starch and soluble sugar were determined by using Starch Kit and Soluble Sugar Kit. The enzyme activities of amylases, SOD, POD and CAT were determined by using Amylases Kit, SOD Kit, POD Kit and CAT Kit. All of the above unmarked Kits are produced from Comin (Suzhou, China).

Protein preparation and western blot analysis

The Arabidopsis proteins were extracted by using Plant Protein Extraction Kit (Solarbio, Beijing, China). 15 μg protein was loaded to 7.5%, 1.5 mM SDS-gels. To measure ICE1 protein, the Anti-ICE1 (1:1000, Agrisera) was used as primary antibody. Anti-Actin (1:1000, Abmart) was used as loading control for ICE1. For all western blots, HRP-anti-rabbit & mouse was used as secondary antibody (1:10000; Abmart). Imaging was carried out using a cooled CCD camera system (Polychromatic fluorescence / chemiluminescence gel imaging system, UVITEC) and Western blots were quantified with Q9 Aliance software.

Histochemical staining and determination of ROS content

The content of H_2O_2 and $O_2^{\cdot-}$ in leaves of Arabidopsis plants could be observed by histochemical staining with DAB and NBT. Specific steps of histochemical staining have been described by predecessors (Wu et al. 2017). The difference to previous study is PH, the PH of NBT and DAB solution were individually adjusted to 7.5 and 5.8. The quantitative content of $H_2O_2$and $O_2^{\cdot-}$in *Arabidopsis thaliana* were determined by spectroscopic method using Hydrogen peroxide (H_2O_2) content Kit and Superoxide anion ($O_2^{\cdot-}$) Kit (Comin).

Y2H and bimolecular fluorescence complementary Imaging Assays

The full-length CDS of *TaUBC24* was cloned into the DNA-binding domain vector pGBKT7 (BD). The full-length CDS of *TaICE1* was amplified and cloned into the activation domain fusion vector pGADT7 (AD). Yeast strain AH109 was cotransformed with TaICE1-AD and BD-CsUBC24. Positive clones were selected on a synthetic dropout medium (SD/-Trp/-Leu). The interactions were tested on SD/-Trp/-Leu/-His/-Ade medium containing X-agalactosidase. Activation was observed after 3 d of growth on selection plates.

To generate *TaUBC24* -GFP^N and*TaICE1* -GFP^C, the full-length CDS of*TaUBC24* and *TaICE1* genes were cloned into the upstream of GFP^N and GFP^C sequences in pCMBIA2300-BiFC vector. The resulted constructs were transiently coexpressed into *N. benthamiana* leaves for 2 d to generate split GFP proteins for bimolecular fluorescence complementation (BiFC) assays. The GFP (excitation wavelength at 488 nm) signals resulted from protein-protein interactions in leaves were observed under the fluorescent microscope.

Experimental design and statistical analysis

All experiments with at least three biological replications were analyzed by Graphpad Prisim 9.0 statistical software using one-way or two-way analysis of variance (ANOVA). The values presented were the means ± one standard deviation (SD) of three biological replicates.P values were corrected with Bonferroni,* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ****$P < 0.0001$.

Results

Molecular cloning and characterization of miR399 and its target UBC24 in Dn1 winter wheat

The precursor of miR399 (*pre-tae-miR399*) from the tillering nodes of Dn1 winter wheat was composed of 126 nt, which contained a 19 nt mature sequence. tae-miR399 was located on A chromosome of wheat (Fig. S1A). There was no mRNA between upstream and downstream 1kb of miR399, which belonged to intergenic miRNA (Fig. S1B). The mature and precursor miR399 sequences of monocots and eudicots were obtained from the miRbase database. The conservation of miR399 was analysed by the WebLogo software (Fig. 1A). The miR399 sequences were found to be highly conserved and have two motifs (5p and 3p). The difference between miR399-3p and miR399-5p was at base 17 (Fig. 1B). According to the prediction by RNAfold, the secondary structure of precursor formed a complex but stable stem-loop (Fig. 1C). The line in Fig. 1C showed the mature sequence of tae-miR399 (tae-miR399: 5'-UGCCAAAGGAGAAUUGCCC-3') (Fig. 1C). A phylogenetic tree was constructed from miRNA precursors using MEGA6. The phylogenetic tree of miR399 showed that the precursor of miR399 was present in both monocots and eudicots, meaning that miR399 was evolutionarily conserved in the common plants listed above (Fig. S1C). To explore the regulation role of tae-miR399, we extracted 2.0 kb of genomic sequence upstream of the transcription start site of the*pre-tae-miR399* and searched for cis-acting elements in the PlantCARE database. We found that the transcription start site of the tae-miR399 precursor contained basic promoter elements, transcription factor such as MYC binding site, MYB binding site and a variety of cis-acting and response elements. It showed that both the expression of tae-miR399 might be induced or inhibited by environmental response factors such as light, low temperature stress and hormones (Fig. 1D).

The target of tae-miR399 in Dn1 was predicted by the psRNATarget software to be the highly reliable *TaUBC24* (TRIAE_CS42_1DL_TGACv1_062114_AA0208970.2). The 2532 bp CDS sequence of *TaUBC24* was cloned from the tillering nodes of Dn1 (Fig. S2A). The ORF of the *TaUBC24* gene encoded a 843aa protein with a predicted molecular weight of 93.774 kDa and a predicted isoelectric point of 4.78. To investigate the orthologous relationship of UBC24 in different species, we obtained UBC24 protein sequences from 16 species, respectively. It could be seen from the phylogenetic tree and multiple sequence alignments that UBC24 from different species was highly conserved, and TaUBC24 was most closely related to TuUBC24, which was considered the ancestral progenitor of the wheat A genomes(Ling et al., 2018) (Fig. S2B C). The subcellular localization analysis showed that TaUBC24 was located in the cytoplasm and nuclear (Fig. S2D). Moreover, we screened the upstream promoter of*TaUBC24* for cis-acting elements with PlantCARE and found that it contained a variety of cis-acting and response elements (Fig. 1D) that responded to light, low temperature stress and hormones. These results are similar to those for the corresponding miRNA precursors. It was indicated that both tae-miR399 and *TaUBC24* could respond to the same environmental factors.

Expression analysis of tae-miR399 and *TaUBC24* at freezing stress in Dn1

Tillering node is an important organ to winter wheat overwintering (K. K. Peng et al., 2021). To investigate the changes in tae-miR399 and*TaUBC24* expression levels in fields under naturally decreasing temperature, the qRT-PCR was performed in Dn1 tillering nodes. The expression of tae-miR399 decreased at 0 and -10 (reached the minimum at 0), and then it increased significantly at -25 (induced more than 9 folds) (Fig. 2A). The expression of *TaUBC24* was opposite to that of miR399 in tillering nodes, reached the maximum at 0 (induced more than 8 folds), then decreased and peaked the minimum at -25 (Fig. 2B).

To eliminate the influence of spatial-temporal differences and other factors in the field on Dn1 tae-miR399, we conducted qPCR experiments on the tillering nodes of Dn1 seedlings cultured in the incubator with short-term freezing treatment. The results showed that the expression pattern of tae-miR399 was similar to that of the field. During cold acclimation at 4, the expression of miR399 decreased, and then increased significantly at -10 (induced more than 15 folds) (Fig. 2C). The expression change of its target gene *TaUBC24* was still opposite to the expression change of tae-miR399. It increased significantly and peaked at 4degC, and then decreased significantly at -10degC, reaching the lowest peak (Fig. 2D). Based on these results, we deduced that tae-miR399 played a positive role in achieving plant tolerance to freezing stress. Meanwhile, from the

expression pattern assays, we speculated that tae-miR399 might regulate *TaUBC24* expression.

tae-miR399 targets *TaUBC24*

To verify the target relationship between tae-miR399 and TaUBC24, the psRNATarget software analysis showed that there are six potential tae-miR399 response elements/binding sites (MREs) in the 5' untranslated region (UTR) of *TaUBC24* (Fig. 3A). The sequence of MRE (2) was the same as that of MRE (3) and (4). The sequence of MRE (1) had the highest basal complementarity (Fig. 3A). Using RNA ligase-mediated 5' RACE (RLM-5' RACE), we found that *TaUBC24* was cleaved at 15 bp downstream of MRE (4) (Fig. 3B-(a)). However, no cleavage was detected at the other five predicted target sites in *TaUBC24* . To independently examine the relationship between tae-miR399 and the potential target site, we performed transient coexpression assays in *Nicotiana benthamiana* leaves. Scince the full length of *TaUBC24* mRNA is 4230bp, it is difficult to clone and vector construction. The partial TaUBC24 containing the six potential MREs (*pTaUBC24*) was cloned with a length of 678 bp. The pBI121-GUS vector (harboring the GUS gene) was introduced into cells of tobacco leaves using the Agrobacterium-mediated transformation system. Control leaves infiltrated with pBI121-GUS exhibited the GUS phenotype revealed by histochemical staining, while leaves infiltrated with pBI121-*pTaUBC24* , in which the target sequence was fused upstream of the GUS gene, showed a similar phenotype. In leaves infiltrated with pBI121-tae-miR399, however, in which the GUS gene was replaced by the precursor of tae-miR399, the GUS phenotype was not observed. Meanwhile, GUS staining was markedly decreased in leaves co-transformed with the strain mixture pBI121-tae-miR399 and pBI121-*pTaUBC24* (Fig. 3C). This was considered to be additional evidence that tae-miR399 could target *TaUBC24* .

To confirm the results of these histochemical observations, we carried out RLM-5' RACE assay using RNA from tobacco leaves infiltrated with the strain mixture pBI121-tae-miR399 and pBI121-*pTaUBC24* to determine the predicted cleavage site in *TaUBC24* . The PCR products were cloned and sequenced. The results showed the cleavage site following at 21 bp downstream of MRE (3) (Fig. 3B-(b)). Taken together, these results show that tae-miR399 targets *TaUBC24* .

Verification of the function of tae-miR399 in the freezing tolerance in transgenic *Arabidopsis thaliana*

To describe the function of tae-miR399 in tolerance against freezing stress, we constructed it into an expression vector under the control of the cauliflower mosaic virus 35S promoter (OEmiR399). After producing Arabidopsis transgenic plants through several cycles of kanamycin resistance selection, PCR analysis of genomic DNA with the primers specific to tae-miR399 and 35S corroborated 9 independent transgenic lines (Fig. S3A). The total RNA of these 9 Arabidopsis lines was extracted for their miR399 expression level by PCR semi-quantitative detection, and the wild type Arabidopsis plant (WT) was used as a control. The results showed that miR399 was expressed in all OEmiR399 transgenic Arabidopsis plants. Although WT also has miR399 endogenously, the expression of miR399 in OEmiR399 transgenic Arabidopsis plants was significantly higher than that in WT. Two lines (OEmiR399-4 and OEmiR399-6) were selected and self-crossed to obtain stable homozygous lines.

To investigate the effects of tae-miR399 on tolerance to freezing stress in Arabidopsis, one-month-old Arabidopsis plants (WT, OEmiR399-4 and OEmiR399-6) were transferred to the 4degC for 3 d for cold acclimation and then the temperature dropped to -10degC for 2 h. Before treatment, there was no obvious difference in the morphological parameters including synchronous growth, leaf size and color among these Arabidopsis lines (Fig. 4A), and the primary roots of OEmiR399 lines were significantly longer than WT (Fig. 4B).After the cold treatment, the leaves of all Arabidopsis lines curled as the temperature decreased. Obviously, the leaf color of WT is darker than that of OEmiR399 lines. Moreover, the tae-miR399 overexpressing plants showed higher survival rates than the rest after recovering culture (Fig. 4C). To verify whether tae-miR399 improved the freezing tolerance of Arabidopsis, physiological indicators of all Arabidopsis lines were determined. The results showed that there was no significant difference in electrical conductivity and MDA content among all lines at 24degC (Fig. 4D and E). With the decrease of temperature, the electrical conductivity and MDA

content of all lines increased. At 4degC and -10degC, the MDA content of OEmiR399 lines were lower than that of WT. The electrical conductivity of OEmiR399 lines were lower than that of WT at -10degC (Fig. 4D and E). Generally, the OEmiR399 lines showed better physiology performance under freezing treatment as compared to other lines.

qRT-PCR analysis of miR399 and *AtUBC24* in all Arabidopsis lines showed that the expression of miR399 in OEmiR399 transgenic Arabidopsis plants was always significantly higher than that of WT, and the expression of its target gene *AtUBC24* was always significantly lower than that of WT (Fig. 5A and B). Moreover, at -10degC, the expression of miR399 in WT significantly increased and the expression of*AtUBC24* in WT decreased obviously. At 4degC, the expression of miR399 in OEmiR399 transgenic Arabidopsis plants decreased and the expression of *AtUBC24* in OEmiR399 transgenic Arabidopsis plants increased (Fig. 5A and B).

CBF signaling pathway are affected in OEmiR399 transgenic Arabidopsis plants under freezing stress

Based on the important role of CBF signaling pathway in plant response to freezing stress, the expression of *CBFs* genes (*AtCBF1* ,*AtCBF2* and *AtCBF3*) and *CORs* genes (*AtCOR15A* ,*AtCOR15B* , *AtCOR47* and *AtCOR413IM*) in Arabidopsis were analyzed. The qRT-PCR results of CBFs showed that before treatment the expressions of *AtCBF2* and *AtCBF3* in OEmiR399 lines were significantly higher than that in WT (Fig. 6B and C). At 4degC, there was no obvious difference in the expression of *AtCBFs* in all Arabidopsis lines (Fig. 6 A, B and C). At -10, the expressions of*AtCBFs* in all Arabidopsis lines increased rapidly, and the expressions of *AtCBFs* in OEmiR399 transgenic Arabidopsis plants were significantly higher than that of WT (Fig. 6 A, B and C). The qRT-PCR results of CORs showed that the expression of most CORs genes selected in all Arabidopsis lines increased with different degrees (Fig. 6 D, E, F and G). At low temperature, the expressions of *AtCOR15A* and*AtCOR15B* in all Arabidopsis lines increased substantially, while the expression of *AtCOR15A* was no obvious difference in all Arabidopsis lines and the expression of *AtCOR15B* in OEmiR399 transgenic Arabidopsis plants was lower than that in WT (Fig. 6D and E). The expressions of *AtCOR47* and *AtCOR413IM* were greatly affected by OEmiR399 (Fig. 6 F and G). Before the cold treatment, the expression of *AtCOR47* in OEmiR399 transgenic Arabidopsis plants was always significantly higher than that in WT. At low temperature, the expressions of *AtCOR47* and *AtCOR413IM* in OEmiR399 transgenic Arabidopsis were higher than that of WT (Fig. 6F and G).

AtICE1 is an upstream regulator of the CBF signaling pathway, the previous study documented that CsUBC24 could inhibit CsICE1 in citrus(R. Wang et al., 2020). To verify whether tae-miR399 affected the CBF signal pathway by regulating AtICE1 at low temperature, we used Western Blot technology to detect the AtICE1 content of all Arabidopsis lines at low temperature. The results showed that with the decrease of temperature, the AtICE1 contents of OEmiR399 transgenic Arabidopsis lines were always higher than that of WT (Fig. 7A and B). The AtICE1 transcription levels of all Arabidopsis lines were detected. The results showed that only at 4, the AtICE1 transcription levels of OEmiR399 transgenic Arabidopsis lines were higher than that of WT (Fig. 7C).

Pi Uptake and Translocation are affected in OEmiR399 transgenic Arabidopsis plants under freezing stress

To verify the role of tae-miR399 in the regulation of phosphorus homeostasis in Arabidopsis under freezing stress, we determined the total phosphorus and inorganic phosphorus (Pi) content of all Arabidopsis lines. The results showed at 24degC, there was no significant difference in the total phosphorus content of all Arabidopsis lines, while the Pi content of OEmiR399 transgenic Arabidopsis plants was significantly higher than that of WT (Fig. 8A and B). At 4degC, the total phosphorus content of OEmiR399 transgenic Arabidopsis plants increased significantly and was significantly higher than that of WT, but Pi content of OEmiR399 transgenic Arabidopsis plants was lower than that of WT (Fig. 8A and B). At -10, the total phosphorus content of OEmiR399 transgenic Arabidopsis plants decreased, and there was no significant difference in the total phosphorus content among all Arabidopsis lines, while the Pi content of OEmiR399

transgenic Arabidopsis plants was still significantly lower than that of WT (Fig. 8A and B).

In the past few decades, the molecular mechanisms of Pi sensing and signal transduction in plants have been well confirmed. It is known that Pi transporters play a key role in Pi uptake and transport (Chiou & Lin, 2011; Liang, Wang, Zhao, Tian, & Liao, 2014). In order to verify whether the change in the phosphorus content of OEmiR399 transgenic Arabidopsis was due to the promotion of Pi transport, we performed the expression of the main phosphorus transporters genes (*AtPHO1* ,*AtPHT1;1* , *AtPHT1;2* , and *AtPHT1;4*) of Arabidopsis. These results showed that the expression of *AtPHO1* in WT gradually increased with the decrease of temperature,while the expression of *AtPHO1* in in the OEmiR399 Arabidopsis plants was significantly lower than that of WT under low temperature stress (Fig. 8C). Under low temperature stress, the expression of *AtPHT1s* in all Arabidopsis gradually increasd. Obviously, at 4, the expressions of *AtPHT1;1*and *AtPHT1;4* in the OEmiR399 Arabidopsis plants were higher than those in WT(Fig. 8D and F). And at -10, only the expression of*AtPHT1;4* in OEmiR399 Arabidopsis plants was significantly higher than that in WT (Fig. 8F). Although the expression of *AtPHT1;2*was induced at low temperature, there was no significant difference in its expression among all Arabidopsis lines (Fig. 8E).

Starch degradation are affected in OEmiR399 transgenic Arabidopsis plants under freezing stress

The soluble sugar contents of Arabidopsis llines were analysed. The results showed that at low temperature, the soluble sugar contents of OEmiR399 transgenic Arabidopsis plants were markedly higher than that of WT (Fig. 9A). The results of starch contents showed that at low temperature, the starch contents of OEmiR399 transgenic Arabidopsis plants were significantly lower than that of WT(Fig. 9B). These findings implied that the higher soluble sugar contents in OEmiR399 transgenic Arabidopsis plants were accumulated through degrading starch. To further validated tae-miR399 fuction on starch degradation under freezing stress, we detected the amylase activities in Arabidopsis lines. The results showed that α-amylase activity of OEmiR399 transgenic Arabidopsis plants was only higher than that of WT at 4 (Fig. 10A). Meanwhile, not only β-amylase activity of OEmiR399 transgenic Arabidopsis plants was higher than that of WT at 4, but also significantly higher than that of WT at -10 (Fig. 10B). The expressions of genes encoding α-amylase (*AtAPL1* and*AtAPL3*) and β-amylase (*AtBAM1* and *AtBAM3*) were also detected, the results showed that low temperature observably induced the expression of amylase genes in all Arabidopsis lines (Fig. 10C, D, E and F). However, not all of these amylase genes were affected by overexpression of tae-miR399. Before cold treatment, only the expression of *AtAPL1* in OEmiR399 transgenic Arabidopsis plants was higher than that in WT(Fig. 10C). At 4,the expressions of all amylase genes in OEmiR399 transgenic Arabidopsis plants were higher than that of WT with variety degrees (Fig. 10). At -10, only the expression of *AtBAM3* in OEmiR399 transgenic Arabidopsis was higher than that in WT, while the expressions of*AtAPL1* and *AtBAM1* slightly lower than that of WT (Fig. 10C, E and F). To explore whether GWD in OEmiR399 transgenic Arabidopsis also regulates starch metabolism under freezing stress, we detected the expression of its coding genes *AtSEX1* and *AtSEX4* in Arabidopsis lines. The results showed that under low temperature stress, the expression of *AtSEX1* in all Arabidopsis lines was not induced (Fig. 10G). And there was no significant difference in the expression level of *AtSEX1* among all Arabidopsis lines (Fig. 10G). The expression of *AtSEX4* was different from that of *AtSEX1* . At low temperature, the expression of *AtSEX4* in all Arabidopsis lines was significantly up-regulated (Fig. 10H). And at 24°C and 4°C, the expression of *AtSEX4* in OEmiR399 transgenic Arabidopsis plants was significantly higher than that in WT(Fig. 10H). However, at -10°C, there was no significant difference in the expression level of*AtSEX4* between WT and OEmiR399 lines(Fig. 10H).

ROS metabolism are affected in OEmiR399 transgenic Arabidopsis plants under freezing stress

To verify the effect of tae-miR399 on oxidative tolerance induced by freezing in Arabidopsis plants, we determined ROS including hydrogen peroxide (H_2O_2) and super-oxygen ion ($O_2^{\cdot-}$) levels in different Arabidopsis lines after freezing stress by histochemical staining and ROS content determination. Nitroblue tetrazolium (NBT) staining for $O_2^{\cdot-}$ and 3, 3'-diaminobenzidine (DAB) staining for H_2O_2 showed ROS accumulated to higher levels in WT than that in OEmiR399 transgenic Arabidopsis plants under freezing stress (Fig. 11). These results indicated that OEmiR399 transgenic Arabidopsis plants exhibited stronger ability to scavenge

ROS and lesser symptoms in response to freezing stress. The activities of SOD, POD and CAT in OEmiR399 transgenic Arabidopsis plants were higher than that in WT under freezing stress (Fig. 12). However, the expression of SOD, POD and CAT encoding genes in OEmiR399 transgenic Arabidopsis were not up-regulated under freezing stress. Moreover, the expression of some SOD, POD and CAT encoding genes in OEmiR399 transgenic Arabidopsis was significantly lower than that in WT (Fig. 12).

TaUBC24 interacted physically with TaICE1

According to the high ICE1 content of OEmiR399 transgenic Arabidopsis and CsUBC24 interacting with CsICE1 in previous study (R. Wang et al., 2020), we supposed TaUBC24 might interact with TaICE1. To confirm the protein-protein interaction between poplar TaUBC24 and TaICE1, the full-length CDS of TaICE1 fused to AD (AD- TaICE1) was tested interaction with the full-length CDS of TaUBC24 fused to BD (BD- TaUBC24). As shown in Fig. 13A, an interaction between the TaUBC24 and TaICE1 was detected in the Y2H assay.

To verify the protein interaction between TaUBC24 and TaICE1 in plant cell, the bimolecular fluorescence complementation (BiFC) was performed. Coding sequences of *TaUBC24* were fused to the N-terminal fragment of green fluorescent protein (GFP) (*TaUBC24* -GFP^{N}), while *TaICE1* was fused to the C-terminal fragment of GFP (*TaICE1* -GFP^{C}). GFP fluorescence was detected in the nucleus of *N. benthamiana epidermal* cells co-transfected with plasmids that harbored either the *TaUBC24* -GFP^{N} and *TaICE1* -GFP^{C} (Fig. 13B). These results showed that TaUBC24 was capable of interacting with TaICE1 in vivo.

Discussion

tae-miR399-*TaUBC24* module has a positive regulatory role in Dn1 response to freezing stress

miR399 is sensitive to environmental temperature(Kim, Ahn, Chiou, & Ahn, 2011). The expression change of miR399 is different in different plants and different low temperature treatments. The expression of miR399 in tobacco was significantly up-regulated at 10 for 1 d (R. S. Hu et al., 2019), but in Arabidopsis, the expression of miR399 at 16 was significantly lower than that at 23 (Kim et al., 2011). However, it has not been reported the expression pattern of miR399 in wheat under low temperature stress. In this study, we found that the expression of miR399 in Dn1 tillering nodes of winter field was not always up or down, it was down-regulated at 0, then gradually increased, and the expression increased rapidly at -25 (Fig. 2A). According to the standards of previous studies, the average temperature of winter wheat cold acclimation period in the north of China is about 5 (Li, Fu, Wang, Sun, & Tong, 2010), and 0 is the critical point of water freezing. At 0, the expression of a little winter wheat cold resistant genes is unchanged or slightly decreased, such as *TaMYB1* , *TabZIP1*and *TaWABI5* (Lv et al., 2018). Previous studies have shown that when plants are exposed to similar environment for a long time, the expression of stress genes may decrease (J. Kilian et al., 2007). Therefore, the expression of tae-miR399 decreased at 0 in this study. We speculate that this may be due to the small temperature difference between 5 and 0, Dn1 did not initiate cold response, and the expression of stress genes returned to the level before cold acclimation due to cold adaptation. When the temperature drops to -10, the physiological and biochemical level and molecular level of winter wheat begins to change significantly (Lu, Guo, Xu, & Cang, 2020; Lv et al., 2018). Previous studies have shown that most cold resistance genes begins to increase significantly at -10, such as *TaWABI5* ,*TaCSD1* and *TaFBA-A10* (Lu, Guo, et al., 2020; Lv et al., 2018; K. K. Peng et al., 2021). In this study, the expression of Dn1 tae-miR399 at -10 was lower than that at 5, but it was higher than that at 0, which was similar to the previous results, indicating that the expression of miR399 was started at -10. At the extremely low temperature of -25, the expression of transcription factors, such as*TaMYB1* , *TabZIP1* and *TaWABI5* , tends to decrease slightly, while the expression of genes encoding key enzymes in metabolism pathway still rises sharply, such as *TaCSD1* ,*TaG6PDH* and *Ta6PGDH* (Lu, Guo, et al., 2020; Lv et al., 2018; Tian et al., 2021). In this study, we found that at -25, the expression of Dn1 tae-miR399 increased significantly, while the target gene *TaUBC24* decreased significantly. According to the function of miR399 in maintaining Pi homeostasis in plants(Bari et al., 2006), these results imply that the Dn1 tillering node still needs to absorb and transport Pi at -25, which may be affected by the high expression of genes encoding phosphatase in Dn1 metabolism

pathway to provide substrate phosphate.

At present, it has been shown that miR399 is involved in plant growth and development. For example, silencing the expression of miR399 can damage the floral meristem and anther development of Citrus (Pegler, Oultram, Grof, & Eamens, 2020), and overexpressing miR399 can affect the fruit quality of strawberry (Y. Wang et al., 2017). However, it has not been reported whether miR399 is involved in the development of tillering nodes in plant seedlings. Combined with previous studies, we found that the date of sampling Dn1 tillering nodes in winter is similar, and it took about three months for the temperature to drop from 5 to -25 (K. K. Peng et al., 2021). Therefore, there might be temporal and spatial differences in gene expression in wheat. To further confirm that the high expression of miR399 in Dn1 under low temperature was indeed induced by low temperature, we treated Dn1 wheat seedlings cultured for about 15 d in the laboratory with short-term low temperature. The results showed that the expression of miR399 in Dn1 seedlings increased rapidly at -10 (Fig. 2C), which indicated that freezing stress could induce the expression of miR399 in Dn1 seedlings and decrease the expression of its target gene *TaUBC24* in response to low temperature stress. However, after cold acclimation at 4 for 30 d, the expression of miR399 decreased slightly, and the expression of its target gene *TaUBC24* increased significantly (Fig. 2C and D). This may be due to the long-term cold acclimation, which make Dn1 adapt to the current temperature.

UBC24 (PHO2) is a ubiquitin-conjugating enzyme, its encoding gene *UBC24* is targeted by miR399. In this study, sequence analysis of miR399 and *TaUBC24* showed that both miR399 and *TaUBC24* belonged to highly conserved families. tae-miR399 precursor could be folded into a perfect stem-loop structure with high negative minimum free energy and minimum free energy index. In the 5'UTR of *TaUBC24* , we predicted six response elements/binding sites (MREs). Similarly, there are five MREs of ath-miR399 in *AtPHO2* of *Arabidopsis thaliana* (Fujii et al., 2005), and six MREs of zm-miR399 in *ZmPHO2* of maize(Du et al., 2018). Using tobacco transient co-transfection and 5'RLM-RACE analysis, we demonstrated that *TaUBC24* , which encodes ubiquitin binding E2 enzyme, is the target gene of tae-miR399 and is negatively regulated by tae-miR399 (Fig. 3). These results indicate that miR399-*UBC24* module is highly conserved in wheat.

tae-miR399-*UBC24* module regulates Arabidopsis CBF signaling pathway in response to freezing stress

In this study, we analyzed the expression pattern and biological function of Dn1 tae-miR399 and its target gene *TaUBC24* under low temperature stress, and provided evidence for tae-miR399 mediated *TaUBC24* expression as a new regulatory mechanism in winter wheat response to low temperature stress. Due to the limitation of experimental conditions, it is very difficult to carry out tissue transfection in wheat. Therefore, *Arabidopsis thaliana* was used for functional gain analysis. The results showed that in transgenic Arabidopsis plants, OEmiR399 lines had a cold tolerance phenotype, and OEmiR399 Arabidopsis plants had a higher survival rate, lower ion permeability and membrane lipid damage than WT (Fig. 4). These results indicates that tae-miR399 has a certain biological function of cold resistance.

Although a large number of cold responsive miRNAs have been identified, further functional analysis is needed to determine their roles in plant cold response, especially resistance to freezing stress. It has found that overexpressing miR394 transgenic Arabidopsis plants show stronger cold tolerance, and the expression of multiple genes in CBF signaling pathway are higher than those of WT, indicating that miR394 is a positive regulator of plant response to low temperature stress (Song, Li, Cao, & Qi, 2019). Overexpressing miR319 in rice has a better survival rate than that of wild-type, the expressions of *CBFs* increase, and the content of ROS is also significantly lower than that of wild-type, which indicates that miR319 is involved in plant cold tolerance (S. T. Wang et al., 2014). At present, the mechanism of miRNA regulating wheat response to low temperature stress is still limited, and the specific mechanism of wheat miRNAs mediated CBF signaling pathway is still unclear. ICE1, as a MYC bHLH protein, also plays a role in CBF signaling pathway by regulating downstream target genes (Deng, Ye, Fan, Pu, & Yan, 2017). The previous study showed that CsUBC24 and CsICE1 interact in citrus, and CsUBC24 mediate degradation of CsICE1 but not regulate the transcription level of *CsICE1* (R. Wang et al., 2020). Therefore, we tested the transcription level of *AtICE1* in OEmiR399 Arabidopsis plants and found that only at 4degC, the expression level of

AtICE1 in OEmiR399 Arabidopsis was higher than that of WT (Fig. 7C). However, the ICE1 protein level of OEmiR399 Arabidopsis plants was always higher than that of WT (Fig. 7A and B). These results suggests that overexpressing tae-miR399 can increase the protein content of AtICE1 by down-regulating the expression of *AtUBC24* . In Arabidopsis, ICE1 plays a key role in the development of leaf stomata and the response to chilling and freezing stress, and can combine with the *CBFs* promoter sequence to promote its expression (Miura et al., 2007). Recent studies have shown that except binding to the promoter sequence of CBFs, ICE1 can also directly initiate the expression of most genes in the CBF signaling pathway, such as*COR47* , *COR413IM* , *MPK* and *HOS* (Tang et al., 2020). In this study, the expression of *CORs* detected in all Arabidopsis lines were up-regulated in different degrees under low temperature. It is worth noting that only the expression levels of*AtCOR47* and *AtCOR413IM* in OEmiR399 Arabidopsis plants were significantly higher than those in WT under cold acclimation and freezing stress (Fig. 6F and G). The expression of *AtCBFs* in OEmiR399 Arabidopsis plants were significantly higher than that in WT under freezing stress (Fig. 6A, B and C). The results indicate that AtICE1 with high protein level promotes the expression of its downstream target genes (*AtCBFs, AtCOR47* and *AtCOR413IM*) by binding to the promoter region under freezing stress. Some studies have shown that plants can activate the expression of *CBFs* in the early stage of cold acclimation, and when the content of CBFs protein reaches a certain level in the later stage, the expression of *CBFs* is inhibited to prevent plants from over chilling defense (Chinnusamy et al., 2003a; Novillo, Alonso, Ecker, & Salinas, 2004). For example, when Arabidopsis was treated at 4 for 24 h, its *AtCBFs* peaked at 3 h and then gradually decreased(Joachim Kilian et al., 2007). In this study, *AtCBFs* also showed similar expression changes. At 3 days of cold acclimation, the expression of *AtCBFs* in WT had returned to the level before untreated, while the expression of *AtCBFs* in OEmiR399 Arabidopsis plants was significantly lower than that at 24 (Fig. 6A, B and C). It implies that during cold acclimation, the AtCBFs protein content of OEmiR399 Arabidopsis plants is significantly higher than that of WT. In addition, we found that TaUBC24 interacts with TaICE1 through Y2H and BiFC experiments (Fig. 13). These results suggested that the interaction between UBC24 and ICE1 may be conserved in plants. Also in wheat, tae-miR399-*TaUBC24* module may mediate the CBF signaling pathway in response to freezing stress by inhibiting the degradation of TaICE1 protein.

tae-miR399-*UBC24* module regulates Pihomeostasis and phosphorus utilization efficiency in Arabidopsis in response to freezing stress

PHO2 down-regulates the expression of PSI-related genes (such as PHTs) by promoting the degradation of PHO1, and negatively regulates Pi uptake and translocation (Chiou & Lin, 2011; Liang et al., 2014). Under phosphorus deficiency, plants adapt complex strategies to enhance the acquisition of phosphorus, for example, the remodeling of the root structure and the reactivation of phosphorus in plants (Chiou & Lin, 2011; Liang et al., 2014). In this study, at 24, the Pi content of OEmiR399 Arabidopsis plants was significantly higher than that of WT, and the primary roots of OEmiR399 Arabidopsis plants were elongated, which was similar to the phenotype of overexpressing miR399 Arabidopsis (Chiou et al., 2006), rice (Hu et al., 2011) and maize(Du et al., 2018). These results imply that overexpressing tae-miR399 may promote the absorption and transport of Pi in Arabidopsis. During the cold acclimation, the total phosphorus content and the expression of*AtPHT1; 1* and *AtPHT1; 4* in OEmiR399 Arabidopsis plants were significantly higher than that in WT (Fig. 8D and F). However, the Pi content of OEmiR399 Arabidopsis plants was significantly lower than that of WT (Fig. 8B). These results may imply that during cold acclimation, the phosphorous transporter in OEmiR399 Arabidopsis plants is active, and the transported Pi has been rapidly converted to organophosphorus. Previous reports have reported that at 5degC, the Pi and organophosphates of *pho2* Arabidopsis mutants are significantly higher than those of WT, and the many phosphatases and other metabolic enzymes activities of *pho2* increase (Hurry, Strand, Furbank, & Stitt, 2000), which is consistent with our research results. Therefore, we speculate that overexpressing tae-miR399 may increase Pi absorption and phosphorus utilization efficiency of Arabidopsis during cold acclimation. The Pi content and the expression of *AtPHTs* in OEmiR399 Arabidopsis plants under freezing stress were similar to cold acclimation, indicating that under freezing stress, the phosphorus utilization efficiency of OEmiR399 Arabidopsis plants is higher than that of WT. These results demonstrate that the function of tae-miR399 in response to Pi signal in Arabidopsis is

conserved, and that Pi signal plays an important and positive role in response to low temperature stress in Arabidopsis. In addition, the expression of *AtPHO1* of OEmiR399 Arabidopsis plants has been at a low level under low temperature in this study, which may be a feedback regulation of increasing AtPHO1 protein content caused by down-regulating*AtUBC24* expression (Fig. 8C).

tae-miR399-*UBC24* module regulates starch degradation in*Arabidopsis thaliana* in response to freezing stress

Pi can regulate plant carbohydrate metabolism, for example, in the dark, phosphorus deficient Arabidopsis will reduce photosynthesis, increase starch and sucrose content (Ciereszko, Johansson, & Kleczkowski, 2005; Muller, Morant, Jarmer, Nilsson, & Nielsen, 2007). OsSPX1 is a Pi-dependent OsPHR2 activity inhibitor, which plays an important role in the Pi signal pathway of rice (Z. Y. Wang et al., 2014), and the decreased expression of *OsSPX1* affects the expression of genes involved in carbohydrate metabolism and sugar transport (K. Zhang et al., 2016). Starch degradation is involved in cold-induced soluble sugar accumulation (Ruelland et al., 2009). In this study, at 4degC and -10degC, the starch content of OEmiR399 Arabidopsis plants was significantly lower than that of WT, while the soluble sugar content was significantly higher than that of WT, which indicated that overexpressing tae-miR399 at low temperature promoted starch degradation in Arabidopsis (Fig. 9A and B). The amylase in plants is mainly divided into α-amylase and β-amylase. In this study, during cold acclimation, the activity of α-amylase and β-amylase in OEmiR399 Arabidopsis plants were higher than that in WT (Fig. 10A and B). However, during freezing stress, only β-amylase activity of OEmiR399 Arabidopsis plants was higher than that of WT (Fig. 10B). These results indicates that during cold acclimation, overexpressing tae-miR399 can promote both α-amylase and β-amylase activities to regulate starch degradation in Arabidopsis, while in freezing stress, overexpressing tae-miR399 only promote β-amylase to mediate starch degradation in Arabidopsis. It is worth noting that the expression of *AtAPL3* and *AtBAM3* in OEmiR399 Arabidopsis plants were similar to the changes in its own enzyme activity (Fig. 10D and F). These results indicate that the activities of α-amylase and β-amylase in OEmiR399 Arabidopsis plants may be mainly regulated by*AtAPL3* and *AtBAM3* . Starch phosphorylation is another way to degrade starch. It has been controversy about whether starch phosphorylase is involved in the starch degradation under low temperature stress. At 2°C, it was no significant change in the enzyme activity of starch phosphorylase in *Arabidopsis thaliana* (R. Yano, Nakamura, Yoneyama, & Nishida, 2005). However, the study by (Berrocal-Lobo et al., 2011) showed that the transcription level and protein level of SEX4 were significantly up-regulated at 4°C, which is consistent with the starch degradation trend. In this study, we found that *AtSEX1* did not participate in the regulation of starch degradation in Arabidopsis under low temperature stress, but the expression of *AtSEX4* in OEmiR399 Arabidopsis plants was induced during the cold acclimation (Fig. 10G and H). These results suggest that during cold acclimation, maybe starch phosphorylase also participate in the starch degradation of Arabidopsis.

tae-miR399-*UBC24* module regulates the ROS metabolism response to freezing stress in Arabidopsis

Low temperature stress can significantly disturb ROS homeostasis in plants, leading to lipid peroxidation, damage to membranes (Ning, Li, Hicks, & Xiong, 2010) and increasing MDA content (J. J. Liu, Wei, & Li, 2014). In this study, under low temperature stress, OEmiR399 Arabidopsis had lower MDA content, indicating that it suffered less oxidative damage than WT. Further histochemical staining and physiological experiment methods to detect ROS content, the results showed that the H_2O_2 content and $O_2^{\cdot-}$ production of OEmiR399 Arabidopsis plants were visibly lower than that of WT under freezing stress (Fig. 11). Also, under freezing stress, the SOD, POD and CAT enzyme activities of OEmiR399 Arabidopsis plants were significantly higher than those of WT (Fig. 12, 13 and 14). These implies that OEmiR399 Arabidopsis plants have a strong ROS scavenging ability. Interestingly, the expression changes of the genes encoding SOD, POD and CAT in OEmiR399 Arabidopsis plants were not consistent with the enzyme activities. The expression of the most genes encoding SOD, POD and CAT in OEmiR399 Arabidopsis plants was significantly up-regulated at 24°C and 4°C, but not significantly changed or down-regulated at -10°C (Fig. 12, 13 and 14). Similary, the expression of genes encoding SOD and APX in bluegrass are significantly down-regulated under

drought stress, while their enzyme activities are significantly up-regulated (Xu, Han, & Huang, 2011). Based on previous studies, we speculate that the changes in the enzyme activity of OEmiR399 Arabidopsis plants under freezing stress may not be caused by mRNA levels, but are regulated at the post-transcriptional level, which is due to the inhibition of enzyme inactivation/activation or degradation/synthesis caused by freezing stress. Homologous or heterologous overexpressing *ICE1* in plants such as tomato and tobacco can increase its antioxidant enzyme activity to enhance the cold tolerance of plants (Feng et al., 2013; Luo et al., 2020). Therefore, in this study, under low temperature stress, the high ICE1 protein levels of OEmiR399 Arabidopsis plants may also be one of the reasons for its stronger ROS scavenging ability.

Combining with our phenotypic data, we can conclude that miR399-*UBC24* module inhibit the degradation of ICE1 and promote the CBF signalling pathway and ROS clearance to initiate plant response to freezing stress. On the other hand, miR399-*UBC24* module can accelerate starch degradation to accumulate a large amount of soluble sugar by promoting the Pi absorption and utilization efficiency, and then enhance freezing tolerance in plants (Fig. 14). These findings clarify the function of miR399-*UBC24* module and suggest that tae-miR399-*TaUBC24* can potentially be used to improve freezing tolerance in crops.

Acknowledgements

This work was financially supported by National Natural Science Foundation of China (31971831, 31701348, 31601236).

Author contributions

K.K.P. and Y.T. did yeast two-hybrid, bimolecular fluorescence complementation, Arabidopsis transformation. K.K.P., Y.T., X.Z.S., Z.P.R. and J.P.X. made the gene cloning, 5' RLM RACE and DNA sequencing. K.K.P., Y.T. and C.H.S. did western blots and co-express tae-miR399 and MREs in *N. benthamiana* . K.K.P., Y.T., Y.Z.B., X.Z.S., Y.S.L., J.Y., D.Z. and Q.H.X. measured the plant physiological and phenotypic indexes and analyzed the data. J.C. designed the experiments. K.K.P. and Y.T. wrote the manuscript. All authors discussed the results and approved the final manuscript.

References

Asif, M. H., Lakhwani, D., Pathak, S., Gupta, P., Bag, S. K., Nath, P., & Trivedi, P. K. (2014). Transcriptome analysis of ripe and unripe fruit tissue of banana identifies major metabolic networks involved in fruit ripening process. *BMC Plant Biol, 14* , 316. doi:10.1186/s12870-014-0316-1

Aung, K., Lin, S. I., Wu, C. C., Huang, Y. T., Su, C. L., & Chiou, T. J. (2006). pho2, a phosphate overaccumulator, is caused by a nonsense mutation in a microRNA399 target gene. *Plant Physiol, 141* (3), 1000-1011. doi:10.1104/pp.106.078063

Babben, S., Schliephake, E., Janitza, P., Berner, T., Keilwagen, J., Koch, M., . . . Perovic, D. (2018). Association genetics studies on frost tolerance in wheat (Triticum aestivum L.) reveal new highly conserved amino acid substitutions in CBF-A3, CBF-A15, VRN3 and PPD1 genes. *Bmc Genomics, 19* , 24. doi:10.1186/s12864-018-4795-6

Baek, D., Chun, H. J., Kang, S., Shin, G., Park, S. J., Hong, H., . . . Yun, D. J. (2016). A Role for Arabidopsis miR399f in Salt, Drought, and ABA Signaling. *Mol Cells, 39* (2), 111-118. doi:10.14348/molcells.2016.2188

Bari, R., Datt Pant, B., Stitt, M., & Scheible, W. R. (2006). PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants.*Plant Physiol, 141* (3), 988-999. doi:10.1104/pp.106.079707

Berrocal-Lobo, M., Ibañez, C., Acebo, P., Ramos, A., Perez-Solis, E., Collada, C., . . . Allona, I. (2011). Identification of a homolog of Arabidopsis DSP4 (SEX4) in chestnut: its induction and accumulation in stem amyloplasts during winter or in response to the cold. *Plant Cell Environ, 34* (10), 1693-1704. doi:10.1111/j.1365-3040.2011.02365.x

Breuil, C., & Saddler, J. (1985). Comparison of the 3, 5-dinitrosalicylic acid and Nelson-Somogyi methods of assaying for reducing sugars and determining cellulase activity. *Enzyme and microbial technology, 7* (7),

327-332.

Chinnusamy, V., Ohta, M., Kanrar, S., Lee, B. H., Hong, X., Agarwal, M., & Zhu, J. K. (2003a). ICE1: a regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis. *Genes Dev, 17* (8), 1043-1054. doi:10.1101/gad.1077503

Chinnusamy, V., Ohta, M., Kanrar, S., Lee, B. H., Hong, X. H., Agarwal, M., & Zhu, J. K. (2003b). ICE1: a regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis. *Genes & Development, 17* (8), 1043-1054. doi:10.1101/gad.1077503

Chiou, T. J., Aung, K., Lin, S. I., Wu, C. C., Chiang, S. F., & Su, C. L. (2006). Regulation of phosphate homeostasis by microRNA in Arabidopsis. *Plant Cell, 18* (2), 412-421. doi:10.1105/tpc.105.038943

Chiou, T. J., & Lin, S. I. (2011). Signaling network in sensing phosphate availability in plants. *Annu Rev Plant Biol, 62* , 185-206. doi:10.1146/annurev-arplant-042110-103849

Ciereszko, I., Johansson, H., & Kleczkowski, L. A. (2005). Interactive effects of phosphate deficiency, sucrose and light/dark conditions on gene expression of UDP-glucose pyrophosphory lase in Arabidopsis.*Journal of Plant Physiology, 162* (3), 343-353. doi:10.1016/j.jplph.2004.08.003

Clough, S. J., & Bent, A. F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana.*The Plant journal : for cell and molecular biology, 16* (6), 735-743. doi:10.1046/j.1365-313x.1998.00343.x

Deng, C., Ye, H., Fan, M., Pu, T., & Yan, J. (2017). The rice transcription factors OsICE confer enhanced cold tolerance in transgenic Arabidopsis. *Plant Signal Behav, 12* (5), e1316442. doi:10.1080/15592324.2017.1316442

Dianjun, X., Yu, Z., & Kuide, Y. I. N. (2007). Transformation of ICE1 Gene Mediated by Agrobacterium Improves Cold Tolerance in Transgenie Rice. [农杆菌介导的转ice1基因提高水稻的耐寒性]. *Chinese Journal of Rice Science, 21* (5), 482-486.

Ding, Y., Lv, J., Shi, Y., Gao, J., Hua, J., Song, C., . . . Yang, S. (2019). EGR2 phosphatase regulates OST1 kinase activity and freezing tolerance in Arabidopsis. *Embo j, 38* (1). doi:10.15252/embj.201899819

Ding, Y., Shi, Y., & Yang, S. (2019). Advances and challenges in uncovering cold tolerance regulatory mechanisms in plants. *New Phytologist* .

Ding, Y. L., Li, H., Zhang, X. Y., Xie, Q., Gong, Z. Z., & Yang, S. H. (2015). OST1 Kinase Modulates Freezing Tolerance by Enhancing ICE1 Stability in Arabidopsis. *Developmental Cell, 32* (3), 278-289. doi:10.1016/j.devcel.2014.12.023

Dionisio-Sese, M. L., & Tobita, S. (1998). Antioxidant responses of rice seedlings to salinity stress. *Plant Science, 135* (1), 1-9. doi:10.1016/s0168-9452(98)00025-9

Dowgert, M. F., & Steponkus, P. L. (1984). Behavior of the Plasma Membrane of Isolated Protoplasts during a Freeze-Thaw Cycle. *Plant Physiology* .

Du, Q. G., Wang, K., Zou, C., Xu, C., & Li, W. X. (2018). The PILNCR1-miR399 Regulatory Module Is Important for Low Phosphate Tolerance in Maize. *Plant Physiology, 177* (4), 1743-1753. doi:10.1104/pp.18.00034

Feng, H.-L., Ma, N.-N., Meng, X., Zhang, S., Wang, J.-R., Chai, S., & Meng, Q.-W. (2013). A novel tomato MYC-type ICE1-like transcription factor, SlICE1a, confers cold, osmotic and salt tolerance in transgenic tobacco. *Plant Physiology and Biochemistry, 73* , 309-320. doi:10.1016/j.plaphy.2013.09.014

Fujii, H., Chiou, T. J., Lin, S. I., Aung, K., & Zhu, J. K. (2005). A miRNA involved in phosphate-starvation response in Arabidopsis.*Curr Biol, 15* (22), 2038-2043. doi:10.1016/j.cub.2005.10.016

Gao, N., Qiang, X. M., Zhai, B. N., Min, J., & Shi, W. M. (2015). Transgenic tomato overexpressing ath-miR399d improves growth under abiotic stress conditions. *Russian Journal of Plant Physiology, 62* (3), 360-366. doi:10.1134/s1021443715030061

Gusta, & L., V. (2004). The effect of water, sugars, and proteins on the pattern of ice nucleation and propagation in acclimated and nonacclimated canola leaves. *Plant Physiology, 135* (3), 1642-1653.

Hejazi, M., Fettke, J., Haebel, S., Edner, C., & Ritte, G. (2010). Glucan, water dikinase phosphorylates crystalline maltodextrins and thereby initiates solubilization. *The Plant Journal, 55* (2), 323-334.

Hu, B., Zhu, C., Li, F., Tang, J., Wang, Y., Lin, A., . . . Chu, C. (2011). LEAF TIP NECROSIS1 plays a pivotal role in the regulation of multiple phosphate starvation responses in rice. *Plant Physiol, 156* (3), 1101-1115. doi:10.1104/pp.110.170209

Hu, R., Li, Z., Xiang, S., Li, Y., Yi, P., Xiao, M., & Zhang, X. (2019). Comparative MicroRNA Profiling Reveals the Cold Response Mechanisms in two Contrasting Tobacco Cultivars. *International Journal of Agriculture and Biology, 22* (4), 757-762. doi:10.17957/ijab/15.1126

Hu, R. S., Li, Z. M., Xiang, S. P., Li, Y. Y., Yi, P. F., Xiao, M. Q., & Zhang, X. W. (2019). Comparative MicroRNA Profiling Reveals the Cold Response Mechanisms in two Contrasting Tobacco Cultivars.*International Journal of Agriculture and Biology, 22* (4), 757-762. doi:10.17957/ijab/15.1126

Huang, T. K., Han, C. L., Lin, S. I., Chen, Y. J., Tsai, Y. C., Chen, Y. R., . . . Chiou, T. J. (2013). Identification of downstream components of ubiquitin-conjugating enzyme PHOSPHATE2 by quantitative membrane proteomics in Arabidopsis roots. *Plant Cell, 25* (10), 4044-4060. doi:10.1105/tpc.113.115998

Hurry, V., Strand, A., Furbank, R., & Stitt, M. (2000). The role of inorganic phosphate in the development of freezing tolerance and the acclimatization of photosynthesis to low temperature is revealed by the pho mutants of Arabidopsis thaliana. *The Plant journal : for cell and molecular biology, 24* (3), 383-396. doi:10.1046/j.1365-313x.2000.00888.x

Jan, N., Mahboob ul, H., & Andrabi, K. I. (2009). Cold resistance in plants: A mystery unresolved. *Electronic Journal of Biotechnology, 12* (3), 15. doi:10.2225/vol12-issue3-fulltext-3

Juan, J. X., Yu, X. H., Jiang, X. M., Gao, Z., Zhang, Y., Li, W., . . . Yang, G. (2015). Agrobacterium-mediated transformation of tomato with the ICE1 transcription factor gene. *Genetics and Molecular Research, 14* (1), 597-608. doi:10.4238/2015.January.30.1

Kaplan, F., & Guy, C. L. (2005). RNA interference of Arabidopsis beta-amylase8 prevents maltose accumulation upon cold shock and increases sensitivity of PSII photochemical efficiency to freezing stress. *The Plant Journal, 44* .

Kaplan, F., Kopka, J., Sung, D. Y., Zhao, W., Popp, M., Porat, R., & Guy, C. L. (2007). Transcript and metabolite profiling during cold acclimation of Arabidopsis reveals an intricate relationship of cold-regulated gene expression with modifications in metabolite content.*The Plant Journal, 50* (6).

Kilian, J., Whitehead, D., Horak, J., Wanke, D., Weinl, S., Batistic, O., . . . Harter, K. (2007). The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. *Plant Journal, 50* (2), 347-363. doi:10.1111/j.1365-313X.2007.03052.x

Kilian, J., Whitehead, D., Horak, J., Wanke, D., Weinl, S., Batistic, O., . . . Harter, K. (2007). The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. *The Plant Journal, 50* (2), 347-363.

Kim, W., Ahn, H. J., Chiou, T. J., & Ahn, J. H. (2011). The role of the miR399-PHO2 module in the regulation of flowering time in response to different ambient temperatures in Arabidopsis thaliana. *Mol Cells, 32* (1), 83-88. doi:10.1007/s10059-011-1043-1

Koc, I., Filiz, E., & Tombuloglu, H. (2015). Assessment of miRNA expression profile and differential expression pattern of target genes in cold-tolerant and cold-sensitive tomato cultivars.*Biotechnology & Biotechnological Equipment, 29* (5), 851-860.

Li, Z., Fu, L., Wang, X., Sun, Y., & Tong, M. (2010). Advance on winter wheat breeding for freezing tolerance in frigid region. [高寒地区冬小麦抗寒育种研究进展]. *Journal of Northeast Agricultural University, 41* (8), 141-147.

Liang, C., Wang, J., Zhao, J., Tian, J., & Liao, H. (2014). Control of phosphate homeostasis through gene regulation in crops. *Curr Opin Plant Biol, 21* , 59-66. doi:10.1016/j.pbi.2014.06.009

Ling, H. Q., Ma, B., Shi, X., Liu, H., Dong, L., Sun, H., . . . Liang, C. (2018). Genome sequence of the progenitor of wheat A subgenome Triticum urartu. *Nature, 557* (7705), 424-428. doi:10.1038/s41586-018-0108-0

Liu, J. J., Wei, Z., & Li, J. H. (2014). Effects of copper on leaf membrane structure and root activity of maize seedling. *Bot Stud, 55* (1), 47. doi:10.1186/s40529-014-0047-5

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., & Shinozaki, K. (1998). Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. *Plant Cell, 10* (8), 1391-1406. doi:10.1105/tpc.10.8.1391

Lu, Q., Guo, F., Xu, Q., & Cang, J. (2020). LncRNA improves cold resistance of winter wheat by interacting with miR398. *Funct Plant Biol, 47* (6), 544-557. doi:10.1071/fp19267

Lu, Q., Xu, Q., Guo, F., Lv, Y., Song, C., Feng, M., . . . Cang, J. (2020). Identification and characterization of long non-coding RNAs as competing endogenous RNAs in the cold stress response of Triticum aestivum. *Plant Biology, 22* (4), 635-645. doi:10.1111/plb.13119

Lundmark, M., Cavaco, A. M., Trevanion, S., & Hurry, V. (2006). Carbon partitioning and export in transgenic Arabidopsis thaliana with altered capacity for sucrose synthesis grown at low temperature: a role for metabolite transporters. *Plant, Cell & Environment, 29* (9).

Luo, P., Li, Z. Y., Chen, W., Xing, W., Yang, J., & Cui, Y. Y. (2020). Overexpression of RmICE1, a bHLH transcription factor from Rosa multiflora, enhances cold tolerance via modulating ROS levels and activating the expression of stress-responsive genes.*Environmental and Experimental Botany, 178* , 11. doi:10.1016/j.envexpbot.2020.104160

Lv, Y., Song, C. H., Lu, Q. W., Tian, Y., Li, H. D., Zhang, D., . . . Cang, J. (2018). The Expression Characteristics of Transcription Factors Regulated by Exogenous ABA in Winter Wheat (Triticum aestivum) under Cold Stress. *Russian Journal of Plant Physiology, 65* (6), 842-848. doi:10.1134/s1021443718060134

Miura, K., Jin, J. B., Lee, J., Yoo, C. Y., Stirm, V., Miura, T., . . . Hasegawa, P. M. (2007). SIZ1-mediated sumoylation of ICE1 controls CBF3/DREB1A expression and freezing tolerance in Arabidopsis.*Plant Cell, 19* (4), 1403-1414. doi:10.1105/tpc.106.048397

Muller, R., Morant, M., Jarmer, H., Nilsson, L., & Nielsen, T. H. (2007). Genome-wide analysis of the Arabidopsis leaf transcriptome reveals interaction of phosphate and sugar metabolism. *Plant Physiology, 143* (1), 156-171. doi:10.1104/pp.106.090167

Ning, J., Li, X., Hicks, L. M., & Xiong, L. (2010). A Raf-like MAPKKK gene DSM1 mediates drought resistance through reactive oxygen species scavenging in rice. *Plant Physiol, 152* (2), 876-890. doi:10.1104/pp.109.149856

Nour-Eldin, H. H., Hansen, B. G., Nørholm, M. H., Jensen, J. K., & Halkier, B. A. (2006). Advancing uracil-excision based cloning towards an ideal technique for cloning PCR fragments. *Nucleic Acids Research, 34* (18), e122-e122.

Novillo, F., Alonso, J. M., Ecker, J. R., & Salinas, J. (2004). CBF2/DREB1C is a negative regulator of CBF1/DREB1B and CBF3/DREB1A expression and plays a central role in stress tolerance in Arabidopsis.*Proc Natl Acad Sci U S A, 101* (11), 3985-3990. doi:10.1073/pnas.0303029101

Pegler, J. L., Oultram, J. M. J., Grof, C. P. L., & Eamens, A. L. (2020). Molecular Manipulation of the miR399/PHO2 Expression Module Alters the Salt Stress Response of Arabidopsis thaliana. *Plants (Basel), 10* (1). doi:10.3390/plants10010073

Peng, K. K., Tian, Y., Cang, J., Yu, J., Wang, D. J., He, F. X., . . . Tan, Y. G. (2021). Overexpression of TaFBA-A10 from Winter Wheat Enhances Freezing Tolerance in Arabidopsis thaliana. *Journal of Plant Growth Regulation* , 13. doi:10.1007/s00344-021-10304-7

Peng, T., Zhu, X., Duan, N., & Liu, J. H. (2014). PtrBAM1, a β-amylase-coding gene of Poncirus trifoliata, is a CBF regulon member with function in cold tolerance by modulating soluble sugar levels.*Plant Cell Environ, 37* (12), 2754-2767. doi:10.1111/pce.12384

Rizwan, M., Ali, S., Ali, B., Adrees, M., Arshad, M., Hussain, A., . . . Waris, A. A. (2019). Zinc and iron oxide nanoparticles improved the plant growth and reduced the oxidative stress and cadmium concentration in wheat. *Chemosphere, 214* , 269-277. doi:10.1016/j.chemosphere.2018.09.120

Ruelland, E., Vaultier, M. N., Zachowski, A., & Hurry, V. (2009). Cold signalling and cold acclimation in plants.

Shabala, S. N., Shabala, S. I., Martynenko, A. I., Babourina, O., & Newman, I. A. (1998). Salinity effect on bioelectric activity, growth, Na+ accumulation and chlorophyll fluorescence of maize leaves: a comparative survey and prospects for screening. *Functional Plant Biology, 25* (5), 609-616. doi:10.1071/PP97146

Shi, Y., Ding, Y., & Yang, S. (2018). Molecular Regulation of CBF Signaling in Cold Acclimation. *Trends in Plant Science, 23* (7), S1360138518300864.

Sofo, A., Dichio, B., Xiloyannis, C., & Masia, A. (2004). Lipoxygenase activity and proline accumulation in leaves and roots of olive trees in response to drought stress. *Physiologia Plantarum, 121* (1), 58-65. doi:10.1111/j.0031-9317.2004.00294.x

Song, X., Li, Y., Cao, X., & Qi, Y. (2019). MicroRNAs and Their Regulatory Roles in Plant-Environment Interactions. *Annu Rev Plant Biol, 70* , 489-525. doi:10.1146/annurev-arplant-050718-100334

Stockinger, E. J., Gilmour, S. J., & Thomashow, M. F. (1997). Arabidopsis thaliana CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proc Natl Acad Sci U S A, 94* (3), 1035-1040. doi:10.1073/pnas.94.3.1035

Tang, K., Zhao, L., Ren, Y., Yang, S., Zhu, J. K., & Zhao, C. (2020). The transcription factor ICE1 functions in cold stress response by binding to the promoters of CBF and COR genes. *J Integr Plant Biol, 62* (3), 258-263. doi:10.1111/jipb.12918

Tian, Y., Peng, K., Bao, Y., Zhang, D., Meng, J., Wang, D., . . . Cang, J. (2021). Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase genes of winter wheat enhance the cold tolerance of transgenic Arabidopsis. *Plant Physiol Biochem, 161* , 86-97. doi:10.1016/j.plaphy.2021.02.005

Wang, J., Ma, X. M., Kojima, M., Sakakibara, H., & Hou, B. K. (2013). Glucosyltransferase UGT76C1 finely modulates cytokinin responses via cytokinin N-glucosylation in Arabidopsis thaliana. *Plant Physiol Biochem, 65* , 9-16. doi:10.1016/j.plaphy.2013.01.012

Wang, J., Sun, J., Miao, J., Guo, J., Shi, Z., He, M., . . . Li, Z. (2013). A phosphate starvation response regulator Ta-PHR1 is involved in phosphate signalling and increases grain yield in wheat. *Ann Bot, 111* (6), 1139-1153. doi:10.1093/aob/mct080

Wang, R., Fang, Y. N., Wu, X. M., Qing, M., Li, C. C., Xie, K. D., . . . Guo, W. W. (2020). The miR399-CsUBC24 Module Regulates Reproductive Development and Male Fertility in Citrus. *Plant Physiol, 183* (4), 1681-1695. doi:10.1104/pp.20.00129

Wang, S. T., Sun, X. L., Hoshino, Y., Yu, Y., Jia, B., Sun, Z. W., . . . Zhu, Y. M. (2014). MicroRNA319 positively regulates cold tolerance by targeting OsPCF6 and OsTCP21 in rice (Oryza sativa L.). *Plos One, 9* (3), e91357.

Wang, Y., Zhang, J., Cui, W., Guan, C., Mao, W., & Zhang, Z. (2017). Improvement in Fruit Quality by Overexpressing miR399a in Woodland Strawberry. *J Agric Food Chem, 65* (34), 7361-7370. doi:10.1021/acs.jafc.7b01687

Wang, Z. Y., Ruan, W. Y., Shi, J., Zhang, L., Xiang, D., Yang, C., . . . Wu, P. (2014). Rice SPX1 and SPX2 inhibit phosphate starvation responses through interacting with PHR2 in a phosphate-dependent manner.*Proc Natl Acad Sci U S A, 111* (41), 14953-14958. doi:10.1073/pnas.1404680111

Xu, L. X., Han, L. B., & Huang, B. R. (2011). Antioxidant Enzyme Activities and Gene Expression Patterns in Leaves of Kentucky Bluegrass in Response to Drought and Post-drought Recovery. *Journal of the American Society for Horticultural Science, 136* (4), 247-255. doi:10.21273/jashs.136.4.247

Yamaguchi-Shinozaki, K., & Shinozaki, K. (1994). A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. *Plant Cell, 6* (2), 251-264. doi:10.1105/tpc.6.2.251

Yano, & R. (2005). Starch-Related α-Glucan/Water Dikinase Is Involved in the Cold-Induced Development of Freezing Tolerance in Arabidopsis.*Plant Physiology, 138* (2), 837-846.

Yano, R., Nakamura, M., Yoneyama, T., & Nishida, I. (2005). Starch-related alpha-glucan/water dikinase is involved in the cold-induced development of freezing tolerance in Arabidopsis.*Plant Physiol, 138* (2), 837-846. doi:10.1104/pp.104.056374

Zhang, K., Song, Q., Wei, Q., Wang, C., Zhang, L., Xu, W., & Su, Z. (2016). Down-regulation of OsSPX1 caused semi-male sterility, resulting in reduction of grain yield in rice. *Plant Biotechnol J, 14* (8), 1661-1672. doi:10.1111/pbi.12527

Zhang, Z., Li, J., Li, F., Liu, H., Yang, W., Chong, K., & Xu, Y. (2017). OsMAPK3 Phosphorylates OsbHLH002/OsICE1 and Inhibits Its Ubiquitination to Activate OsTPP1 and Enhances Rice Chilling Tolerance.*Dev Cell, 43* (6), 731-743.e735. doi:10.1016/j.devcel.2017.11.016

Zhao, L. Y., Yang, T. Y., Xing, C. H., Dong, H. Z., Qi, K. J., Gao, J. Z., . . . Huang, X. S. (2019). The beta-amylase PbrBAM3 from pear (Pyrus betulaefolia) regulates soluble sugar accumulation and ROS homeostasis in response to cold stress. *Plant Science, 287* , 13. doi:10.1016/j.plantsci.2019.110184

Figure legends

Fig. 1 Bioinformatic analysis of tae-miR399 and TaUBC24. (A) Base conservation analysis of miR399. (B) Sequence alignment analysis of miR399. (C) RNA secondary structure analysis of pre-tae-miR399, the straight line represented the mature sequence. (D) Cis-acting element analysis of upstream promoter sequence of tae-miR399 and its targets*TaUBC24* . The mature miR399 sequences were of monocots (*A. tauschii* , *B. distachyom* ,*O. sativa* , *H. vulgare* , *S. bicolor* , *P. vulgaris* , *T. aestivum* and *Z. mays*) and eudicots (A. thaliana, G. max, G. raimondii, N. tabacum, M. truncatula, P. trichocarpa, *S. tuberosum* , *G. hirsutum* , *M. domestica* , *C. melo* , *M. esculenta* , *C. cardunculus* ,*L. usitatissimum* , *P. persica* , *S. lycopersicum* ,*F. vesca* , *P. abies* , *C. sativa* , *A. officinalis* , *B. napus* , *V. vinifera* , *A. caerulea* ,*R. communis* , *A. lyrate* , *V. unguiculate* , *T. cacao* and *V. vinifera*) were obtained from the miRbase database.

Fig. 2 The expression levels of tae-miR399 and its targets*TaUBC24* under low temperature stress. (A) Expression of Dn1 tae-miR399 in field under low temperature. (B) Expression of Dn1*TaUBC24* in field under low temperature. (C) Expression of Dn1 tae-miR399 in green house under low temperature. (D)

Expression of Dn1 *TaUBC24* in green house under low temperature. All values in the figure are mean ± SD (n = 3), *P* values (the field samples versus 5, the indoor samples versus 22) were calculated with one-way ANOVA, **P* <0.05, ** *P* <0.01, ****P* <0.001, **** *P* <0.0001.

Fig. 3 tae-miR399 targets *TaUBC24* . (A) Alignment between tae-miR399 and six MREs in the 5' UTR of *TaUBC24* . (B) The model of cDNA structure and mRNA cleavage site of *TaUBC24* determined by 5' RACE. tae-miR399 complementary sites (red lines) with the nucleotide positions of *TaUBC24* indicated. The RNA sequence of each complementary site from 5' to 3' and the miRNA sequence from 3' to 5' are shown in the expanded regions. The black arrow indicated a cleavage site verified by RLM 5'-RACE, with the frequency of cloned PCR products shown above the alignment. (a) The cleavage site of *TaUBC24* in wheat. (b) The cleavage site of *pTaUBC24* in *N. benthamiana* . (C) β-Glucuronidase (GUS) phenotype observed by histochemical staining. (a) pBI121- GUS (b) pBI121-tae-miR399 (c) pBI121-*pTaUBC24* (d) the mixture of pBI121-tae-miR399 and pBI121-*pTaUBC24*

Fig. 4 Phenotype and survival rate of Arabidopsis lines under freezing stress. (A) Phenotypes of Arabidopsis lines under freezing stress. (B) Root phenotype of Arabidopsis seedlings. (C) Survival rate of Arabidopsis lines under freezing stress. The values are mean ± SD (n = 3), *P* values (versus WT) were calculated with one-way ANOVA, **P* <0.05, ** *P* <0.01, ****P* <0.001, **** *P* <0.0001. (D) Electronic conductivity of leaves in Arabidopsis lines under freezing stress. (E) MDA content of Arabidopsis lines under freezing stress. The values are mean ± SD (n = 3), *P* values (versus 24) were calculated with two-way ANOVA, * *P* <0.05, ** *P* <0.01, *** *P* <0.001, **** *P* <0.0001.

Fig. 5 The gene expression level of miR399 and *AtUBC24* in Arabidopsis lines under freezing stress. (A) The expression of miR399 in Arabidopsis lines under freezing stress. (B) The expression of *AtUBC24* in Arabidopsis lines under freezing stress. All values in the figure are mean +- SD (n = 3), *P* values (versus 24) were calculated with two-way ANOVA, * *P* <0.05, ***P* <0.01, *** *P* <0.001, *****P* <0.0001.

Fig. 6 The gene expression level of CBF signal pathway in Arabidopsis lines under freezing stress. (A) The expression of *AtCBF1* in Arabidopsis lines under freezing stress. (B) The expression of *AtCBF2* in Arabidopsis lines under freezing stress. (C) The expression of *AtCBF3* in Arabidopsis lines under freezing stress. (D) The expression of *AtCOR15A* in Arabidopsis lines under freezing stress. (E) The expression of *AtCOR15B* in Arabidopsis lines under freezing stress. (F) The expression of *AtCOR47* in Arabidopsis lines under freezing stress. (G) The expression of *AtCOR413IM* in Arabidopsis lines under freezing stress. All values in the figure are mean +- SD (n = 3), *P* values (versus 24) were calculated with two-way ANOVA, * *P* <0.05, ***P* <0.01, *** *P* <0.001, *****P* <0.0001.

Fig. 7 The protein level and transcription level of AtICE1 in Arabidopsis lines under freezing stress. (A) Protein level of AtICE1 in Arabidopsis lines under freezing stress. (B) Labeled AtICE1 bands were quantified using Q9 Aliance software. (C) Transcription level of *AtICE1* in Arabidopsis lines under freezing stress. All values in the figure are mean +- SD (n = 3),*P* values (protein content versus WT, expression of *AtICE1* versus 24) were calculated with one-way or two-way ANOVA, **P* <0.05, ** *P* <0.01, ****P* <0.001, **** *P* <0.0001.

Fig. 8 Phosphorus content and transcript levels of selected PSI genes in Arabidopsis lines under freezing stress. (A) Total phosphorus content of Arabidopsis lines under freezing stress. (B) The content of inorganic phosphorus in Arabidopsis under freezing stress. (C) The expression of *AtPHO1* in Arabidopsis lines under freezing stress. (D) The expression of *AtPHT1;1* in Arabidopsis lines under freezing stress. (E) The expression of *AtPHT1;2* in Arabidopsis lines under freezing stress. (F) The expression of *AtPHT1;4* in Arabidopsis lines under freezing stress.

Fig. 9 Contents of starch and soluble sugar in Arabidopsis lines under freezing stress. (A) Soluble sugar content in Arabidopsis under freezing stress. (B) Starch content of Arabidopsis lines under freezing stress. All values in the figure are mean +- SD (n = 3),*P* values (versus 24) were calculated with two-way ANOVA, **P* <0.05, ** *P* <0.01, ****P* <0.001, **** *P* <0.0001.

Fig. 10 Expression and activity of starch hydrolase in Arabidopsis lines under freezing stress. (A) Activity

of α-amylase in Arabidopsis under freezing stress. (B) Activity of β-amylase in Arabidopsis under freezing stress. (C) Expression of *AtAPL1* in Arabidopsis lines under freezing stress. (D) Expression of *AtAPL3* in Arabidopsis under freezing stress. (E) Expression of *AtBAM1* in Arabidopsis under freezing stress. (F) Expression of *AtBAM3* in Arabidopsis under freezing stress. (G) Expression of *AtSEX1* in Arabidopsis lines under freezing stress. (H) Expression of *AtSEX4* in Arabidopsis under freezing stress. All values in the figure are mean ± SD (n = 3), *P* values (versus 24) were calculated with two-way ANOVA, **P* <0.05, ** *P* <0.01, ****P* <0.001, **** *P* <0.0001.

Fig. 11 ROS content in Arabidopsis lines under freezing stress. (A) DAB staining of Arabidopsis leaves under freezing stress. (B) NBT staining of Arabidopsis leaves under freezing stress. (C) H_2O_2 content of Arabidopsis lines under freezing stress. (D) O_2^- content of Arabidopsis lines under freezing stress. All values in the figure are mean +- SD (n = 3), *P* values (versus 24) were calculated with two-way ANOVA, * *P* <0.05, ** *P* <0.01, ****P* <0.001, **** *P* <0.0001.

Fig. 12 Expression and activity of SOD, POD and CAT in Arabidopsis lines under freezing stress. (A) Activity of SOD in Arabidopsis under freezing stress. (B) Expression of *AtSOD1* in Arabidopsis lines under freezing stress. (C) Expression of *AtSOD2*in Arabidopsis under freezing stress. (D) Expression of *AtSOD3* in Arabidopsis under freezing stress. (E) Activity of POD in Arabidopsis under freezing stress. (F) Expression of *AtPER3* in Arabidopsis lines under freezing stress. (G) Activity of CAT in Arabidopsis under freezing stress. (H) Expression of *AtCAT2* in Arabidopsis lines under freezing stress. (I) Expression of *AtCAT3* in Arabidopsis lines under freezing stress. All values in the figure are mean +- SD (n = 3), *P* values (versus 24) were calculated with two-way ANOVA, **P* <0.05, ** *P* <0.01, ****P* <0.001, **** *P* <0.0001.

Fig. 13 TaUBC24 physically interacts with TaICE1 protein. (A) Yeast two-hybrid (Y2H) assays to determine interactions between TaUBC24 and TaICE1. (B) Bimolecular fluorescence complementation (BiFC) assays for determine the interactions of TaUBC24 with TaICE1.

Fig. 14 Proposed working model for the underlying mechanism used by tae-miR399-*UBC24* model to regulate plant response to freezing stress.

Hosted file

`Figure1.pdf` available at https://authorea.com/users/414023/articles/522179-mir399-ubc24-module-enhances-freezing-tolerance-through-regulating-cbf-signaling-pathway-and-starch-degradation

Hosted file

`Figure2.pdf` available at https://authorea.com/users/414023/articles/522179-mir399-ubc24-module-enhances-freezing-tolerance-through-regulating-cbf-signaling-pathway-and-starch-degradation

Hosted file

`Figure3.pdf` available at https://authorea.com/users/414023/articles/522179-mir399-ubc24-module-enhances-freezing-tolerance-through-regulating-cbf-signaling-pathway-and-starch-degradation

Hosted file

`Figure4.pdf` available at https://authorea.com/users/414023/articles/522179-mir399-ubc24-module-enhances-freezing-tolerance-through-regulating-cbf-signaling-pathway-and-starch-degradation

Hosted file

`Figure5.pdf` available at https://authorea.com/users/414023/articles/522179-mir399-ubc24-module-enhances-freezing-tolerance-through-regulating-cbf-signaling-pathway-and-starch-

degradation

Hosted file

Figure6.pdf available at https://authorea.com/users/414023/articles/522179-mir399-ubc24-module-enhances-freezing-tolerance-through-regulating-cbf-signaling-pathway-and-starch-degradation

Hosted file

Figure7.pdf available at https://authorea.com/users/414023/articles/522179-mir399-ubc24-module-enhances-freezing-tolerance-through-regulating-cbf-signaling-pathway-and-starch-degradation

Hosted file

Figure8.pdf available at https://authorea.com/users/414023/articles/522179-mir399-ubc24-module-enhances-freezing-tolerance-through-regulating-cbf-signaling-pathway-and-starch-degradation

Hosted file

Figure9.pdf available at https://authorea.com/users/414023/articles/522179-mir399-ubc24-module-enhances-freezing-tolerance-through-regulating-cbf-signaling-pathway-and-starch-degradation

Hosted file

Figure10.pdf available at https://authorea.com/users/414023/articles/522179-mir399-ubc24-module-enhances-freezing-tolerance-through-regulating-cbf-signaling-pathway-and-starch-degradation

Hosted file

Figure11.pdf available at https://authorea.com/users/414023/articles/522179-mir399-ubc24-module-enhances-freezing-tolerance-through-regulating-cbf-signaling-pathway-and-starch-degradation

Hosted file

Figure12.pdf available at https://authorea.com/users/414023/articles/522179-mir399-ubc24-module-enhances-freezing-tolerance-through-regulating-cbf-signaling-pathway-and-starch-degradation

Hosted file

Figure13.pdf available at https://authorea.com/users/414023/articles/522179-mir399-ubc24-module-enhances-freezing-tolerance-through-regulating-cbf-signaling-pathway-and-starch-degradation

Hosted file

Figure14.pdf available at https://authorea.com/users/414023/articles/522179-mir399-ubc24-module-enhances-freezing-tolerance-through-regulating-cbf-signaling-pathway-and-starch-degradation