Multi-omics analysis reveals a link between Brassica -specific miR1885 and rapeseed tolerance to low temperature

Pengfei Xu[1], Wenting Zhang[2], Xuan Wang[3], Yantao Zhu[4], Wanqi Liang[1], Yuke He[2], and Xiang Yu[1]

[1]Shanghai Jiao Tong University School of Life Sciences and Biotechnology
[2]Institute of Plant Physiology and Ecology Shanghai Institute for Biological Sciences
[3]Shanghai Normal University
[4]Hybrid Rapeseed Research Center of Shaanxi Province

July 1, 2022

Abstract

Brassica crops include various edible vegetable and plant oil crops, and their production is limited by low temperature. The key regulators of low-temperature resistance in *Brassica* remain largely unexplored. To identify post-transcriptional regulators of plant response to low temperature, we performed small RNA profiling, and found that 16 known miRNAs responded to cold treatment in *Brassica rapa*. The cold response of seven of those miRNAs were further confirmed by qRT-PCR and/or northern blotting analyses. In parallel, a genome-wide association study of 220 accessions of *Brassica napus* identified four candidate MIRNA genes, all of which were cold-responsive, at the loci associated with low temperature resistance. Specifically, these large-scale data analyses revealed a link between miR1885 and the plant response to low temperature in both *B. rapa* and *B. napus*. Using 5' rapid amplification of cDNA ends approach, we validated that miR1885 can cleave its putative target gene transcripts, *Bn.TIR.A09* and *Bn.TNL.A03,* in *B. napus*. Furthermore, overexpression of miR1885 in *B. napus* decreased the mRNA abundance of *Bn.TIR.A09* and *Bn.TNL.A03* and resulted in increased sensitivity to low temperature. Knocking down of miR1885 in *B. napus* led to increased mRNA abundance of its targets and improved rapeseed tolerance to low temperature. Together, our results suggested that the loci of miR1885 and its targets could be potential candidates for the molecular breeding of low temperature-tolerant *Brassica* crops.

INTRODUCTION

Brassica crop species include *Brassica napus* (rapeseed),*Brassica rapa* , *Brassica oleracea* , *Brassica juncea* , and *Brassica nigra* . Among them, *B. napus* arose approximately 7500 years ago by allopolyploidy between its ancestors,*B. rapa* and *B. oleracea* (Chalhoub et al., 2014). *B. rapa* and *Arabidopsis thaliana* share a common ancestor, but*B. rapa* has a larger genome than that of *A. thaliana* due to genome triplication (Wang et al., 2011). Both *B. napus* and*B. rapa* are important food sources. *B. napus* mainly provides edible plant oil and *B. rapa* is a valuable oil and vegetable crop. However, *Brassica* crops are negatively affected by low temperature, especially those that over-winter in frigid environments. Worldwide, extensive economic losses of *Brassica*crops are attributed to low temperature, often in combination with other abiotic or biotic stresses (Sanghera et al., 2011).

Previous studies have identified many genes involved in the plant response to low temperature (Ding et., 2019). Cold induced the expression of the transcription factor gene *CBF* (encoding C-repeat binding factor), and CBF triggered the expression of *COR*(COLD RESPONSIVE) genes (Shi et al., 2018). CBF/COR-dependent factors allowed plants to withstand subsequent freezing stress (McClung and Davis, 2010). In addition, many important genes, which contributed to low-temperature response in *CBF* -independent man-

1

ner, were also found (Bolt et al., 2017; Liu et al., 2018). Although breeding for low-temperature resistance is possible, the genetic improvement of crops is still hindered by the lack of elite genetic resources related to low-temperature resistance.

MicroRNAs (miRNAs) are regulators of plant adaptation to abiotic or biotic stress (Biggar and Storey, 2015). They are approximately 20–24 nucleotides (nt) in length and originate from pre-miRNAs with stem-loop structures (Song et. al., 2019). The mature miRNAs are loaded into the RNA-induced silencing complex that regulate target transcripts involved in various aspects of plant growth, development, and stress responses, including the response to cold or freezing stress (Chen, 2009; Anjali and Sabu, 2020). For instance, overexpression of miR156 increased cold tolerance in species including rice, *A. thaliana,* and pine (Zhou and Tang, 2019). In rice, OsmiR319b down-regulated *OsPCF6* and *OsTCP21* , resulting in enhanced cold tolerance (Wang et al., 2014), and overexpressing OsmiR1320 improved cold tolerance, although OsmiR1320 was repressed under cold stress (Sun et al., 2022). In *A. thaliana,* plants with knocked-down miR165/166 displayed a drought- and cold-resistant phenotype (Yan et al., 2016). Other known cold-responsive miRNAs included miR172, miR396, miR845, and miR168 (Gupta et al., 2014; Zeng et al., 2018). However, miRNAs in response to low temperature stress have not been well studied in *Brassica* crops.

The plant *R* (RESISTANCE) gene encodes immune proteins involved in the response to pathogen infection (Jones and Dangl, 2006). Most of the identified *R* genes contain a nucleotide-binding site-leucine-rich repeat (NBS-LRR) domain with a coiled-coil (CC) or a Toll/Interleukin-1 receptor (TIR) domain at the amino terminus (Dangl and Jones, 2001). Emerging evidence has shown that cold and pathogen attack induced the expression of some common genes, such as *PR* (PATHOGENESIS-RELATED) genes, which played roles in the response to pathogen infection and low temperature stress (Snider et al., 2000; Seo et al., 2008). Some R genes negatively regulated plants tolerance to low temperature. In *A. thaliana* , the *rpp4-1d* mutant plant with a gain-of-function mutation in *RPP4* (TIR-NB-LRR protein PERONOSPORA PARASITICA 4) was chilling-sensitive (Huang et al., 2010). Mutations in *HSP90* suppressed the chilling-sensitive phenotype of *rpp4-1d* mutant plant, and HSP90 interacted with the RPP4 protein in planta (Bao et al., 2014). Interestingly, a significant increase in *HSP90* mRNA was detected in *B. napus* exposed to low temperature (Krishna et al., 1995). In addition, a gain-of-function mutant of *CHS3* , encoding a TIR-NB-LRR-LIM protein, resulted in enhanced defense responses and chilling-sensitivity phenotype (Yang et al., 2010). Other studies found that some *R* genes played positive roles in plants tolerance to low temperature. A point missense mutation of *CHS1* , encoding a TIR-NB protein, resulted in a chilling-sensitive phenotype (Wang et al., 2013; Zbierzak et al., 2013). *R* genes were also regulated by genes that contributed to cold tolerance. In tobacco, overexpression of *SgRVE6* (*REVEILLE*) improved plant tolerance to low temperature and up-regulated the expression of genes encoding NB-LRR proteins (Chen et al., 2020).

Although many studies have investigated the molecular mechanisms underlying low-temperature resistance, the roles of miRNAs in low-temperature resistance in *Brassica* crops are largely unknown. In this study, we constructed and sequenced small RNA libraries from *B. rapa* under cold stress and performed a genome-wide association study (GWAS) related to the phenotype of cold resistance using a *B. napus* population. Combing the results from both small RNA sequencing and the GWAS, we found that *Brassica* -specific miR1885 was responsive to cold stress in both *B rapa* and *B napus* . Previous studies have revealed that heat, as well as *Turnip mosaic virus* (*TuMV*) and *Plasmodiophora brassicae* infection, induced miR1885 accumulation (He et al., 2008; Yu et al., 2011, Paul et al., 2021). Here, we further demonstrated that miR1885 was also involved in the response to low-temperature stress through its target genes and functioned as a negative regulator of *B. napus* in low temperature tolerance, providing potential genetic candidates for cold-resistant rapeseed breeding.

RESULTS

Genome-wide analysis of small RNAs responsive to cold stress in *B. rapa*

To study the cold response of *B. rapa* , the degree of cold tolerance was estimated by evaluating morphological traits under cold stress. After 7 days of cold treatment (4 °C), the plants stopped growing and their

2

leaves drooped (Figure 1A). Therefore, to identify genome-wide cold-responsive miRNAs, we performed cold treatment for 7 days (B_V7) on 3-week-old seedlings of *B. rapa* in a growth chamber, and plants grown at 22 °C (B_V0) was used as control for small RNA sequencing.

Totally, 17.96 million (B_V0) and 17.17 million (B_V7) raw reads were obtained from these two samples (Figure 1B). Among them, most of reads were shared between the two libraries, while 2.82 million reads were B_V0-specific, and 2.23 million reads were B_V7-specific (Figure 1B). Small RNA length distribution analysis showed that 24-nt small RNAs were most abundant, and 21-nt small RNAs were second most abundant (Figure 1C).

To learn the genomic loci that produced these small RNAs, 11.46 million (B_V0) and 11.36 million (B_V7) clean reads were mapped to the *B. rapa* reference genome (http://brassicadb.cn/#/). We found that the small RNAs were generated from exons, introns, miRNAs, rRNAs, snRNAs, snoRNAs, tRNAs, and repeat regions (Table S1). Notably, focusing on small RNAs from no-coding gene regions, most abundant small RNAs were produced from ribosomal RNAs (rRNAs) (B_V0, 44.67% and B_V7, 48.10%), followed by unknown RNAs (B_V0, 27.65% and B_V7, 25.22%), and miRNA precursors (B_V0, 8.50% and B_V7, 8.71%) (Figure S1).

Identification of *B. rapa* miRNAs responsive to cold stress

To identify known miRNAs in our sequence libraries, we aligned the sequenced reads with mature miRNAs in miRBase and the *B. rapa* reference genome, allowing no more than two mismatches. In total, 77 mature miRNAs related to 184 conserved miRNA precursors were found in our libraries. Among them, 52 miRNAs were conserved with those in *Arabidopsis*, while 16 miRNAs were *Brassica*-specific miRNAs (Table S2). To further identify novel miRNA candidates, the flanking sequences of unknown small RNAs in genome were extracted for RNA structure prediction. Based on the plant miRNA criteria (Axtell et al., 2018), we identified 11 highly credible novel miRNAs (Table S3). Among them, novel-miR1 and novel-miR11 were identified with more than 100 reads.

Among the 77 known miRNAs, 16 miRNAs were differentially expressed between the cold treatment and the control (>1.5-fold) (Table 1). Among these mature miRNAs, bra-miR157a~c, bra-miR161, bra-miR393f, bra-miR396b, bra-miR319c, bra-miR156k, bra-miR1140, bra-miR158a, and bra-miR156a~f were down-regulated; and bra-miR168d, bra-miR403, bra-miR390a~b, bra-miR398b~c, bra-miR408, and bra-miR1885 were up-regulated under cold treatment. Among them, the *Brassica*-specific bra-miR1885 was induced more than four-fold by cold stress. Among the novel miRNAs, only novel-miR11 was cold-responsive. Taken together, the small RNA profiling provided cues to cold-responsive miRNAs in *B. rapa*.

Validation of cold responsiveness of mature miRNAs in *B. rapa*

To validate the cold-responsive miRNAs detected by small RNA sequencing, we further analyzed the cold responsiveness of mature bra-miR1885, bra-miR398, bra-miR408, bra-miR161, bra-miR157, bra-miR393, and bra-miR168 using qRT-PCR. We found that the abundance of bra-miR1885, bra-miR398 and bra-miR168 were significantly up-regulated by cold stress, whereas bra-miR161, bra-miR157 and bra-miR393 were significantly down-regulated, which were consistent with the small RNA sequencing data (Figure 2A). Among these miRNAs, bra-miR398 and bra-miR168 were conserved miRNAs that were induced more than three-fold by cold treatment (Figure 2A). We then performed northern blotting analyses to further confirm the cold responsiveness of bra-miR398, bra-miR168, bra-miR1140 and bra-miR1885. Consistently, the accumulation of bra-miR398 and bra-miR168 was much higher in B_V7 than in B_V0, and *Brassica*-specific miR1140 was repressed after cold treatment (Figure 2B). In addition, the results of northern blot analyses further confirmed the abundance of mature bra-miR1885 was increased in both leaf and the shoot apical meristem (SAM) under cold stress (Figure 2C).

Next, to identify the potential cold-regulated *cis*-elements in the *bra-MIR1885* promoter, we extracted the promoter sequence of *bra-MIR1885* using Promoter 2.0 Prediction Server (https://services.healthtech.dtu.dk/service.php?Promoter-2.0), and analyzed the *cis*-elements in the putative *bra-MIR1885* promoter sequence using PlantCare

3

(http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). These analyses revealed that the promoter contained light-responsive, defense-responsive, stress-responsive, and low temperature-responsive elements (LTR: CCGAAA) (Figure S2). A previous study has revealed that LTR motif (CCGAAA) within the promoter region of *CsINV5* was the core *cis* element in response to low temperature (Qian et al., 2018). This result suggested that the *bra-MIR1885* promoter contained a low temperature-responsive element that potentially regulated the expression of *bra-MIR1885* under cold stress.

Genome-wide association study to identify low temperature resistance-associated loci containing MIRNA genes in *B. napus*

B. napus is closely related to *B. rapa* and both are *Brassica* crops. To identify MIRNA genes associated with low temperature stress in *Brassica* crops, we constructed a *B. napus* population. Low-temperature resistance was divided into with 5 levels (Grade 1 indicates most cold-resistant, while Grade 5 indicates most cold-sensitive) and was measured in the field in January of 2017 and 2018, and the phenotypic data of rapeseed population showed a nearly skewed distribution (Xu et al., 2021). The genome re-sequencing of 220 *B. napus* accessions generated 2.13 Tb clean reads, and single nucleotide polymorphism (SNP) genotyping of *B. napus* population identified 3.80 million highly quality SNPs (MAF > 0.05, geno < 0.2) (Xu et al., 2021). Overall, the SNPs were not evenly distributed across the whole genome. The SNP density in the A sub-genome (5.25/Kb) was higher than that in the C sub-genome (4.74/Kb) (Figure S3A). A principal component analysis (PCA) was used to assess the genetic relationship in the association panel. Based on the PCA, the *B. napus* population was roughly divided into three groups corresponding to winter (W), semi-winter (SW), and spring (S) ecotypes (Figure S3B). We further analyzed the linkage disequilibrium (LD) throughout the genome. The LD was estimated as r^2 (the squared Pearson correlation coefficient) between all pairs of SNP markers. The average distance over which LD decayed to $r^2 = 0.2$ of its maximum value was 100 kb, indicating that SNPs located in the 200-kb genomic region around each peak SNP represented a GWAS-quantitative trait locus (QTL) (Figure S3C).

The GWAS was performed using a mixed linear model, and the SNPs significantly associated with low-temperature resistance were identified at a threshold of $p < 10^{-6}$. As a result, we identified 32 highly credible GWAS-QTLs (Figure 3A). To discover candidate MIRNA genes within these QTLs, we collected 163 pre-miRNA sequences specific to the Cruciferae family from four species (*B. napus* , *B. rapa* , *B. oleracea,* and *Arabidopsis*) according to previous reports (Jones-Rhoades and Bartel, 2004; Alves-Junior et al., 2009; Yu et al., 2011; He et al., 2018; Zhang et al., 2018; Li et al., 2019). All the pre-miRNA sequences were mapped to the *B. napus* reference to identify their location (Table S4). We found that four candidate MIRNA genes, *bna-MIR166C* , *bna-MIR1885* , *bna-MIR168A* and *bna-MIR845A,* were in the QTLs associated with low-temperature resistance.

A previous study proved that knock-down of miR165/166 expression conferred a cold-resistant phenotype in *A. thaliana* (Yan et al., 2016). Conserved miR845a was known to be differentially expressed in leaves of winter turnip rape under cold stress (Zeng et al., 2018). To further determine whether these four miRNAs were responsive to low temperature, we measured the abundance of mature miRNAs in *B. napus* under cold stress by qRT-PCR. From this analysis, we found that all four of them were cold-responsive. bna-miR1885 and bna-miR168a were up-regulated at all these time points, while bna-miR845a and bna-miR166c were down-regulated (Figure 3B and Figure 4A).

To identify if any genetic variation in cis-regulatory element contribute to the *MIR1885* transcription in the population of rapeseed, we studied the LD of SNPs in the promoter of *bna-MIR1885* gene locus and found that they exhibited significant linkage (Figure S3D). We identified 22 SNPs and 7 INDELs in *bna-MIR1885* promoter region, which were found to be significant associated with cold resistance. We further investigated the five variants located in the 200-bp genomic region around the low temperature-responsive (LTR: CCGAAA) element identified above. The *B. napus* accessions were classified into three haplotype groups based on the genotypes of these variants (Figure 3C). We found that 179 accessions contained the H1 haplotype, which variants were identical with the genome reference, and this haplotype was associated with low temperature-sensitive phenotype. 24 accessions carried H2 haplotype, which contained mutated

4

LTR cis-element and was associated with low temperature-resistant phenotype. Consistently, heterozygous H3 haplotype had an average resistance degree, which less than H1 haplotype, but higher than H2. Next, we randomly selected 4 accessions with low temperature-resistant variant alleles and low temperature-sensitive variant alleles, respectively, to measure their abundance of miR1885 after cold treatment by qRT-PCR. The qRT-PCR data indicated that the abundance of miR1885 was significantly higher in low temperature-sensitive accessions with normal LTR in the promoter (H1 haplotype), as compared to low temperature-resistant accessions with mutated LTR in their promoter (H2 haplotype) (Figure S3E). In total, these results suggested that genetic variation in the LTR region at *MIR1885* promoter in the rapeseed population potentially affected the miR1885 expression and associated with cold resistance.

Identification of miR1885 target genes and their cold responsivity in *B. napus*

Because bra-miR1885 and bna-miR1885 shared the same mature sequence (Figure 4B), we used miR1885 to represent the two miRNAs in *Brassica* crops hereafter. We first predicted the candidate target genes of miR1885 in *B. napus* using a plant small RNA target analysis tool (psRNATarget). Two *R* genes, *Bn.TIR.A09* (BnaA09g14980D) and *Bn.TNL.A03* (BnaA03g56180D), were identified as highly credible targets, and the miR1885 target sites were within the TIR domain. To verify the miR1885 cleavages of the predicted target genes, we performed a 5' rapid amplification of cDNA ends (5'-RACE) assay in *B. napus* and detected miR1885 cleavage sites frequently within *Bn.TIR.A09* and *Bn.TNL.A03* (Figure 4C and Figure S4), indicating that they were targeted by miR1885 in *B. napus* .

Next, we examined the transcript levels of these two target genes under cold stress, using three low temperature-responsive marker genes (*Bn.CBF1.C03* , *Bn.HSP70.A01* and *Bn.ERF105.C09*) as positive controls (Wang and Hua, 2009; Bolt et al., 2017; Liu et al., 2018). The target genes were down-regulated under cold stress in *B. napus* (Figure 4D), while the expression of *Bn.CBF1.C03* ,*Bn.HSP70.A01* were induced and *Bn.ERF105.C09* were repressed with cold treatment, consistent with those reported in previous studies (Figure 4E).

Overexpression of miR1885 in *B. napus* led to down-regulation of its target genes *Bn.TIR.A09* and *Bn.TNL.A03*

To further verify the function of miR1885, we constructed a vector with the *bra-MIR1885* gene under the control of the AA6 promoter (Patent WO 2007/069894) and transformed it into *B. napus* cv. K407 (Figure 5A). In total, seven positive transgenic lines (T_0) were screened by PCR (Figure 5B and C). The expression level of pri-miR1885 was confirmed by qRT-PCR. We found that pri-miR1885 was overexpressed in the leaves of X2, X8, X11, X12, and X15 lines (Figure 5D). Compared with WT, there were no obvious morphological abnormalities among miR1885-OE lines at seeding stage (Figure 5B). We then checked the abundance of mature miR1885 in the transformed lines by qRT-PCR and northern blotting analyses. As expected, mature miR1885 was found to be over-accumulated in the X2, X8, X11, X12, and X15 lines (Figure 5E and 5F). And then, we conducted qRT-PCR analyses to validate the influence of the miR1885 on the expression level of the two target genes in the transgenic lines. As expected, transcript levels of *Bn.TIR.A09* and *Bn.TNL.A03* were lower in the transgenic lines than in wild type (Figure 5G and 5H).

Next, we checked the RNA levels of miR1885 and its targets in miR1885-OE lines and WT with or without cold treatment by qRT-PCR. As expected, the abundance of miR1885 was increased in miR1885-OE lines and WT after cold treatment (Figure 6A). In addition, the RNA levels of *Bn.TIR.A09* were further decreased in miR1885-OE under cold stress (Figure 6B), but those of *Bn.TNL.A03* were unchanged (Figure 6C), suggesting that there was additional cold-triggered regulation of *Bn.TNL.A03* at the transcriptional level, while the cold response of *Bn.TIR.A09* was majorly contributed by miR1885-mediated post-transcriptional regulation.

Given that CBF/CORs played critical roles in plant response to low temperature, we investigated whether miR1885 can affect the cold induction of CBF/COR factors. To do this, we analyzed the expression levels of *Bn.CBF1.CO3* , *Bn.CBF2.Ann* and *Bn.COR15.A03* in the miR1885-OE under cold treatment. However, there were no significant differences at the expression levels of *Bn.CBF1.CO3* ,*Bn.CBF2.Ann* and

5

Bn.COR15.A03 between WT and miR1885-OE lines after cold treatment (Figure 6D-F). This result suggested that miR1885 regulated low temperature tolerance in *Brassica* through CBF/CORs-independent pathway.

miR1885 negatively regulated low temperature tolerance in *Brassica*

A previous study reported that knockdown of *CHS1* , encoding a TIR-NB protein, led to a chilling-sensitive phenotype in *A. thaliana* (Zbierzak et al., 2013). We found that two TIR-NB transcripts were the targets of miR1885 and both miR1885 and these two TIR-NB genes were cold-responsive. Therefore, we further tested the effects of miR1885 and its targets on low-temperature resistance in *B. napus* . WT and miR1885-OE plants were grown for 6 weeks at 22 degC, and then were transferred to 4 degC for 30 days or 2 degC for 7 days. We found that the miR1885-OE plants were more sensitive as compared to WT under 2 degC for 7 days (Figure 7A) but showed no apparent phenotypic difference under 4 degC (Figure S5A). This result indicated miR1885 functioned as negative regulator in rapeseed response to low temperature. We further performed phenotypic studies using WT and miR1885-OE seeds during post-germination under normal and cold environment. The seeds were harvested at the same season and the cold environment was 6 degC. The hypocotyl length (NHL) of WT and miR1885-OE showed no difference after growing for 2 days on the germination bed without cold treatment. However, after 7 days cold treatment, the miR1885-OE exhibited shorter NHL compared with WT (Figure S5B-D), indicating that miR1885 also played critical role in cold response in the early developmental stage, when no significant phenotypic difference between WT and miR1885-OE was observed under normal condition.

To confirm that whether miR1885 and their targets negatively regulated low temperature tolerance in *Brassica* , the miR1885 knockdown plants (*STTM1885*) generated in *Brassica napus cv.* 'Westar' were used for further study (Cui et al., 2020). The qRT-PCR results showed that the accumulation of miR1885 was reduced in *STTM1885* plants. Compared with those in wildtype Westar, the expression levels of *Bn.TNL.A03* and *Bn.TIR.A09* were increased in *STTM1885* plants (Figure 7B). To study morphological responses to cold stress, we then moved the Westar and *STTM1885* plants into 2 degC environment. After 14 days cold treatment, a cold-tolerant phenotype was observed in *STTM1885* plants (Figure 7C). This finding further suggested that attenuating miR1885 resulted in enhanced cold tolerance of rapeseed potentially via their targets.

We further tested whether overexpression of miR1885 affected plant growth at freezing temperature compared to its background winter ecotype K407, while the spring ecotype Westar was used as negative control (Figure S6A). The abundance of mature miR1885 in wild-type *B. napus* under freezing stress was examined by real-time PCR. Based on previous studies, we set the freezing temperature at -10 degC (Xu and Cai, 2019; Ljubej et al., 2021). We found that miR1885 was up-regulated under freezing stress, and its expression level was approximately 2.4-fold than in wild type at 2.5 h (Figure S6B). The wild type and miR1885-OE lines were subjected to a -10 degC treatment for 2.5 h, and then allowed to recover at 22 degC for 7 days. The survival rate of wild type K407 was 78.00%, and those of miR1885-OE were much lower, ranging from 22.33% to 55.33%, whereas 100% of spring ecotype Westar died (Figure S6A and Figure S6C). Accumulation of free proline (Pro) during cold or freezing stress is thought to protect plants (Xin and Browse, 1998). To test whether miR1885-OE had altered Pro content, we determined the Pro content in wild type and miR1885-OE with or without freezing treatment (Figure S6D). As we expected, the Pro content was higher in wild type and miR1885-OE grown at -10 degC than in those grown at 22 degC. In plants grown at -10 degC, more Pro accumulated in the wild type plants than in the miR1885-OE. This finding was consistent with the freezing-sensitive phenotype of transgenic lines subjected to a -10 degC treatment. These results demonstrated that overexpression of miR1885 increases plant sensitivity to freezing at the seedling stage.

DISCUSSION

At present, many MIRNA genes functioning in stress response have been identified by small RNA profiling and RNA-seq (Ahmed et al., 2020; Ma et al., 2022; Zhu et al., 2022). However, the MIRNA genes that function in the response to cold stress in crops remain largely unexplored. In this study, we used small

6

RNA profiling and GWAS to identify cold-responsive candidate miRNAs or MIRNA genes associated with low-temperature resistance. The results from these two methods provide mutual support for the roles of miR1885 in the cold response of *Brassica* crops.

We identified up-regulated or down-regulated miRNAs in small RNA sequencing libraries of *B. rapa* and candidate MIRNA genes from GWAS loci associated with low-temperature resistance in *B. napus*. The small RNA profiling suggested that miR1885 was drastically triggered by cold stress. Using GWAS, we found *MIR1885* gene was in the loci associated with low-temperature resistance. We further confirmed that miR1885 was induced by cold stress in both *B. rapa* and *B. napus*.

MiR1885 is a *Brassica*-specific miRNA that targets the *R* gene family. In *A. thaliana*, the TIR-NB-LRR protein RPP4 and CHS1 were involved in plant resistance to low temperature stress (Huang et al., 2010; Zbierzak et al., 2013). Here, two *R* genes, *Bn.TIR.A09* and *Bn.TNL.A03*, were predicted as targets for cleavage by miR1885 in *B. napus*. These *R* genes belonged to two different subgroups: *Bn.TIR.A09* encoded a protein with the TIR motif lacking NBS-LRR domain; while *Bn.TNL.A03* encoded a protein with the entire TIR-NBS-LRR structure. We found that these two targeted genes were down-regulated in wild-type *B. napus* under cold treatment, opposite to the expression pattern of miR1885 (Figure 6). These results demonstrated that miR1885 negatively regulated these *R* genes under cold temperatures.

Overexpression of miR1885 in *B. napus* attenuated rapeseed sensitivity to low temperature at the seedling stage. While knocking down of miR1885 in *B. napus* (*STTM1885* lines) improved plant tolerance to low temperature (Figure 7). The RNA levels of miR1885-cleaved R genes *Bn.TIR.A09* and *Bn.TNL.A03,* which increased in *STTM1885* rapeseed and decreased in miR1885-OE lines, may contribute to rapeseed response to cold stress. Although a link between miR1885 and cold response has been revealed, the hiding mechanism is still uncovered. In addition, we have checked some CBF-dependent genes in miR1885-OE lines, including *CBFs* and *CORs*, and found that the transcript levels of these genes in miR1885-OE in response to cold stress were not different with that of wild type, suggesting that miR1885-targets regulated plant resistance to low temperature independent on CBFs pathway. How miR1885 and its cascade target genes modulated plant resistance to low temperature remains to be further investigated.

In rice, OsmiR1320 was repressed by cold stress. However, overexpressing OsmiR1320 enhanced plant tolerance to cold stress, indicating that OsmiR1320 played positive role in cold response (Sun et al., 2022). On the contrary, we found that, as a negative regulator of cold response, miR1885 was induced under cold stress potentially via its low-temperature response (LTR) cis-element in *MIR1885* promoter. Therefore, *MIRNA* transcription could be regulated incoherently by cold stress. The genetic mutation in and close to this LTR in the rapeseed population may contribute to their diversity of cold response (Figure 3C and Figure S3E). Genetic editing in this LTR could be used for detaching the cold response of *MIR1885*, and therefore enhance rapeseed resistance to cold stress.

In summary, using multi-omics data, we identified miR1885 involved in plant response to low temperature stress by small RNA sequencing and GWAS. Overexpression of miR1885 in *B. napus* increased plant sensitivity to low temperature, while knockdown of miR1885 improved plant tolerance to low temperature. Our findings suggested that *MIR1885* and its target genes can be used as genetic resource for improving resistance to low temperature in the breeding of *Brassica* crops.

METHODS AND MATERIALS

Plants materials, cold treatment, and freezing tolerance assay

The wild-type *B. rapa* variety 'Bre' was grown under a 16-h light/8-h dark (22 degC) photoperiod for 3 weeks. To analyze the miRNAs abundance in 'Bre' under cold stress, some 'Bre' plants were grown at 4 degC for 7 days and some were grown at 22 degC for 7 days as the control. The aboveground parts of treated and untreated were then harvested, frozen in liquid nitrogen, and stored at -80 degC until extraction of total RNA. Small RNA sequencing was performed using the Illumina GAII sequencer at BIG (ShenZhen, China).

The wild-type *B. napus* variety 'K407', a winter ecotype, was grown under a 16-h light/8-h dark (22 degC)

7

photoperiod with light intensity of 250 µmol m^{-2} s^{-1}. For the cold resistance assay, the plants were grown under standard conditions (22 °C) for 6 weeks before transferring to a cold environment (4 °C or 2 °C, 16-h/8-h light/dark photoperiod). After cold treatment, the cold resistance of plants was determined. The freezing tolerance assay was performed in a freezing chamber, after cold acclimation for 24 h, in which wild-type and transgenic lines were subjected to a -10 degC treatment for 2.5 h. The plants were then allowed to recover at 22 degC for 7 days before determining the survival rate. The Pro content was measured immediately after the freezing treatment using a commercial assay kit (A107-1-1, Nanjing JianCheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instructions.

Identification of known and novel miRNAs in *B. rapa*

We aligned the clean reads with known miRNAs in the miRBase (https://www.mirbase.org/), allowing two mismatches using TopHat (http://ccb.jhu.edu/software/tophat/index.shtml). The potential miRNAs were mapped to *B. rapa* reference genome (http://brassicadb.cn/#/) to extract the 600-bp flanking sequence. The flanking sequence of miRNAs were also aligned with known pre-miRNAs to confirm their identities of miRNA family. The flanking sequence of unknown small RNAs was also used to predict secondary structures using RNAfold software (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). Novel miRNAs were identified according to the plant miRNA criteria (Axtell et al., 2018). The novel miRNA sequences were named using the following format: novel-miR-number.

Real time PCR

Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. High-quality total RNA (A260/280 = 2.00) was reverse-transcribed into cDNA. Small RNA qRT-PCR was conducted using an miRNA First Strand cDNA Synthesis kit (stem-loop method) (Sangon Biotech, Shanghai, China). A total of 3 µg total RNA was used for first-strand cDNA synthesis with the stem-loop primer and *U6* reverse primer in a 20-µL reaction volume. The *U6* snoRNA was used as an internal control. For qRT-PCR analysis of coding genes, 2 µg total RNA treated with DNase I was reserve-transcribed into cDNA using M-MuLV (Takara, Otsu, Japan) with oligos (dT). The internal control was *ACT7*. qRT-PCR was performed using SYBR mix (YEASEN, China) on a Bio-CFX96 instrument. Three biological replicates and three technological replicates were analyzed. Real-time PCR and reverse transcriptase (RT)-PCR were performed using gene-specific primer pairs (Table S5).

Northern blot analyses

A 30–45 µg aliquot of high-quality total RNA (A260/280=2.00) was separated on a 19% polyacrylamide denaturing gel, and then transferred to a Hybond membrane (GE healthcare) for 2 h at 200 mA at 4 °C. In brief, after crosslinking for 5 min with ultraviolet irradiation, the membrane was hybridized overnight at 45 °C with DNA probes (Yu et al., 2011). Washing and autoradiography of the Hybond membrane were performed according to the instructions of the North2South Chemiluminescent Hybridization and Detection kit (No.17097, Pierce, Rockford, IL).

Genome-wide association analysis (GWAS)

A diversity panel, consisting of 220 *B. napus* inbred lines was used for the GWAS in this study (Xu et al., 2021). More specifically, the population included winter, semi-winter and spring oilseed rape. The accessions originated from Asia, Europe, and North America. The *B. napus* population was grown at the Farm Station in Yangling, Shaanxi province, China (107deg59'–108deg08'E; 34deg14'–34deg20'N), where the local temperature ranged from -15 to 10 degC in winter (https://lishi.tianqi.com/xianyang/202101.html). Low-temperature resistance was measured in the field in January of 2017 and 2018. The grade of low-temperature resistance was evaluated with criteria including grade 1 (the plants are normal, and the leaves are green), grade 2 (a small part of leaves are necrotic), grade 3 (a half of leaves are necrotic), grade 4 (most of leaves are necrotic), and grade 5 (all leaves are necrotic). The association analysis for low-temperature resistance was carried out using a MLM with the software package TASSEL 5 (https://www.softpedia.com/get/Science-CAD/TASSEL.shtml). The population structure was calculated using PCA. The software PopLDdecay

8

(https://github.com/BGI-shenzhen/PopLDdecay) was used to calculate LD. The parameter r^2 =0.2 was read as LD length. The threshold of the GWAS was set to P-value $< 10^{-6}$.

5'-RACE

The 5'modified RACE was performed using the FirstChoice RLM-RACE Kit (Invitrogen). In brief, total RNA was isolated from young leaves of $B. napus$. Then, 8–10 μg high-quality total RNA (A260/280[?]2.00) was directly ligated to the 5'-RACE adapter without calf intestinal phosphatase and tobacco acid pyrophosphatase treatment. The first-strand cDNA was synthesized using M-MLV reverse transcriptase according to the manufacturer's instructions. The PCR amplification was performed using the 5' outer primer and gene-specific outer primer, and 0.1 μL PCR product was used as the template for nested PCR. Nested PCR amplification was performed using the 5' inner primer and gene-specific inner primer. The PCR products were gel-purified and then cloned and sequenced.

Plasmid construction, genetic transformation, and phenotypic analysis

The plant overexpression vector was generated using pCAMBIA1301 harboring the AA6 promoter and the tAA6 terminator. The bra-$MIR1885$ genomic DNA fragment was isolated from 'Bre' seedlings and cloned into the pCAMBIA1301 binary vector. The binary construct was introduced into $Agrobacterium$ $tumefaciens$ strain GV3101. Genetic transformation of the bra-$MIR1885$ gene construct into $B. napus$ was performed as described elsewhere (Bhalla and Singh, 2008). Seedlings exhibiting resistance to hygromycin were transplanted and grown in a greenhouse at 22 °C under a 16-h/8-h light/dark photoperiod. The genomic DNA extracted from T_0 plants was used for PCR amplification with specific primers. The seeds (T_1) were harvested separately from positive plants for further analyses.

The wild-type and transgenic lines were grown in the field, and the genomic DNA extracted from plants was used for PCR amplification with specific primers. Each transgenic line was grown in a row of 10 plants, with 30-cm spacing between plants and 40 cm spacing between rows. The phenotypes of transgenic and wild-type plants were measured in the field.

AUTHOR CONTRIBUTIONS

P.X., W.Z., X.Y., and Y.H. conceived the project; P.X., W.Z. and Y.H. designed the experiments; P.X., X.W. and Y.Z. performed the experiments; P.X. and W.Z. analyzed and interpreted the data; P.X., W.L., X.Y., and Y.H. wrote the manuscript.

ACKNOWLEDGEMENTS

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All the data and materials that support the findings of this study are available upon request from the corresponding author. The sequencing data generated in this study have been deposited in the National Center for Biotechnology Information Sequence Read Archive database under the accession PRJNA748869 and PRJNA745269.

TABLES

Table 1. The miRNAs that were down- or up-regulated by more than 1.5-fold change under cold treatment.

9

B_V0 and B_V7 were the two libraries derived from 22 °C and 4 °C treatments, respectively. RPM: Reads per million.

FIGURE LEGENDS

Figure 1. Phenotypes of *B. rapa* after cold treatment and global view of small RNA reads in sequencing libraries.

(A) Phenotypes of wild-type *B. rapa* plants grown in soil at 22 °C (left) and 4 °C (right) for 7 days. Red scale bar represents 5 cm. **(B)** Number of total reads in small RNA library of *B. rapa* . B_V0 and B_V7 were libraries derived from plants grown at 22 °C and 4 °C, respectively. Shared small RNAs were presented in both libraries. Specific small RNAs were those detected only in B_V0 or B_V7 library. **(C)** Length distribution of unique and raw reads in B_V0 and B_V7 small RNA libraries.

Figure 2. Abundance of differentially expressed miRNAs between cold-stress and normal conditions.

(A) qRT-PCR analysis of seven differentially expressed miRNAs in B_V0 and B_V7 libraries. **(B)** Relative abundance of three differentially expressed miRNAs under normal condition and cold condition detected by northern blotting. **(C)** Relative abundance of miR1885 in leaf (top) and SAM (bottom) of *B. rapa* plants after cold treatment for 0, 7, 14, 21 and 28 days, respectively. SAM: shoot apical meristem. "D" represents days. Error bar represents mean $\pm$ SE; $n = 3$. *U6* was used as the endogenous reference gene in expression analyses. * indicates $P < 0.05$; ** indicates $P < 0.01$; Student's *t* -test.

Figure 3. Genome-wide association study of low-temperature resistance in a panel of *B. napus* accessions.

(A) Manhattan plots and Q-Q plots for genome-wide association study of low temperature resistance phenotype measured in Year 2017 (top) and Year 2018 (bottom), respectively. **(B)** qRT-PCR analysis of the abundance of three candidate miRNAs after cold treatment for 0, 10, 20 and 30 days, respectively. Error bar represents mean $\pm$ SE; $n = 3$. *U6* was used as the endogenous reference gene. * indicates $P < 0.05$; ** indicates $P < 0.01$; Student's t-test. **(C**) Haplotypes of candidate SNPs within *MIR1885* promoter for low-temperature resistance in *B. napus* population. LTR represents low-temperature element (CCGAAA). Five variants, including four SNPs and the one-bp deletion, which were significantly associated with low-temperature resistance, were marked with dark bold vertical lines. The five variants located in the 200-bp genomic region around LTR were used for analysis. 'Hap.' denotes the haplotypes of *MIR1885* ; 'Number' denotes the number of lines belonging to each haplotype group. 'Degree' denotes the average grade of low-temperature resistance.

Figure 4. qRT-PCR analysis of the abundance of miR1885 and its targets under cold stress in *B. napus*.

(A) qRT-PCR analysis of the abundance of miR1885 in *B. napus* under cold stress. *U6* was used as endogenous reference gene. **(B)** Alignment of miR1885 mature sequence between *B. rapa* and *B. napus* . Short black line represents the perfect matches. **(C)** Validation of miR1885 target genes and cleavage sites using 5'-RACE PCR. Predicted TIR, NBS, and LRR domains encoded by target genes are labeled (Left). Reverse complementary matches of miR1885 and their target sites were showed and vertical arrows indicated 5 termini of miRNA-guided cleavage products, as identified by 5' RACE, with frequency of clones shown (Right). **(D**) The transcript levels of *Bn.TIR.A09* and *Bn.TNL.A03* in *B. napus* under cold treatment. "D" represents days. **(E)** Relative transcript levels of cold-related genes in wild-type *B. napus* under cold treatment. *ACT7* was used as endogenous reference gene. Error bar represents mean +- SE; $n = 3$. * indicates $P < 0.05$; ** indicates $P < 0.01$; Student's t-test.

Figure 5. Transgenic *B. napus* plants overexpressing miR1885.

(A) Construct with *bra-MiR1885* under control of AA6 promoter. pCaMV35S, CaMV35S promoter; Hyg R, hygromycin-resistance gene; pAA, AA6 promoter; LB, left border of T-DNA; t35S, CaMV35S terminator;

tAA6, AA6 terminator. *Eco* RI, *Nco* I and *Bst* EII are restriction sites. **(B)** Plants of T_1 transgenic lines and wild type in the field. Red scale bar = 10 cm. X2–X16, transgenic plants. **(C)** Detection of transgene in rapeseed transgenic lines by PCR using specific primer spanning AA6 promoter and*MiR1885* fragment. M, DNA marker. **(D)** Real-time PCR showing expression of pri-miR1885 in transgenic lines. *ACT7* was used as endogenous reference gene. **(E)** Relative abundance of mature miR1885 in transgenic lines. *U6* was used as endogenous reference gene. **(F)** Northern blot analysis of accumulation of mature miR1885 in *MIR1885* -OE lines. *U6* was used as endogenous reference gene. **(G-H)** Relative transcript levels of *Bn.TIR.A09* **(G)** and *Bn.TNL.A03***(H)** in miR1885-OE lines as compared to WT. *ACT7* was used as endogenous reference gene. Error bar represents mean +- SE; $n = 3$. * indicates $P < 0.05$; ** indicates $P < 0.01$; Student's t-test.

Figure 6. Relative transcript levels of miR1885 and its targets in transgenic and wild-type plants with or without cold treatment.

(A-C) qRT-PCR analysis of RNA levels of miR1885 **(A)**,*Bn.TNL.A03* **(B)** and *Bn.TIR.A09* **(C)**,*Bn.CBF1.C03* **(D)** , *Bn.CBF2.Ann* **(E)** and*Bn.COR15.A03* **(F)** in transgenic plants after 4 degC cold treatment for 30 days. Error bar represents mean +- SD; $n = 3$. * indicates $P < 0.05$ and ** indicates $P < 0.01$ under normal condition; + indicates $P < 0.05$ and ++ indicates $P < 0.01$ under 4 degC. ns indicates no significance. Student's t-test. *U6* and*ACT7* were used as endogenous reference gene for checking miR1885 and targets expression levels, respectively.

Figure 7. Effects of overexpressing and knocking down of miR1885 in *B. napus* after cold treatment. **(A)** Overexpression of miR1885 increased the sensitivity of cold in *B. napus.* The cold treatment was 2 degC for 7 days. **(B)** Expression of miR1885 and its targets in miR1885 knock down plants (STTM1885). Error bar represents mean +- SD; $n = 3$. * indicates $P < 0.05$; ** indicates $P < 0.01$; Student's t-test.*U6* and *ACT7* were used as endogenous reference gene for measuring miR1885 and targets expression levels, respectively. **(C)** Knocking down of miR1885 increased the resistance to cold in *B. napus* as compared to its background. The cold treatment was 2 degC for 14 days. Red scale bar was 5 cm.

Supplementary datasets

Table S1. Distribution of small RNAs among different categories of genomic loci. rRNA: ribosomal RNA; snRNA: small nuclear RNA; snoRNA: small nucleolar RNA; tRNA: transfer RNA; unann: small RNAs without any specific annotation.

Table S2. Differential expression analysis of identified known miRNAs in *Brassica rapa* by small RNA sequencing. RPM: reads per million.

Table S3. Putative target genes of novel miRNAs in *B. rapa* . The miRNA target genes were predicted by psRNAtarget.

Table S4. Location information of pre-miRNAs in *Brassica napus* reference genome.

Table S5. List of primers used in this study.

Supplementary Figures

Figure S1. Proportion of small RNAs generated from different categories of genomic loci in B_V0 and B_V7 small RNA libraries in*Brassica rapa* .

Figure S2. Cis-element analysis of *bra-MIR1885* promoter. G-box, GA-motif, P-box, TC, TCT, AAAC, ATCT, Box 4, I-box and LTR represent *cis* -elements. A06: chrA06. 24219516 and 24221516: Location information. Red or green bar represent position.

Figure S3. Analysis of the single nucleotide polymorphism (SNP) density distribution, principal component analysis (PCA), and linkage disequilibrium (LD) of *B. napus* population. **(A)** SNP density distribution (number of SNPs in 0.1 Mb sliding windows across each chromosome). **(B)** PCA analysis. Blue, green, and red points represent winter (W), semi-winter (SW) and spring (S) ecotypes, respectively. **(C)** Genome-wide average LD decay estimated from*B. napus* . **(D)** LD heatmap of SNPs in *bna-MIR1885*promoter. Numbers

indicate physical position where *bna-MIR1885* promoter locates. Deeper red color indicates stronger linkage relationship. (**E**) Comparison of the miR1885 relative expression abundance between the two groups with extremely different phenotypes. ES: Low-temperature sensitive accessions; ER: Low-temperature resistant accessions. *U6* was used as an internal control. Data are presented as boxplot. Significant differences were determined by Student's t-test: * indicates $p < 0.05$.

Figure S4. 5'-RACE assays of *Bn. TIR.A09* (BnaA09g14980D) and *Bn. TNL.A03* (BnaA03g56180D). PCR products of *Bn.A09.TIR* and *Bn.A03.TNL* were separated in 2% agarose gel. DNA ladders are labeled on the side. PCR products were cloned into T vectors for Sanger sequencing. Red line represents target fragment; black line represents inner primer.

Figure S5. Phenotypes of transgenic plants overexpressing miR1885 after cold treatment. (A) Phenotypes of transgenic lines grown at 22 degC or 4 degC. Plants were grown 22 degC for 6 weeks (upper panel), and then transferred to 4 degC for an additional 30 days (middle panel), and then allowed to recover at 22 degC for 7 days. (**B**) Post-germination phenotypes of miR1885-OE and WT after cold treatment. Seeds were grown on the germination bed at 22 degC for 2 days and then transferred to 6 degC for 7 days. (**C**) Seeds were grown on the germination bed at 22 degC for 7 days. (**D**) Quantitative analysis of net hypocotyl lengths from WT and *MIR1885* -OE lines. $n = 10$-15 plants. Similar results were obtained in three independent experiments.

Figure S6. Freezing sensitivity of miR1885 over-expressing transgenic plants.

(**A**) Phenotypes of transgenic lines grown at 22 degC and -10 degC. Plants were grown at 22 degC for 6 weeks (upper panel), and then transferred to -10 degC for 2.5 h (middle panel), and then allowed to recover at 22 degC for 7 days. (**B**) Real-time PCR showing the expression pattern of miR1885 under freezing treatment. *U6* was used as endogenous reference gene. Error bar represents mean +- SD; $n = 3$. (**C**) Survival rates of transgenic lines and wild-type plants after freezing treatment (-10 degC in a growth chamber for 2.5 h, $n = 15$–20 plants). * indicates $P < 0.05$; ** indicates $P < 0.01$ (Student's t -test).(**D**) Proline content in transgenic plants and wild type after freezing treatment. Error bar represents mean +- SD. * indicates $P < 0.05$, significant difference from plants subjected to -10 degC before freezing treatment (0 h). + indicates $P < 0.05$, significant difference from plants subjected to -10 degC for 2.5 h (Student's t -test).

REFERENCE

Ahmed, W., Li, R., Xia, Y., Bai, G., Siddique, K.H.M, Zhang, H., Zheng Y., Yang X. Guo P. (2022). Comparative Analysis of miRNA Expression Profiles Between Heat-Tolerant and Heat-Sensitive Genotypes of Flowering Chinese Cabbage Under Heat Stress Using High-Throughput Sequencing. Genes **11:** 264.

Alves-Junior, L., Niemeier, S., Hauenschild, A., Rehmsmeier, M., and Merkle, T. (2009). Comprehensive prediction of novel microRNA targets in Arabidopsis thaliana. Nucleic Acids Research **37:**4010-4021.

Anjali, N.N., and Sabu, K.K. (2020). Role of miRNAs in Abiotic and Biotic Stress Management in Crop Plants. In: Sustainable Agriculture in the Era of Climate Change–Roychowdhury, R., Choudhury, S., Hasanuzzaman, M., and Srivastava, S., eds. Cham: Springer International Publishing. 513-532.

Axtell, M., Meyers, B. (2018). Revisiting criteria for plant microRNA annotation in the era of big data. Plant Cell **30:** 272–284.

Bao, F., Huang, X., Zhu, C., Zhang, X., Li, X., and Yang, S. (2014). Arabidopsis HSP90 protein modulates RPP4-mediated temperature-dependent cell death and defense responses. New Phytologist **202:** 1320-1334.

Bhalla, P.L., and Singh, M.B. (2008). Agrobacterium-mediated transformation of Brassica napus and Brassica oleracea. Nature Protocols **3:** 181-189.

Biggar, K.K., and Storey, K.B. (2015). Insight into post-transcriptional gene regulation: stress-responsive microRNAs and their role in the environmental stress survival of tolerant animals. Journal of Experimental Biology **218:** 1281-1289.

Bolt, S., Zuther, E., Zintl, S., Hincha, D.K., and Schmulling, T. (2017). ERF105 is a transcription factor gene of Arabidopsis thaliana required for freezing tolerance and cold acclimation. Plant, Cell & Environment **40:** 108-120.

Chalhoub, B., Denoeud, F., Liu, S., Parkin, I.A.P., Tang, H., Wang, X., Chiquet, J., Belcram, H., Tong, C., Samans, B., Correa, M., Da Silva, C., Just, J., Falentin, C., Koh, C.S., Le Clainche, I., Bernard, M., Bento, P., Noel, B., Labadie, K., Alberti, A., Charles, M., Arnaud, D., Guo, H., Daviaud, C., Alamery, S., Jabbari, K., Zhao, M., Edger, P.P., Chelaifa, H., Tack, D., Lassalle, G., Mestiri, I., Schnel, N., Le Paslier, M.-C., Fan, G., Renault, V., Bayer, P.E., Golicz, A.A., Manoli, S., Lee, T.-H., Thi, V.H.D., Chalabi, S., Hu, Q., Fan, C., Tollenaere, R., Lu, Y., Battail, C., Shen, J., Sidebottom, C.H.D., Wang, X., Canaguier, A., Chauveau, A., Berard, A., Deniot, G., Guan, M., Liu, Z., Sun, F., Lim, Y.P., Lyons, E., Town, C.D., Bancroft, I., Wang, X., Meng, J., Ma, J., Pires, J.C., King, G.J., Brunel, D., Delourme, R., Renard, M., Aury, J.-M., Adams, K.L., Batley, J., Snowdon, R.J., Tost, J., Edwards, D., Zhou, Y., Hua, W., Sharpe, A.G., Paterson, A.H., Guan, C., and Wincker, P. (2014). Early allopolyploid evolution in the post-Neolithic Brassica napus oilseed genome. Science **345:**950-953.

Chen, S., Huang, H.-A., Chen, J.-H., Fu, C.-C., Zhan, P.-L., Ke, S.-W., Zhang, X.-Q., Zhong, T.-X., and Xie, X.-M. (2020). SgRVE6, a LHY-CCA1-Like Transcription Factor From Fine-Stem Stylo, Upregulates NB-LRR Gene Expression and Enhances Cold Tolerance in Tobacco. Front Plant Sci **11** . 1276

Chen, X. (2009). Small RNAs and Their Roles in Plant Development. Annual Review of Cell and Developmental Biology**25:** 21-44.

Cui, C., Wang, J., Zhao, J., Fang, Y., He, X., Guo, H., and Duan, C. (2020). A Brassica miRNA Regulates Plant Growth and Immunity through Distinct Modes of Action. Molecular Plant **13:** 231-245.

Dangl, J.L., and Jones, J.D.G. (2001). Plant pathogens and integrated defence responses to infection. Nature **411:** 826-833.

Ding, Y, Shi, Y., Yang, S. (2019). Advances and challenges in uncovering cold tolerance regulatory mechanisms in plants. New Phytologist **222:** 1690-1704.

Gupta, O.P., Meena, N.L., Sharma, I., and Sharma, P. (2014). Differential regulation of microRNAs in response to osmotic, salt and cold stresses in wheat. Molecular Biology Reports **41:**4623-4629.

He, J., Xu, M., Willmann, M.R., McCormick, K., Hu, T., Yang, L., Starker, C.G., Voytas, D.F., Meyers, B.C., and Poethig, R.S. (2018). Threshold-dependent repression of SPL gene expression by miR156/miR157 controls vegetative phase change in Arabidopsis thaliana. Plos Genetics**14:** e1007337-e1007337.

He, X., Fang, Y., Feng, L., and Guo, H. (2008). Characterization of conserved and novel microRNAs and their targets, including a TuMV-induced TIR–NBS–LRR class R gene-derived novel miRNA in Brassica. FEBS Letters **582:** 2445-2452.

Huang, X., Li, J., Bao, F., Zhang, X., and Yang, S. (2010). A Gain-of-Function Mutation in the Arabidopsis Disease Resistance Gene RPP4 Confers Sensitivity to Low Temperature. Plant Physiology**154:** 796-809.

Jones-Rhoades, M.W., and Bartel, D.P. (2004). Computational Identification of Plant MicroRNAs and Their Targets, Including a Stress-Induced miRNA. Molecular Cell **14:** 787-799.

Jones, J.D.G., and Dangl, J.L. (2006). The plant immune system. Nature **444:** 323-329.

Krishna, P., Sacco, M., Cherutti, J.F., and Hill, S. (1995). Cold-Induced Accumulation of hsp90 Transcripts in Brassica napus. Plant Physiology **107:** 915-923.

Li, X., Lian, H., Zhao, Q., and He, Y. (2019). MicroRNA166 Monitors SPOROCYTELESS/NOZZLE for Building of the Anther Internal Boundary1. Plant Physiology **181:** 208-220.

Liu, J., Pang, X., Cheng, Y., Yin, Y., Zhang, Q., Su, W., Hu, B., Guo, Q., Ha, S., Zhang, J., and Wan, H. (2018). The Hsp70 Gene Family in Solanum tuberosum: Genome-Wide Identification, Phylogeny, and Expression Patterns. Scientific Reports **8:** 16628.

Ljubej, V., Radojčić Redovniković, I., Salopek-Sondi, B., Smolko, A., Roje, S., and Šamec, D. (2021). Chilling and Freezing Temperature Stress Differently Influence Glucosinolates Content in Brassica oleracea var. acephala. Plants **10:** 1305.

Ma, X., Zhao, F., Zhou, B. (2022). The Characters of Non-Coding RNAs and Their Biological Roles in Plant Development and Abiotic Stress Response. International Journal of Molecular Science **23:** 4124.

McClung, C.R., and Davis, S.J. (2010). Ambient Thermometers in Plants: From Physiological Outputs towards Mechanisms of Thermal Sensing. Current Biology **20:** R1086-R1092.

Paul, P., Chhapekar, S.S., Rameneni, J.J., Oh, S.H., Dhandapani, V., Subburaj, S., Shin, S.Y., Ramchiary, N., Shin, C., Choi, S.R., and Lim, Y.P. (2021). MiR1885 Regulates Disease Tolerance Genes in Brassica rapa during Early Infection with Plasmodiophora brassicae. International Journal of Molecular Sciences **22:** 9433.

Qian, W., Xiao, B., Wang, L., Hao, X., Yue, C., Cao, H., Wang, Y., Li, N., Yu, Y., Zeng, J., Yang Y., Wang, X. (2018). CsINV5, a tea vacuolar invertase gene enhances cold tolerance in transgenic Arabidopsis. BMC Plant Biology **18:** 228

Sanghera, G.S., Wani, S.H., Hussain, W., and Singh, N.B.(2011). Engineering cold stress tolerance in crop plants. Curr Genomics**12:** 30-43.

Seo, P.J., Lee, A.K., Xiang, F., and Park, C.M. (2008). Molecular and functional profiling of Arabidopsis pathogenesis-related genes: insights into their roles in salt response of seed germination. Plant & cell physiology **49:** 334-344.

Shi, Y., Ding, Y., and Yang, S. (2018). Molecular Regulation of CBF Signaling in Cold Acclimation. Trends in Plant Science **23:**623-6.

Snider, C.S., Hsiang, T., Zhao, G., and Griffith, M. (2000). Role of Ice Nucleation and Antifreeze Activities in Pathogenesis and Growth of Snow Molds. Phytopathology **90:** 354-361.

Song, X., Yan, L., Cao, X., Qi,Y. (2019). MicroRNAs and Their Regulatory Roles in Plant-Environment Interactions. Annual Review of Plant Biology **70:** 489-525.

Sun, M., Shen, Y., Chen, Y., Wang, Y., Cai, X., Yang, J., Jia, B., Dong, W., Chen, X., Sun, X. (2022). Osa-miR1320 targets the ERF transcription factor OsERF096 to regulate cold tolerance via JA-mediated signaling. Plant physiology kiac208.

Wang, S., Sun, X., Hoshino, Y., Yu, Y., Jia, B., Sun, Z., Sun, M., Duan, X., and Zhu, Y. (2014). MicroRNA319 Positively Regulates Cold Tolerance by Targeting OsPCF6 and OsTCP21 in Rice (Oryza sativa L.). Plos One **9** . e91357

Wang, X., and Wang, H., and Wang, J., and Sun, R., and Wu, J., and Liu, S., and Bai, Y., and Mun, J.-H., and Bancroft, I., and Cheng, F., and Huang, S., and Li, X., and Hua, W., and Wang, J., and Wang, X., and Freeling, M., and Pires, J.C., and Paterson, A.H., and Chalhoub, B., and Wang, B., and Hayward, A., and Sharpe, A.G., and Park, B.S., and Weisshaar, B., and Liu, B., and Li, B., and Liu, B., and Tong, C., and Song, C., and Duran, C., and Peng, C., and Geng, C., and Koh, C., and Lin, C., and Edwards, D., and Mu, D., and Shen, D.,

and Soumpourou, E., and Li, F., and Fraser, F., and Conant, G., and Lassalle, G., and King, G.J., and Bonnema, G., and Tang, H., and Wang, H., and Belcram, H., and Zhou, H., and Hirakawa, H., and Abe, H., and Guo, H., and Wang, H., and Jin, H., and Parkin, I.A.P., and Batley, J., and Kim, J.-S., and Just, J., and Li, J., and Xu, J., and Deng, J., and Kim, J.A., and Li, J., and Yu, J., and Meng, J., and Wang, J., and Min, J., and Poulain, J., and Wang, J., and Hatakeyama, K., and Wu, K., and Wang, L., and Fang, L., and Trick, M., and Links, M.G., and Zhao, M., and Jin, M., and Ramchiary, N., and Drou, N., and Berkman, P.J., and Cai, Q., and Huang, Q., and Li, R., and Tabata, S., and Cheng, S., and Zhang, S., and Zhang, S., and Huang, S., and Sato, S., and Sun, S., and Kwon, S.-J., and Choi, S.-R., and Lee, T.-H., and Fan, W., and Zhao, X., and Tan, X., and Xu, X., and Wang, Y., and Qiu, Y., and Yin, Y., and Li, Y., and Du, Y., and Liao, Y., and Lim, Y., and Narusaka, Y., and Wang, Y., and Wang, Z., and Li, Z., and Wang, Z., and Xiong, Z., and Zhang, Z., and The Brassica rapa Genome Sequencing Project, C.** (2011). The genome of the mesopolyploid crop species Brassica rapa. Nature Genetics **43:**1035-1039.

Wang, Y., Zhang, Y., Wang, Z., Zhang, X., and Yang, S. (2013). A missense mutation in CHS1, a TIR-NB protein, induces chilling sensitivity in Arabidopsis. The Plant Journal **75:** 553-565.

Xin, Z., and Browse, J. (1998). eskimo1 mutants of Arabidopsis are constitutively freezing-tolerant. Proceedings of the National Academy of Sciences **95:** 7799.

Xu, P., and Cai, W. (2019). Function of Brassica napus BnABI3 in Arabidopsis gs1, an Allele of AtABI3, in Seed Development and Stress Response. Fronters in Plant Science **10** . 00067

Xu, P., Zhu, Y., Zhang, Y., Jiang, J., Yang, L., Mu, J., Yu, X., and He, Y. (2021). Global Analysis of the Genetic Variations in miRNA-Targeted Sites and Their Correlations With Agronomic Traits in Rapeseed. Fronters in Genetics **12** . 741858.

Yan, J., Zhao, C., Zhou, J., Yang, Y., Wang, P., Zhu, X., Tang, G., Bressan, R.A., and Zhu, J.-K. (2016). The miR165/166 Mediated Regulatory Module Plays Critical Roles in ABA Homeostasis and Response in Arabidopsis thaliana. Plos Genetics **12:** e1006416.

Yang, H., Shi, Y., Liu, J., Guo, L., Zhang, X., and Yang, S.(2010). A mutant CHS3 protein with TIR-NB-LRR-LIM domains modulates growth, cell death and freezing tolerance in a temperature-dependent manner in Arabidopsis. The Plant Journal **63:** 283-296.

Yu, X., Wang, H., Lu, Y., de Ruiter, M., Cariaso, M., Prins, M., van Tunen, A., and He, Y. (2011). Identification of conserved and novel microRNAs that are responsive to heat stress in Brassica rapa. J Exp Bot**63:** 1025-1038.

Zbierzak, A.M., Porfirova, S., Griebel, T., Melzer, M., Parker, J.E., and Dörmann, P. (2013). A TIR–NBS protein encoded by Arabidopsis Chilling Sensitive 1 (CHS1) limits chloroplast damage and cell death at low temperature. The Plant Journal **75:** 539-552.

Zeng, X., Xu, Y., Jiang, J., Zhang, F., Ma, L., Wu, D., Wang, Y., and Sun, W. (2018). Identification of cold stress responsive microRNAs in two winter turnip rape (Brassica rapa L.) by high throughput sequencing. BMC Plant Biology **18:** 52.

Zhang, X.D., Sun, J.Y., You, Y.Y., Song, J.B., and Yang, Z.M.(2018). Identification of Cd-responsive RNA helicase genes and expression of a putative BnRH 24 mediated by miR158 in canola (Brassica napus). Ecotoxicology and Environmental Safety **157:** 159-168.

Zhou, M., and Tang, W. (2019). MicroRNA156 amplifies transcription factor-associated cold stress tolerance in plant cells. Molecular Genetics and Genomics **294:** 379-393.

Zhu, M., Wang, X., Zhou, Y., Tan, J., Zhou, Y., Gao, F. (2022). Small RNA Sequencing Revealed that miR4415, a Legume-Specific miRNA, was Involved in the Cold Acclimation of Ammopiptanthus nanus

by Targeting an L-Ascorbate Oxidase Gene and Regulating the Redox State of Apoplast. Fronters in Genetics 13:870446.

Table 1. The miRNAs that were down- or up-regulated by more than 1.5-fold change under cold treatment. B_V0 and B_V7 are the two libraries derived from 22 and 4 treatments, respectively. RPM: Reads per million.

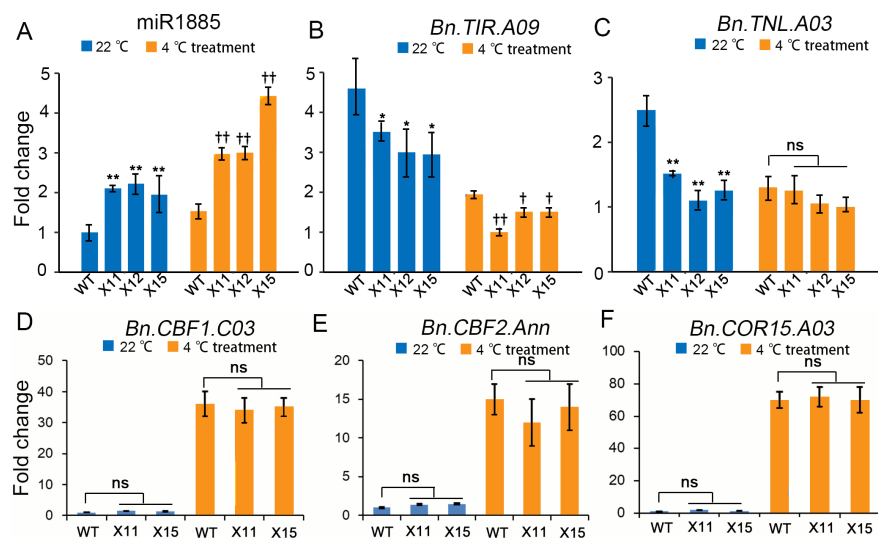| miRNA	B_V0	B_V7	RPM_B_V0	RPM_B_V7	BV7/B_V0	Up/Down	Up/Down	Up/Down
bra-miR157a~c	2865	870	2500.10	765.55	0.31	Down		
bra-miR161	263	68	251.12	87.89	0.35	Down	Down	
bra-miR393f	214	78	186.74	68.64	0.37	Down	Down	
bra-miR396b	1641	826	1431.99	726.84	0.51	Down	Down	
bra-miR319c	332	170	289.71	149.59	0.52	Down	Down	
bra-miR156k	11291	6530	9852.92	5746.05	0.58	Down	Down	
bra-miR1140	8382	5042	7314.42	4436.69	0.61	Down	Down	
bra-miR158a	193	118	168.42	103.83	0.62	Down	Down	
bra-miR156a~f	2319	1503	2023.64	1322.56	0.65	Down	Down	
bra-miR159a	21003	14654	18327.95	12894.73	0.70	Down	Down	
bra-miR168d	2863	4101	2498.35	3608.66	1.44	Up	Up	
bra-miR403	770	1142	671.93	1004.90	1.50	Up	Up	
bra-miR390a~b	288	474	251.32	417.09	1.66	Up	Up	
bra-miR398b~c	1146	2706	1000.04	2381.13	2.38	Up	Up	
bra-miR408	192	505	167.55	444.37	2.65	Up	Up	
bra-miR1885	91	373	79.41	328.22	4.13	Up	Up	Up
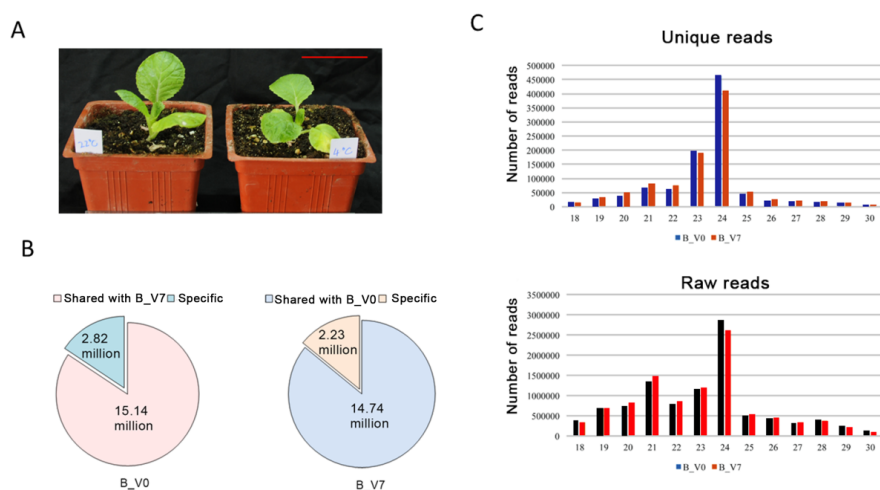
Figure 1.tiff
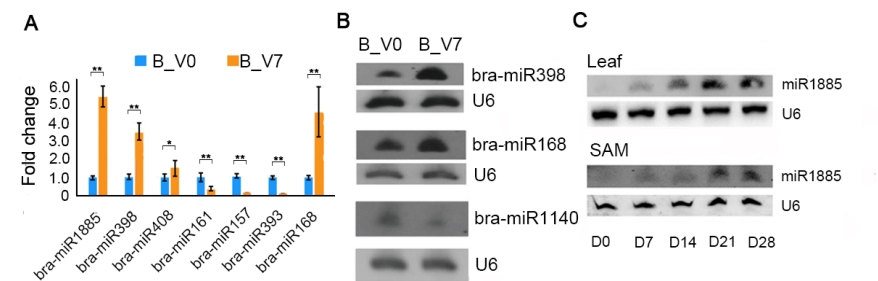
Figure 2.tiff

Figure 3.tiff

17
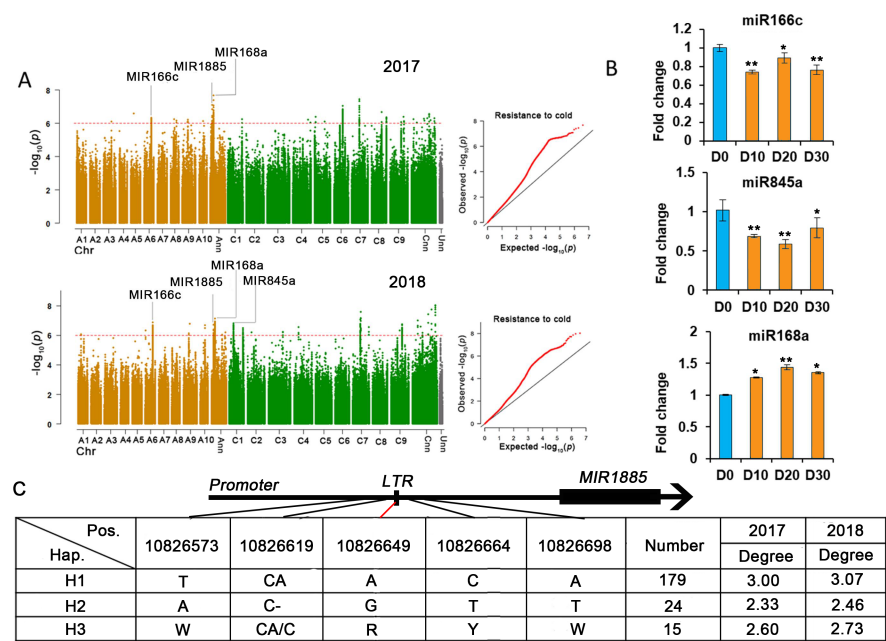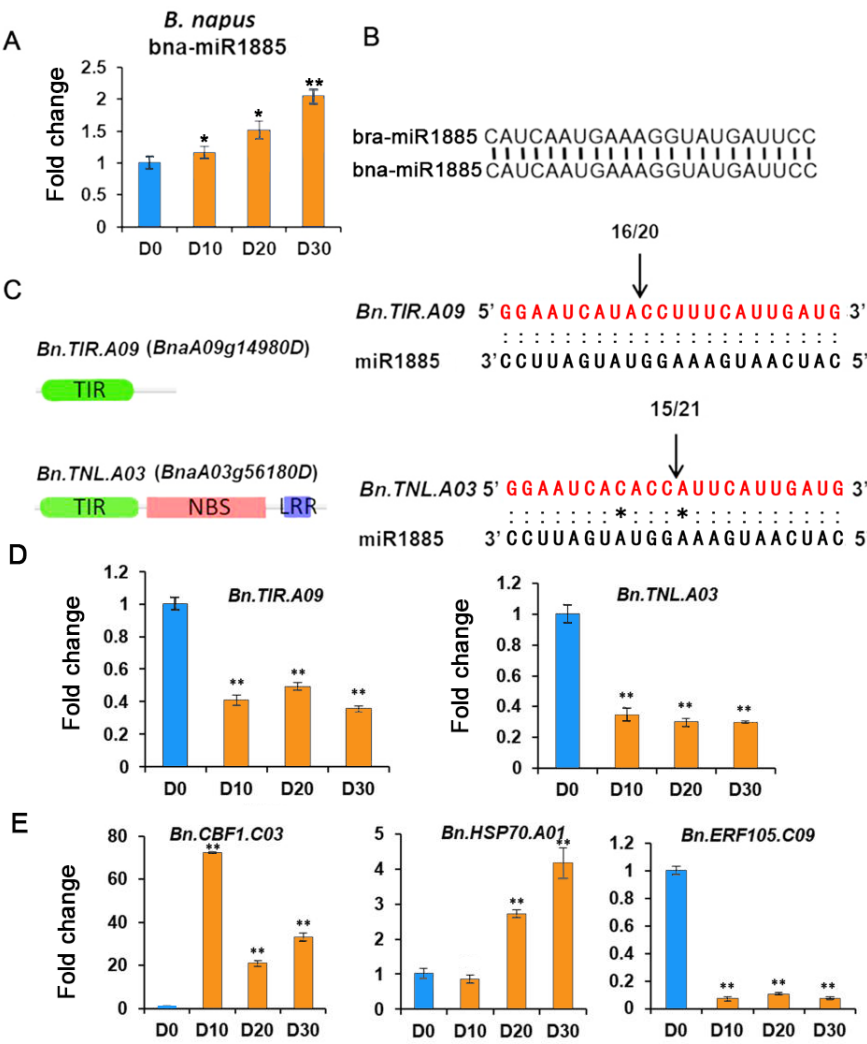
Figure 4.tiff
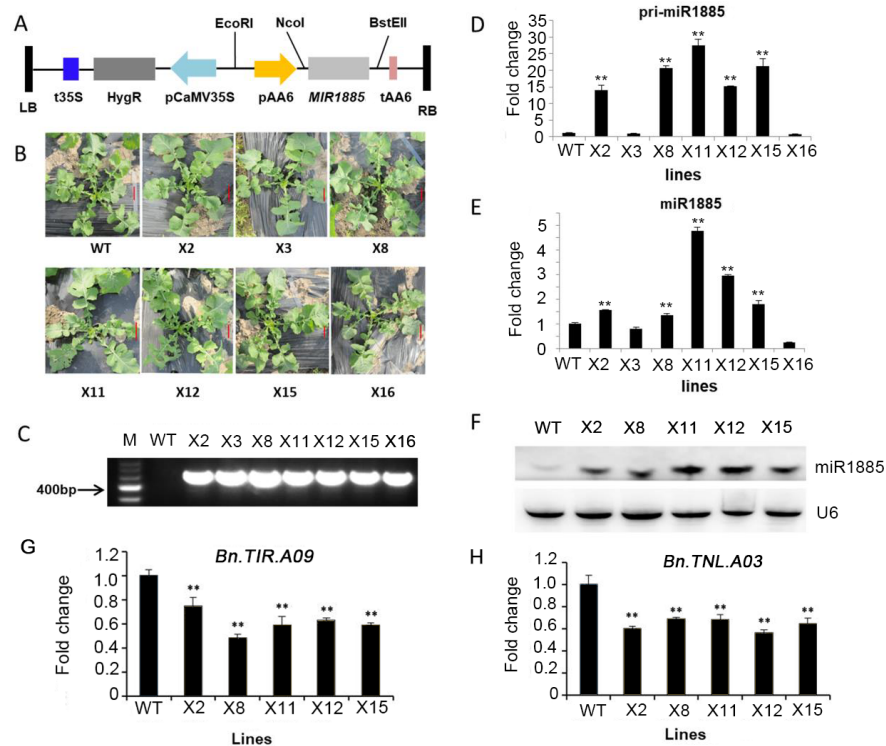
Figure 5.tiff

Figure 6.tiff

Figure 7.tiff