INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
19F–051/201x/00–0–000–000
DOI: 10.17957/IJAB/15.1126
http://www.fspublishers.org

Full Length Article

Comparative MicroRNA Profiling Reveals the Cold Response Mechanisms in two Contrasting Tobacco Cultivars

Risheng Hu[1,2], Zeming Li [1], Shipeng Xiang[2], Yangyang Li[2], Pengfei Yi[1], Minqi Xiao[3] and Xianwen Zhang[4*]
[1]*College of Agronomy, Hunan Agricultural University, Changsha, China*
[2]*Central-South Agricultural Experiment Station of China Tobacco, Changsha, China*
[3]*Central-South University, Changsha, China*
[4]*College of Bioscience and Biotechnology, Hunan Agricultural University, Changsha, China*
[*]For correspondence: zzuliuqi@hotmail.com

Abstract

Cold in late spring seriously affects the yield and quality of tobacco. It is widely known that microRNAs (miRNAs) act in the plant growth, development and stress response. However, the role of miRNAs in tobacco response to cold stress remains to be elucidated. In the study, comparative analysis of miRNA profile between NC567, a cold-tolerant tobacco cultivar, and Taiyan8, a cold-sensitive cultivar, with and without cold treatment was performed. For each sample, more than 22 M raw reads were obtained. The alignment against the miRbase identified 164 conserved miRNAs. A total of 25 cold-regulated (COR) conserved miRNAs in NC567 and 13 in Taiyan8 were discovered. Further analysis revealed the down-regulation of 15 nta-miR169 family members only in NC567, up-regulation of 7 nta-miR399 members just in Taiyan8, and the contrasting expression patterns of miR6154a and b in two cultivars. Finally, with Gene Ontology (GO) analysis of target genes, the cold response model was postulated, which demonstrated the positive role of miR169 family members and the negative effect of miR399. Moreover, the conserved mechanism of miR477a, miR169t and miR156f in response to cold stress was also revealed in tobacco. The study provides important information for understanding and improving the tobacco tolerance to cold stress.

Key words: Tobacco; miRNA profile; Cold stress; Gene ontology; Cold response model

Introduction

Cold stress adversely affects the plant survival, production, and even distribution. During cold stress, some biochemical and physiological changes such as sugars, amino acids, and cold-regulated (COR) genes in plants have been identified. The current studies have shown that C-repeat (CRT)-binding factors (CBFs) play critical roles in the cold acclimation by regulating COR target genes (Thomashow, 2010). At the same time, the CBF signaling pathway is regulated by ICE1 (Inducer of CBF Expression 1) (Wang *et al.*, 2017).

MicroRNAs (miRNAs) are a type of noncoding small RNAs (sRNAs) with 20 to 24 nucleotides, which generally down-regulate target gene expression at post-transcriptional level leading to the target degradation in eukaryotic organisms (Rogers and Chen, 2013). Recently, the important roles of many miRNAs in plant growth and stress responses have been demonstrated (Zhao *et al.*, 2015; Li *et al.*, 2016b). The latest study revealed that heterologous expression of osa-miR393a could enhance switchgrass (*Panicum virgatum* L.) tolerance to cold (Liu *et al.*, 2017). It was reported that the overexpression of miR529a enhanced rice (*Oryza sativa* L.) resistance to oxidative stress (Yue *et al.*, 2017).

In past decades, with the development and wide application of the next-generation sequencing (NGS) technology, more and more miRNAs related to plants growth and stress response were identified (Bertolini *et al.*, 2013; Sun *et al.*, 2015). In wheat (*Triticum aestivum*), six miRNAs were characterized as COR sRNAs in spike tissues, and miR167 functions in the auxin-signaling pathway (Tang *et al.*, 2012). Genome-wide investigation uncovered miRNA expression patterns under cold stress in *Prunus dulcis* Mill. (Karimi *et al.*, 2016), *Citrullus lanatus* L. (Li *et al.*, 2016a), *Medicago sativa* L. (Shu *et al.*, 2016), common wheat (Song *et al.*, 2017) and eggplant (*Solanum aculeatissimum*) plants (Yang *et al.*, 2017a). To date, the researches on tobacco (*Nicotiana tabacum* L.) have demonstrated the tissue specific expression atlas of miRNAs (Baksa *et al.*, 2015) as well as the roles of miRNAs in the floral development (Burklew *et al.*, 2014). Even though recent study revealed miRNA expression profile involved in response to water, drought and cadmium stresses in tobacco (Yin *et al.*, 2014, 2015; Bukhari *et al.*, 2015; He *et al.*, 2016). Our previous study reported cold response mechanisms at the transcriptome and proteome level in tobacco (Hu *et al.*, 2016; Hu *et al.*, 2017), few data of cold-related miRNA profile in tobacco are available.

To cite this paper: Hu, R., Z. Li, S. Xiang, Y. Li, P. Yi, M. Xiao and X. Zhang, 201x. Comparative MicroRNA profiling reveals the cold response mechanisms in two contrasting tobacco cultivars. *Intl. J. Agric. Biol*. 00: 000-000

Purpose of this study was to investigate genome-wide miRNAs profiling in tobacco (*Nicotiana tabacum* L.) under cold stress using NGS technology. The genotype-specific and conserved response mechanisms to cold were unveiled based on the comparative analysis. The COR miRNAs expression was confirmed by qRT-PCR (quantitative real-time reverse transcription). The data would be valuable for understanding and further improving cold tolerance in tobacco.

Materials and Methods

Plant Growth and Clod Treatment

The cold-tolerant NC567 and cold-sensitive Taiyan8 cultivars of tobacco were grown. Growth conditions, cold treatment and sampling were carried out according to the previously reported methods (Hu *et al.*, 2016). The third leaf from the top of tobacco seedlings with 6-8 leaves from 3 different plants was collected at 0 h (the control) and 24 h after cold treatment, respectively, and immediately frozen in liquid nitrogen and stored at -80℃ for further use.

RNA Isolation, sRNA Library Construction and Sequencing

The leaf samples from 3 independent plants of each time point were mixed, and total RNA was isolated from each sample with Trizol reagent (Invitrogen, Carlsbad, CA). The RNA quality and quantity were determined using gel electrophoresis and Agilent 2100 Bioanalyzer (Agilent, MN). The sRNA cDNA libraries were constructed using sRNA Sample Prep Kit (Illumina, CA, USA). Then 4 sRNA libraries were constructed and submitted for Illumina sequencing according to the HiSeq 2500 platform sequencing protocols. The miRNA sequence raw data were deposited to the NCBI Gene Expression Ominus (NCBI GEO) with accession number GSE108733.

Identification of Conserved and Novel miRNAs

After raw reads processing, clean reads were generated from independent libraries. The unique sRNAs with length of 18 to 35 nt were mapped to the tobacco reference genome sequences (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/715/135/GCF_000715135.1_Ntab-TN90/GCF_ 000715135.1_Ntab-TN90_genomic.fna.gz) using the CLC Genomics Workbench program 6.1. The further analysis of these unique sRNA sequences and identification of novel miRNAs was performed based on the previous study (Yang *et al.*, 2017b).

Differential Expression Analysis of COR miRNAs

The expression level of all the miRNAs was normalized to one million against total clean reads of miRNAs in each sample, and was assigned with Transcripts Per Million (TPM). The differential expression of miRNAs for both tobacco cultivars was calculated as: fold-change = (TPM in "24 h"/TPM in "CK"). The *P*-value significance threshold was set by the false discovery rate (FDR). The miRNAs with fold-change of TPM ≥ 2 or ≤ 0.5 together with *P*-value < 0.05 were determined as up- or down-regulated miRNAs between cold-treated and untreated samples for both tobacco cultivars using R DEGseq package.

Prediction and Annotation of Target Genes for miRNAs

The target prediction of differentially expressed known miRNAs was performed with the plant sRNA target server (http://plantgrn.noble.org/psRNATarget/) according to the established criteria (Unver and Budak, 2009). Additionally, with *P*-value < 0.05, functional annotation of the predicted target genes was conducted using Blast2GO software (http://www.blast2go.org/).

Quantitative Real-time PCR (qRT-PCR) Validation

Quantitative real-time PCR (qRT-PCR) was carried out to validate the quality of high-throughput sequencing. Total RNAs were isolated from all samples independently and converted to cDNA as described above. The amplification reactions were conducted on a BioRad iQ5 sequence detection system (BioRad, USA) following the protocol (Naya *et al.*, 2014). According to the mature miRNA sequences, the forward and reverse primers specific to the candidate miRNAs were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). For each condition, qRT-PCR was performed with three replicates. The internal reference gene, UBQ4 (GenBank accession CN949766), was selected, and the relative expression data of the target genes between the treated sample and the control were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The *t*-test was carried out for the significant analysis between the sequencing data and qRT-PCR.

Results

sRNA Library Sequencing

After construction of four libraries of the sRNA, they were sequenced by Illummina HiSeq 2500 Sequencing System. More than 22 M raw reads were obtained for each sample, that is, 22.4 M and 28.7 M for the control (CK) and the cold-treated (CT) samples of NC567, and 24.2 M and 23 M for CK and CT samples of Taiyan8, respectively. After raw reads processing, the data demonstrated that the length of clean reads was between 18 and 30 nt, and 24 nt was more than 30% of all sRNAs, then 30 nt was about 15%, and sRNAs with length of 21 to 23 nt were about 13%, respectively (Fig. 1).

Identification of Conserved and Novel miRNAs

To investigate the repertoire of miRNAs in tobacco, all reads in the sRNA libraries were mapped to the Rfam database (http://rfam.xfam.org/) and the reference genome sequence of tobacco to identify the known RNAs. The further alignment of the remaining sRNAs with the miRbase database identified 164 conserved miRNAs with the top largest number in the families of miR156, miR169, miR172 and miR6151. After the genome mapping, the novel miRNAs were predicted among the un-annotated sRNA sequences and the total of 1834 novel miRNAs were identified in both the tobacco cultivars.

Differentially Expressed miRNAs for Both Tobacco Genotypes

The miRNAs expression level in four samples from NC567 and Taiyan8 is indicated by TPM. The criteria of fold-change of miRNA expression ≥ 2 or ≤ 0.5 and *P*-value < 0.05 was used to characterize the differentially expressed miRNAs in tobacco under cold stress that are considered as cold-regulated (COR) miRNAs. A total of 25 COR conserved miRNAs in NC567 and 13 in Taiyan8 were discovered. Among them, there were 4 and 10 up-regulated, and 21 and 3 down-regulated miRNAs in NC567 and Taiyan8, respectively, and 16 members from nta-miR169 only in NC567 and 7 members from nta-miR399 family only in Taiyan8 were identified as COR miRNAs (Table 1). Five COR miRNAs commonly identified in two tobacco cultivars included nta-miR6154a and b, nta-miR477a, nta-miR169t and nta-miR156f. At the same time, 97 novel miRNAs were considered COR in both tobacco cultivars.

Prediction and Functional Analysis of Target Genes

The target gene prediction of the conserved miRNAs in tobacco was performed using psRNATarget, and altogether 1031 genes were identified. The functional classification of these genes for the COR miRNAs characterized in two tobacco cultivars with *P*-value < 0.05 detected 20 genes corresponding to the down-regulated miRNAs and 4 genes to the up-regulated miRNAs in NC567, and 17 genes related to the down-regulated miRNAs and 27 genes to the up-regulated miRNAs in Taiyan8. In NC567, the target genes for the down-regulated miRNAs were mainly related to transcription (CCAAT-binding factor complex), and reproduction regulation (anther and flower development, regulation of timing from vegetative to reproductive stage), which was similar to that in Taiyan8. However, there were more target genes for the up-regulated miRNAs in Taiyan8 than that in NC567, and most of these genes in Taiyan8 are mainly related to catalysis (oxidoreductase, lignin catabolic, and copper ion binding process) and cell wall modification (Fig. 2). The results demonstrated different molecular mechanism in contrasting tobacco genotypes response to cold stress.

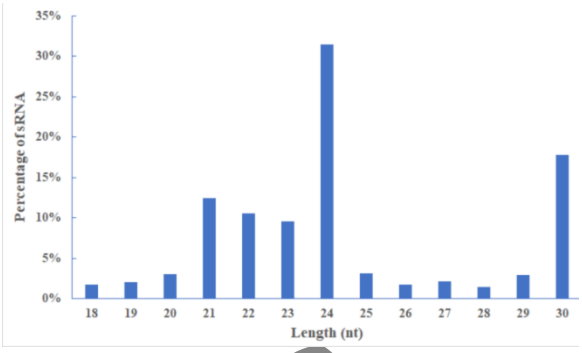

Fig. 1: Distribution of sRNA length from 4 samples
The length of clean reads from 4 samples is mainly between 18 and 30 nt, and 24 nt is more than 30% of all sRNAs, then 30 nt is about 15%, and sRNAs with length of 21 to 23 nt are about 13%

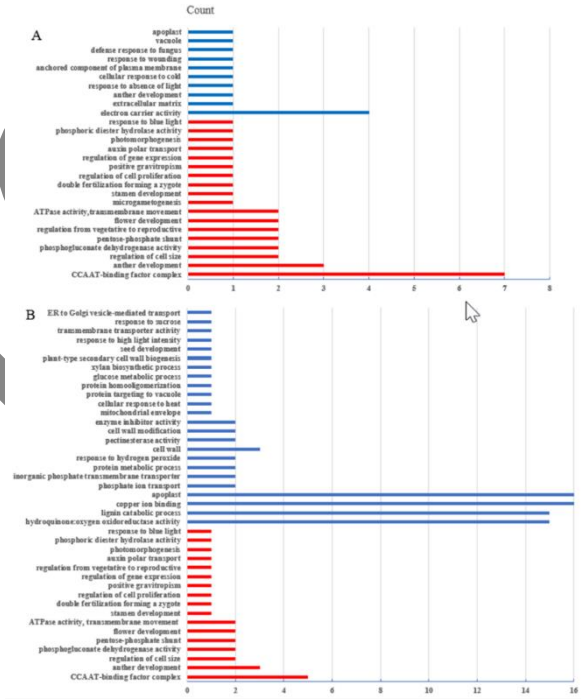

Fig. 2: Functional classification of target genes for candidate miRNAs
In NC567 (A), the target genes for the down-regulated miRNAs are mainly involved in transcription (CCAAT-binding factor complex), and reproduction regulation (anther and flower development, regulation of timing from vegetative to reproductive stage), which is similar to that in Taiyan8 (B). Most of the target genes for the up-regulated miRNAs in Taiyan8 are mainly related to catalysis (oxidoreductase, lignin catabolic, and copper ion binding process) and cell wall modification. The red bar represents the target genes for the down-regulated miRNAs, and the green for the target genes of the up-regulated miRNAs in tobacco under cold stress

Confirmation of miRNA Expression

To confirm the miRNAs expression level by high-throughput sequencing, 8 COR miRNAs were randomly selected to detect their expression using qRT-PCR. As shown in Fig. 3, the expression of the miRNAs obtained by qRT-PCR was in accord with the results from sequencing data based on *P*-value < 0.01 of the *t*-test.

Table 1: Differentially expressed miRNAs in tobacco under cold stress

Symbol	NC567_CK	NC567_24 h	Regulated	Taiyan8_CK	Taiyan8_24 h	Regulated
nta-miR6154b	3.1	1.51	Down	1.86	4.18	Up
nta-miR6154a	3.1	1.51	Down	1.86	4.18	Up
nta-miR6145f	5.35	11.83	Up	-	-	
nta-miR477a	21.98	5.36	Down	10.92	4.49	Down
nta-miR408	7.33	24.34	Up	-	-	
nta-miR399g	-	-		3.33	9.61	Up
nta-miR399f	-	-		3.33	9.61	Up
nta-miR399e	-	-		3.33	9.61	Up
nta-miR399d	-	-		3.33	9.61	Up
nta-miR399c	-	-		3.33	9.61	Up
nta-miR399b	-	-		3.33	9.61	Up
nta-miR399a	-	-		3.33	9.61	Up
nta-miR398	5.64	12.51	Up	-	-	
nta-miR397	-	-		0.8	2.48	Up
nta-miR171a	0.56	1.79	Up	-	-	
nta-miR169t	269.14	100.38	Down	135.82	55.01	Down
nta-miR169p	1.69	0.55	Down	-	-	
nta-miR169o	1.69	0.55	Down	-	-	
nta-miR169m	1.69	0.55	Down	-	-	
nta-miR169l	1.69	0.55	Down	-	-	
nta-miR169k	1.69	0.55	Down	-	-	
nta-miR169j	1.69	0.55	Down	-	-	
nta-miR169i	1.69	0.55	Down	-	-	
nta-miR169h	1.69	0.55	Down	-	-	
nta-miR169g	1.69	0.55	Down	-	-	
nta-miR169f	1.69	0.55	Down	-	-	
nta-miR169e	1.69	0.55	Down	-	-	
nta-miR169d	1.69	0.55	Down	-	-	
nta-miR169c	1.69	0.55	Down	-	-	
nta-miR169b	1.69	0.55	Down	-	-	
nta-miR169a	1.69	0.55	Down	-	-	
nta-miR156f	1.13	0.41	Down	3.73	1.08	Down
nta-miR1446	1.13	0.28	Down	-	-	

Note: "-" represents non-significant difference of expression change

Only the expression of nta-miR408 in Taiyan8 has not significant change according to the sequencing data, but showed more than 2-fold change by qRT-PCR. Taken together, qRT-PCR should be sensitive and reliable to identify transcript abundance.

Discussion

Our previous study elucidated a close relationship between the up-regulation of osa-miR169a expression and the floral transition in wild rice (*Oryza rufipogon* Griff.) plant (Chen *et al.*, 2013), and further study presented the induction of miR169 family members in *Arabidopsis*, maize, and soybean under cold stress, which in turn leads to early flowering phenotype (Xu *et al.*, 2014). The present results demonstrated that 15 members from nta-miR169 family only in NC567 were down-regulated by cold stress, but these members in Taiyan8 showed insignificant expression change (Table 1). Therefore, the data provided the evidence for more cold-tolerance ability of NC567 due to the down-regulation of miR169 members under cold condition than that in Taiyan8.

More recently, the study showed that miR399 exhibits lower expression in plants at 16°C than in those at 23°C, and proposed that the miR399 accumulation may lead to the early flowering of *Arabidopsis* (Kim *et al.*, 2011).

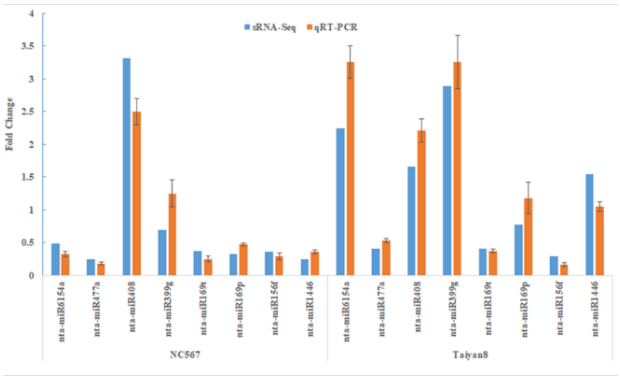

Fig. 3: Expression confirmation of candidate miRNAs by qRT-PCR

The 8 COR miRNAs were randomly selected. The $2^{-\Delta\Delta CT}$ method was used for the experiment and the UBQ4 was selected as the internal reference gene. The *t*-test was carried out for the analysis of significant difference

In this paper, seven members from nta-miR399 family were up-regulated only in Taiyan8, but showed no obvious changes in NC567 after cold treatment (Table 1), which suggests the up-regulation of nta-miR399 expression may result in cold-sensitivity and early flowering in Taiyan8 under cold stress.

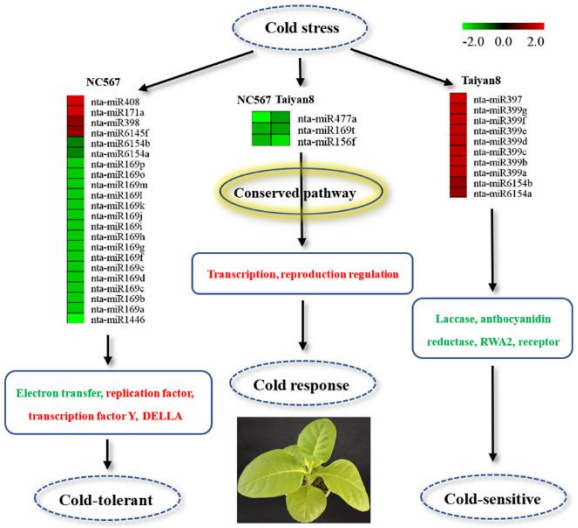

Fig. 4: Cold response model in tobacco

Cold treatment induced miR408, miR171a, miR398 and miR6154f, and repressed miR6154b and b, and many members from miR169 family as well as miR1446 in NC567, then further down-regulated the genes related to electron transfer, and up-regulated the genes encoding replication factor, transcription factor Y, and DELLA protein, which leads to cold-tolerant phenotype. In the contrast, miR397, and all members from miR399 family together with miR6154b and a were induced under cold stress in Taiyan8, then repressed the genes encoding laccase, anthocyanidin reductase, RWA2, and receptors, which consequently result in cold-sensitive phenotype in tobacco. At the same time, cold stress repressed the expression of miR477a, miR169t and miR156f and subsequently elevated the target genes involved in transcription factor and reproduction regulation, which consists of the conserved pathway in tobacco response to cold stress. Color scale bar (representing the log2 fold changes) is shown at the upper right. Red (to miRNAs and target genes) indicates the up-regulation, green for the down-regulation

More interestingly, two members from nta-miR6154 family exhibited contrasting expression patterns, that is, both of nta-miR6154a and nta-miR6154b were down-regulated in NC567, but up-regulated in Taiyan8 by cold (Table 1). Little information about the function of miR6154 family members is available. Further analysis showed that one target gene of nta-miR6154a encodes *REDUCED WALL ACETYLATION 2* (*rwa2*, LOC107800019), suggesting the enhanced expression of *rwa2* in NC567 and its decreased expression in Taiyan8 was due to the nta-miR6154a-mediated target mRNA degradation. A recent study revealed great importance of *rwa2* in cell wall acetylation, and that reduction of cell wall acetylation results in global stress responses in *Arabidopsis* (Nafisi *et al.*, 2015). Consequently, the contrasting expression patterns of nta-miR6154a in two cultivars suggested the better tolerance to cold stress in NC567 than that in Taiyan8.

Consequently, the cold response model at the miRNA level in tobacco was postulated (Fig. 4). The model shows that cold treatment induced miR408, miR171a, miR398 and miR6154f, and repressed miR6154b and a, as well as many members from miR169 family and miR1446 in NC567, then down-regulated their target genes related to electron transfer, and up-regulated the genes encoding replication factor, transcription factor Y and DELLA protein, respectively. Previous studies revealed that the elevated expression of DELLA protein leads to the enhanced cold tolerance in plant (Zhou *et al.*, 2017), therefore, the induction of target genes resulting from the down-regulation of specific miRNAs under cold stress may account for the tobacco cold-tolerant phenotype. Meanwhile, in Taiyan8, miR397, and all members from miR399 family together with miR6154b and a were induced by cold stress, then repressed the target genes encoding laccase, anthocyanidin reductase, RWA2, and receptors, which may account for the cold-sensitive phenotype in tobacco due to the positive roles of laccase (Dhakar *et al.*, 2014) and *rwa2* (Nafisi *et al.*, 2015) in cold adaptation. According to the present data, cold stress repressed the expression of miR477a, miR169t and miR156f in both cultivars, and subsequently elevated the target genes mainly encoding transcription factors such as squamosa promoter-binding-like protein, which may consist of the conserved pathway in tobacco response to cold stress. Previous studies showed that squamosa promoter-binding-like proteins play important roles in controlling the earliness of heading time in Chinese cabbage (Wang *et al.*, 2014) and drought response in rice (Mutum *et al.*, 2013). As a result, the elevated expression of the members from the squamosa promoter-binding-like protein family may be fatal for tobacco survival under cold stress.

Conclusion

Combined with the previous studies on the miRNA family members in plants, the present data provided firm evidence for the possible roles of the miRNAs characterized above in tobacco response to cold stress, and further revealed the importance of the cold response model in resistance to cold stress for tobacco. Although the function of the candidate miRNAs identified here needs further elucidated, the present study provides important clues for understanding and improving the tobacco tolerance to cold stress.

Acknowledgements

The work was supported by the Key Science and Technology Program of Hunan Provincial Tobacco Corporation (Grant No: 17-20Aa04) and the Key Science and Technology Program of China National Tobacco Corporation (Grant No: 110201302002).

References

Baksa, I., T. Nagy, E. Barta, Z. Havelda, E. Varallyay, D. Silhavy, J. Burgyan and G. Szittya, 2015. Identification of *Nicotiana benthamiana* microRNAs and their targets using high throughput sequencing and degradome analysis. *BMC Genom.,* 16: 1025

Bertolini, E., W. Verelst, D.S. Horner, L. Gianfranceschi, V. Piccolo, D. Inze, M.E. Pe and E. Mica, 2013. Addressing the Role of microRNAs in Reprogramming Leaf Growth during Drought Stress in Brachypodium distachyon. *Mol. Plant*, 6: 423–443

Bukhari, S.A., S. Shang, M. Zhang, W. Zheng, G. Zhang, T.Z. Wang, I.H. Shamsi and F. Wu, 2015. Genome-wide identification of chromium stress-responsive micro RNAs and their target genes in tobacco (Nicotiana tabacum) roots. *Environ. Toxicol. Chem.*, 34: 2573–2582

Burklew, C.E., F. Xie, J. Ashlock and B. Zhang, 2014. Expression of microRNAs and their targets regulates floral development in tobacco (Nicotiana tabacum). *Funct. Integr. Genom.*, 14: 299–306

Chen, Z., F. Li, S. Yang, Y. Dong, Q. Yuan, F. Wang, W. Li, Y. Jiang, S. Jia and X. Pei, 2013. Identification and Functional Analysis of Flowering Related microRNAs in Common Wild Rice (*Oryza rufipogon* Griff.). *PLoS One*, 8: e82844

Dhakar, K., R. Jain, S. Tamta and A. Pandey, 2014. Prolonged Laccase Production by a Cold and pH Tolerant Strain of Penicillium pinophilum (MCC 1049) Isolated from a Low Temperature Environment. *Enzyme Res.*, 2014: 120708

He, X., W. Zheng, F. Cao and F. Wu, 2016. Identification and comparative analysis of the microRNA transcriptome in roots of two contrasting tobacco genotypes in response to cadmium stress. *Sci. Rep.*, 6: 32805

Hu, R., X. Zhu, S. Xiang, Y. Zhan, M. Zhu, H. Yin, Q. Zhou, L. Zhu, X. Zhang and Z. Liu, 2016. Comparative transcriptome analysis revealed the genotype specific cold response mechanism in tobacco. *Biochem. Biophys. Res. Commun.*, 469: 535–541

Hu, R., X. Zhu, S. Xiang, X. Zhang, Z. Liu, L. Zhu, Y. Cao, C. Yang and J. Lai, 2017. Comparative proteomic analysis reveals differential protein and energy metabolisms from two tobacco cultivars in response to cold stress. *Acta Physiol. Plant.*, 40: 19

Karimi, M., F. Ghazanfari, A. Fadaei, L. Ahmadi, B. Shiran, M. Rabei and H. Fallahi, 2016. The Small-RNA Profiles of Almond (Prunus dulcis Mill.) Reproductive Tissues in Response to Cold Stress. *PLoS One*, 11: e0156519

Kim, W., H.J. Ahn, T.J. Chiou and J.H. Ahn, 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: 83–88

Li, H., Y. Dong, J. Chang, J. He, H. Chen, Q. Liu, C. Wei, J. Ma, Y. Zhang, J. Yang and X. Zhang, 2016a. High-Throughput MicroRNA and mRNA Sequencing Reveals That MicroRNAs May Be Involved in Melatonin-Mediated Cold Tolerance in Citrullus lanatus L. *Front. Plant Sci.*, 7: 1231

Li, W., T. Wang, Y. Zhang and Y. Li, 2016b. Overexpression of soybean miR172c confers tolerance to water deficit and salt stress, but increases ABA sensitivity in transgenic Arabidopsis thaliana. *J. Exp. Bot.*, 67: 175–194

Liu, Y., K. Wang, D. Li, J. Yan and W. Zhang, 2017. Enhanced Cold Tolerance and Tillering in Switchgrass (Panicum virgatum L.) by Heterologous Expression of Osa-miR393a. *Plant Cell Physiol.*, 58: 2226–2240

Livak, K.J. and T.D. Schmittgen, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C (T)) method. *Methods*, 25: 402–408

Mutum, R.D., S.C. Balyan, S. Kansal, P. Agarwal, S. Kumar, M. Kumar and S. Raghuvanshi, 2013. Evolution of variety-specific regulatory schema for expression of osa-miR408 in indica rice varieties under drought stress. *FEBS J.*, 280: 1717–1730

Nafisi, M., M. Stranne, L. Fimognari, S. Atwell, H.J. Martens, P.R. Pedas, S.F. Hansen, C. Nawrath, H.V. Scheller, D.J. Kliebenstein and Y. Sakuragi, 2015. Acetylation of cell wall is required for structural integrity of the leaf surface and exerts a global impact on plant stress responses. *Front. Plant Sci.*, 6: 550

Naya, L., S. Paul, O. Valdes-Lopez, A.B. Mendoza-Soto, B. Nova-Franco, G. Sosa-Valencia, J.L. Reyes and G. Hernandez, 2014. Regulation of Copper Homeostasis and Biotic Interactions by MicroRNA 398b in Common Bean. *PLoS One*, 9: e84416

Rogers, K. and X. Chen, 2013. Biogenesis, turnover, and mode of action of plant microRNAs. *Plant Cell*, 25: 2383–2399

Shu, Y., Y. Liu, W. Li, L. Song, J. Zhang and C. Guo, 2016. Genome-wide investigation of micrornas and their targets in response to freezing stress in *Medicago sativa* L., based on high-throughput sequencing. *G3 (Bethesda)*, 6: 755–765

Song, G., R. Zhang, S. Zhang, Y. Li, J. Gao, X. Han, M. Chen, J. Wang, W. Li and G. Li, 2017. Response of microRNAs to cold treatment in the young spikes of common wheat. *BMC Genom.*, 18: 212

Sun, X., G. Fan, L. Su, W. Wang, Z. Liang, S. Li and H. Xin, 2015. Identification of cold-inducible microRNAs in grapevine. *Front. Plant Sci.*, 6: 595

Tang, Z., L. Zhang, C. Xu, S. Yuan, F. Zhang, Y. Zheng and C. Zhao, 2012. Uncovering small RNA-mediated responses to cold stress in a wheat thermosensitive genic male-sterile line by deep sequencing. *Plant Physiol.*, 159: 721–738

Thomashow, M.F., 2010. Molecular basis of plant cold acclimation: insights gained from studying the CBF cold response pathway. *Plant Physiol.*, 154: 571–577

Unver, T. and H. Budak, 2009. Conserved microRNAs and their targets in model grass species Brachypodium distachyon. *Planta*, 230: 659–669

Wang, D.Z., Y.N. Jin, X.H. Ding, W.J. Wang, S.S. Zhai, L.P. Bai and Z.F. Guo, 2017. Gene regulation and signal transduction in the ICE-CBF-COR signaling pathway during cold stress in plants. *Biochemistry*, 82: 1103–1117

Wang, Y., F. Wu, J. Bai and Y. He, 2014. BrpSPL9 (Brassica rapa ssp. pekinensis SPL9) controls the earliness of heading time in Chinese cabbage. *Plant Biotechnol. J.*, 12: 312–321

Xu, M.Y., L. Zhang, W.W. Li, X.L. Hu, M.B. Wang, Y.L. Fan, C.Y. Zhang and L. Wang, 2014. Stress-induced early flowering is mediated by miR169 in Arabidopsis thaliana. *J. Exp. Bot.*, 65: 89–101

Yang, X., F. Liu, Y. Zhang, L. Wang and Y.F. Cheng, 2017a. Cold-responsive miRNAs and their target genes in the wild eggplant species Solanum aculeatissimum. *BMC Genom.*, 18: 1000

Yang, Y., X. Zhang, Y. Su, J. Zou, Z. Wang, L. Xu and Y. Que, 2017b. miRNA alteration is an important mechanism in sugarcane response to low-temperature environment. *BMC Genom.*, 18: 833

Yin, F., J. Gao, M. Liu, C. Qin, W. Zhang, A. Yang, M. Xia, Z. Zhang, Y. Shen, H. Lin, C. Luo and G. Pan, 2014. Genome-wide analysis of water-stress-responsive microRNA expression profile in tobacco roots. *Funct. Integr. Genom.*, 14: 319–332

Yin, F., C. Qin, J. Gao, M. Liu, X. Luo, W. Zhang, H. Liu, X. Liao, Y. Shen, L. Mao, Z. Zhang, H. Lin, T. Lubberstedt and G. Pan, 2015. Genome-wide identification and analysis of drought-responsive genes and microRNAs in tobacco. *Intl. J. Mol. Sci.*, 16: 5714–5740

Yue, E., Z. Liu, C. Li, Y. Li, Q. Liu and J.H. Xu, 2017. Overexpression of miR529a confers enhanced resistance to oxidative stress in rice (Oryza sativa L.). *Plant Cell Rep.*, 36: 1171–1182

Zhao, Y., S. Lin, Z. Qiu, D. Cao, J. Wen, X. Deng, X. Wang, J. Lin and X. Li, 2015. MicroRNA857 Is Involved in the Regulation of Secondary Growth of Vascular Tissues in Arabidopsis. *Plant Physiol.*, 169: 2539–2552

Zhou, M., H. Chen, D. Wei, H. Ma and J. Lin, 2017. Arabidopsis CBF3 and DELLAs positively regulate each other in response to low temperature. *Sci. Rep.*, 7: 39819

(Received 17 April 2019; Accepted 30 April 2019)