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Assessment of miRNA expression profile and differential expression pattern of target genes in cold-tolerant and cold-sensitive tomato cultivars

Ibrahim Koc^a, Ertugrul Filiz^b and Huseyin Tombuloglu^{c*}

^aDepartment of Molecular Biology and Genetics, Faculty of Science, Gebze Technical University, Gebze, Kocaeli, Turkey; ^bDepartment of Crop and Animal Production, Cilimli Vocational School, Duzce University, Cilimli, Duzce, Turkey; ^cDepartment of Biology, Faculty of Science and Arts, Fatih University, Buyukcekmece, Istanbul, Turkey

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MicroRNAs (miRNAs) are small non-coding RNAs about 21 nt in length. These short transcripts regulate developmental and stress responses in plants. Cold stress is one of the most restraining abiotic factors adversely affecting the plant yield. In the present study, some cold stress-related miRNAs (miR167, miR169, miR172, miR393 and miR397) in tomato (*Solanum lycopersicum*) were assessed at early time points (0, 1, 4 and 16 h) of cold exposure. Relative expression of miRNAs was measured by stem–loop quantitative reverse transcription polymerase chain reaction. The results showed that miR167, miR169, miR172 and miR393 were activated in the early time points of cold treatment. Especially, miR172 was found to have highest expression level. Furthermore, target genes of selected miRNAs were identified and their expression profiles were assessed between cold-sensitive and cold-tolerant cultivars of tomato. It was found that inferred expression patterns of target genes were differentiated between the cultivars. Analysis of *cis*-acting elements showed that miRNAs had stress-responsive elements. Meanwhile, since no miR393 sequence is available, putative miR393 sequence and its secondary structure were predicted in tomato. These results may provide a framework for further analysis in terms of understanding the response of miRNAs against cold stress in tomato.

Keywords: cold stress; miRNA; stem–loop qRT–PCR; tomato; *cis*-acting element; target genes

Introduction

Plant microRNAs (miRNAs), which are non-coding short transcripts, play a key role in development and stress response in plants. To date, hundreds of miRNAs have been found and characterized. miRNAs are often located in gene families and conserved between closely related species.[1–3] They repress gene expression by base-pairing with nearly perfect complementarity against coding sequences, leading to cleavage or translational repression of mRNAs.[4–6] Thus, miRNAs regulate the gene expression post-transcriptionally.[7] miRNAs have been shown to be involved in the response to various stress conditions.[8–10] In *Arabidopsis*, miR398 was identified to target two closely related Cu/Zn superoxide dismutase genes, and down-regulation of miR398 led to improved tolerance of transgenic lines under oxidative stress.[11] miR395 and miR399 were found to be involved in sulphate and phosphate starvation.[12,13] In another study, miR393 was strongly induced by cold stress.[8] In addition, miRNAs regulate many developmental processes, including leaf development, auxin signalling, phase transition, flowering and genome maintenance.[14–21] The knowledge about miRNAs mostly comes from *Arabidopsis* and accumulation of new data is still an ongoing

process. To date, miRNAs have been identified and detected using high-throughput sequencing, cloning and bioinformatic approaches.[22–24] Conventional technologies such as northern hybridization and microarray analysis have been widely used for investigation of miRNAs but it was reported that it may not be sensitive enough to detect less abundant miRNAs.[25] Among the many techniques, stem–loop reverse transcription polymerase chain reaction (stem–loop RT–PCR) provides more specificity and sensitivity since it uses linear primers due to base-stacking and spatial constraint of the stem–loop structure.[25,26] Furthermore, detection sensitivity is increased by a pulsed RT reaction.[27]

Cold stresses, such as chilling and freezing, affect the agricultural productivity around the world.[28] Numerous physiological and molecular changes occur during cold acclimation.[29] Among them, repression of genes, mRNA export and mRNA degradation have been found to be of central importance for the cold-stress response.[29,30] However, knowledge on the role of miRNA in cold stress is still limited compared to other abiotic stresses. Therefore, elucidating the cold-stress-regulated miRNAs and determination of their expression profile could improve our understanding of miRNAs and their

*Corresponding author: htombuloglu@fatih.edu.tr

functions in biological systems. Recently, expression profiles of all known miRNAs under cold stress in *Arabidopsis*, poplar and rice were validated with microarray analyses.[19,24,28] Zhou et al. [7] established a machine-learning transcriptome-based approach for annotating cold-inducible miRNAs in *Arabidopsis*. They found that miR165/166, miR169, miR172, miR393, miR396 and miR397 were over expressed in cold stress. They also formed a sketch pathway, implying possible regulatory role of miR169. Tang et al. [31] found up-regulation of miR167 and role of *trans*-acting short-interfering RNA–auxin response factor (tasiRNA–ARF) in regulating the auxin-signalling pathway and possibly in the developmental response to cold stress in wheat. In rice, Jian et al. [32] found several cold-regulated miRNAs by direct cloning and sequencing. Zhang et al. [33] identified 28 cold responsive miRNAs in *Brachypodium*.

Plants from temperate regions are tolerant to chilling but not to freezing; however, they can increase their freezing tolerance by being exposed to chilling temperatures, by a process known as cold acclimation.[34,35] On the contrary, tropical and subtropical plants, such as rice, maize and tomato are sensitive to cold stress and largely lack the capacity for cold acclimation.[35] Tomato suffers from chilling injuries at all stages of growth and development.[36] Prolonging cold temperature can further reduce the plant production and growth.

It was predicted that there are many cold-induced miRNAs in plant species but knowledge about them is still limited. In this study, we investigated cold-induced miRNAs (miR167, miR169, miR172, miR397 and miR393) [7,8,31] by a sensitive method, stem–loop qRT–PCR, in cold-treated tomato plants. In order to understand the expression profiles of miRNA target genes, we also retrieved the microarray data of three tomato cultivars from a public database.

Materials and methods

Plant growth and cold treatment

Tomato (*Solanum lycopersicum* var. H-2274) plants were grown in soil under greenhouse conditions with a 16:8 h (light:dark) photoperiod at $25\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$. After 4 weeks of growth, plants were subjected to cold treatment ($4\text{ }^{\circ}\text{C}$ for 1, 4 and 16 h) with a light intensity of $20\text{ }\mu\text{mol}/(\text{m}^2\text{ s})$. [37] Then plant leaves were quickly harvested and stored at $-80\text{ }^{\circ}\text{C}$. Control plants were not subjected to cold treatment.

Preparation of RNA and cDNA

Total RNA of the leaf samples were extracted using Trizol® (Invitrogen) reagent, according to the manufacturer's instructions. Extracted RNA was dissolved in

Rnase-free water and stored at $-80\text{ }^{\circ}\text{C}$. RNA integrity was observed in a 2% agarose gel; three bands corresponding to ribosomal RNA (28S, 18S and 5S) were apparent. RNA concentration was calculated by using Cubit flour metric system (Invitrogen). An amount of 500 ng of total RNA was treated with 5 U of Rnase-free recombinant DNase I® (Roche) and incubated at $37\text{ }^{\circ}\text{C}$ for 15 min. Reaction was stopped by adding $2\text{ }\mu\text{L}$ of 0.2 mol/L ethylenediaminetetraacetic acid (pH 8.0) to a final concentration of 8 mmol/L and heating to $75\text{ }^{\circ}\text{C}$ for 10 min. Stem–loop RT primers were designed according to Varkonyi-Gasic et al. [25]. RT reaction contained 30 ng RNA, $1\text{ }\mu\text{L}$ stem–loop RT primer ($1\text{ }\mu\text{mol/L}$) and 0.25 mmol/L deoxyribonucleoside triphosphates. The mixture was incubated at $65\text{ }^{\circ}\text{C}$ for 5 min and then $5\times$ reverse transcriptase buffer, $1\text{ }\mu\text{L}$ 20 units RiboLock RNase inhibitor, $1\text{ }\mu\text{L}$ RevertAid M–MuLV RT (Thermo Scientific™ RevertAid™ First Strand Synthesis Kit) and Rnase-free water were added into the mixture up to $20\text{ }\mu\text{L}$ per sample. This mixture was incubated for 30 min at $16\text{ }^{\circ}\text{C}$, followed by 60 cycles at $30\text{ }^{\circ}\text{C}$ for 30 s, $42\text{ }^{\circ}\text{C}$ for 30 s and $50\text{ }^{\circ}\text{C}$ for 1 s for pulsed RT. Finally, reverse transcriptase was inactivated by incubation at $70\text{ }^{\circ}\text{C}$ for 15 min.

Stem–loop reverse transcription–PCR

Prior to quantification analysis, miRNAs of interest were amplified by conventional PCR (Techne® TC512 Gradient Thermal Cycler) and checked in a 2% agarose gel (Figure 1(a)). For quantification analysis, primer-specific regions of targeted miRNAs were amplified using Luminaris™ Color HiGreen qPCR Master Mix kit (Thermo Scientific) and real-time PCR (qPCR) was performed in a RotorGene PCR machine (Qiagen). A miRNA-specific primer and a universal reverse primer were used for amplification of individual miRNAs (Table 1). Then, $0.5\text{ }\mu\text{mol/L}$ of each forward and reverse primer with $2\text{ }\mu\text{L}$ cDNA was mixed with $10\text{ }\mu\text{L}$ of qPCR Master Mix. The following program was set up: $95\text{ }^{\circ}\text{C}$ for 10 min, 40 cycles of $95\text{ }^{\circ}\text{C}$ for 15 s, $58\text{ }^{\circ}\text{C}$ for 30 s, $72\text{ }^{\circ}\text{C}$ for 30 s and melting analysis at a temperature gradient from $57\text{ }^{\circ}\text{C}$ to $95\text{ }^{\circ}\text{C}$ with an increment of $0.5\text{ }^{\circ}\text{C}/\text{min}$.

Relative quantity of individual transcripts was calculated by using a mathematical model, including an efficiency correction and crossing point (Cp) deviation of an unknown sample versus a control.[38] Tomato actin gene was used as internal control for qPCR analysis. The fold change (FC) in the expression of each miRNAs was computed according to Pfaffl [38].

Expression analysis of target gene under cold stress

Mature miRNA sequences were obtained from miRBase (<http://www.mirbase.org>).[39] Target predictions were

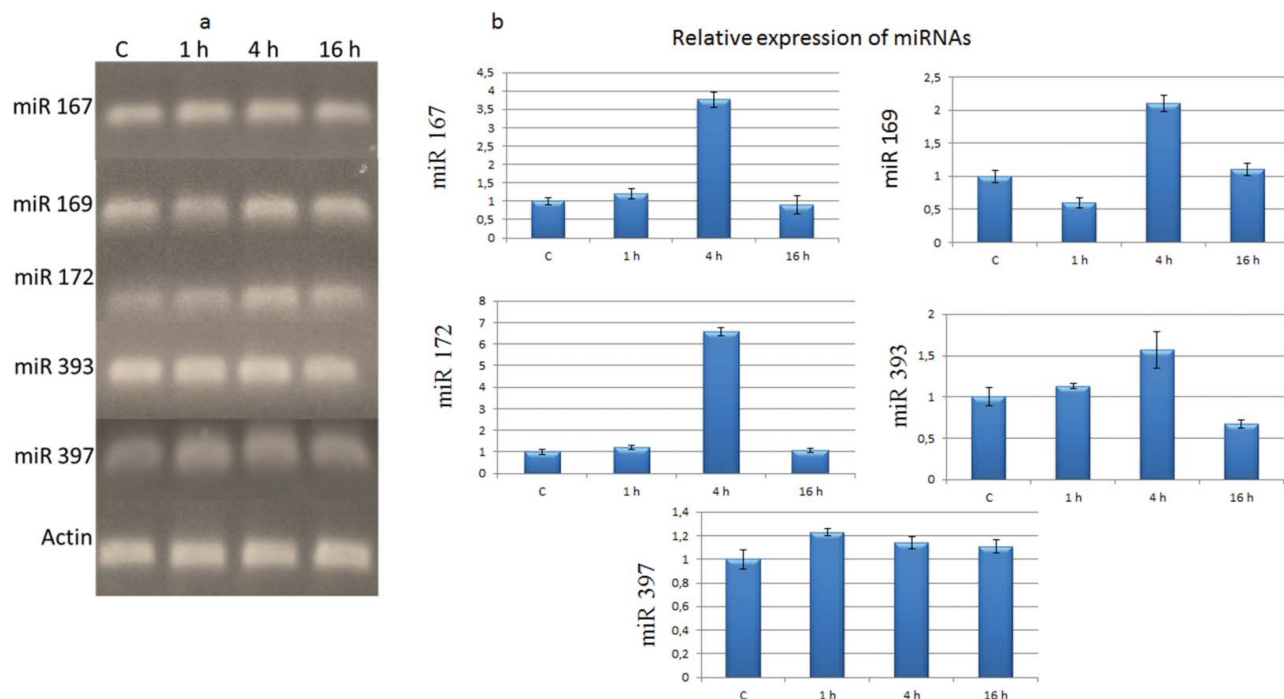


Figure 1. Expression of miRNAs in tomato at 1, 4 and 16 h of cold stress. (a) Agarose gel showing amplification of miRNAs. C – control. (b) Relative expression level of tomato miRNAs.

performed using the psRNATarget (<http://plantgrn.noble.org/psRNATarget/>) [40] with default settings. We also extracted the expression profile of five miRNAs' target genes from microarray data for three cold-treated tomato cultivars (LA1777, LA3969 and LA4024) in the Tomato Functional Genomics Database (<http://ted.bti.cornell.edu/>). The data was normalized using Print-tip lowess normalisation method.[36] We also compiled some target genes, which were reported from previous studies. However, expressions of some target genes were not available in databases. Thus, we excluded these targets. Probe sequences were further used as query sequences for

the BLASTN search against SGN tomato whole-genome chromosome database (<http://solgenomics.net/tools/blast/index.pl>) and NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi#>). Gene annotation analysis was performed using the Tomato Functional Genomics Database. The microarray data was deposited in the Tomato Functional Genomics Database with accession number E060,[36] for three tomato genotypes. The cold tolerance level of these genotypes was reported by Liu et al. [36]. In this manner, we were able to comparatively investigate how the expression profile of target genes differs among cold-tolerant and cold-sensitive tomato cultivars. Statistical analysis was

Table 1. Primers used for stem-loop qRT–PCR.

Primer name	Sequence
Universal reverse primer	GTGCAGGGTCCGAGGT
miR167-F	TCGCGTGAAGCTGCCAGCAT
miR167-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACTAGATC
miR169-F	GCGGCGGCAGCCAAGGATGACT
miR169-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACTCGGCA
miR172-F	CGGCGCAGAATCTTGATGATG
miR172-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACATGCAG
miR393-F	GCGCGGTCCAAAGGGATCGCA
miR393-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACGATCAA
miR397-F	CGGCGTCATTGAGTGCAGCG
miR397-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACCATCAA

carried out using the data obtained from three separate sets of biological samples. One-way analysis of variance with the *post hoc* test was performed using SPSS 18.0 for Windows. $P < 0.05$ was considered to be statistically significant.

Isolation of sly-miR393 and cis-elements of miRNAs

The pre-miRNA sequences for each of miRNA were obtained from miRBase and mapped to the sequences of tomato genome (<http://www.phytozome.net/>) using the BLAST search. Blast parameters were adjusted as follows: an expected value cutoff of 1; a low-complexity sequence filter; 1000 descriptions and alignments, and automatically adjusted parameters for short input sequences to improve the accuracy of outputs. For each miRNA, 1 kb of upstream putative promoter sequence was retrieved.[19] PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/>) was used to identify and categorize the *cis*-acting elements. The secondary structures of putative miR393 sequence in tomato and minimal folding free energies (MFEs ΔG in kcal/mol) were predicted by using the web-based software Mfold 2.3 (<http://mfold.rutgers.edu/?q=mfold/RNA-Folding-Form>). Then, adjusted MFE and MFE index (MFEI) were calculated according to the previous report.[41]

Results and discussion

Expression patterns of tomato miRNAs

The obtained results showed that miR167 was induced by approximately four FC comparing to the control group at 4 h of cold treatment (Figure 1(b)). After 16 h of cold treatment, its transcript accumulation was decreased to control level. Transcript accumulation of miR169 was decreased at 1 h and then increased at 4 h of cold treatment. Expression of miR169 was not altered in comparison to control after 16 h of cold exposure. miR172 was induced to a highest extent among the tested miRNAs. Especially, its expression was significantly increased over six times at 4 h. Similar to miR167, its expression pattern showed a decrease in expression at 16 h of cold treatment as that in the control. The transcript abundance of miR393 was nearly 1.6 FC increased at 4 h. The expression level of miR393 was lower than that in the control at 1 h, whereas cold treatment did not significantly change the expression of miR397.

When we compared the expression profile of miRNAs with that in the control group, it was miR172 that stood out as the most induced one (Figure 1(b)). Following miR172, the levels of expression of miR167 and miR169 were higher than that of other miRNAs. Interestingly, it was indicated that 4 h of cold treatment induced expression of miR172, miR167, miR169 and miR393. After 4 h,

the expression of those miRNAs decreased but nevertheless they showed a nearly constant expression profile compared to the control. We suggest that 4 h of cold treatment was a suitable duration for up-regulation of miR167, miR169, miR172 and miR393 in tomato plants.

Isolation of sly-miR393 and cis-element analysis of miRNAs

We should note that miR393 expression in tomato was previously reported [42,43] but no sequence was deposited in any database. Using *Solanum tuberosum* (potato) and *Arabidopsis* mature miR393 sequences, which are well conserved, we identified putative mature sly-miR393 sequence location (100% identity with mature miRNA sequences of potato and *Arabidopsis*). Additionally, we tried to elucidate the sly-miR393 sequence by blasting stem-loop miRNA sequences of potato and *Arabidopsis* against the tomato genome (<http://www.phytozome.net/>), and only potato miRNA sequences showed 93% identity (100% coverage; data not shown) with tomato sequence in the same location as those of mature sequences of both plants. The matched sequences were obtained and further screened for the potential hairpin structure by an RNA-fold prediction program. The output of the program, which gave rise to the characteristic stem-loop structure, is shown in Figure 2(a). The MFE of putative sly-miR393 was -39.30 kcal/mol and the MFEI was 1.22. This finding indicated that the miRNA precursor sequence had a significantly higher MFEI value than other non-coding or coding RNAs, e.g. tRNA, rRNA.[44] Furthermore, putative mature sly-miR393 sequences are in the stem portion of hairpin structures (Figure 2(a)).

The gene expression level largely depends on *cis*-elements existing in the promoter region.[45] *Cis*-elements analysis could also be an effective approach for the miRNAs as for protein-coding genes.[46,47] In the analysis, unknown *cis*-elements were not included. *Cis*-elements were heterogeneously distributed among miRNAs. The investigation revealed that abscisic acid (ABA)-response elements (ABRE, miR172 and miR393), ethylene-responsive element (ERE, miR172) and MYB-binding site involved in drought inducibility (miR167 and miR172), and defence- and stress-responsive elements (TC-rich repeats, miR167, miR172 and miR393), heat-stress-responsive elements (HSE, miR169, miR172 and miR397), light-responsive elements (G-Box, miR172, miR393 and miR397), and auxin-responsive elements AuxRR-core (miR167 and miR169) were present in miRNAs (Supplementary Table S1). When comparing miRNA *cis*-elements, it was found that these *cis*-acting regulatory elements were mainly related to two physiological processes: light cycle and hormonal/environmental responses (Figure 2(b) and Supplementary Table S1). miR172 had 11 *cis*-acting elements related to hormonal/

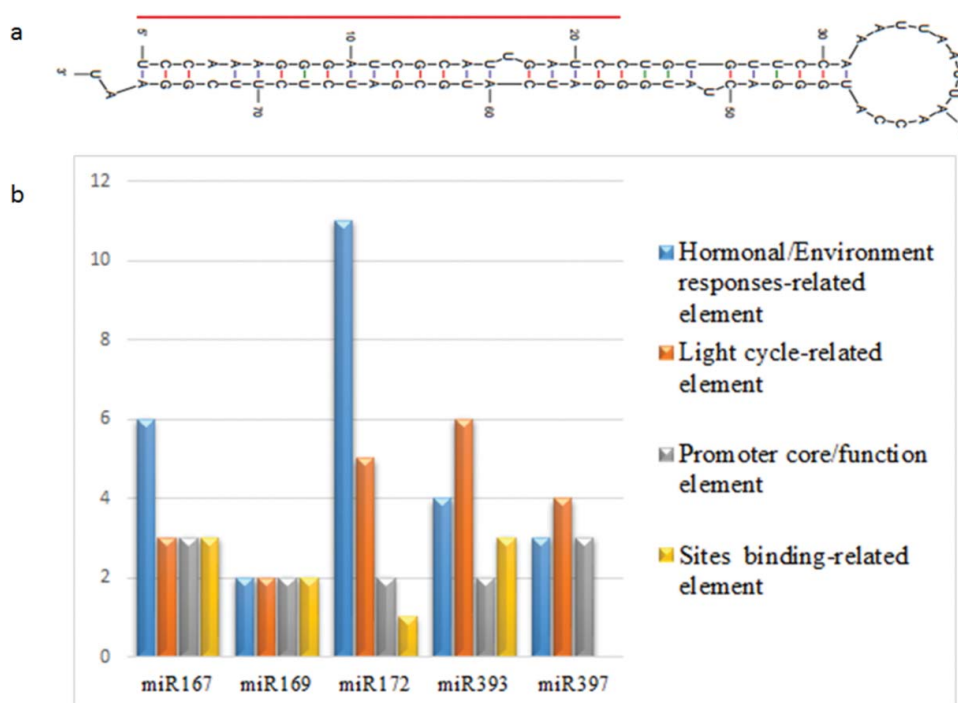


Figure 2. Secondary structure of putative pre-miR393 in tomato (a); horizontal red line indicates mature sequences in the stem region. (b) Graphical representation of *cis*-acting elements in promoter region.

environmental responses, while there were two *cis*-acting elements in miR169. On the other hand, miR393 had six *cis*-acting elements related to light cycle, whereas miR169 had two *cis*-acting elements. In the analysis, it the least number of *cis*-acting elements were found in miR397, which coincided with the low level of expression. In addition, some other *cis*-acting elements were also found, such as those possibly involved in regulation in response to methyl jasmonate, gibberellic acid, ethylene and salicylic acid. The transcription levels of miR172 and miR167 were higher than those of other miRNAs. Presumably, this could be related to higher number of hormonal/environmental responsive *cis*-acting elements contained in them.

Predicted target genes

We also identified potential target genes. Currently, bioinformatics tools facilitate revealing of target sequences based on the high degree of homology between miRNAs and target genes.[48] Most target genes are highly conserved across plants. The targets found in the analysis are shown in Table 2. Moreover, we investigated the target genes from which they had been previously identified in the literature. *AFR6* targeted by miR167 [49] and *TIR* targeted by miR393 [31,50] were included in target gene expression analysis.

The inferred expression of homeobox–leucine zipper protein 22 (*HD–ZIP III*; SGN-U216828) and

annexin (SGN-U214256) targeted by miR167 were more induced in cold-sensitive (LA4024) than in cold-tolerant tomato cultivars (LA1777 and LA3969) under cold treatment (Table 2). Similarly, *HD–ZIP III* expression was increased in cold-stored potato tubers.[35] SGN-U214038 and SGN-U214039 encoding multi-drug resistance protein mtdK showed higher FC in cold-tolerant cultivar LA1777 (4.9 and 5.5 FC, respectively). However, their expression in cv. LA3969 (1.6 FC) displayed lower pattern than cv. LA1777 but higher than cv. LA4024. SGN-U215435 (receptor-like protein kinase; *RLK*) was highly up-regulated in all cultivars. Similarly, in *Arabidopsis*, *RLK1* was induced by several environmental stresses, such as dehydration, high salt and cold treatment.[51] Expression of *AFR8* (SGN-U240026 and SGN-U227556) targeted by miR167 was repressed in cv. LA1777 and cv. LA4024. On the other hand, they were not significantly changed in cv. LA3969. Interestingly, the LA3969 cultivar showed higher expression profile of *ARF6* (SGN-U230670) than others (1.7 FC). Following this observation, *ARF6* transcript accumulation was decreased in LA1777, but it was not changed in LA4024. Although SGN-U214629 (ATP synthase I–like protein) was slightly up-regulated in cold-tolerant cultivars, it displayed constant expression level in the cold-sensitive cultivar. These findings suggest that gene expression profiles of cultivars may become differentiated in response to cold stress depending on the level of tolerance.

Table 2. Putative miRNA target genes, expectation value and expression level of three tomato genotypes.

miRNA	Target gene ID	Description	Expectation	Fold change of gene expression		
				LA1777	LA3969	LA4024
miR167	SGN-U216828	Homeobox-leucine zipper protein 22	3	1.51*	1.85*	2.20*
	SGN-U214256	Annexin	3	1.60*	1.50*	1.93*
	SGN-U214039	Multi-drug resistance protein mdtK	2.5	5.50*	1.60*	1.03
	SGN-U214038	Multi-drug resistance protein mdtK	2.5	4.90*	1.64*	0.90
	SGN-U215435	Receptor-like protein kinase	3	5.70*	4.58*	5.0*
	SGN-U240026	Auxin response factor 8	3	0.77*	1.10	0.69*
	SGN-U227556	Auxin response factor 8	3	0.41*	1.24	0.61*
	SGN-U230670	Auxin response factor 6**		0.70*	1.67*	1.03
	SGN-U214629	ATP synthase I-like protein	3	1.34*	1.24	0.92
miR169	SGN-U227057	Nuclear transcription factor Y subunit A-3	2.5	0.26*	0.55*	0.22*
	SGN-U241720	Nuclear transcription factor Y subunit A-3	2.5	0.54*	0.40*	0.25*
	SGN-U217121	Multi-drug resistance protein ABC transporter family	3	5.80*	2.70*	2.46*
miR172	SGN-U242104	AP2-like ethylene-responsive transcription factor	0.5	0.37*	1.06	1.52*
	SGN-U232455	AP2-like ethylene-responsive transcription factor	1.5	3.24*	1.54*	1.42*
	SGN-U224195	AP2-like ethylene-responsive transcription factor	1.5	0.44*	0.85	0.71*
	SGN-U226730	AP2-like ethylene-responsive transcription factor	1.5	0.71*	0.90	0.69*
	SGN-U218022	AP2-like ethylene-responsive transcription factor	1.5	0.48*	1.68*	1.26*
	SGN-U214382	AP2-like ethylene-responsive transcription factor	1.5	1.03	1.52*	0.99
	SGN-U219598	Serine/threonine kinase receptor	2.5	1.07	2.40*	3.0*
miR393	SGN-U213940	Auxin F-box protein 5	2	0.08*	0.15*	0.09*
	SGN-U223813	Auxin F-box protein 5	2	0.20*	0.20*	0.08*
	SGN-U221001	B3 domain-containing protein	3	0.88	0.99	0.72
	SGN-U231414	U-box domain-containing protein	3	0.74	0.93	0.61*
	SGN-U236908	Transport inhibitor response 1**		0.17*	0.13*	0.10*
miR397	SGN-U216616	Laccase 7	0.5	1.34*	0.96	1.03
	SGN-U216617	Laccase	1	0.69*	0.78	0.68*
	SGN-U226352	Laccase 3	1	0.81	0.87	0.64*
	SGN-U223145	Laccase 5	3	0.41*	0.44*	0.33*
	SGN-U214046	Translocase of chloroplast 34	2	1.34*	0.82	1.13
	SGN-U214047	Translocase of chloroplast 34	2	1.33*	1.10	0.94
	SGN-U224659	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase	3	0.36*	0.30*	0.16*

* $P < 0.05$ was considered to indicate differential expression of genes. **Target genes were included from previous reports.

Moreover, we retrieved two nuclear transcription factors Y subunit A-3 (NF-Y) targeted by miR169, which was repressed in all genotypes. Similarly, miR169 was induced in response to drought stress and led to repression of the expression of three nuclear factor Y subunit genes.[52] Contrary, multi-drug resistance protein ABC transporter was highly induced in all genotypes, especially in the most cold-tolerant tomato cultivar (LA1777; 5.8 FC). Multi-drug resistance-associated proteins (MRPs or ABCs) are involved in cellular detoxification by transporting toxic compounds from cytosol into the vacuole.[53] Considering that, cold resistance of tomato genotypes may likely have similar mechanisms in the way MRPs are regulated.

AP2-like transcription factor clade targeted by miR172 has many members. In the analysis, we found six *AP2*-like transcription factors in tomato as targets of miR172. Expression of *AP2*-like transcription factors differed based on the cultivars. *AP2*-like transcription factors such as SGN-U242104 and SGN-U218022 in cv. LA1777, SGN-U224195 and SGN-U226730 in cv. LA1777 and cv. LA4024 were down-regulated. However, SGN-U232455 in all cultivars, SGN-U218022 in cv. LA3969 and in cv. LA4024, and SGN-U214382 in cv. LA3969 showed over-expression. On the other hand, serine/threonine-protein kinase receptor (SGN-U219598) expression was highly induced in the LA3969 and LA4024 cultivars, but was not changed in cv. LA1777.

TIR and *TIR*-like are involved in the auxin-signalling pathway, and are targeted by miR393.[32,52] SGN-U236908 (*TIR*) was repressed in all three tomato genotypes. The degradation of *TIR* mRNAs may subsequently prevent depression of auxin-signalling pathway.[7] Auxin F-box proteins 5 are another target of miR393. Auxin signalling primarily depends on F-box protein receptors and *Arabidopsis* has four redundant F-box proteins of the *TIR1/AFB2* auxin receptor clade.[54] Therefore, F-box proteins are closely related with *TIR* and *TIR*-like proteins. In addition to down-regulation of *TIR*, F-box protein (SGN-U213940 and SGN-U223813) accumulations were also decreased in all genotypes. As regards to SGN-U221001 (B3 domain-containing protein) and SGN-U231414 (U-box domain-containing protein) targeted by miR393, they showed slight changes in the expression levels.

SGN-U216617 (laccase) targeted by miR397 exhibited low level of expression in cv. LA1777 and cv. LA4024, while SGN-U216616 (laccase 7) was over-expressed in cv. LA1777. On the other hand, SGN-U223145 (laccase 5) was repressed in all cultivars, while SGN-U226352 (laccase 3) was not significantly changed in cold-tolerant cultivars. Translocase of chloroplast 34 (Toc34) (SGN-U214046 and SGN-U214047) expression levels were significantly changed in cold-tolerant cv. LA1777. Toc34 is a member of the translocase of the outer membrane complex, which mediates the initial stage of protein import into chloroplasts.[55] Although studies

on Toc34 are very limited, cold conditions did not seem to affect the function of Toc34. Interestingly, SGN-U224659 (1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase; PIP2) was repressed in all cultivars. Contrary to our finding, heat stress activated PIP2 accumulation in tobacco.[56]

Comparative analysis

Cold stress is one of the most severe abiotic stresses adversely affecting plants yields by restraining sowing time, causing tissue damage, and stunting growth.[28] Tomato is an important vegetable crop grown worldwide and prone to cold stress. Stress-related functions of miRNAs may provide further insight in response to cold stress. However, little is known about the relation between the miRNAs and cold response.[19]

In this study, miR167a, miR169a, miR172a and miR393 were up-regulated in response to initial phase of cold stress in tomato. Liu et al. [19] found that miR167a, miR393a and miR397a were up-regulated in cold-treated *Arabidopsis* based on the RT-PCR analysis. Besides, they identified that miR169 and miR172 were induced as a stress response by microarray analysis. Contrary to our finding, miR169 was not detected in cold-stored potato tubers by deep sequencing and degradome analysis.[36] Meanwhile, miR397 was not induced in tomato under cold treatment. On the other hand, the expression levels of miR172, miR393 and miR397 were 1.88, 1.56 and 2.08 FC, respectively, in *Arabidopsis*.[19] In comparison to tomato, *Arabidopsis* miR397 was induced, whereas tomato miR397 showed nearly constant expression. In regard to miR393, it showed similar expression in both plants.

The expression of *ARF8*, targeted by miR167, was higher in the cold-tolerant LA3969 cultivar, while it was at a low level in other cultivars. Although *ARF6*, targeted by miR167, was induced in cv. LA3969, it did not show significant expression level in cv. LA1777 and cv. LA4024. Therefore, up- and/or down-regulation of miR167 might play a potential role in cold resistance by affecting auxin-signalling pathways.[28] Also, miR393 targets *TIR1* and other closely related F-box proteins in *Arabidopsis*.[12] These proteins are auxin receptors that target repressors of *ARF*.[31] The different expression profiles of *ARF6* and *ARF8*, and down regulation of *TIR1* and F-box protein in cold-tolerant tomato cultivars suggested that auxin perception and signalling may change in stress conditions based on tolerance. In addition to miR167, there could be a key regulatory role of miR393 in the auxin-signalling pathway under cold stress in tomato.

NF-Y is post-transcriptionally regulated by miR169.[57] NF-Y is also known as heme-activated protein or CCAAT-binding factor. Moreover, CCAAT box is present in about 25% of eukaryotic gene promoters.[57,58] Over-expression of *NFYB1* in *Arabidopsis* and maize

conferred significantly increased drought resistance.[59] In drought stress, expression of NF-Y genes appears to be decreased in wheat leaves.[60] Similarly, NF-Y expression was down-regulated in response to cold stress in tomato genotypes. This result demonstrated that understanding the biological roles of NF-Y may have potential applications in enhancing stress tolerance in plants. Furthermore, laccase has been proposed as the target of miR397.[8,61] Expression analysis showed that accumulation of four laccase transcripts was differentiated in all cultivars. Laccase is involved in lignin metabolism and it has been reported to be induced under cold conditions in winter wheat.[8] However, it is not clear what the role of laccase is in response to cold.[62]

Stem–loop qRT–PCR analysis showed that miR172 was quickly activated by cold stress. Previous reports on miR172 and *AP2* showed that miR172 act on both inhibition of the translation of *AP2* and degradation of *AP2* transcript.[4] An important role of *AP2* is related to the flowering time and phase transition.[14] The expression profile of *AP2*-like fell within three categories in the analysis: up-regulated, down-regulated or slightly changed in the three tomato genotypes, which suggested the obscure behaviour of this transcription factor. *AP2* transcript has been observed to be significantly reduced in tomato leaves agroinfected with tomato leaf curl New Delhi virus as miR172 transcript increased.[63] On the other hand, *AP2* expression showed similar expression profile under cold and control conditions in wheat.[31] These data indicate that the detailed investigation on the role of *AP2* under cold stress in tomato is needed.

Analysis of the promoter elements of miRNAs revealed that they contain various hormone-regulated *cis*-acting elements, such as ABRE, ERE, GARE and methyl responsive elements. miR172 and miR393 had GATA-box, which has been proposed to regulate light response [64,65] and to be related to cold stress.[8,66] In plants, most ABA-responsive genes have the conserved ABREs in their promoters, which are significant *cis*-acting elements for genes responsive to abiotic stress in *Arabidopsis*. [67] ABA plays an important role in the process of cold acclimation. Although miR393 has an ABRE motif, its expression was not as highly induced as miR172 in tomato. Nevertheless, expression of a number of genes induced by both dehydration and cold has revealed broad variation in the timing of their induction and differences in their responsiveness to ABA.[68] Another significant cold-related motif is the LTRE/DRE/C-repeats.[7] Interestingly, this motif could not be found in our miRNAs. CBFs (C-repeat binding factor) can bind many cold-inducible genes, which have C-repeats/DRE regulatory element in promoters, and can activate their expression. [36] It is known that tomato also has a complete CBF cold-response pathway but its CBF regulon differs from

that of *Arabidopsis*, [37] and the CBF cold-response pathway in tomato is not as important as in cold-acclimated plants.[36] At this point, the absence of LTRE/DRE/C-repeats in the promoter of five analysed miRNAs suggested that ABA-dependent signalling pathways and/or ABA-independent pathways may get involved in the regulation of cold tolerance of tomato. Despite the genome sequencing of tomato, low numbers of miRNAs (48) have been deposited from tomato.[69] In this study, we only analysed five miRNAs. It is also considered that the miRNA members in the same miRNA family often share the same *cis*-acting motifs in their promoter regions, suggesting that miRNA members of the same miRNA family may respond to the same biotic/abiotic or phytohormone stimuli.[70]

Conclusions

In this study, we analysed some cold-stress-related miRNAs (miR167, miR169, miR172, miR393 and miR397) in tomato at early time points (0, 1, 4 and 16 h) of cold exposure by stem–loop qRT–PCR. In addition, we retrieved the expression profile of predicted target genes from three tomato genotypes, which were obtained from a public database. Different expression profiles of target genes were observed among cultivars. The result suggested that this differentiation among cultivars may highlight cold tolerance of tomato genotypes. Among the miRNAs assessed, miR167, miR169, miR172 and miR393 were up-regulated in early time points of cold stress in tomato. Moreover, the accumulation of the miR172 transcript was larger than that of others. Thus, it can be proposed that these miRNAs are activated in response to the initial phase of cold exposure in tomato. The level of miR397 was not significantly changed under cold conditions. Analysis of *cis*-elements showed that hormonal/environmental responses regulatory elements were predominant in the tested miRNAs. Furthermore, we proposed putative sly-miR393 with its secondary structure. Expression of predicted target genes showed different expression profile in three genotypes. Thus, we assumed that cold tolerance of tomato genotypes may affect the expression of target genes. However, further studies such as over-expression and/or RNAi strategies are needed to elucidate the precise role of these genes in tomato.

Disclosure statement

No potential conflict of interest was reported by the authors.

Supplemental data

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