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MicroRNA319 family members play an important role in *Solanum habrochaites* and *S. lycopersicum* responses to chilling and heat stresses

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Abstract

The microRNA319 (miR319) family is involved in plant development and responses to abiotic stresses. Previous work showed that miR319 responded to chilling stress in the chilling-tolerant wild tomato (*Solanum habrochaites* L.) genotype LA1777. Here, the precursors of sha-miR319a, b, c, and d were cloned from LA1777 and the putative target genes *tosinte branched/cycloidea/proliferating cell factors* (*TCP3* and *TCP29*) were validated using 5′-RLM-RACE. Expression patterns revealed a negative correlation of sha-miR319 with *TCP3* and *TCP29* in LA1777. Four tomato (*S. lycopersicum*) genotypes with varying sensitivities to chilling and heat stresses were selected to characterize expression patterns of miR319 and target genes under extreme temperatures. The involvement of miR319 in the chilling tolerance of tomato might be mediated by the repression of *TCP3* and *TCP29* expression. Initial stages of heat stress resulted in the up-regulation of miR319a, b, and d and led to a decrease in *TCP3, TCP29*, and *TCP2* expression, whereas the down-regulation of miR319c in the later stages of heat stress may have been responsible for the subsequent up-regulation of *TCP3, TCP29,* and *TCP2*. *Cis*-elements found in the promoter regions of the miR319 family members indicated a potential role of miR319 in the regulation of stress tolerance and development processes. This study provides insights into the role of miR319-mediated regulatory mechanisms in responses to temperature stress in tomato genotypes.

Additional key words: abiotic stresses, target gene expression, *TCPs*.

Introduction

MicroRNAs (miRNAs) are a class of small non-coding, single-stranded RNAs of 19 - 24 nucleotides (nt) in length that are widely distributed in plants and animals (Bartel 2004). In plants, miRNAs are highly complementary to messenger RNAs (target mRNAs) and suppress the expression of target genes through cleavage or translational repression (Dugas and Bartel 2004). Studies from a diversity of plant species have shown that miRNAs play important roles not only in plant growth and development (Palatnik *et al*. 2003, Wang *et al*. 2009, Zhu *et al*. 2009, 2011), but also in stress responses (Sunkar and Zhu 2004, Sunkar *et al*. 2006, Liu *et al*. 2008, Wang *et al*. 2012, Phookaew *et al*. 2014, Huang *et al*. 2018).

One of the most conserved miRNA families in plants is the miR319. Some of the teosinte branched/cycloidea/proliferating cell factors (TCP) family members have been reported to be the target genes of miR319 (Palatnik *et al*. 2003, Nag *et al*. 2009, Wu *et al*. 2017). Various aspects of miR319-regulated *TCPs* have been studied in plant growth and development, including regulation of leaf (Nath *et al*. 2003, Palatnik *et al*. 2003, Koyama *et al*. 2007, Schommer *et al*. 2008, Koyama *et al*. 2010, Xie *et al*. 2017) and flower (Nag *et al*. 2009) morphogenesis, cell proliferation (Sarvepalli and Nath 2011, Schommer *et al*. 2014), secondary cell wall biosynthesis (Sun *et al*. 2017), and phytohormone biosynthesis and signal

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Abbreviations: CBF - C repeat binding factor; miRNA - microRNA; qPCR - quantitative PCR; 5′-RLM-RACE - 5′ RNA ligase-mediated rapid amplification of cDNA-end; TCP - tosinte branched/cycloidea/proliferating cell factors.

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transduction (Schommer *et al.* 2008, Koyama *et al.* 2010). Furthermore, expression of miR319-mediated target genes has been associated with responses to abiotic and biotic stresses. For example, *OsmiR319b* impacts leaf morphogenesis and leads to enhanced salinity and drought tolerance by controlling the expression of *PCF5, PCF6, and PCF7*, and *TCP21* (Yang *et al.* 2013, Zhou *et al.* 2013), and miR319 may indirectly affect active oxygen scavenging by targeting *OsPCF6* and *OsTCP21* to regulate plant chilling tolerance (Wang *et al.* 2014). Evidence of miR319a regulation of *GAMYB* genes in response to chilling stress has previously been found in tomato plants (Valiollahi *et al.* 2014), while, heat stress has been shown to cause an increase in the expression of miR319 in radish (Wang *et al.* 2015) and miR319b has been found to negatively regulate the responses of tomato to root-knot nematode (Zhao *et al.* 2015).

Solanum lycopersicum is an economically important crop that suffers damage induced by chilling and heat, however, the *S. habrochaites* has been identified as chilling tolerant (Venema *et al.* 1999, Foolad and Lin 2000, Cao *et al.* 2015). A large number of miRNAs has been identified in tomato (Cao *et al.* 2014, Zhao *et al.* 2015, Omidvar *et al.* 2015, Zhou *et al.* 2016), but mechanisms of expression, function, and regulation under temperature stress conditions are poorly understood. Currently, four members of the miR319 family in *S. lycopersicum* have been identified and listed in *miRBase 21.0* (http://www.mirbase.org/), comprising miR319a, miR319b, miR319c-3p (the primary form of miR319c, referred to as miR319c hereinafter), and miR319c-5p, while miR319d (initially named sha-miR319b_stu) has been found in *S. habrochaites* (Cao *et al.* 2014, Fan *et al.* 2015), and *S. lycopersicum* (Gu *et al.* 2014). However, little is known about other miR319 family members in *S. habrochaites*.

There is increasing evidence of miR319-mediated responses to chilling and heat stresses (Chen *et al.* 2012, Wang *et al.* 2015, Zhou *et al.* 2016), but the expression mechanisms and target gene responses are unclear, as previous studies have tended to focus on the stress responses in just one member of the miR319 family (Zhou *et al.* 2010, 2013, Valiollahi *et al.* 2014). Here, we identified target genes, expression patterns, and upstream promoter regions of members of miR319 in *S. habrochaites* and *S. lycopersicum* under chilling and heat stresses to provide insights into the role of miR319 in the regulation of temperature stress tolerance in tomato.

Materials and methods

Plants and treatments: We used *Solanum habrochaites* L. genotype LA1777 and four *Solanum. lycopersicum* L. genotypes with varying sensitivities to chilling and heat stresses (LA2683, Pole red Siberian, LA3475, and Stupice; Table 1 Suppl.). Following soaking and pre-germination treatments, tomato seeds were sown in plastic plots and raised in growth chambers (*RDN-560E-4*, *Dongnan Instruments*, Ningbo, China) set at day/night temperatures of 25/18 °C, an air humidity of 60 ± 5 %, a 14-h photoperiod, and an irradiance of 280 ± 20 μmol m^{-2} s^{-1}.

Thirty days after sowing, vigorous and uniform seedlings were selected for the temperature stress treatments, where seedlings of LA1777, LA2683, Pole red Siberian, and LA3475 genotypes were exposed to chilling stress (4 °C) and LA1777, Stupice, Pole red Siberian, and LA2683 genotypes were exposed to heat stress (40 °C) in the growth chambers. Three composite samples of second leaves were collected from five seedlings of each genotype after 0, 1, 4, 8, and 24 h of exposure to the chilling and heating treatments. Leaf samples were immediately frozen in liquid nitrogen and stored at -80 °C prior to RNA isolation.

Expression of miR319 and target genes in LA1777: Roots, stems, and second fully expanded leaves were collected from five 30-d-old seedlings, along with three replicate samples of five opened flowers. Samples were frozen in liquid nitrogen and stored at -80 °C prior to RNA isolation.

Total RNA was extracted using *TRIzol* reagent (*Bioteke*, Beijing, China). For cDNA synthesis, 1 μg of total RNA was used for reverse transcription using a *PrimeScript* reverse transcription (RT) reagent kit (*Takara*, Dalian, China) according to the manufacturer's recommendations. Stem-loop RT primers for miR319 (Table 2 Suppl.) were designed according to the criteria described by Tang *et al.* (2006) and were amplified using *SYBR Premix Ex Taq* (*Takara*) in a *Quantstudio3* real-time PCR system (*Applied Biosystems*, Foster City, USA) according to the manufacturer's instructions. There were three replicate PCR reactions for each sample.

Real-time quantitative (q)PCR reactions of miRNAs and target genes were performed with an initial cycle at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and at 60 °C for 30 s. Tomato uracil riched non-coding small nuclear RNA6 (*U6snRNA*) and *Actin* were used as reference genes of miRNAs and mRNAs, respectively. The reactions were repeated three times per sample and expressions were calculated using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001). Data were analyzed using analysis of variance (*ANOVA*) in *SPSS 18.0* and figures were drawn using *Origin7.0*.

Cloning miR319d: Total RNA of LA1777, LA2683, Pole red Siberian, LA3475, and Stupice genotypes were transcribed into cDNA using miR319d stem-loop RT primers (Table 2 Suppl.). PCR was performed using

Premix Taq (*Takara*) according to the manufacturer's recommendations, and PCR products were cloned into pMD19-T vector (*Takara*) prior to sequencing.

Three pairs of primers (sly-pre-aF and sly-pre-aR; sly-319bpre1F and sly-319bpre2R; and, sly-319cpre3F and sly-319cpre1R) were designed based on *miRBase 21.0*, to clone the precursors of miR319a, b, and c (MIR319a, b, and c, respectively) in LA1777 genotype. We searched for the possible precursor of miR319d from the *Tomato Genome SL3.0* (https://www.solgenomics.net/) using *Blast* tools; about 200 nt extended sequences were selected for secondary structures and analyzed using *RNAfold* software (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). Only sequences with a stable hairpin structure were considered as precursors of miR319d (MIR319d). Forward (sha-319-3-F) and reverse (sha-319-3-R) primers were designed based on 200 bp up- and down-stream of 5′ and 3′ sequence of MIR319d position, respectively.

Verification of target genes: To verify the target genes, a modified 5′ RNA ligase-mediated RACE (5′-RLM-RACE) assay was carried out according to the *FirstChoice RLM-RACE* procedure (*Ambion*, CA, USA). The nested PCR products were cloned into *pEASY Blunt Simple* cloning vector (*Trans*, Beijing, China) and sequenced to validate the cleavage sites and confirm the putative target genes.

Approximately 2500 bp upstream precursor sequences of each member of MIR319 were selected as promoter regions and analysis was carried out using the *PlantCARE* (http://bioinformatics.psb.ugent.be/webtools/plantcare/html).

Results

A sequence comparison showed mismatches between sha-miR319d and the known members of sly-miR319 (Fig. 1*A*). We isolated, characterized, and cloned miR319d from *S. habrochaites* LA1777 and *S. lycopersicum* LA2683, Pole red Siberian, LA3475, and Stupice genotypes (Fig. 1*B* and Fig. 1*A* Suppl.).

To investigate all members of miR319 in *S. habrochaites*, we cloned and sequenced 169, 292, and 195 bp DNA fragments, where similarity between sly-MIR319a, sly-MIR319b, and sly-MIR319c was 95.37, 98.36, and 100.00 %, respectively (Fig. 1*C-E*). Therefore, we named the obtained sequences sha-MIR319a, sha-MIR319b, and sha-MIR319c (*GenBank* accession numbers MH230178, MH230179, and MH230180), respectively. The sequence of miR319d mapped to the tomato genome, where the extended sequence SL3.0ch03 (69596746-69596918) formed a stable hairpin structure, amplified sha-MIR319d (*GenBank* accession number MH230181) using the LA1777 DNA template (174 bp in length) and shared 98.21 and 95.43 % similarity to stu-MIR319b and sly-MIR319d, respectively (Fig. *1F*). There was a stable hairpin structure in sha-MIR319a, b, c, and d (Fig. 1*B-E* Suppl.) and the minimum free energy (MFE) was -348.53, -479.49, -338.49, and -363.17 kJ mol^{-1}, respectively. Minimal folding free energy index (MFEI) is an important feature for miRNA identification (Bonnet *et al*. 2004) and it was 1.01, 1.07, 0.87, and 1.06, respectively. MFE of sly-MIR319d was -353.97 kJ mol^{-1} and MFEI was 1.03 (Fig. 1*F* Suppl.).

Several putative miR319 target genes were predicted using high-throughput degradome sequencing (Cao *et al.* 2014) and *psRNATarget* web server (http://plantgrn.noble.org/psRNATarget/) (Dai and Zhao 2011) (Table 3 Suppl.), and we validated the cleavage sites of target genes *TCP3* (*Solyc12g014140.1.1*, International Tomato Annotation, *ITAG v. 2.40*) and *TCP29* (*Solyc08g048370.2.1*). Further cloning and sequencing showed the cleavage site was predominantly located between the tenth and eleventh C-nucleotides, and there was correspondence between sequences of miR319 members (Fig. 2). There were two mismatches between corresponding sites of *TCP3* and miR319a, miR319c, and miR319d, and between corresponding sites of *TCP29* and miR319c and miR319d. There was greater mismatch between *TCP3* and miR319b, *TCP29* and miR319b, and *TCP29* and miR319a. The specific bands of *TCP3* and *TCP29* were detected at 280 and 230 bp (Fig. 2 Suppl.).

We found that expression of miR319 tended to be greatest in stems of LA1777 and lowest in its leaves. Expression of miR319a was consistently greater than that of the other members in roots, stems, and flowers, and expression of miR319b-d was consistently low in roots, leaves, and flowers (Fig. 3*A*). The lowest expressions of *TCP3* and *TCP29* were in the stems and highest in the flowers, where expression was negatively correlated with that of miR319. There was some variation in pattern of target gene expression, where expression of *TCP3* was higher in leaves than in roots, but that of *TCP29* was higher in roots (Fig. 3*B*).

To investigate the role of miR319 in response to chilling, tomato genotypes with varying sensitivities to chilling (Fig. 4*A-D*) were used to explore expressions of the members of miR319 (Fig. 4*E-H*). After a 24-h treatment, expressions of all four members were lower in the LA1777 genotype than in the three cultivated tomato genotypes, including the chilling tolerant LA2683, and expressions of miR319c and d were higher after 24 h than those of miR319a and b. In contrast, we found that transcription of miR319b was higher after 24 h than those of all other members in each of the three cultivated genotypes (Fig. 4*F-H*). There were temporal differences

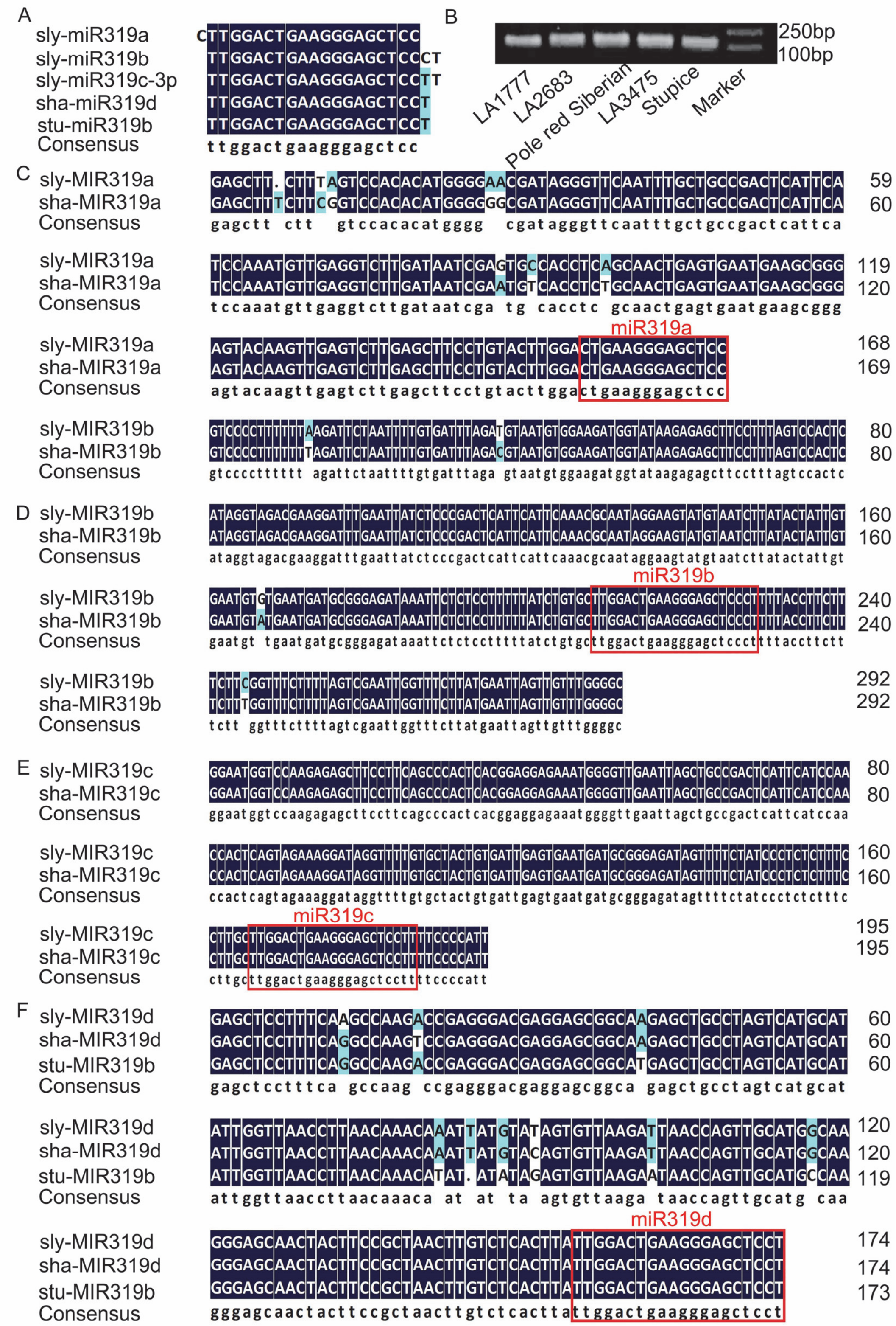

Fig. 1. Cloning and sequence analysis of miR319d and sha-MIR319. *A* - The sequence similarity alignment between sly-miR319, sha-miR319d and *S. tuberosum* stu-miR319b. *B* - Electrophoresis of stem-loop reverse transcription PCR products of miR319d in different tomato genotypes. *C* - Sequence comparison of sha-MIR319a and sly-MIR319a. *D* - Sequence comparison of sha-MIR319b and sly-MIR319b. *E* - Sequence comparison of sha-MIR319c and sly-MIR319c. *F* - Sequence comparison of sha-MIR319d, sly-MIR319d (tomato genome SL3.0ch03:69596746-69596918) and stu-MIR319b. The *dark blue colour* indicates the same bases and the *light blue colour* indicates different bases, respectively. *Red rectangle* indicates mature miR319 regions.

in rates of expression of the members of miR319 among the cultivated genotypes of varied cold tolerance, where miR319b increased more rapidly and to a higher level in the chilling-tolerant LA2683, whereas miR319a expressions was higher in the chilling sensitive genotype LA3475 than in the more chilling tolerant genotypes within 8 h of treatment (Fig. 4*F-H*).

Expressions of *TCP3* and *TCP29* were lower after chilling in all four tomato genotypes (Fig. 4*I-L*). *TCP2* expression in the wild genotype LA1777 was inhibited during chilling (Fig. 4*I*), whereas in the cultivated genotypes LA2683, Pole red Siberian, and LA3475, there was a temporal increase (Fig. 4*G-L*).

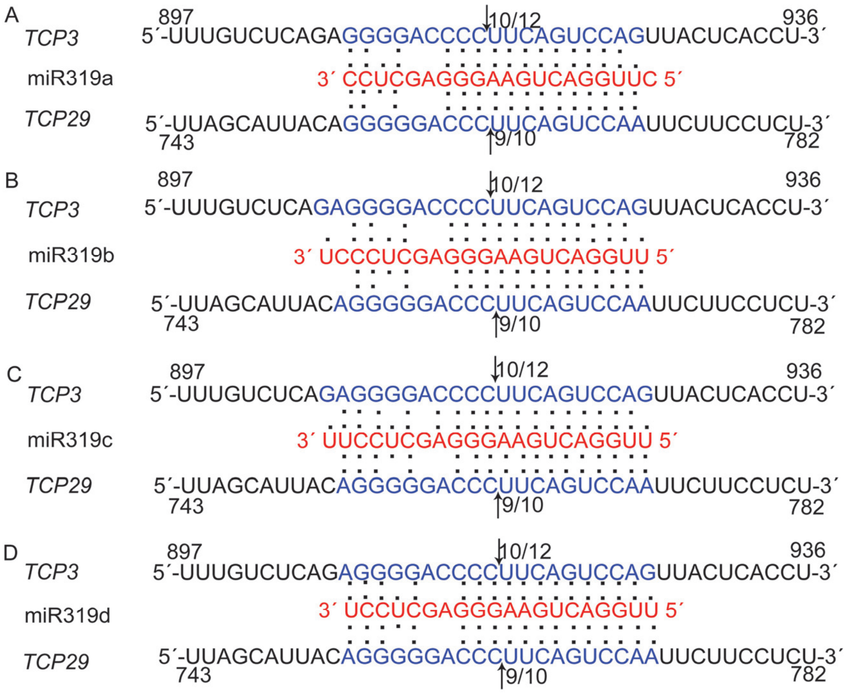

Fig. 2. The miR319 guided cleavage and the target sites validated by 5′ RACE analysis. *A* - Corresponding sites of *TCP3* and *TCP29* to miR319a. *B* - Corresponding sites of *TCP3* and *TCP29* to miR319b. *C* - Corresponding sites of *TCP* and *TCP29* to miR319c. *D* - The corresponding sites of *TCP* and, *TCP29* to miR319d. The numbers indicate the fraction of cloned PCR products terminating at the position.

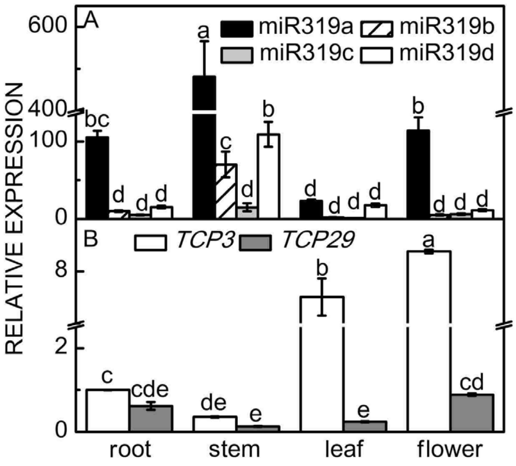

Fig. 3. The qPCR analysis of the expressions of miR319 (*A*) and target genes (*B*) in LA1777 genotype. The reference gene for miRNAs was *U6snRNA* and for target genes was *Actin*. Means ± SEs, *n* = 3. Different letters indicated significant difference ($P \leq 0.05$).

We found visible effects of heat stress in all four genotypes, albeit reduced in the heat-tolerant Stupice (Fig. 5*A-D*) and these were reflected in expressions of miR319 (Fig. 5*E-H*). Expressions of miR319c decreased from 1 to 8 h in response to heat in all genotypes (Fig. 5*E-H*).

Target gene expressions were consistently lower in LA1777 than in the cultivated genotypes following heat treatment (Fig. 5*I-L*) and the expressions of *TCP3* and *TCP29* were inhibited following heat treatment in the wild genotype (Fig. 5*I*). Heat triggered a great decrease in the expression of *TCP3* within 1 h of treatment in the four genotypes (Fig. 5*I-L*). We also detected a large decrease in the expression of *TCP29* within 1 h of treatment in the cultivated genotypes, except in the heat-sensitive LA2683 (Fig. 5*J-L*). Expressions of *TCP2* initially decreased and then peaked after 8 h of exposure to heat (Fig. 5*I-L*). We analyzed the temporal expression patterns of miR319 members and target genes, and found a negative correlation between expression of miR319a, b, and d and the target genes after the first hour of heat treatment (Fig. 3*A-D* Suppl). There was a negative correlation between increased expressions of miR319a, b, and d and the target genes up to 8 h of treatment in the cultivated genotypes (Fig. 3*E-F* Suppl.). In order to elucidate miR319 family function in a stress response, the 2 500 bp sequence upstream of each member of miR319 was analyzed. A number of *cis*-acting, stress related elements were identified from each of the four members of miR319 where expression of miR319b was associated with the greatest number of elements (Table 1).

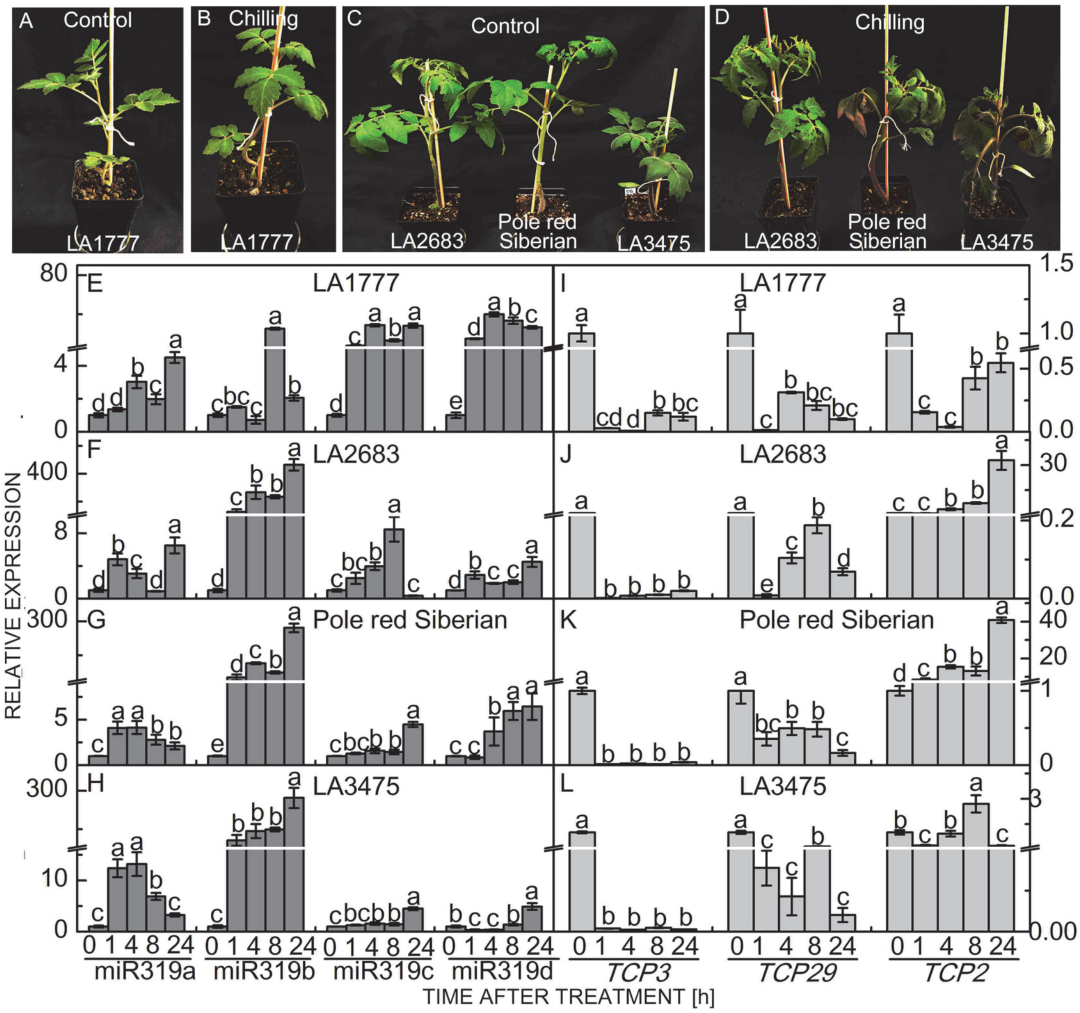

Fig. 4. Different tomato genotypes (*A-D*) and relative expressions of miR319 (*E-H*) and target genes (*I-L*) in these genotypes under chilling stress (4 °C for 0 - 24 h). Reference genes for miRNAs and target genes were *U6snRNA* and *Actin*, respectively. Means ± SEs, *n* = 3. Different letters indicate significant differences ($P \leq 0.05$).

Discussion

We used three key methods to identify miRNAs and, based on the conserved sequence and secondary structures in *S. lycopersicum*, we cloned sha-MIR319a, sha-MIR319b, and sha-MIR319c from *S. habrochaites* genotype LA1777 and used bioinformatics prediction to clone sha-MIR319d. High sensitivity, stem-loop RT PCR has been used previously to identify miRNAs (Varkonyi-Gasic *et al*. 2007, Li *et al*. 2009) and using this technique, we cloned miR319d in the wild genotype *S. habrochaites* and *S. lycopersicum* genotypes to confirm miR319d as a new member of miR319 in cultivated tomato. We found that 5′ fragments of cleaved *TCP3* and *TCP29* enriched the miR319 target members in the tomato genotypes.

We explored expression patterns of miR319 and target genes under chilling and heat stresses with the aim of identifying the mechanism of miR319-regulated temperature stress tolerance in tomato. We found that expression of all four miR319 members increased as soon as 1 h after chilling in the chill-tolerant tomato genotypes LA1777 and LA2683, whereas expression of miR319c and miR319d increased later in moderately chill-tolerant genotype Pole red Siberian and chill-sensitive genotype LA3475. These results indicated a positive correlation between the expression of miR319 members and chilling tolerance in tomato, because more rapidly elevated expression of miR319 led to improvement in tolerance. We found that the increase in miR319d was greater in LA1777, while that of miR319b was greater in the cultivated *S. lycopersicum* genotypes. When we assessed heat stress, we found a similar trend of miR319 expression in the genotypes, where was the up-regulation of miR319a, b, and d and down-regulation of miR319c. This also indicated the variability in response by different members of miR319 to heat stress.

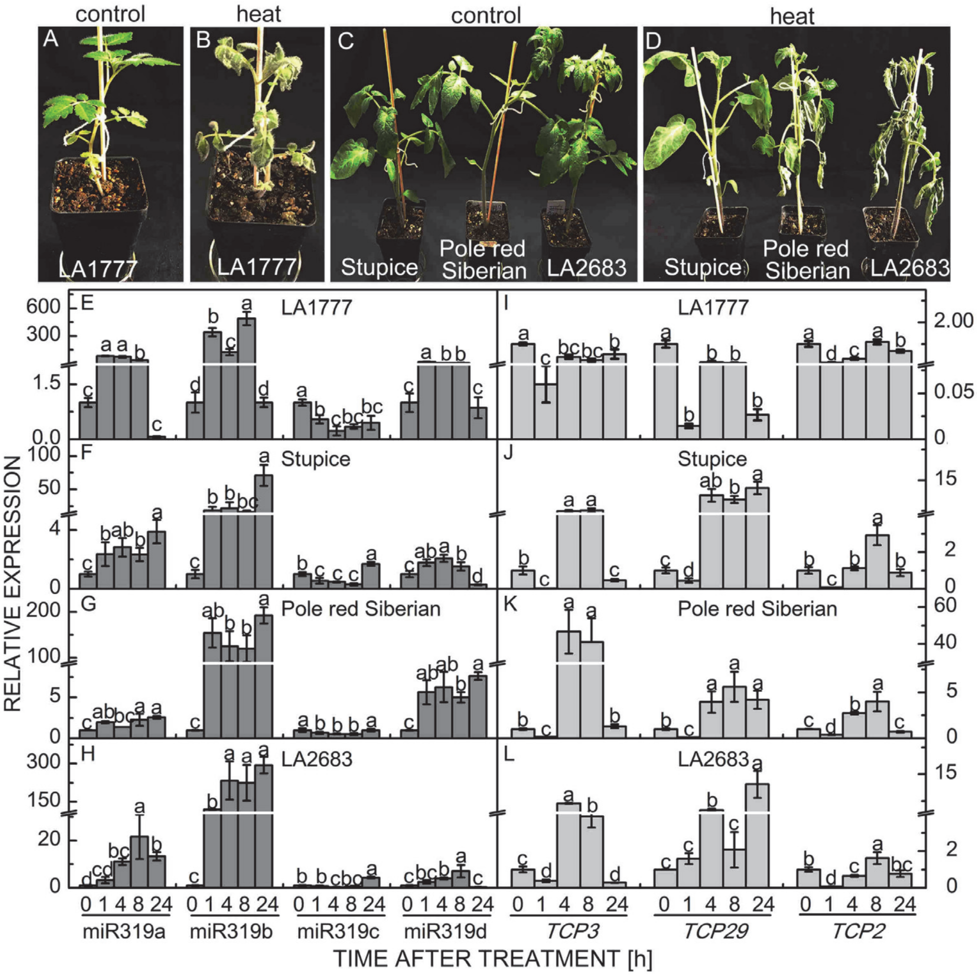

Fig. 5. Different tomato genotypes (*A-D*) and relative expression levels of miR319 (*E-H*) and target genes (*I-L*) in these genotypes under heat stress (40 °C for 1 - 24 h). Reference genes for miRNAs and target genes were *U6snRNA* and *Actin*, respectively. Means ± SEs, $n = 3$. Different letters indicate significant differences ($P \leq 0.05$).

Generally, target mRNA expression is negatively correlated with miRNA expression. However, expression patterns of miRNA and target genes may be complex due to variable environmental conditions, as it was shown for miR408 and its targets by Ma *et al.* (2015). Also, a complementary relation between miR393 and target *AFB1* has not been found in *Arabidopsis* (Chen *et al.* 2011). In this study, the expressions of *TCP3* and *TCP29* were lower under chilling stress, whereas those of *TCP2*, which is a miR319 target in tomato, were not negatively related to miR319. This illustrated that miR319 regulated chilling tolerance through the inhibition of *TCP3* and *TCP29*, but not *TCP2*. For heat stress, where there were negative correlations between miR319a, b, and d and the target genes *TCP3*, *TCP29*, and *TCP2* after 1 h of treatment, and a negative correlation between decrease in miR319c after 4 and 8 h of treatment and of expression of *TCP3*, *TCP29,* and *TCP2.* Thus, there was regulation of *TCP3*, *TCP29*, and *TCP2* by miR319a, b, and d at the initial stage of heat stress, whereas longer term regulation of the target genes was mediated by miR319c.

To further explore the roles of *TCP3* and *TCP29* under chilling and heat stresses, we identified their functional interactions using *STRING* software (https://string-db.org/) (Fig. 4 Suppl.). Calcineurin B-like interacting protein kinases 6 (CIPK6) has been predicted to interact with TCP3 and a recent study demonstrated that tomato CIPK6 regulates the role of Ca^{2+} in reactive oxygen species (ROS) signaling pathways (Gutiérrez-Beltrán *et al*. 2017). ANT2 (involved in anthocyanin synthesis) and chilling and heat responsive GAMYB-like proteins (Wang *et al*. 2012, Valiollahi *et al*. 2014) are predicted to interact with *TCP29*. Anthocyanins may serve as antioxidants and regulate stress resistance (Zhang *et al*. 2010, Meng *et al*. 2014) and *AtTCP3* is involved in anthocyanin synthesis in *Arabidopsis* (Li and Zachgo 2013). In this study, we found MBSI, which is the MYB binding site involved in flavonoid biosynthetic gene regulation, in the miR319a and miR319b promoter regions, suggesting the involvement of miR319 with *TCP3* and *TCP29* in temperature stress regulation as a result of ROS and Ca^{2+} signaling and synthesis of anthocyanins. However, these suggested mechanisms of *TCP3*- and *TCP29*-mediated temperature stress regulation require confirmation.

Promoters contain various functional elements that control the initiation of gene transcription by activating RNA polymerase (Mitsuhara *et al.* 1996). For example, the C-repeat binding factors (CBFs) bind to the C-repeat (CRT) and dehydration-responsive element (DRE) in the promoter of cold responsive genes (*COR*); the plant specific transcription factor WRKY binds to W-box, to regulate responses to cold, heat, salinity, drought, oxidative stress, salicylic acid, abscisic acid (ABA), and ethylene (Jiang *et al*. 2017); and, anaerobic induction elements (AREs) participate in hypoxic, low-temperature, and dehydration stress responses (Dolferus *et al*. 1994, Liu *et al*. 2008). In our study, heat shock response element (HSE) was found in the promoter region of all miR319 members and it may account for the direct induction of miR319a, b, and d, and the inhibition of miR319c by heat stress. We identified other, non-temperature stress response elements (Table 1) that indicated the participation of miR319 in response to chilling stress *via* various signal transduction pathways, while elements related to gibberellic acid, auxin, ethylene and ABA implied a potential regulatory role of miR319 in plant development processes.

Table 1. Types and number of known stress-related elements in the upstream regions of miR319 in *S. lycopersicum.* For abbreviations see text.
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Element	Function	miR319a	miR319b	miR319c	miR319d
ARE	stress	1	1	2	1
MBS	drought stress	1		2	2
HSE	heat stress	3	2	3	2
TC-rich repeats	defense and stress	1	4	1	1
Box-W1	fungal elicitor	1	2	2	1
AuxRR-core	auxin	1			
TGA-element	auxin		1	1	
CGTCA-motif	methyl jasmonate	1	1	1	3
TGACG-motif	methyl jasmonate	1	1	1	3
GARE-motif	gibberellic acid	1			
TATC-box	gibberellic acid			1	
ERE	ethylene	2	8	2	1
ABRE	abscisic acid	1		4	
TCA-element	salicylic acid		1	4	
MBSI	flavonoid biosynthesis regulation	1	1		
W box	WRKY binding site	1	2	1	1

Chilling and heat stress responses in plants are complex processes, involving multiple interactions among genes. We propose a hypothetical model of miR319 regulation of temperature stress in tomato plants (Fig. 5 Suppl.) in which exposure of tomato to chilling induces expression of miR319 that, in turn, inhibits expression of target gene *TCPs*; in contrast, exposure to heat induces expression of miR319a, b, and d, but decreases expression of miR319c that regulates expression of *TCPs*. We suggest that changes in expression of *TCPs* in tomato plants may indirectly affect ROS and Ca^{2+} signaling and anthocyanin synthesis through interactions with CIPK6, GAMYB-like proteins, and ANT2, resulting in enhanced chilling or heat tolerance. Detailed mechanisms of *TCPs* in chilling or heat stress remain to be determined.

In conclusion, we cloned miR319a, b, c, and d from the wild tomato (*S. habrochaites*) genotype LA1777 and found a negative correlation between expression of miR319 family members and *TCP3* and *TCP29* in different plant parts. Up-regulated miR319 family members, which led to repressed *TCP3* and *TCP29*, might promote tolerance to chilling in cultivated tomato genotypes. Expression of miR319a, b, and d increased with heat exposure and inhibited expression of *TCP3, TCP29,* and *TCP2* at the early stage of heat stress, while a negative response of miR319c to heat led to increased expression of *TCP3, TCP29,* and *TCP2* at the later stage of heat stress. The analysis of *cis*-elements in the promoter regions of miR319 members provided further basis for the study of tomato miR319 family members under various abiotic stresses and plant development.

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