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Microarray-based Analysis of Tomato miRNA Regulated by *Botrytis cinerea*

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Abstract *Botrytis cinerea* Pers.:Fr. is an important pathogen in tomato plants that causes stem rot of tomatoes grown indoors for an extended period. MicroRNAs (miRNAs) have recently been reported as a class of gene expression regulators linked to stress responses; however, data on the role of miRNAs in plant responses to biotic stresses are still limited. In this study, three *Botrytis* stress-responsive miRNAs were identified using microarray analysis and the effects of *Botrytis* infection were surveyed in tomatoes. Two downregulated miRNAs and one upregulated miRNA were detected. These stress-responsive miRNAs regulated metabolic, morphological, and physiological adaptations of tomato seedlings at the post-transcriptional level. The presence of stress-related elements in the miRNA promoter regions further supports our results. These findings extend the current view about miRNAs as ubiquitous regulators under stress conditions.

Keywords MicroRNA · Microarray · *Botrytis cinerea* · Gene expression regulators · Tomato

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Introduction

Botrytis cinerea causes gray mold diseases in a broad range of plant species and is one of the most comprehensively studied necrotrophic plant pathogens. Necrotrophs kill their host cells by secreting toxic compounds or lytic enzymes, and they produce an array of pathogenicity factors that can subdue host defenses (van Kan 2006; Choquer and others 2007). To limit the spread of pathogens, host cells generate signaling molecules to initiate defense mechanisms in the surrounding cells. The plant hormones abscisic acid (ABA) and ethylene have been validated to participate in this process (Audenaert and others 2002; Boller 1991; Bleecker and Kende 2000). In spite of extensive research efforts, the biochemical and genetic bases of plant resistance to *Botrytis* is still not fully understood. Recent studies have provided compelling evidence showing that microRNAs (miRNAs) are hypersensitive to abiotic or biotic stress, as well as to diverse physiological processes (Sunkar and Zhu 2004; Liu and others 2005).

miRNAs are approximately 21-nucleotide noncoding RNAs that play several important roles in post-transcriptional gene regulation by degrading target mRNAs in plants. Plant miRNA-guided gene regulation is involved in multiple developmental processes, including organ polarity (Bonnet and others 2004), leaf growth (Chuck and others 2007a), sex determination (Chuck and others 2007b), and male or female sterility (Millar and Gubler 2005). That some plant miRNAs respond to stress conditions and some miRNA targets are stress-related genes suggest that miRNAs play an important role in the stress response of plants (Phillips and others 2007; Covarrubias and Reyes 2010).

miR398, whose expression is transcriptionally downregulated by oxidative stress, is the first miRNA to have

been associated with stress tolerance. Compared with wild-type plants, the decreased *miR398* expression in transgenic lines led to improved tolerance under oxidative stress conditions in *Arabidopsis* (Sunkar and others 2006). In addition, *miR395* and *miR399* were reported to be involved in sulfate and inorganic phosphate starvation responses, respectively (Jones-Rhoades and Bartel 2004; Fujii and others 2005). In rice, *miR169g* was confirmed as the only member induced by drought in the *miR169* family (Zhao and others 2007). Moreover, *miR399* and *miR398* were determined to be involved in bacterial infections (Navarro and others 2006; Jagadeeswaran and others 2009). Thus far, however, the expression profiles of miRNAs under fungal stress conditions have not been reported. Efforts to identify fungus-responsive miRNAs and determine their expression patterns could certainly improve our understanding of their functions in stress adaptation.

Microarray technology has been applied in the high-throughput detection of gene expression and proven to be a useful tool in miRNA expression assays (Liu and others 2008). In the current study, the effects of 173 probes in tomato leaves under *Botrytis*-stress conditions were analyzed using an miRNA microarray representing all plant miRNAs in the miRBase Registry (version 9.1) (Griffiths-Jones and others 2006). Five miRNAs from three miRNA families were found to be involved in stress response. The results were further confirmed by detecting their expression patterns and analyzing the *cis*-elements in their promoter sequences.

Materials and Methods

Plants, *Botrytis* Inoculation, and RNA Extraction

Seedlings of *Solanum lycopersicum* were used as host plants and grown in a greenhouse under a 16-h light/8-h dark cycle and at a temperature range of 22–28°C. Six-week-old plants were inoculated using a suspension medium containing *Botrytis* conidia (2×10^6 spores/ml), 5 mM glucose, and 2.5 mM KH_2PO_4. The inoculation suspension was applied on both leaf surfaces using a soft brush. After inoculation, the plants were kept at 100% relative humidity to ensure spore germination. The *Botrytis* spores appeared in the leaves 7 dpi (days postinoculation). Therefore, pools of leaves were harvested from more than one plant at 0, 1, 3, 5, and 7 dpi, respectively. Samples were used in the analysis of the temporal expression patterns of the miRNAs.

Total RNAs were extracted from leaf tissues using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA), followed by RNase-free DNase treatment (Takara, Dalian, China). The RNA concentrations were quantified by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

miRNA Microarray Assay

Total RNAs isolated from the 7-dpi leaves of *Botrytis*-inoculated and mock-inoculated plant leaves were sent to CapitalBio Corporation (Beijing, China) for noncoding RNA microarray analysis. The purification of miRNA samples, labeling, and hybridization analysis were executed following the manufacturer's instructions. Briefly, 60 μg of total RNAs was used to extract small-sized RNA with an miRNA isolation kit (Ambion, Austin, TX, USA). Fluorescein-labeled miRNAs were hybridized to each noncoding RNA microarray slide, which contained probes that were complementary to 173 nonredundant plant miRNAs registered in miRBase 9.1 (Griffiths-Jones and others 2006). Each probe was spotted in triplicate in each slide and every sample was assayed in duplicate. The data were extracted using LuxScan (CapitalBio), and the differential miRNAs were selected using SAM (Significance Analysis of Microarrays ver. 3.0).

Validation of Mature miRNA Expression Profile Via Stem–loop Reverse Transcription (RT)-PCR

The expression profiles of three *Botrytis*-responsive mature miRNAs were assayed by stem–loop RT-PCR. The stem–loop RT primers were designed following the methods described by Chen and others (2005) and Varkonyi-Gasic and others (2007). Five hundred nanograms of total RNAs was used for initiating the RT of the mature miRNAs. The RT product was amplified using a miRNA-specific forward primer and a universal reverse primer. The stem–loop RT reactions were performed using M-MLV Reverse Transcriptase (Takara, Japan) according to the supplier's protocol. Primers were then added to perform PCR. U6, one of the uniformly expressed small RNAs, was used as the inner control for stem–loop RT-PCR.

RT-PCR was performed to investigate the expression of target genes as follows: Total RNAs were isolated from 3 g of fresh tomato leaves from more than one individual plant, and oligo$(dT)_{15}$ was then used in the primer for cDNA synthesis with reverse transcriptase. One microliter of cDNA was used to amplify the target genes and inner control with the gene-specific primers. The reactions were amplified for 3 min at 94°C, followed by 23 cycles of 94°C for 25 s, 56°C for 25 s, and 72°C for 30 s. The sequences of stem–loop RT primers and all specific PCR primers are given in Table 1.

Table 1 Primers used in this study for RT-PCR assay

Target	Primer	Sequence
miRNA		
Sly-miR160	RT	GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACCGGCAT
	Forward primer	CCTTGCCTGGCTCCCTGT
	Reverse primer	GTGCAGGGTCCGAGGT
Sly-miR171a	RT	GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACGATATT
	Forward primer	GGGTGATTGAGCCGTGCC
	Reverse primer	GTGCAGGGTCCGAGGT
Sly-miR169	RT	GTCGTATCCAGTGCAGGGTCCGAGGTA TTCGCACTGGATACGACTCGGCA
	Forward primer	GGGCAGCCAAGGATGACT
	Reverse primer	GTGCAGGGTCCGAGGT
U6	RT	GTCGTATCCAGTGCAGGGTCCGAGGTA TTCGCACTGGATACGACAAAATATGGAAC
	Forward primer	TGCGGGTGCTCGCTTCGGCAGC
	Reverse primer	GGGCAGCCAAGGATGACT
mRNA		
SCL6-like	Forward primer	CACCGCAGGCGAAACGACAC
	Reverse primer	AACCCATCCAAGACCTCAAGCAG
ARF17-like	Forward primer	ATCCCACAGTTTCTTCCTCCA
	Reverse primer	AGCTTTGCCTTGATTTCTTCA
NF-YA5	Forward primer	CACCTGTATGACATATTCTGTGTGGAG
	Reverse primer	TGCAAATTGGGTATTGGCTATG
Actin	Forward primer	ACCGAAGCCCCTCTTAACCC
	Reverse primer	GTATGGCTGACACCATCACC

Quantitative Analysis of the Mature miRNA Expression Profile

SYBR® Green PCR was performed as per the manufacturer's instructions (Takara, Japan). Briefly, 2 µl of cDNA template was added to 12.5 µl of 2× SYBR Green PCR master mix (Takara), 1 µM concentration of each primer, and ddH_2O to a final volume of 25 µl. The reactions were amplified for 10 s at 95°C, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. All reactions were performed in triplicate, and the controls (no template and no RT) were included for each gene. The threshold cycle (C_T) values were determined automatically by the instrument, and the fold change in each gene was calculated using the following equation: $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_T = (C_{T,target} - C_{T,U6})_{Infection} - (C_{T,target} - C_{T,U6})_{Mock}$.

Analysis of *Cis*-acting Elements in miRNA Promoter

The pre-miRNA sequences retrieved by Moxon and others (2008) were mapped onto the tomato BAC sequences (bacs.v481.seq) obtained from the SOL Genomics Network (ftp://ftp.sgn.cornell.edu/tomato_genome/bacs) using ClustalW. A fragment of the 2,500-bp upstream genomic sequence of the pre-miRNA was used for predicting the *cis*-elements using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/; Lescot and others 2002). The −75 CAAT-box and −30 TATA-box were recognized as the milestones to confirm the transcription start site (TSS). The 1,500-bp DNA sequence upstream of the TSS was truncated to identify *cis*-acting motifs via PlantCARE.

Results

Identification of Different miRNA Expressions

miRNA microarray analysis of the samples from the 7-dpi leaves of *Botrytis*-inoculated and mock-inoculated plant leaves was performed to investigate the potential involvement of miRNAs in *Botrytis*-infected tomatoes. SAM and the criteria of fold change greater than 2 and q less than 0.001 were used to examine the effects of *Botrytis* treatment. Three miRNAs on the microarray showed differential expression

profiles in response to *Botrytis* stress (Table 2; Supplementary Table 1). According to the retrieved precursor sequences of the three miRNAs (Moxon 2008), only two sequences were retrieved as the precursors of *miR169* and *miR160*. However, three sequences were found in the tomato BACs as the precursors of *miR171a*. Of these, only one miRNA, s*ly-miR169*, was upregulated near the twofold in *Botrytis*-infected leaves. The other two miRNAs, namely, *sly-miR160* and *sly-miR171a*, were downregulated.

Expression Profiles of Stress-inducible miRNAs During Stress Treatment

Semiquantitative RT-PCR analysis was performed at five time points in mock and infected leaves to determine the differential expression profiles of miRNAs. The results are consistent with the microarray data (Fig. 1). The differentially expressed miRNAs were classified into two groups, according to their expression profiles, between the mock treatment and the experimental treatment. Only *sly-miR169* was detected to be slowly upregulated at all five time points in the infected leaves. *sly-miR160* and *sly-miR171a* showed rapid downregulation in 1-dpi leaves (Fig. 1). These results were also validated by RT-quantitative PCR (qPCR) analysis. Furthermore, similar to the microarray data, the RT-qPCR findings showed that the three differentially expressed miRNAs changed by twofold in the 7-dpi leaves. However, *sly-miR160* and *sly-miR171a* showed rapid downregulation by approximately sevenfold and approximately sixfold in the 1-dpi leaves, respectively (Fig. 2).

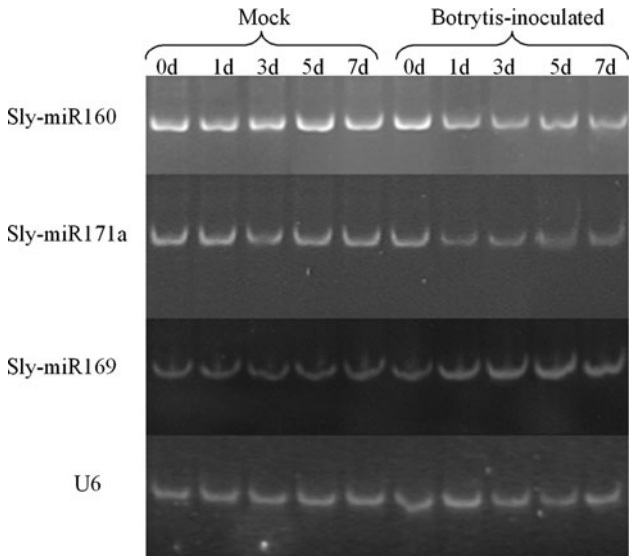

Fig. 1 Time course of altered expression of selected miRNAs by RT-PCR analysis. The expression profile of *Sly-miR160*, *Sly-miR171a*, and *Sly-miR169* in *Botrytis*-infected and mock leaves at 0, 1, 3, 5, and 7 days. Small RNA (U6) was used as the inner control

Therefore, the expression profiles of these three miRNAs in the 1-dpi leaves were further analyzed 0, 3, 6, 9, and 12 h after inoculation. As shown in Fig. 3, the expressions of *sly-miR160* and *sly-miR171a* were gradually downregulated within 12 h after inoculation, whereas *sly-miR169* was gradually upregulated. These results strongly suggest that *sly-miR169*, *sly-miR160*, and *sly-miR171a* contribute to the *Botrytis* stress response of tomatoes.

Table 2 *Botrytis*-response miRNAs identified by microarray analysis

miR family	Members of identified miRNAs	miR family members	Fold change
miR160	ath-miR160a/b/c	Sly-miR160a	−2.33
	osa-miR160a/b/c/d/e/f		
	ptc-miR160a/b/c/d/e/f/g		
	sbi-miR160a/b/c/d/e		
	zma-miR160a/b/c/d/e/f		
	gma-miR160		
miR171	ath-miR171b/c	Sly-miR171a/b	−2.03
	osa-miR171b/c/d/e/f		
	ptc-miR171a/b/e/f/g/h/i		
	mtr-miR171		
	sbi-miR171a/b/d		
	zma-miR171b/d/e/f/i/j		
miR169	ath-miR169a–m	sly-miR169c	1.93
	osa-miR169a–o		
	ptc-miR69a–x		
	gma-miR169		
	mtr-miR169a/b		
	sbi-miR69a–i		
	zma-miR169a–k		

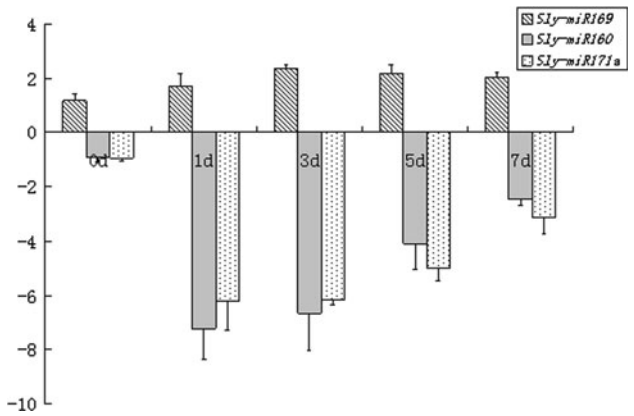

Fig. 2 Quantitative analyses of *Sly-miR160*, *Sly-miR171a*, and *Sly-miR169* through stem–loop real-time RT-PCR at 0, 1, 3, 5, and 7 days using the $2^{-\Delta\Delta Ct}$ method. U6 RNA was used as the inner control. *Y* axis indicated the fold changes of miRNA expression in the stress samples compared with the mock samples. Data were averages of three independent biological experiments. Standard deviation (SD) is shown as *error bar* ($n = 3$)

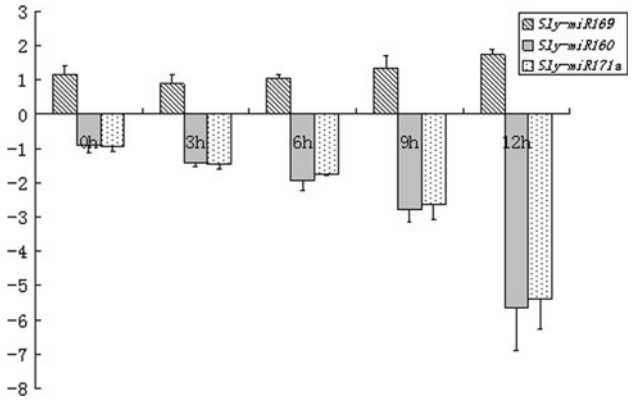

Fig. 3 Quantitative analyses of *Sly-miR160*, *Sly-miR171a*, and *Sly-miR169* through stem–loop real-time RT-PCR at 0, 3, 6, 9, and 12 h using the $2^{-\Delta\Delta Ct}$ method. U6 RNA was used as the inner control. *Y* axis indicated the fold changes of miRNA expression in the stress samples compared with the mock samples. Data were averages of three independent biological experiments. Standard deviation (SD) is shown as *error bar* ($n = 3$)

RT-PCR Analysis of miRNA Targets

Previous research has identified and validated *ARF17-like*, *SCL6-like*, and *NF-YA5* as the target genes of *miR171a*, *miR160*, and *miR169*, respectively (Maity and de Crombrugghe 1998; Mantovani 1999; Moxon and others 2008; Zhao and others 2009). The transcript levels of these target genes were also investigated via RT-PCR 0, 12 h, and 7 days after *Botrytis* infection to systematically analyze the effects of the treatment on the miRNA pathways in tomato plants. Figure 4 illustrates that the expression levels of these target mRNAs changed at different time points. The expressions of *ARF17-like* and *SCL6-like* were most

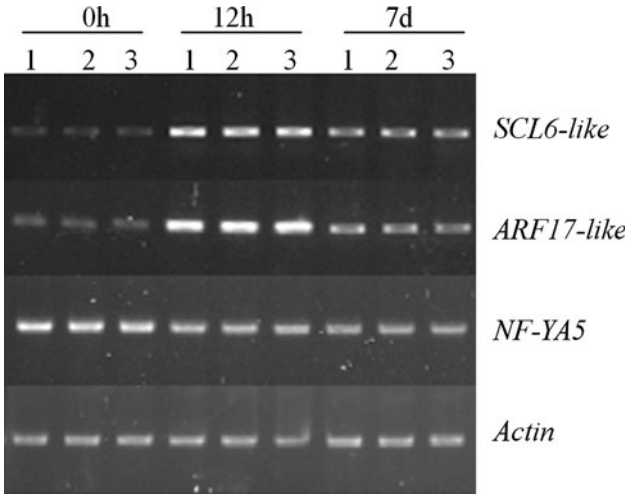

Fig. 4 The expression of SCL6-like, ARF17-like, and NF-YA5 were detected using RT-PCR at 0, 12, and 7 days. Actin was used as the inner control. Biological replicates are indicated with *1*, *2*, and *3*

severely upregulated at the 12-h time point after inoculation but were moderate at 7 dpi. *NF-YA5* showed significant and constitutive downregulation in expression levels at the different detection time points.

Stress-relevant *Cis*-elements in the Promoters of miRNA Genes

Cis- and *trans*-acting elements involved in stress-induced gene expression have been analyzed extensively (Jaglo-Ottosen and others 1998; Kasuga and others 1999; Zhang and others 2005). To identify the *cis*-acting elements in the upstreams of these three miRNAs, a total of six pre-miRNA sequences were first retrieved for the three miRNAs by Moxon and others (2008). Among them, four are the precursors of *miR171a*, and the other two pre-miRNAs are the precursors of *miR169* and *miR160*, respectively. Then the −75 CAAT-box and −30 TATA-box were identified in the 2,500-bp upstream genomic sequences of these six pre-miRNAs as the milestones to confirm the TSS. Finally, the 1,500-bp DNA sequence upstream of the TSS was truncated to identify *cis*-acting motifs via PlantCARE. As shown in Table 3, several known stress-responsive elements were identified in promoters of pre-miRNAs: fungal elicitor (Box-W1), wound-responsive element (WUN-Motif), ABA-response elements (ABREs), auxin response elements (TGA-element), and MYB binding site involved in drought inducibility (MBS), among others.

Reported promoters longer than 1,500 bp of tomato protein-coding genes deposited in GenBank were retrieved and analyzed via PlantCARE. Eighteen promoters were found and the stress response relevance of the genes was investigated. Sixteen were found to be stress-related and

Table 3 Kinds and numbers of known stress-related elements in the upstream regions of miRNA genes and 18 promoters of tomato protein-coding gene

Element	ABRE	ARE	AuxRR-core	Box-W1	CGTCA-motif	ERE	Hse	MBS	TC-rich repeats	TCA-element	TGACG-motif	WUN-motif	Total	References
Responsive	ABA	Anaerobic	Auxin	Fungal	MeJA	EI	Heat	Drought	Defense and stress	Salicylic acid	MeJ	Wound		
Promoter of miRNA gene														
miR171a-1			1	1		2	2			1			7	
miR171a-2		2		1	1	3	0	2	1	2	1		13	
miR171a-3		1			1			1	2	1	1		7	
miR171a-4	3	1	1				1	1	1	2			10	
miR160		3			1				1	3	1		9	
miR169		1		1	1	1	3		1		1	1	10	
Promoter of non-stress-related gene														
ERF1		1					1						2	
hmg2				1		1			1				3	
Promoter of stress-related gene														
Ivi									4	1			5	Ji and others (2005)
AOC	2			1		1		2	1				7	Wu and others (2011)
Lehsp23.8		2			1	1	1	1			1		7	Lu and Shono (1999)
LeftsH6					2			2		1	2		7	Sun and others (2006)
TOMSSF	1	1		2		1	1	1	1				8	Xu and Huang (2008)
Ltp1	1	1		1	1			1	2		1		8	Li and others (2006)
ER24					2		2	2		1	2		9	Zinn and others (2010)
Polygalacturonase		2		2		1	1		2	1			9	Kagan-Zur and others (1995)
JERF3						3	1	1	4		1		10	Wu and others (2008)
Prosystemin	2			1	1		5				1		10	Schmidt and Baldwin (2006)
XSP10	2	1		1	1		1	1	3		1		11	Krasikov and others (2011)
ACS7		1		1	2		2	1	2		2		11	Shiu and others (1998)
Tubulin 8-like	1					1	4	1	1	3			11	Oakley and others (2007)
2-ODD	2	1			1		2	1	4		1		12	Mita and others (2006)
LEA	2	1			2	1	2		2		2		12	Aghaei (2009)
HDC-like	1	1		2	1	1			4	1	1	1	13	Höcker and others (1998)

two were non-stress-related. As seen in Table 3, no more than three stress-responsive elements were found in the two non-stress-related promoters. At least 5 were found in the 16 stress-related promoters. No less than seven stress-responsive elements were found in all promoters of the six pre-miRNAs, indicating that the three miRNAs participate in stress responses.

Discussion

B. cinerea Pers.:Fr. is an important pathogen in tomato plants that causes stem rot of tomatoes grown indoors for a prolonged period. The pathogen gains access through leaf wounds caused by cultivation practices (Verhoeff 1967; Peng and others 1996; O'Neill and others 1997). Many efforts to characterize the resistance mechanism of plants have been carried out (Kuzniak and Skødowska 2004; Asselbergh and others 2007). However, little is known about the relationship between miRNAs and *Botrytis* infection. In this study, of the three differential expressions of miRNAs, *sly-miR160* and *sly-miR171a* were identified as downregulated miRNAs in treatment leaves, and this drop happened mostly within the first 12 h after the inoculation.

Data from previous research showed that stress treatment triggers rapid changes in the transcript levels of plant miRNAs at early stages of stress (Zhao and others 2007; Liu and others 2008; Ding and others 2009). In *Arabidopsis*, some miRNAs (*miR156*, *miR167*, *miR168*, and *miR396*, among others) increased from 2 to 24 h after exposure to high-salinity treatment. *miR167a* levels accumulated after 2 h of drought stress, whereas most miRNA levels were higher after 6 h of inoculation and declined with longer stress during cold-stress treatment (Liu 2008). Moreover, even 0.5 h after stress treatment, the transcript levels of some miRNAs were evidently altered (Zhao and others 2007; Ding and others 2009). In this study, the expressions of the miRNAs were immediately changed within 6 h after the inoculation. Very likely, this rapid change of the miRNAs quickly regulated the correlative functional genes to respond to the environmental stimuli, which was the *Botrytis* stress.

The miR169 family is a large and conservative miRNA family in many plant species. In *Arabidopsis*, there are 14 members in the family and most of them were involved in stress resistance (Li and others 2010). The miR169 family members *miR169b*, *d*, *e*, *f*, *i/j*, and *k/l* were identified as heat-responsive miRNAs, and *miR169a*, *c*, and *e* were found to be cold-induced miRNAs (Li and others 2010). In this study, only one putative member of the miR169 family was identified in the tomato genome by predicting the precursor and promoter of *miR169*. The expression of this *Sly-miR169* was induced in *Botrytis*-infected leaves. Accordingly, as the target gene of *miR169*, the expression of *NF-YA5* was downregulated at the same time. Therefore, we speculated that the upregulation of *Sly-miR169* would downregulate the expression of *NF-YA5*, which might promote the stress tolerance of plants.

The *cis*-element Box-W1 is located in the promoter regions of *miR169* and *miR171a*. It is a member of the W box family [(T)TGAC(C/T)], which is a major class of *cis*-acting elements responsible for pathogen inducibility of many plant genes (Raventós and others 1995; Rushton and others 1996; Wang and others 1998); it is also the binding site for members of the WRKY family of transcription factors (Rushton and others 1996). Moreover, the WRKY transcription factors have been implicated in gene expression in response to wounding (Hara and others 2000). Wound- and pathogen-induced signaling consists of networks with some shared components (Romeis and others 1999). The wound-responsive element WUN-motif is located in the promoter of miR169, but not in the promoters of miR160 and miR171a (Table 3), indicating that the only upregulation of miR169 among these three miRNAs was very likely contributed to these two elements.

Although many stress-related elements were also found in the upstream of the *miR171a* and *miR160*, these two miRNAs were detected as downregulated miRNAs, but not upregulated. The reason is that downregulation is a much more complicated process. This type of regulation cannot be simply answered through stress-related *cis*-element analysis. It is more relevant to suppressive regulators and the coresponsive feedback signals. Stress-related *cis*-elements found in *miR171a* and *miR160* did not stop their downregulation. One reasonable guess is that there is an unknown feedback suppressive regulation pathway. There is a similar case in soybean that although serials of *cis*-elements were found in its promoter, the expression of *LEA* (late embryogenesis abundant) was downregulated in responding to the salt stress anyway (Aghaei and others 2009).

In conclusion, 173 miRNA expression profiles were analyzed in *Botrytis*-infected and mock tomato leaves, of which three were differentially regulated. Data on miRNA expression patterns and the biochemical function of their targets are essential and a valuable contribution to our understanding of the function of these tiny noncoding genes as well as their cooperation in complex biological networks. The results of the RT-qPCR and promoter analysis suggest that *sly-miR169*, *sly-miR160*, and *sly-miR171a* play important roles in the *Botrytis* stress response of plants.

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