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Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*

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ABSTRACT

High-salinity, drought, and low temperature are three common environmental stress factors that seriously influence plant growth and development worldwide. Recently, microRNAs (miRNAs) have emerged as a class of gene expression regulators that have also been linked to stress responses. However, the relationship between miRNA expression and stress responses is just beginning to be explored. Here, we identified 14 stress-inducible miRNAs using microarray data in which the effects of three abiotic stresses were surveyed in *Arabidopsis thaliana*. Among them, 10 high-salinity-, four drought-, and 10 cold-regulated miRNAs were detected, respectively. miR168, miR171, and miR396 responded to all of the stresses. Expression profiling by RT-PCR analysis showed great cross-talk among the high-salinity, drought, and cold stress signaling pathways. The existence of stress-related elements in miRNA promoter regions provided further evidence supporting our results. These findings extend the current view about miRNA as ubiquitous regulators under stress conditions.

Keywords: microRNA; microarray; stress; gene expression regulators; *Arabidopsis thaliana*

INTRODUCTION

MicroRNAs (miRNAs) are a highly conserved class of small noncoding RNAs that regulate gene expression by post-transcriptional degradation or translational repression (Carrington and Ambros 2003; Bartel 2004). With the discovery of large numbers of miRNAs in both plants and animals, the important roles of these special small RNAs have been widely recognized (Llave et al. 2002; Park et al. 2002; Ambros 2004). Many processes, such as leaf development, auxin signaling, phase transition, flowering, and genome maintenance, are regulated in similar ways by different miRNAs (Aukerman and Sakai 2003; Palatnik et al. 2003; Vaucheret et al. 2004; Guo et al. 2005; Mallory et al. 2005).

Recently, there has been strong evidence leading to the proposal that miRNAs are hypersensitive to abiotic or biotic stress as well as to diverse physiological processes (Sunkar and Zhu 2004; Lu et al. 2005). The first report linking miRNA and stress tolerance was miR398, expression of which is transcriptionally down-regulated by oxidative stresses. In *Arabidopsis*, miR398 was found to target two closely related Cu/Zn superoxide dismutase coding genes, cytosolic *CSD1* and chloroplastic *CSD2*, and a reduced level of miR398 led to improved tolerance of transgenic lines compared with the wild-type plants under oxidative stress conditions (Sunkar et al. 2006). Additionally, miR395 and miR399 were identified to be involved in sulfate and inorganic phosphate starvation responses, respectively (Jones-Rhoades and Bartel 2004; Fujii et al. 2005). In rice, miR169g was confirmed as the only member induced by drought among the miR169 family (Zhao et al. 2007). Furthermore, in line with the hypothesis of miRNA's response to environmental stimuli, 21 miRNAs belonging to 11 miRNA families in *Arabidopsis* were predicted to be up-regulated under UV-B stress condition (Zhou et al. 2007). However, systematic expression analysis for miRNAs under abiotic stress conditions in *Arabidopsis* has not been reported. Hence, efforts to identify novel stress-regulated miRNAs and determine their expression patterns could improve our understanding of their functions in stress adaptation.

Currently, a variety of biochemical, molecular, and bioinformatic approaches and technologies have been developed for miRNA analysis and detection (Schena et al. 1995; Eisen

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and Brown 1999). Using tiling path microarray analysis as a tool, it is now possible to perform high-throughput profiling of the expression of all the known miRNAs to examine their expression profiles under different environmental stresses (Garzon et al. 2006; Zhao et al. 2007). In this work, we analyzed the effects of 117 miRNAs under high-salinity, drought, and low-temperature stress conditions with miRNA chips representing nearly all known miRNAs cloned or identified in *Arabidopsis*. Fourteen stress-inducible miRNAs were detected, and our results were further confirmed by detecting their expression patterns and analyzing the *cis*-elements in their promoter sequences.

RESULTS AND DISCUSSION

Arabidopsis miRNA microarray experiments

In order to test whether the expression of any of the currently known miRNAs was regulated by abiotic stresses, we prepared a miRNA microarray containing 117 probes that were complementary to known *Arabidopsis* miRNAs. In addition, eight short oligos possessing no homology with any existing miRNA sequences were designed as negative controls, and a transcriptional repressor Hex as positive control was also included. Total RNA was extracted from the whole plants, and low-molecular-weight miRNAs, which were used for preparation of labeled probes, were obtained. Microarrays were hybridized with Cy3 probe pairs of high-salinity-, drought-, and cold-treated plants and unstressed plants as described in Materials and Methods. The hybridized microarray was scanned by a separate laser channel for Cy3 emissions. The ratio of the treated and untreated plants' fluorescent signal intensities was then measured as a relative measure to determine changes in the differential expression of the oligomer sequence represented by miRNA spots on the microarrays. The quality of the microarray data was assessed, and standard quality-control measures indicated that the microarray used in this analysis showed a similar distribution of intensities. None of the negative control probes gave a detectable signal in any of the samples. The q value calculated from different samples was used as a measure of biological reproducibility, and for each gene, it was the lowest false discovery rate at which that gene was called significant.

Identification of stress-response miRNAs

The miRNA populations from different stress treatment samples were compared to a mock sample. Significance analysis of microarrays (SAM) and a criterion of fold change >1.5 and q value <0.001 were used to examine the effects of various stress treatments. We found that 14 miRNAs (miR156, miR159, miR165, miR167, miR168, miR169, miR171, miR172, miR319, miR393, miR394, miR396, miR397, and miR408) on the microarray showed differential expression profiles in response to abiotic stresses (Table 1). No statistically significantly down-regulated miRNAs were found during this process. Among them, 10 high-salinity-, four drought-, and 10 cold-regulated miRNAs were detected (Fig. 1). miR165, miR319, and miR393 were up-regulated by both high salinity and cold, miR167 was induced by both high salinity and drought, and miR168, miR171, and miR396 were observed to respond remarkably to all three stresses.

Based on the characteristic of their targets, the identified miRNAs could be classified into three major categories (Table 2). The first category includes eight miRNAs—miR156, miR159, miR165, miR169, miR171, miR172, miR319, and miR396—which target transcription factors involved in further regulation of gene expression and signal transduction that probably function in stress responses (Jones-Rhoades and Bartel 2004; Sunkar and Zhu 2004). Thus, it appears that the induction of these miRNAs would lead to the repression of some corresponding transcription factor genes which, in turn, target a set of specific protein coding genes and play defensive roles against stresses. The second category includes miR167, miR168, miR393, and miR394, which are related to direct response to stresses or

TABLE 1. Stress-response miRNAs identified by microarray analysis

Treatment	miRNAs	Fold change
300 mM NaCl	miR396	2.99
	miR168	1.91
	miR167	1.87
	miR165	1.82
	miR319	1.81
	miR159	1.63
	miR394	1.58
	miR156	1.56
	miR393	1.55
	miR171	1.50
	miR158	1.46
	miR169	1.30
200 mM mannitol	miR396	2.59
	miR168	1.91
	miR167	1.84
	miR408	1.73
	miR171	1.55
	miR157	1.42
	miR393	1.36
4°C	miR396	3.95
	miR397	2.08
	miR172	1.88
	miR169	1.79
	miR408	1.73
	miR168	1.71
	miR171	1.60
	miR393	1.56
	miR319	1.54
	mIR165	1.50
	miR400	1.36

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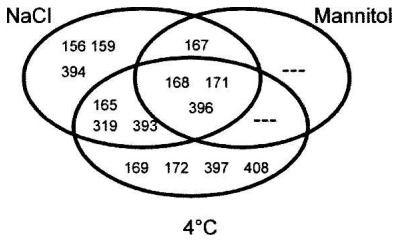

FIGURE 1. Venn diagram illustrates common and unique differential miRNAs expression under three treatment conditions.

external stimuli. miR393 and miR394 target the messages of F-box proteins, which were recently reported to be differentially regulated by stress conditions and play significant roles in the abiotic stress-response pathway (Jones-Rhoades and Bartel 2004; Navarro et al. 2006; Jain et al. 2007). miR168 has been proven to target mRNA of ARGONAUTE 1 (AGO1), which might be the only member of the AGO family that is regulated by a miRNA (Vazquez et al. 2004). The direct role of AGO in plant abiotic stresses has not yet been demonstrated. However, one of its associated partners, Hsp90, is stress-sensitive (Tahbaz et al. 2001; Liu et al. 2005; Leung and Sharp 2007). miR167 targets two auxin response factors, ARF6 and ARF8 (Wu et al. 2006). Plant auxin regulates many agronomically important aspects of plant growth and development as well as responses to environmental stress conditions (Fedoroff 2002; Himmelbach et al. 2003; Achard et al. 2006). Hence, miR167 might play a potential role in stress-resistance progress by affecting auxin signaling pathways. The last category includes miR397 and miR408, whose target genes are hydrolase and oxidoreductase coding genes, which respond to many stresses (Kimura et al. 2003; Apel and Hirt 2004). However, a few of the miRNAs reported to be regulated by high salinity, drought, or cold stress were missed in our results, such as miR389 and miR400 (Sunkar and Zhu 2004). We speculate that this might have been caused by the differences between our treatment methods and theirs or by the technical differences between RNA gel blot and microarray analysis. Some other miRNAs failed to be detected, perhaps because they are required at low levels at this stage or their expression is limited to specific cell types or particular growth conditions, which make it difficult to identify the changes under stress conditions.

In addition, most miRNAs were proven to be involved in the regulation of important developmental processes as shown in Table 2. Stressed plants often resemble abnormal developmental phenotypes, which can be viewed as entering a particular developmental phase (Cooper et al. 2003; Sunkar et al. 2007). Furthermore, there is evidence that many of the miRNA target genes are involved in both development and stress regulation, so we speculate that these miRNAs might be co-regulated by both environmental factors and developmental cues.

Expression profiles of stress-inducible miRNAs during stress treatments

In microarray data, we found that miRNAs belonging to the same family have similar expression patterns, suggesting that they could not be differentiated in microarray analysis because of a potential cross-hybridization problem. To determine which locus is responsive to stress conditions, we performed semiquantitative RT-PCR analysis using specific primers designed to amplify fold-back precursor transcripts in *Arabidopsis*. Two-week-old wild-type *Arabidopsis* seedlings were subjected to stress treatments of

TABLE 2. Putative target genes of stress-inducible miRNAs and their function annotations

miRNAs	Targets	Function description
miR156	SBP family of transcription factors	Vegetative phase change; root development
miR159	MYB and TCP transcription factors	SD flowering time; anther development leaf
miR165	Class III HD-ZIP transcription factors	Development
miR167	ARF6 and ARF8	Gynoecium and stamen development
miR168	ARGONAUTE1	Plant development
miR169	CBF	—
miR171	SCL transcription factors	Floral development
miR172	AP2 transcription factors	Flowering time; floral organ identity
miR319	TCP transcription factors	Morphogenesis of shoot lateral organs
miR393	F-box protein; bHLH transcription factors	Bacterial disease resistance
miR394	F-box protein	—
miR396	GRL transcription factors; Rhodenase-like protein; Kinesin-like protein B	—
miR397	Laccases; β-6 tubulin	—
miR408	Peptide chain release factor; plantacyanin	—

SBP, squamosa promoter binding protein; SD, short day; TCP, TEOSINTE BRANCHED1, CYCLOIDEA, and PCF; HD-ZIP, class III homeodomain-leucine zipper; ARF, auxin response factors; CBF, CAAT binding factor; SCL, scarecrow-like; AP2, APETALA2; bHLH, basic helix–loop–helix; GRL, growth regulating factor;

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300 mM NaCl, 200 mM mannitol, or 4°C, respectively. To ensure the RT-PCR was accurate, elongation factor 1-α (ef1-α) gene was used as internal control, and varying PCR cycle numbers were determined to quantitatively amplify each transcript (Fig. 2). The RT-PCR results were ultimately consistent with the microarray data for all miRNAs tested except miR156h and miR167d. The induction of miR393 reported here was in agreement with previous studies using Northern blotting (Sunkar and Zhu 2004).

To further analyze the temporal expression patterns of these miRNAs, time course analysis by RT-PCR was performed to detect whether they can be regulated by stress after an extended treatment time (Fig. 3). Our data showed that the levels of miR156h, miR167a, miR167c, miR167d, miR168, and miR171b were gradually increased from 2 to 24 h after exposure to high-salinity treatment, while expression of miR396a peaked at 24 h. miR167a levels accumulated after 2 h of drought stress and were greatly increased after 24 h of treatment, while miR168 first increased then returned to a normal level after 6 h. During cold-stress treatment, most miRNA levels were higher after 6 h and declined with longer stress; the exception was miR156a, which had maximal expression at 24 h. The expression of additional members of miRNA families was also analyzed, but these transcripts were undetectable or did not change (data not shown). Members of the same miRNA family might have different functional roles in spite of their high similarity in the sequence. Taken together, the newly identified stress-response miRNAs were prone to being induced by all treatments, indicating the existence of great cross talk between high-salinity, drought, and cold stress signaling processes. Our results revealed that a single miRNA has the potential to regulate multiple functionally related mRNAs in response to stress.

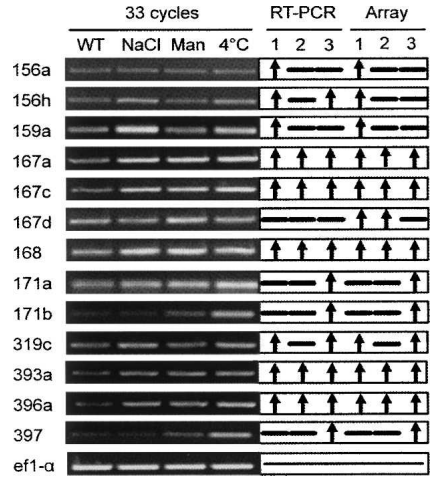

FIGURE 2. RT-PCR validation of the microarray. (RT-PCR) results from RT-PCR analysis; (Array) results from microarray; (Man) mannitol treatment; (lane *1*) 300 mM NaCl treatment; (lane *2*) 200 mM mannitol treatment; (lane *3*) 4°C treatment; (↑) up-regulated compared to WT; and (—) no significant difference.

Stress-relevant *cis*-elements exist in the promoters of miRNA genes

Cis- and *trans*-acting elements involved in stress-induced gene expression have been analyzed extensively (Jaglo-Ottosen et al. 1998; Kasuga et al. 1999; Zhang et al. 2005). To further elucidate the inducibility of these products, we analyzed the 1000-base-pair (bp) upstream promoter sequence of 20 miRNAs by using the PlantCARE database (http://intra.psb.ugent.be:8080/PlantCARE) (Lescot et al. 2002). Among the elements listed in Table 3, we identified several known stress-responsive elements, such as the ABA-response elements (ABREs), anaerobic induction elements (AREs), MYB binding site involved in drought-inducibility (MBS), heat-stress-responsive elements (HSEs), low-temperature-responsive elements (LTRs), and defense- and stress-responsive elements (TC-rich repeats). Some other regulatory elements were also identified, such as those possibly involved in regulation in response to gibberellic acid (GA), ethylene, salicylic acid (SA), or methyl jasmonate (MeJA). In plants, most ABA-responsive genes have the conserved ABREs in their promoters, which are significant *cis*-elements for genes responsive to abiotic stress in *Arabidopsis* (Mundy et al. 1990; Xu et al. 1996). Of the 20 miRNAs analyzed, six had ABREs, suggesting these miRNAs might be involved in ABA-mediated stress-response processes. Sixteen miRNAs had AREs, which respond to hypoxic, low-temperature, and dehydration stress (Dolferus et al. 1994). The presence of ABREs and AREs suggests that these miRNA might be regulated by stress conditions as protein coding genes. For example, miR393 was reported to be strongly up-regulated by cold, dehydration, and NaCl treatments. We found seven AREs and two ABREs in the promoters of three miR393 family members (miR393a, -b, and -c), which might provide the evidence of the induction of this miRNA. Likewise, the responses of miR167, miR168, and miR396 to high-salinity, drought, and cold treatments are correlated with the enrichment of AREs and ABREs in their promoters. Stress-related elements also existed in miR408 promoters and the role of this miRNA needs to be determined experimentally. Taken together, analysis of stress-response enrichment *cis*-elements provides additional evidence that these 14 miRNA genes are very likely to be involved in the responses to abiotic stresses.

In conclusion, we analyzed 117 miRNAs expression profiles under three abiotic stress conditions in *Arabidopsis* and found that 14 of them were differentially regulated by one or more stress conditions. Information about miRNA expression patterns and the biochemical function of their targets is essential and provides a valuable contribution toward our understanding of the function of these tiny non-coding genes and their cooperation in complex biological

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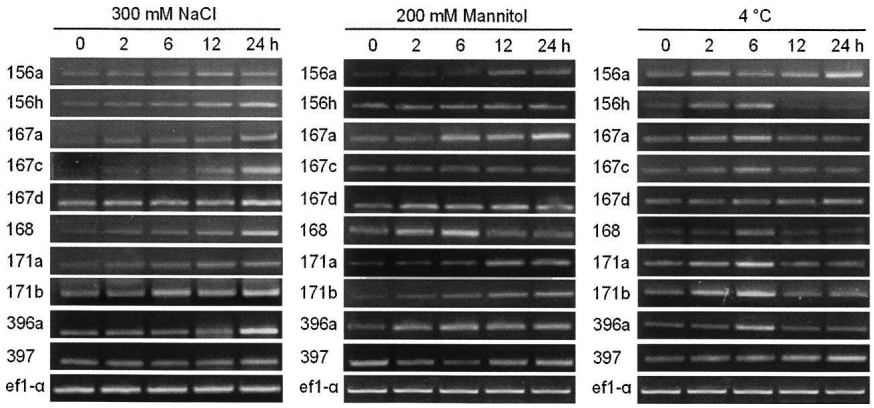

FIGURE 3. Time course of altered expression of selected miRNAs by RT-PCR analysis. PCR amplification cycles: ef1-α, 25 cycles; miRNA precursor, 33 cycles.

networks. Based on the RT-PCR and promoter analysis, we speculate that miR167, miR168, and miR396 might play important roles in plant abiotic stress responses. However, due to the potential uncertainty in this experiment, it is not feasible to just estimate the exact effects of these miRNAs in plants. These data provide a starting point for future studies, and continued efforts are needed to confirm the function of miRNAs in stress response and stress adaptation.

MATERIALS AND METHODS

Plant materials

A. thaliana ecotype Columbia seeds were surface-sterilized and sown on MS-agar plates. Seeds were stratified at 4°C for 2 d and then transferred to 22°C for 2 wk. For different stresses, seedlings were transferred to blotting paper without stress treatment or saturated with 300 mM NaCl, or 200 mM mannitol, or treated with cold (4°C). For RNA extraction and microarray experiment, the whole plants were frozen and stored in liquid nitrogen immediately after harvest.

MicroRNA microarray construction

Mature miRNA sequences were downloaded in the miRBase (http://microrna.sanger.ac.uk/, as of June 2005). There were 117 miRNAs from *Arabidopsis* after redundant sequences were discarded. In addition, eight short oligos were designed to possess no homology with any existing RNA sequence, and their corresponding synthetic miRNAs were produced by in vitro transcription using a miRNA Probe Construction Kit (Ambion). Various amounts of synthetic miRNAs were spiked into the RNA samples. All miRNA probe sequences were designed to be complementary to the full-length mature miRNA. To facilitate probe immobilization on aldehyde-modified glass slides, the probe sequences were concatenated up to 40 nt and modified with 5′-amino-modifier C6. Oligonucleotide probes were synthesized at MWG Biotech and dissolved in EasyArray spotting solution at 40 μM concentration. Each probe was printed in triplicate using a SmartArray microarrayer (CapitalBio Corporation).

RNA extraction and miRNA microarray experiments

Total RNA was isolated from different *Arabidopsis* seedlings with TRIZOL reagent (Invitrogen) and the low-molecular-weight RNA was isolated using the miRNA Isolation Kit (Ambion). Target RNA labeling was performed as described (Thomson et al. 2004). In brief, 4 μg of low-molecular-weight RNA was labeled with 500 ng of 5′-phosphate-cytidyl-uridyl-cy3-3′ (Dharmacon) with 2 units of T4 RNA ligase (NEB). The labeling reaction was performed at 4°C for 2 h. Labeled RNA was precipitated with 0.3 M sodium acetate and 2.5 volumes of ethanol and was then resuspended in 15 μL of hybridization buffer containing 3× SSC, 0.2% SDS, and 15% formamide. Hybridization was performed using LifterSlip (Erie), which allowed for even dispersal of hybridization solutions between the microarray and coverslip. The hybridization chamber was laid on a three-phase tiling agitator (BioMixer II) to prompt the microfluidic circulation under the coverslip. Hybridization was performed in a water bath at 42°C overnight. After the array had been hybridized at 42°C overnight, it was washed with two consecutive washing solutions (0.2% SDS, 2× SSC at 42°C for 5 min, and 0.2% SSC for 5 min at room temperature).

Image acquisition and data processing

Clustering arrays were scanned with a confocal LuxScan scanner. The scanning setting was adjusted to obtain a visualized equal intensity of U6 spots across arrays. Data was extracted from the TIFF images using LuxScan 3.0 software (CapitalBio). Low-intensity spots were removed for which fewer than 30% of the signal pixels exceeded the median background plus two times its standard deviation. Then normalization was performed based on the mean array intensity for inter-array comparison. For each sample, consideration of two hybridizations was carried out and each miRNA probe had three replicate spots on a microarray. The mean intensity value of each probe was used for cluster analysis. The raw data were Log 2 transformed and median centered by arrays and genes using the Adjust Data function of CLUSTER 3.0 software and then further analyzed with hierarchical clustering with average linkage (Eisen et al. 1998). Original microarray data are deposited at the Gene Expression Omnibus. To determine the significant differentially expressed miRNAs, Significance Analysis of Microarrays (SAM, version 2.1) was performed using two-class unpaired comparison in the SAM procedure (Tusher et al. 2001).

RT-PCR analysis

Total RNA isolated with RNase Plant Mini Kit (Qiagen) was processed. Reverse transcription reactions were performed in 20 μL using 5 μg of RNA by M-MLV (NEB). RT-PCR conditions for elongation factor 1-α gene ef1-α amplification were as follows: 94°C for 5 min, 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and 72°C for 5 min, 25 cycles. Primers for ef1-α were as follows: forward, 5′-GTATGGTTGTTACCTTTGCTCCCACAG and reverse,

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TABLE 3. Kinds and numbers of known stress-related elements in the upstream regions of miRNA genes

Element	Function	156a	156h	159a	165	167a	167c	167d	168	171a	171b	319a	319b	319c	393a	393b	393c	396a	396b	397	408
MBS	Drought	1						1	1	2	1		1				1	1			2
HSE	Heat stress	1				1		1		1			1					1	1		
LTR	Low temperature		1		1	1														1	1
TC-rich repeat	Defense and stress				1			1	1	1	1	2	1	1	3	2	1			3	
ARE	Stress	1		1	1	2	1	1	2		1	2		1	3	2	2		1	2	4
TGA element	Auxin	2		1					1		1		1		1	1				1	1
AuxRR core	Auxin							1	1					1			1				
GARE motif	GA		2		1	1				2	1										2
P Box	GA					1															1
ABRE	ABA			1			1		6							2			3		2
ERE	Ethylene									1						1					
CGTCA motif	MeJA		1	3	1										2		2		1	1	5
TGACG motif	MeJA		1	3	1										2			1	1	1	1
TCA element	SA				1	1	2		1	1		3		1					1	2	
Box W1	Fungal elicitor	1													2		2				

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3′-CATCATTTGGCACCCTTCTTCACTGC (500 bp). For miRNA precursor amplification, essentially the same conditions were used except the number of PCR cycles was increased to 33. The primer pairs used for RT-PCR and predicted amplicon sizes were as follows:

156a, 5′-AGAATTTGGTATGCAGAGACAGAT; 3′-CGGTTTCTG GACTAATTGGAA (298 bp);
156h, 5′-TGACACGATCACACAACATGG; 3′-CCACCGTCACT GCTTACTTA (209 bp);
159a, 5′-CACGCTAAACATTGCTTCGGAAT; 3′-ATCCCATAAG CCCTAATCCTTGT (290 bp);
159c, 5′-ACCAAGTTTTGAAGAACAGAGACT; 3′-CATAGAGA GTGCGCGGTGTT (169 bp);
167a, 5′-GTGTAGTCAACTGTGTGCGTT; 3′-GCACAACTTGT TGCTCAGGT (234 bp);
167c, 5′-TTCATGCTACAATCATTAGCAGGT; 3′-AGTCGTCTT CATGTCTGTATGT (205 bp);
167d, 5′-GAGTTGTGGCCATTAAGAGCT; 3′-CTTCTTGTTAAT GTTTGCTCTCTCCT (216 bp);
168, 5′-TGATAGTAGAGTCTCACCATCG; 3′-GAAGGAGAAGC GTAGAAATCTTC (205 bp);
171a, 5′-TCCAAAATAGAGACGAGAGAGT; 3′-CTCCTCCTCAC ACTTCACAT (215 bp);
171b, 5′-CGAGTGCCTGTAGAGTAAAAAC; 3′-TTCTGGAGCT AAGTGGAGATT (278 bp);
319c, 5′-AAACACTCGTGGTAGAGAAACGAT; 3′-AGAGGTTG AAAATGCAAATCCAGT (238 bp);
393, 5′-CAAAGAGATAGCATGATCCAA; 3′-AAGAGGAACACG ATCCATTGAC (214 bp);
396a, 5′-AGGGTTTCGTCTGCTCTACAT; 3′-TCTGATTATGGA ATCAATCACGCT (242 bp);
397, 5′-CCCCTGGGTTTGAATGAACAT; 3′-AGAACTCTCAAG GTCTTTTAAGTG (180 bp).

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