

Differential and dynamic regulation of miR398 in response to ABA and salt stress in *Populus tremula* and *Arabidopsis thaliana*

Xiaoyun Jia · Wang-Xia Wang · Ligang Ren ·
Qi-Jun Chen · Venugopal Mendu · Benjamin Willcut ·
Randy Dinkins · Xiaoqing Tang · Guiliang Tang

Received: 5 February 2009 / Accepted: 22 May 2009 / Published online: 17 June 2009
© Springer Science+Business Media B.V. 2009

Abstract MicroRNAs (miRNAs) are endogenous small RNAs of ~22 nucleotides (nt) that play a key role in down regulation of gene expression at the post-transcriptional level in plants and animals. Various studies have identified numerous miRNAs that were either up regulated or down regulated upon stress treatment. Here, we sought to understand the temporal regulation of miRNAs in different plant species under abscisic acid (ABA) and salt (NaCl) stress. Our results showed that the regulation of miR398 in response to ABA and salt stress was more dynamic in plants than previously reported. In poplars, miR398 was first induced upon 3–4 h of ABA or salt stress. However, this induction declined after 48 h and finally accumulated again over a prolonged stress (72 h). We referred to this kind of regulation as dynamic regulation. In contrast, such dynamic regulation of miR398 under salt stress was completely absent in *Arabidopsis*, in which miR398 was steadily and unidirectionally suppressed. Interestingly, ABA treatment caused a deviate dynamic regulation of miR398 in *Arabidopsis*, showing an opposite response as compared to that in poplars. We referred to the difference in regulation between *Arabidopsis* and poplars as differential

regulation. Furthermore, the expression of the miR398 target, copper superoxide dismutase1 (CSD1), was in reverse correlation with the miR398 level, suggesting a control of this specific target expression predominantly by miR398 under abiotic stress. Together, these data consistently show a correlated regulation between miR398 and its representative target, CSD1, by ABA and salt stresses, and raise the possibility that regulation of miRNAs in plants is twofold: a dynamic regulation within a plant species and a differential regulation between different plant species.

Keywords miRNA · Abiotic stress ·
Dynamic regulation · ABA · Salt stress

Abbreviations

ABA	Abscisic acid
APS	ATP sulfurylase
CSD	Cu/Zn superoxide dismutase
miRNA	MicroRNA
qRT-PCR	Quantitative reverse transcription PCR
RISC	RNA-induced silencing complex

Electronic supplementary material The online version of this article (doi:10.1007/s11103-009-9508-8) contains supplementary material, which is available to authorized users.

X. Jia · W.-X. Wang · L. Ren · Q.-J. Chen · V. Mendu ·
B. Willcut · X. Tang · G. Tang (✉)
Department of Plant and Soil Sciences and KTRDC, Gene
Suppression Laboratory, University of Kentucky, Lexington, KY
40546-0236, USA
e-mail: gtang2@uky.edu

R. Dinkins
Forage-Animal Production Unit, USDA-ARS, Lexington, KY
40546, USA

Introduction

MicroRNAs (miRNAs) are a class of ~22 nt, endogenous non-protein-coding small RNAs that down regulate gene expression at the post-transcriptional level in plants and animals (Bartel 2004; Chen 2005; Lai 2005; Nilsson 2007; Tang et al. 2008; Zhang et al. 2006, 2007). Mature miRNAs are derived from stem-loop regions of RNA precursors by Dicer-like enzymes of the RNase III family (Bartel 2004; Chen 2005; Du and Zamore 2005; Kim 2005; Murchison and Hannon 2004; Tang 2005). The mature

miRNA is initially a double-stranded duplex, presumably associated with Dicer, and is further delivered to downstream proteins, including a key component Argonaute (AGO), to form an RNA-induced silencing complex (RISC). Only one strand, designated “miRNA,” of the miRNA duplex is incorporated into a RISC that further guides the target mRNA for cleavage or translational repression in a sequence-dependent manner. However, the other strand, termed “miRNA*,” is cleaved by the miRNA associated RISC and/or excluded from the RISC. Not all miRNA*s are non-functional, and some are assembled into RISCs to regulate gene expression under cellular conditions (Okamura et al. 2008).

Plant responses to adverse environmental conditions have a close relationship with plant self-defense, adaptation, and cellular signaling. Recently, a number of miRNAs have been implicated to play important roles in abiotic stresses or other specific environmental stress conditions (Jones-Rhoades and Bartel 2004; Lu et al. 2005; Marsit et al. 2006; Shukla et al. 2008; Sunkar et al. 2006, 2007; Sunkar and Zhu 2004). For example, miR169g played a role in drought and submergence stress (Zhao et al. 2007). MiR398 and its target genes were differentially expressed in response to high sucrose levels and other type biotic and abiotic stresses (Dugas and Bartel 2008; Jagadeeswaran et al. 2009; Sunkar et al. 2006). In addition, miR399, miR395 and miR398 have been shown to be induced by nutrient deficient stress (e.g., phosphate, sulfate, copper deficiency), and the induction caused down-regulation of the respective target genes (Abdel-Ghany and Pilon 2008; Chiou et al. 2006; Jones-Rhoades and Bartel 2004). Finally, a number of miRNAs were regulated by mechanical, cold, heat, dehydration, salinity, and mechanical stresses in woody plant poplars (Lu et al. 2005).

The response of miRNAs is likely controlled by the promoter elements of the miRNA genes. *Cis*- and *trans*-acting elements involved in stress-induced gene expression have been identified in the promoter region of several miRNAs. For example, miR-169g was induced by drought and its upstream region contained two dehydration-responsive elements (DREs), suggesting that the expression of miR-169g may be regulated directly by DRE-binding transcription factors (Zhao et al. 2007). In addition, some AREs (anaerobic induction elements) and ABREs (ABA-response elements) were found in the promoter of miR167, miR168, miR393, and miR396, implicating that these miRNAs are involved in various stress-response processes. However, the regulatory role of miRNA and its targets can diverge at different development stages (Carlolano et al. 2007) or different stress stages. In maize, the expression of miR159, miR395, miR474, and miR528 were suppressed at the early stage of water submergence and induced after 24 h post-submergence, showing a dynamic

regulation of miRNAs during water stress (Zhang et al. 2008). Here, we reported that ABA and salt stress induced a dynamic regulation of miR398 and its target gene CSD1 in Poplars and a differential regulation of the miRNA and the target between poplar and Arabidopsis plants, thus expanding our understanding of the regulatory role of miRNAs in abiotic stresses.

Materials and methods

Plant materials and stress conditions

Different plant species used in this study are *P. tremula* and *Arabidopsis thaliana* (Col-0). The plants were grown under normal conditions in a growth chamber or tissue culture unless indicated for stress treatments. For stress treatments, 1.5 month old in vitro cultured *P. tremula* plantlets or 2 week old Arabidopsis seedlings were subjected to the following stress treatments: (1) salt stress: salt stress was applied by immersing plant roots in a MS liquid growth medium containing 300 mM NaCl. (2) ABA treatment: ABA was applied by immersing plant roots in a MS liquid growth medium containing 100 μ M ABA. The plant leaves were sampled for RNA extraction at different time point.

Design of plant miRNA platform and miRNA array

Plant miRNA probes were selected according to the same criteria as discussed for animal miRNA array platform (Tang et al. 2007). About 188 of the plant miRNA antisense probes (20 μ M) were printed in duplicate using the Genetix Qpix2 robot as described previously (Tang et al. 2007). The array platform included 4 external controls (MAC2, MAC3, MAC4, and MAC5, where MAC represents miRNA array control). The MAC2-5 probes sequences are: ATGGA CCCGTCTACAGAGGCA (MAC2), ATCCGGGGCTGC CGGCTTCGA (MAC3), AGCTAGTCCTGGAACCCG GCA (MAC4), and ATCTCCCCAAGAAAGCCGGCA (MAC5), corresponding to the four synthetic 21 nt small RNA oligo sequences UGCCUCUGUAGACGGGUCC AU, UCGAAGCCGGCAGCCCCGGAU, UGCCGGGUU CCAGGACUAGCU, and UGCCGGCAGCCCCGGAGGC UU, respectively.

Total RNAs were isolated from treated plant leaves using TRIZOL reagent (Invitrogen). 100 μ g of total RNA from each sample was separated on a 15% denaturing PAGE and small RNAs (14–28 nt) were recovered from the gel and used for the array as described previously (Tang et al. 2007). Briefly, the small RNAs were dephosphorylated with antarctic phosphatase (New England Biolabs), and then radiolabeled with gamma-³²P-ATP and PNK. The

radiolabeled small RNAs were hybridized to the miRNA array membrane and subsequently detected using a phosphorimager (Typhoon 9400, Amersham). The obtained data were normalized to external controls and clustered using Cluster 3.0 (de Hoon et al. 2004). Clustering analyses were performed with a hierarchical method using an average linkage and Euclidean distance metric to illustrate relationships among the experimental data. The clustering data were visualized using Java TreeView (Saldanha 2004).

Northern blot validation

Northern blot analysis was performed as previously described (Andrali et al. 2007; Tang 2005; Tang et al. 2003). Briefly, 20 µg of total RNA isolated from plant leaves was separated on a denaturing 15% Urea-PAGE gel, electro-transferred to Hybond-N⁺ membrane (Amersham Biosciences), blotted, and probed using DNA oligonucleotides labeled with γ -P³²-ATP. After washing, Northern blots were placed on phosphorimager screens, and subsequently scanned using the Typhoon Scanner. The radioactive signals were quantified using the ImageQuant software package.

Analysis of target gene expression by qRT-PCR

Total RNA was extracted from treated plant samples using a RNeasy Mini Kit (Qiagen). A total of 1 µg of RNA was used for reverse transcription (RT) reaction by using High-Capacity cDNA Archive Kit (Applied Biosystem). qRT-PCR was performed with an Applied Biosystem-step one instrument using the SYBR Green PCR master mix kit (Applied Biosystems) according to the manufacturer's instructions. As an internal control, the expression level of actin in Arabidopsis and poplars was determined. Primers for the target genes were designed using the Genscript real-time PCR primer design program. The primer sequences of the target genes along with the internal control actins are listed in Table S5. The relative quantity of gene expression was detected using 2-DDCT method (Livak and Schmittgen 2001).

Results

Differential regulation of various miRNAs in response to short-time ABA treatment and salt stress in poplar plantlets

Long-term stress allowed us to examine both the expression of miRNAs and the consequences of the stressed plants. However, long-term stress often causes

considerable damages that eventually lead to cell and plant death (Lichtenthaler 1998). Mechanisms for plants to adapt to abiotic stresses should be better explored for their physiological changes or gene expressions under short-term stress conditions when the plant can survive the stress. In order to explore the regulatory mechanism of miRNAs in abiotic stress, the global response of miRNAs to short-term (≤ 4 h in this study) ABA and salt stress were examined in poplar plantlets (*Populus tremula*) by a miRNA array (for array layout see Table S1). Specifically, 1.5 month old in vitro cultured poplar plantlets were subjected to 4 h ABA treatment (100 µM) or 3 h salt stress (300 mM NaCl), and then RNA was extracted from leaves for a miRNA array analysis. Compared to the non-stressed control (normal growth conditions), a significant regulation of miRNAs was revealed in response to ABA and salt stress (Fig. 1a). The array results showed that over 15 abundant miRNAs were regulated by ABA and salt stress (Table 1). Most of the miRNAs were induced after short-term stress. On the other hand, two miRNAs, miR167h and miR169a, were suppressed, as revealed by clustering analysis (Fig. 1b). The pattern of miRNA regulation between ABA treatment and salt stress was quite similar.

To confirm the expression of identified miRNAs, six stress-regulated miRNAs (miR168, miR169, miR395, miR398, miR399, and miR408) were further validated by Northern blot analysis (Fig. 1c). As observed in the original array, the expression of miR168, miR395, miR398, miR399, and miR408 were induced in response to ABA and salt stress. In contrast, the expression of miR169 was suppressed under the same stress conditions.

Among the 15 stress-responsive miRNAs revealed by our analysis, several were the previously identified specific abiotic stress-associated miRNAs. MiR395 was a sulfate starvation-induced miRNA (Jones-Rhoades and Bartel 2004), miR399 was a phosphate starvation induced miRNA (Bari et al. 2006; Chiou et al. 2006; Fujii et al. 2005), and miR408 was induced by tension and compression stresses (Lu et al. 2005). Additionally, miR168 was responsive to all of the high-salinity, drought, and cold stress in Arabidopsis. Our results showed that these previously identified miRNAs were also induced by ABA and salt stress (Fig. 1), indicating these miRNAs are widely involved in different types of stress and may exert very different functions by regulating different targets. In addition, the array also revealed that a number of other miRNAs, such as miR164a, miR166a and miR472a/b, were responsive to ABA and salt stress (Table 1). These miRNAs were abundantly expressed in the poplars. Strikingly, miR398, suppressed by oxidative stress in Arabidopsis as previously described (Sunkar et al. 2006), showed an opposite response to ABA and salt stress in poplars (Fig. 1b, c).

Fig. 1 Regulation of miRNAs in response to abiotic stresses in poplar plantlets. **a** The expressions of miRNAs were screened for non-stressed control, ABA and NaCl treated poplar plantlets by miRNA array analysis. The highly regulated miRNAs were highlighted by their names on the array map. **b** Hierarchical clustering of 15 differentially expressed miRNAs based on their expression profiles using Gene Cluster 3.0 (average linkage and Euclidean distance as similarity measure). The data obtained for each miRNA were normalized to external controls and median centered prior to clustering. The highlighted miRNAs on the array map were marked as bolded. **c** Northern blot validation of the stress-regulated miRNAs from the array data. U6 was used as a loading control. MiR168 was slightly, and miR395, miR398, miR399 and miR408 were significantly, up-regulated upon ABA or NaCl stress. In contrast, the expression of miR169 was slightly down-regulated by both types of stress

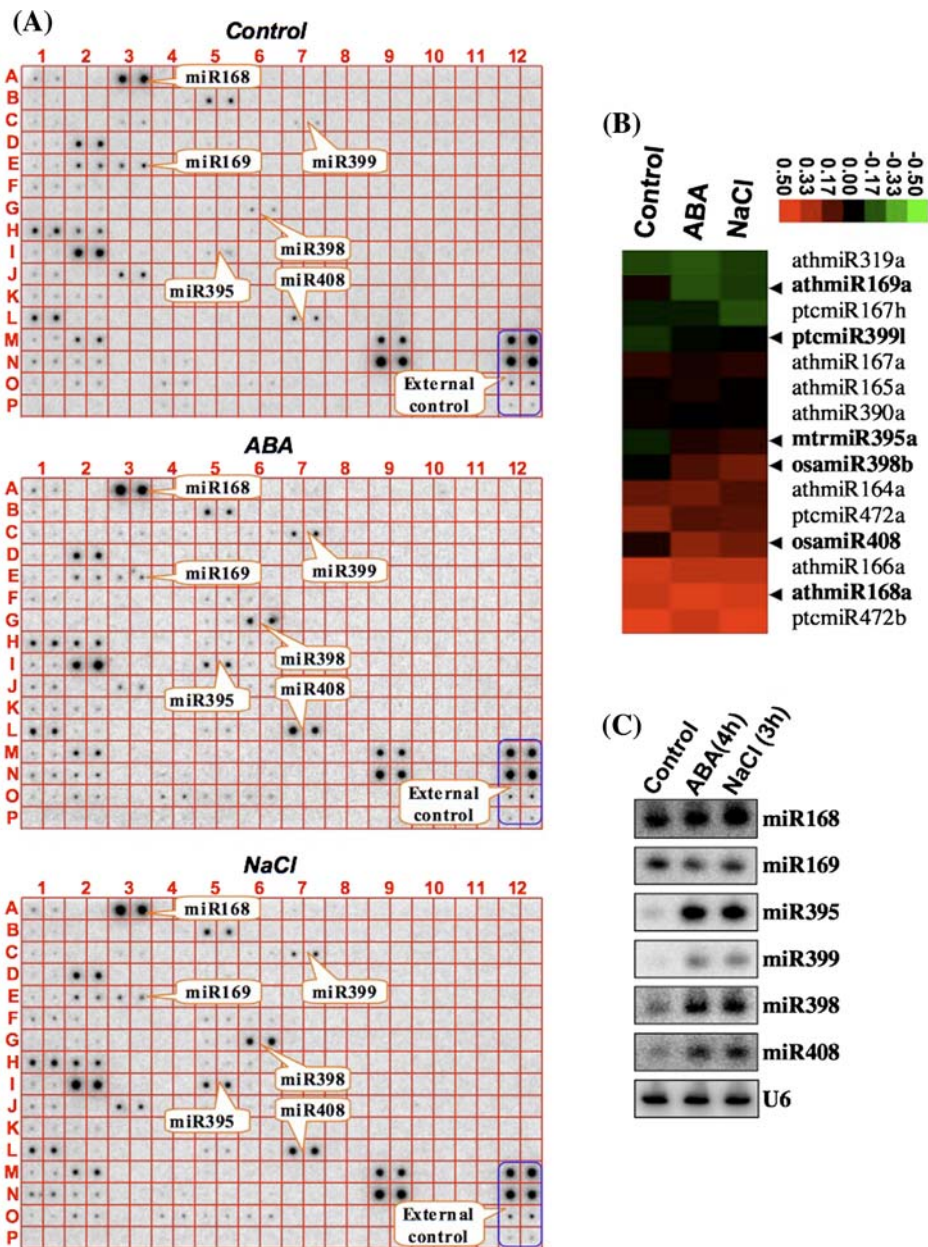


Table 1 ABA and salt stress regulated miRNAs in poplar plantlets

This table is generated from the quantitative data of Fig. 1. It lists 15 NaCl and ABA regulated miRNAs in poplar plants. Bolded miRNAs were validated by Northern blot analysis in this study

Category	ABA treatment	NaCl stress
Up regulated	ath-miR164a, athmiR165a, athmiR166a, ath-miR167a, ptcmiR167h, athmiR168a, athmiR319a, athmiR390a, mtrmiR395a, osamiR398b, ptcmiR399l, osamiR408, ptcmiR472a, ptcmiR472b	ath-miR164a, athmiR165a, athmiR166a, ath-miR167a, athmiR168a, athmiR319a, athmiR390a, mtrmiR395a, osamiR398b, ptcmiR399l, osamiR408, ptcmiR472a, ptcmiR472b
Down regulated	athmiR169a	ptcmiR167h, athmiR169a

Long-term ABA and salt stress revealed a dynamic regulation of miR398 and its target gene CSD1 in poplar plantlets

As shown in Fig. 1, miR398 was significantly up-regulated by short-term (i.e., 3–4 h) ABA and salt treatments in poplars. Previous study has reported that the expression of miR398 decreased upon a long-term (i.e., 8–12 h) oxidative stress in *Arabidopsis* (Sunkar et al. 2006), suggesting that different types of abiotic stress differentially regulate miR398 level. To examine how miR398 is responsive to ABA and salt stress over a long time period, poplar plantlets were stressed by immersing the root system inside a liquid medium containing 100 μ M ABA or 300 mM NaCl for over 72 h, while the plantlets grown in the normal medium served as a control. The leaves from the treated plants were sampled at different time points (0, 3 or 4, 48, and 72 h) and total RNA was extracted and analyzed for the expression of miR398. Figure 2a showed that the expression of miR398 was markedly induced fivefold at 4 h of ABA treatment as measured by Northern blot. Intriguingly, this induction of miR398 dramatically decreased after 48 h, and increased again at 72 h stress. Similar results were observed in salt stressed plants (Fig. 2c). These data indicated that the expression of miR398

was dynamically regulated at different stress stages and this regulation was built up gradually during a continuous stress exercise, suggesting that an adaptive process to abnormal conditions happened within the stressed plants.

To investigate the role of the dynamic change of miR398 level over a longer-term stress, the expression of miR398 target gene CSD1 was examined using quantitative reverse transcription PCR (qRT-PCR). As shown in Fig. 2b, the expression of CSD1 mRNA was significantly reduced at 4 h, increased at 48 h, and followed by a reduction at 72 h of ABA treatment, demonstrating a reverse correlation with the expression of miR398. Similarly, the CSD1 level also had a consistent but reverse change during salt stress (Fig. 2d) as compared to the expression of miR398 (Fig. 2c). Taken together, these results demonstrated that the regulation of miR398 by long-term ABA or salt stress was a dynamic process that was reflected by dynamic changes in the expression of both the miRNA and the miRNA target gene.

While the regulation of the miR398 expression was dynamic over a long-term ABA and salt stress, we next sought to understand whether other miRNAs also showed a similar dynamic regulation. MiR395 was chosen to address this question because it was also induced by short-term

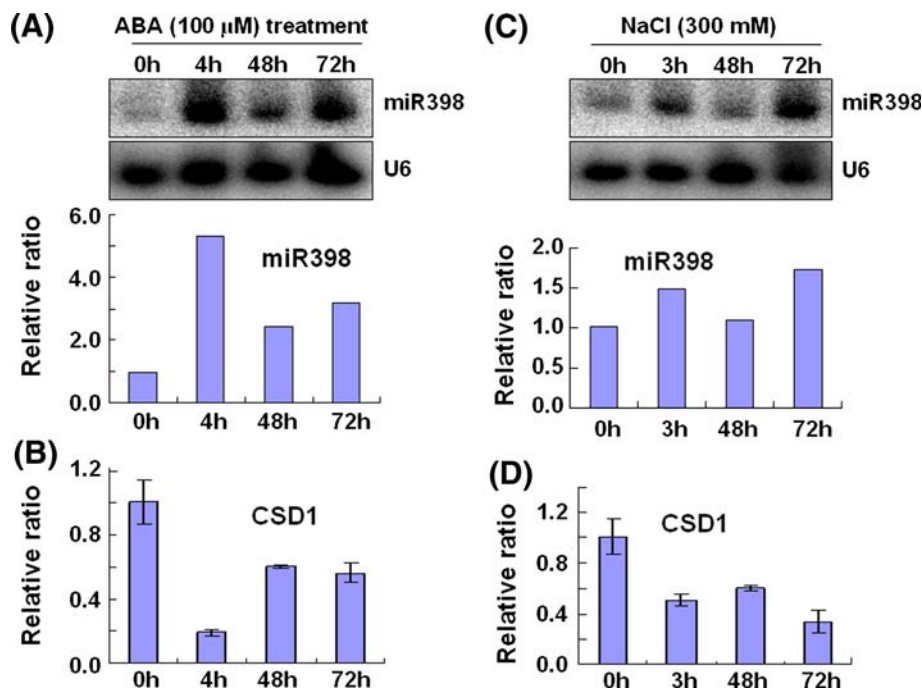


Fig. 2 MiR398 and its target gene were dynamically regulated in poplar plantlets by either ABA treatment or salt stress. Poplar plantlets were subjected to either long-term ABA treatment (100 μ M) or salt stress (300 mM NaCl). The leaves from stressed plantlets were sampled for analysis at different time points (0, 3/4, 48, and 72 h). a Effect of ABA treatment on the expression of miR398 analyzed by Northern blot. U6 served as an RNA loading control. MiRNA levels were obtained by normalizing to U6 and then to that of wild type

plants. The normalized miRNA levels in leaves without treatment were set arbitrarily to 1. b Effect of ABA treatment on the expression of miR398 target gene CSD1 evaluated by qRT-PCR. The CSD1 mRNA level was normalized to the internal control Actin. The presented data are the average of two independent experiments \pm SD. c and d Effect of salt stress on the expression of miR398 (by Northern blot) and its target gene CSD1 (by qRT-PCR)

stress as revealed by miRNA array and Northern blot validation (Fig. 1). Rather different from the response of miR398, the expression level of miR395 showed a continuous induction over 72 h ABA or salt stress (Fig. 3a, c). Correlated with the induction of miR395, the expression of ATP sulfurylase1 (APS1), one of the miR395 targets, was steadily repressed (Fig. 3b, d). Therefore, no dynamic regulation of miR395 and its target was observed during the stress treatment, suggesting the regulation of different miRNAs was controlled by different molecular mechanism even under the same stress environment.

MiR398 and its target genes were differentially regulated by ABA and salt stress in poplars and Arabidopsis

To determine whether the dynamic regulation of miR398 and its target gene in poplars is conserved in other plant species, we conducted similar experiments in Arabidopsis. As shown in Fig. 4a, the expression of miR398 did show a dynamic regulation under ABA treatment in Arabidopsis. However, this dynamic regulation was in the opposite direction when compared to the miR398 response in poplars. In Arabidopsis, the expression of miR398 was initially inhibited almost fivefold. About 24 h later, miR398 level was gradually increased, and then further elevated after 48 h of ABA treatment. Due to the death of Arabidopsis plants after 72 h of treatment with ABA, a long time examination of miR398 expression was not available. Nevertheless the data clearly showed that miR398 was subjected to a deviate dynamic regulation under ABA

treatment. Similarly, examination of the miR398 targets, CSD1 and CSD2, confirmed the reverse correlation with miR398 in expression (Fig. 4b). In conclusion, the dynamic regulation of miRNA and the target gene was also present in Arabidopsis, but the regulation of the same miRNA and the target gene in Arabidopsis was clearly different from that in poplars in terms of the expression levels (induction or suppression) and the trend of the expression.

In contrast, miR398 and its target CSDs exhibited a completely different response to salt stress in Arabidopsis. As shown in Fig. 4c, the expression of miR398 was steadily down-regulated over 48 h salt stress. Consistently, CSD1 and CSD2 were steadily enhanced at mRNA level by salt stress (Fig. 4d), exhibiting a reverse correlation with the expression of miR398. Interestingly, previous study showed that oxidative stress also repressed the miR398 level and increased the expression of CSD1 and CSD2 in Arabidopsis (Sunkar et al. 2006). These results demonstrated that the expression of miR398 was similarly regulated by salt stress and oxidative stress in Arabidopsis. However, miR398 and its target genes lost dynamic regulation under salt stress in Arabidopsis.

Discussion

Responses of miR398 to abiotic stress are considerably plastic and multitudinous in plants

Plants are extremely sensitive and also adapt very differently to various abiotic stresses. Increasing evidence points

Fig. 3 MiR395 was up-regulated by both ABA and salt stress in poplar plantlets. Total RNA was extracted from stressed poplar plantlets and analyzed for the expression of miR395 and its target gene APS1. **a** and **b** Effect of ABA treatment on the expression of miR395 (by Northern blot) and APS1 (by qRT-PCR). **c** and **d** Effect of salt stress on the expression of miR395 (by Northern blot) and APS1 (by qRT-PCR)

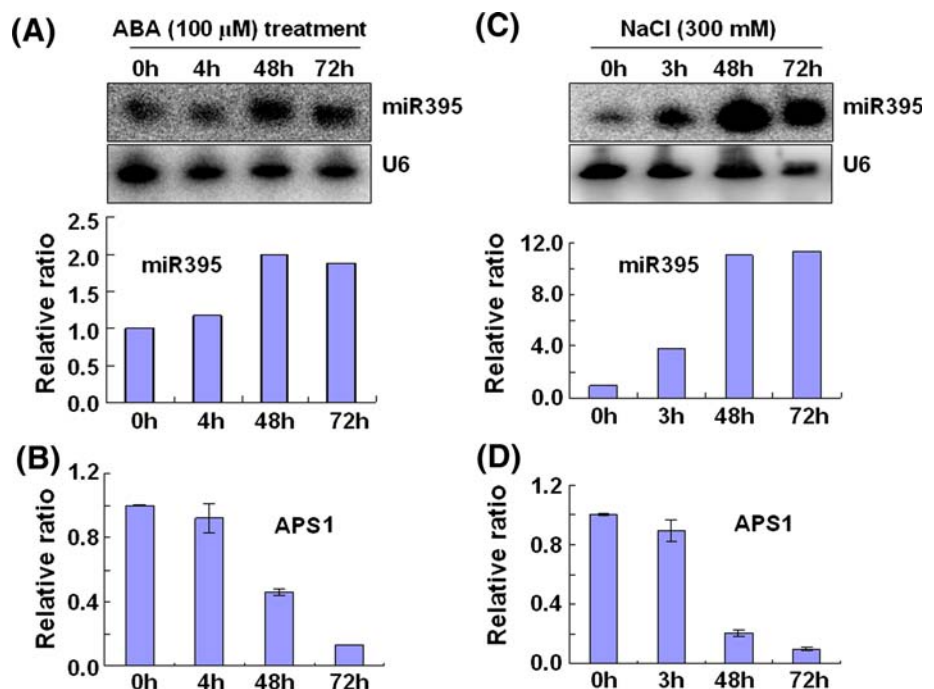
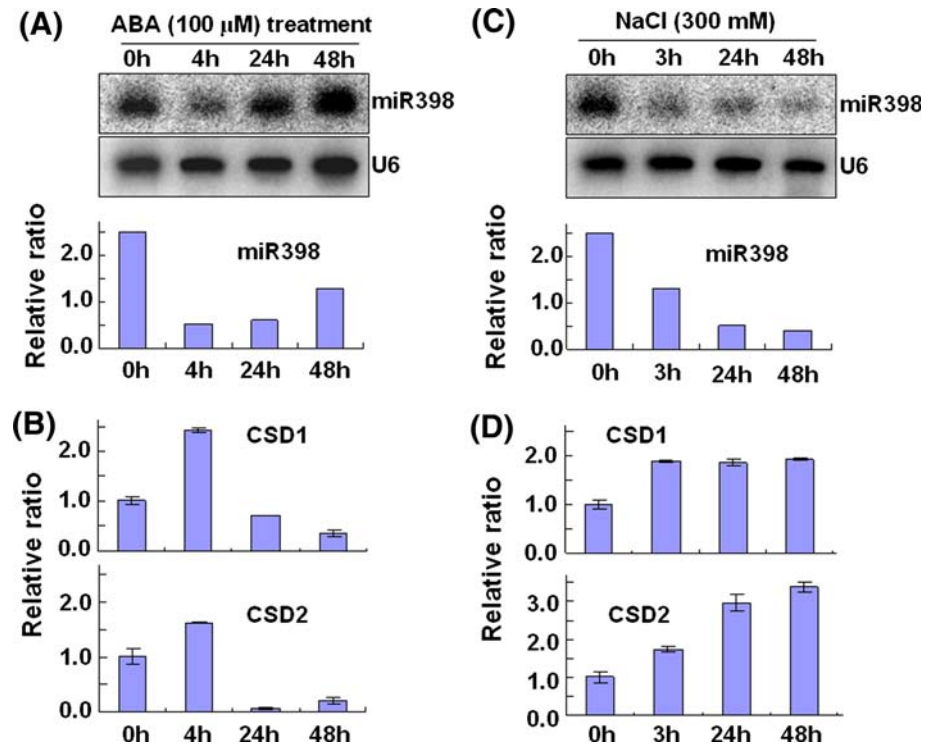


Fig. 4 Regulation of miR398 and its target genes by ABA and salt stress in Arabidopsis. Two week old Arabidopsis seedlings (Col-0) were treated with ABA or salt and total RNA was extracted from stressed leaves at different time points (0, 3/4, 24, and 48 h). **a** Effect of ABA treatment on the expression of miR398 analyzed by Northern blot. **b** Effect of ABA treatment on the expression of miR398 target genes, CSD1 and CSD2, measured by qRT-PCR. **c** and **d** Effect of salt stress on the expression of miR398 (by Northern blot) and CSD1/2 (by qRT-PCR)



to an important role of miRNAs in stress tolerance. In Arabidopsis, the expression of miR398 is highly regulated under different abiotic stress conditions. For example, miR398 level decreased during high Cu^{2+} , high Fe^{3+} , methyl viologen, high-intensity light, ozone fumigation, salt stress and *P. syringae* infection, and increased during low Cu^{2+} and high sucrose levels (Dugas and Bartel 2008; Jagadeeswaran et al. 2009; Sunkar et al. 2006). In poplars, however, miR398 level was first increased (4 h), then declined (48 h) and then accumulated again with longer stress (72 h; Fig. 2). Such a dynamic regulation was also observed for other miRNAs in poplar plantlets that were under cold treatment. For example, *ptc-miR474c* was up-regulated under 4–12 h cold stress, but down-regulated to non-treatment level under 16–20 h cold stress, and up-regulated again after 24 h cold treatment (Lu et al. 2008). Therefore, the functions of miR398 under different abiotic stresses may be variable in different plant species and the response of miR398 is rather plastic and multitudinous.

In addition to miR398, we also examined the regulation of other miRNAs and their target for comparison purposes. Several studies have reported that miRNAs regulate the expression of stress responsive protein-coding genes at post-transcriptional level, showing a reverse correlation between the miRNA and the target expression. For example, miR395 targets three ATP sulphurylases (APS1, APS3, and APS4) and a low-affinity sulphate transporter (SULTR2;1), and APS1 has shown to be induced by sulfate deprivation (Chiou et al. 2006; Fujii et al. 2005; Jones-

Rhoades and Bartel 2004). Such an induction was considered important for sulfate homeostasis in plants. On the other hand, Dalmay's group recently showed that the mRNA level of another miR395 target, SULTR2;1, strongly increases while miR395 was induced in roots, suggesting that the expression regulation of miR395 and its targets is more complicated than previously thought (Kawashima et al. 2009). Our results showed that the expression of miR395 was in a reverse correlation with its specific target APS1 over 72 h ABA or salt stress (Fig. 1). This suggests that ABA and salt stresses also affect sulfate homeostasis by likely sharing a common signaling pathway via miR395. It is still not known yet whether other members of miR395 targets in poplars are also under a tight control by miR395 at the post-transcriptional level or are subjected to complex regulations such as transcriptional regulation in addition to the post-transcriptional regulation by miR395.

In addition, miR408 level was induced by short-term tension and compression stresses in xylem tissue in poplars (Lu et al. 2005). A similar induction by ABA and salt stress was observed in our study. Furthermore, miR168, a miRNA playing a role in the auto-regulatory loop of plant Argonaute 1 (AGO1) in Arabidopsis, was also slightly up-regulated by ABA and salt stress, suggesting a regulatory role of miR168 in abiotic stress. This observation is supported by recent studies in Arabidopsis and poplars (Liu et al. 2008). Thus, the highly conserved responses of a few miRNA species (e.g., miR168, miR395, miR399, miR398,

and miR408) to various stresses including nutrient, abiotic, mechanical, and physiological stresses suggest a common pathway for plant sensing and responding to various environmental changes. The underlying mechanisms remain to be investigated in further detail.

Different plant species have rather distinct expression responses of miR398 under different types of abiotic stresses

In this study, we found that miR398 had a different response to ABA and salt stress in poplars and Arabidopsis. First, in poplars, the expression of miR398 was dynamically regulated by salt stress instead of steadily suppressed as in Arabidopsis. Second, although ABA treatment caused the dynamic regulation of miR398 in both poplars and Arabidopsis, the regulation trend of miR398 was completely opposite. The results indicated that different plant species are very different in the response of miRNAs under different types of abiotic stresses. Poplar plants represent a perennial woody plant species and have a high level of stress resistance over the long life cycle, which may be partially reflected by the dynamic change of miR398 and the target gene CSD1 in metabolism. In contrast, Arabidopsis represents annual herbaceous plants with a short life cycle and may have lost the dynamic regulation of miR398 under salt stress during the evolution.

Why are the expressions of miR398 and its target so different between poplar and Arabidopsis? In addition to the major physical and structural differences between woody and herbaceous plants, which may make the difference in stress responses, there are other possibilities for such differences. For example, in the case of ABA treatment, the rate of ABA sensing, uptake and regulating could be different between the two species. Other explanation for differential regulation of stress-responsive miRNAs in Arabidopsis and poplar can simply be that these plants undergo different levels of cellular stress when they receive the same stress treatment. The dynamic regulation of miR398 and its target may reflect an auto-regulation between miR398 and the target gene through a coordination between transcriptional and post-transcriptional regulations, which may exist in the perennial woody plants but not in the annual herbaceous plants. Nevertheless, the current study should be extended to more plant species to reach a more conclusive argument regarding the regulation of miR398 and its target gene in plants.

In summary, we have dissected in more detail the regulation of miR398 and miR395 and their corresponding target genes in plants. The result demonstrated that a dynamic regulation of miR398 and its target genes CSDs may be a general phenomenon in response to abiotic stresses in plant species. This argument was supported by a

recent study that demonstrated a dynamic regulation of a number of miRNAs under water submergence stress in maize (Zhang et al. 2008). Furthermore, miR398 and the target genes have distinct responses to different types of abiotic stresses in different plant species. These suggest that a fine-tuning role of miR398 in the plant abiotic stresses exists in different ecotypes of plants but exerts with distinct regulatory machineries for plants to adapt to a changing environment.

Acknowledgments We thank the University of Kentucky Advanced Genetics Technologies Center for printing the array, and Professor Arie Altman, at Robert H. Smith Institute of Plant Sciences and Genetics in Agriculture, Hebrew University of Jerusalem (Rehovot, Israel), for providing *P. tremula* material. G.T. is supported by the Kentucky Tobacco Research and Development Center (KTRDC), the USDA-NRI grants 2006-35301-17115 and 2006-35100-17433, the NSF MCB-0718029 (Subaward No. S-00000260), and an award from the Kentucky Science and Technology Corporation under Contract # KSTC-144-401-08-029.

References

- Abdel-Ghany SE, Pilon M (2008) MicroRNA-mediated systemic down-regulation of copper protein expression in response to low copper availability in Arabidopsis. *J Biol Chem* 283:15932–15945. doi:10.1074/jbc.M801406200
- Andrali SS, Qian Q, Ozcan S (2007) Glucose mediates the translocation of NeuroD1 by O-linked glycosylation. *J Biol Chem* 282:15589–15596. doi:10.1074/jbc.M701762200
- Bari R, Datt Pant B, Stitt M, Scheible WR (2006) PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. *Plant Physiol* 141:988–999. doi:10.1104/pp.106.079707
- Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116:281–297. doi:10.1016/S0092-8674(04)00045-5
- Cartolano M, Castillo R, Efremova N, Kuckenbergh M, Zethof J, Gerats T, Schwarz-Sommer Z, Vandenbussche M (2007) A conserved microRNA module exerts homeotic control over *petunia hybrida* and *antirrhinum majus* floral organ identity. *Nat Genet* 39:901–905. doi:10.1038/ng2056
- Chen X (2005) MicroRNA biogenesis and function in plants. *FEBS Lett* 579:5923–5931. doi:10.1016/j.febslet.2005.07.071
- Chiou TJ, Aung K, Lin SI, Wu CC, Chiang SF, Su CL (2006) Regulation of phosphate homeostasis by MicroRNA in Arabidopsis. *Plant Cell* 18:412–421. doi:10.1105/tpc.105.038943
- de Hoon MJ, Imoto S, Nolan J, Miyano S (2004) Open source clustering software. *Bioinformatics* 20:1453–1454. doi:10.1093/bioinformatics/bth078
- Du T, Zamore PD (2005) MicroPrimer: the biogenesis and function of microRNA. *Development* 132:4645–4652. doi:10.1242/dev.02070
- Dugas DV, Bartel B (2008) Sucrose induction of Arabidopsis miR398 represses two Cu/Zn superoxide dismutases. *Plant Mol Biol* 67:403–417. doi:10.1007/s11103-008-9329-1
- Fujii H, Chiou TJ, Lin SI, Aung K, Zhu JK (2005) A miRNA involved in phosphate-starvation response in Arabidopsis. *Curr Biol* 15:2038–2043. doi:10.1016/j.cub.2005.10.016
- Jagadeeswaran G, Saini A, Sunkar R (2009) Biotic and abiotic stress down-regulate miR398 expression in Arabidopsis. *Planta* 229:1009–1014. doi:10.1007/s00425-009-0889-3

- Jones-Rhoades MW, Bartel DP (2004) Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. *Mol Cell* 14:787–799. doi:10.1016/j.molcel.2004.05.027
- Kawashima CG, Yoshimoto N, Maruyama-Nakashita A, Tsuchiya YN, Saito K, Takahashi H, Dalmay T (2009) Sulphur starvation induces the expression of microRNA-395 and one of its target genes but in different cell types. *Plant J* 57:313–321. doi:10.1111/j.1365-313X.2008.03690.x
- Kim VN (2005) MicroRNA biogenesis: coordinated cropping and dicing. *Nat Rev Mol Cell Biol* 6:376–385. doi:10.1038/nrm1644
- Lai EC (2005) miRNAs: whys and wherefores of miRNA-mediated regulation. *Curr Biol* 15:R458–R460. doi:10.1016/j.cub.2005.06.015
- Lichtenthaler HK (1998) The stress concept in plants: an introduction. *Ann N Y Acad Sci* 851:187–198. doi:10.1111/j.1749-6632.1998.tb08993.x
- Liu HH, Tian X, Li YJ, Wu CA, Zheng CC (2008) Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*. *RNA* 14:836–843. doi:10.1261/rna.895308
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* 25:402–408
- Lu S, Sun YH, Shi R, Clark C, Li L, Chiang VL (2005) Novel and mechanical stress-responsive MicroRNAs in *Populus trichocarpa* that are absent from *Arabidopsis*. *Plant Cell* 17:2186–2203. doi:10.1105/tpc.105.033456
- Lu S, Sun YH, Chiang VL (2008) Stress-responsive microRNAs in populus. *Plant J* 55:131–151. doi:10.1111/j.1365-313X.2008.03497.x
- Marsit CJ, Eddy K, Kelsey KT (2006) MicroRNA responses to cellular stress. *Cancer Res* 66:10843–10848. doi:10.1158/0008-5472.CAN-06-1894
- Murchison EP, Hannon GJ (2004) miRNAs on the move: miRNA biogenesis and the RNAi machinery. *Curr Opin Cell Biol* 16:223–229. doi:10.1016/j.ceb.2004.04.003
- Nilsen TW (2007) Mechanisms of microRNA-mediated gene regulation in animal cells. *Trends Genet* 23:243–249. doi:10.1016/j.tig.2007.02.011
- Okamura K, Phillips MD, Tyler DM, Duan H, Chou YT, Lai EC (2008) The regulatory activity of microRNA* species has substantial influence on microRNA and 3' UTR evolution. *Nat Struct Mol Biol* 15:354–363. doi:10.1038/nsmb.1409
- Saldanha AJ (2004) Java treeview—extensible visualization of microarray data. *Bioinformatics* 20:3246–3248. doi:10.1093/bioinformatics/bth349
- Shukla LI, Chinnusamy V, Sunkar R (2008) The role of microRNAs and other endogenous small RNAs in plant stress responses. *Biochim Biophys Acta* 1779:743–748
- Sunkar R, Zhu JK (2004) Novel and stress-regulated microRNAs and other small RNAs from *Arabidopsis*. *Plant Cell* 16:2001–2019. doi:10.1105/tpc.104.022830
- Sunkar R, Kapoor A, Zhu JK (2006) Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in *Arabidopsis* is mediated by downregulation of miR398 and important for oxidative stress tolerance. *Plant Cell* 18:2051–2065. doi:10.1105/tpc.106.041673
- Sunkar R, Chinnusamy V, Zhu J, Zhu JK (2007) Small RNAs as big players in plant abiotic stress responses and nutrient deprivation. *Trends Plant Sci* 12:301–309. doi:10.1016/j.tplants.2007.05.001
- Tang G (2005) siRNA and miRNA: an insight into RISCs. *Trends Biochem Sci* 30:106–114. doi:10.1016/j.tibs.2004.12.007
- Tang G, Reinhart BJ, Bartel DP, Zamore PD (2003) A biochemical framework for RNA silencing in plants. *Genes Dev* 17:49–63. doi:10.1101/gad.1048103
- Tang X, Gal J, Zhuang X, Wang W, Zhu H, Tang G (2007) A simple array platform for microRNA analysis and its application in mouse tissues. *RNA* 13:1803–1822. doi:10.1261/rna.498607
- Tang G, Tang X, Mendu V, Jia X, Chen QJ, He L (2008) The art of microRNA: various strategies leading to gene silencing via an ancient pathway. *Biochim Biophys Acta* 1779:655–662
- Zhang B, Pan X, Cobb GP, Anderson TA (2006) Plant microRNA: a small regulatory molecule with big impact. *Dev Biol* 289:3–16. doi:10.1016/j.ydbio.2005.10.036
- Zhang B, Wang Q, Pan X (2007) MicroRNAs and their regulatory roles in animals and plants. *J Cell Physiol* 210:279–289. doi:10.1002/jcp.20869
- Zhang Z, Wei L, Zou X, Tao Y, Liu Z, Zheng Y (2008) Submergence-responsive microRNAs are potentially involved in the regulation of morphological and metabolic adaptations in maize root cells. *Ann Bot (Lond)* 102:509–519. doi:10.1093/aob/mcn129
- Zhao B, Liang R, Ge L, Li W, Xiao H, Lin H, Ruan K, Jin Y (2007) Identification of drought-induced microRNAs in rice. *Biochem Biophys Res Commun* 354:585–590. doi:10.1016/j.bbrc.2007.01.022