Analysis of miRNA expression under stress in *Arabidopsis thaliana*

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Abstract

MicroRNAs (miRNAs) are a class of small, 21-24 nucleotides long, non-coding RNAs involved in the post-transcriptional regulation of gene expression. Using the array analysis on *Arabidopsis thaliana* infected with the Oil-seed Rape Mosaic Virus (ORMV), we have found 28 up-regulated miRNAs. From them, six were selected for further validation by Northern blot analysis: miRNA172a, miRNA161, miRNA167a&b, miRNA168a&b, miRNA171a, and miRNA159. In addition, 29 miRNAs were detected in plants exposed to drought stress, 13 of those detected miRNAs were up-regulated and 16 down-regulated miRNAs. Out of 29 differentially expressed miRNAs during the abiotic stress, six miRNAs (167a&b, 168a&b, 173, 171b&c, 399d and 447c) were chosen for Northern blot and RT-PCR analysis to confirm the array results. Interestingly, four out of these six miRNAs, 171b&c, 168a&b, 399d, and 447c, showed very high abundance of pri-miRNAs and pre-miRNAs. Furthermore, mature forms of miRNAs171b&c, 399d, and 447c, were not detectable in the rosette leaves, indicating that miRNA processing is tissue specific. In conclusion, using the array analysis we show that 28 miRNAs are involved in the plant response to viral infection and 29 miRNAs are involved in the regulation of drought stress. We also demonstrate that at least some miRNAs involved in the stress response in *Arabidopsis thaliana* are regulated at the maturation level. One such example is miRNA 171b&c. This miRNA is transcribed in all tissues, evidenced by its detected pri and pre-miRNA forms; however, its mature form is constitutively or transiently expressed depending on the tissue type.

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KEY WORDS: *Arabidopsis thaliana*, miRNA, ORMV, drought, microarray

INTRODUCTION

MicroRNAs (miRNAs) are non-coding RNAs that negatively regulate gene expression at the post-transcriptional level by binding to the target mRNAs [1]. There are two post-transcriptional mechanisms of gene expression regulation by miRNAs [2, 3]. First is the cleavage of target mRNA and second is the inhibition of protein translation [2, 4]. Cleavage of the target mRNA is most often seen in plants due to their almost perfect complementarities to miRNAs. MiRNA genes are similar to the protein coding genes in that they may contain introns and that they are transcribed by RNA polymerase II [3, 4]. MiRNAs are heavily involved in the developmental and stress response processes [5, 6]. Therefore, much effort has been put into small RNA research in hopes of discovering more about their role in development and stress and through that in learning more about the post-transcriptional regulation. Different organisms have developed various mechanisms on how to survive stressful changes in the environment. For plants, as sessile organisms, surviving the environmental stresses depends not only on the plasticity of its developmental and defense mechanisms but also on their ability to quickly adapt to environmental changes [7, 8]. Regulation of gene expression is essential mechanism for any organism when dealing with stress. One good example of such regulation is the RNA silencing in plants as an antiviral defense mechanism. Systemic infection by plant viruses resembles developmental defects because in both cases plants experience loss of proper control of gene expression that regulates growth [9]. Several silencing suppressors that affect miRNA biogenesis also provide evidence for the transient presence of the miRNA/miRNA* duplex [10]. When expressed in *Arabidopsis thaliana*, the *Beet yellows virus* p21 and the *Tomato bushy stunt virus* p19 silencing suppressors bind the duplex but not single-stranded miRNAs leading to increased abundance of miRNA* and reduced miRNA-mediated cleavage of target mRNAs [10-13]. One important miRNA family heavily involved in RNA silencing during viral infection is the miRNA168 [14]. This miRNA has been reported to target *AGO1* protein, an

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integral part of the RISC complex and miRNA biogenesis process [14]. The induction of *AGO1* mRNA level has been reported to be a part of the host defense mechanism, whereas the induction of miR168 (which overlaps spatially with virus-occupied sectors) is mediated mainly by the Tabomavirus p19 RNA-silencing suppressor [14]. In addition, viral infection induces the stabilization of specific small RNA (sRNA) species and it causes the 21nt's small RNA enrichment that leads to accumulation of novel miRNA/like small RNAs from miRNA precursors [15]. The increase in accumulation seen for sRNAs initiating with a 5'U is mainly caused by virus-enriched miRNAs, whereas the increase seen for sRNAs starting with a 5'G reflects the accumulation of miRNA* sequences [15]. On the other hand many plant genes are regulated by stresses such as drought, soil salinity and extreme temperatures [7, 8, 16-18]. Plants that are under abiotic stress conditions modify certain physiological processes in plants such as the time of flowering, the rate of growth, and the rate of grain development [6]. For example, drought stress reduces days to flowering in wheat [19]. On the other hand drought stress delays flowering in rice [20]. In maize, it increases anthesis to silking interval [21]. In addition, with the help of functional analyses it has been determined that miRNAs play essential role in plant resistance to abiotic as well as biotic stresses [22]. The ability to understand the small RNA regulatory pathway, in plants which are undergoing stress, could be a new tool on how to genetically improve stress tolerance in plants. It has already been shown that with the manipulation of miRNA guided gene regulation plants can be engineered to be more resistant to stress [22]. Initial clues suggest that small RNAs are involved in plant stress responses stemming from studies showing stress regulation of miRNAs and endogenous siRNAs, as well as from target predictions for some miRNAs [6]. Subsequent studies have demonstrated an important functional role for these small RNAs in abiotic stress responses [6]. Roots of plants exposed to drought can continue growth deeper in the soil layers in order to acquire water and nutrients [23]. Experimental data show that miRNAs are highly involved in the root regulation and consequently plants drought response [23]. It has been shown that miRNAs 160 and 164 including their targets are involved in the root growth regulation. Over-expression of miRNA160, which targets auxin response factors (ARFs), manifested increased number of lateral roots [23]. However, over-expression of miRNA160-resistant ARF16 protein leads to reduced lateral roots and reduced fertility of the plant [23]. Furthermore the transgenic *Arabidopsis thaliana* that is over-expressing miR164, targeting NAC transcription factors mRNA, displays reduced lateral roots phenotype [24]. On the other hand, over-expression of miRNA164-resistant NAC1 resulted in increased number of lateral roots [24]. All of this indicates strong miRNA involvement in the drought stress regulation. From the earliest discovery and documentation miRNAs have been indicated to be heavily involved in the regulation of development and stress conditions in various species including plants [25, 1]. In this study, we show miRNA expression in plants under biotic and abiotic stress conditions. We demonstrate how miRNA expression during the ORMV infection causes uniform up-regulation of all detected miRNAs involved in the stress regulation. On the other hand, we show that plants under drought stress conditions show differential miRNA expression. More specifically, from the total number of miRNAs picked by the array analysis, approximately half of them are up-regulated and the other half are down-regulated genes. All of this leads to a conclusion that plant defense to viral infection and drought stress is highly dependent on miRNAs and their silencing mechanisms.

MATERIALS AND METHODS

Arabidopsis thaliana Growth Conditions in Soil (ORMV infection)

Arabidopsis thaliana plants used for viral infection were grown in soil (Col-0 ecotype) under standard growth chamber conditions with the short day supplemental light of 8 hours photoperiod and 16 hours dark period at 22°C. The virus particle isolation and plant inoculation was based on the established protocol [26]. The ORMV infection and the mock treatment using NaCl were performed on the *Arabidopsis thaliana* plants (6-8 leaves growth stage). Plants were grown under standard growth chamber conditions including long day of 16 hours photoperiod (supplemental light) and 8 hours dark period. One drop of ORMV particles diluted in H_2O (infected plants) and NaCl (mock treated plants) was applied to the leaf surface and rubbed into the leaf with a brush. Three leafs per plant were inoculated. Plant was collected 3 days post inoculation (3DPI), 7 days post inoculation (7DPI), and 3 weeks post inoculation (3WPI). A pool of approximately 20 plants was frozen and ground to powder in N_2. From that pool an aliquot of approximately 200 mg was taken and total RNA was isolated using the miRVana Ambion isolation kit and hybridized on the miRCURY™ LNA Array.

Arabidopsis thaliana Growth Conditions in Soil (Drought treatment)

Arabidopsis thaliana plants used for drought stress were grown in soil (Col-0 ecotype) under standard growth chamber conditions with the long day supplemental light of 16 hours photoperiod and 8 hours dark period at 22°C. Plants were plated on an agarose medium for 9 days after which they were transferred to soil. Plants were grown for 4 weeks post germination and at this point they were watered last time.

TABLE 1. RT-PCR Primers.

Primer Name	Direction	Primer Sequence
167a&b rev.	5´- 3´	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAGATC
167a&b fwd.	5´- 3´	TCGCTTGAAGCTGCCAGCAT
168a&b rev.	5´- 3´	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTCCCG
168a&b fwd.	5´- 3´	TCGCTTCGCTTGGTGCAGGT
171b&c rev.	5´- 3´	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGTGAT
171b&c fwd.	5´- 3´	TCGCTTGAGCCGTGCCAAT
173 rev.	5´- 3´	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGATT
173 fwd.	5´- 3´	TCGCTTTCGCTTGCAGAGAGA
399b&c rev.	5´- 3´	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGGGC
399b&c fwd.	5´- 3´	TCGCTTGCCAAAGGAGAGTT
399d rev.	5´- 3´	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGGGGC
399d fwd.	5´- 3´	TCGCTTGCCAAAGGAGATTT
universal reverse	5´- 3´	GTGCAGGGTCCGAGGT

This was taken as a zero point or control and the time of first collection. The next collection was 8 days after, followed by collections of plants based on their phenotype and viability. Some plants were collected without any subsequent watering and some were watered to stay viable for collection. Plants without rehydration were collected at 11 days, and 13 days of drought. Some plants were re-hydrated and collected on day 11 and 13 to observe the recovery process. Total RNA was isolated with Trizol (Sigma-Aldrich Chemie GmbH Taufkirchen, Germany) using a standard manufacturers protocol followed by the Qiagen clean-up kit (Qiagen GmbH Hilden, Germany). Finally, samples were hybridized on the Exiqon miRCURY™LNA Array (Exiqon A/S Vedbaek, Denmark).

Arabidopsis thaliana Seedlings Growth Conditions

Plants were grown under standard growth chamber conditions with the long day supplemental light of 16 hours photoperiod and 8 hours dark period at 22°C on a ½ MS cultivation medium containing: 2.2g/L MS powder (Duchefa Biochemie Haarlem, Netherlands), 10 g/L sucrose, 7 g/L plant agar (Duchefa Biochemie Haarlem, Netherlands).

RNA Isolation, Northern Blot, RT-PCR

Small RNAs were isolated using the mirVana miRNA isolation kit (Ambion Austin, USA) and analyzed by Northern blot hybridization according SequaGel Sequencing system Kit (EC-833) (National Diagnostics Atlanta, USA) according to manufacturer's instructions. Stem-loop RT followed by end-point PCR, a highly sensitive RT-PCR method for detection and verification of miRNAs was performed according to Varkonyi-Gasic et al. protocol from 2007 [27].

RESULTS

To gain insight into miRNA expression in *Arabidopsis thaliana* during ORMV infection and drought stress we have used the Exiqon miRCURY™ LNA Array screen. To validate array results we performed Northern blot hybridizations and RT PCR. RNA samples used in the first screen were extracted from the *Arabidopsis thaliana* (Col-0, WT) untreated, mock treated and ORMV infected plants. For the second screen RNA samples were extracted from *Arabidopsis thaliana* (Col-0, WT) untreated plants, drought stressed plants at two different time points (11 and 13 days) and recovered by rehydration (11 and 13 day rehydrated) drought treated plants. RT PCR, as a more sensitive validation method compared to Northern blot, was performed only for the drought treated plants due to such poor condition of the samples collected after 13 days of drought treatment. First array screen reported 28 uniformly up-regulated miRNAs due to ORMV infection. Out of these 28 up-regulated miRNAs (Figure 1A) 6 were chosen for further analysis: miRNA 172a, 161, 167a&b, 168a&b, 171a and 159 (Figure 1B). Selection for these miRNAs was based on several important criteria. Primarily it was based on the candidate's strength of the response from untreated to treated samples (Figure 1B). Another important criteria was miRNAs overall expression level obtained from the ASRP database (http://asrp.cgrb.oregonstate.edu). Further selection criteria were their target protein identity and function, known activity during the plant stress response and the localization of their mature form in *Arabidopsis thaliana* based on previously published data. Figure 2A represents Northern blot results for selected miRNAs and the corresponding graphical representation of their signal volume quantifications. For three miRNA 161, 168a&b and 171a a good correlation between the array and Northern blot analysis was observed; i.e. all three miRNAs showed up-regulated expression in ORMV infected samples compared to the untreated and mock treated samples (Figure 2A and B). The other three miRNAs (159, 167a&b, and 172a) tested, display consistent up-regulation in the infected plants as seen in the Figure 2C and D, although the expression signal differentiation from untreated/mock treated plants to ORMV treated plants is not as strong as seen on the

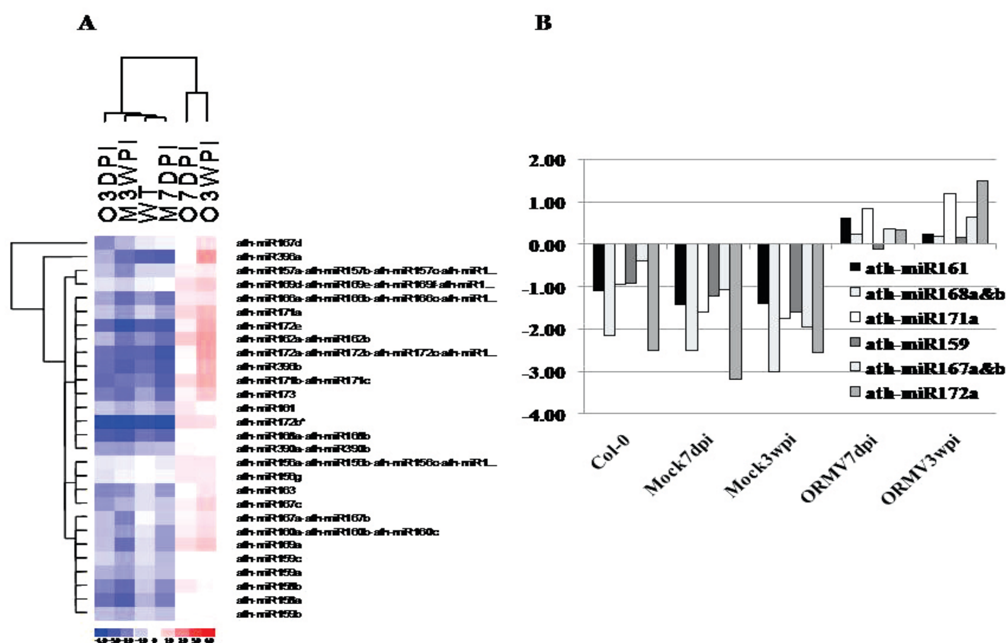

FIGURE 1. Heat map of differentially expressed miRNAs due to ORMV infection of *Arabidopsis thaliana*. Each row represents a miRNA and each column represents a specific sample. O3DPI: Arabidopsis plants infected with ORMV and collected 3 days post inoculation (3DPI). M3WPI: mock treated plants collected 3 weeks post inoculation (3WPI). Col-0; untreated plants. M7DPI: mock treated plants collected 7 days post inoculation (7DPI). O7DPI; ORMV infected plants collected 7DPI. O3WPI: ORMV infected plants collected three weeks post inoculation (3WPI). The miRNA clustering tree is shown on the left, and the sample clustering tree appears at the top. The color scale shown at the bottom illustrates the relative expression level of a miRNA across all samples: red color represents an expression level above mean, blue color represents expression lower than the mean. Gray color means that the specific miRNA on a given slide has been flagged (i.e. the signal was below background). The clustering is performed on log2 (Hy3/Hy5) ratios which passed the filtering criteria on variation across samples; standard deviation > 0.50 (column C-H, Expression matrix). (B) Graphical representation of miRNAs 161, 168a&b, 171a, 159, 167a&b, and 172a expression based on their relative values obtained as a function of log2(Hy3/Hy5) from the Exiqon expression matrix.

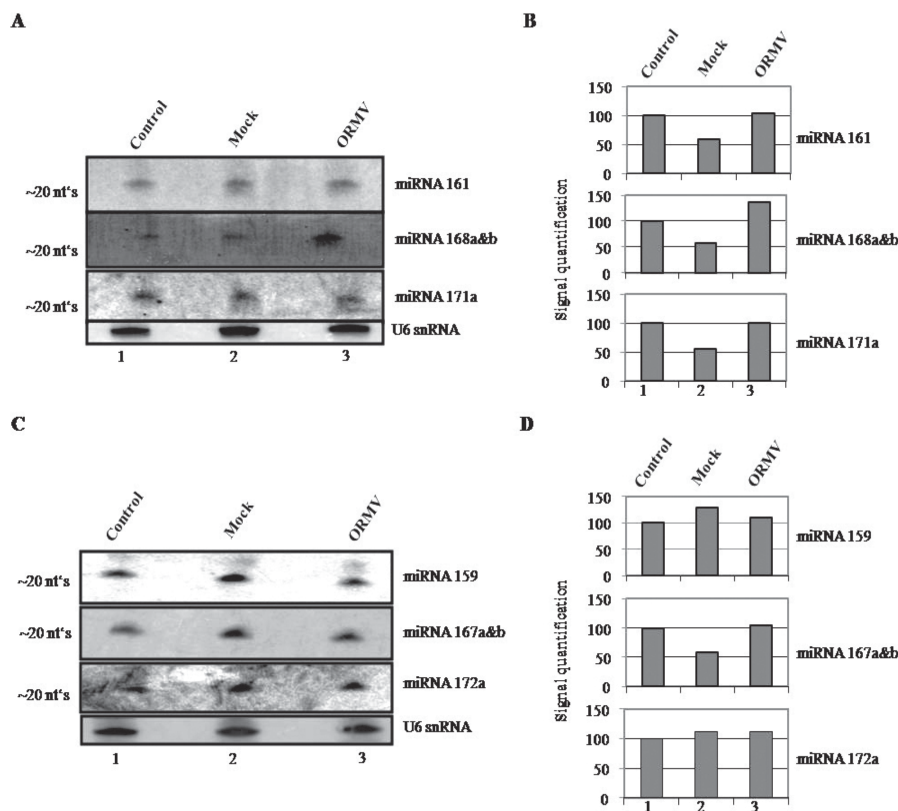

FIGURE 2. Expression analysis of selected miRNAs by Northern blot. (A) Northern blots for miRNA161, 168a&b and 171a. Lane 1, control plants without any treatment. Lane 2, mock treated plants 7 days post inoculation (7DPI). Lane 3, ORMV infected plants collected seven days after infection (7DPI). (B) Quantification of Northern blotting signals. Bars are marked 1-3 corresponding to Lanes 1-3 on the Northern blot (2A). To normalize expression of each miRNA each blot was hybridized with the U6 snRNA probe. Y axis values are Northern blot quantification volume. (C) Northern blots for miRNAs 159, 167a&b and 172a. Lane 1, control plants without any treatment. Lane 2, mock treated plants 7 days post inoculation (7DPI). Lane 3, ORMV infected plants collected seven days after infection (7DPI). (D) Quantification of Northern blotting signals. Bars are marked 1-3 corresponding to Lanes 1-3 on the Northern blot (2C). To normalize expression of each miRNA each blot was hybridized with the U6 snRNA probe. Y axis represents Northern blot quantification volume.

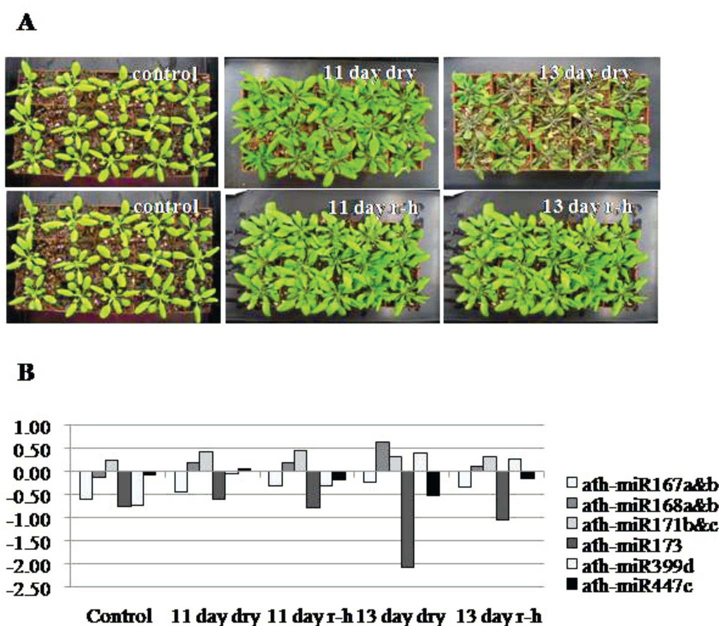

FIGURE 3. Images of control and drought treated plants used for miRNA analysis. (A) From left to right are the *Arabidopsis thaliana* Col-0: Control is 0 days drought, Day 11 dry is collection after 11 days of drought, Day 11 r-h is collection after 10 days of drought plus one day of recovery with rehydration, Day 13 dry is collection after 13 days of drought, Day 13 r-h is collection after 12 days of drought plus one day of recovery with rehydration. (B) Graphical representation of miRNAs 167a&b, 168a&b, 171b&c, 173, 399d and 447c expression based on their relative values obtained as a function of log2(Hy3/Hy5) from the Exiqon expression matrix.

array. In summary, from the plants tested we have reported 28 uniformly up-regulated miRNAs when infected with the ORMV compared to the untreated and mock treated plants. The second array analysis was performed on the RNA extracted from *Arabidopsis thaliana* drought stressed plants (Figure 3A). Out of 29 differentially expressed miRNAs reported by the array (data not shown) six miRNAs were chosen for further analysis. MiRNAs 167a&b, 168a&b, 171b&c, 399d, 173 and 447c have been selected and their expression was analyzed by Northern blot hybridization first, followed by the RT-PCR as a more sensitive method. MiRNAs were selected based on the same criteria mentioned previously for selection in the ORMV infected plants. From the graphical representation of the array signal (Figure 3B) it can be seen that miRNA 399d, miRNA 167a&b and miRNA 168a&b show greatest up-regulation at 13 day drought collection point whereas miRNA447c and miRNA173 are both down-regulated at the same collection point. MiRNA 171b&c shows consistent up-regulation in all drought stressed plants. Unlike the ORMV infected plants, where all miRNAs were uniformly up-regulated, drought treated plants show both the up-regulated and down regulated trend of expression. MiRNA 168a&b, on the array, shows the greatest up-regulation from control to 13 days drought treated samples (Figure 3B). From Northern blot result (Figure 3A) for miRNA 168a&b it can be seen that the overall expression differentiation has a trend of expression as seen on the array (Figure 3B). On the other hand miRNA 447c and miRNA 173 show the greatest down-regulation on day 13 of the drought treatment (Figure 3B). Interestingly, all six miRNAs selected respond to rehydration as shown in Figure 3B and 4C for 13 days dry and 13 day r-h collection points. To further validate the Northern blot results we have performed RT-PCR on selected miRNAs (Figure 4C). For this purpose a new batch of biological repeats was used (soil and growth conditions were the same as previously described for the drought treated plants in Materials and Methods) only this time with one time collection point at 9 days drought and rehydration. All five tested miRNAs show increased expression after 9 days of drought treatment and upon rehydration the levels of miRNA expression go down. The most notable change from untreated to treated plants can be seen for miRNA 168a&b (Figure 4A and B) and miRNA 399d (Figure 3B). Based on all three methods employed we can confirm differential expression of miRNAs induced by drought stress in *Arabidopsis thaliana*. MiRNA 171b&c, on the array, shows consistent up-regulation from the control samples (Figure 3B). MiRNA 399d shows greatest up-regulation in the plants under drought stress whereas miRNA 447c shows down-regulation in drought stress plants compared to the untreated samples (Figure 3B). However, on the Northern blots performed for miRNAs 171b&c, 447c and 399dwe never detected mature forms (data not shown). Instead, for these three miRNAs, we only detected the pre and pri-miRNAs. The subsequent quantification of the same blots showed a trend of expression as seen on the array (data not shown). Therefore, it can be concluded that the signal obtained on the array comes not only from the mature miRNAs but also from the pre-miRNA and pri-miRNA. Since the mature forms were not detected with Northern blot there could be two explanations for this, the first being that the levels of mature miRNAs is so low in the tissue tested that it was below detection levels for the Northern blot. Another explanation may be that the mature forms were not present in the tissues tested here, the plant shoot, but in some other

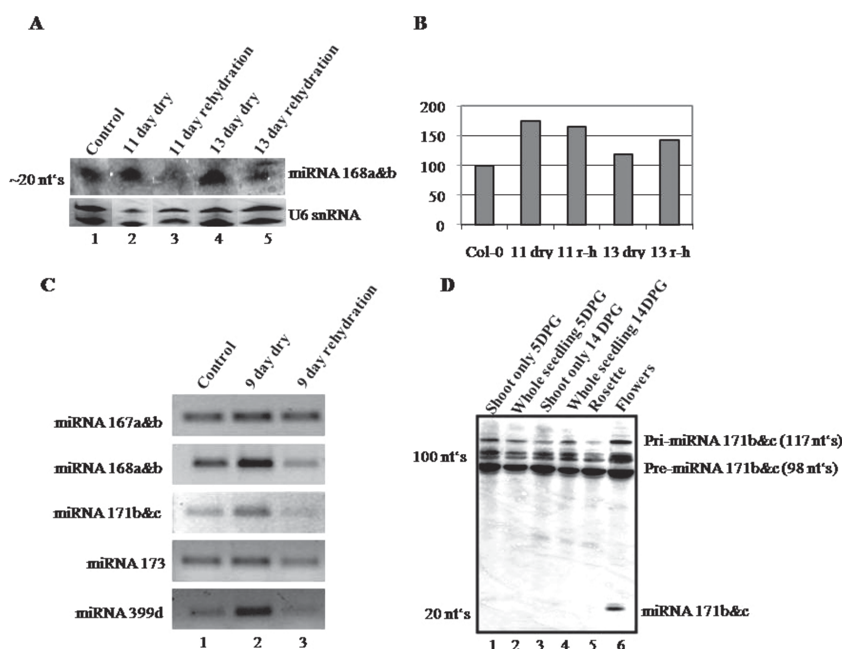

FIGURE 4. (A) Northern blot probed with the anti-sense (mature miRNA complement) miRNA168a: Lane1. WT control (Col-0); Lane 2, 11 days drought WT; Lane 3, 11 days re-hydration WT; Lane 4, 13 days drought WT; Lane 5, 13 days re-hydration WT. (B) Northern blot signal quantification graph for miRNA168a&b. To normalize the quantification signal each blot was hybridized with the U6 snRNA probe. (C) RT-PCR analysis of five selected miRNAs. PCR products were resolved on 2% agarose gels. Lane 1, untreated control sample. Lane 2, 9 days drought treated sample. Lane 3, 9 days re-hydrated sample (8 days drought plus one day rehydration). (D) Northern blot probed with the anti-sense oligo (mature miRNA complement) miRNA 171b&c. Lane 1. Seedlings shoot tissue 5 days post germination (DPG); Lane 2. Whole seedling (shoot and root) 5 DPG; Lane 3. Seedling shoot tissue 14 DPG; Lane 4. Whole seedling (shoot and root) 14 DPG; Lane 5. Grown plant rosette tissue; Lane 6. Flowers tissue.

parts of the plant such as flowers or roots. To ensure that the signal detected on Northern blot is actually pri and pre-miRNAs, another set of hybridizations was done only this time blots were probed with the sense miRNA sequence oligos for miRNA171b&c, 399d and 447c. The Northern blots probed with sense sequences of miRNAs (data not shown) have confirmed that the signal detected with anti-sense probe is indeed that from pri-miRNAs and pre-miRNAs. This additionally confirms that the signal detected on the array does come from pri and pre-miRNA sequences and not solely from the mature forms. In order to get more answers regarding the localization of mature miRNAs, another Northern blot was performed probing only for the miRNA 171b&c in different types of untreated tissues. From Figure 4D it can be seen that the mature form of miRNA 171b&c in high enough abundance detectable by Northern blot was found only in the flowers of *Arabidopsis thaliana* plants tested here. Also, it can be seen from the same blot that the levels of pri and pre-miRNA 171b&c is very high in all of the tissues analyzed. Thus confirming the hypothesis that miRNA 171b&c is transcribed in all plants tissues but it is constitutively processed to its mature form only in the flowers (Figure 4D).

DISCUSSION

We report here 28 up-regulated miRNAs due to ORMV infection in *Arabidopsis thaliana* plants. Out of six miRNAs (161, 168a&b, 171a, 159, 167a&b, and 172a) selected for further validation, four miRNAs 161, 167a&b, 168a&b, and 171a showed a good correlation between the array and Northern blot signal and two miRNAs 159 and 172a display higher expression (Northern blot) in the mock samples then in the infected plants as seen on the array. One explanation for this could be the dynamics of a viral infection; more specifically, how fast a plant defense response works and the factors involved in those processes may vary from plant to plant even when grown under same the conditions. In addition, based on the array and Northern blot analysis of miRNAs 161, 168a&b, 167a&b and 171a, we might speculate that they have a more involved role in the plants defense to viral infection as opposed to miRNAs 159 and 172a. This can be further confirmed by looking at their target proteins and their functions during plants stress response. MiRNA 171a targets transcription factors highly involved in the plants development and flowering [2, 28]. MiRNA 168a&b target argonaute 1 (AGO1) protein mRNA [14]. *AGO1* is an essential component of the RISC complex necessary for the silencing of target mRNAs by miRNAs [2]. MiRNA 167a&b target auxin response factors thought to control transcription in response to the phytohormone auxin [29]. Transcriptional regulation is important for many of the diverse developmental responses to auxin signals, which include cell elongation, division, and differentiation in both roots and shoots [2]. Finally, miRNA161 targets PPR (pentatricopeptide) repeat proteins that include various proteins with pentatricopeptide motif in its structure [2]. One very important factor that plays an essential role in miRNA expression during viral infection is the silencing suppressor. Interestingly five of the six, above mentioned, miRNAs have been reported as targets of viral silencing suppressors [30]. Although, ORMV suppressor has not been dis-

covered yet, it is possible to speculate on its existence and its effect on plant response especially since other plant viruses from the same genus have been already documented to have them. Our findings also show that ORMV infection enriches miRNA expression of 21 nucleotides long miRNAs with a 5'-terminal uridine. Additionally, our array screen detected one miRNA* with 5'-terminal guanine, miRNA172*. The increase in accumulation seen for small RNAs initiating with a 5'U is mainly caused by virus-enriched miRNAs, whereas the increase seen for small RNAs starting with a 5'G reflects the accumulation of miRNA* sequences [15]. These findings could be an indication of existence of specific miRNA-associated effector complexes, which requires the specific 5'-terminal, formed upon virus infection as previously suggested by Hu et al. 2011 [15]. Furthermore, we have found that miRNAs analyzed by Northern blot have very strong pri and pre-miRNA signals. This in turn could cause the accumulation of novel miRNA/like sRNAs (ml-siRNAs) from miRNA precursors as seen previously by Hu et al. 2011 [15]. For example we find high pre and pri-miRNA expression for miRNA159. Ml-siRNAs of miR159 precursors are located towards the loop of the precursors, separated by one phase from the miRNA sequence at the lower stems [31, 32]. Since miRNA159 is generated by sequential DCL cleavage of the precursors starting at the loop [31, 32], the ml-siRNAs are likely generated during normal miRNA processing. However, whereas miRNA159 ml-siRNAs may be unstable under normal conditions, it may be stabilized in virus-infected tissues explaining its high pre and pri-miRNA expression during viral infection [31, 32].

The second array data we report here show 29 differentially expressed miRNAs induced by drought stress in *Arabidopsis thaliana*. From those selected for further validation miRNA168a&b shows the greatest up-regulation from control to 13 days drought treated samples. MiRNA171b&c, on the array, shows consistent up-regulation from the control plants. Furthermore, the array and Northern blot results for miRNA399d show consistent up-regulation, however, for miRNA447c they show consistent down regulation. Significant evidence that these miRNAs are directly involved in drought stress response is that all six miRNAs selected show a strong response to rehydration (13 Days dry and 13 Day r-h collection points). By analyzing the up-stream promoter sequence of several miRNAs a number of known stress-responsive elements have been identified: the abscisic acid (ABA)-response elements (ABREs), anaerobic induction response 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) [33]. For us an interesting stress responsive element is the abscisic acid which is produced when plants are undergoing different stresses such as dehydration and cold [33]. In this study two miRNAs reported, miRNA168a&b and miRNA167a&b, have also been shown to have the promoter regions highly enriched with the AREs and ABREs [33] further confirming their involvement in the drought stress response. We have also seen on our Northern hybridizations that certain miRNAs are only detected as pri and pre-miRNAs. MiRNA 399d, 171b&c and 447c Northern blot signal quantification was obtained from the pri- and/or pre-miRNA bands and not from the mature miRNAs. Some miRNAs, such as miRNA 399d, directly respond to stress and external environmental changes. MiRNA399d family is involved in the regulation of phosphate levels in plants [34]. When the Pi levels are low there is an up-regulation of miRNA399d which targets two different genes: a phosphate transporter [34] and a putative ubiquitin conjugating enzyme (UBC24) thus maintaining the Pi homeostasis [34]. Interestingly miRNA399d is also a tissue specific miRNA and its mature forms are found only in the plant roots [35, 36] thus explaining its lack of presence in the shoot of *Arabidopsis thaliana* samples analyzed here. In addition, looking at the previously published data about miRNA171b&c it can be seen that the localization of mature form of miRNA171b&c is in flowers [28] explaining its lack of presence in the shoot of *Arabidopsis thaliana* samples analyzed here. Therefore, several miRNAs analyzed in this study show only presence of pre-miRNAs and/or pri-miRNAs when analyzed on the Northern blot posing the question if some of these miRNAs are transiently expressed during stress and/or development? To answer this question another blot was performed on miRNA 171b&c (Figure 4C). The mature form of miRNA 171b&c in high enough abundance to be detected by the Northern blot was found only in the flowers confirming its tissue specific constitutive expression. On the other hand, it is interesting to note that although miRNA 171b&c has been previously reported [28] to be involved in the plant development it does not show detectable expression of its mature form in either 5 or 14 DPG seedlings. Therefore, our data suggest that the mature form of miRNA 171b&c, in tissues other then flowers, is transient during the plants stress response and during plants development.

CONCLUSION

In conclusion, we have shown that miRNAs are indeed involved in the plants response to viral infection and drought as indicated by the number of differentially expressed miRNAs. We have also shown that each miRNA has its unique mechanism of action during stress response largely dependent on the stress type and target protein identity and its function. Furthermore, some miRNAs such as miRNA 171b&c and 399d have a constitutive

and/or transient tissue specific processing. It is undeniable that miRNAs play a significant role in plants stress response and each with its own unique mechanism of action.

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DECLARATION OF INTEREST

The authors declare no conflict of interest.

REFERENCES

[1] Bartel DP. MicroRNAs: genomics biogenesis, mechanism, and function. Cell 2004; 116: 281-297.
[2] Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel DP. Prediction of plant micro RNA targets. Cell 2002; 110: 513-520.
[3] Kim VN. Micro RNA biogenesis: coordinated cropping and dicing. Nat Rev Mol Cell Biol 2005; 6(5): 376-85.
[4] Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 2008; 9(2): 102-14.
[5] Jones-Rhoades MW, Bartel DP, Bartel B. MicroRNAs and their regulatory roles in plants. Annu Rev Plant Biol.2006; 57: 19-53.
[6] Sunkar R, Chinnusamy V, Zhu J, Zhu JK. Small RNAs as big players in plant abiotic stress responses and nutrient deprivation. Trends Plant Sci. 2007; 12(7): 301-9.
[7] Bartels D, Sunkar R. Drought and salt tolerance in plants. Crit Rev Plant Sci. 2005; 24: 23–58.
[8] Fowler S, Thomashow MF. Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. Plant Cell 2002; 14(20): 1675–1690.
[9] Vance KW, Campo MS, Morgan IM. A novel silencer element in the bovine papilloma virus type 4 promoter represses the transcriptional response to papilloma virus E2 protein. J Virol. 2001; 75(6): 2829-38.
[10] Chapman EJ, Prokhnevsky AI, Gopinath K, Dolja VV, Carrington JC. Viral RNA silencing suppressors inhibit the micro RNA pathway at an intermediate step. Genes Dev.2004; 18: 1179-1186.
[11] Silhavy D, Molnar A, Lucioli A, Szittya G, Hornyik C, Tavazza M, Burgyan J. A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs. EMBO J. 2002; 21: 3070–3080.
[12] Vargason JM, Szittya G, Burgyan J, Hall TM. Size selective recognition of siRNA by and RNA silencing suppressor. Cell 2003; 115: 799–811.
[13] Ye K, Malinina L, Patel DJ. Recognition of small interfering RNA by a viral suppressor of RNA silencing. Nature 2003; 426: 874–878.
[14] Várallyay E, Válóczi A, Ágyi A,Burgyán J, Havelda Z. Plant virus mediated induction of miR168 is associated with repression of ARGONAUTE1 accumulation. The EMBO Journal 2010; 29: 3507-3519.
[15] Hu Q, Hollunder J, Niehl A, Kørner CJ, Gereige D, Windels D. Specific impact of tobamovirus infection on the Arabidopsis small RNA profile. PLoS ONE 2011; 6(5): e19549.
[16] Zhu JK. Salt and drought stress signal transduction in plants. Annu Rev Plant Biol. 2002; 53: 247–273.
[17] Seki, M., M. Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y et al. Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full length cDNA microarray. Plant J. 2002; 31(3): 279–292.
[18] Shinozaki K, Yamaguchi-Shinozaki K. Gene networks involved in drought stress response and tolerance. J Exp Bot. 2007; 58: 221–227.
[19] Kato K, Yokoyama H. Geographical variation in heading characters among wheat landraces, Triticum aestivum L., and its implication for their adaptability. Theor Appl Genet.1992; 84: 259–265.
[20] Boonjung H, Fukai S. Effect of soil water deficit at different growth stages on rice growth and yield under upland conditions. Phenology, biomass production and yield. Field Crop Res. 1996; 48: 47–55.
[21] Ribaut JM, Hoisington DA, Deutsch JA, Jiang C and Gonzalez-de-Leon D. Identification of quantitative trait loci under drought conditions in tropical maize: 1 flowering parameters and the anthesis-silking interval. Theor Appl Genet. 1996; 92: 905–914.
[22] Sunkar R, Kapoor A, Zhu JK. Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in Arabidopsis is mediated by down regulation of miR398 and important for oxidative stress tolerance. Plant Cell 2006; 18: 2051-2065.
[23] Wang JW, Wang LJ, Mao YB, Cai WJ, Xue HW, Chen XY. Control of root cap formation by micro RNA targeted auxin response factors in Arabidopsis. Plant Cell 2005; 17: 2204–2216.
[24] Guo HS, Xie Q, Fei JF, Chua NH. Micro RNA directs mRNA cleavage of the transcription factor NAC1 to down regulate auxin signals for Arabidopsis lateral root development. Plant Cell 2005; 17: 1376–1387.
[25] Ambros V. Micro RNA pathways in flies and worms: growth, death, fat, stress and timing. Cell 2003; 113: 673-676.
[26] Ruggenthaler P. Characterization of the endogenous function of MPB2C-a novel microtubules associated protein in plants. PhD Dissertation, University of Vienna, 2008.
[27] Varkonyi-Gasic E, Wu R, Wood M, Walton EF, Hellens RP. Protocol: a highly sensitive RT-PCR method for detection and quantification of miRNAs. Plant Methods 2007; 3:12.
[28] Llave C, Xie Z, Kasschau KD, Carrington JC. Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. Science 2002; 297(5589): 2053-6.
[29] Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B, Bartel DP. MicroRNAs in plants. Genes Dev. 2002; 16: 1616–1626
[30] Chen MH, Sheng J, Hind G, Handa AK, Citovsky V. Interaction between the tobacco mosaic virus movement protein and host cell pectin methylesterases is required for viral cell-to-cell movement. EMBO J. 2000; 19: 913–920.
[31] Addo-Quaye C, Snyder JA, Park YB, Li YF, Sunkar R, Axtell MJ. Sliced microRNA targets and precise loop-first processing of MIR319 hairpins revealed by analysis of the Physcomitrella patens degradome. RNA 2009; 15(12): 2112-21.
[32] Bologna NG, Mateos JL, Bresso EG, Palatnik JF.A loop-to-base processing mechanism underlies the biogenesis of plant microRNAs miR319 and miR159. EMBO J. 2009; 28(23): 3646-56.
[33] Liu HH, Tian X, Li YJ, Wu CA, Zheng CC. Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*. RNA2008; 14: 836-843.
[34] Chiou TJ. The role of microRNAs in sensing nutrient stress. Plant Cell Environ.2007; 30(3): 323-32.
[35] Pant BD, Buhtz A, Kehr J, Scheible WR. MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. Plant J. 2008; 53(5): 731-8.
[36] Bari R, Datt Pant B, Stitt M, Scheible WR. PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. Plant Physiol. 2006; 141(3): 988-99.