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A common set of developmental miRNAs are upregulated in *Nicotiana benthamiana* by diverse begomoviruses

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Abstract

Background: Begomoviruses are single-stranded DNA viruses that cause economically important diseases of many crops throughout the world and induce symptoms in plants, including enations, leaf curling and stunting, that resemble developmental abnormalities. MicroRNAs (miRNAs) are small endogenous RNAs that are involved in a variety of activities, including plant development, signal transduction and protein degradation, as well as response to environmental stress, and pathogen invasion.

Results: The present study was aimed at understanding the deregulation of miRNAs upon begomovirus infection. Four distinct begomoviruses *African cassava mosaic virus* (ACMV), *Cabbage leaf curl virus* (CbLCuV), *Tomato yellow leaf curl virus* (TYLCV) and *Cotton leaf curl Multan virus*/Cotton leaf curl betasatellite (CLCuV/CLCuMB), were used in this study. Ten developmental miRNA were studied. *N. benthamiana* plants were inoculated with begomoviruses and their miRNA profiles were analysed by northern blotting using specific miRNA probes. The levels of most developmental miRNA were increased in *N. benthamiana* by TYLCV, CLCuMV/CLCuMB and CbLCuV infection with a common pattern despite their diverse genomic components. However, the increased levels of individual miRNAs differed for distinct begomoviruses, reflecting differences in severity of symptom phenotypes. Some of these miRNA were also common to ACMV infection.

Conclusions: Our results have shown a common pattern of miRNAs accumulation upon begomovirus infection. It was found that begomoviruses generally increase the accumulation of miRNA and thus result in the decreased translation of genes involved in the development of plants. Identification of common miRNAs that are deregulated upon begomovirus infection may provide novel targets for control strategies aimed at developing broad-spectrum resistance.

Background

MicroRNAs (miRNAs) are endogenous, approx. 22 nt RNAs that can play important regulatory roles in animals and plants by targeting mRNA for cleavage or translational repression [1]. miRNA are the second most abundant class of RNA among short RNAs [2] that play a very important role in multicellular organisms and influence the output of many protein-coding genes. The first miRNAs were discovered during a study of nematode larval development. Two approximately 22 nt RNAs (the *lin-4* and *let-7* RNAs) control developmental timing by binding to their respective mRNA targets preventing their translation [3,4].

In mid-2002, four groups reported RNAs with miRNA characteristics among the tiny RNAs present in *Arabidopsis* [5-8]. An important difference between plant and animal miRNAs is that the regulatory targets of plant miRNAs can be predicted with a fair degree of confidence, simply by identifying mRNAs with near perfect complementarity [9].

The discovery of miRNAs in plants is still an ongoing process. Much focus has been directed toward miRNA identification in *Arabidopsis* and rice, but many species which are important economically or evolutionarily have yet to be examined. Initial cloning of small RNAs from

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Arabidopsis and rice has revealed that plants are extremely rich in endogenous small RNAs and that only a small portion of cloned small RNAs correspond to miRNAs [6,10]. The majority of endogenous small RNA species represent small interfering RNAs (siRNAs). The difference between miRNAs and siRNAs lies in their biogenesis. miRNAs originate from the processing of single-stranded precursors that form a hairpin structure, whereas siRNAs are generated from long double-stranded RNAs (dsRNAs) or single-stranded RNAs that form hairpin structures [6].

Plant miRNAs have a high degree of sequence complementarity to their target mRNAs and direct the slicing of the target mRNAs in the middle of the complementary regions [10,11]. This has been demonstrated by the detection of 3' cleavage products that have 5' ends that start in the middle of the complementary regions. This is probably mediated by AGO1 [12,13]. However, plant miRNAs also regulate gene expression by translational repression [14-17].

Geminiviruses are an important group of plant viruses with small circular, single-stranded (ss) DNA genomes that replicate in the nucleus of infected cells [18]. Viruses of the family *Geminiviridae* are divided into four genera based on insect vectors and genome organization [19]. Whitefly-transmitted geminiviruses are classified in the genus *Begomovirus* and constitute the largest genus that causes economically-important diseases throughout the warmer parts of the world [19-21]. Begomoviruses originating from the New World are invariably bipartite, with genomes consisting of two ssDNA components, known as DNA A and DNA B, of approximately equal size (~2.8 kb). Although a few bipartite begomoviruses are known in the Old World, the vast majority of begomoviruses are monopartite with a genome that is a homolog of the DNA A component of the bipartite viruses, and most of these interact with ssDNA satellites [22]. Some begomoviruses, such as Tomato yellow leaf curl virus (TYLCV), are monopartite while a DNA satellite resembling betasatellite was found associated with Tomato leaf curl virus (ToLCV) [23].

Plant virus infections can result in disease symptoms that may include chlorosis and/or necrosis, curling, stunting and altered plant stature and morphology, presumably caused by interference of the infection with developmental processes [24]. In recent years, it has been proven experimentally that short RNA (sRNA), and particularly miRNAs, play important roles in plant development and are implicated in host-pathogen interactions [25,26].

Recent studies in plants and animals suggest that viruses can suppress gene expression and use endogenous RNA-silencing pathways to regulate host gene expression, presumably to benefit virus replication [26-29]. However, the underlying mechanisms that control these activities remain unclear. Epstein-Barr virus and other DNA viruses encode miRNAs that directly down- or up-regulate host and/or viral mRNAs [28].

Several studies have demonstrated that viral suppressors of RNA silencing can interfere with miRNA-mediated regulation of host genes [30,31]. These studies showed that viral proteins interfere with miRNA pathways, although it is unclear whether it is part of the virus replication strategy or a side effect due to the overlap of the siRNA and miRNA pathways.

Transgenic expression in plants of the AC4 protein from ACMV, a suppressor of post-transcriptional gene silencing (PTGS) [32], was correlated with decreased accumulation of host miRNAs and increased development abnormalities in *Arabidopsis thaliana* [33]. Down- regulation of miRNA correlated with an up-regulation of target mRNA level. Another study showed that infection of *N. tabacum* by plant RNA viruses representative of the *Tobamoviridae, Potyviridae*, and *Potexviridae* families altered accumulation of certain miRNAs [34].

In the present study the accumulation of ten miRNAs were studied as a result of the infection of four viruses belonging to different types of *begomovirus*. *African cassava mosaic virus* (ACMV; representing a bipartite virus from the Old World), *Cabbage leaf curl virus* (CbLCuV: representing a bipartite virus from New World), Tomato yellow leaf curl virus (TYLCV; representing a monopartite virus) and *Cotton leaf curl Multan virus*/cotton leaf curl Multan betasatellite (CLCuMV/CLCuMB: representing a betasatellite requiring monopartite virus) were used in this study. The accumulation of miRNA as a result of the infection of each virus was then compared to assess the possible role of specific miRNAs in virus pathogenicity.

Results

Virus infection

Potato virus X (PVX) infection of *N. benthamiana* resulted in mild symptoms including mild vein yellowing, very mild vein thickening and a faint mosaic that appeared approx. 10 dpi. Additionally, at approx. 15 dpi, *N. benthamiana* plants infected with PVX ceased to show symptoms, indicative of recovery (Figure 1 panel B). Infection of CLCuMV/CLCuMB resulted in downward leaf curling, vein yellowing, stunting, vein swelling, and the formation of small enations on the veins on the undersides of leaves (Figure 1 panels C and D). *N. benthamiana* plants infected with CbLCuV showed mild symptoms after 16 dpi that include mild leaf curling and deformed leaves at the margins (Figure 1 panel G). TYLCV infection in *N. benthamiana* resulted in a stunted growth, severe downward leaf curling and vein

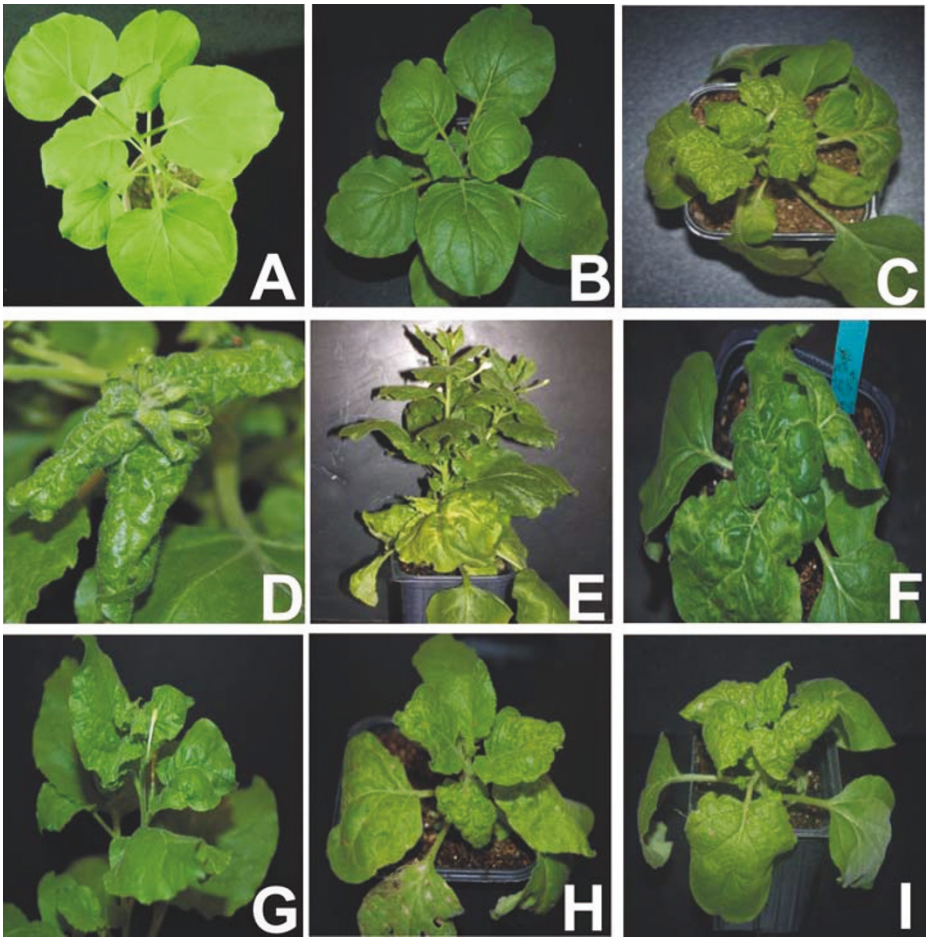

Figure 1 Photographs of a healthy *N. benthamiana* plant (panel A), and *N. benthamiana* plants infected with PVX (panel B), CLCuMV/CLCuMB (panels C and D), ACMV (panels E and F) CbLCuV (panel G) and TYLCV (panels H and I). Photographs were taken at approx. 21 dpi.

yellowing (Figure 1 panels H and I). The presence of each virus was confirmed by PCR using specific primers designed to the replication-associated gene of each virus (data not shown).

Effects of virus infection on miR156 levels

Upon infection of *N. benthamiana* by ACMV and CbLCuV the level of miR156 decreases as compared to *N. benthamiana* while the infection of TYLCV and CLCuMV resulted in an increased accumulation of miR156 (Figure 2). PVX infection, which was used in this study as a control and a representative RNA virus, resulted in an increased accumulation of miR156 when compared with un-inoculated *N. benthamiana* plant (Figure 2).

- miR159

Infections of all begomoviruses under study resulted in an increase in the accumulation of miR159 when compared with healthy *N. benthamiana* plants. The highest increase was observed in case of the infection of CbLCuV (Figure 2). PVX infection also resulted in elevated level of miR159 as compared to healthy control (Figure 2).

- miR160

Infections of CbLCuV, TYLCV and CLCuMV resulted in a slight increase while ACMV resulted in decrease in the levels of miR160 when compared with healthy control plants (Figure 2). PVX infection also resulted in a slight decrease in the level of miR160 (Figure 2).

-miR164

N. benthamiana plants infected with ACMV, CLCuMV, CbLCuV and TYLCV showed an increase in the level of miR164 when compared with un-inoculated healthy plant. A maximum increase was observed in the infection of CbLCuV (Figure 2). Infection of PVX also resulted in an increase accumulation of miR164 (Figure 2).

-miR165 and miR166

Infection of *N. benthamiana* plants with ACMV, CLCuMV, CbLCuV and TYLCV resulted in an increase

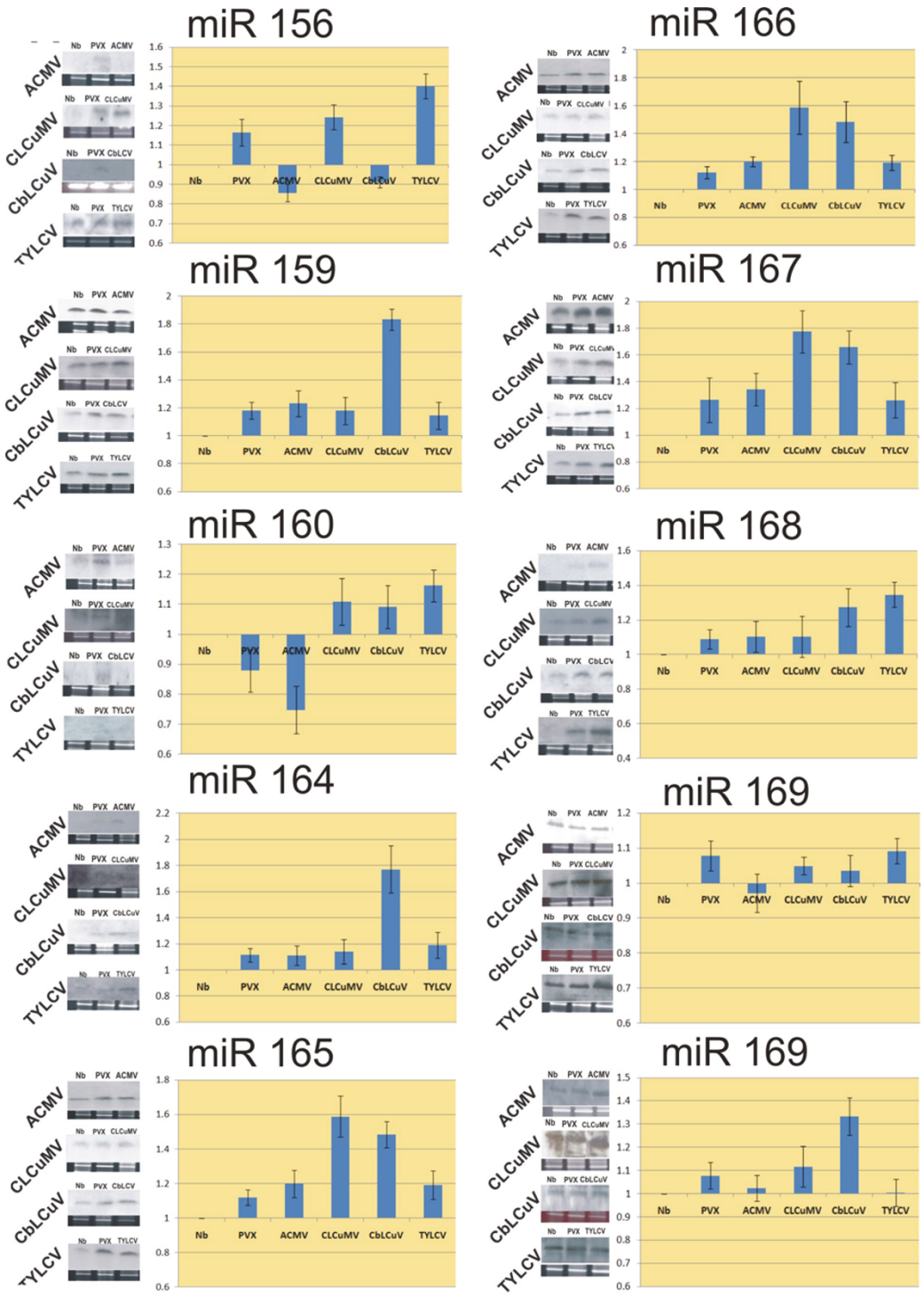

Figure 2 Effects of virus infection on the levels of selected miRNAs. Northern blot analysis to detect the accumulation of miR156, miR159, miR160, miR164, miR165, miR166, miR167, miR168, miR169 and miR170 after infection with begomoviruses and PVX. Shown below each blot is the rRNA band of the ethidium bromide stained agarose gel that was used to normalize the data for loading. The bar graphs show the average (with standard error) levels calculated for each miRNA levels for *N. benthamiana* plants infected with ACMV, CLCuMV/CLCuMB, CbLCuV, TYLCV and PVX. The relative levels of miRNA for virus infected plants were calculated by taking the levels in healthy *N. benthamiana* as 1.0. All samples were taken at 21 dpi.

in the accumulation of miR165/166 when compared with the healthy control plants. The highest increase was observed in plants infected with CLCuMV (Figure 2). Infection of PVX also resulted in an increase in the accumulation of miR165/166 when compared with healthy *N. benthamiana* plants (Figure 2). However, this increase in accumulation of miR165/166 was less than for the begomovirus infection.

-miRNA 167

Infection with ACMV, CLCuMV, CbLCuV and TYLCV showed an increase in the accumulation of miR167 when compared with healthy control. Plants inoculated with PVX showed an increase in the level of miR167 though the increase was less than that caused by ACMV, CLCuMV and CbLCuV infections (Figure 2).

- miR168

Infection of *N. benthamiana* plants ACMV, CbLCuV, CLCuMV and TYLCV showed an increase in level of miR168 when compared with healthy plants. Infection of PVX also resulted in increased accumulation of miR167 which was comparable with ACMV and CLCuMV infections. A significant increase was observed for CbLCuV and TYLCV infections (Figure 2).

-miR 169 and miR170

Infection of ACMV does not alter the level of miR169 significantly in *N. benthamiana* plants however, a slight decrease was observed when compared with healthy control plants (Figure 2). A slight increase in miR169 was observed in case of the infection of CLCuMV, CbLCuV and TYLCV (Figure 2). PVX infection in *N. benthamiana* also resulted in an increase in level of miR169 (Figure 2). A heat diagram summarizing the miRNA profiles in response to infection with the selected begomoviruses is given in Figure 3.

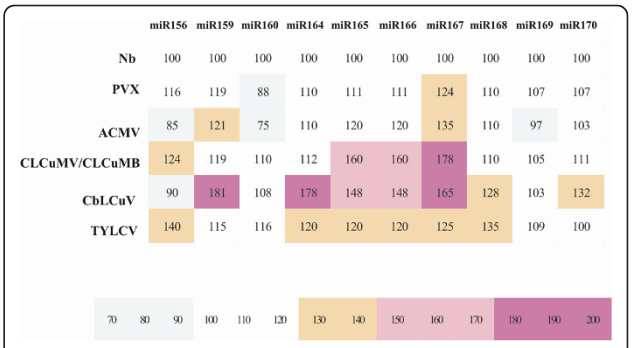

Figure 3 A heat diagram summarizing the levels of miRNAs detected in *N. benthamiana* upon virus infection. The signal of each band was quantified using imaj J and normalized with ethidium bromide stained RNA. The levels of miRNAs in *N. benthamiana* were taken as 100 and rest of the miRNA levels were calculated relative to that and color scheme is given which is also shown.

Discussion

Recent studies of both animal and plant viruses have shown that viruses alter the RNA silencing pathway to regulate host gene expression [35,36]. One of the limitations at present is that the mechanisms controlling such activities are unclear. However, a generally accepted concept is that RNA silencing is a natural defense response of plants against invading viruses. To counter RNA silencing viruses encode certain proteins that can block the RNAi pathway and are referred to as suppressor of gene silencing [36,37]. It has been demonstrated that viral suppressors of RNA silencing can interact/interfere with the miRNA pathway [30,31], although it remains unclear whether these interactions are the part of the survival strategy of viruses or just side effects (collateral damage) of their infection cycle. In the work presented here the interaction begomoviruses ACMV, CLCuMV and its associated CLCuMB, TYLCV and CbLCuV with selected host miRNAs was studied. With the exception of CbLCuV, these viruses are well characterized as far as suppressors of gene silencing are concerned. TrAP and AC4 encoded by ACMV [32], the V2 protein encoded by TYLCV [38], the TrAP, C4 and V2 proteins of CLCuMV and βC1 encoded CLCuMB have been shown to have suppressor of gene silencing activity (Amin et al., manuscript in preparation).

miR156 has been shown to act on SQUAMOSA PROMOTER BINDING PROTEIN (SPL), which is believed to be a transcription factor [39,40]. The accumulation of miR156 upon infection by selected begomovirus showed that these viruses can be divided into two groups. Infections by the bipartite viruses (ACMV and CbLCuV) lead to a decrease in miR156 while infections by the monopartite viruses (CLCuMV and TYLCV) lead to an increase. It has been shown that SPL3 is a target of miR156 and constitutive expression of miR156 results in a prolonged juvenile vegetative phase and delayed flowering [40].

miR159 was identified independently by two groups [8,41] and is thought to target mRNAs coding for MYB proteins which are known to bind to the promoter of the floral meristem identity gene LEAFY (LFY; [9]). The LFY genes play an important role during the transition from the vegetative to the reproductive phase, as it is both necessary and sufficient for the initiation of individual flowers [42]. LFY is extensively expressed during the vegetative phase of plant growth [43]. Thus, the reduction in the expression of the LFY gene plays an important role in the transition from the vegetative phase to the sexual phase. A uniform pattern of up-regulation was observed for the accumulation of miR159 as a result of the infection of begomoviruses. These findings are in line with the recent findings where it was shown that upon infection of Tomato leaf curl New

Delhi virus (ToLCNDV) the level of miR159 was increased. In the same studies it was also shown that the symptom development may also be due to the upregulation of this miRNA [44].

TYLCV REn interacts with at least two host-encoded proteins, PCNA and the RBR that play an important role in altering the cell cycle [45]. A major function of RBR proteins is to control the expression of many genes required for cell cycle progression, by regulating the activity of E2F transcription factors [46]. The study here has shown a further way that REn may influence the cell-cycle of the host, by up-regulating miR159. CbLCuV has been shown to alter expression of cell cycle-associated genes, preferentially activating genes expressed during the S and G2 phases as well as inhibiting genes active in G1 and M phases. A limited set of core cell cycle genes associated with cell cycle reentry, late G1, S, and early G2 had increased RNA levels, while core cell cycle genes linked to early G1 and late G2 had reduced transcripts [47].

miR160 is encoded on chromosome 2 in *Arabidopsis* [8] and targets mRNAs coding for auxin response factor (ARF) proteins [9,31]. ARFs are a major class of transcription activators and repressors that facilitate the auxin signal by binding to specific cis-elements in the upstream regions of auxin-inducible genes [48]. The study presented here has shown a basic difference in the infection patterns of OW bipartite and monopartite viruses. ACMV infection resulted in decreased accumulation of miR159, whereas for the OW monopartite virus infection resulted in the increased accumulation of miR160. The major role in these interaction could be of βC1 in case CLCuMV/CLCuMB and TrAP/V2 of TYLCV because the phenotypes produced by V2 of TYLCV and βC1, when expressed from the PVX vector in *N. benthamiana*, produced virus-like symptoms [49,50]. These symptoms suggest that the auxin response has been disturbed. The finding that CLCuMV/CLCuMB, TYLCV and CbLCuV infections of *N. benthamiana* resulted in an increase in the levels of miR160 also suggest a general behavior of begomoviruses infection in reducing the response to auxin in infected plants, although this was not the case for ACMV. This suggestion will need further experimental confirmation. Earlier studies with the *Curtovirus* BCTV showed that infection reduces auxin levels, but the authors were unable to show a correlation between reduced auxin and visible symptoms [51].

The miRNA miR164 negatively regulates several genes that encode NAC-like transcription factors [7,31,52,53]. These genes include CUP-SHAPED COTYLEDON 1 (CUC1) and CUC2, which are expressed in, and are necessary for, the formation of boundaries between meristems and emerging organ primordia [54-56]. Failure to establish organ boundaries leads to severe developmental consequences [57]. Infections of all four begomoviruses resulted in an increased accumulation of miR164, suggesting that the viruses down regulate the NAC-like transcription factors. This effect upon miR164 may be one of the contributing factors to the induction of disease symptoms for these viruses. We have observed that with the exception of CLCuMV, there is a significant increase in the levels of miR164 upon PVX-mediated expression of the TrAP genes of all viruses under study (Amin et al, unpublished data). Therefore, TrAP might be the gene responsible for this interaction of viruses with miR164. It has been shown that the TrAPs of ToLCNDV, *Papaya leaf curl virus* (PaLCuV) and CLCuKoV can counter a HR induced by NSP (ToLCNDV) or V2 (PaLCuV and CLCuKoV [58,59]. Virus up-regulation of miR164 may provide a possible explanation of this phenomenon. A recent study has shown that oxygen responsive elements 1 (ORE1), which is a NAC-like transcription factor, positively regulates aging-induced cell death in *Arabidopsis thaliana* leaves. ORE1 expression is up-regulated concurrently with leaf aging by ethylene insensitive 2 (EIN2) but is negatively regulated by miR164. miR164 expression gradually decreases with aging through negative regulation by EIN2, which leads to the up-regulation of ORE1 expression [60] and thus to the cell death. Up-regulation of miR164 thus will counter the cell death, and thus possibly also HR associated cell death due to NSP and V2.

A uniform pattern of up-regulation was observed with relation to the accumulation of miRNA165/166. miR165 is found on chromosome 1 in *Arabidopsis* and regulates HD-ZIPIII transcription factor genes, PHABULOSA (PHB) and PHAVOLUTA (PHV; [9]). It has been shown in *Arabidopsis* that the establishment of leaf polarity requires the generation and perception of positional information along the radial axis of the plant [61]. The results presented here also showed that in general, begomovirus infection resulted in increased accumulation of miR165/166. The genes which upon inoculation produced virus like symptoms may be involved in this interaction. It has been earlier reports that transgenic *Arabidopsis* expressing the TYLCCNB βC1 exhibited virus-like symptoms. These morphological changes were paralleled by a reduction in miR165/166 levels and an increase in PHB and PHV transcript levels. Two factors, ASYMMETRIC LEAVES 1 (AS1) and ASYMMETRIC LEAVES 2 (AS2), are known to regulate leaf development as an AS1/AS2 complex [62]. βC1 is able to partially complement *as2* mutation. We also observed significant decrease in the levels of miR165/166 upon PVX-mediated expression of CLCuMB βC1 (Amin et al., unpublished data), CLCuMV/CLCuMB infection led to an increased accumulation.

The results show that begomovirus infection generally increases the accumulation of miR167. It has been shown that miR167 targets ARF 6 and ARF8 [9,63]. ARF proteins regulate embryogenesis, root development and floral organ formation [63-67]. ARF6 and ARF8 regulate flower maturation [68]. Infection of plants with some begomoviruses, as well as constitutive expression of some of their genes in plants results in severe developmental defects. For example, transgenic expression of ACMV AC4 in *Arabidopsis* resulted in stunted plants with severe developmental defects, such as narrow rosette leaves and lack of development of reproductive tissue [33]. Similarly the transgenic expression of βC1 in *N. benthamiana* as well as expression on βC1 from PVX resulted in severely twisted plants [49,69,70]. Thus, the results presented here suggest a possible mechanism for the induction of these virus-like symptoms.

With the exception of CbLCuV, infection of *N. benthamiana* plants with all viruses under study resulted in a slightly increased accumulation in the levels of both miR169 and miR170 which suggests that begomovirus infection does not significantly affect the levels of these miRNAs. It has been shown that miR169 and related miRNAs are strongly dependent on P or N status in *Arabidopsis* and rapeseed (*Brassica napus*) phloem sap, flagging them as candidate systemic signaling molecules [71].

The study presented here has shown that, in general, begomovirus infection (assuming that the four viruses originating from distinct classes of begomoviruses [NW, OW, bipartite, monopartite and betasatellite requiring] are representative) increases the accumulation of miRNAs. This finding is in agreement with an earlier study of RNA viruses from three distinct families (*Tobamoviridae*, *Potyviridae*, and *Potexviridae*) that examined a similar range of miRNAs [34]. However, it is difficult to reconcile the presence for all these viruses (both the begomoviruses and the RNA viruses) of proteins that apparently bind (and presumably inactivate/down-regulate) miRNAs with a system that ultimately leads to miRNA up-regulation.

Methods

Virus Infections

Agroinoculable infectious clones of begomovirus isolates CLCuMV-His[PK:Mul] (AJ496461), ACMV-[CM:DO3:98] (DNA-A, AY211885; DNA-B, AF112353), CbLCuV-[US:Flo:96] (DNA-A, U65529; DNA-B, U65530) and TYLCV-Mld[ES:72:97] (AF071228) were used to infect *N. benthamiana* plants. Three leaves per plants were inoculated. Samples were collected at 21 dpi. The whole experiment was done on two independent biological replicates.

miRNA Analysis

Total RNA was isolated from leaves using TRIzol reagent (Invitrogen, Carlsbad, CA), 1/5 volume of 5× RNA loading dye (95% deionized formamide, 0.025% bromophenol blue, 0.025%Xylene cyanol FF, 5 mM EDTA (pH8), 0.025% formaldehyde; 20 μl of 10 mg/ml ethidium bromide was added per 2 ml of dye) was added to 30 μg of total RNA. After heating that at 65°C for 5 min, sample was placed on ice for 2 min before loading it to TBE gel (15%TBE; 7 M urea). Gel was run in 1× TBE buffer at 150-180 volts for 2 to 2.5 hours. After removing gel from the cassette it was photographed under UV trans-illumination and details of the samples were documented.

RNA was transferred to Hybond N+ (Amersham) by a semi-dry blotting system (Bio-Rad) at 10-12 volts for 45-60 min. The membrane was air dried, UV cross-linked and stored at 4°C between two sheets of wet Whatman filter paper.

Oligonucleotide primers complementary to *Arabidopsis* miRNAs (miR156, miR159, miR160, miR164, miR165, miR166, miR167, miR168, miR169 and miR170) were end-labeled using a DIG olig-labeling kit according to manufacturer instructions (Roche, USA). The sequences of oligo-nucleotides used for end labeling are listed in Table 1. Blot was hybridized with probe at 42°C for 12-16 hours. Washing was done with 2XSSC, 1% SDS and 1× SSC, 0.1%SDS for 30 min each. The blot was developed by using the CDP-Star method according to the manufacturer instruction (Roche) and image was taken on X-ray film (hyper film, Amersham, UK).

The intensity of bands was quantified by using software ImageJ. Data from these analyses were used to normalize the intensity of each band, based on rRNA loaded in each well. For virus infection the values for the miRNA species in non-infected *N. benthamiana* plants were set at 1 and other data calculated relative to this value. The data shown in Figure 2 is the average of

Table 1 Name and sequence of oligonucletides used for end labeling

miRNA	Sequence
miR156	5′-TGACAGAAGAGAGTGAGCAC-3′
miR159	5′-TTTGGATTGAAGGGAGCTCTA-3′
miR160	5′-TGCCTGGCTCCCTGTATGCCA-3′
miR164	5′-TGGAGAAGCAGGGCACGTGCA-3′
miR165	5′-TCGGACCAGGCTTCATCCCCC-3′
miR166	5′-TCGGACCAGGCTTCATTCCCC-3′
miR167	5′-TGAAGCTGCCAGCATGATCTA-3′
miR168	5′-TCGCTTGGTGCAGGTCGGGAA-3′
miR169	5′-CAGCCAAGGATGACTTGCCGA-3′
miR170	5′-TGATTGAGCCGTGTCAATATC-3′

two independent biological replicates along with standard deviation (SD).

Experimental design

A group of 15 *N. benthamiana* plants were agro-infiltrated with infectious clones of ACMV, CLCuMV/CLCuMB, CbLCuV and TYLCV in two independent experiments. Potato virus X (PVX) was used as reference for virus infection with an RNA genome. All plants were kept in the same green house for duration of each experiment.

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Authors' contributions

IA, BP performed the experiments. IA, RWB, SM and CMF conceived the study and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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