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Brief Report

Changes in host microRNA expression during citrus tristeza virus induced disease

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Abstract

The physical effects of disease induced by citrus tristeza virus (CTV) on its citrus host have long been known, but not how disease is induced either by the virus, or its effects on the host at the genetic level. In this study we aimed to examine the latter, by deliberate inoculation of *Citrus macrophylla* with a mutant T36-based infectious clone known to induce stem pitting and other growth abnormalities. The microRNA populations of disease-expressing plants were compared with plants infected with asymptomatic, wild-type T36, and with un-inoculated controls. We found that while virus infection induced change in expression of a large number of miRNAs, the onset of disease correlated with the downregulation of miR164, a miRNA associated with vascular differentiation. This is the first evidence of specific host miRNAs associated with the induction of a CTV disease syndrome.

Keywords: citrus tristeza virus, disease, microRNA, miR164, symptom expression

Introduction

Many viruses that infect perennials cause diseases of development, permanent physiological changes in the growth and differentiation of cells and tissues. Citrus tristeza virus (CTV: genus *Closterovirus*, family Closteroviridae) causes such alterations in citrus growth. For example, stem pitting diseases of citrus are localized changes in the pattern of vascular tissue differentiation, resulting in phloem hyperplasia and a corresponding lack of xylem element formation (Brlansky et al. 2002). This leads to twisted, weakened branches and reduced fruit quality. Yet understanding how viruses induce diseases of development is difficult due to the localized nature of the symptoms; pitting may occur on one stem, or one side of a stem, and not another, while the pits themselves are surrounded by asymptomatic cells (Schneider 1957). Another disease phenotype in citrus caused by CTV, referred to as seedling yellows, results in the stunting and chlorosis of new growth, and ranges in severity from mild chlorosis of the leaves, through to stunting of the leaf petioles, compression of shoots on susceptible hosts and, in severe cases, sieve tube necrosis (Moreno et al. 2008). Histological studies have shown that the induction of the seedling yellows disease syndrome increases cambial activity in the roots, and to a lesser extent the shoots, inducing phloem hyperplasia, and reducing xylem formation (Schneider 1957).

How then, does the virus induce such changes? It has been shown that specific sequence variants of one of the CTV-encoded suppressors of silencing, p23, is involved in the induction of seedling yellows, and it has been speculated to induce disease through interference with host microRNA pathways (Albiach Marti et al. 2010).

MicroRNAs (miRNA) function as post-transcriptional regulators of gene expression and are, in effect, the switches by which a plant directs its growth and development. There are multiple means by which viruses can interfere with host miRNA activity, including expression of suppressors of RNA silencing (Kasschau et al. 2003; Lewsey et al. 2007), viral structural and movement proteins (Bazzini et al. 2009), and production of viral-derived small RNAs that mimic miRNA activity (Moissiard and Voinnet 2006). Of these, suppressors of silencing are the most well understood, and have been shown to act directly by sequestering miRNA and miRNA/miRNA* complexes, preventing their incorporation into the RNA-induced silencing complex (Kasschau et al. 2003; Lewsey et al. 2007; Shiboleth et al. 2007), or indirectly, by targeting components of the silencing pathway, such as the Argonaute1 protein and inhibiting miRNA cleavage of mRNAs (Zhang et al. 2006; Lewsey et al. 2007).

In this study, we compared the patterns of miRNA expression in *C. macrophylla* infected with a mutant that induces severe stem pitting and seedling yellows-like

symptoms to asymptomatic seedlings, and to uninfected seedlings through small RNA sequencing and real-time PCR (RT-qPCR). We found that changes in expression of most known miRNAs correlated with CTV infection rather than the expression of a specific phenotype. Only one, miR164, was found to be differentially expressed between symptomatic and asymptomatic plants. This is the first evidence of specific host miRNAs associated with the induction of a CTV disease syndrome.

Materials and Methods

Inoculations and small RNA sequencing

Six-month old *C. macrophylla* were graft-infected with a T36 isolate-based infectious clone containing non-synonymous mutations in the p61 (positions 17 and 531), p65 (position 222), p25 (position 14), and p27 (position 4) ORFs, which produces severe yellowing and stem pitting symptoms on this host. As a control, an equal number of plants were inoculated with wild-type T36, which is largely asymptomatic in *C. macrophylla*. Uninfected *C. macrophylla* acted as a negative control. Plants were maintained under greenhouse conditions and tested for CTV presence by ELISA at 3 months post-inoculation. Symptoms first appeared at 4 months post-inoculation, and were allowed to develop for 2 further months, at which time small RNA was extracted from 2 g samples of peeled bark tissue from single symptomatic, asymptomatic, or uninfected tissue as per Harper (2013).

Small RNA libraries from single symptom-expressing, asymptomatic, and uninfected *C. macrophylla* were constructed using the ABI SOLiD small RNA expression kit (Applied Biosystems Inc., Foster City, CA) as per the manufacturer's protocol and sequenced using a SOLiD 5500xl platform at the Interdisciplinary Center for Biotechnology Research, University of Florida. The resulting reads for each sample were trimmed to remove adapters, and reads with a length of less than 19 nt and greater than 25 nt were discarded. Each sample library was then depleted by removal of CTV specific reads, plant snoRNA databases (Brown et al. 2003), Arabidopsis tRNA and mitochondrial sequences (Unseld et al. 1997), and the *C. sinensis* chloroplast (Bausher et al. 2006). The sequence of the mutant infectious clone was also derived from siRNA reads and deposited in the NCBI GenBank database under accession MK018120.

Analysis of miRNA expression

The reads resulting from the depleted libraries were sorted, counted and annotated against miRBase 19 (Kozomara and Griffiths-Jones 2011), then each sample library was normalized by scaling (number of copies per 10^6 reads). MicroRNA expression of mutant and wild-type samples was calculated using Kal's z-test using the normalized data against the uninfected reference sample, with a false discovery rate (FDR) q-value threshold of 0.1; only samples that showed a greater than 1 fold change were considered for further analysis. Target genes of the identified miRNAs in the *C. sinensis* genome were predicted using psRNATarget server (Dai and Zhao 2011) with an expectation value of 2.0; BLASTX searches were conducted on the identified targets to locate homologues of known function.

Quantification of miRNAs by real-time PCR

Enriched small RNA samples from 3 symptomatic, 2 asymptomatic, and 3 uninfected plants were polyadenylated using the Ambion Poly (A) tailing kit, as per the manufacturer's instructions (Invitrogen, Carlsbad, CA). Reverse transcription was performed using 50 µl of polyadenylated small RNA with Invitrogen Superscript III (Invitrogen) with a 3' poly(T) adapter containing specific reverse priming and probe binding sites (5'-CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGC AATTTTTTTTTTTVN-3') developed from the methods of Shi and Chang (2005) and Kang et al. (2012). RT-qPCR was performed using 400 nM each of miRNA sequence-specific forward (Table 1) and general adapter-specific reverse (5'-CAGTGCAGGGTCCGAGGT-3') primers and 100 nM of Taqman probe (5'-FAM-CAGAGCCACCTGGGCAATTT-BHQ1-3'), in a total reaction volume of 20 µl. The expression levels of known and putative miRNAs from symptomatic and asymptomatic samples were calculated against uninfected control values as per Livak and Schmittgen (2001), and samples normalized against the geometric mean of Ct values of the five most invariant miRNAs (miR156, mirR159, miR395, miR858, and miR3948). Samples, tested in technical replicates of three, of each type (three symptomatic and two asymptomatic biological replicates) were averaged to calculate the normalized mean expression, and standard error calculated.

Histological analysis

Tissue samples from the main taproot of *C. macrophylla* infected with the clone, with wild type T36, or an uninfected negative control, were collected and thin sections made on a cryo-microtome (Harris Manufacturing Co., North Billerica, MA). Sections were stained with Azure A, mounted on glass slides, and examined on an Olympus BX61 light microscope (Olympus America Inc., Melville, NY).

Results

Symptom Development

C. macrophylla infected with mutant virus developed vein clearing and leaf cupping within 4 months of infection, followed by the emergence of shortened internodes and yellowing of newly developing flush. New growth of these plants remained stunted throughout the experiment, whilst older leaves developed vein corking symptoms by 6 months post-inoculation. These symptoms were similar to seedling yellows symptoms induced in sour orange, grapefruit, and lemons by severe isolates of CTV. Severe stem pitting also was observed on the wood of these plants when sampled. The T36 samples exhibited only mild

vein clearing and slight leaf cupping symptoms over the course of the experiment.

When the infected plants were cross-sectioned and stained with Azure A we observed that those plants expressing the severe disease syndrome had fewer layers of xylem tissue and hyperplasic phloem in the stem. This cytopathology was even more pronounced in the root sections. In addition, phloem fibers were either distorted, or in many cases, absent from root tissue sections (Figure 1). Plants infected with wild-type T36 did not exhibit these changes and were visually indistinguishable from uninfected controls.

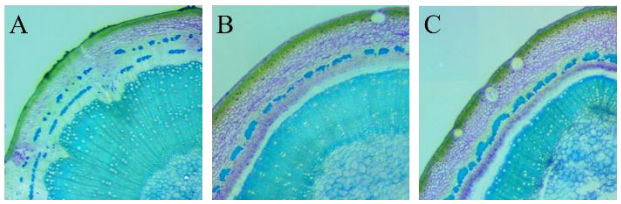

Fig. 1. Cytopathological effects of the CTV mutant (A) on *C. macrophylla* taproot sections, as compared to wild-type T36 CTV (B), and an uninfected control (C). Observe the distortion of the phloem fibers (dark blue) and stem pitting ingrowths into the xylem (light blue) in symptomatic tissue.

Correlation of severe symptoms with miRNA expression

Comparison of normalized miRNA expression levels in 1) CTV-infected tissues expressing severe symptoms, and 2) asymptomatic tissue, to 3) uninfected tissue indicated that the majority of miRNAs expressed in all three tissue types were stable, with less than 1-fold variation (data not shown). Differential expression, defined as greater than one-fold change (FDR q=0.1) relative to the uninfected control was observed in the small RNA libraries for 21 miRNAs (Table 1) regardless of phenotype, of which 15 were downregulated and 6 upregulated in diseased tissue versus 11 and 9 respectively in asymptomatic tissue. The expression of 8 of the aforementioned miRNAs differed between severely symptomatic and asymptomatic tissues; 6 miRNAs: miR166e, -167a & b, 169a, -390b, -473 and -482a were downregulated in the severely symptomatic tissue yet upregulated in asymptomatic tissue, while miR171 and -396b were the opposite (Table 1).

To confirm these findings, we quantified the expression of 17 differentially expressed miRNAs in symptomatic and asymptomatic samples using real-time RT-qPCR and normalized against levels in uninfected *C. macrophylla* controls (Table 2); 4 miRNAs could not be analyzed due to lack of amplification in one or more sample types. Real-time RT-qPCR quantification indicated that only 10 of the 17 miRNAs tested had greater than 1-fold (mean normalized expression, Log2) change from the uninfected control, and of those, 4 were upregulated, 6 downregulated, and 1, miR164, differentially expressed (Table 2).

Table 1
Differentially expressed miRNAs present in diseased (SYM) and asymptomatic tissue (WT) versus uninfected (HLY) *C. macrophylla* as identified by sequencing.

	miRNA	Variant(s)	Citrus Target Gene Ref(s)	Activity	Target Protein	Change in miRNA abundance		
						SYM vs HLY	WT vs HLY	WT vs SYM
miRNAs downregulated in SYM Tissue	MIR1515		1g013686	Translation	Putative serine/threonine-protein kinase	-5.30	-1.44	-3.67
	MIR160		1g008088	Cleavage	Auxin response factor	-12.37	-2.02	-6.12
	MIR160	a, b, c	1g008078	Cleavage	Auxin response factor	-5.89	-1.92	-3.06
	MIR166	e	None			-5.36	1.13	-6.08
	MIR167	a, b	1g008134	Translation	Long chain acyl-CoA synthetase	-1.65	1.38	-2.34
	MIR169	a	1g001072	Cleavage	Zinc finger protein	-1.72	5.94	-10.20
	MIR172	a	1g001809	Translation	Putative transcription factor NPL8-like	-12.38	-1.70	-7.30
	MIR390	b	1g037313	Cleavage	LRR receptor-like serine/threonine protein kinase	-1.37	1.54	-2.12
	MIR3954		1g037457	Cleavage	NAC domain-containing protein	-2.54	-1.05	-2.42
	MIR399	e	None			-1.99	-1.02	-1.94
	MIR473		1g37028	Cleavage	GRAS family transcription factor	-2.46	3.59	-8.81
	MIR479		1g008484	Translation	Laccase 11-like	-2.67	-1.09	-2.46
	MIR482	a	1g016717	Translation	Palmitoyl-protein thioesterase 1-like	-3.45	2.62	-9.03
	MIR530	a	1g041930	Cleavage	Phosphate transporter PHO1-like	-6.39	-6.25	-1.02
	MIR396	a	1g023105	Cleavage	Growth-regulating factor 5-like	-1.17	-1.20	1.02
miRNAs upregulated in SYM Tissue	MIR164	a, b	1g047710	Cleavage	Cup-shaped cotyledon 2-like	1.17	2.00	-1.72
	MIR169	b, c	1g037423	Cleavage	Nucleolar GTP-binding protein 1-like	1.49	2.83	-1.91
	MIR171		1g005237	Cleavage	Scarecrow-like protein 6-like	1.36	-3.03	4.12
	MIR3951		1g019390	Cleavage	Hypothetical Protein	4.39	1.60	2.74
	MIR396	b	1g014868	Cleavage	Growth-regulating factor 2-like	1.44	-1.20	1.73
	MIR477	a, c	1g37028	Cleavage	GRAS family transcription factor	2.55	1.78	0.52
	MIR530	b	1g035505	Cleavage	Hypothetical Protein	*	*	1.51

Table 2
Normalized mean expression (Log2) of miRNAs in CTV infected symptomatic and asymptomatic *C. macrophylla* against uninfected controls, as determined by real-time RT-qPCR.

miRNA	Fold Change Asymptomatic vs. Uninfected (Log2)	Fold Change Symptomatic vs. Uninfected (Log2)
miR160	-4.38 ± 1.06	-7.00 ± 1.25
miR164a	1.10 ± 1.41	-2.67 ± 0.29
miR166e	-3.77 ± 0.0	-2.17 ± 0.66
miR167a	-3.39 ± 1.43	-2.92 ± 0.26
miR169c	-1.94 ± 1.12	-3.01 ± 1.40
miR171	-0.64 ± 1.51	-1.46 ± 1.51
miR172	-4.75 ± 1.34	-4.93 ± 1.51
miR390	-1.20 ± 0.0	-3.32 ± 1.09
miR396a	2.14 ± 0.0	1.49 ± 0.77
miR399e	-2.52 ± 0.0	-1.57 ± 1.28
miR473	3.79 ± 1.91	2.77 ± 0.25
miR477	0.86 ± 0.21	-0.67 ± 0.49
miR479	3.00 ± 2.35	3.33 ± 1.26
miR482a	7.16 ± 0.0	7.94 ± 1.28
miR530a	-0.94 ± 2.18	-1.43 ± 0.49
miR1515	3.05 ± 0.0	1.25 ± 0.94
miR3954	-2.27 ± 0.73	-2.93 ± 0.89

With the exception of the latter, and in contrast to the small RNA library data above, this suggests that changes in expression of most miRNAs in these plants is due to CTV infection, and not the expression of a specific phenotype. The differential expression of miR164, which targets a transcription factor required for regulation of plant tissue development, was found to significantly different ($F(1,4) = 40.32$, MS=12.5, $p=0.008$) between severely symptomatic and asymptomatic tissue types.

Finally, the differences in the expression of miR164 between sequence data and real-time RT-qPCR, in which sequence data suggested that miR164 was upregulated relative to uninoculated controls in both symptomatic and asymptomatic tissue while real-time RT-qPCR data showed differential regulation, are illustrative of the need for replication and validation when examining RNAseq data, and of the potential for bias depending on the platform used (Tian et al. 2010).

Discussion

It has been shown that virus infection alters accumulation and expression of host miRNAs, leading to symptom induction (Bazzini et al. 2009). The involvement of miRNAs in this process has been most clearly illustrated by ectopic expression of DCL (dicer) mutants, which alter miRNA processing, producing virus-like developmental abnormalities (Mlotshwa et al. 2005). Here we studied the change in miRNA expression levels during the induction of the severe disease syndrome by CTV. We found that while the expression of many miRNAs changes after CTV infection, most of these do not correlate with disease induction, but are instead due to viral presence and affect pathways as diverse as NBS-LRR resistance, hormone response factors, and regulators of growth and development. Change in expression of only one miRNA, miR164, correlated with the seedling yellows symptoms. Ruiz-Ruiz et al. (2011) found that miR164 was upregulated in CTV-infected Mexican lime and sweet orange relative to mock-inoculated controls and downregulated in sour orange. This indicates differential interaction between a single CTV isolate and different citrus species. Here, we found that individual CTV isolates also show differential interaction with a single host species, particularly when one isolate causes disease and the other does not.

Members of the miR164 family have multiple roles in plant development, and have been associated with processes as varied as leaf margin development (Nikoviks et al. 2006), leaf maturation and senescence (Breeze et al. 2011), and vascular cell division (Yang et al. 2015), and are highly expressed in vascular tissue, meristems, lateral roots, and in hydathodes (Bazzini et al. 2009; Stauffer and Maizel 2014).

In citrus is has been shown that corky split vein symptoms, the result of boron deficiency, are associated with downregulation of microRNAs 156 and 164, and subsequent upregulation of their target genes, SPL3/4 and CUC2 respectively, which in turn led to vascular hypertrophy and cambium cell proliferation (Yang et al. 2015). In this study we found similar downregulation of miR164 during the induction of severe symptoms, although expression of miR156 was unaffected (data not shown). This suggests that just as there are phenotypic similarities between the two syndromes, such as increased vascular hypertrophy and cell proliferation, there are common pathways. If so, how does CTV induce change in the miR164 pathway?

Infection by both oilseed rape mosaic virus and tobacco mosaic virus has been shown to increase miR164 expression in *Arabidopsis* (Bazzini et al. 2009), suggesting that viruses can affect this pathway, although the means by which they do so remains unknown. Viral suppressors of silencing (Kasschau et al. 2003; Lewsey et al. 2007; Shiboleth et al. 2007), virus structural and movement associated proteins (Bazzini et al. 2009), and the viral sequence itself (Moissiard and Voinnet 2006) all were suggested to be involved in this process. It may also be that the titer of the effector is important, for Fagoaga et al. (2005) reported that stem pitting is induced in CTV p23-expressing transgenic citrus once the transgene product has accumulated past a threshold level, regardless of the strain the transgene was derived from. This does not seem to be true of seedling yellows, in which specific sequence variants of p23 are required for seedling yellows induction (Albiach-Marti et al. 2010). Further research is required to determine how p23 and/or other CTV gene products interact with the miR164 pathway, and on a more

fundamental level, the role these specific miRNAs and their host gene targets correlate with disease expression.

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