MicroRNAs Suppress NB Domain Genes in Tomato That Confer Resistance to *Fusarium oxysporum*

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Abstract

MicroRNAs (miRNAs) suppress the transcriptional and post-transcriptional expression of genes in plants. Several miRNA families target genes encoding nucleotide-binding site–leucine-rich repeat (NB-LRR) plant innate immune receptors. The fungus *Fusarium oxysporum* f. sp. *lycopersici* causes vascular wilt disease in tomato. We explored a role for miRNAs in tomato defense against *F. oxysporum* using comparative miRNA profiling of susceptible (Moneymaker) and resistant (Motelle) tomato cultivars. slmiR482f and slmiR5300 were repressed during infection of Motelle with *F. oxysporum*. Two predicted miRNA targets each of slmiR482f and slmiR5300 exhibited increased expression in Motelle and the ability of these four targets to be regulated by the miRNAs was confirmed by co-expression in *Nicotiana benthamiana*. Silencing of the targets in the resistant Motelle cultivar revealed a role in fungal resistance for all four genes. All four targets encode proteins with full or partial nucleotide-binding (NB) domains. One slmiR5300 target corresponds to *tm-2*, a susceptible allele of the Tomato Mosaic Virus resistance gene, supporting functions in immunity to a fungal pathogen. The observation that none of the targets correspond to I-2, the only known resistance (*R*) gene for *F. oxysporum* in tomato, supports roles for additional *R* genes in the immune response. Taken together, our findings suggest that Moneymaker is highly susceptible because its potential resistance is insufficiently expressed due to the action of miRNAs.

Introduction

MicroRNAs (miRNAs) are single-stranded RNA molecules of approximately 20–24 nucleotides in length that are endogenously transcribed from single-stranded non-coding RNA species [1,2]. Plant miRNAs were first identified in 2002 [1,3] and have been shown to play vital roles in multiple biological processes, including leaf morphogenesis and polarity, floral organ identity, hormone signaling and stress responses [4,5,6,7,8,9,10,11]. miRNAs primarily act on their target miRNAs by influencing mRNA degradation or translational inhibition. In contrast to animals, plant miRNAs are not deadenylated prior to miRNA-guided transcript cleavage and degradation. Although there are several examples of translational inhibition of miRNAs by miRNAs in animals [12,13], this phenomenon has only recently been reported in plants [14].

Expression of miRNA genes is regulated by external stimuli, including abiotic (e.g., drought, temperature, salinity) and biotic (e.g., pathogens such as viruses, bacteria and fungi) stresses. During pathogen attack, recognition of microbe-associated molecular patterns (MAMPs) by plant pattern-recognition receptors leads to pattern-triggered immunity (PTI) resulting in changes in gene expression that result in altered hormone and metabolite levels [15]. Pathogens have evolved effectors to sabotage PTI. In return, plants acquired disease resistance (*R*) genes, to recognize the presence or action of specific effectors, directly or indirectly, and to activate effector-triggered immunity (ETI), a fast and strong form of immunity [16].

A role for miRNAs in regulating genes important for plant defense has been demonstrated for the response to several pathogens [17]. In tomato (*Solanum lycopersicum*), the levels of miR319/miR159 and miR172 are induced during *Tomato leaf curl New Delhi virus* (ToLCNDV) disease progression [18]. miR393, miR160 and miR167 are up-regulated in leaves challenged with the virulent bacterial pathogen *Pseudomonas syringae pv. tomato* (*Pst*) DC3000 [19]. Similarly, miR393, miR319, miR158, miR160, miR167, miR165/166 and miR159 are induced, while miR390, miR408 and miR398 are repressed, in *Arabidopsis thaliana* (Arabidopsis) leaves infected with *Pst* DC3000 [20].
Author Summary

*Fusarium oxysporum* is a fungal pathogen that represents a species complex, with members that infect numerous crops. In spite of its importance to agriculture, very little is known about roles of small RNAs in plant immunity against *F. oxysporum*. In this study, we set up a screen for tomato microRNAs (miRNAs) that correlate with resistance to *F. oxysporum f.sp. lycopersici* by performing deep sequencing of small RNAs from a resistant and susceptible tomato cultivar. We focused on two miRNAs that are uniquely down-regulated in the resistant cultivar during fungal infection. All predicted targets of these miRNAs encode proteins with NB domains, a motif associated with pathogen resistance in plants. Using a heterologous system, we validated that the miRNAs could regulate expression of four targets. Silencing of the target genes in tomato resulted in decreased immunity to *F. oxysporum* in the normally resistant cultivar. The finding that none of our targets correspond to I-2, the only known resistance (*R*) gene for *F. oxysporum* in tomato, supports roles for additional *R* genes in the immune response. Our results suggest that the potential resistance of the susceptible cultivar is insufficiently expressed due to the action of miRNAs.

flagellin-derived peptide, flg-22, induces expression of miR393, a negative regulator of mRNAs for the F-box auxin receptors TIR1, AFB2, and AFB3 [21]. miR482a, a member of the miR482/2118 superfamily, targets mRNAs for *R* proteins, with nucleotide-binding site (NB) and leucine-rich repeat (LRR) motifs, for degradation both directly and through generation of secondary small interfering RNAs (siRNAs) in *Nicotiana benthamiana* infected with *Pst DC3000* [22,23]. miR5300 was first identified as a novel tomato miRNA [24] and later classified as a member of the miR482/2118 superfamily [23]. However, regulation of predicted target genes by miR5300 has not yet been reported [25].

Strains of the ascomycete fungus *Fusarium oxysporum* are ubiquitous soil inhabitants [26,27]. Accumulating data indicate that *F. oxysporum* is a large species complex, with more than 120 formsae speciales causing disease in vegetables, fruit trees, wheat, corn, cotton and ornamental crops [26,27]. *F. oxysporum* infects vascular bundles in the plant host, leading to wilt symptoms. Germination of dormant spores in soil results in adherence and invasion of plant roots by fungal hyphae. The hyphae then move from the root cortex to the xylem where production and dissemination of microconidia spores is critical for disease progression [26].

Previous work has demonstrated that the I-2 gene of tomato confers resistance to race 2 strains of *F. oxysporum* f.sp. *lycopersici* (hereafter referred to as *F. oxysporum*; [28]). The I-2 locus encodes a coiled-coil (CC) NB-LRR protein that recognizes the avr2 gene product of *F. oxysporum* [29]. The near-isogenic tomato cultivars Moneymaker and Motelle are susceptible (i-2/i-2) and resistant (I-2/I-2) genotypes, respectively, for I-2 and the response to *F. oxysporum* infection [30,31,32].

In this study, we explored a possible role for tomato miRNAs in the differential resistance of Moneymaker and Motelle to *F. oxysporum*. Our results indicate that different miRNAs contribute to plant immunity in tomato by influencing mRNA stability or translation of at least three NB domain-containing proteins distinct from I-2.

Results

Identification of microRNAs induced by *F. oxysporum* in tomato roots

We investigated microRNA (miRNA) production in roots of tomato during infection with the wilt fungus *F. oxysporum* through construction of small RNA libraries and deep sequencing. We took advantage of two near-isogenic cultivars that show differential interaction with *F. oxysporum* – Moneymaker (susceptible) and Motelle (resistant) [30,31,32]. We generated a total of four libraries, including: Moneymaker treated with water (MM_H2O), Moneymaker treated with *F. oxysporum* (MM_Foxy), Motelle treated with water (Mot_H2O) and Motelle treated with *F. oxysporum* (Mot_Foxy). Our goal was to identify miRNAs that were either upregulated in Moneymaker or down-regulated in Motelle after infection with *F. oxysporum*. Such a pattern of expression would presumably lead to upregulation of potential target mRNAs required for plant defense in Motelle, but not Moneymaker, after infection.

Using Illumina sequencing, we obtained a total of more than 27 million high quality small RNA sequences from the four libraries that could be mapped to the tomato genome. Of these, 5,743,067 were from MM_H2O, 5,492,955 from MM_Foxy, 4,392,583 from Mot_H2O and 5,497,730 from Mot_Foxy (Table S1). Among all size classes, 24-, 21- and 22-n small RNA species were the three most abundant (Fig. 1A). These sizes are similar to those previously identified in tomato [22,23]. Within the miRNA population of sequences, more than 98% of the reads began with a uracil. It has been demonstrated that Argonate proteins recruit small RNAs based on the 5′ terminal nucleotide: AGO2 and AGO4 recruit small RNAs with 5′ terminal adenosine, whereas AGO1 and AGO5 recruit small RNAs with a 5′ terminal uracil and cytosine, respectively [33,34,35]. We identified 82 predicted miRNAs with at least one raw sequence read in one of the four libraries (Table S2). miRNAs were considered for further analysis if there were at least 12 raw sequence reads and at least a two-fold change between the *F. oxysporum*-infected and control plant libraries. Based on these criteria, we identified 18 unique miRNA sequences corresponding to plant disease resistance, stress responses, transcription factors, and others (Fig. 1B). Notably, among all of the regulated miRNAs identified, miR103 and miR398 are associated with disease resistance in other plant species [36,37]. miR398 is also implicated in the regulatory network for additional abiotic stresses, including salinity, water deficit, oxidative stress, high levels of abscisic acid, ultraviolet light, copper and phosphate deficiency and high sucrose [11,38,39,40,41,42,43,44]. In contrast to the other regulated miRNAs, functions for miR3900 have not been previously reported in any plant species, including tomato.

As stated above, our objective in this study was to identify miRNAs that were present at increased levels in Moneymaker or decreased levels in Motelle after infection with *F. oxysporum*. Our results showed that the majority of miRNAs (15) were present at increased levels in Motelle plants after infection with *F. oxysporum* and we did not identify any miRNAs that were increased (or decreased) at least two-fold in Moneymaker after infection (Fig. 1B). In contrast, smiR398, smiR5300 and smiR482f were all suppressed at least two-fold in Motelle plants after *F. oxysporum* treatment (Fig. 1B), consistent with our original hypothesis.

Northern blot analysis was performed to analyze expression of the three miRNAs that were demonstrated to be down-regulated in Motelle by deep sequencing. We analyzed 14 additional miRNAs (17 total) in order to avoid excluding other possible candidates due to issues with sequencing data. The 13 miRNAs that could be detected using northern analysis are presented in Fig. 2. A subset of the small RNA northern blot results was
consistent with the deep sequencing data. A caveat to this analysis is that subfamily members (e.g., slmiR482a-f; [23, 45, 46, 47] that share significant homology will cross-hybridize during this analysis. Of interest, both slmiR482f and slmiR5300 were decreased in Motelle plants treated with *F. oxysporum* as detected by both methods (Fig. 1B and Fig. 2). The reduction observed during northern analysis (Fig. 2; 53% for slmiR482f and 58% for slmiR5300) was similar to that obtained during deep sequencing (Table S2; 72% for slmiR482f and 61% for slmiR5300). Deep sequencing data showed that slmiR398 was induced by 1.89-fold in Moneymaker by *F. oxysporum* infection, but suppressed by 71% in Motelle (Table S2). However, these expression trends were essentially reversed in the small RNA northern blot analysis. slmiR398 levels were similar in Moneymaker and Motelle controls, elevated in Motelle treated with *F. oxysporum* and barely detectable in Moneymaker under the same conditions (Fig. 2). Thus, the northern results for slmiR398 were reversed relative to those from deep sequencing for infected Moneymaker and Motelle plants. Deep sequencing data indicated that expression of slmiR403 was reduced by 26% in Moneymaker plants, but induced four-fold in Motelle, after treatment with *F. oxysporum* (Fig. 1B). Although the small RNA northern results also detected slight reduction of slmiR403 in Moneymaker, slmiR403 levels were slightly reduced in Motelle treated with *F. oxysporum* (Fig. 2).

Based on our original hypothesis, the results from both deep-sequencing and small RNA northern blot analysis pointed to slmiR482f and slmiR5300 as potential regulators of plant defense genes in tomato. *Fusarium oxysporum* infection of tomato plants induces the expression of several defense-related genes, including pathogenesis-related (PR) proteins and resistance genes. Of the 21 miRNAs that were negatively regulated in Motelle, but not Moneymaker, after *F. oxysporum* infection, six of them are known to be involved in plant defense responses. These include miR162, miR167, miR398, and miR482. miR162 is known to target the Arabidopsis thaliana RACK1 gene, which plays a role in pathogen defense. miR167 is involved in the regulation of the auxin response factor (ARF) family of transcription factors, which are important for plant development and defense responses. miR398 is known to target genes involved in copper homeostasis and has been shown to be induced in response to pathogen infection. miR482 is known to target genes involved in RNA degradation and has been shown to be induced in response to pathogen infection.

**Figure 1.** Properties of miRNAs expressed in resistant and susceptible tomato cultivars treated with water or the fungal pathogen *Fusarium oxysporum*. In total, four small RNA libraries were subjected to deep sequencing: susceptible tomato cultivar Moneymaker treated with water; Moneymaker treated with *Fusarium oxysporum f. sp. lycopersici* (*F. oxysporum*); resistant tomato cultivar Motelle treated with water and Motelle treated with *F. oxysporum*. The sequence length of small RNAs (A) for the combined data from the four libraries was determined as described in the Materials and Methods. Relative expression levels of known miRNAs (B) were determined by dividing normalized reads for *F. oxysporum* treatment by those for water treatment for each cultivar. The red bracket indicates the miRNAs that were negatively regulated in Motelle, but not Moneymaker, after *F. oxysporum* infection. Putative roles/targets of miRNAs in various plant species (information from miRBase.org): miR156, miR156c, miR156j and miR157d: Squamosa-promoter Binding Protein (SBP)-like transcription factors; miR166gilmf: HD-Zip transcription factors, including Phabulosa (PHB) and Phavoluta (PHV) that regulate axillary meristem initiation and leaf development; miR167b, miR167d and miR167fijeqhac: Auxin Response Factors (ARF transcription factors); miR396abcd: Growth Regulating Factor (GRF) transcription factors, rhodenase-like proteins, and kinesin-like protein B; miR394ab, F-box proteins; miR827abc: Unknown; miR403bdf: Virus defense; miR162 and miR162abc: Unknown; miR530: Unknown; miR398abc: copper superoxide dismutases and cytochrome C oxidase subunit V; miR482f: NB domain proteins; miR5300, Unknown.

PLOS Pathogens | www.plospathogens.org 3 October 2014 | Volume 10 | Issue 10 | e1004464
found several potential targets in the tomato genome (Fig. S1). Interestingly, all top putative targets for either miRNA encode proteins with full or partial NB domains (Fig. 3B). The binding site for both slmiR482f and slmiR5300 miRNAs is in the P-loop region of the NB domain in each target (indicated by red arrow in Fig. 3B). For slmiR482f, the top two putative targets were Solyc08g075630 (NB and CC domains) and Solyc08g076000 (NB and three LRR domains) (Fig. 3A, B). Solyc08g075630 has an atypical arrangement, with the CC domain following the NB domain (Fig. 3B). For slmiR5300, the top two putative targets were Solyc09g008650 (NB and three LRR domains) (Fig. 3A, B). Solyc09g008650 contains a truncated NB domain and overlapping DUF3542 and CC motifs (Fig. 3B). We analyzed available RNAseq data, as well as all three reading frames of genomic sequence at the Sol Genomics database (http://solgenomics.net/organism/Solanum_lycopersicum/genome) downstream from this gene, but could not find sequence corresponding to the rest of the NB domain. DUF3542 is a domain of unknown function found in eukaryotes and viruses [51]. Interestingly, the CC-NB-LRR domain protein-encoding gene Solyc09g018220 is tm-2 [52,53]. Tomato cultivars Motelle and Moneymaker contain tm-2 (http://tgc.ifas.ufl.edu/vol43/p79.html), the susceptible allele of the Tm-2 locus [53]. Tm-2 is required for durable resistance of tomato to Tomato mosaic virus (ToMV) [52].

We next tested the possibility that the presence of slmiR482f or slmiR5300 would suppress expression of the target genes, leading to reduced levels of the encoded mRNAs and proteins. Before quantitating expression of putative target miRNAs, we first determined the expression levels of several control genes in our four RNA preparations using qRT-PCR (Fig. 4A). These included I-2, required for Fusarium resistance [29], several I-2-homologous genes identified in the Sol Genomics database, and Mi-1, required for resistance to nematodes and other pests, but not Fusarium. Mi-1 was chosen because Motelle and Moneymaker are also near-isogenic for this gene [30]. The results for I-2 and Mi-1 were in agreement with previous findings and the genotypes

Figure 2. Northern blot analysis of miRNAs. Oligonucleotide probes were used to quantitate levels of several miRNAs identified during deep sequencing using northern blot analysis. Root total RNA samples (40 μg each) were from Moneymaker treated with water (MM-H2O), Moneymaker infected with F. oxysporum (MM-Foxy), Motelle treated with water (Mot-H2O) and Motelle infected with F. oxysporum (Mot-Foxy). U6 RNA served as a loading control for each blot. Blots were imaged using a Phosphorimager and miRNA species quantitated using Imagequant software, with normalization to the amount of U6 RNA. The numbers below each blot indicate the amount of miRNA in each sample relative to the corresponding water-treated control. Note that due to significant sequence homology, slmiR482 subfamily members cannot be quantitatively distinguished from one another using northern analysis.

doi:10.1371/journal.ppat.1004464.g002
of the two cultivars [30]. I-2 was not detectable in Moneymaker, but levels increased more than 3-fold in Motelle after infection with *F. oxysporum*. Mi-1 levels were similar in both cultivars and did not change significantly after *F. oxysporum* treatment.

Expression of the four I-2-homologous genes varied, but none exhibited a significant difference between water control and *F. oxysporum* exposure.

We checked the mRNA levels of the putative targets under water or *F. oxysporum* treatment conditions in both tomato cultivars using qRT-PCR (Fig. 4B) and northern blot analysis (Fig. S2). The results of qRT-PCR showed that putative slmiR482f target Soly08g075630 was induced by almost two-fold in Motelle, but unchanged in Moneymaker, after treatment with *F. oxysporum* (Fig. 4B). The results from northern analysis of Soly08g075630 closely mirrored those from qRT-PCR (Fig. S2). Both qRT-PCR and northern analysis demonstrated that Soly08g076000 mRNA levels were not significantly changed by *F. oxysporum* treatment in either cultivar, although levels of Soly08g076000 were elevated in Motelle relative to Moneymaker (Fig. 4B, Fig. S2). The results from qRT-PCR and northern analysis revealed significant upregulation of slmiR5300 putative target Soly05g008650 in Motelle (3–4 fold), but not Moneymaker, after infection with *F. oxysporum* (Fig. 4B, Fig. S2). Likewise, slmiR5300 target Soly09g018220 exhibited 3.4- (Fig. 4B) or 5.9-fold (Fig. S2) up-regulation by *F. oxysporum* infection in Motelle compared to water, while levels in Moneymaker were unchanged. Taken together, these results support regulation of at least three of the four predicted target genes at the mRNA abundance level by their respective miRNAs.

The four target genes are affected by slmiR482f or slmiR5300 in co-expression studies

The psRNATarget algorithm results predicted that both slmiR482f predicted targets and one slmiR5300 target (Soly05g008650) were regulated at the translational level, while the second slmiR5300 target (Soly05g008650) is regulated at the mRNA cleavage step. The results from our mRNA analysis were consistent with pre- or post-transcriptional regulation of certain target genes, as three targets exhibited elevated transcript levels in Motelle, but not Moneymaker, with fungal infection, while one target (Soly08g076000) was relatively unchanged. In order to further probe the possible mechanism for regulation of targets by the four miRNAs, as well as determine specificity of the miRNA/target interaction, we conducted Agrobacterium-mediated transient co-expression experiments in *N. benthamiana*. We used a binary construct to co-express the FLAG-tagged putative target protein gene and the respective miRNA gene. The presence of the FLAG tag would allow us to detect differences in protein levels and thus, possible translational or post-translational regulation of the target by the miRNA. Vectors with no insert, only a target gene, or containing the miRNA gene slmiR166 that does not recognize our predicted targets, were used as negative controls.
We first performed qRT-PCR to check the mRNA levels of targets during co-expression (Fig. 5A). In the presence of slmiR482f, expression of its both putative target genes Solyc08g075630 and Solyc08g076000 were not significantly decreased. In contrast, slmiR5300 targets Solyc05g008650 and Solyc09g018220 were greatly suppressed by the presence of the miRNA; in the case of Solyc09g018220 transcript levels were reduced by almost 90% (Fig. 5A).

We investigated possible translational control of target gene expression by checking levels of the target proteins, using western blot analysis with antibody against the FLAG-tag that was placed at the N-terminus of each target in our constructs. Our results showed that protein levels of all targets were down-regulated by the presence of the corresponding miRNA (Fig. 5B). Proteins corresponding to slmiR482f target gene Solyc08g076000 and slmiR5300 target gene Solyc05g008650 were difficult to detect (Fig. 5B). These results strongly suggest that slmiR482f and slmiR5300 are responsible for the down-regulation of their respective protein targets. The observation that levels of Solyc08g075630 and Solyc08g076000 proteins were greatly reduced, while transcript amount was only slightly affected, suggests that slmiR482f silences these two targets mainly via translational inhibition. These results are consistent with the predictions from the psRNATarget algorithm described above.

On the other hand, both mRNA and protein levels of Solyc05g008650 and Solyc09g018220 are predicted targets of slmiR5300.
Figure 5. Co-expression of slmiRNAs and predicted targets in *Nicotiana benthamiana* leaves. **A. Levels of target mRNAs.** qRT-PCR was used to determine relative levels of predicted tomato mRNAs in *N. benthamiana* leaves expressing only empty vector; target mRNA and the appropriate miRNA (slmiR482f or slmiR5300); or target mRNA and empty vector. Values were normalized to *N. benthamiana* actin. Errors are expressed as the standard error. *** indicates significant differences when compared to the corresponding control plants in the same treatments at p<0.001. **B. Target protein levels.** Total protein isolated from the samples in (A) was electrophoresed on SDS-PAGE gels and blotted onto nitrocellulose membranes. A FLAG antiserum was used to detect the tagged target proteins during western analysis as described in the Materials and Methods (top panels). A duplicate gel was Coomassie-stained and used as a loading control (bottom panels). Similar
target genes are influenced at the mRNA abundance and/or protein level by co-expression of slmiR482f or slmiR5300.

The four miRNAs target cleavage sites were then validated by RNA Ligase-Mediated 5’ Rapid Amplification of cDNA Ends (RACE) [54,55] using total RNA isolated from the N. benthamiana leaves used for the co-expression studies described above. The 5’ end of the 3’ derived cleavage product without enzymatic pretreatment can be ligated directly to an RNA adaptor with T4 RNA ligase. Gene-specific 5’ RACE primers were designed to yield predicted products of between 300–400 bp if miRNA-guided cleavage occurred in vivo. In this way, miRNA-guided cleavage can be detected by sequence analysis of the cloned PCR products. With each primer, a major PCR product of the size predicted to be generated from a template resulting from a miRNA-guided cleavage event was detected. In all cases, at least 80% of the 5’ ends of inserts terminated at a position corresponding to the miRNA (Fig. 5C). With the exception of SolyC05g008650, all of the predicted miRNA-mRNA interactions contain mismatched positions. These findings show that perfect base pairing during the miRNA-mRNA interaction is not a strict requirement to guide cleavage of target RNAs, a result which has been reported by several groups [54,56].

Silencing of slmiR482f and slmiR5300 target genes renders the Motelle cultivar susceptible to F. oxysporum

We investigated a possible role for the four target proteins in resistance to F. oxysporum using a TRV-based virus-induced gene silencing (VIGS) system to down-regulate expression of each gene in the resistant tomato cultivar Motelle. For these studies, Phytoene Desaturase (PDS) TRV-silenced plants (TRV-PDS) were used as a positive control for silencing. The photobleached phenotype was consistently observed on the third and fourth leaves above the inoculated leaves 3–4 weeks after TRV infiltration [57]. Therefore, treatment with F. oxysporum was carried out four weeks after TRV infection.

Transcript levels of genes were checked using qRT-PCR prior to F. oxysporum infection. All VIGS plants were tested for expression levels of all four miRNA target genes, as well as Mi-1 and I-2, in order to detect possible off-target effects of the VIGS constructs (Fig. 6). The results demonstrated down-regulation of the corresponding mRNA for all four VIGS-target genes, with reductions ranging from ~60–95% compared to control Motelle plants not treated with a TRV vector (Fig. 6). The VIGS was specific for the silenced genes, with the exception of one VIGS plants (slmiR5300) caused lower levels of I-2 expression than the water-treated control. However, the observation that the other three plants were not significantly different is consistent with a specific effect on the SolyC08g076000 gene.

The VIGS constructs for all four target genes corresponded to the extreme 3’ end of the ORF and a portion of the 3’ untranslated region. The VIGS constructs for three out of four target genes did not display nucleotide identity with any other genes in the tomato genome using BLAST. However, the SolyC08g076000 VIGS construct exhibited significant nucleotide identity with a region of tomato gene SolyC02g014230 (38 identical nucleotides in the longest stretch). In order to determine whether the SolyC08g076000 VIGS construct down-regulated expression of SolyC02g014230, we performed qRT-PCR on the same RNA samples used in Fig. 6. The results revealed that SolyC02g014230 mRNA levels were not decreased in the SolyC08g076000 VIGS plants (Fig. S3).

Having demonstrated that the VIGS constructs reduced expression of the appropriate genes in tomato, we assessed disease phenotypes for control and VIGS plants. Scoring was performed four weeks after F. oxysporum infection. Control Motelle plants treated with water and VIGS control Motelle plants carrying an empty TRV vector did not exhibit disease symptoms after infection with F. oxysporum (Fig. 7A). As expected, Moneymaker plants infected with F. oxysporum displayed severe wilting symptoms (Fig. 7A). Disease symptoms, including leaf wilting and discoloration, were observed in all VIGS plants inoculated with F. oxysporum but not in water-treated controls (Fig. 7C). Water-treated controls are plant or leaf #1 in each panel. All F. oxysporum-infected plants carrying a target VIGS construct grew more slowly than control plants treated with water (Fig. 7C) and exhibited wilting at the top leaves. In particular, line 2 of TRV-SolyC05g008650 and line 4 of TRV-SolyC09g018220 exhibited especially severe disease symptoms (Fig. 7C).

We quantified the degree of F. oxysporum infection in tomato leaves by amplifying the rRNA Intergenic Spacer Region (IGS) from genomic DNA isolated from leaves using qPCR [58]. In the control plants, levels of F. oxysporum were significantly elevated in Moneymaker after infection (Fig. 7B, left panel), while they were relatively unchanged in Motelle with or without TRV vector (Fig. 7B, center and right panels). The extremely high fungal load in Moneymaker after infection with F. oxysporum is in agreement with the severe disease symptoms of these plants (Fig. 7A). For plants carrying a VIGS construct, our data indicate that F. oxysporum levels were elevated in inoculated plants for each of the four target genes (Fig. 7D). The greatest levels were observed in line 2 of TRV-SolyC05g008650 and line 4 of TRV-SolyC09g018220 (Fig. 7D), consistent with disease severity symptoms. However, the phenotypes observed in VIGS plants were not as severe as those of the control Moneymaker plants after infection (Fig. 7A).

Discussion

In this study, we exploited the availability of near-isogenic susceptible and resistant cultivars of tomato towards F. oxysporum to identify miRNAs important for plant defense. The results with these two cultivars guided our experiments, allowing us to focus on miRNAs that were down-regulated in the resistant Motelle cultivar during infection. We were able to quickly narrow down to a small group of miRNAs and identify two (slmiR482f and slmiR5300) that correlated with disease. Knock-down of the target genes (SolyC08g076360 and SolyC05g076000 for slmiR482f and SolyC05g008650 and SolyC09g018220 for slmiR5300) caused the resistant Motelle cultivar to become susceptible to F. oxysporum. Our study provides a platform for differentially expressed miRNAs in tomato after F. oxysporum infection and demonstrates that plant miRNAs are involved in defense against F. oxysporum.
Due to extensive DNA sequence homology with another gene (Solyc02g014230), we were not able to produce a VIGS construct that was specific for the slmiR482f target Solyc08g076000. However, this construct resulted in susceptibility of the Motelle cultivar to *F. oxysporum* infection, and accumulation of fungal biomass comparable to that observed during knockdown of the

**Figure 6.** qRT-PCR to assess the degree of gene silencing and to determine possible off-target effects in VIGS plants. Leaflets were harvested four weeks after VIGS and prior to infection with *F. oxysporum*. Total RNA was isolated and subjected to qRT-PCR to evaluate expression of the four predicted miRNA target genes, along with *Mi-1* and Mot-I-2 controls. The silenced gene is indicated in the title for each horizontal panel, while the gene transcript being measured is shown under the y-axis at the bottom of the figure. Plants treated with water or the TRV empty vector are included as negative controls. mRNA levels are expressed relative to the Motelle plant not treated with TRV for each VIGS construct. Values were normalized using tomato 18S rRNA. Errors are expressed as the standard error. Significant differences are indicated by asterisks. Among all of the VIGS plants, only VIGS Solyc08g075630 Plant #2 exhibits evidence of an off-target reduction in Mot-I-2 transcript levels relative to the water-treated control. * and *** indicate significant differences when compared to the corresponding control plants in the same treatments at p<0.05 and p<0.001, respectively.

doi:10.1371/journal.ppat.1004464.g006

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other three miRNA target genes. Furthermore, qRT-PCR results demonstrated that Solyc08g076000 mRNA levels were greatly reduced, while Solyc02g014230 levels were relatively unchanged in the VIGS plants. Although we cannot rule out an effect on expression of Solyc02g014230 at an earlier time point that might affect plant defense, this finding supports an active role for Solyc08g076000, but not Solyc02g014230, in resistance to F. oxysporum in tomato.

Tomato is one of the most economically important crops and a model system for fruit development. Although whole-genome sequencing of domesticated tomato has made it possible to characterize the entire family of miRNAs, only a small number of miRNAs have been implicated in tomato-specific processes, such as fruit development and ripening [59,60,61]. Our study showed that some disease resistance or abiotic stress-associated miRNAs, such as smiR482f and smiR398, are suppressed in the resistant tomato cultivar Motelle after F. oxysporum treatment. In addition we determined that smiR5300, for which no functions had been previously ascribed [24,25], was also suppressed in Motelle during F. oxysporum infection. SmiR482 and smiR3500 are members of the miR482/2118 superfamily and members of this family have been shown to target the p-loop motif in the mRNA of the NB-LRR encoding R genes [23]. The miR482 family has six members, including miR482a-f [23,45,46,47].

Plant defense responses can be activated very rapidly by pathogen infection. Studies in both plant and animal systems have demonstrated that some small RNAs are induced quickly and specifically by various pathogens and diseases [62,63,64]. Interestingly, employing the psRNATarget algorithm, the top targets predicted for smiR482f (Solyc08g075630 and Solyc08g076000) and smiR5300 (Solyc05g008650 and Solyc09g018220) encode proteins with partial or full NB-domains. Surprisingly, Solyc09g018220 is allelic with the susceptible allele of the ToMV R gene tm-2. This finding implicates tm-2 in resistance to fungal attack in tomato and suggests that susceptible disease resistance alleles could have roles in immunity. It is intriguing to speculate that plants are able to use susceptible disease resistance alleles to broaden the pathogen recognition spectrum. Since smiR482f should also target both the functional Tm-2 and broken Tm-2 alleles, it is likely that these genes can also perform the role demonstrated here for tm-2 in F. oxysporum resistance. The discovery of the need for additional full-length or truncated genes for a presumed single gene resistance has broad implication in breeding for resistance and transfer of these traits to plants from different families. It remains to be seen whether the interfamily barrier seen in the transfer of R genes is due to the absence of these additional genes [65].

None of the four miRNAs target I-2 and the three non-tm-2 targets are not homologs of known R proteins in tomato or other plant species (Fig. S4). Surprisingly, silencing each of their respective genes resulted in susceptibility of Motelle tomato to F. oxysporum. Although silencing of Solyc09g018220/tm-2 resulted in the most severe symptoms, the phenotypes of some single gene VIGS Motelle plants were not as drastic as those observed in the susceptible tomato cultivar Moneymaker lacking I-2. This could be because down-regulation of one NB-domain containing protein is not sufficient to completely abolish effective disease resistance in tomato. Alternatively, the residual transcript levels of target genes in VIGS plants may have produced this outcome. Taken together these results support the requirement for multiple proteins carrying the NB domain, including tm-2, in resistance of tomato to F. oxysporum.

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Arabidopsis miR172 regulates cell-fate specification as a translational repressor of APETALA2 [74] and miR156/157 inhibits translation of the SBP box gene, SPL3 [75]. It is worth noting that miRNAs have been demonstrated to generate secondary siRNAs from the 3’-UTR side of the target RNA sequence, and these secondary siRNAs can regulate gene expression in plants [22,45,76,77,78]. In particular, slmiR482a, a miR482/2118 superfamily member, targets the LRR1 miRNA as a siRNA-mediated secondary target [23].

To conclude, our results support the notion that the miR482/2118 superfamily-mediated reduction of gene expression involves multiple NB-domain-encoding genes, including tm-2, and occurs via mRNA cleavage and/or translational control mechanisms in tomato. It remains to be determined whether introduction of artificial miRNAs that silence mature and/or precursor forms of slmiR482f and slmiR5300 could up-regulate target gene expression in the susceptible Moneymaker plants. In this scenario, we would expect that silencing of miRNAs will enhance resistance to F. oxysporum and would therefore be a useful molecular tool to uncover functional roles for the increasing number of discovered miRNAs in tomato.

Materials and Methods

Inoculation of tomato plants, small-RNA library construction and deep sequencing

Two tomato near-isogenic cultivars (cv.) Motelle (I-2/I-2) and Moneymaker (I-2/2-I-2) that exhibit different susceptibilities to the root pathogen F. oxysporum were used for plant infection and library construction. The wild-type Fusarium oxysporum 5sp lyopersici strain used for all experiments is FGSC 9935 (also referred to as FOL 4287 or NRRRL 34936). Profiling experiments were performed on two-week-old tomato seedlings grown at 25°C with a 16/8-h light/dark cycle. Plants were removed from soil and roots incubated in a solution of F. oxysporum conidia at a concentration of 1x10^5/ml for 30 min. Control tomato plants were treated with water. Plants were then replanted in soil and maintained in a growth chamber at 25°C for 24 h with constant light. Plants were removed from soil, and roots rinsed and excised using a razor blade. Roots were immediately frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated from roots using either a method involving hot phenol extraction [79] or Trizol (#15596-081, Qagen, Grand Island, NY) according to the manufacturer’s recommendations. Small RNA libraries for deep sequencing were constructed as described [80] and sequenced using an Illumina GSII sequencer at Los Alamos National Laboratory (Los Alamos, NM).

Northern blot analysis and quantitative RT-PCR

For small RNA northern blot analysis, 40 μg total tomato root RNA was resolved on 7 M urea/15% denaturing polyacrylamide gels in 1x Tris/Boric Acid/EDTA (TBE). miRNA-specific oligonucleotide probes (Table S3) were end-labeled using [32P]-32P-ATP (#M0201, New England Biolabs, Ipswich, MA); oligonucleotide probes were labeled according to the manufacturer’s recommendations). Blots were stripped and reprobed using a U6 RNA oligonucleotide probe to provide a loading control. All blots were imaged using a PhosphorImager (Molecular Dynamics/GE Life Sciences, Pittsburgh, PA) and band intensities quantified using Imagequant software (GE Life Sciences). Expression of target or control miRNAs was determined using northern blot analysis or quantitative reverse transcriptase PCR (qRT-PCR). For northern analysis, 20 μg of total RNA was resolved on 1.2% agarose gels and processed as described [81].
were grown in liquid LB medium with selection [82]. After 40 h, leaves were harvested, and protein extraction was performed [83]. Proteins were separated on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes (Millipore, Billerica, MA). Membranes were blocked using 5% milk in 1x TBST and then incubated with Anti-FLAG (DYKDDDDK) Antibody (#635691, Clontech, Mountain View, CA) followed by a secondary horseradish peroxidase (HRP)-conjugated goat anti-Rabbit polyclonal antibody (#A0504, Sigma, St. Louis, MO) according to the manufacturer’s recommendations. Reactive species were visualized using SuperSignal West Pico Chemiluminescent Substrate manufacturer’s recommendations. Reactive species were visualized using SuperSignal West Pico Chemiluminescent Substrate.

Validation of miRNA targets

Target validation was done using a RNA ligase-mediated rapid amplification of cDNA ends (5′RACE) assay as described [54,55], with slight modification using the FirstChoice RLM-RACE Kit (#AM1700, Invitrogen, CA). Total RNA was isolated from N. benthamiana leaves used for co-expression of miRNAs with predicted mRNA target genes. Poly(A)+ mRNA was prepared by two rounds of purification with an Oligotex mRNA Midi Kit (#70042 Qiagen) and directly ligated to the FirstChoice RLM-RACE Kit RNA Oligo adaptor without further modifications. Gene-specific primers were designed approximately 400 nucleotides to the 3′ side of predicted target sites (Table S3). The conditions used for this amplification step were those for gene-specific RACE recommended by the manufacturer.

Virus-Induced Gene Silencing (VIGS) constructs and Agrobacterium-mediated virus infection

VIGS was used to suppress expression of the predicted mRNA targets using TRV-based vectors (pTRV1 and pTRV2) [84]. Gene-specific VIGS constructs could only be developed for three of the four target genes in tomato, due to high nucleotide identity between Solyc08g076000 and Solyc02g014230 (see Results). slmiR482f target genes Solyc08g075630 and Solyc08g076000 and slmiR5300 target genes Solyc03g008650 and Solyc09g018220 were amplified using gene-specific primers (Table S3) and cloned into the pTRV2 vector. A vector carrying a fragment of the Phytoene Desaturase (PDS) gene was used as a positive control for silencing [57]. All TRV-VIGS constructs were transformed into A. tumefaciens strain GV3101. Bacterial cultures were grown as described above. Equal volumes (OD600 = 1) of A. tumefaciens carrying pTRV1 and suspensions containing pTRV2-derived constructs or pTRV2 empty vector were mixed prior to infiltration into leaves of 2 to 3-week-old tomato plants. pTRV2 empty vector was used as the negative control in this study [57,84,85,86]. Plants were maintained at 20°C for four weeks, until photobleaching symptoms were observed in the leaves of PDS TRV-silenced plants. At this time, leaflets were harvested from several plants for isolation of RNA and qRT-PCR analysis of the target genes to assess the degree of silencing. The same plants were then treated with F. oxysporum or water as described above for the small RNA library construction. After four more weeks, plants were scored for disease symptoms. Genomic DNA was isolated from leaves [87,88] and relative levels of F. oxysporum determined using qPCR of the rRNA intergenic spacer (IGS) sequence of F. oxysporum [58] using specific primer sequences (Table S3).

Supporting Information

Figure S1 All predicted targets of slmiRNA482f and slmiRNA5300 in the tomato genome. Alignments were made using ClustalW2 with slmiRNA482f or slmiRNA5300 and predicted target sequences from the Sol Genomics database (http://solgenomics.net). The nucleotides shown in red in each mRNA target are mismatches with the corresponding miRNA.

Figure S2 mRNA levels for predicted target genes in tomato cultivars infected by F. oxysporum. Twenty µg of total root RNA were used for northern blot. Blots were stripped and reprobed using an 18S RNA probe as a loading control. Blots were imaged and bands quantitated as described in Figure 2.

Figure S3 Expression of Solyc02g014230 is not reduced in VIGS-Solyc08g076000 tomato plants. Leaves from plants subjected to VIGS using the Solyc08g076000 construct were harvested three weeks after VIGS. Total RNA was isolated and subjected to qRT-PCR to check expression of the Solyc02g014230 gene.

Figure S4 Phylogenetic analysis of the I-2 gene family and the four miRNA targets. All protein sequences were obtained from the Sol Genomics database, except for I-2, which was taken from reference [28]. Alignment and tree building were performed using MEGA5.2.2 [90].

Figure S5 DNA sequence alignment of I-2 and other genes used for design of I-2-specific primers for qRT-PCR analysis. ClustalW2 was used to align the DNA sequences of I-2, Mi-1 and sequences homologous to I-2 in the Sol Genomics database. The regions of I-2 used to design 5′ and 3′ primers for qRT-PCR are indicated with pink shading. There is a minimum of 6/20 (5′ primer) or 7/21 (3′ primer) mismatches when comparing primers for I-2 and the other genes in the alignment.

Table S1 MicroRNAs identified using deep sequencing. (XLSX)

Table S2 MicroRNAs with at least one raw sequence read in at least one library. (XLS)

Table S3 Primers used in this study. (DOC)

Table S4 Sequence reads for members of the miRNA482 a-f family. (XLSX)

Acknowledgments

We are indebted to Dr. Hailing Jin for advice on small RNA library construction and many helpful discussions over the course of this project. We thank members of the Dr. Xuemei Chen and Dr. Wenbo Ma laboratories for DNA vectors, N. benthamiana plants and protocols.

Author Contributions

Conceived and designed the experiments: KAB IK JES CSH SO. Performed the experiments: SO GP HSA JES. Analyzed the data: KAB IK JES SO GP. Contributed reagents/materials/analysis tools: CH JES IK. Wrote the paper: KAB IK SO GP JS.
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