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Analysis of phytoplasma-responsive sRNAs provide insight into the pathogenic mechanisms of mulberry yellow dwarf disease

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The yellow dwarf disease associated with phytoplasmas is one of the most devastating diseases of mulberry and the pathogenesis involved in the disease is poorly understood. To analyze the molecular mechanisms mediating gene expression in mulberry-phytoplasma interaction, the comprehensive sRNA changes of mulberry leaf in response to phytoplasma-infection were examined. A total of 164 conserved miRNAs and 23 novel miRNAs were identified, and 62 conserved miRNAs and 13 novel miRNAs were found to be involved in the response to phytoplasma-infection. Meanwhile, target genes of the responsive miRNAs were identified by sequencing of the degradome library. In addition, the endogenous siRNAs were sequenced, and their expression profiles were characterized. Interestingly, we found that phytoplasma infection induced the accumulation of mul-miR393-5p which was resulted from the increased transcription of *MulMIR393A*, and mul-miR393-5p most likely initiate the biogenesis of siRNAs from *TIR1* transcript. Based on the results, we can conclude that phytoplasma-responsive sRNAs modulate multiple hormone pathways and play crucial roles in the regulation of development and metabolism. These responsive sRNAs may work cooperatively in the response to phytoplasma-infection and be responsible for some symptoms in the infected plants.

Mulberry tree is a perennial woody plant that has long been planted, and is of considerable economic importance to sericulture[1]. It is susceptible to a number of diseases[2], among which yellow dwarf disease associated with aster yellows phytoplasma is one of the most devastating diseases[3]. Phytoplasmas are obligate endocellular parasites lacking cell walls, and have devastating effects on more than several hundred plant species worldwide[4]. Infected plants show dramatic changes in morphology including yellowing, phyllody, stunting, proliferation, and witches' broom[5]. These abnormal developments in plants can cause devastating losses in agriculture and forestry[6]. Because phytoplasmas were unculturable in pure cultures in laboratory broth, these organisms remain one of the most poorly characterized plant pathogens, and the mechanisms underlying their pathogenicities are not yet understood[7].

When faced with pathogen invasion, plants activate sophisticated response mechanisms to reprogram gene expression[8]. MicroRNAs (miRNAs) and endogenous small interfering RNAs (siRNAs) are two main categories of small regulatory RNAs which globally regulate plant immunity by inhibiting target gene expression at transcriptional or post-transcriptional level[9]. Increasing evidence indicated that miRNAs serve as one important mechanism for mediating gene expression in plant-microorganism interactions[10–14]. As for the phytoplasma-responsive miRNAs, only one study has explored in Mexican lime (*Citrus aurantifolia* L.) infected by '*Candidatus Phytoplasma aurantifolia*'[15]. Though some of the pathogen-responsive miRNA families are deeply conserved among various plant species, their function may be specie specific[16]. Moreover, every plant has its own specie-specific miRNAs which play important roles in the regulatory networks associated with the stress resistance, and individual miRNAs of the same family may be expressed differentially and have different functions in response to the same stress[13,16]. Therefore, the phytoplasma responsive miRNAs have not been fully explored, and how their gene expression reprogrammings achieved are largely unknown. As far as we know, there was no miRNA information reported in mulberry so far. Characterization of the phytoplasma responsive miRNAs in mulberry may provide a novel platform to better understand the plant-phytoplasma interactions.

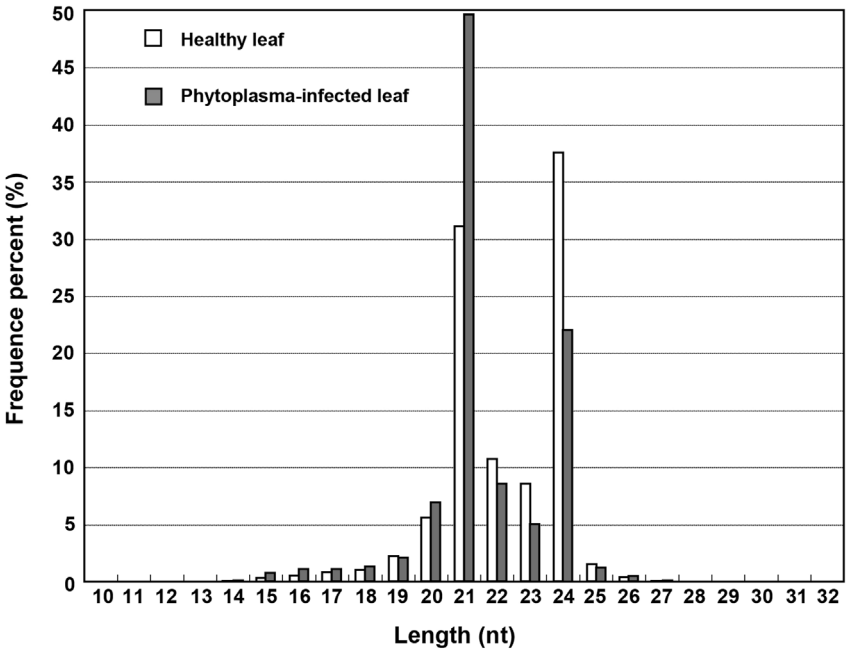

Figure 1 | Length distribution of the small RNA in mulberry.

SiRNAs are distinguished with miRNAs by the structure of their precursors and by their targets, and they arise from a long double-stranded RNAs and typically direct the cleavage of transcripts to which they are completely complementary[17]. Though thousands of endogenous siRNAs have been sequenced[18–20], their biological roles are largely unknown except for the functions of some chromatin-associated siRNAs in DNA methylation and transcriptional gene silencing and the roles of transacting siRNAs (ta-siRNA) in plant development and hormone signaling[21]. Except for the *nat-siRN-AATGB2*, which was specifically induced by the effector *avr-Rpt2* of *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000)[21], the AtlsiRNA1, which was strongly induced by *Pst* (*avrRpt2*), and some siRNAs were found differentially expressed in response to *Heterodera schachtii* infection[22], limited siRNAs involved in plant biotic stress has been reported. Therefore, identification of entire set of siRNAs and their expressions in response to biotic stress is needed to unravel the complex siRNA-mediated regulatory networks controlling gene expression in plant-microorganism interactions.

In the present study, based on the transcriptome information attained, we employed the next generation high throughput sequencing technology to characterize the miRNAs and siRNAs in mulberry. By comparing the normalized expression levels of sRNAs from the healthy and infected leaf libraries, the phytoplasma-responsive miRNAs and siRNAs were identified. In addition, the potential target genes of the responsive miRNAs were identified by sequencing of the degradome library and their functions involved in the response of mulberry to phytoplasma infection were discussed.

Results

Illumina sequencing of small RNAs. To examine the phytoplasma-responsive sRNAs, two sRNA libraries were constructed from phytoplasma-infected leaves and healthy leaves, respectively, and were then subjected to Solexa deep sequencing. The resulting raw sequence reads were cleaned and yielded 28 208 781 and 26 814 061 transcriptome-matching reads from the healthy and phytoplasma-infected leaf libraries, respectively. More than 60% of these mapped small RNAs were 20–24 nt in length with 24 and 21 nt as the major size groups (Fig. 1), consistent with the size of Dicer-like cleavage products. The matched sRNA sequences were clustered into several RNA categories such as known miRNAs, repeats, rRNAs, tRNAs, snRNAs, snoRNAs and unannotatated sRNAs. The known miRNAs account for 41.25% of all sequence reads in the healthy leaf library and 41.85% in the phytoplasma-infected leaf library. However, the proportion of sRNA unique sequences derived from known miRNAs represented only a very small fraction (0.64% and 0.79%, respectively) of the total unique sequences (Table 1). The highest proportion of unique sequences was unannotatated sRNA sequence, which probably include some novel miRNA candidates and lots of siRNAs as well as other type RNAs.

Table 1 | Distribution of the sequence reads in the small RNA libraries of mulberry leaves.

	Healthy leaf		Phytoplasma-infected leaf	
RNA class	Reads	Unique sequences	Reads	Unique sequences
Total	28208781 (100%)	7513906 (100%)	26814061	4812269
miRNAs	11636122 (41.25%)	48348 (0.64%)	11222483 (41.85%)	38056 (0.79%)
Repeats	10 (0.00%)	7 (0.00%)	5 (0.00%)	4 (0.00%)
rRNA	3212490 (11.39%)	104485 (1.39%)	2370547 (8.84%)	108431 (2.25%)
tRNA	537157 (1.90%)	13514 (0.18%)	659539 (2.46%)	16373 (0.34%)
snRNA	24485 (0.09%)	3861 (0.05%)	18057 (0.07%)	3820 (0.08%)
snoRNA	4054 (0.01%)	1396 (0.02%)	2936 (0.01%)	1019 (0.02%)
Unann[a]	12794463 (45.36%)	7342295 (97.72%)	12540494 (46.77%)	4644566 (96.53%)

[a]Contains all of the unclassified sequences that possibly include new miRNAs.

Identification and expression profiling of conserved miRNAs. When the sRNA sequences obtained from our libraries were aligned to plant miRNAs in the miRBase database, 164 miRNA members showed perfect matches to the known miRNAs. These miRNAs identified have been shown conserved in many plant species. These conserved miRNAs varied significantly in expression abundances in both libraries (Table 2), and a total of 62 conserved miRNAs identified were found to be significantly responsive to phytoplasma infection, among which, 37 miRNAs were significantly decreased and 25 miRNAs were increased in the infected leaf ($P < 0.05$, fold 2.0). These differentially expressed miRNAs included some highly expressed miRNAs such as mul-miR156a-5p and mul-miR157a-5p, also some low abundance miRNAs such as mul-miR394a-5p and mul-miR4343a. Moreover, many differentially expressed miRNA-3p sequences, such as mul-miR1023b-3p, mul-miR1157-3p and mul-miR157d-3p, were identified, and these miRNAs (3p) may be the authentic miRNAs since the corresponding miRNA-5p sequences were not detected (Table 2). Real-time PCR analysis for 24 individual miRNAs covering different expression patterns were performed, and the results demonstrated that there was a very strong correlation between PCR data and read frequencies, which indicated that the sequencing profiles are quantitative and reliable (Fig. 2A).

Identification and expression profiling of novel miRNAs. All the non-annotated clean tag sequences were mapped to our mulberry transcriptome database, and only 23 sequences perfectly matched transcriptome sequences able to fold into hairpin structures. These sequences comprise 20 putative new miRNA families (Table 3). The lengths of novel miRNAs identified vary from 20 to 23 nt with a peak at 21 nt, and most begin with a 5′ uridine which is a characteristic feature of miRNAs. These newly identified mulberry miRNA precursors have negative folding free energies (from -21.0 to -105.0 kcal mol^{-1} with an average of about -46.9 kcal mol^{-1}) according to Mfold, which are similar to the free energy values of rice and Arabidopsis[23] and much lower than the folding free energies of tRNA (-27.5 kcal mol^{-1}) and rRNA (-33 kcal mol^{-1})[24]. Of these miRNAs, 3 candidates contained both miRNA-5p and miRNA-3p sequences. Moreover, other 5 miRNA-3p sequences were also detected (Table 3). The detection of miRNA-3p is a strong clue for the formation of precursor hairpin structures and represents further evidence supporting them as novel miRNAs. Compared to the conserved miRNAs, most of the novel miRNAs exhibited relatively low abundance as indicated by their frequencies (Table 2, 4). Among the 23 novel miRNAs, only 7 had at least 20 transcripts per million (TPM) in both libraries with the highest abundance 506 TPM for mul-miRn12-5p. Therefore, the expression levels of the majority of the novel miRNAs identified were low.

In order to identify the novel miRNAs involved in response to phytoplasma-infection, normalized expression profiles of the novel miRNAs in healthy and infected leaves were compared (Table 4). Among 13 differential novel miRNAs, 7 miRNAs were significantly decreased and 6 miRNAs were up-regulated more than 2.0 fold changes ($P < 0.05$) in the infected leaf. Real-time PCR analysis to determine the expression patterns of the 13 differential miRNAs (Fig. 2B) reveals a very strong correlation between qRT-PCR data and read frequencies, demonstrating that the profiles of these novel miRNAs detected by Illumina sequencing are reliable.

Targets of phytoplasma-responsive miRNAs. To understand the functions of phytoplasma-responsive miRNAs, target genes of the phytoplasma-responsive miRNAs including conserved and novel miRNAs were predicted. In total, 42 differentially expressed miRNAs were predicted to match 122 target genes (Table 5). The predicted miRNA targets were further experimentally verified by mRNA degradome sequencing. In total, we obtained 25 850 468 raw reads from the degradome libraries. After removing the reads without the adaptor, 25 721 355 clean reads were obtained, among which the percent of 20–21 nt reads was 99.39%. The distinct sequences were aligned to the mulberry transcriptome database, and 6913 912 sequences could be matched to the transcriptome without mismatch. The matched sequences were further analyzed by the CleaveLand pipeline, and 69 target genes of 28 phytoplasma-responsive miRNAs were qualified (Table 5), of which 8 genes were cleaved by two unconserved miRNAs and 61 genes were cleaved by 26 conserved miRNAs. Among these identified targets, 27 belonged to category 0, 5 were in category I, 13 were in category II, 15 were in category III and 9 were in category VI. These results indicated that most of these targets were efficiently cleaved by miRNA. The representative 'target plots' (tplots) of identified targets included all five categories were showed in Figure 3.

In most cases, the identified miRNAs were predicted to cleave two or more different targets. For example, three members of SPL transcription factor family genes were predicted to be cleaved by mul-miR157a-5p, and mul-miR160a-5p was predicted to slice three genes belonging to the auxin response factor. Inconstantly, some miRNAs were only predicted to cleave one target such as mul-miR1030a, mul-miR164c-3p, mul-miR6463, and mul-miR6475. Unfortunately, we could not detect the cleavage signature for most of miRNAs in this degradome library. The low identification frequency might be because that their target mRNAs have not been annotated in our mulberry transcriptome data.

The expressions of the targets of phytoplasma-responsive miRNAs were investigated by real-time PCR in response to phytoplasma-infection (Fig. 4). The results indicated that most of the target genes showed a negative correlation with the corresponding miRNA expression, and this is consistent with miRNA function in guiding the cleavage of target mRNAs. But it should be noted that a similar expression pattern between the target gene and its corresponding miRNAs was also observed. For instance, mul-miR169a-3p was positively correlated with its target gene encoding phosphatidylinositol 4-kinase. Of course, the putative target genes were bioinformatically predicted, and are subjected to experimental verification.

GO function analysis of targets. To better understand miRNA functions, we subjected the identified target genes to Gene Ontology (GO) analysis. The result of GO analysis demonstrated that the target genes of the phytoplasma-responsive miRNAs were classified into 10 categories according to their ontologies in Arabidopsis based on KEGG functional annotations (Fig. 5). The first category of the predicted target genes encoding an array of transcription factors involved in regulation of gene expression. In the second category, target genes associated with signaling pathway, indicating that there were many signaling pathways involved in phytoplasma infection. Target genes involved in metabolic process belong to the third category, suggesting that phytoplasma infection may change diverse metabolic processes in infected plant. The other categories included stress response, development, DNA and RNA processing, and so on. In most cases, targets of the same miRNA were with similar functions (Table 5), however, some miRNAs, such as mul-miR394a-5p, target many genes with different functions. So these miRNAs might be play potential roles in the expression control of genes related to diverse biologic processes, and the regulation networks of miRNAs involved in the response of mulberry to phytoplasma-infection were intricate.

Identification and expression profiling of siRNAs. According to the standard that siRNAs were a pair of perfectly complementary sRNAs with 2 nt overhangs at the 3′-end, 315 910 and 271 367 unique siRNA sequences were identified in the healthy and infected leaf small RNA libraries, respectively. The siRNAs identified varied in length from 20 to 24 nt, and the 24 nt siRNAs, which were considered as long siRNAs, were the major size groups (Fig. 6). In order to determine the siRNAs responsive to phytoplasma

Table 2 | Expression profiling of conserved in mulberry miRNAs

MiRNA-name	Sequence (5′-3′)	Normalized value		Fold-change (log_2 IL/HL)	P-value	Significance lable
		Healthy leaf (HL)	Infected leaf (IL)			
mul-miR1023b-3p	ACAGAACUGAAGAAGAGUGCAUA	25.7721	13.3885	−0.94482	1.55E-25	
mul-miR1030a	UCUGCAUUUGCACCUGCACCU	1.2053	8.9505	2.892576	6.38E-42	**
mul-miR1122	UCAGAUACAUCCGGAUUUGCA	0.9571	1.5663	0.710619	0.0444562	
mul-miR1134	GAAGAACAAAAGAAUGAAGAAGAU	3.4386	2.4987	−0.46064	0.0438324	
mul-miR1144b	UGCGGAAGUGUGGCGGAACGGCAG	1.6661	0.9323	−0.83761	0.01748	
mul-miR1157-3p	UUCAGGUAGUGGGAACCAGGC	221.8813	103.6769	−1.09769	1.10E-263	**
mul-miR1310	AGGCAUCGGGGGCGCAACGC	16.1297	15.477	−0.05959	0.5436935	
mul-miR1521a	CUAUUAUGGACAAUGUUGGA	43.2489	28.8282	−0.58518	4.43E-19	
mul-miR156a-5p	UGACAGAAGAGAGUGAGCAC	867.3186	6523.48	2.911008	0	**
mul-miR157a-5p	UUGACAGAAGAUAGAGAGCAC	5519.133	322078.7	5.866828	0	**
mul-miR157d-3p	GCUCUCUAUGCUUCUGUCAUCC	1.418	31.1404	4.456858	1.34E-203	**
mul-miR159a-3p.1	UUUGGAUUGAAGGGAGCUCUG	1764.699	1693.253	−0.05962	1.89E-10	
mul-miR160a-5p	UGCCUGGCUCCCUGUAUGCCA	4.7148	10.5542	1.162549	2.03E-15	**
mul-miR160b-3p	GCGUAUGAGGAGCCAUGCAUA	56.4363	58.6633	0.055835	0.2761912	
mul-miR162-3p	UCGAUAAACCUCUGCAUCCA	103.6911	97.7472	−0.08516	0.0281548	
mul-miR162a-3p	UCGAUAAACCUCUGCAUCCAG	110.1785	103.0057	−0.09712	0.0100218	
mul-miR164a-5p	UGGAGAAGCAGGGCACGUGCA	616.7583	140.7471	−2.1316	0	**
mul-miR164c-3p	CAUGUGCCCGUCUUCGCCAUC	5.1757	0.4848	−3.41629	1.87E-28	**
mul-miR165a	UCGGACCAGGCUUCCCCCCC	132.5828	104.3109	−0.346	5.59E-22	
mul-miR165a-3p	UCGGACCAGGCUUCCCCC	69.8364	53.8523	−0.37497	4.68E-14	
mul-miR166a-3p	UCGGACCAGGCUUCAUUCCCC	22928.82	18439.32	−0.31438	0	
mul-miR166h-3p	UCUCGGACCAGGCUUCAUUCC	7061.206	3301.962	−1.09659	0	**
mul-miR167d-5p	UGAAGCUGCCAGCAUGAUCUG	5533.773	3360.961	−0.71939	0	
mul-miR167h-3p	AGGUCAUCUUGCAGCUUCAAC	19.2493	17.2671	−0.15678	0.0857988	
mul-miR168a-5p	UCGCUUGGUGCAGGUCGGGAA	1734.637	1280.858	−0.43752	0	
mul-miR168a-3p	CCCGCCUUGCAUCAACUGAAU	30.9124	24.0918	−0.35964	1.41E-06	
mul-miR169a-3p	UGGCAAGUUGUUCUUGGCUAC	12.3366	41.247	1.741344	8.12E-101	**
mul-miR169q-5p	UGAGCCAGGAAUGACUUGCCG	100.9969	104.7585	0.052756	0.168986	
mul-miR170-5p	UAUUGGCCUGGUUCACUCAGA	1.3825	1.3799	−0.00272	0.9963935	
mul-miR171b	UGAUUGAGCCGUGCCAAUAUC	101.5287	60.9009	−0.73735	3.22E-63	
mul-miR171b-3p	CGAGCCGAAUCAAUAUCACUC	8.2953	15.477	0.89976	6.79E-15	
mul-miR172a-3p	AGAAUCUUGAUGAUGCUGCAU	2493.763	365.3307	−2.77105	0	**
mul-miR172e-3p	GAAUCUUGAUGAUGCUGCAU	2461.326	359.662	−2.77472	0	**
mul-miR1854-5p	UGUGAGUUUUGUAGAUUCGGA	24.7086	11.0017	−1.16729	5.43E-34	**
mul-miR1863a	CGCUCUGAUACCAUGUUAGUUUAC	23.8933	13.8733	−0.7843	9.08E-18	
mul-miR1873	ACUAACAUGGUAUCAGAGCGGGAG	71.7507	39.2704	−0.86955	1.95E-59	
mul-miR1919a	AGAGAGUCUUCUGUGGACGGG	6.7	3.5056	−0.9345	1.39E-07	
mul-miR2078	GGUUCGCUGCCUGUGACGU	65.157	40.0536	−0.70199	5.94E-38	
mul-miR2086-3p	UACACUGAAUGCAGAAAUGGACA	7.7635	5.967	−0.3797	0.0111255	
mul-miR2087-5p	GAAGAAAGAACCGGCAGUCAU	12.2302	0.3356	−5.18756	2.86E-86	**
mul-miR2108a	UUAAUAGUGUUUGUAAGUCGG	38.9949	28.1196	−0.47171	3.21E-12	
mul-miR2111a-5p	UAAUCUGCAUCCUGAGGUUUA	4.2185	1.1188	−1.91478	5.67E-13	**
mul-miR2119	CGAAAGGGAGCUUGUAGGGAA	4.7857	3.8413	−0.31714	0.0928318	
mul-miR2199	UGAUAACUCGACGGAUCGC	715.4864	607.7035	−0.23556	1.88E-54	
mul-miR2595	UACAGUUUUCUUCUUUUUUCC	2.0915	0.7459	−1.48748	2.36E-05	**
mul-miR2610a	CGAUGUGAGACUGUACGGCUU	11.9466	10.1812	−0.23069	0.0494168	
mul-miR2645	UUUAUAGAUGAUGAGCAUUAU	1.9143	3.2819	0.777714	0.0015907	
mul-miR2661	UGAGGUUUAAGAAAAUGGCAC	2.5169	2.0139	−0.32166	0.2182677	
mul-miR2670f	AGGGUCUGUUUGGUUGGGGGA	75.6857	38.8229	−0.96311	5.57E-74	
mul-miR2873c	CAAUAUGAGUUGUGUUUGGAA	53.9903	46.4682	−0.21646	8.34E-05	
mul-miR2916	UGGGGGCUCGAAGACGAUCAG	243.6475	224.6582	−0.11706	4.22E-06	
mul-miR319g	UUGGACUGAAGGGAGCUCCUC	26.8002	27.3364	0.02858	0.7018003	
mul-miR3437-5p	AAAAAACACAGGAUCAACGGACA	5.1402	2.9089	−0.82135	3.59E-05	
mul-miR3515	AAUGUGAGCAAAGAACGGUAU	1.1698	0.6713	−0.80123	0.0562362	
mul-miR3522b	UGAGACCUAAAUGAAGAAGAUGAC	0.8862	1.2307	0.473775	0.2161161	
mul-miR3626-5p	GGUAGUUCGACCGUGAAAUUUAA	9105.604	10815.07	0.248217	0	
mul-miR3627-5p	UGUCGCAGGAGAGAUGGCGAAU	2.3397	11.7849	2.332544	2.85E-43	**
mul-miR3628-3p	CCAAGCAGAGCUCUUCGCAUC	1.737	2.2003	0.341102	0.2213622	
mul-miR3710	UGCGGCACGUGACGGGCCUCC	3.9704	2.797	−0.5054	0.0183083	
mul-miR390a-5p	AAGCUCAGGAGGGAUAGCGCC	308.0956	229.3573	−0.42578	4.23E-71	
mul-miR390a-3p	CGCUAUCUAUCCUGAGUUUCA	5.4593	4.3261	−0.33565	0.058158	
mul-miR391-5p	UGUCGCAGGAGAGAUGGCGAA	3.4032	15.6634	2.202434	3.40E-53	**
mul-miR3933	AGAAGACAAAAUGCACGACUCUA	34.032	20.0268	−0.76496	1.14E-23	
mul-miR393-5p	UCCAAAGGGAUCGCAUUGAUCC	1.4534	3.9531	1.443553	9.68E-09	**
mul-miR393a-3p	AUCAUGCUAUCUCUUUGGAUU	5.8847	9.5472	0.698109	9.02E-07	
mul-miR3946-5p	UUGUAGACAGAGAGAGAGAGAGAC	24.7086	28.5298	0.207456	0.0059993	
mul-miR394a-5p	UUGGCAUUCUGUCCACCUCC	1.0989	8.5403	2.958227	5.74E-41	**
mul-miR3954	CUGUACAGAGAAAUCACAGCA	139.7437	103.043	−0.43954	4.06E-35	

Table 2 | Continued

MiRNA-name	Sequence (5′-3′)	Normalized value		Fold-change (log_2 IL/HL)	P-value	Significance lable
		Healthy leaf (HL)	Infected leaf (IL)			
mul-miR395a-3p	UGAAGUGUUUGGGGGAACUCC	10.5995	0.8951	−3.5658	7.59E-59	**
mul-miR396b-5p	UUCCACAGCUUUCUUGAACUU	153.3211	132.5051	−0.21051	1.08E-10	
mul-miR396b-3p	GCUCAAGAAAGCUGUGGGAGA	954.0292	107.6301	−3.14795	0	**
mul-miR397a-5p	UCAUUGAGUGCAGCGUUGAUG	29.5653	53.1065	0.844984	1.26E-42	
mul-miR397b-3p	UCAGCGCUGCACUCAAUUAUG	1.8788	1.1561	−0.70055	0.0304262	
mul-miR398a-5p	GGCGUGACCCCUGAGAACACAAG	13.6482	1.8274	−2.90085	2.22E-62	**
mul-miR399k-3p	UGCCAAGAAGAGUUGCUCUGU	14.3927	7.3469	−0.97013	1.52E-15	
mul-miR403a-3p	UUAGAUUCACCCACAAACUCG	15.7752	19.542	0.30892	0.0008772	
mul-miR408b-5p	CAGGGAACGGACAGAGCAUGG	1508.963	530.1323	−1.50913	0	**
mul-miR415	AAGGAGCAGAGCAGAGCAG	17.1223	5.6687	−1.59479	1.12E-37	**
mul-miR418	UAAUCUGAUGAUAGAUGGACG	62.0729	20.9219	−1.56895	5.93E-129	**
mul-miR419	UGAUGAAUGUAUGGAUGAUGGAU	9.8551	7.2723	−0.43846	0.0010722	
mul-miR4343a	AAAAAACUUACGGACAAGACGACU	2.3042	1.0442	−1.14187	0.0002923	**
mul-miR4371a	AAGAGAGGACAGUGACAAGCAAGU	8.1535	3.6921	−1.14298	7.09E-12	**
mul-miR4376-5p	UACGCAGGAGAGAUGACGCUGU	2564.095	1769.519	−0.53509	0	
mul-miR4376a-3p	AGCAUCAUUUUCCUGCAUAGU	1.2053	1.0442	−0.20699	0.579823	
mul-miR4385	AAUCGAUGGUAGAAAGUGAUGGGC	1.1698	0.5967	−0.97118	0.024715	
mul-miR4401b	UCACAAGACCUUGCUGAAGAA	9.6069	6.1535	−0.64266	4.93E-06	
mul-miR4414a-3p	AUCCAACGAUGCAGGAGCUAGCC	92.7371	37.3312	−1.31276	3.71E-147	**
mul-miR4414a-5p	AGCUGCUGACUCGUUGGUUCA	162.8216	71.4923	−1.18743	8.25E-220	**
mul-miR447b-5p	ACUCUCACUCAAGGGCUUCA	4.0767	8.5403	1.066885	2.71E-11	**
mul-miR472b-3p	UUUUCCCAACACCACCCAUACC	60.903	87.2676	0.518933	4.45E-30	
mul-miR473a-5p	ACUCUCCCCCUUAAGGCUUCCA	181.5392	697.6191	1.942158	0	**
mul-miR477a-5p	ACUCUCACUCAAGGGCUUCAG	4.2894	9.3981	1.131593	2.29E-13	**
mul-miR479	CGUGAUAUUGAUUCGGCUCAUA	4.8921	14.7684	1.593988	5.47E-33	**
mul-miR482a-3p	UUCCCAAGGCCGCCCAUUCCGA	201.8166	361.3403	0.840313	1.57E-276	
mul-miR482a-5p	GGAAUGGGCUGUUUGGGAAGA	10799.72	20081.4	0.894866	0	
mul-miR5020a	CUGGAAGAAGCUGAGCGUGCA	27.4383	11.6357	−1.23763	4.89E-41	**
mul-miR5021	UGAGGAAGAAGAAGAAAUGA	52.1114	35.8394	−0.54005	7.94E-20	
mul-miR5029	AUGAGAGAAAAACACUGCAUA	4.4312	2.8716	−0.62584	0.0024964	
mul-miR5054	GUGCCCCACGGUGGGCGCCA	1.1344	3.5056	1.627732	3.68E-09	**
mul-miR5059	CGUUCCUGGGCAGCAACACCA	35.45	31.849	−0.15454	0.0214399	
mul-miR5065	UAGGCAAUUCACUUAGAUCUG	2.3397	4.1769	0.836109	0.0001483	
mul-miR5072	GUUCCCCAGUGGAGUCGCCA	2.2333	5.333	1.25577	2.07E-09	**
mul-miR5077	UUCACGUCGGGUUCACCA	203.6954	291.4143	0.516658	1.49E-95	
mul-miR5085	AAGGACAUUUGGUUGUGGCUC	83.3074	92.9736	0.158376	0.0001338	
mul-miR5138	AAACGAAUCGUUGGCCGCUA	1.2762	1.7528	0.457807	0.1517183	
mul-miR5139	AACCUGGCUCUGAUACCA	51.0479	34.5341	−0.56383	6.68E-21	
mul-miR5224a	UUGAUGGACAUGAAGACGUUAU	119.5372	160.1772	0.422209	2.38E-37	
mul-miR5225a	ACUGUCGCAGGAGAGAUGACGC	81.5703	33.4153	−1.28754	6.81E-126	**
mul-miR5234	GUCUUGUUAUGGAUGGCAGAA	6.0619	12.0086	0.986226	1.29E-13	
mul-miR5236a	UGAAUUUUCGGGCAGAUCGGGUU	4.1476	1.8647	−1.15333	8.88E-07	**
mul-miR5247	GCAGGAGCCAUCUCUGAUCGA	1.2053	0.6713	−0.84436	0.0426108	
mul-miR5266	CGGGGGACGGACUGGGGG	530.8631	339.1504	−0.64641	3.68E-256	
mul-miR5280	AAUUAUUAAACGGGCCGUGACGGG	43.8161	20.9592	−1.06388	5.08E-51	**
mul-miR5291a	GUUUGGUAGGAUGGAUGGAUGGAG	2.7651	2.0139	−0.45734	0.0729034	
mul-miR529-3p	GCUGUACCCCCUCUCUUCUCC	3.1905	6.2654	0.973624	1.23E-07	
mul-miR529b	AGAAGAGAGAGAGUACAGCUU	275.7297	874.2055	1.664718	0	**
mul-miR530-3p	AGGUGCAGAUGCAGAUGCAG	15.208	44.2678	1.541427	5.81E-91	**
mul-miR530-5p	UGCGUUUGCACCUGCACCUUA	4.3603	62.7656	3.847475	0	**
mul-miR535a	UGACAACGAGAGAGAGCACGC	54.0257	592.2266	3.454432	0	**
mul-miR5368	AGGGACAGUCUCAGGUAGACA	7.8699	15.5515	0.982637	4.25E-17	
mul-miR5369	UGAGAACAGUAGGAUGUCAUC	7.1254	4.3634	−0.70752	1.87E-05	
mul-miR5386	CGUCAGCUGUCGGCGGACUG	81.0386	161.6316	0.996028	1.41E-165	
mul-miR5485	UGACAGAGUGGUAUCAGAGCA	17.4414	6.8994	-1.33797	6.69E-30	**
mul-miR5500	AUCACUGAUGAAACUUCGGGCGGC	2.2688	0.9696	−1.22647	0.000143	**
mul-miR5509	AAGGCCUUUUGCUCUUGGCAU	1.418	0.9323	−0.60499	0.0997003	
mul-miR5519	UGGUAGACGCUACGGACUUAG	26.6584	68.9937	1.371874	1.99E-118	**
mul-miR5521	AAGUGGCUGGAUGUUGAUGAG	2.2688	2.3868	0.073148	0.7729617	
mul-miR5559-5p	UACUUGGUGAAGUGUUGGAUAA	0.1772	7.3096	5.366342	6.12E-54	**
mul-miR5564c-5p	CAAUUCGUCGAACGCCGCACC	1.5243	0.7459	−1.03109	0.0068859	**
mul-miR5565g-3p	ACACAAGUGGAUUGAUGAUGAAUC	1.5243	0.5221	−1.54575	0.0002156	**
mul-miR5658	AUGAUGCUGAUAAUGAUGACAA	1.5598	0.4848	−1.6859	6.78E-05	**
mul-miR5665	AUUGGUGAACAAGAUCGUGAU	5.7429	1.7901	−1.68174	1.35E-14	**
mul-miR5741a	AACGAGGACUAAGUUGAUGGUUU	3.8995	2.536	−0.62073	0.0048851	
mul-miR5751	UUAAUUCUGAUUGAGAUGGUUUU	21.4827	12.2324	−0.81247	4.83E-17	
mul-miR5813	AGCAGGACGGUGGUCAUGGA	314.5829	295.3674	−0.09093	4.52E-05	

Table 2 | Continued

MiRNA-name	Sequence (5′-3′)	Normalized value		Fold-change (log_2 IL/HL)	P-value	Significance lable
		Healthy leaf (HL)	Infected leaf (IL)			
mul-miR6021	UUGGAAGAGGAUGCAUUGAAC	10.0678	0.1119	−6.49139	1.86E-77	**
mul-miR6145b	UAUGAGACGUAUGCACUAGCC	0.5317	1.0442	0.973714	0.032467	
mul-miR6171	ACUGUGGAUCGCAGAAGGUUU	21.4827	25.6209	0.254146	0.0015546	
mul-miR6196	AGGCGAGUGAGACGGAGAUGA	7.0545	3.804	−0.89103	2.07E-07	
mul-miR6203	AGAGAGAUUAAGAAGACCUGGUAA	2.2333	0.7832	−1.51172	9.71E-06	**
mul-miR6204	AGGAGAAGAAUUAGAAGCUUUCGA	5.991	2.9835	−1.00579	1.21E-07	**
mul-miR6213	AAGCAGAUUGAUACAGACUGGUU	20.561	9.2862	−1.14675	8.30E-28	**
mul-miR6223-5p	UUCUUGAGGAGGAGCGUACUG	5.8492	3.8786	−0.5927	0.0009253	
mul-miR6300	GUCGUUGUAGUAUAGUGGU	112.0573	127.3213	0.184237	2.27E-07	
mul-miR6429	GAGUAGAAAAUGCAGUACUAG	3.7931	2.4614	−0.6239	0.0053003	
mul-miR6440b	GGAGUUUGAUUGAGUUCGGUU	0.6026	29.4249	5.609693	3.52E-217	**
mul-miR6444	AGGAAAAUCAAGAGAUAAUGU	7.1254	4.4007	−0.69524	2.51E-05	
mul-miR6449	UCAUGAUUCGGAUCACGGUUU	8.6498	8.0928	−0.09603	0.4769051	
mul-miR6462c-5p	AAGUGGACAGAAAAUGGAAUAAAA	10.7768	5.706	−0.91738	4.59E-11	
mul-miR6463	UGUGAUGAUCAUUGGACAACC	0.1418	3.5056	4.627732	1.19E-24	**
mul-miR6464	UAAUGCUUGUUGGGUAUUU	8.9688	5.1838	−0.7909	1.21E-07	
mul-miR6466-5p	UUUGGAUGACAUUUGACGA	2.5169	1.7901	−0.49161	0.0676547	
mul-miR6475	UCUUGAGAAGUAGAGACGUCU	1.8079	10.2931	2.509291	2.81E-41	**
mul-miR6478	CCGACCUUAGCUCAGUUGGUA	22.298	20.2506	−0.13895	0.1001727	
mul-miR6483	UAUUGUAGAAAUCUUCGGGAU	3.9704	3.6175	−0.13429	0.5044449	
mul-miR774b-5p	UGAGAUGAAGAUUAUAGUGAA	38.4632	44.7153	0.21729	0.0003217	
mul-miR780.2	UUUUCGUGAAUAUAUGGUCGU	56.5781	23.4205	−1.27247	2.43E-86	**
mul-miR827-5p	UUUGUUGAUGGUCAUUUAACU	6.5228	6.8994	0.08098	0.5892211	
mul-miR829.1	CCUCUGAUAACAAAUGAUGGACAU	1.0989	0.1119	−3.29578	8.66E-07	**
mul-miR845b-3p	CGCUCUGAUACCAACUGUGACG	126.9817	80.7412	−0.65324	6.83E-64	
mul-miR858b	UUCGUUGUCUGUUCGACCUUG	52.0051	25.2479	−1.04249	2.55E-58	**
mul-miR893	AUUGGGUACUUGUGGUUGGC	3.4741	2.9835	−0.21963	0.3141346	
mul-miR894	GUUUCACGUCGGGUUCACCA	584.1443	788.9517	0.433612	1.40E-185	
mul-miR952a	AACAAGCAUCAUCGUUGGUU	12.372	11.673	−0.0839	0.4559723	

infection, we looked for differential siRNAs by comparing the normalized expression levels of the siRNAs between the healthy and infected leaf libraries. A total of 14 598 siRNAs were identified to be significantly responsive to phytoplasma infection. Among these differential siRNAs, 10 745 siRNAs were significantly decreased and 3853 siRNAs were up-regulated in the infected leaf ($P < 0.05$, fold 2.0) (Fig. 6; Supplementary Text).

***MulMIR393A* is induced by phytoplasma infection.** It was reported that Arabidopsis miR393 plays a role in plant PAMP-triggered immunity in Arabidopsis[10]. Though phytoplasma lacks cell wall and flagellum, and has no PAMPs like flg22[4,6], our data showed that mul-miR393 was also induced by phytoplasma infection (Table 2). Therefore, mul-miR393 was induced by phytoplasma through some pathway other than flg22. MiR393 is potentially encoded by two genes, *AtMIR393A* and *AtMIR393B*, in Arabidopsis[18]. To study mul-miR393 function in the response to phytoplasma infection, we cloned the two genes encoding mul-miR393, *MulMIR393A* and *MulMIR393B*, in mulberry. Sequence analysis showed that limited homology exists between the *MulMIR393A* and *MulMIR393B*, but the mature mul-miRNA393-5p sequences produced are identical (Fig. 7A). Meanwhile, the promoters of *MulMIR393A* and *MulMIR393B* were cloned, and cis-acting regulatory elements analysis revealed that the two promoters were different, such as the promoter of *MulMIR393A* has a cis-acting element involved in salicylic acid responsiveness, but this was not found in the promoter of *MulMIR393B*. GUS activity analysis indicated that *MulMIR393A*::*GUS* showed induced GUS activity, but in contrast *MulMIR393B*::*GUS* showed no induced GUS activity after application of salicylic acid (SA) (Fig. 7B), confirming the cis-acting regulatory elements analysis results above. Therefore, *MulMIR393A* and *MulMIR393B* have different regulators. Previous studies showed that miR393 is a developmentally regulated miRNA which arises predominantly from *AtMIR393B* in aerial organs of Arabidopsis[25]. But when the plants were treated with flg22, the expressed level of miR393 was up-regulated mainly from activated transcription of *AtMIR393A*[10]. Northern blot and real-time PCR analysis results showed that the transcript level of *MulMIR393A* was increased in the infected leaves, but the transcript level of *MulMIR393B* was unaltered (Fig. 7C, D). Therefore, the mul-miR393 accumulation resulted from the increased transcripts of *MulMIR393A* in responding to phytoplasma infection.

Discussion

So far, miRNAs and their regulatory functions have been extensively characterized in model plant species, but to our knowledge, it was the first report about the sRNAs of mulberry. In present study, solexa high-throughput sequencing of small RNAs revealed a diverse and complex small RNA population. Among the 62 conserved phytoplasma-responsive miRNAs identified in mulberry, only 6 miRNAs, including miR157a, miR172a-3p, miR4414, miR473, miR477a, and miR479, were also differentially expressed in *Candidatus Phytoplasma aurantifolia* infected Mexican lime (*Citrus aurantifolia* L.)[15]. Therefore, the effect of phytoplasma infection on the expression of miRNAs depends on the pathogen and host types. Furthermore, it was found that the miRNAs of the same family were differentially regulated in the response to phytoplasma-infection. For instance, the expression level of mul-miR160a-5p was up-regulated by phytoplasma-infection, but the expression level of mul-miR160b-3p showed no obvious change in the infected leaves (Table 2). So the gene expression programmings of the miRNAs, which share a high degree of sequence similarity and belong to the same family, were controlled by different regulator[16].

Phytoplasma-associated disorders are believed to be the auxinic diseases of plants[26]. It was reported that many auxin response factors

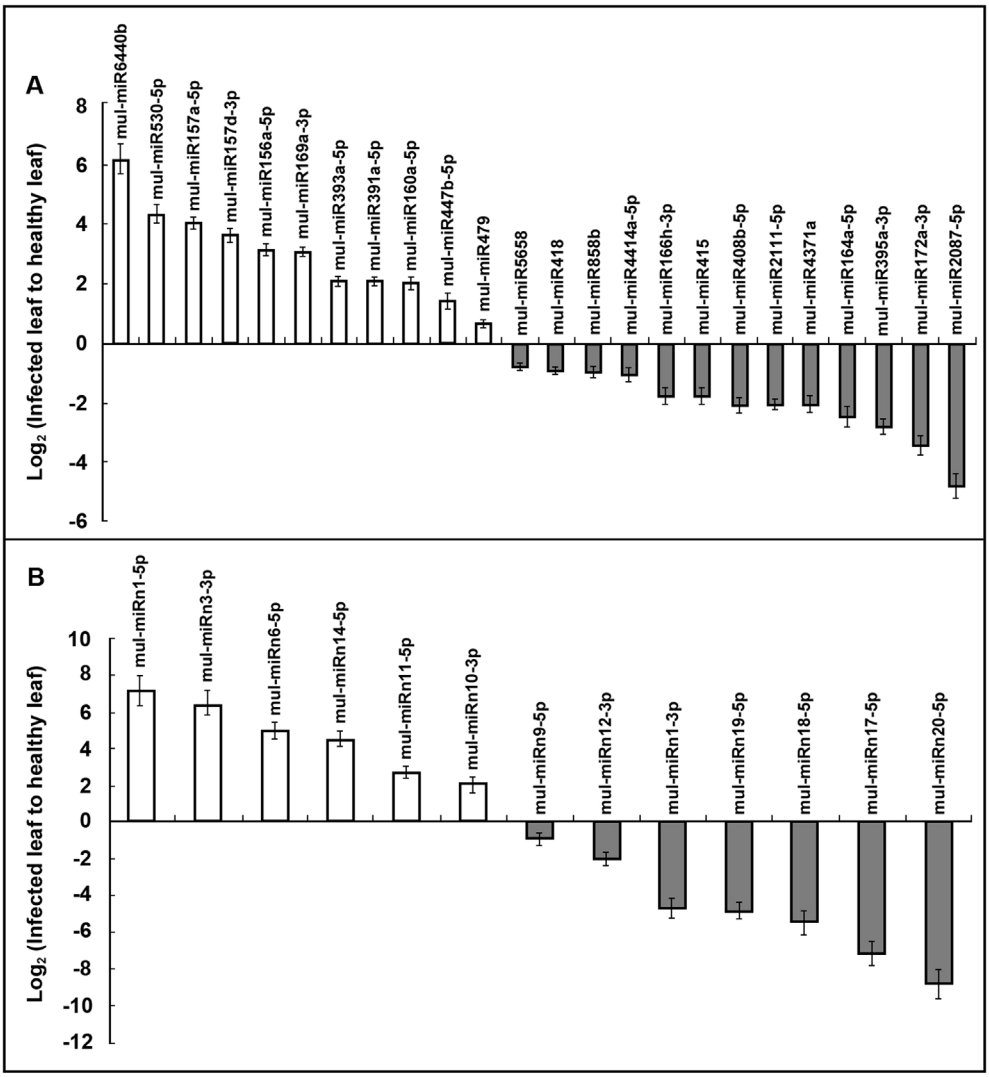

Figure 2 | MiRNA abundance analysis by real-time PCR. (A) Conserved miRNA abundance analysis by real-time PCR. (B) Novel miRNA abundance analysis by real-time PCR. The relative miRNA abundance was evaluated using comparative Ct method taking *U6* as the reference. The $\log_2$ values of the ratio of phytoplasma-infected samples to the healthy samples are plotted. Values are given as mean ± SD of three experiments in each group.

were down-regulated by the effector (tengu) of Onion yellows phytoplasma, and suggested that the symptoms caused by phytoplasma may be involved in an auxin-related pathway[5]. In plants, a group of miRNAs regulate and fine-tune multiple hormone signaling pathways coordinately[27]. Our results showed some miRNAs, which target the genes associated with auxin signaling and metabolism, were differentially expressed between the healthy and infected leaves. MiR393 was the first miRNA identified which targets auxin receptor mRNAs and subsequently represses auxin signaling in Arabidopsis[10], and we found that mul-miR393 was up-regulated in the infected leaves (Table 2). In addition to miR393, target analyses showed that mul-miRn10-3p was also involved in auxin signaling pathway by regulating auxin response factor (Table 5). Moreover, miR160, miR164 and miR166, which were associated with auxin signaling pathway by regulating different transcription factors[13], which were found differentially expressed in healthy and infected leaves (Table 2). Furthermore, the differential mul-miR169a-3p and mul-miR6204 were predicted to target the genes coding for O-fucosyltransferase family protein and SAUR-like auxin-responsive protein family, respectively, which were associated with auxin metabolism (Table 5). Therefore, these differentially expressed miRNAs may play a role in altering auxin signaling and metabolism in infected plants.

Beside the auxin signaling pathway, miRNAs were also involved in regulating other hormone signaling pathways. For instance, miR164, miR166 and miR398 were reported involved in abscisic acid signaling pathway[28–30]. MiR166 and miR172 were found to be down-regulated by gibberellin[31] and cytokinin[32], respectively. The expression levels of these miRNAs were differential between healthy and infected leaves (Table 2). In addition, mul-miR2111a-5p was predicted to target the gene coding ACC oxidase 1 associated with ethylene metabolism, and mul-miR2595 and mul-miRn20-5p were predicted to target the pentatricopeptide repeat-containing protein gene involved in the response to SA stimulus (Table 5). Therefore, it is more likely that the phytoplasma infection-induced alteration in hormonal signaling triggers reprogramming of the plant growth and developmental pattern, leading to some symptoms in infected plants. This is in accord with early reports that phytoplasma infection disturbs hormonal balance in the host plants[4,33–36].

Symptoms induced in infected plants suggest that phytoplasma infection may modulate developmental processes within the plant host[37]. This was confirmed by the reports that the expression of effector protein (phyllogen) of phytoplasmas in Arabidopsis can alter floral development, resulting in the production of leaf-like flowers[38,39]. In Arabidopsis, many Squamosa Promoter-Binding Protein-Like (SPL) genes are post-transcriptionally regulated by miR156. Meanwhile, some SPL genes, such as AtSPL9, in turn positively regulate the expression of miR172. This forms the miR156-AtSPL9-miR172 regulatory pathway[40]. Our results showed that

Table 3 | Novel miRNAs in mulberry by Illumina sequencing

MiRNA-name	Sequence (5′-3′)	Precursor sequence (5′-3′)	Energy (kcal mol^{-1})
mul-miRn1-5p	UAUAACAAUGACUUUAUAAAG	GACAUUUAUUUUAUAUAACAAUGACUUUAUAAAGGUGUAAC-	−39.70
mul-miRn1-3p	UUUUGAAGUCGUUGUUAUACA	AAAAAAUUUAUCUAUUGUAAACUGAUGUUGCAUCUUUUUGA-AGUCGUUGUUAUACAGAACAAAUGU	
mul-miRn2-5p	UCUGGGACAAGUAACACUACAU	CCAAAGUCAUUCUGGGACAAGUAACACUACAUUAAAUUAGA-UCUUUAAUAUUACAUUUUCAAUUUCAGGGCUAAAUCUUAGUU-GAAUAAGCGAAUUUAGUAAUUCAAACAUACAUGCGAUUUUUA-GCCAAAACAAAUGUAAGAGAUACAUUGCUUCAGUCUUUAA	
mul-miRn3-3p	GUGGUGAUCAAUUGGACCUUU	UGGAAUCGUCUUAGGUCUAAUUCCUAUUACUUUGGCUGGAU-UAUUCGUAACGGCAUAUUUACAAUAUAGACGUGGUGAUCAA-UUGGACCUUUGAUUAAUUAA	−21.80
mul-miRn4-5p	CGGGCCUGGGAGGUUUGGUA	UUUGAGCUUUAUGAAGUUGUCGGGCCUGGGAGGUUUGGUA-	−51.80
mul-miRn4-3p	UUCCAAAUCCACCCAUGCCCAC	GGAGUAAUAAGUAAUUACCAUUUAGUUUUUUGUUCACUUAAU-UGAUAUUAUAAUUGUAUGUUUUAAUUUAGUUCUCCUUCCAAA-UCCACCCAUGCCCACAAUUUCCUCAGGCUUCUCUC	
mul-miRn5-3p	UCCCUUUGGAUGUCGUCCUGC	CUGUGGCUUUUGCUGGGGACAGUGACAAAGGGAUGCUUUA-UGUGUUGAGAGGGUGUAAGAAACUUCGCAAGCUUGAGAUC-AGGGACUGCCCCUUUGGUGAUGUUGCUCUCUUGACGGACG-UGGGAAAGUAUGAAACAAUGCGAUCCCUUUGGAUGUCGUC-CUGCGAAGUUACCC	−58.30
mul-miRn6-5p	UAAGGCCGCGUACAUAUGGG-AUU	GUAAGGCCGCGUACAUAUGGGAUUAAGCUUUCUCCCGAGC-CUGUCAUAGUACGGGAGCCUUUU	−21.00
mul-miRn7-3p	UUGUAGAAGUCUUAAGAGAAG	AUGGUUUUUUCUUCUCUUAACACUUCUAUAAAUAUCUUUAUC-CGUCAGAUGAUCAAGUAAAUUGCGGGGAGCGAAAUUUGUAG-AAGUCUUAAGAGAAGAGAAAAACCA	−42.30
mul-miRn8-5p	UCAUGAUUCGUGGUUCGCUA	UCAUGAUUCGUGGUUCGCUACAAUAUUUUCUCUUACAAAAAA-UACACAAAUUAAAAAUAAUUCGAAUCACAUAGUUUGAAUCUA-CCAACUAAACGUCUUUUUUUUUUAGUUGAUCAUAAUUGUACU-CUUUUUUUUUCUCUCUUGUUAAAUCAGAGAACUCUACUCCUU-ACGAAACACUCCAUUGGAGGAAGUGCCUUUAAGUUUGAAAG-UGCAAUCCGUGGCUGGCUAGACGAAACUAUCAUGUGAUGCA-UAUGGACCCCCCAUGCCUGAUGCCACACAAGUUAAACAGCA-GGAUACGAAUCUUGU	
mul-miRn9-5p	GGGGUUACCUGAGAAUACAUG	GAGUCUCACAGGGGUUACCUGAGAAUACAUGAAUAAAUUA-UUUAUAUUUAUUUAUCUCACAAUGUGGUGGUCGUUUCAUGU-GUUCUCAGGUCGCCCCUGUAUGGCUUU	−50.70
mul-miRn10-3p	GGUGCAGAUGCAGAUGCAGG	GUCGCCUUUAUCUGCGUUUGCACCUGCACCUUAAUUGGCUUC-UUAAUUUUCUUCAUCUCAUUUGCCGAUCACUAACUCUACUUG-AGAUCAGCCAAACCCUCAAACUAAUCAACGGCGAUAAGACG-AAGGACCAUAGCAAGAGGUGCAGAUGCAGAUGCAGGUGAAU-GCCAU	−60.52
mul-miRn11-5p	UGUUAGGAUCCUGGUUGAGUC	UAUGUCACUCUGUUAGGAUCCUGGUUGAGUCUCGAGUCCUG-GGACUCAACUAGGAUCCUAACACACUCUUACU	−40.60
mul-miRn12-5p	UGUGGGGGGAUCGGUAAGUGU	GUUUAGCAGGAAGUUAUAGGUGUGGGGGGAUCGGUAAGUG-	−55.80
mul-miRn12-3p	UCUUGCCGAGACCUCCCAUACC	UUAAAUCUGUCGUGUUUUUAGCGGAUAAUUCUUGCCGAG-ACCUCCCAUACCGGUCAUUUCCUGCUGUUUCU	
mul-miRn13-3p	UUCUGUGUUCUUCUACGGCGA	CCUUGGUGAGUAGUAGUAGUAUAGAACAAGUGAAACAAAA-CUGUGUUUGUUAGAAAUGGUUUUGAUUGAUGAGGAAUGAAU-GAAAGAAAGAGAGAGAGUGAUUAGAGAACUGACGGUGCCA-UCUCUCCUGCGACAGUUUUUUCGUUUCUGUGUUCUUCUAC-GGCGACGAUGACAAG	−42.70
mul-miRn14-5p	UCUACCAGCCGAGUAGAAGUA	ACAUUGAAUCUCUACCAGCCGAGUAGAAGUAACAAUCGAG-GGAAAUGAGAUACUUACUUCUACUCGGCUGGUCGAGAUCC-AAUAU	−45.10
mul-miRn15-5p	CAGAGUGCACAAAUACAUUAG	CAGAGCCAGAGUGCACAAAUACAUUAGAAGACAAUUGGA-CUUCUACUGCUUUUGUGCACUCUGGCUCUGA	−44.70
mul-miRn16-3p	UAAUGUAUUUGCACACUCUGG	AUCAUCAGGUUCUCUUUUCUCCAACCGUAACAUGUCUAAAA-GAGGGUGCGAAUCUGGCAGCUCAUAUACUUGGUGCUCUGU-UCUUAGUCGGUUCACAUUCUUCAGCUUGGGCAUAGGAAUA-GAAGCAGCUUCUGGUCCUAAAGCAACUAAGGCCUUCGACA-UUUCAGCACCUUGAAGUUCCAUGUUUCUUUGCAUGUAGUU-CUGUACAUUCUGAGUGAACUCUUCAAUGUUAAGCUUGAUUG-UAGGAAUUUCAUUAGGAUCUUCAGAGAAAAAGUCCUCUAUG-UCCCCUAAUGUAUUUGCACACUCUGGCUCUGGUGUU	−76.05
mul-miRn17-5p	AUGACACAAGAUCAUCCUCCA	AUUUCACUUUAUGACACAAGAUCAUCCUCCAAAUAGAAAUA-GAAACAAAUCAUUGGUUUACCUUGAUUAUUCUUAUUGGCUUA-CGCCUAGAAGUUAGAGGGAUAAUAUCAAGAUAACGGGGAAG-UCUAGGGGUUUUGCGGUUUUGUGGGUGUUUAUAGAGUUACA-ACAUGGCGGUUGAUUGUUGAGUGUUGAUUUGUUUCUAUUUCU-AUUUGAAGGAUGAUCUUGUGUCAUGUAGUGAAAUG	−100.5

Table 3 | Continued

MiRNA-name	Sequence (5′-3′)	Precursor sequence (5′-3′)	Energy (kcal mol^{-1})
mul-miRn18-5p	UUCUGCAAUCAUAACUCGGAG	UCAUUAGUUAUUCUGCAAUCAUAACUCGGAGGAAAGAUUCU-GGUGUUGGCCGGAUUGCAGAUUGCUUUACA	−22.7
mul-miRn19-5p	AACUCGGUGCUGGAAUCGGUC	UGGAACUCGGUGCUGGAAUCGGUCAUGUCGGGCGUGCCAAU-GCUAGCUUGGCCCAUGGGAGCCGACCAAUUCAUAAACGCG-ACAUUAUUAGACCAAUUGAAGUUGGCAAUUAGAGUGGGUG-AAGGAGAUCGAAAUGUUUCUGAUUCGGUCGAUUUGACUCG-AGUUUUG	−49.42
mul-miRn20-5p	AGGAGGAGGAGGAGGAGUUU	GAUGACGAGGAGGAGGAGGAGGAGGAGUUUGAGAUGGAC-GAGUUGUCUUGUUUCAGAGGUCUUGUUCUCGAUACCUCCUA-CAGGCCAAU	−30.7

mul-miR156 was up-regulated and its target gene SPL9 was down-regulated, meanwhile the mul-miR172 was down-regulated (Table 2; Fig. 2A). Therefore, the miR156-SPL9-miR172 regulatory pathway may be also conserved in mulberry and responsive to phytoplasma infection. SPL family of transcription factors play important roles in flower and fruit development, plant architecture and phase transition[40]. AP2 domain-containing transcription factor, which is the target of mul-miR172, takes part in regulating flowering time and floral organ identity[41]. Therefore, the expression changes of miR156 and miR172 might disturb the plant development process and be responsible for the mulberry yellow dwarf symptoms such as development of green leaf-like structures instead of flowers and sterility of flowers.

In addition, our results showed some other miRNAs, which target the genes associated with plant growth and development, were also differentially expressed. For example, the expression level of miR166h-3p, which targets the class III HD-Zip protein 6 (Table 2, 5) involved in the regulation of shoot meristem initiation, vascular development[42], was down-regulated by phytoplasma infection, and this probably resulted in an abnormal plant development such as meristem defects and was partially responsible for the symptoms such as dwarf and witches' broom. Meanwhile, miR160, miR162, miR164, miR165, miR168, and miR319 were reported to modulate plant development through different pathway[41,43–48]. These up or down-regulation of these miRNAs (Table 2) may disorder the expressions of many genes involved in diverse development processes and change the architecture of infected plants causing some symptoms in the infected plants.

Our data showed that some differentially expressed miRNAs target the genes associated with CHO metabolism, protein metabolism, lipid metabolism, and so on. For example, the up-regulated mul-miR160a-5p may down-regulate its target, beta-amylase gene (Table 2, 5), and responsible for the accumulation of starch in the infected leaves. Similarly, the up-regulated mul-miR529b may down-regulate its target, chlorophyll synthase gene (Table 2, 5), resulting in the blockage of the biosynthesis of chlorophyll and the decoloration symptoms. Thus, the differential expressions of these miRNAs might disturb the normal metabolism process and partly responsible for some of the mulberry yellow dwarf symptoms. Based on the information obtained, the complex regulatory networks of miRNAs involved in the response of mulberry to phytoplasma-infection is outlined in Figure 8.

Unlike miRNAs, the lengths of siRNAs are more variable, ranging from 21 to 30 nt. The biogenesis of the siRNAs depends on specific RNA-dependent RNA polymerases and Dicer-like (DCL) proteins and RNA polymerase IV[49]. The siRNAs produced by different DCLs

Table 4 | Novel miRNA abundance analysis by Illumina sequencing

	Normalized value				
miRNA-name	Healthy leaves (HL)	Infected leaves (IL)	Fold-change (log_2 IL/HL)	P-value	Significance lable
mul-miRn1-3p	1.0989	0.01	−6.77992	0.132485	**
mul-miRn1-5p	0.01	0.7086	6.14690	0.143462	**
mul-miRn2-5p	21.6954	32.8932	0.6004	1.46E-15	
mul-miRn3-3p	0.01	29.1638	11.50996	7.30E-245	**
mul-miRn4-5p	290.7251	219.9219	−0.40266	9.14E-61	
mul-miRn4-3p	94.4398	78.91425	−0.25911	1.42E-32	
mul-miRn5-3p	52.1114	48.4447	−0.10526	0.055345	
mul-miRn6-5p	0.01	1.268	6.986411	2.37E-11	**
mul-miRn7-3p	17.9731	16.4466	−0.12805	0.173085	
mul-miRn8-5p	7.3381	5.8178	−0.33493	0.028247	
mul-miRn9-5p	41.6891	19.0572	−1.12933	2.22E-53	**
mul-miRn10-3p	12.372	37.9652	1.617599	7.55E-84	**
mul-miRn11-5p	0.01	1.0442	6.706254	1.77E-09	**
mul-miRn12-5p	506.0126	267.5089	−0.91959	0	
mul-miRn12-3p	156.38231	72.49928	−1.1090	3.54E-42	**
mul-miRn13-5p	16.7324	15.9618	−0.06802	0.480784	
mul-miRn14-5p	0.01	3.804	8.571373	1.40E-32	**
mul-miRn15-5p	1.5243	0.8578	−0.82943	0.024299	
mul-miRn16-3p	58.1379	31.9235	−0.86486	4.54E-48	
mul-miRn17-5p	6.0619	0.01	−9.24363	2.48E-50	**
mul-miRn18-5p	1.9852	0.01	−7.63314	5.78E-17	**
mul-miRn19-5p	1.0989	0.01	−6.77992	1.04E-09	**
mul-miRn20-5p	2.3397	0.01	−7.87018	7.25E-20	**

Table 5 | Predicted targets for the differential miRNAs

MiRNA-name	Predicted target annotations in mulberry transcriptome data	Putative GO_process	Degradome sequencing		
			Read	Score	Class
mul-miR1030a	SWIB complex BAF60b domain-containing protein	Transcription regulation	52	4	0
mul-miR156a-5p	Homeobox protein BEL1-like protein	Transcription regulation	57	4	0
	Squamosa promoter-binding-like protein 7	Transcription regulation	62	3	I
	Promoter-binding protein SPL9	Transcription regulation	26	3	0
	Promoter-binding protein SPL10	Transcription regulation	334	2.5	III
	Homolog A-like transcriptional coactivator KELP	Transcription regulation	No		
	UDP-glycosyltransferase-like protein	Metabolic process	No		
mul-miR157a-5p	SPL domain class transcription factor	Transcription regulation	119	1	
	Squamosa promoter-binding-like protein 7	Transcription regulation	45	3	0
	Promoter-binding protein	Transcription regulation	54	2	0
	Promoter-binding protein SPL10	Transcription regulation	1435	3	III
	Dioxygenase-like protein transcriptional coactivator KELP	Transcription regulation	No		
mul-miR160a-5p	Auxin response factor 10 (ARF10)	Auxin signaling; transcription regulation	65	0.5	0
	ARF18-like	Auxin signaling; transcription regulation	56	1	0
	ARF16	Auxin signaling; transcription regulation	1282	3	IV
	Beta-amylase	Metabolic process	1611	2	II
mul-miR164a-5p	NAC domain protein	Transcription regulation	139	3	0
	CBL-interacting serine/threonine-protein kinase 25-like	Signalling pathway; stress response	4353	4	III
mul-miR164c-3p	C2 domain-containing protein	Calcium-mediated signalling	No		
mul-miR166h-3p	Class III HD-Zip protein 6	Transcription regulation; development	102	2	0
	Homeobox leucine-zipper protein	Transcription regulation; transcription	No		
mul-miR169a-3p	Phosphatidylinositol 4-kinase	Signalling pathway	No		
	Serine/threonine-protein kinase BRI1-like 1	Signalling pathway	No		
	O-fucosyltransferase family protein	Hormone metabolism	No		
	Nuclear transcription factor Y subunit A-1	Transcription regulation	109	3	0
	Nuclear transcription factor Y subunit A-9-like	Transcription regulation	1091	3	III
	Nuclear transcription factor Y subunit A-3-like	Transcription regulation	3524	3	IV
mul-miR172a-3p	Ethylene-responsive transcription factor RAP2-7-like	Transcription regulation	979	1.5	IV
	AP2 domain-containing transcription factor	Transcription regulation	82	2.5	0
	S-locus lectin protein kinase-like protein	Signalling pathway	1979	1.5	III
mul-miR172e-3p	Transcription factor bHLH123	Transcription regulation	No		
	S-locus lectin protein kinase-like protein	Signalling pathway	234	3	II
	NAC domain protein, IPR003441	Transcription regulation	No		
	Trehalose 6-phosphate synthase	Carbohydrate metabolic process	No		
	Ethylene-responsive transcription factor RAP2-7-like	Transcription regulation	979	2.5	IV
	AP2 domain class transcription factor	Transcription regulation	82	1.5	0
mul-miR2087-5p	PCI domain-containing protein-like	Transcription regulation; development	No		
mul-miR2111a-5p	Myb-like transcription factor family protein	Transcription regulation	123	3	0
	ACC oxidase 1	Hormone metabolism	No		
	Disease resistance protein (TIR-NBS-LRR class) family	Stress response	451	3	0
	C2H2-like zinc finger protein	Transcription regulation	2561	4	III
mul-miR2595	RNA processing factor1	RNA processing	No		
	MIF4G domain and MA3 domain-containing protein	RNA.processing	No		
	FAR1-related protein	Response to red or far red light	478	2	II
	Tir-nbs-lrr resistance protein	Stress response	3271	2.5	IV
	FKBP12 interacting protein 37	Development	No		
	Pentatricopeptide repeat-containing protein	RNA processing; stress response	373	3	III
	Transcription factor MYB884	Transcription regulation	422	2	III
mul-miR393-5p	Transport inhibitor response protein	Signaling pathway	678	4	III
	Auxin receptor 1 (TIR1)	Signaling pathway	721	3.5	II
mul-miR394a-5p	OSIGBa0145C02.4	Unknown	No		
	Aluminum-activated, malate transporter 12	Transportion; stomatal movement	No		
	Methyltransferase PMT18-like	DNA processing	No		
	F-box family protein	Signaling pathway; development	1522	2.5	II
mul-miR395a-3p	AP2 domain-containing transcription factor	Transcription regulation	334	4	I
	Zinc finger (CCCH-type) family protein	Transcription regulation	1724	3	IV
mul-miR415	ARF domain class transcription factor (IAA2)	Transcription regulation	77	4	0
	NBS-LRR resistance protein gene	Stress response	142	3	0
	Transcription factor BPE	Transcription regulation	1150	4	0
	Ubtilisin-like protease-like	Metabolic process	2951	3	IV
	ADP-ribosylation factor 1-like, transcript variant 2	Carbohydrate metabolic process	77	4	0
mul-miR4414a-3p	Uncharacterized protein	Unknown	No		
mul-miR4414a-5p	Peptide deformylase	Protein biosynthesis	No		
mul-miR447b-5p	Psb P-like protein	Photosystem II reaction	No		
	ATP binding microtubule motor family protein	Microtubule-based movement	No		
	Chitinase-like protein LA-b	Stress response	38	4	0
mul-miR473a-5p	Aminopeptidase M1	Hormone metabolism	1251	2.5	II

Table 5 | Continued

MiRNA-name	Predicted target annotations in mulberry transcriptome data	Putative GO_process	Degradome sequencing		
			Read	Score	Class
mul-miR477a-5p	Nucleic acid binding protein	Spliceosome assembly; stimulation of apoptosis	No		
mul-miR5020a	RNA recognition motif-containing protein	Stress response	631	3	II
	Galactosyltransferase family protein	Protein biosynthesis	No		
mul-miR5072	Heteroglycan glucosidase 1	Carbohydrate metabolic process	No		
mul-miR529b	Xyloglucan endotransglycosylase/hydrolase precursor	Carbohydrate metabolic process	5556	4	III
	Chlorophyll synthase	Chlorophyll biosynthesis	9002	4	IV
	Cysteine-rich repeat secretory protein 6	Stress response	No	No	
	SPL domain class transcription factor	Transcription regulation	119	0.5	0
mul-miR530-3p	AWPM-19-like family protein	Unknown	No		
	Zinc finger family protein	Transcription regulation	732	4	III
	Esterase/lipase/thioesterase family protein	Metabolic process	323	3	II
	Beige-related and WD-40 repeat-containing protein	Unknown	No		
	Transcription regulator	Transcription regulation	541	2.5	II
	TIR-NBS-LRR type disease resistance protein	Stress response	152	3	II
mul-miR530a	SWIB complex BAF60b domain-containing protein	Unknown	1152	4	0
	Zinc knuckle (CCHC-type) family protein	Transcription regulation; growth	101	3	I
mul-miR5565g-3p	Unknown	Unknown	No		
mul-miR5658	Anthranilate N-benzoyltransferase protein	Metabolic process	63	4	I
	C2H2L domain class transcription factor	Transcription regulation	53	3	0
	Uncharacterized protein	Unknown	No		
	Eukaryotic translation initiation factor 2c	Transcription regulation; protein synthesis	No		
	Putative RNA recognition motif-containing protein	Stress response; ethylene biosynthetic process	No		
	Trihelix transcription factor GT-2-like	Transcription regulation	No		
mul-miR5665	RAB geranylgeranyl transferase alpha subunit 1	Protein synthesis, response to cadmium ion	65	2	I
	Shikimate kinase	Development	No		
mul-miR6021	Transcription factor	Transcription regulation	No		
mul-miR6204	SAUR-like auxin-responsive protein family	Hormone metabolism	No		
	E3 ubiquitin-protein ligase LOG2-like	Metabolic process	2011	4	III
mul-miR6213	ARM repeat-containing protein-like protein	Unknown	No		
mul-miR6463	Lactosylceramide 4-alpha-galactosyltransferase	Protein synthesis	No		
mul-miR6475	Tir-nbs-lrr resistance protein	Stress response	79	3	0
mul-miR858b	Transcription factor MYB32-like	Transcription regulation	76	3.5	0
	MYB domain protein 51-2	Transcription regulation	23040	3.5	III
mul-miRn17-5p	Bystin-like	Ribosome biogenesis and assembly	No		
	ATP-dependent RNA helicase DHX8/PRP22	RNA processing	No		
mul-miRn19-5p	40S ribosomal protein S20-2	Protein biosynthesis	No		
mul-miRn10-3p	Auxin response factor 19	Auxin signaling; transcription regulation	1271	3	II
	Squamosa promoter binding protein-like 14	Transcription regulation	656	2	III
	Leucine-rich receptor-like protein kinase family protein	Signaling pathway	155	2	0
	S-formylglutathione hydrolase	Response to cadmium;methanal catabolism	No		
	Photosystem II reaction center PsbP family protein	PS.lightreaction.photosystem II	No		
	Protein kinase superfamily protein	Signaling pathway	No		
mul-miRn11-5p	Nucleoside-triphosphatase	Catalysis of the hydrolysis of various bonds	No		
mul-miRn18-5p	Haloacid dehalogenase-like hydrolase superfamily protein	Unknown	No		
mul-miRn20-5p	Aminoacyl-tRNA synthetase-like	Protein synthesis	231	3.5	0
	Chaperone protein dnaJ 13-like	Development	1518	3.5	III
	C2H2-type zinc finger protein	Transcription regulation	42	3.5	0
	Galactose oxidase	Carbohydrate metabolic process	No		
	Calmodulin binding protein	Transcription; stress response	No		
	F-box/kelch-repeat protein SKIP6	Unknown	322	2	II
	Splicing factor 1	RNA.processing	No		
	Dicer-like protein	RNA.processing	No		
	AP2 domain class transcription factor	Transcription regulation	No		
	Zinc finger-homeodomain protein 1	Transcription regulation	4541	2	IV
	Pentatricopeptide repeat-containing protein	Stress response	No		

may have different lengths and functions[50]. In addition to the relatively limited number of miRNAs, thousands of differentially expressed siRNAs were involved in the response of mulberry to phytoplasma-infection. The lengths of these siRNAs are more variable (Fig. 6), and the targeted genes of these differentially expressed siRNAs were associated with a wide range of functions in hormone networks, development and metabolism. It was reported that the 22-nt forms of miRNAs are important for triggering secondary siRNA production in plants[51,52]. Previous research showed that miR393 is necessary to initiate the production of the secondary siRNAs from the transport inhibitor response 1/auxin signaling F-box 2 (*TIR1/AFB2*) clade of auxin receptors (TAARs) transcripts in Arabidopsis[25].

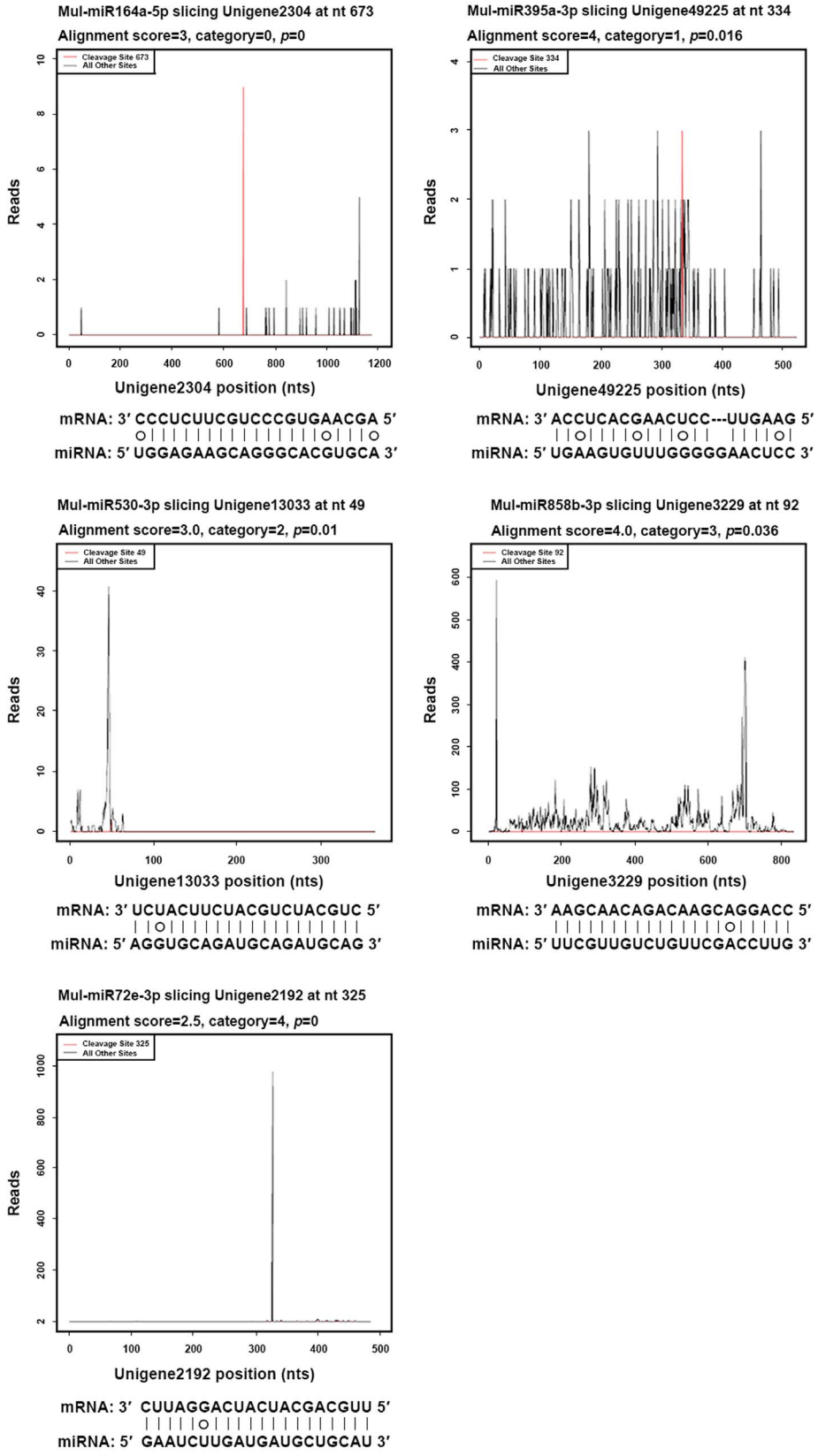

Figure 3 | Target plots (t-plots) of miRNA targets in different categories confirmed by degradome sequencing. (A) T-plot (top) and mRNA: miRNA alignments (bottom) for category 0 target, Unigene 2304 (NAC domain protein) transcripts. (B) As in (A) for Unigene 49225 (AP2 domain-containing transcription factor), a category I target for mul-miR395a-3p. (C) As in (A) for Unigene 13033 (TIR-NBS-LRR type disease resistance protein), a category II target for mul-miR530-3p. (D) As in (A) for Unigene 3229 (MYB domain protein 51-2), a category III target for mul-miR858b. (E) As in (A) for Unigene 2192 (Ethylene-responsive transcription factor RAP2-7-like), a category IV target for mul-miR72e-3p. The solid lines and dot in mRNA: miRNA alignments indicate matched and mismatch RNA base pairs, respectively.

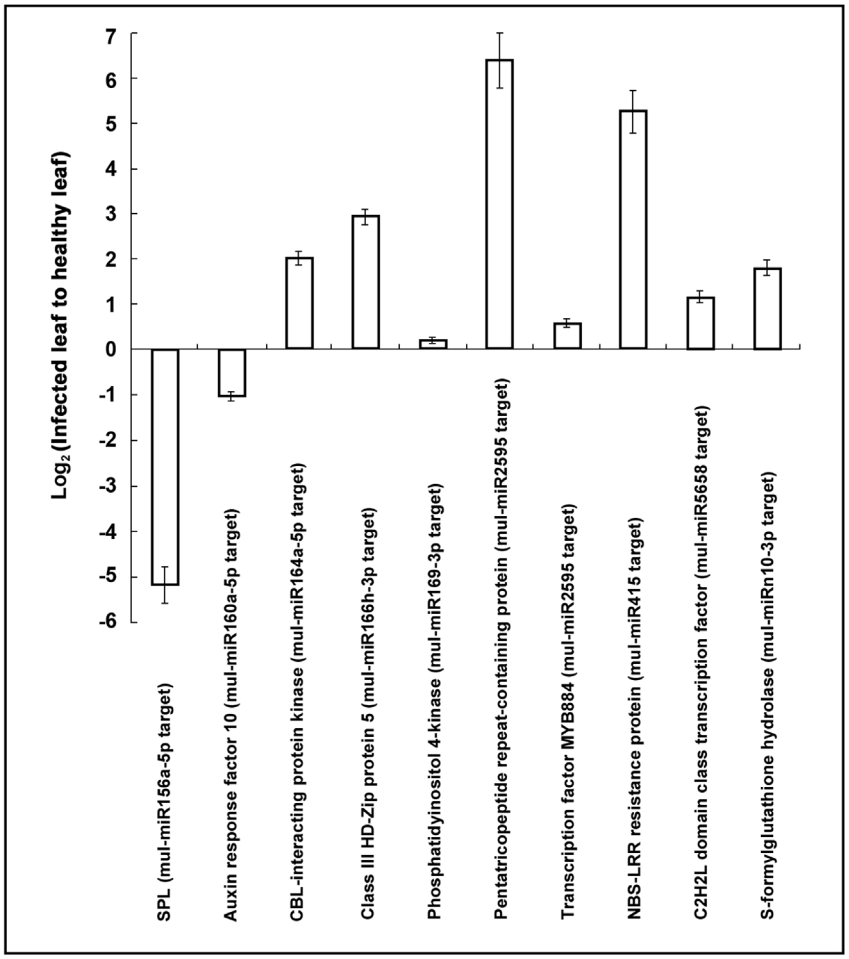

Figure 4 | Predicted target abundance analysis by real-time PCR. The relative gene expression was evaluated using comparative Ct method taking actin (Accession No. DQ785808) as the reference gene. The $\log_2$ values of the ratio of phytoplasma-infected leaves to the healthy leaves are plotted. Values are given as mean ± SD of three experiments in each group.

Sequencing results showed that mul-miR393 is 22-nt long, so we wondered whether it can trigger the production of the secondary siRNAs. Our small RNA library sequencing data showed there were some siRNAs matching to the *TIR1* mRNA downstream of the mul-miR393-5p binding sites (Figure 9). Thus, mul-miR393 might also trigger the production of the secondary siRNAs. However, we can't predict which one is necessary to trigger the production of these siRNAs, mul-miR393a or mul-miR393b. Future studies will be carried out to explore the functions of these phytoplasma-responsive siRNAs, and identify the pathways connected with the response against phytoplasma-infection. In conclusion, the information provided here would be particularly useful for understanding the function of sRNAs in mulberry and be aid to reveal the mechanisms underlying of phytoplasma pathogenicity and the symptoms they cause in host plants.

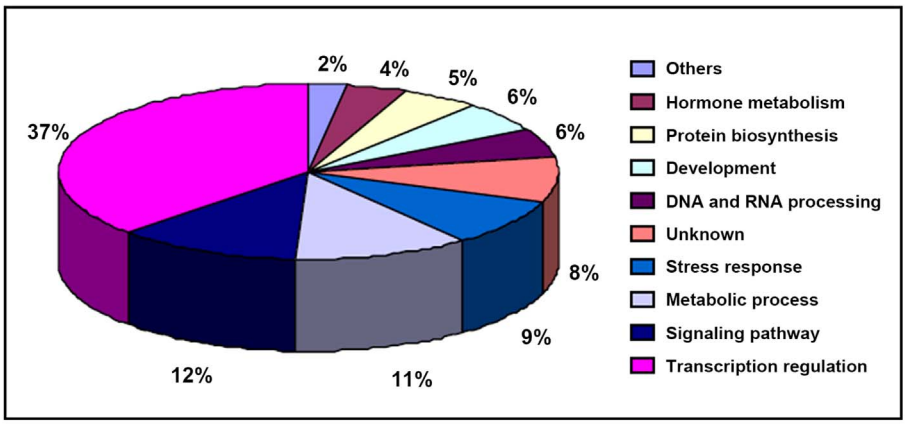

Figure 5 | Percentage distribution of the predicted target genes for the differentially expressed miRNAs in various categories.

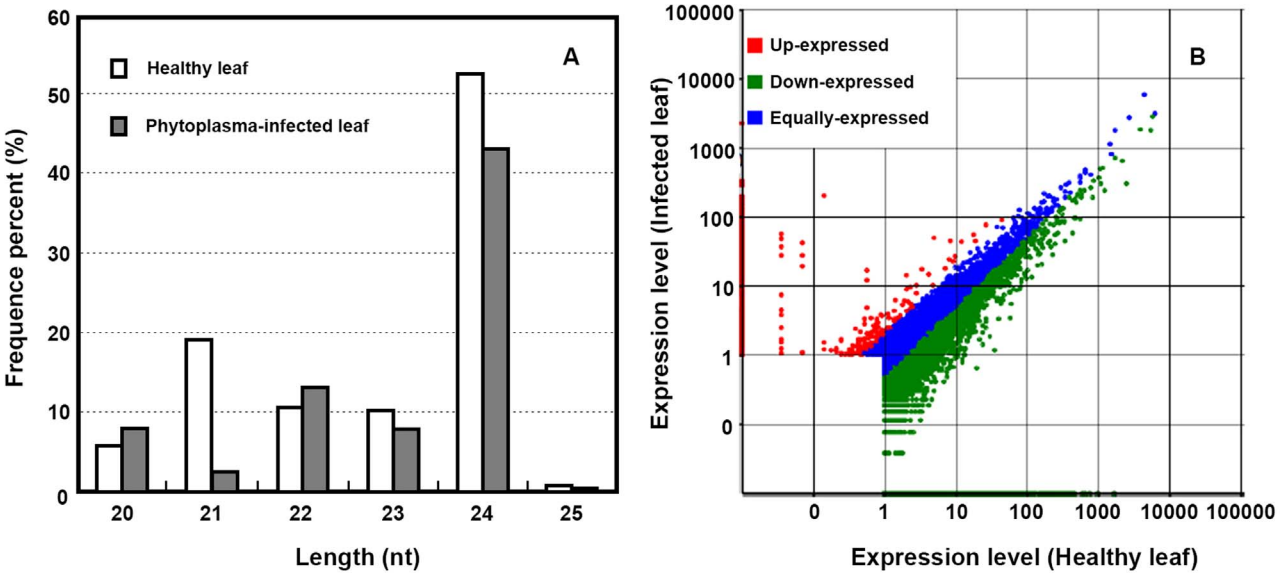

Figure 6 | **Length distribution of the siRNAs in mulberry(A) and scatter plots display the levels of siRNAs in healthy and infected leaf libraries (B).**

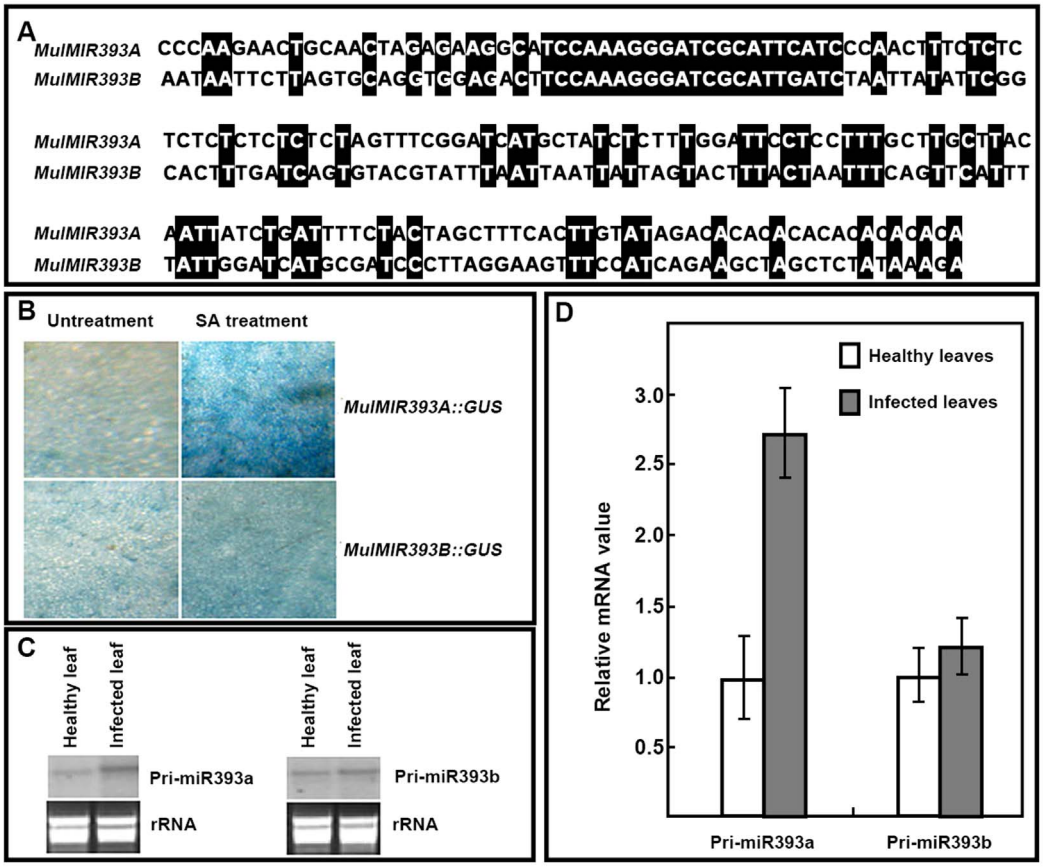

Figure 7 | **Comparison of nucleotide sequences of *MulMIR393A* and *MulMIR393B*, *MulMIR393A*::*GUS* and *MulMIR393B*::*GUS* activity, and pri-miR393a pri-miR393b abundance analysis.** (A) Comparison of nucleotide sequences of *MulMIR393A* and *MulMIR393B*. Identical nucleotides were black shaded. (B) *MulMIR393A*::*GUS* and *MulMIR393B*::*GUS* activity analysis by histochemical staining. Histochemical staining for GUS activity was performed 24 h later after application of SA with X-gluc as a substrate. (C) Pri-miR393a and pri-miR393b abundance analysis by northern blot. (D) Pri-miR393a and pri-miR393b abundance analysis by real-time PCR. The relative gene expression was evaluated using comparative Ct method taking actin (Accession No. DQ785808) as the reference gene. Values are given as mean ± SD of three experiments in each group.

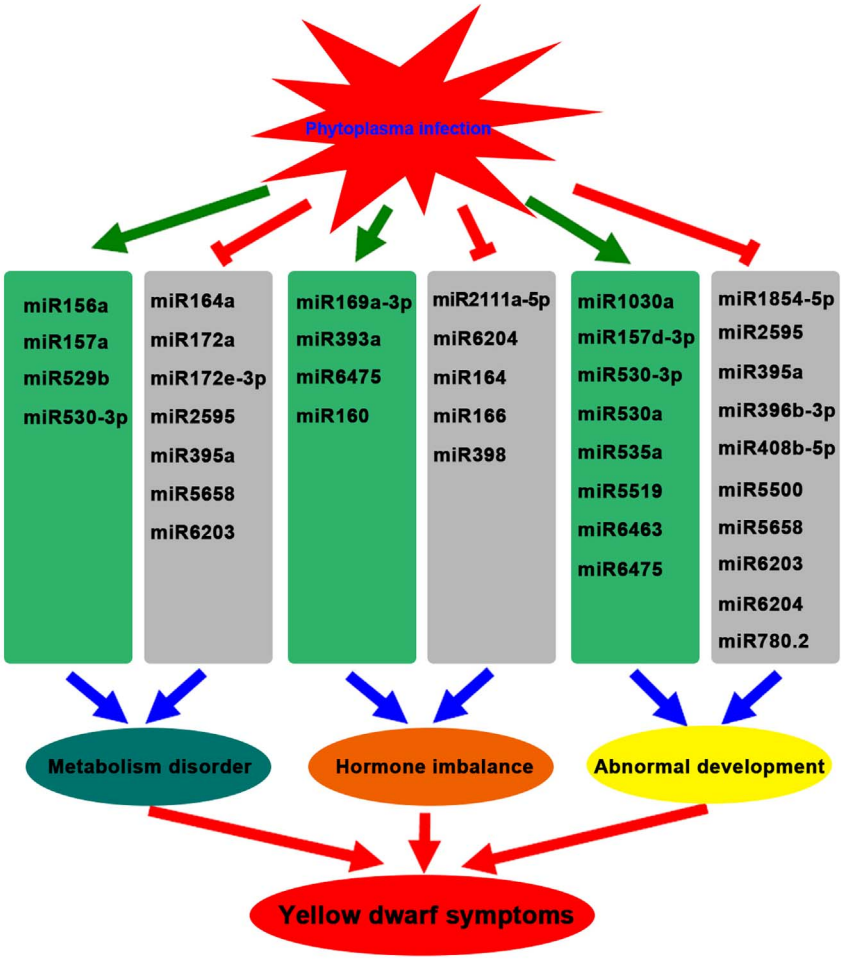

Figure 8 | Complex regulatory networks of phytoplasma-responsive microRNAs. Phytoplasma-responsive miRNAs target different genes involved in diverse biological processes, contribute to fine-tuning in defense responses and trigger metabolism, hormone and development perturbation and promote disease development.

Methods

Plant material. One-year-old cutting seedlings derived from the same mother tree, Husang 32 (*Morus multicaulis* Perr.), were used as rootstocks which were grafted with the scions collected from healthy and phytoplasma-infected mulberry trees (Husang 32), respectively. All the grafts were incubated in a growth chamber at 26°C, humidity 90% and under 12 h of light. Six weeks after infection, the plant showing typical symptoms as yellowing of the leaves, stunting, and witches'-broom were confirmed by PCR assay with an amplified fragment of the 16S rRNA gene (GenBank Accession No. EF532410) and TEM detection as described before[1]. The plants that were phytoplasma-positive by PCR assay and TEM detection were used as treatments while the plants grafted with the scions collected from healthy trees were used as controls.

Small RNA library construction and high-throughput sequencing. Leaves were collected from the infected plants and the controls, and RNAs were isolated from the leaves of healthy and infected plants separately using TRIzol kit (Invitrogen) following the manufacturer's instructions. Total RNAs were separated on 15% denaturing PAGE and small RNAs between 14 and 30 nt were enriched and ligated with 5′ and 3′ adaptors. The ligation products were purified and amplified after reverse transcription. The amplification products were purified and used for sequencing by Solexa machine.

Conserved miRNAs in mulberry. The raw sequences tags from HiSeq sequencing were cleaned by getting rid of adaptors, low-quality tags, as well as contaminants to generate clean reads. Length distribution of clean tags is then summarized, and the trimmed sequences longer than 18 nt were used for further analyses. The remaining clean reads were annotatated into different categories through standard bioinformatics analysis. Subsequently, the sequences were compared with a miRBase database v19.0 (http://www.mirbase.org/). The sequences with identical or related sequences from other plants were identified as conserved miRNAs, and the other sequences were analyzed for novel miRNA candidates.

Novel miRNA candidates. To discover novel miRNAs, all non-annotated clean tag sequences were mapped to our mulberry transcriptome database. The characteristic hairpin structure of miRNA precursor was used to predict novel miRNA using the Mireap algorithm (http://sourceforge.net/projects/mireap/) by exploring the

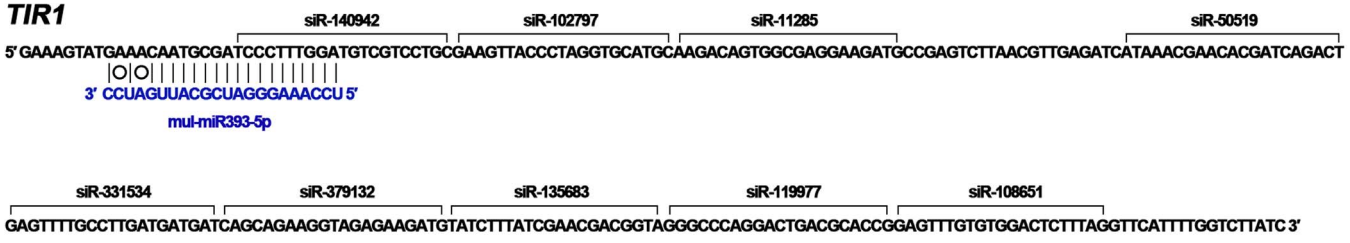

Figure 9 | The production of secondary siRNAs triggered by mul-miR393-mediated cleavage of *TIR1* mRNA.

secondary structure, the dicer cleavage site, the minimum free energy, and other criteria provided by before[53].

Identification of siRNAs. To identify siRNAs, tags from clean reads were aligned with each other. A pair of perfectly complementary sRNAs with 2 nt overhangs at the 3′-end were considered as siRNAs.

Differential expression analyses of miRNAs and siRNAs. The expression of miRNAs and siRNAs in healthy and infected leaf samples were normalized to obtain the expression of transcripts per million, and only the sRNA with an expression value of ≥1, it was considered in future differential expression analysis. Based on the normalized expression analysis, the fold-change and P-value were calculated according to the following equation. Normalized expression = (Actual sRNA sequencing reads count/Total clean reads count) ×1,000,000; Fold change = Log_2 (x/y); P-value formula:

$$P(x|y) = \left(\frac{N_2}{N_1}\right)^y \frac{(x+y)!}{x!\,y!\left(1+\frac{N_2}{N_1}\right)^{(x+y+1)}} \qquad C(y \le y_{min}|x) = \sum_{y=0}^{y \le y_{min}} p(y|x)$$

$$D(y \ge y_{max}|x) = \sum_{y \ge y_{max}}^{\infty} p(y|x)$$

The N_1 and N_2 represent total count of clean reads of a given sRNA in the sRNA library of phytoplasma infected and healthy leaves, respectively. The x and y represent normalized expression level of a given sRNA in the sRNA library of phytoplasma infected and healthy leaves, respectively.

Prediction and validation of targets of differential miRNAs. The sequences from the mulberry transcriptome data were chosen to predict the miRNA targets, and the plant small RNA target analysis server, psRNATarget (http://plantgrn.noble.org/psRNATarget/), was used to predict the target genes following the rules suggested by Allen *et al.*[54]. All the predicted miRNA targets were experimentally verified by mRNA degradome sequencing. A degradome sequencing library was constructed using mRNAs isolated from the healthy and infected leaves according to the protocols described previously[55,56] and then were submitted for parallel analysis of RNA end (PARE) sequencing on a Solexa Analyzer. All the adaptor sequences and low quality sequencing reads were removed from the raw sequencing reads to obtain the clean reads with the length of 20 and 21 nt, which were used to identify potentially cleaved targets with the software package, CleaveLand 3.0, as previously described[55,56]. The clean reads were mapped to the mulberry transcriptome unigene sequences obtained, and only the perfect matching reads were kept and extend to 35–36 nt by adding 15 nt of upstream of the sequence. All resulting reads were reverse-complemented and aligned to the miRNA identified and scored by previously described method[54]. All targets identified were classified into five categories based on the abundance of the resulting mRNA tag relative to the overall profile of degradome reads that matched the target[57]. When a target mRNA was predicted or identified, a BlastN search against a reference *Arabidopsis thaliana* database downloaded from TAIR (http://www.arabidopsis.org/; version TAIR10) was used to provide the gene ontologies, and then GO analysis was performed for the matched Arabidopsis accession entries based on their TAIR GO categories.

Quantitative real-time PCR analysis for miRNAs and mRNAs. RNA was extracted using the TRIzol® reagent following the manufacturer's recommendations (Invitrogen) and digested with DNase I. Real-time PCR was performed using the PrimeScript™ miRNA qPCR Starter Kit Ver.2.0 for miRNAs and the SYBR Premix Ex TaqTM kit for mRNA according to the manufacturer's protocol on the Rotor-Gene 3000A system. The U6 and actin genes were amplified as reference genes for miRNA and mRNA normalization, respectively. The primers (Supplementary table 1, 2) used to amplify the genes were designed based on our available mulberry transcriptome data. Comparative cycle threshold (Ct) method[58] was used to evaluate the relative gene expression level. All samples were assayed in triplicate.

RNA gel blotting. Twenty micrograms of total RNA of each sample isolated as above were separated on 1.2% formaldehyde denatured agarose gel, and then were blotted onto nylon Hybond N membrane (Hybond N, Amersham). The blots were hybridized with digoxigenin-labeled RNA probes prepared using the PCR DIG Probe Synthesis Kit (Roche, Germany). Prehybridization, hybridization, membrane washing, and detection were carried out as described previously[59].

Promoter analysis of MulMIR393A and MulMIR393B genes. The promoter sequences of *MulMIR393A* and *MulMIR393B* genes were cloned using Tail-PCR method and analyzed using the program online (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to search for cis-acting motifs. Then they were cloned into the vector pBI121, which harbors the promoter 35S and the *GUS* gene, to replace 35S, respectively. The pBI121 vector containing *MulMIR393A*::*GUS* or *MulMIR393B*::*GUS* was introduced into *Agrobacteriun tumefaciens* strain GV3101. Tobacco (*Nicotiana benthamiana*) plants were used for transformation by infection of leaf sections with *A. tumefaciens* strain harboring the constructs described above. Selected transgenic shoots were treated with SA by dipping the leaves into a solution containing 1 mM SA, and the leaves treated with water were used as control. Histochemical staining for GUS activity was performed 24 h later using the method described by Jefferson[60] with X-gluc as a substrate.

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Author contributions

Y.P. and X.L. were responsible for design, sample preparations, and contributed to write the manuscript. Y.Q. and F.Y. were responsible for high-throughput deep sequencing analysis. C.Z. and Y.Y. were responsible for PCR analysis. H.L. and H. were responsible for mul-miR393 analysis. All authors edited the manuscript and reviewed the manuscript.

Additional information

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