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ORIGINAL ARTICLE

Identification of miRNAs and miRNA-mediated regulatory pathways in *Carica papaya*

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Abstract Plant microRNAs (miRNAs) post-transcriptionally regulate target gene expression to modulate growth and development and biotic and abiotic stress responses. By analyzing small RNA deep sequencing data in combination with the genome sequence, we identified 75 conserved miRNAs and 11 novel miRNAs. Their target genes were also predicted. For most conserved miRNAs, the miRNA-target pairs were conserved across plant species. In addition to these conserved miRNA-target pairs, we also identified some papaya-specific miRNA-target regulatory pathways. Both miR168 and miR530 target the *Argonaute 1* gene, indicating a second autoregulatory mechanism for miRNA regulation. A non-conserved miRNA was mapped within an intron of *Dicer-like 1* (*DCL1*), suggesting a conserved homeostatic autoregulatory mechanism for *DCL1* expression. A 21-nt miRNA triggers secondary siRNA production from its target genes, nucleotide-binding site leucine-rich repeat protein genes. Certain phased-miRNAs were processed from their conserved miRNA precursors, indicating a putative miRNA evolution mechanism. In addition, we identified a *Carica papaya*-specific miRNA that targets an ethylene receptor gene, implying its function in the ethylene signaling pathway. This work will also advance our understanding of miRNA functions and evolution in plants.

Keywords Argonaute · Dicer-like · miRNA · Phased-miRNA · Nucleotide-binding site leucine-rich repeat (NB-LRR) · SPL

Abbreviations

miRNA	microRNA
DCL1	Dicer-like 1
AGO	Argonaute
SE	Serrate
CPL1	C-terminal Domain Phosphatase-like
HEN1	Hua Enhancer 1
RISC	RNA-induced silencing complex
tasiRNAs	Transacting siRNA
HYL1	Hyponastic leaves 1
APS	ATP sulfurylase
SULTR	Sulfate transporter

Introduction

Regulation of gene expression is crucial for all organisms. As key post-transcriptional regulators of eukaryotic gene expression, plant miRNAs can cleave target transcripts and/or repress translation (Eulalio et al. 2008). Generally, miRNAs are 20–24 ntRNA molecules. Unlike siRNAs, which originate from double-stranded RNAs depending on RNA-dependent RNA polymerases (RDRs) or RNA amplification mechanisms, miRNAs are derived from their own primary transcripts (called pri-miRNAs) that are transcribed by RNA polymerase II (Lee et al. 2004). The pri-miRNA typically forms an imperfect fold-back structure, which

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is processed into a stem-loop precursor (pre-miRNA) and further diced as an RNA duplex by Dicer-like 1 (DCL1; or occasionally by DCL4) (Rajagopalan et al. 2006) in combination with the double-stranded RNA-binding protein Hyponastic Leaves 1 (HYL1) and the C2H2-zinc finger protein SE (Kim 2005; Kurihara et al. 2006; Fang and Spector 2007). In this process, C-terminal Domain Phosphatase-like 1 (CPL1) is required to dephosphorylate HYL1 (Manavella et al. 2012a). The mature miRNA duplex (miRNA/miRNA*) is further methylated on the 3′ terminal nucleotides of each strand by the *S*-adenosyl methionine-dependent methyltransferase Hua Enhancer 1 (HEN1) to prevent its uridylation and subsequent degradation (Li et al. 2005; Yang et al. 2006). The miRNA is then incorporated into the RNA-induced silencing complex (RISC) to recognize its target transcripts with fully or partly complementary sequences. The remaining strand, miRNA*, is often degraded.

Some miRNAs also can trigger secondary siRNA biogenesis. Noncoding *TAS1-4* genes serve as the precursors of transacting siRNAs (tasiRNAs). After cleavage by miRNAs, the remaining transcript is converted into dsRNA by RDR6, which is then processed by DCL4 into tasiRNAs in a 21-nt phase relative to the cleavage site. miR173 triggers *TAS1* and *TAS2* to produce tasiRNAs, which in turn can target mRNAs encoding pentatricopeptide repeat proteins (Howell et al. 2007; Montgomery et al. 2008). miR390 is the initiator of *TAS3* tasiRNAs, which target *Auxin Response Factor* transcripts (Allen et al. 2005; Axtell et al. 2006). One of miR828's targets is *TAS4*, which can produce tasiRNAs that target the mRNAs of *MYB* transcript factors (Rajagopalan et al. 2006). Recent studies revealed that many 22-nt miRNAs can induce disease defense genes that encode proteins with nucleotide-binding site (NBS) and leucine-rich repeat (LRR) motifs (termed NB-LRR proteins) to give rise to secondary siRNAs (Zhai et al. 2011; Shivaprasad et al. 2012).

Papaya is cultivated in tropical climates worldwide. Unfortunately, papaya is susceptible to biotic stresses (e.g., papaya ringspot virus) and is also affected by abiotic stresses (e.g., low temperatures occurring periodically in the subtropics). Therefore, it is important to discover the genes that can improve papaya's resistance to biotic and abiotic stresses.

Many studies have established that plant miRNAs not only play important roles in development (Flynt and Lai 2008; Garcia 2008; Yang et al. 2009), but also regulate a vast array of other biological functions, including hormonal control, immune responses, and adaptation to a variety of biotic and abiotic stresses (Sunkar et al. 2007; Pedersen and David 2008; Voinnet 2008; Liang et al. 2010, 2012b). Hundreds of miRNAs have been identified in many plant species (http://www.mirbase.org). Although the genome sequencing of papaya has been completed (Ming et al. 2008), little is known about miRNA regulation in *Carica papaya*. Genome survey sequences and expressed sequence tags were used to identify conserved miRNAs in plant species with unknown genome sequences (Zhang et al. 2006). However, this approach is not suitable for identifying non-conserved miRNAs. Small RNA deep sequencing technology is an efficient strategy that has been used for miRNA discovery in diverse plant species, including maize (Zhang et al. 2009), cotton (Pang et al. 2009), and rubber tree (Lertpanyasampatha et al. 2012). By deep sequencing, Aryal et al. (2012) annotated 60 papaya miRNAs. In the present study, we identified 75 conserved and 11 novel papaya miRNAs. The putative targets of these miRNAs were also predicted or/and validated experimentally. In addition to the canonical plant miRNA regulatory pathways, papaya has also evolved novel miRNA-mediated cascades. The present work provides an insight into the specific regulation of miRNAs.

Materials and methods

Plant materials and RNA preparation

The plant materials used in this study were obtained from papaya cultivar in the XTBG (Xishuangbanna Tropical Botanical Garden). Leaves of age 1–2 weeks and female flowers of age 1–3 weeks were sampled, respectively. Total RNA was extracted with the TRizol reagent (Invitrogen).

Computational analysis of sequencing data

The small RNA sequencing data was downloaded from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under GEO accession number GSM712525 and GSM712526. The trimmed sequencing data were mapped to the papaya genome (http://www.plantgdb.org/CpGDB/) using SOAP (Li et al. 2008a). Reads that do not perfectly match the papaya genome were used for comparative analysis with conserved plant miRNAs. Conserved plant miRNAs were retrieved from miRBase (http://www.mirbase.org/). The miRNA prediction pipeline was performed with Perl scripts combined with miREAP (https://sourceforge.net/projects/mireap/). We used miREAP to evaluate the pairing of the miRNA and miRNA* with the parameters set to allow a maximal distance of 200 nucleotides between miRNA and miRNA* (-d 200), extending 10 nucleotides at the end of the precursor (-f 10).

miRNA Northern blotting

For small RNA gel blots, 20 μg of total RNA was separated on a 15 % polyacrylamide gel with 7 M urea. DNA

oligonucleotides complementary to miRNAs were end-labeled using T4 polynucleotide kinase and used for hybridizations. miRNA gel blot hybridizations were performed as described by Liang et al. (2012a).

Real-time quantitative RT-PCR

For the production of cDNA from mRNA, 0.5 μg of total RNA was reverse-transcribed using an oligo(dT)18 primer according to the reverse transcription protocol (Fermentas). A 20-μl reaction mixture was used for the production of cDNA. After heat inactivation, a 1-μl aliquot was used as a template for real-time quantitative RT-PCR. A pair of gene-specific primers was used to amplify the target cDNA. All quantitative RT-PCR analyses were performed using a SYBR Premix Ex Taq™ kit (Takara) on a Roche LightCycler 480 real-time PCR machine, according to the manufacturer's instructions.

5′ RACE

Following the manufacturer's instructions for the SMARTer™ RACE cDNA Amplification kit (Clontech), 1 μg of total RNA isolated from papaya flowers was used for reverse transcription. Gene-specific primers (designed according to the protocol) and the UPM primer (provided by kit) were used to conduct PCRs, and purified PCR products were cloned into the pMD-18T vector (Takara) and sequenced.

Agrobacterium tumefaciens infiltration in *Nicotiana benthamiana*

The constructs, 35S-intron, 35S-miR535, 35S-SPL, and vector, were transformed into *A. tumefaciens* strain EHA105 by electroporation and selected on Luria–Bertani medium containing rifampicin at 50 μg/ml and spectinomycin at 100 mg/ml. Agrobacterial cells were then infiltrated into leaves of *N. benthamiana*. For coinfiltration experiments, equal volumes of an agrobacterium culture containing 35S-MIR535 or vector (OD600 = 1.75) and 35S-SPL (OD600 = 0.25) were mixed before infiltration into *N. benthamiana* leaves.

Results

Conserved miRNAs in papaya

Recently, Aryal et al. (2012) annotated 60 papaya miRNAs (24 conserved and 36 novel miRNAs). In their work, miRNAs were identified based on stem-loop structure using algorithm miRDeep which calls miRNA from aligned reads containing both guide (miRNA) and passenger (miRNA*) strands. In fact, for many canonical miRNAs, the abundance of miRNA* strands is so low that they are undetectable. Meanwhile, due to a number of gaps existing in the draft genome, some reads fail to be aligned with genome sequence. Hence, some authentic miRNAs were missing in their annotated papaya miRNAs.

Here, we employed a new strategy to identify papaya miRNAs as described in Fig. 1. Deep sequencing data of small RNAs from leaves and flowers was compared with all known plant miRNAs in the miRNA database (Griffiths-Jones et al. 2006). Small RNAs that were completely identical to known plant miRNAs were mapped to the papaya genome and their putative precursors were predicted using the Vienna RNA package (Hofacker 2003). After filtering false miRNAs according to miRNA criteria (Meyers et al. 2008), 67 conserved miRNAs were identified and their corresponding loci in the genome were identified (Online Resource 1). To identify the potential conserved

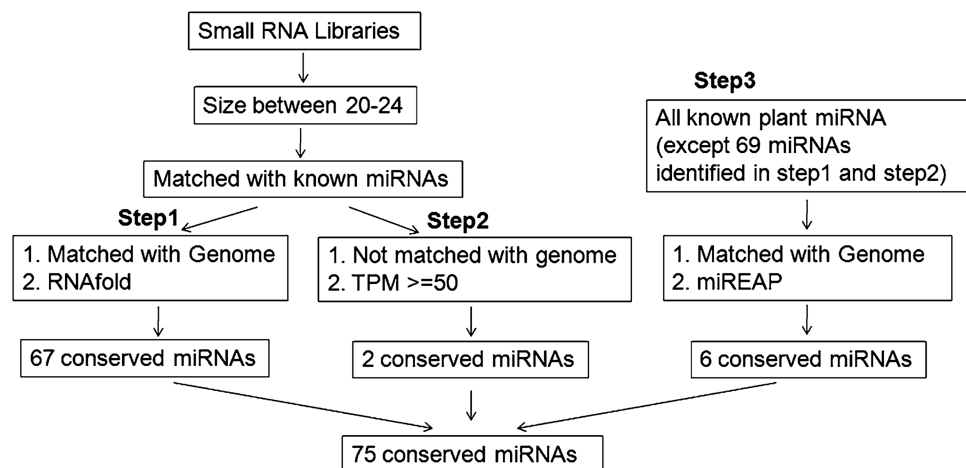

Fig. 1 Strategy for identification of conserved miRNAs

miRNAs from among the small RNAs (which may exist in the gaps of genome draft map) that did not match the genome, small RNAs with read abundances greater than 50 TPM (transcripts per million) were compared with all known plant miRNAs. Two conserved miRNAs (miR168a and miR168b) were discovered. Some spatiotemporally or conditionally expressed miRNAs may not be included in the libraries; therefore, we used all known plant miRNAs, except those miRNAs with mature sequences identical to the 69 conserved miRNAs identified above, as query sequences to discover the loci of other known miRNAs in the papaya genome, using an miRNA prediction pipeline, miREAP. Only 6 conserved miRNA was found. Ultimately, total 75 conserved miRNAs were identified (Table 1). These conserved miRNAs bore a canonical stem-loop structure in their precursors (Online Resource 1). We examined the expression of three conserved miRNAs (Fig. 2), revealing that both miR156 and miR535 were highly expressed in leaves, and miR395 was undetectable because of its low abundance.

Candidate non-conserved miRNAs in papaya

Many species-specific miRNAs have been discovered across plant species. As a tropical species, papaya may have evolved unique miRNAs. Therefore, we also predicted papaya-specific miRNAs. After excluding conserved miRNAs, we mapped those small RNAs of 20–24 nucleotides in the small RNA libraries to the papaya genome using SOAP. Furthermore, we removed the sequences that matched perfectly with known protein-encoding RNAs or noncoding RNAs (rRNA, tRNA, snRNAs and snoRNA) in the RNA database (Rfam; Gardner et al. 2011). Subsequently, the remaining small RNAs were processed for miRNA discovery using miREAP. Finally, 11 candidate miRNA-producing loci were identified (Table 2 and Online Resource 1). We then compared our predicted novel miRNA with those identified by Aryal et al. and found that only two novel miRNAs were overlapped (Table 2), suggesting a major difference between miREAP and miRdeep. We confirmed the expression of two selected papaya-specific miRNAs (miR-c2 and miR-c11) by northern blotting (Fig. 2). Further experiments were required to clarify if the remaining candidates are authentic miRNAs.

Targets of papaya miRNAs

To investigate the functions of an miRNA, it is crucial to predict its target(s). We employed WMD3 (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) and psRNATarget (http://plantgrn.noble.org/psRNATarget/) to predict the targets of all identified papaya miRNAs (Tables 1, 2). For the conserved miRNAs, the miRNA-target pairs are conserved in several plant species, including *Arabidopsis*, poplar, and rice. For example, miR156 targets *SPL* transcription factors (Schwarz et al. 2008), while miR159 regulates the *MYB* gene family (Achard et al. 2004; Reyes and Chua 2007) miR172 targets *AP2-like* genes (Chen 2004), whereas both miR160 and miR167 target *Auxin Response Factor* transcription factors (Wang et al. 2005). The same trend was observed in many other miRNA families, including miR164, miR166, mi169, miR171, and miR396, which target various families of transcription factors, such as *NAC* domain containing transcription factors (Guo et al. 2005), *basic-leucine Zipper* genes (Williams et al. 2005), *CCAAT-binding* transcription factors (Li et al. 2008b), *Scarecrow-like* genes (Llave et al. 2002), and *Growth Regulation Factor* genes (Liu et al. 2009; Yang et al. 2009). These transcription factors play crucial roles in plant development. miR393 targets *F-box* genes whose products are involved in the transduction of auxin signals (Navarro et al. 2006). Two classes of genes, *APS* and *SULTR*, are targeted by miR395 in *Arabidopsis* and rice (Kawashima et al. 2009; Liang et al. 2010). In papaya, *APS* genes, but not *SULTR* genes, are targeted by miR395. miR397, miR398, and miR408 regulate the homeostasis of copper in *Arabidopsis*, and their homologous target genes were identified in papaya, suggesting their potential functions in copper metabolism. However, we could not predict the conserved targets for miR319, miR390, miR399, and miR3627. More than one target gene was predicted for most conserved miRNAs. Considering the fact that the targets of conserved miRNAs were homologous across diverse plants species, we excluded the predicted targets whose homologues were not targeted by the corresponding homologous miRNAs in *Arabidopsis* and rice. For the 11 non-conserved miRNAs, the putative targets of 8 miRNAs were predicted and no target was predicted for the remainder. The targets of miR-c4 and miR-c10 were experimentally validated, as shown in the results below.

miRNA auto-regulation pathways in papaya

miRNA precursors are processed into mature miRNAs by DCL1 or DCL4 with other factors. In plants, *DCL1* is targeted by conserved miR162. Similarly, we also identified one miR162 gene in papaya. Target prediction analysis indicated that the papaya miR162-*DCL1* duplex (Fig. 3c) is completely identical to that in *Arabidopsis*, implying conservation of the miR162 function in papaya.

The post-transcriptional regulation of the *DCL1* gene by its own product was established in *Arabidopsis*, where the 14th intron of the *DCL1* gene contains an miRNA gene (Ath-miR838) (Rajagopalan et al. 2006). Here, a 21-nt length RNA sequence (designated miR-c7) perfectly matching with an intron of the *DCL1* gene

Table 1 Conserved miRNAs and their targets

Family	Member	Sequence (5′-3′)	Length	Previously identified[e]	Predicted targets	Target products	Functional conservation	
							Ath	Osa
miR156	a	TGACAGAAGAGAGTGAGCAC	20	Yes	16409298[a] 16420023[a] 16405577[a]	SPL	Yes	Yes
	b	TGACAGAAGAGAGTGAGCAC	20	Yes				
	c	TGACAGAAGAGAGTGAGCAC	20	Yes				
	d	TGACAGAAGAGAGTGAGCAC	20	Yes				
	e	TGACAGAAGAGAGAGAGCAC	20	No				
	f	TTGACAGAAGATAGAGAGCAC	21	Yes				
	g	TTGACAGAAGATAGAGAGCAC	21	Yes				
miR159		TTTGGATTGAAGGGAGCTCTA	21	Yes	16418773[a] 16417950[a] 16417599[a] 16406850[a]	MYB	Yes	Yes
miR160	a	TGCCTGGCTCCCTGTATGCCA	21	Yes	16421034[a] 16422316[a] 16424448[a]	ARF	Yes	Yes
	b	TGCCTGGCTCCCTGTATGCCA	21	Yes				
	c	TGCCTGGCTCCCTGTATGCCA	21	Yes				
	d	TGCCTGGCTCCCTGAATGCCA	21	Yes				
miR162		TCGATAAACCTCTGCATCCAG	21	No	16422899[a]	DCL1	Yes	Yes
miR164	a	TGGAGAAGCAGGGCACGTGCA	21	Yes	16404615[a] 16408087[a] 16405845[a] 16408594[a]	NAC domain containing protein	Yes	Yes
	b	TGGAGAAGCAGGGCACGTGCA	21	Yes				
	c	TGGAGAAGCAGGGCACGTGCA	21	Yes				
miR166	a	TCGGACCAGGCTTCATTCCCG	21	Yes	16405536[a] 16422471[a]	Homeobox-leucine zipper	Yes	Yes
	b	TCGGACCAGGCTTCATTCCCC	21	Yes				
	c	TCGGACCAGGCTTCATTCCCC	21	Yes				
	d	TCGGACCAGGCTTCATTCCCC	21	Yes				
	e	TTGGACCAGGCTTCATTCCCC	21	No				
miR167	a	TGAAGCTGCCAGCATGATCTA	21	Yes	6462[b] 21134[b]	ARF	Yes	Yes
	b	TGAAGCTGCCAGCATGATCTA	21	Yes				
	c	TGAAGCTGCCAGCATGATCTT	21	Yes				
	d	TGAAGCTGCCAGCATGATCTGA	22	Yes				
miR168	a	TCGCTTGGTGCAGGTCGGGAA	21	No	1673.1[c] 16410499[a]	AGO1	Yes	Yes
	b	TCGCTTGGTGCAGGTCGGGAT	21	No				
miR169	a	TAGCCAAGGATGACTTGCCTG	21	No	28033[b] 28624[b]	HAP2	Yes	Yes
	b	TAGCCAAGGATGACTTGCCTG	21	No				
	c	TAGCCAAGAATGACTTGCCTG	21	No				
	d	CAGCCAAGGATGACTTGCCGG	21	No				
	e	CAGCCAAGGATGACTTGCCGG	21	No				
	f	CAGCCAAGGATGACTTGCCGG	21	No				
	g	CAGCCAAGGATGACTTGCCGG	21	No				
	h	TAGCCAAGGATGACTTGCCGG	21	No				
	i	GAGCCAAGGATGACTTGCCAG	21	No				
	k	CAGCCAAGAATGACTTGCCG	20	Yes				
miR171	a	TGATTGAGCCGTGCCAATATC	21	Yes	16416165[a] 16421072[a]	SCARECROW-like	Yes	Yes
	b	TGATTGAGCCGTGCCAATATC	21	Yes				
	c	TGATTGAGCCGTGCCAATATC	21	Yes				
	d	TGATTGAGCCGTGCCAATATC	21	No				
	e	TGATTGAGCCGTGCCAATATC	21	Yes				
miR172	a	GGGAATCTTGATGATGCTGCA	21	Yes	16422502[a] 16408542[a] 16406148[a] 16404201[a]	AP2-like	Yes	Yes
	b	GGGAATCTTGATGATGCTGCA	21	Yes				
	c	AGAATCTTGATGATGCTGCAT	21	No				

Table 1 continued

Family	Member	Sequence (5′-3′)	Length	Previously identified[e]	Predicted targets	Target products	Functional conservation	
							Ath	Osa
miR319	a	TATGAATGATGCGGGAGATAT	21	No				
	b	TGTGAATGATGCGGGAGATAA	21	No				
miR390	a	AAGCTCAGGAGGGATAGCGCC	21	Yes				
	b	AAGCTCAGGAGGGATAGCGCC	21	Yes				
miR393		TCCAAAGGGATCGCATTGATC	21	Yes	16425377[a] 16428059[a]	AFB TIR1	Yes	Yes
miR394	a	TTGGCATTCTGTCCACCTCC	20	Yes	16420029[a]	F-box family protein	Yes	Yes
	b	TTGGCATTCTGTCCACCTCC	20	Yes				
miR395	a	CTGAAGTGTTTGGGGGAACAC	21	No	16420138[a]	APS	Yes	Yes
	b	CTGAAGTGTTTGGGGGAACTC	21	Yes				
	c	CTGAAGTGTTTGGGGGAACTC	21	Yes				
	d	CTGAAGTGTTTGGGGGAACTC	21	Yes				
	e	CTGAAGTGTTTGGGGGAACTC	21	Yes				
	f	CTGAAGTGTTTGGGGGAACTC	21	Yes				
miR396	a	TTCCACAGCTTTCTTGAACTT	21	No	16421514[a] 16409350[a] 16407578[a] 16410960[a]	Growth-regulating factor	Yes	Yes
	b	TTCCACAGCTTTCTTGAACTG	21	Yes				
	c	TTCCACAGCTTTCTTGAACTG	21	No				
miR397		TCATTGAGTGCAGCGTTGATG	21	No	16427516[a] 16427517[a] 16411960[a] 16427332[a] 16422717[a] 16410537[a] 16415257[a] 16422794[a] 16426867[a]	Laccase	Yes	Yes
miR398	a	TGTGTTCTCAGGTCGCCCCTG	21	No	3201500[d] 32623[b]	CSD CSS	Yes	Yes
	b	TGTGTTCTCAGGTCACCCCTT	21	No				
miR399	a	TGCCAAAGGAGATTTGCCCAG	21	No				
	b	TGCCAAAGGAGATTTGCCCGG	21	No				
	c	TGCCAAAGGAGATTTGCCCGG	21	No				
	d	TGCCAAAGGAGAATTGCCCTG	21	No				
	e	TGCCAAAGGAGAATTGCCCTG	21	No				
	f	TGCCAAAGGAGAGTTGCCCTG	21	No				
miR403		CTAGATTCACGCACAAACTCG	21	No	16408280[a]	AGO2	Yes	Yes
miR408		CTGCACTGCCTCTTCCCTGGC	21	Yes	16412738[a]	Laccase	Yes	
miR530		TGCATTTGCACCTGCACCTTA	21	No	16423020[a] 16410499[a]	CCHC-type RNA helicase AGO1		
miR535		TGACAACGAGAGAGAGCACGC	21	Yes	16409298[a] 16420023[a] 16405577[a]	SPL		Yes
miR3627		TTGTCGCAGGAGCGATGGCACC	22	No				

[a] PACid

[b] PUT-167a-Carica_papay

[c] Supercontig

[d] gi

[e] 'Yes' and 'No' indicate whether the miRNA was identified by Aryal et al. (2012)

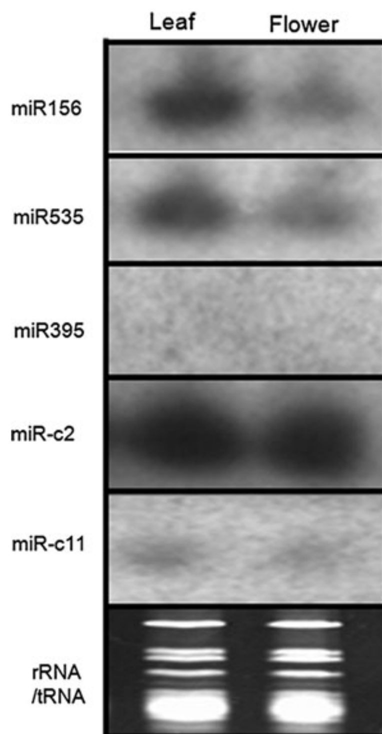

Fig. 2 Northern blotting analysis of miRNAs. RNA was extracted from leaves and flowers. rRNA staining is shown as a loading control. 20 μg of total RNA was used for northern blotting

was found in papaya. This intron is 340 bp long and its secondary structure contains a stem-loop region according to RNAfold prediction. Thus, this intron may be an miRNA-producing locus. To further confirm that this intron can be processed into mature miRNAs, the predicted stem-loop region (Fig. 3a) was sub-cloned downstream of a 35S promoter in an expression vector to generate a 35S::Intron construct. Subsequently, agrobacterium-mediated transient expression experiments were conducted in *N. benthamiana* leaves. Compared with the empty vector, the 35S::Intron construct accumulated a significantly higher level of miR-c7, as shown by northern blotting (Fig. 3b). Therefore, this intron is potentially recognized and then sliced by DCL1. We compared the sequences of Ath-miR838 and Cpa-miR-c7, founding that both precursor and mature sequences displayed a very low similarity. It suggested that only the DCL1 auto-regulatory manner, but not the intron miRNA, was conserved.

miRNAs are incorporated into the RISC containing AGO proteins and silence targets with complementary sequences. Many eukaryotes have evolved diverse AGOs to regulate many cellular processes (Hutvagner and Simard 2008; Mallory and Vaucheret 2010). In *Arabidopsis*, ten AGO protein-encoding genes were annotated. By comparative analysis of the genomic sequence, we identified ten AGO protein-encoding gene loci in papaya (Online Resource 2). To date, two conserved miRNA (miR168 and miR403) have been confirmed to directly regulate *AGO1* and *AGO2*, respectively, in plants (Vaucheret et al. 2004; Allen et al. 2005). In papaya, two miR168 and one miR403 mature sequences were identified and they also mediated the cleavage of *AGO1* and *AGO2*, respectively (Fig. 3d, e). Interestingly, we found that *AGO1* was also predicted to be targeted by miR530 in papaya. The cleavage of *AGO1* was verified by 5′ rapid identification of cDNA ends experiments (Fig. 3e); however, we could not find a similar miR530-recognition site in the *AGO1* genes of other plants species, such as *Oryza sativa*, *Populus trichocarpa*, and *Glycine max*, in which the miR530 gene was identified. Thus, the cleavage of *AGO1* by miR530 may be specific to papaya.

Both miR156 and miR535 target the same *SPL* genes

miR156 targets several *SPL* family genes (Wu and Poethig 2006; Gandikota et al. 2007; Schwarz et al. 2008). As a conserved miRNA, miR156 was also predicted to target three *SPL* genes in papaya. Interestingly, these three *SPL* genes were also targeted by miR535, according to our prediction results. miR535 has been identified in nine plant species (miRbase), including monocotyledons and dicotyledons. However, there are no reports of its targets and functions. To confirm our prediction, 5′ RACE experiments were used to identify the cleavage sites in *SPL* genes. As shown in Fig. 4a, two types of cleavage products, with only one nucleotide shift, were recovered from the 5′ RACE products. Usually, the target cleavage mediated by a plant miRNA occurs in the 10th nucleotide from the 5′ end of the miRNA. As expected, these two cleavage sites perfectly matched with the 10th nucleotides of miR156 and miR535, respectively. Thus, these two cleavage products might result from the recognition of miR156 and miR535. However, it is possible that both types of cleavage products were caused by miR156. To exclude this possibility, we performed a transient assay by co-expressing the miR535 gene and one *SPL* gene in *N. benthamiana* leaves. Three days after incubation, the *SPL* mRNAs were efficiently cleaved in the presence of 35S:MIR535 compared with that in the absence of 35S:MIR535 (Fig. 4b).

Recent reports revealed that both miR156 and miR529 targeted *SPL* genes in maize and rice (Zhang et al. 2009; Jeong et al. 2011). We compared those miR156-targeted *SPL* gene sequences from *Arabidopsis*, rice and papaya, and found that all miR156-targeted sites could also be recognized by both miR535 and miR529. However, we could not find miR529 sequences in the papaya small RNA libraries. miR529 was identified in *Oryza sativa*,

Table 2 Papaya-specific miRNAs and their targets

Family	Sequence (5′-3′)	Length	Reads in leaf	Reads in flower	miRNA*	Predicted targets	Target products
miR-c1	TCAATGACACAAGCTCGGAGCA	22	122	21	Yes	2642[b]	Unknown protein
miR-c2	GGGGATGTAGCTCAGATGGT	20	17,866	10,891		16409098[a]	Chromatin remodeling factor
miR-c3	GCCGGCCGGGGGACGGACTGGG	22	249	604	Yes		
miR-c4	CTTTTCAAGACTTCAGCTTCA	21	37	66	Yes	16414446[a]	Ethylene receptor EIN4
miR-c5	AGACCTTGGGATGCGGATTACC	22	24	42	Yes		
miR-c6	TTTTGCATGACTCAGGAGATGT	22	336	184	Yes	16417828[a] 16415122[a]	F-box/RNI-like superfamily
miR-c7	AAGGAATAGCAACTTCTGTCA	21	1	0		16421957[a]	Histone acetyltransferase
miR-c8	TACGCAGGAGAGATGGCGCCGT	22	48	27	Yes		
miR-c9	CATTCTTCTTTGTGAGTCGGG	21	28	5	Yes	16426812[a]	3-hydroxyacyl-CoA dehydrogenase
miR-c10	TCTGGTAGAGATTTGAGCATG	21	78	34	Yes	16415515[a] 16415528[a] 16415521[a] 16415526[a]	NB-LRR domain-containing protein
miR-c11	TAAAGTGGAATTGGGATAATA	21	159	119	Yes	16407110[a]	Alpha-1,4-glucan-protein synthase

Bold letter indicates that the miRNA has been identified by Aryal et al. (2012)

[a] PACid

[b] PUT-167a-Carica_papay

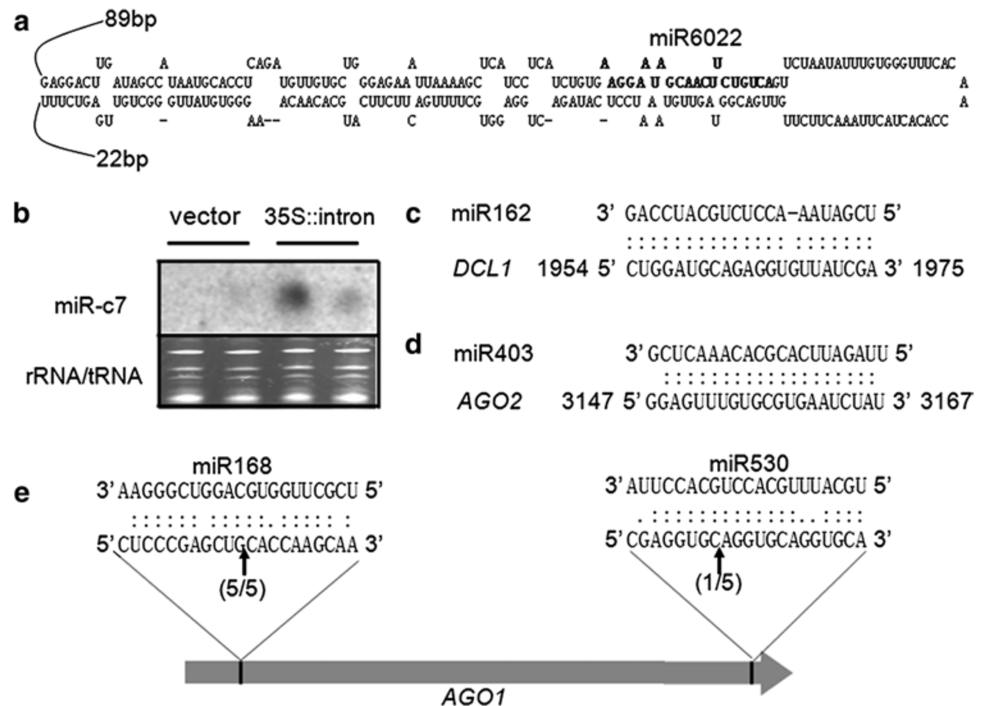

Fig. 3 miRNA auto-regulation pathways in papaya. **a** The precursor sequence of miR-c7. **b** Transition expression analysis of miR-c7 in *N. benthamiana* leaves. **c** The target sequence of miR162 in the *DCL1* gene. **d** The target sequence of miR403 in the *AGO2* gene. **e** The target sites of miR168 and miR530 in the *AGO1* gene. *Arrows* indicate the 5′ termini of mRNA fragments isolated from plants, as identified by cloned cleavage products, with the frequency of the clones shown in parenthesis

Zea mays, and *Sorghum bicolor*, but not in *Arabidopsis*, *Medicago truncatula*, and *Populus trichocarpa*, whereas miR535 was found in *Oryza sativa* and *Vitis vinifera*, but not in *Arabidopsis*, *Medicago truncatula*, and *Populus trichocarpa*. However, miR156 is present in all plant species.

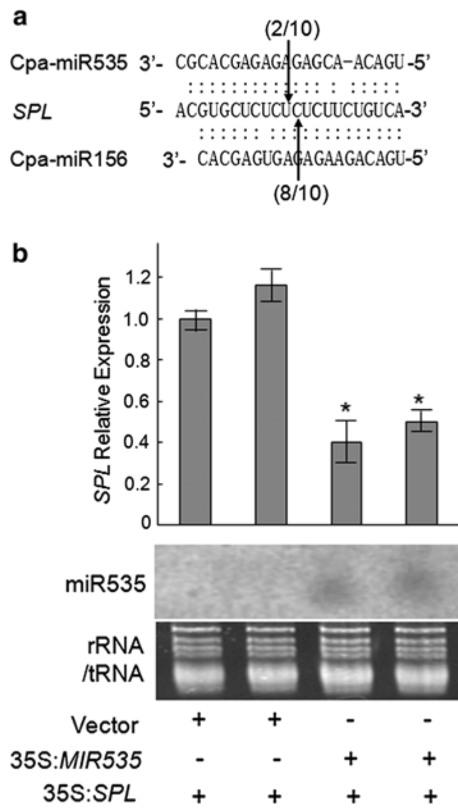

Fig. 4 miR535 mediates the cleavage of an *SPL* gene. **a** The recognition sites of miR535 and miR156 in an *SPL* gene (PACid: 1640929). *Arrows* indicate the 5′ termini of mRNA fragments isolated from plants, as identified by cloned cleavage products, with the frequency of the clones shown in parenthesis. **b** Effect of miR535 on its *SPL* target transcript in coinfiltration assays. 50 μg of total RNA from *N. benthamiana* leaf samples collected at 48 h after infiltration was loaded and hybridized. rRNA and tRNA were used as a loading control. Expression of the *SPL* gene was determined by real-time PCR. *Error bars* indicate ± SE obtained from three biological repeats. *Values marked by an asterisk* are significantly different from the corresponding control value with Student's *t* test ($P < 0.01$; $n = 3$)

Phased-miRNAs produced from miR159 and miR169 precursors

A recent study suggested that multiple distinct phased-miRNAs could originate from the same precursor (Zhang et al. 2010). After we predicted novel miRNAs, we found that several miRNAs arose from a single miRNA precursor. These miRNAs were arranged in phase and unique RNA reads followed one another in tandem. For example, we identified two other miRNAs that mapped to the miR159 precursor (Fig. 5a). In contrast to the high level of miR159 mature sequences, the read numbers of miR159.2 and miR159.3 were relatively low. To confirm the sequencing data, the expressions of miR159.2-5p and miR159.3-3p were confirmed by northern blotting analysis (Fig. 5c). Comparative analysis of phased-miRNA sequences across different plant species found that they displayed lower similarity to their counterparts, whereas miR159 mature sequences were highly conserved (Fig. 5b). Similarly, the miR169b precursor can produce miR169b and miR169b.2 (Online Resource 3).

A 21-nt miRNA triggers secondary siRNA production from Nucleotide-Binding Site Leucine-rich repeat (*NB-LRR*) genes

Occasionally, an sRNA's interaction can trigger the production of secondary siRNA, which involves the generation of double-stranded RNA by RDRs. In *Arabidopsis*, *TAS1-4* genes were identified as the precursors of tasiRNAs. After miRNA-mediated cleavage, a *TAS* gene is converted into double-stranded RNA by RDR6, which is further processed into tasiRNAs by DCL4. Here, four *NB-LRR* genes were predicted to be targeted by a 21-nt miRNA (miR-c10) and their cleavage products were recovered by 5′ RACE experiments. Cuperus et al. (2010) and Chen et al. (2010) revealed that 22-nucleotide miRNAs are sufficient to trigger secondary siRNA biogenesis in plants. Further research confirmed that it is the miRNA-duplex structure that determines the production of plant secondary siRNAs (Manavella et al. 2012b). The miR-c10/miR-c10* duplex contains two asymmetrically positioned bulged bases (Fig. 6a), which should be sufficient for the initiation of secondary miRNAs. To confirm our hypothesis, we aligned all small RNAs in the libraries to the four *NB-LRR* genes, and found that many small RNAs completely matched with them (Fig. 6b). By contrast, only four small RNAs (raw data) were mapped to an *ARF* gene (PACid: 16421034) targeted by miR160. These results suggest that 21-nt length miR-c10 triggers secondary siRNA production from its *NB-LRR* targets.

A papaya-specific miRNA acts in the ethylene signaling pathway

In tomato, miR1917 was identified to target *CTR4* which encodes a key regulator in ethylene signaling pathway (Moxon et al. 2008). Here, we identified a papaya-specific miRNA, miR-c4 (Fig. 7a), which was predicted to target a gene encoding an ethylene receptor protein (highly similar to *Arabidopsis* Ethylene Response 1). The predicted target sequence spans the coding region and 3′ UTR (Fig. 7c). To validate our prediction, we conducted 5′ RACE. As expected, the target cleavage site was mapped to the miR-c4 recognition sequence (Fig. 7b). By a BLASTp search, we discovered three other genes encoding ethylene receptor proteins. However, target prediction analysis indicated that they were not potential targets of miR-c4. In addition, we did not find the homologous miRNA of miR-c4 in other plant species, which suggests that miR-c4 serving in the ethylene signaling pathway is specific in papaya.

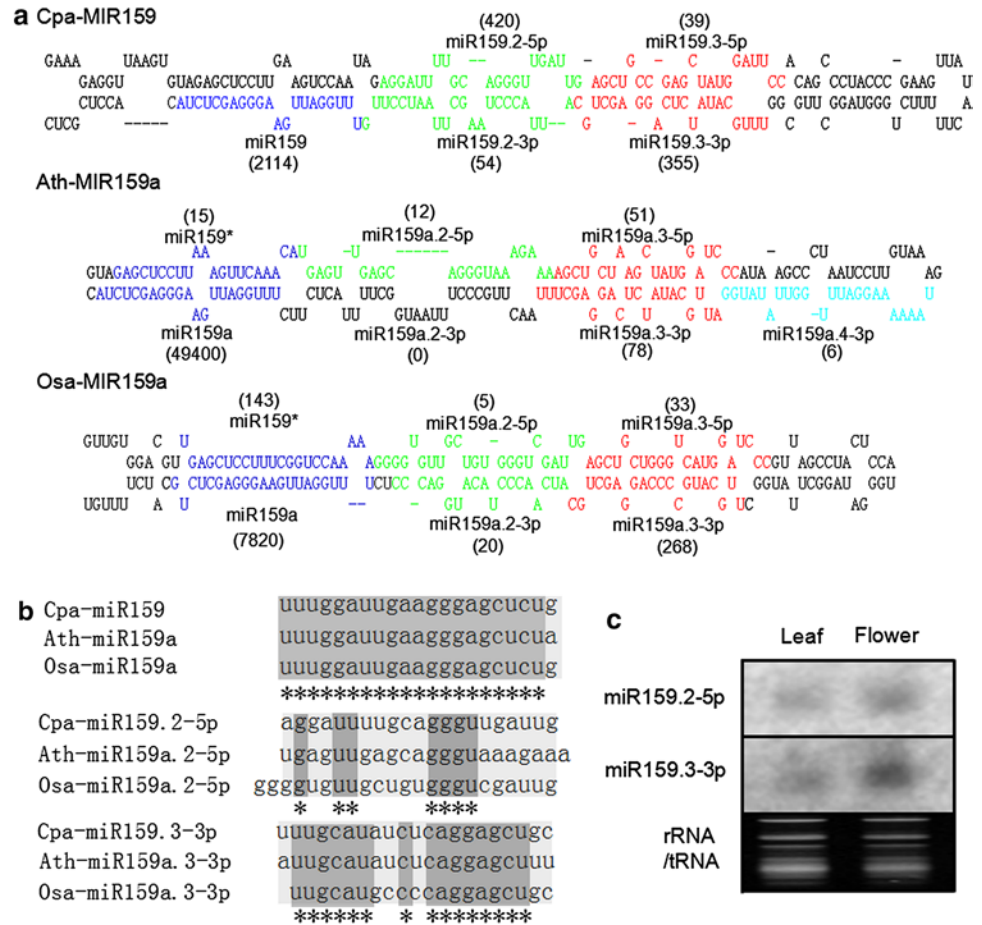

Fig. 5 Phased-miRNAs from the miR159 precursor. **a** Phased-miRNAs in miR159 precursors from *Carica papaya*, *Arabidopsis thaliana*, and *Oryza sativa*. *The number in parenthesis* indicates normalized read number (transcripts per million). **b** Conservation analysis of miRNA sequences in different species. **c** Northern blotting analysis of two phased-miRNAs

Discussion

We conducted an extensive survey of the miRNAs in leaves and flowers of papaya and 75 conserved miRNAs and 11 novel miRNAs were identified. The prediction of these miRNAs suggests that they may play roles in the development or regulation of stress responses.

We showed that papaya has evolved a variety of miRNA-target regulatory pathways. In addition to the conserved miRNA auto-regulation pathways, we also found papaya-specific *AGO1* auto-regulation pathways. In fact, species-specific miRNA auto-regulation pathways have been observed for *DCL2* and *SGS3* (*Suppressor of Gene Silencing*); the former was specifically cleaved by miR1507 in *Medicago truncatula* (Zhai et al. 2011) and by miR1515 in *Glycine max* (Li et al. 2010), and the latter is cleaved by miR2118 in *Glycine max* (Song et al. 2011). In plants, a major method of blocking the invasion of viruses is to generate siRNA to silence viral RNAs. This process involves the participation of DCL and AGO proteins. Therefore, the miRNA auto-regulation pathways are usually important for plants' resistance to viruses. The four DCL proteins of *Arabidopsis* can generate virus-derived small interfering RNAs, with DCL1 being specific to DNA viruses (Blevins et al. 2006). The conserved miR168-*AGO1* and miR403-*AGO2* regulation pathways represent two layers of RNA-mediated defense and counter-defense in the interactions between plants and their viruses (Várallyay et al. 2010; Harvey et al. 2011). Papaya is a perennial plant and the continuous growth allows pathogens to maintain high populations. It is possible that these papaya-specific miRNA auto-regulation pathways serve to defend against papaya-specific viruses.

We revealed that papaya miR535 directly regulates the target genes of miR156, which is analogous to the case of miR529 in rice. In contrast to the high conservation of miR156, both miR529 and miR535 are less conserved. Jeong et al. (2011) revealed that miR529, which is highly abundant in the panicle, may specifically function in panicle branching in rice. However, the expression patterns of miR156 and miR533 are very similar in the leaves and flowers of papaya. Perhaps more detailed tissue expression patterns are required to investigate the function of miR535. Alternatively, miR535 and miR156 may function redundantly to regulate *SPL* genes.

Fig. 6 Secondary siRNA triggered by miR-c10. **a** The miR-c10/miR-c10* duplex. **b** Secondary miRNAs originating from four target genes of miR-c10. Arrows indicate the 5′ termini of mRNA fragments isolated from plants, as identified by cloned cleavage products, with the frequency of the clones shown in parenthesis

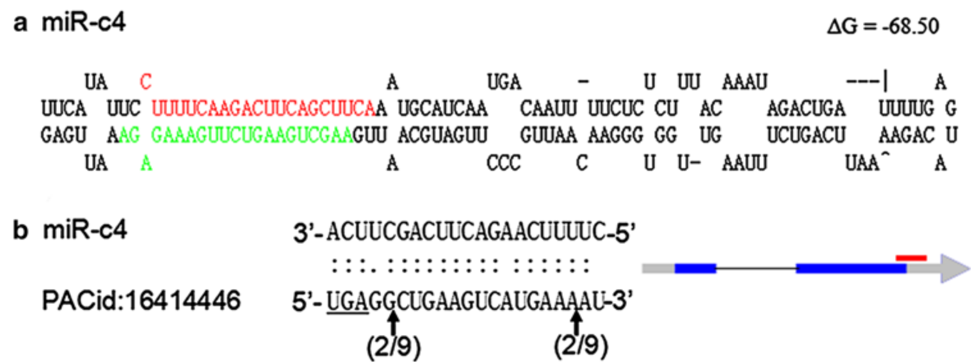

Fig. 7 miR-c4 and its target. **a** Precursor sequence of miR-c4. **b** Recognition site of miR-c4 in its target gene. Arrows indicate the 5′ termini of mRNA fragments isolated from plants, as identified by cloned cleavage products, with the frequency of the clones shown in parenthesis. The triple nucleotides indicate the stop codon

Like canonical miRNAs, phased-miRNAs are processed by DCL1/DCL4 and incorporated into AGO-associated RISC (Zhang et al. 2010; Jeong et al. 2011). Although multiple miRNAs were produced from the same precursors, they targeted different genes because of their different mature sequences. For example, miR319.2, originating from *Arabidopsis* miR319 precursors, targets one *RAP2.12* mRNA, whereas canonical miR319 targets several *TCP* genes (Sobkowiak et al. 2012). The lack of conservation of these phased-miRNAs implies that their target genes are also not conserved. Indeed, the predicted target genes of these phased-miRNAs in papaya were completely different from those in *Arabidopsis* (Online Resource 4). To date, only the function of *Arabidopsis* miR319.2 has been established. These phased-miRNA precursors often contain a long stem-loop structure and only produce a conserved canonical miRNA. Their non-conserved functions suggest that these phased-miRNAs are by-products of miRNA processing. Further investigation is required to understand the functions and evolution of these phased-miRNAs.

Recent research revealed that several miRNAs mediated the cleavage of *NB-LRR* defense genes and simultaneously triggered the production of phased, transacting siRNAs in *Medicago truncatula* (Zhai et al. 2011) and *Solanum lycopersicum* (Shivaprasad et al. 2012). When plants are subjected to pathogen infection, the miRNA-mediated silencing cascade is suppressed, which then leads to the release of *NB-LRRs* to defend against the pathogen. Our results indicated that miRNA-mediated regulation of *NB-LRR* exists in papaya, suggesting a conserved miRNA regulation cascade against pathogens in plants.

In plants, several miRNAs were characterized to function in hormone signaling pathways. For example, miR160, miR167, and miR390 mediate auxin signaling transduction by regulating *ARF* genes (Allen et al. 2005; Wang et al. 2005; Axtell et al. 2006; Marin et al. 2010). miR164 also acts in the auxin pathway through cleavage of *NAC-Domain Containing Transcription Factors* (Guo et al. 2005). miR393 is involved in auxin signaling transduction through regulation of auxin receptors, the *TIR/AFB* family genes (Navarro et al. 2006). In addition, miR159 plays a role in the crosstalk of ABA and GA signaling by targeting *MYB* genes (Achard et al. 2004; Reyes and Chua 2007). Our results uncovered that miR-c4 mediates the regulation of ethylene signaling pathways, which may represent a new expression regulation during fruit development and ripening.

Although there is still more to be learnt about the biological significance of these miRNA-mediated pathways, their identification advances our understanding of plant miRNA function and evolution.

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