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Short communication

MicroRNA (miR396) negatively regulates expression of ceramidase-like genes in *Arabidopsis*

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Abstract

MicroRNAs (miRNAs) are 21–23 nucleotide (nt), endogenous RNAs that regulate gene expression by targeting mRNAs for direct cleavage or translational repression in plants. In *Arabidopsis*, miR396 is encoded by two different loci (*MIR396a* and *MIR396b*) and sequence analysis suggests it may target three ceramidase-like genes (*Atceramidase-like 1*, *Atceramidase-like 2* and *Atceramidase-like 3*). To demonstrate the biological function of miR396, we inserted the synthetic precursors, *MIR396a* or *MIR396b*, under the control of the enhanced cauliflower mosaic virus (CaMV) *35S* promoter, into a plant transformation vector (pOCA30) and transformed the constructs into *Arabidopsis*. The promoter increased miR396 levels by more than 2-fold, indicating appropriate maturation of the synthetic precursor *MIR396a* or *MIR396b* transcript in transgenic plants. Microarray analysis showed that the transcript levels of two ceramidase-like genes (*Atceramidase-like 1*, *Atceramidase-like 2*) were decreased by more than 2-fold and lactosylceramide 4-α-galactosyltransferase increased by more than 2-fold in transgenic plants compared with the empty vector-transformed plants. Northern blot analysis showed that the mRNA levels of the two ceramidase-like genes were significantly reduced in transgenic plants. These results indicated that miR396 probably plays a crucial role in the ceramide metabolism pathway by negatively regulating the expression of ceramidase-like genes in *Arabidopsis*.

Keywords: miR396; miR396 precursor; Ceramidase; Ceramide

1. Introduction

MicroRNAs (miRNAs) are 21–23 nucleotide, single-stranded non-coding RNAs that are processed by Dicer-like enzymes from long RNA precursors containing an imperfect stem-loop secondary structure [1,2]. Mature miRNA derives from the double-stranded portion of hairpin structures that range in length of 70–200 nucleotides [3–6]. The importance of plant miRNAs in various developmental processes, such as root, leaf and flower development, has been demonstrated [7]. For example, in *Arabidopsis*, miR164 regulates *NAC1* transcription that functions in lateral root development [8]; miR165 plays a crucial role in the control of leaf morphogenesis [9]; miR172 regulates flowering time and floral organ identity by translational repression of apetala2 (ap2) [10]. In addition, some miRNAs also play important roles in plant responses to environmental conditions. For example, miR393 promotes plant disease resistance through suppression of auxin signaling [7,11–13]. Although miRNAs play important roles in plants, the targets and function of only a limited number of miRNAs have been demonstrated.

Forward genetics, direct cloning and bioinformatic prediction followed by experimental validation have been used to discover miRNAs [7,14]. Jones-Rhoades and Bartel identified 92 miRNAs, including miR396, using a bioinformatic approach in *Arabidopsis* and rice [3]. In *Arabidopsis*,

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miR396 has two loci (*MIR396a* and *MIR396b*) and their expression patterns in different developmental stages have been detected by both Northern blot analysis and a PCR-based assay [3]. The miR396-target genes, including six growth-regulating factor (GRF) genes and two additional genes (*At4g27180* and *At2g40760*) encoding a Kinesin-like protein B and a Rhodenase-like protein, respectively, have been predicted by a refined computational procedure [3]. Based on 5′ rapid amplification of cDNA ends (RACE), Jones-Rhoades and Bartel verified that miR396 could direct the cleavage of six GRF genes [3,7]. In addition, using EST analysis, Zhang et al. have demonstrated that the miR396 family, which is found in 15 different plant species, is highly conserved [15]. However, the biological functions of the miR396 family are not well understood.

Ceramides (the most simple sphingolipids), sphinganine and sphinganine-1-phosphate (S1P) play crucial roles as second messengers in regulating the biological functions of some enzymes and proteins [16,17]. Some plant sphingolipid genes have been cloned, expressed and functionally analyzed in yeast [18–21]. The functions of S1P and its related signaling molecules have attracted research interest in higher plants [22–24]. It was found that the cellular concentration of S1P was increased under drought stress and abscisic acid treatments, which led to the closure of guard cells [22–24]. In addition, the exogenous application of S1P inhibited light-induced stomatal opening and stimulated the closure of open stomata [25]. It has also been reported that sphinganine-1-phosphate lyase (SPL) plays important roles during leaf development and senescence in *Arabidopsis*. The level of transcription of AtSPL was dynamically changed during leaf development and senescence, and steadily increased from immature leaves to mature leaves [26]. AtSPL transcripts reached their highest levels at the final stage of leaf senescence [26]. This implies the role of AtSPL in regulating the cellular content of S1P in the leaf tissues [23]. In *Arabidopsis* leaf tissues, the S1P content is from 5 to 46 pg/g dry weight under normal growth conditions, and increases by 1.3- to 2.4-fold following drought stress [23].

In order to understand the biological function of miR396, we generated transgenic *Arabidopsis* plants that constitutively over-expressed *MIR396a* or *MIR396b* in this study. Using both the microarray and Northern blot hybridizations approach we demonstrated that miR396 negatively regulates the expression level of ceramidase-like genes in *Arabidopsis*. These results implied miR396's function in the ceramide metabolism pathways.

2. Materials and methods

2.1. Plant material

All the experiments were performed on the Columbia ecotype of *Arabidopsis thaliana*. Seeds were surface sterilized and sown on plates containing Murashige and Skoog (MS) media containing 0.9% agar. Plates were transferred to a tissue culture box at 28 °C for about 5–7 days after being stratified in darkness at 4 °C for 2 days and then the seedlings were further transferred to soil and grown under normal conditions (14 h light, 23 °C/10 h dark, 20 °C).

2.2. Plasmid construction and Arabidopsis transformation

The 544 bp genomic sequence containing the *MIR396a* foldback sequence was amplified from *Arabidopsis* genomic DNA using two primers (5′-TGCTGTAAAAGAATGACCCTT-3′ and 5′-AAACTCATAGACAGAAGTTAGGGTT-3′) and cloned into pUCm-T vector (Sangon), producing a recombinant pT-MIR396a. The sequence of the amplified DNA fragment was verified by sequencing and then sub-cloned into pOCA30 between the CaMV *35S* promoter and the *NOS* 3′poly (A) signal to generate the *35S:MIR396a* construct. The *MIR396b* sequence was amplified by PCR using two primers (5′-TCTTTCAGTCCCACGCTACT-3′ and 5′-TGGATCTAAAGAGTTATCCTGTGT-3′) and the *35S:MIR396b* construct was generated by the same procedure used for the *35S:MIR396a* construct.

Arabidopsis transformation was performed using the floral-dip procedure as previously described [27].

2.3. Northern blot hybridizations

Total RNA was extracted from plant tissues with Trizol reagent (Invitrogen) and separated on 1.5% formaldehyde-MOPS agarose gels before being blotted onto Nylon membranes. Hybridization was performed at 68 °C with PerfectHyb™ Plus buffer (Sigma–Aldrich). The probes were labeled with ^{32}P-dATP using the Klenow fragment (Takara).

For the analysis of small RNAs, 20 μg of total RNA was separated on a denatured 15% polyacrylamide gel-containing 7 M urea and transferred onto Nylon membranes. Hybridization was performed at 35 °C with PerfectHyb™ Plus buffer (Sigma–Aldrich). The probes were labeled with ^{32}P-dATP by terminal deoxynucleotide transferase (Takara).

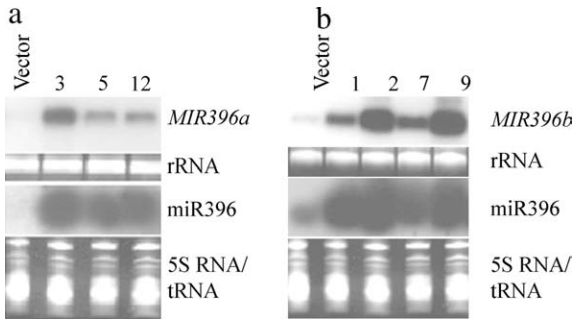

Fig. 1. Northern analysis of *MIR396a* (a) and *MIR396b* (b) transgenic plants.

Table 1
Description of the Atceramidase-like family.

Name	Gene locus	Gene annotation
CER1	At5G58980	Neutral/alkaline non-lysosomal ceramidase-like 1
CER2	At1G07380	Neutral/alkaline non-lysosomal ceramidase-like 2
CER3	At2G38010	Neutral/alkaline non-lysosomal ceramidase-like 3

Table 2
Microarray analysis of transgenic plants.

AGI code	Gene annotation	Log ratio
At5G58980	Neutral/alkaline non-lysosomal ceramidase-like 1	−6.2
At1G07380	Neutral/alkaline non-lysosomal ceramidase-like 2	−2.1
At2G38010	Neutral/alkaline non-lysosomal ceramidase-like 3	−1.6
At4g22330	Alkaline phytoceramidase	−2.3
At3g09020	Lactosylceramide 4-α-galactosyltransferase	3.8

2.4. Affymetrix microarray analysis

The plants transformed with the empty vector and *miR396* transgenic plants were grown for about 20 days under the same conditions as described above. Total RNA was extracted from whole plants (except the roots) with TRIzol reagent (Invitrogen). The purified RNA was used for hybridization on *Arabidopsis* whole genome microarray gene chips (Affymetrix).

3. Results

3.1. Expression of miR396 and its precursors increased in transgenic plants

By comparative genomic approaches, we have known that miR396 has two loci, *MIR396a* and *MIR396b*, which are located on chromosomes 2 and 5 in *Arabidopsis* [3]. Both miR396a and miR396b are 21 nt in length and differ only in their last nucleotide [3].

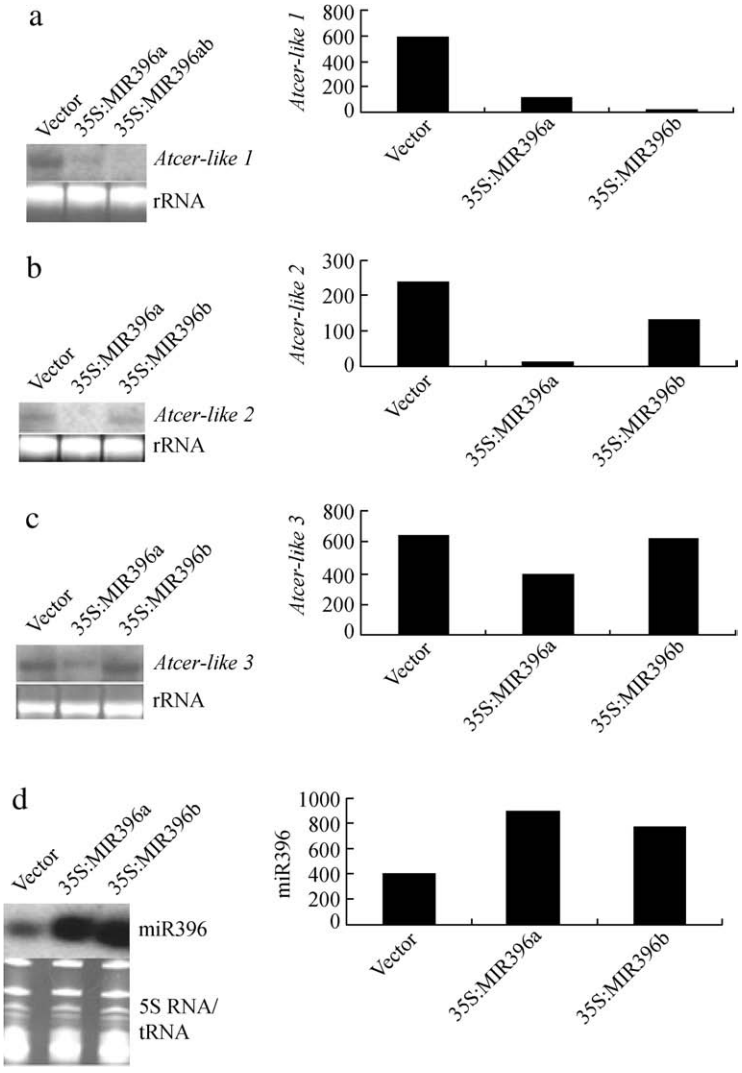

Fig. 2. Northern analysis of *Atcer-like 1*, *Atcer-like 2* and *Atcer-like 3* in transgenic plants transformed with *35S:MIR396a* and *35S:MIR396b*.

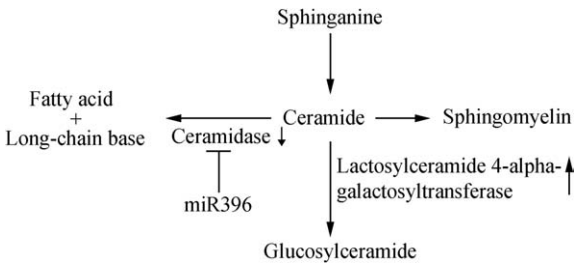

Fig. 3. The role of miR396 in the ceramide metabolism pathway in *Arabidopsis*.

In this study, transgenic plants that over express the synthetic precursors of miR396 were constructed, named *35S:MIR396a* and *35S:MIR396b*, respectively. Northern blot analysis demonstrated that under the control of the promoter miR396 precursor transcript levels increased by more than 2-fold in transgenic *35S:MIR396a* and *35S:MIR396b* plants compared with the plants transformed with the empty vector, indicating appropriate maturation of the synthetic precursor transcripts for *MIR396a* and *MIR396b* in *Arabidopsis* (Fig. 1).

3.2. Microarray experiments demonstrated that the expression of ceramidase-like genes decreased

It has been reported that isoforms of ceramidase differ mainly in their catalytic pH optima [28]. The ceramidase with acidic pH optima is named acid ceramidase. Likewise, the ceramidase with activity at neutral to alkaline pH is called neutral and alkaline ceramidase [28]. In *Arabidopsis*, three ceramidase-like genes all encode neutral and alkaline ceramidases (Table 1). MiR396 shares nearly perfect complementarity with a region present in the ceramidase-like genes, therefore miR396 might negatively regulate the expression of the ceramidase-like genes. Therefore, we used the Affymetrix microarray chip to analyze the genome of *miR396* transgenic plants. The results showed that, in both the transgenic plants, the expression of *Atceramidase-like 1* was decreased by 6.2-fold; the expressions of *Atceramidase-like 2* and alkaline phytoceramidase were decreased by more than 2-fold; the expression of *Atceramidase-like 3* was decreased by 1.6-fold; and the expression of lactosylceramide 4-α-galactosyltransferase was increased by more than 2-fold (all compared to the plants transformed with an empty vector) (Table 2). These results imply that ceramide was mainly converted into glucosylceramide when ceramidase decreased.

3.3. Northern blot verified miR396 negatively regulates ceramidase-like genes

In *Arabidopsis*, there are three members in the ceramidase-like gene family, *Atceramidase-like 1*, *Atceramidase-like 2* and *Atceramidase-like 3*. Microarray analysis showed that the expression of these genes in *miR396* transgenic plants was down-regulated by 6.2, 2.1 and 1.6-fold, respectively, compared with those in empty vector transgenic plants (Table 2). Likewise, Northern blotting showed that the transcript levels of *Atceramidase-like 1* and *Atceramidase-like 2* decreased by more than 8-fold and 2-fold, respectively, in transgenic *35S:MIR396a* and *35S:MIR396b* plants; the *Atceramidase-like 3* expression level in transgenic *35S:MIR396a* plants was down-regulated, but not markedly decreased in transgenic *35S:MIR396b* plants (Fig. 2). Conversely, miR396 levels increased by more than 2-fold in transgenic *35S:MIR396a* and *35S:MIR396b* plants compared with the plants transformed with the empty vector (Fig. 2). This demonstrated that miR396 negatively regulates the expression of the ceramidase-like genes.

4. Discussion

It is known that miR396 is highly conserved among 15 different plant species, for both the primary and mature miRNA sequences. This is especially true for mature sequences and their complementary miRNA sequence [15]. MiR396 has high sequence similarity to the mRNAs from the ceramidase-like genes and therefore probably has crucial and conserved functions in plant development

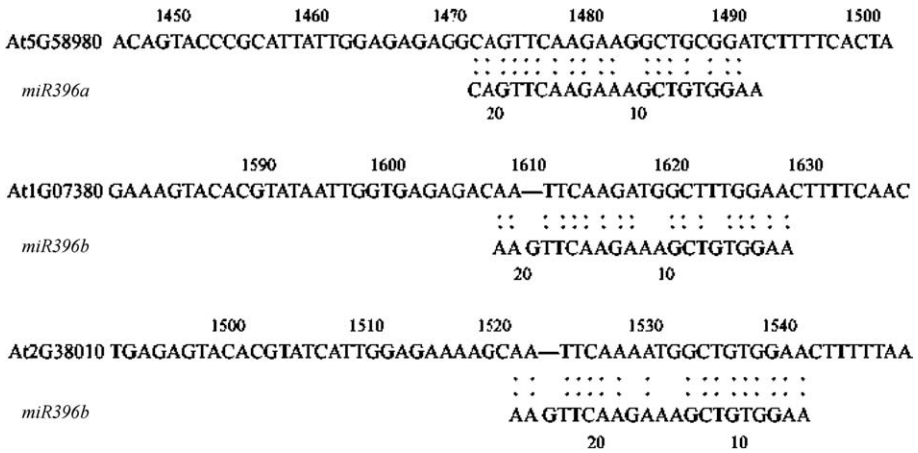

Fig. 4. miR396 is nearly perfectly complementary with the regions of the Atceramidase-like genes in *Arabidopsis*.

related to the ceramide metabolic pathway [15]. In this study, we found that one of miR396's important functions is to negatively regulate the expression of three ceramidase-like genes.

In plants, ceramide is synthesized from sphinganine and is converted to complex sphingolipids by three pathways: conversion to glucosylceramide by glucosylceramide synthase, incorporation into fatty acids by ceramidase, and conversion to sphingomyelin by ceramide kinase [29] (Fig. 3). Accompanied with decreasing ceramidase levels, lactosylceramide 4-α-galactosyltransferase levels increased in *miR396* transgenic plants, suggesting that ceramide was mainly converted to glucosylceramide when the pathway for the formation of fatty acid was repressed (Fig. 3). This implies that miR396 plays an important role in the ceramide metabolic pathway by negatively regulating the expression of ceramidase-like genes in *Arabidopsis*.

MicroRNAs have been demonstrated to affect both target-direct cleavage and translational repression in plants when miRNAs share nearly perfect complementarity with their targets. Given that the miR396 sequence has nearly perfect complementarity with the regions of the three ceramidase-like genes (Fig. 4) and negatively regulates their expression (Fig. 2), we deduce that miR396 probably targets the ceramidase-like genes in *Arabidopsis*.

It has been reported that the important function of plant miRNAs is that they negatively regulate their targets by cleavage and translational repression. A good approach for studying miRNAs' function is to over-express miRNA precursors, because it increases miRNA levels, which in turn decreases target mRNA levels [8]. In this study, over-expression of miR396 precursors in transgenic plants leads to increased miR396 levels and decreased transcript levels of ceramidase-like genes in *Arabidopsis*. The mechanism by which miR396 negatively regulates the ceramidase-like genes requires further investigation.

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