Title

Specificity of plant microRNA target *MIMICs*: cross-targeting of miR159 and miR319.

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Running title: *MIM319/MIM159* specificity.

Summary

Plant microRNA (miRNA) target *MIMICs* (*MIMs*) are non-coding RNA transcripts that can inhibit endogenous miRNAs, as they contain a miRNA binding site that forms a three nucleotide (nt) mismatch loop opposite the miRNA cleavage site upon miRNA binding. This loop renders the *MIMs* non-cleavable, presumably leading to sequestration of the miRNA and thus enabling the endogenous targets to be deregulated. Arabidopsis miR319 and miR159 are two closely related but distinct miRNA families, as they are functionally specific for two different sets of targets, *TCP* and *MYB* genes, respectively. Being offset by one nt, *MIM319* and *MIM159* should have specificity to their respective miRNA families. However, *MIM319* and *MIM159* plants appear indistinguishable, having highly similar developmental defects reminiscent of a loss-of-function *mir159* mutant. In both *MIM319* and *MIM159* plants, miR159 and miR319 levels are reduced, and correspondingly, both *MYB* and *TCP* mRNA levels are elevated, implying that these *MIMs* are inhibiting both miR159 and miR319. These data demonstrate that *MIMs* are able to inhibit closely related miRNAs, including those with cleavage sites not opposite the three nt loop. This highlights that *MIMs* can have unintended off-target effects and that their use should include corresponding molecular analysis to investigate their impact on closely related miRNAs.

Key words: miR159, miR319, *MIMICs*, functional analysis, off-targets, Arabidopsis.

Introduction

Plant microRNAs (miRNA) are small RNAs of approximately 21 nucleotides (nts) in length. Generally, they act as negative regulators of gene expression by guiding the RNA Induced Silencing Complex (RISC) via base pairing to highly complementary binding sites within target mRNAs. Repression of target mRNA expression then occurs through mechanisms including target mRNA degradation, where RISC-mediated cleavage of the phosphodiester backbone of the target mRNA occurs opposite nts 10 and 11 of the miRNA, resulting in the irreversible destruction of the transcript (Llave et al., 2002).

However, a class of miRNA targets known as *MIMICs* (*MIMs*) has been discovered, which are non-coding transcripts that contain a miRNA binding site with a three-nt bulge opposite nts 10 and 11 of the miRNA (Franco-Zorrilla et al., 2007). Consequently, the miRNA is unable to cleave the phosphodiester backbone of the *MIM* transcript which presumably leads to the sequestration of the miRNA on the *MIM* transcript facilitating its degradation. Hence, *MIMs* can inhibit miRNA activity causing loss-of-function miRNA effects (Franco-Zorrilla et al., 2007). Artificial *MIMs*, and variations thereof, have now been designed as tools to target miRNAs of interest, being stably introduced into the plant via *Agrobacterium* transformation (Todesco et al., 2010; Yan et al., 2012) or transiently with the use of viral vectors (Sha et al., 2014, Yan et al., 2014), enabling the *in vivo* functional roles of miRNAs to be elucidated.

In terms of their inhibitory effects, the position of the three-nt loop has been shown to be critical. An artificial *MIM* targeting Arabidopsis miR172 (*MIM172*) in which the loop was positioned opposite nts 10 and 11 resulted in strong attenuation of miR172 activity, whereas a *MIM172* with the loop opposite positions 11 and 12 was ineffective at perturbing miR172 activity (Todesco et al., 2010). In this way, the position of the loop could help define specificity for closely related, but offset, miRNAs.

One such instance is the miR159 and miR319 families, that share 17/21 nucleotides in sequence, but are offset by a single nt (Fig. 1; Palatnik et al., 2007). Despite their similarity, they have distinct target genes; miR159 is specific for a class of genes encoding MYB transcription factors (Palatnik et al., 2007), predominantly *MYB33* and *MYB65* (Allen et al., 2007). By contrast, miR319 mainly targets genes encoding TCP transcription factors, predominantly *TCP2* and *TCP4* (Palatnik et al., 2007). MiR319 can also target *MYB33* and *MYB65*, however due to its low abundance and limited expression domain, regulation of *MYB33/65* by miR319 is negligible (Palatnik et al., 2007). By comparison, miR159 has a widespread expression domain and is often found to be the most abundant miRNA as determined by deep sequencing, and hence is the major regulator of *MYB33/65* (Palatnik et al., 2007; Allen et al., 2007; Rajagopalan et al., 2006). Highlighting the robustness of miR159 repression of *MYB33/65*, miR159 must be reduced to less than 5% of wild-type levels for *MYB33/65* deregulation to occur (Allen et al., 2007).

Curiously however, a *MIM* designed to target miR319 not only resulted in deregulation of the *TCP* targets, but also, and to a greater extent, the *MYB* target genes (Franco-Zorrilla et al., 2007). As miR319 and miR159 are offset by a single nucleotide (Palatnik et al., 2007), the loop within *MIM319* would not be opposite nt 10 and 11 if bound to miR159 (Figure 1), and hence miR159 would be predicted not to be affected. However, countering this is the deregulation of the *MYB33/65* genes in *MIM319* plants, which implies the activity of the highly abundant and redundant miR159 family members must be perturbed in *MIM319* plants. Moreover, *MIM319* and *MIM159* plants result in similar severe pleiotropic defects, suggesting that the *MIMs* could be cross-targeting both miR319 and miR159 (Todesco et al., 2010). This would go against the notion that the three-nt loop must be opposite nts 10 and 11 of the miRNA to perturb its activity. This study aimed to resolve this conundrum.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used in all experiments and is referred to as wild-type. The *mir159ab* mutant is in a Col-0 background and represents a T-DNA insertion loss-of-function mutant, which has been described previously (Allen et al., 2007). Plants were grown on soil (Debco Plugger Mix soil mixed with 3.5 g/L Osmocote Extra Mini fertilizer) in 22 °C growth cabinets under long-day photoperiods (16 hours light/ 8 hours dark, 150 $\mu\text{mol}/\text{m}^2/\text{sec}$).

Generation of binary vectors and transgenic plants

The artificial target *MIMs* *MIM159* and *MIM319* (Todesco et al., 2010), were obtained from the European Arabidopsis Stock Centre (NASC). They were sub-cloned into pDONR/Zeo using the Gateway BP Clonase II enzyme mix (Invitrogen) and then recombined into the Gateway compatible destination vector pMDC32 through Gateway LR Clonase II enzyme mix (Invitrogen) according to the manufacturer's instructions. All expression vectors were sequenced to verify their integrity, transformed into *Agrobacterium tumefaciens* GV3101 cells by electroporation (Hellens et al., 2000) and then transformed into Col-0 using the floral dip method (Clough and Bent, 1998).

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from whole plants using TRIzol (Invitrogen) according to the manufacturer's instructions except for the modifications described in Li et al. (2014). RQ1 DNase (Promega) was used to treat RNA samples, except those used for TaqMan assays, as described in Reichel et al. (2015). Treated RNA was then purified using the Spectrum Plant Total RNA Kit (Sigma) according to the manufacturer's instructions. 1-5 μg of total RNA

was used for cDNA synthesis using SuperScript III reverse transcriptase (Invitrogen) with an oligo dT primer (Invitrogen) according to the manufacturer's instructions. The cDNA was diluted 50X in nuclease-free water and used for qRT-PCR as described in Li et al. (2014). *CYCLOPHILIN 5* (At2g29960) was used to normalize mRNA levels and the Rotor-Gene Q software (QIAGEN) was used to carry out comparative quantitation. The values for each gene are derived from the average of triplicate assays. Gene specific primers are identical to those previously described (Alonso-Peral et al., 2010).

TaqMan assays for mature miRNA analysis

TaqMan MicroRNA Assays (Applied Biosystems) were used to quantitate mature miRNA levels according to the protocol described by Allen et al. (2010). Each cDNA was assayed in triplicate on a Rotor-Gene Q real-time PCR machine (QIAGEN) using the same cycling conditions as described above. Expression of mature miRNAs was normalized to *snoR101* and comparative quantitation analysis was carried out using the Rotor-Gene Q software (QIAGEN). The values for each set of triplicates were averaged and the standard error of the mean (SEM) was calculated. For both qRT-PCR and TaqMan assays, statistically significant changes were determined using Student's t-test analysis.

Results

Firstly, we generated multiple *MIM319* and *MIM159* primary transformants and determined the frequency and severity of phenotypes elicited by the transgenes. Consistent with what has been previously reported, both *MIM319* and *MIM159* plants generated phenotypes reminiscent of the loss-of-function *mir159ab* mutant, with upward leaf curl and a smaller rosette stature, although *MIM159* was able to induce a higher frequency of transformants with this phenotype (Fig. 2). Transcript profiling was then carried out on the different phenotypic categories of primary transformants, which had been classified as having no phenotype (NP; indistinguishable from wild-type), moderate (M; reduced rosette size with moderate leaf curl) or severe (S; rosette size similar to *mir159ab* with most leaves displaying strong curl) morphological defects. In agreement with previous data, both *MYB33/65* and *TCP* targets were elevated in *MIM319* plants, with the *MYB* targets having a much greater fold change than the modest changes observed for the *TCP4* gene (Fig. 3; Franco-Zorrilla et al., 2007). Similar fold-level changes of the *MYB* and *TCP* genes were also observed in *MIM159* plants (Fig. 3). Consistent with the increases in *MYB33/65*, levels of *CPI*, which serves as a marker for MYB protein activity (Alonso-Peral et al., 2010), were also elevated in both *MIM319* and *MIM159* plants. In all measurements, the fold-level changes correlated strongly with the severity of the phenotypes.

In accordance with the elevation of targets from both miR159 and miR319 families, measurement of miRNA levels by ABI Taqman assays found that the abundance of both miR319 and miR159 had decreased in both *MIM319* and *MIM159* plants (Fig. 4). This is clearest for *MIM319* S plants, where the abundance of mature miR319 and miR159 both fall to approximately 30% of wild-type levels. For *MIM159* S plants, the cross-targeting of miR319 does not appear as strong, where the abundance of miR319 levels has only been reduced to 75% of wild-type levels (Fig. 4). Nevertheless, the data strongly suggests that both

MIMs are able to inhibit the activity of both miRNAs to some extent. Interestingly, the measurement of *MIM* RNA levels showed that the steady-state RNA levels of *MIM319* is much higher than *MIM159* (Fig. 4). These high *MIM319* RNA levels might partially explain the strong effect *MIM319* is having on miR159, where miR159 function only becomes compromised in plants with very high *MIM319* levels.

In conclusion, based on this molecular data and the fact that both *MIMs* generate phenotypes reminiscent of *mir159ab*, the phenotype observed in *MIM319* plants is likely to be overwhelmingly caused by the inhibition of miR159 function (Palatnik et al., 2007).

Discussion

Given the ubiquity and diversity of plant miRNAs, methodologies to understand their function will become increasingly important. For RNAi approaches that target protein coding mRNAs in plants, unintended off-targeting of sequence-related genes can produce misleading results (Senthil-Kumar and Mysore, 2011). Similarly, we have clearly demonstrated here that gain-of-function transgenic *MIM* approaches can also result in unintended off-target effects. Therefore, as for standard RNAi approaches which often require molecular analysis to confirm specificity of the particular construct, similar molecular analyses are likely needed when using *MIMs* to discriminate the function of closely related miRNAs.

Clearly this has to be taken as a case-by-case approach. Previously, it was shown for *MIM172* that a three-nt loop opposite position 10-11 was necessary for miRNA inhibition, as a three-nucleotide loop opposite nts 11-12 was insufficient to result in miR172 inhibition (Todesco et al., 2010). However, this does not appear to be the case for miR159/miR319, where a three nt loop opposite nt 10-11 is not mandatory for inhibition, but rather poor central

complementarity is sufficient to confer an inhibitory effect. Such variability between different miRNA families has also been demonstrated for the efficacy of different *MIMs* and associated technologies (Reichel et al., 2015), again highlighting variable complex factors controlling *MIM* - miRNA interactions.

Although it is clear that the *MIM319* can interact and inhibit miR159 activity, it appears that *MIM319* is much less efficient at inhibiting miR159 than the *MIM159*. For instance, the *MIM* transcript levels in *MIM319* M plants, is much higher than those in the *MIM159* S plants; therefore, it appears that more *MIM319* transcript is required to have the same inhibitory effect as the *MIM159*. In our study, both *MIMs* were under the strong constitutive 2X 35S promoter in pMD32 (Curtis and Grossniklaus, 2003). Interestingly, when *MIM319* is expressed in a virus-based system, using a *Tobacco rattle virus* vector, no plants with upwardly curled leaves were observed, instead the plants exhibited a phenotype that would be expected from loss-of-function of miR319 (Yan et al., 2014). This suggests that the strength at which the *MIM* transgene is being transcribed may play an important part of whether it can impose off-target effects, where cross-targeting is more likely to occur in transgenic plants with very high *MIM* expression levels (Fig. 4).

Interestingly, despite the *MIM159* plants resembling *mir159ab* loss-of-function mutants, miR159 levels in the *MIM159* plants were much higher than that in *mir159ab* (Fig. 4). This indicates that measuring miRNA levels in plants expressing *MIMs* may be informative to some extent, but not necessarily an absolute indicator of miRNA activity. Previous studies have found that the levels of most miRNAs were reduced in their corresponding *MIM* plant lines, but in some instances this reduction appeared negligible despite the *MIM* conferring a strong phenotype (e.g. *MIM156*, Todesco et al. 2010). As the *MIMs* are probably sequestering miRNAs, the measured miRNA levels in these lines might not all correspond to active miRNAs.

MiR159 and miR319 are both ancient miRNAs, and their function has been implicated in many developmental and environmental processes (Palatnik et al., 2007; Allen et al., 2007; Nag et al., 2009) in multiple plant species (Ori et al., 2007; Sha et al., 2014). Therefore, genetic tools with such *MIM319/MIM159* binding sites will have to be used with caution to ensure that only the intended targets are affected. More broadly, there are many plant miRNA families with multiple family members that have isoforms off-set from one another by one or several nts. In Arabidopsis, examples include the miR170/171 families and the miR169 family amongst many others (Fig S1). Again, if *MIMs* are to be used to investigate the role of particular family members, careful analysis should be employed to ensure the desired result is achieved, as it cannot be assumed that because the cleavage site of the miRNA is not opposite the loop of the target *MIM*, its function will not be inhibited.

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Figure Legends

Figure 1: Sequences of *MIM159*, *MIM319* and targeted miRNAs.

MIM transcripts are highly complementary to their targeted miRNA but contain a 3 nt mismatch loop opposite nts 10-11. MiR159 and miR319 have 17 out of 21 nts in common, but are offset by one nucleotide. Mismatches between *MIMs* and miRNAs are indicated in red. Nts 10 and 11 of the miRNA (site that demarcates cleavage on the target transcript) are highlighted in bold.

Figure 2: Phenotypic analysis of 4-week-old Arabidopsis plants expressing *MIM159* and *MIM319*.

(A) Wild-type (Col-0) plants and *mir159ab* double mutant plants were used as controls. (B) Col-0 plants expressing *MIM159* and (C) *MIM319*. NP: no phenotype, M: moderate, S: severe phenotype. Numbers indicate the portion of primary transgenic lines falling into the phenotypic categories shown. Scale bar= 10 mm.

Figure 3: Transcript profiling of 4-week-old Arabidopsis plants expressing *MIM159* and *MIM319*.

qRT-PCR analysis of *MYB33*, *MYB65*, *CPI* and *TCP4* levels measured relative to *CYCLOPHILIN*. RNA was extracted from 4-week-old primary transformants using rosette tissue. Measurements are the average of three technical replicates with error bars representing the standard error of the mean (SEM). Asterisks mark statistically significant changes compared to the wild-type control as determined by student t-test analysis.

Figure 4: Analysis of *MIM* and mature miRNA levels in *MIM159* and *MIM319* plants.

qRT-PCR analysis of *MIM* RNA levels measured relative to *CYCLOPHILIN*, and ABI TaqMan assays of mature miR159 and miR319 levels measured relative to *snoR101*. RNA was extracted from 35-day-old primary transformants using tissue from whole plants. Measurements are the average of three technical replicates with error bars representing the SEM. NP: no phenotype, M: moderate, S: severe phenotypes. Asterisks mark statistically significant changes compared to wild-type.

Supplementary Figure1: Alignment of sequence related miRNAs from Arabidopsis that are off-set by one or more nucleotides. MiRNA sequences were obtained from miRBase (<http://www.mirbase.org/>).