Inhibiting plant microRNA activity: molecular SPONGEs, target MIMICs and STTMs all display variable efficacies against target microRNAs

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Introduction

The discovery of the importance of small noncoding RNAs (sRNAs) in controlling gene expression has led to a paradigm shift in our understanding of the information contained in the genome and how it is regulated. Central to this has been the in-depth elucidation of a class of endogenous 20–24 nucleotides (nt) sRNAs known as microRNAs (miRNAs). They are incorporated into RNA-induced silencing complexes (RISCs), which they guide to complementary miRNAs that are subsequently silenced, mainly through cleavage and translational repression mechanisms (Voinnet, 2009). Cleavage occurs at the phosphodiester bond opposite nt 10 and 11 of the miRNA in the miRNA–mRNA duplex and is catalysed by the RNAse H-like domain of ARGONAUTE (AGO) (Voinnet, 2009). In both plants and animals, miRNAs have been shown to play crucial roles in many different biological processes, including development, disease and environmental responses (Sunkar et al., 2012), making miRNAs strong targets for biotechnology (Zhou and Luo, 2013).

In plants, the biological roles of most miRNAs remain unknown, as accurately defining their function has remained challenging. Much of the functional analysis of miRNAs has been based on gain-of-function approaches that have obvious limitations. Firstly, methodologies using the strong constitutive 35S promoter in activation tagging mutagenesis or miRNA overexpression will generally lead to miRNA expression levels and patterns that are not representative of the in vivo condition and therefore may misrepresent the endogenous role of the miRNA (Voinnet, 2009). An alternative approach has been the use of miRNA-resistant target transgenes, but this approach, even when using the target gene promoter, can also potentially misrepresent miRNA function due to transgenic artefacts (Li and Millar, 2013). Therefore, conclusions from these strategies must be drawn with caution and need to be confirmed by miRNA loss-of-function approaches. However, creating loss-of-function mirna mutants is problematic as most miRNAs belong to gene families consisting of multiple redundant members, so the generation of combinatorial loss-of-function mutants has only been achieved for miRNA families with few members (Allen et al., 2007; Sieber et al., 2007).

To address this deficiency, a number of transgenic methodologies have been developed that generate loss-of-function outcomes. This has included using artificial miRNAs against endogenous miRNAs, which can target either single or multiple family members (Eamens et al., 2011) or RNAi approaches that target the primary-miRNA transcript and its promoter (Vaistij...
et al., 2010). More widely used has been the transgenic expression of decoy targets, which are RNA molecules with high complementarity to particular miRNA families (Ivashuta et al., 2011; Todesco et al., 2010; Yan et al., 2012). These decoys act by competing for miRNA binding thereby reducing the number of miRNAs available for the repression of primary targets, which results in their deregulation. In plants, transcripts that contain a single noncleavable miRNA binding site called target MIMics (MIMs) can act as such decoys. MIMs are based on the nonprotein coding gene INDUCED BY PHOSPHATE STARVATION 1 (IPS1) that inhibits miR399 (Franco-Zorrilla et al., 2007). IPS1 contains a highly complementary binding site for miR399 but has a 3 nt mismatch loop at the miRNA cleavage site. Due to this loop, it is thought that IPS1 cannot be cleaved but instead sequesters the miR399-loaded RISC leading to the de-repression of the primary-miRNA target gene (Franco-Zorrilla et al., 2007). The IPS1 gene was modified to generate 73 artificial MIMs to individually inhibit the majority of known miRNA families in Arabidopsis. From this experiment, the expression of 15 MIMs resulted in obvious morphological phenotypes, some of which were similar to phenotypes caused by expressing the corresponding miRNA-resistant target gene or the corresponding miRNA loss-of-function mutant (Todesco et al., 2010).

As some MIMs did not generate strong loss-of-function phenotypes, short tandem target mimics (STTMs), composed of two MIM binding sites separated by a 48 nt spacer, were generated as a subsequent modification of the MIM approach (Yan et al., 2012). STTMs were optimized against the miRNA family miR165/166, and it was empirically determined that a spacer of 48 nt and the two binding sites are both required for strong miR165/166 inhibition, generating a stronger phenotype than a MIM165/166 decoy. This STTM design was then applied to two other miRNA families, mir156/157 and mir160, and two transacting small interfering RNAs and in each case produced strong loss-of-function phenotypes, indicating the robustness of the approach. However, although the STTM165/166 resulted in complete destruction of mir165/166 (Yan et al., 2012), Arabidopsis plants expressing a STTM396 only had moderately decreased miR396 levels (Liang et al., 2014) suggesting that the efficacy of the approach may vary depending on the miRNA targeted.

In animal systems, the use of ‘miRNA sponges’ has proven very effective at inhibiting miRNA action (Ebert et al., 2007). Sponges (SPs) are synthetic transcripts that contain multiple binding sites to a miRNA of interest, either in a nonprotein coding RNA or in the 3’ UTR of a reporter gene. They compete for miRNA binding, which results in the perturbation of the endogenous miRNA-target miRNA interaction. As their specificity is mainly determined by the degree of complementarity to the miRNA, they are able to target entire miRNA families and thus, like plant MIMs or STTMs, can overcome problems of functional redundancy due to multiple miRNA family members. Although SPs with perfect complementarity to a miRNA were shown to inhibit miRNA activity to some extent, SPs with bulges to positions 9–12 of the target miRNA had a greater efficacy (Ebert et al., 2007).

Here, we have investigated the use of miRNA SPs, synthetic transcripts with multiple miRNA binding sites as a methodology for inhibiting miRNA action in plants. We found that SPs containing 15 miRNA binding sites with mismatches at the cleavage site (cmSPs) could strongly perturb miRNA activity; however, their efficacies varied dramatically depending on the miRNA family targeted. Likewise, from direct comparisons of cmSPs to MIMs and STTMs, we found that the silencing efficacy of MIMs and STTMs also varies, demonstrating that different miRNA families respond differently to the three approaches and that no one approach works equally well for the miRNAs tested.

**Results**

**Design of miRNA SPONGES to target the miR159 and miR165/166 families**

Firstly, we generated SPs that aimed to specifically perturb miR159 and miR165/166. SPs were designed largely according to Ebert et al. (2007), where they have multiple miRNA binding sites separated by 4 nt spacers. Furthermore, they contained unique primer binding sites which were used for measuring SP transcript levels by qRT-PCR (Figure 1a). All SP constructs were under the transcriptional control of a dual 35S CaMV promoter in the binary vector pMDC32 (Curtis and Grossniklaus, 2003), to enable strong constitutive expression. SPs with two types of miRNA binding sites were created for both miR159 and miR165/166. Firstly, to investigate the impact of endogenous target site abundance on miRNA regulation, SPs containing binding sites identical to the endogenous miRNA binding sites of the targets of miR159 (MYB33/65) or miR165/166 (PHB/PHV) were made [termed wild type (wt) SP159 and wtSP165/166]. Secondly, to examine the role of cleavage, SPs containing binding sites with mismatches at the cleavage site, which is opposite nts 10–11 of the respective miRNA, were generated [termed central mismatch (cm) SP159 and cmSP165/166] (Figure 1b,c). cmSPs are different from the original SPs by Ebert et al. (2007), which contained a bulge at the cleavage site as opposed to two central mismatches. wtSPs contained fifteen miRNA binding sites, whereas cmSPs contained three (3×), seven (7×) or fifteen (15×) miRNA binding sites, respectively. In contrast to MIMs and STTMs, SPs contain no additional nts between position 10 and 11, hence no asymmetric mismatch loop forms at the cleavage site when bound with respective miRNAs.

WTSP165/166 and cmSP165/166 were transformed into wild-type (Col-0) Arabidopsis plants, while wtSP159 and cmSP159 were transformed into wild-type and mir159a mutant plants. The mir159a allele is a strong loss-of-function mutant, where total miR159 levels are reduced to approximately 10% of wild type (Allen et al., 2010). Hence, the use of wild-type and mir159a plants enables the impact of miRNA abundance on the efficacy of the SPs to be ascertained. The GUS reporter gene, cloned into the same vector as the SPs, was used as a transgenic control.

**cmSP159 transgene can strongly inhibit miR159 activity**

To evaluate the efficacy of miRNA inhibition by the transgene, the frequency and severity of morphological defects were scored for multiple independent primary transformants of the same construct. For SP159 analysis, primary transformants were grown side-by-side and classified as having weak (leaf curling with little or no growth reduction), moderate (leaf curling with growth reduction) or severe (leaf curling with strong reduction in size similar to a strong loss-of-function mir159ab double mutant; Figure 2a) phenotypes.

All wtSP159 (Col-0), wtSP159 (mir159a) and cmSP159 (3×) primary transformants were indistinguishable from wild type. In contrast, 64% (74 of 116) of cmSP159 (7×) and 79% (84 of 106) of cmSP159 (15×) primary transformants displayed moderate and
MicroRNA decoys display variable inhibitory efficacies

Figure 1 Structure and sequences of SP159, SP165/66 and their corresponding targeted miRNAs. (a) SPs are composed of multiple miRNA binding sites separated by 4 nt spacers and two unique sequences that serve as priming sites for qRT-PCR. They are driven by a dual 35S promoter and harbour a poly(A) tail. (b) wtSP159 contains binding sites that are identical to the endogenous mir159 binding site in MYB33/65, while cmSP159 contains binding site with mismatches at the cleavage site (lower case). The endogenous mismatches have been corrected to maintain a similar free energy (ΔG). wtSP159 can target both mir159a and mir159b. (c) wtSP165/166 contains binding sites that are identical to the endogenous miR165/166 binding site in PHB/PHV, while cmSP165/166 contains mismatches at the cleavage site. Original mismatches have been corrected to minimize changes in free energy (ΔG). Tables indicate ΔG values and the number of mismatches to the corresponding cmSP (mismatched nucleotides are underlined). ΔG values were calculated using the DInA melt Web Server (http://mfold.rna.albany.edu/?q=DINAMelt/Two-state-melting).

weak phenotypes (Figure 2b). CmSP159 (15×) caused stronger effects than cmSP159 (7×), as a higher percentage of plants showed a moderate phenotype; however, neither construct could induce severe phenotypes as observed in mir159ab. CmSP159 (15×) showed a better efficacy in mir159a where 76% (65 of 86) of plants displayed moderate phenotypes, compared to Col-0 plants with only 25% (27 of 106) of plants showing moderate symptoms (Figure 2c). Thus, the efficacy of cmSP159 appears to be influenced by both the abundance of miR159 and the number of miRNA binding sites. All GUS transformants had a wild-type phenotype (Figure 2a).

As cmSP159 (15×) had the strongest efficacy, we decided to further test its impact and that of wtSP159 on miR159 regulation by measuring transcript levels of its target genes, MYB33 and MYB65, and the downstream gene CP1, which acts as a marker for MYB33/65 protein activity (Alonso-Peral et al., 2010). For MYB33/MYB65, gene-specific primers spanning the miRNA binding site were used to quantify intact (un sliced) target mRNA. Consistent with previous studies (Allen et al., 2007), the mRNA levels of MYB33 and MYB65 were ∼fivefold and ∼sevenfold higher, respectively, in mir159ab than in wild-type plants (Figure 2d). In wtSP159 (Col-0) plants, MYB33 and MYB65 transcript levels remained unchanged compared to the controls, which was also reflected in the unaltered CP1 levels. For wtSP159 (mir159a) plants, MYB33, MYB65 and CP1 transcript levels were ∼1.5- to 2-fold higher compared to the controls (Figure 2d). Thus, wtSP159 may be having an effect on miR159 silencing in mir159a plants; however, any perturbation of miR159 activity is not strong enough to enable deregulation of MYB33 or MYB65 expression
A cmSP165/166 transgene strongly perturbs miR165/166 activity

Similar to wtSP159, expression of wtSP165/166 did not result in any phenotypic abnormalities. However, 103 of 105 cmSP165/166 (15x) and 75 of 92 cmSP165/166 (7x) primary transgenants displayed phenotypic abnormalities of adaxialized leaves (Figure 3a), the expected phenotype of a miR165/166 loss-of-function mutant (Xu et al., 2007). Plants were classified as having weak (weakly adaxialized organs), moderate (strongly adaxialized organs) or severe (trumpet- and cup-shaped leaves with fasciated stems and flowers) phenotypes. Moderate and severe lines were sterile, and the latter did not produce extended internodes in the inflorescence resulting in floral clumps (Figure S1a).

CmSP165/166 (15x) clearly showed the strongest efficacy with 75 of 105 plants showing severe abnormalities, whereas of 92 cmSP165/166 (7x) transformants, two showed severe, 17 showed moderate and 56 showed weak symptoms. CmSP165/166 (3x) did not appear to strongly perturb miRNA activity, as only 3 of 121 transformants showed weak defects, while all other transformants had no phenotypic abnormalities.

Next, transcript levels of the miR165/166 target genes PHB, PHV and ATHB-15 (Emery et al., 2003; McConnell et al., 2001) were measured using qRT-PCR.
were measured in wtSP165/166 and cmSP165/166 (15x) plants using qRT-PCR. Consistently, the transcript levels of these genes were slightly elevated in wtSP165/166 plants, but were strongly up-regulated in cmSP165/166 (15x) plants (Figure 3b), strongly correlating with the morphological defects. Additionally, transcript levels of cmSP165/166 (15x) were higher than that of wtSP165/166 and positively correlated with phenotypic severity (Figure 3b). Together, these data demonstrate that cmSPs with 15 miRNA binding sites are most effective in inhibiting miRNA activity.

**Utilization of cmSP165/166 to inhibit miR165/166 function in a tissue-specific manner**

As many plant miRNAs are known to be expressed in a spatiotemporal manner, it is often of interest to analyse miRNA function in a specific tissue. Thus, we aimed to test whether cmSPs can be used for tissue-specific inhibition of miRNA activity. To do this, cmSP165/166 (15x) was cloned downstream of the pOp6 promoter (pOp6-cmSP165/166) to utilize the pOp6-LhG4 transactivation system (Figure 4a) (Craft et al., 2005). The pOp6-cmSP165/166 construct as well as a pOp6-GUS control were then transformed into the LhG4 enhancer trap line HET:59a (Rutherford et al., 2005). As shown by GUS staining (Figure 4b), transgene expression in the HET:59a line occurs predominantly in the shoot apical region (SAR) and young petioles. Expression of cmSP165/166 in these tissues induced strong developmental defects in leaves and floral tissues. Primary pOp6-cmSP165/166 transformants showed ectopic growth of fasciated flowers resembling the floral phenotype induced by cmSP165/166 under the 35S promoter (Figure 4f). Furthermore, an outgrowth of adaxial-like tissue with trichomes was apparent on the abaxial side of the leaf, which was not observed in plants constitutively expressing cmSP165/166 (Figure 4d,e). These phenotypes indicate that using the pOp6/LhG4 system, we have successfully expressed cmSP165/166 in a spatial manner generating a tissue-specific miR165/166 loss-of-function phenotype.

**Application of cmSPs targeting different miRNAs reveals varying efficacy**

To further test the applicability of cmSPs as miRNA inhibitors, additional 35S-cmSP (15x) transgenes were generated aiming at specifically inhibiting individual miRNA families. Firstly, of 42 primary transformants generated with a cmSP164 transgene, four plants showed moderate defects characterized by serrated rosette leaves (Figure 5a), which is similar to the phenotype reported for a mir164abc triple mutants (Sieber et al., 2007). However, the disruption of phyllotaxis and floral organ development as seen in mir164abc was not evident in cmSP164 transformants indicating that miR164 activity might not be completely inhibited.
Next, of 50 primary transformants expressing a cmSP390 transgene, 29 showed developmental defects characterized by narrow leaves and downwardly curled leaf margins. Two plants also appeared smaller in rosette size and were thus classified as severe (S), while the other 27 plants showing leaf defects were classified as moderate (M) (Figure 5b). These morphological phenotypes resemble that of the ago7 mutant. AGO7 specifically loads miR390 and triggers the production of transacting siRNAs (tasiRNAs), which regulate AUXIN RESPONSE FACTOR (ARF) genes controlling leaf development (Adenot et al., 2006; Garcia et al., 2006; Hunter et al., 2006). Consistently, transcript levels of ARF3 and ARF4 were significantly increased in cmSP390 S plants compared to Col-0, while no changes were observed in cmSP390 plants that did not display a mutant phenotype (NP). Thus, both the molecular and morphological phenotypes indicate that cmSP390 could inhibit miR390 activity.

However, other cmSP (15x) transgenes which would be predicted to result in strong developmental defects failed to generate any transgenic plants displaying the predicted phenotype. For instance, cmSP156 (15x) and cmSP172 (15x) transgenes failed to result in any phenotype (>30 primary transformants; data not shown), despite other approaches successfully generating stronger loss-of-function phenotypes when targeting these miRNAs (Todesco et al., 2010; Yan et al., 2012). Therefore, the SP approach appears highly variable in its ability to inhibit miRNA-mediated activities, where strong (cmSP166, cmSP159), weak (cmSP164, cmSP390) and no (cmSP156, cmSP172) inhibition occurred for different target miRNAs.

CmSP165/166 and STTM165/166 have a stronger efficacy than MIM165/166

To appraise the efficacy of cmSPs (15x), they were compared to other decoy technologies such as MIMs and STTMs (Todesco et al., 2010; Yan et al., 2012). All decoys were subcloned into pMDC32 that contains the dual 35S promoter, the same binary vector backbone as the cmSPs, enabling direct efficacy comparisons through analysis of multiple independent primary transformants for each construct.

Firstly, we compared the efficacies of cmSP165/166, MIM165/166 and STTM165/166 by scoring the severity and frequency of developmental abnormalities (leaf adaxialization; Figure 6a) in multiple independent primary transformants for each construct. For cmSP165/166, the majority (64%) of primary transformants displayed a severe phenotype (Figure 6b). By contrast, none of the MIM165/166 plants displayed such a phenotype, but mainly displayed moderate and weak phenotypes based on the classification (Figure 6b). Consistent with this, the miR165/166 target transcripts PHB, PHV and REV were much higher in cmSP165/166 than in MIM165/166 plants (Figure S2). The previously reported STTM165/166-48 (a MIM165 and MIM166 binding site in tandem separated by a 48 nt spacer) conferred a strong efficacy with 24% of 82 primary transformants showing a severe phenotype (Figure 6b), an efficacy similar to what was previously reported, where 30% of primary transformants resulted in strongly adaxialized leaves (Yan et al., 2012).

The number of miRNA binding sites and the strength of ΔG interaction influence decoy efficacy

To test whether the higher efficacy of cmSP165/166 and STTM165/166-48 compared to MIM165/166 is due to the increased number of miR165/166 binding sites or the sequence of the binding sites, we generated a SP construct with 15 MIM165/166 binding sites [SP165/166 (15x·MIM)]. Furthermore, the miRNA binding site of MIM165/166 has an additional mismatch to miR165/166 when compared to the binding sites of STTM165/166-48 (Figure S3) resulting in a weaker ΔG interaction (Table S1). Hence, another SP construct containing 15 MIM165/166 sites with this mismatch corrected (MIM-MC) was also generated [named SP165/166 (15x·MIM-MC)] to test whether strengthening the ΔG interaction can increase efficacy.

For SP165/166 (15x·MIM), approximately 9% of 90 primary transformants displayed a severe phenotype implying it has a stronger efficacy than MIM165/166 (Figure 6b). This indicates that increasing the number of miRNA binding sites can increase efficacy. Additionally, for SP165/166 (15x·MIM-MC), approximately 14% of 99 primary transformants displayed a severe phenotype (Figure 6b), suggesting it has a stronger efficacy than SP165/166 (15x·MIM) and that the increase of the ΔG interaction increases decay efficacy.
phenotype. Significant changes compared to the controls. S: severe, NP: no replicates with error bars representing the SEM. Asterisks mark statistically.

CYCLOPHILIN classified according to their phenotype. mRNA levels were normalized to transformants (T2). RNA was extracted from rosettes of 5-week-old plants defects. Scale bar indicate the portion of primary transgenic lines showing developmental.

10 mm. (d) qRT-PCR analysis of ARF3 and ARF4 transcript levels in the rosettes of the progeny of the primary transformants (T2). RNA was extracted from rosettes of 5-week-old plants classified according to their phenotype. miRNA levels were normalized to CYCLOPHILIN. Measurements equal the average of three technical replicates with error bars representing the SEM. Asterisks mark statistically significant changes compared to the controls. S: severe, M: moderate phenotype. Numbers.

Figure 5 Analysis of 4-week-old Arabidopsis plants expressing cmSP164 and cmSP390. Aerial view of wild-type (Col-0) plants (a), cmSP164 plants (b) and cmSP390 plants (c). S: severe, M: moderate phenotype. Numbers indicate the portion of primary transgenic lines showing developmental defects.

According to their phenotype. Consistent with previous measurements, a tight negative correlation between phenotypic severity and miRNA levels can be observed for all decoy constructs. While miR166a levels in decoy plants showing no and mild phenotypes are similar to wild type, they are strongly reduced in plants with moderate and severe phenotypes (Figures 6c, S2).

**MIM159 has an efficacy stronger than cmSP159 and STTM159**

To appraise the efficacy of cmSP159 (15×), it was compared to MIM159 (Todesco et al., 2010) and STTM159, which contained two MIM159 binding sites separated by a 48 nt spacer (based on the design from Yan et al., 2012). All constructs were cloned into pMDC32 and transformed into Col-0; MIM159 was also transformed into mir159a.

Primary transformants were grown side-by-side and classified as having weak, moderate or severe phenotypes (Figure 7a), using the criteria described before. In accordance with previous results, none of the cmSP159 (15×) transformants displayed severe defects. In contrast, approximately 50% MIM159 (Col-0) and MIM159 (mir159a) transformants showed severe abnormalities, with strong leaf curl and stunted growth (Figure 7b), implying that MIM159 has a stronger efficacy than cmSP159 (15×). However, STTM159 was also unable to induce severe phenotypes, where its efficacy also appeared weaker than that of cmSP159 (15×), as only approximately 1% (one of 82) of STTM159 primary transformants displayed moderate phenotypes. This shows that MIM159 is considerably more potent in inhibiting mir159 than both STTM159 and cmSP159 (15×), again demonstrating that the number of miRNA binding sites does not necessarily determine the efficacy of miRNA inhibition. Finally, measurement of miR159a levels demonstrates the negative correlation between miRNA levels and phenotypic severity, where the stronger the phenotype, the stronger the reduction in miR159 levels (Figure 7c).

**Discussion**

Here, we have demonstrated that molecular SPs with multiple miRNA binding sites can be effective inhibitors of miRNA activity and thus represent a strategy in plants for generating strong loss-of-function miRNA phenotypes. Moreover, we have evaluated the efficacies of different technologies used for inhibiting miRNA activity, cmSPs, MIMs and STTMs, and have found that no one approach guarantees the strongest inhibition, but rather their efficacies can vary dramatically depending on the miRNA family targeted. As the reasons for this variation are unclear, we suggest that multiple approaches should be used when attempting to inhibit a plant miRNA family, thus increasing the likelihood of a strong loss-of-function outcome.

**CmSPs can strongly perturb miRNA action**

By generating multiple transformants for any given construct and then measuring the severity and frequency by which they induced a miRNA loss-of-function phenotype, we quantitatively measured the efficacy of each decoy construct. As all constructs were in the same vector backbone (pMDC32) and driven by the same promoter (2 × 35S promoter) (Curtis and Grossniklaus, 2003), differences observed should be independent of transcription.

It was found that cmSPs (15×) were able to generate loss-of-function phenotypes, albeit with varying efficacies. Additionally, these cmSPs (15×) appeared to work better than existing...
approaches for some miRNA families. For instance, the cmSP165/166 could strongly inhibit miRNA activity at a frequency greater than MIM165/166 or STTM165/166-48, and a cmSP390 (15xMIM) could induce loss-of-function phenotypes, whereas no phenotype was reported for a MIM390 construct (Todesco et al., 2010). This latter finding demonstrates that cmSPs can inhibit miRNAs that are incorporated into AGOs other than AGO1.

By contrast, expression of wtSPs at levels many fold higher than endogenous miRNA target transcripts does not appear to impact miRNA action. This implies that efficiently cleaved endogenous plant miRNAs are unlikely to act as competitive inhibitors against one another. Supporting this is our recent report showing that cmSPs can inhibit miRNAs that are incorporated into AGOs other than AGO1.

CmSPs, MIMs and STTMs all display varying efficacies

By directly comparing the silencing efficacy of MIMs, STTMs and cmSPs, we clearly show that all three approaches are variable in their effectiveness in the inhibition of miRNA-mediated activity.

Figure 6 Phenotypic analysis of different decoy constructs targeting miR165/166. (a) Rosette tissue of 4-week-old wild-type (Col-0) plants and representative examples of 4-week-old primary transformants having a weak, moderate or severe phenotype. Scale bar = 10 mm (b) Percentage of primary transformants of different decoys falling into the respective phenotypic categories. All plants were grown side-by-side under the same conditions. n = number of primary transformants analysed. (c) qRT-PCR analysis of miR166a levels, normalized to snoR101, in different decoys. RNA was extracted from 4-week-old rosette tissue. Measurements are the average of three technical replicates with error bars representing the SEM. Asterisks mark statistically significant changes compared to wild type. NP: No phenotype, W: weak, M: moderate, S: severe phenotype; MIM-MC: MIM mismatch corrected.

For instance, although cmSP165/166 (15xMIM) and STTM165/166 were more effective at inhibiting miR165/166 than MIM165/166, MIM159 was more effective at inhibiting miR159 than both cmSP159 and STTM159 (Figure 7). Additionally, it has been reported that MIM156 is highly efficient at inhibiting miR156 (Wu et al., 2009), and a STTM156 transgene can also induce strong phenotypes (Yan et al., 2012), whereas a cmSP156 construct failed to induce any phenotypic defects. Therefore, from this study of the efficacy of multiple approaches targeting multiple miRNA families, it is clear that no one approach guarantees the strongest outcome. Most unexpected was the weak efficacy of the STTM159; the general rules for STTM design were followed [two MIM159 binding sites linked by the empirically determined 48 nt spacer of identical sequence to Yan et al. (2012)]; however, the STTM159 had a much weaker efficacy than both MIM159 and cmSP159. This weaker efficacy also strongly contrasts to the robust silencing outcomes of other STTMs, which has been reported (Yan et al., 2012). Similarly, MIM165/166 appears weak by comparison to many other MIMs targeting different miRNA families (Todesco et al., 2010), which again suggests strong variation in any one given method. Therefore, this raises the question of why these approaches vary so dramatically against
different miRNA families and what the factors controlling these variable efficacies are.

Factors influencing decoy efficacy

Firstly, a strong $\Delta G$ interaction between the miRNA and its target has been shown to be an important determining factor in miRNA target recognition in plants, where a strong $\Delta G$ value is a strong determinant of specificity (Schwab et al., 2005). On the one hand, a strong $\Delta G$ interaction would facilitate target recognition that would be considered beneficial, but on the other hand, it may encourage cleavage even with two central mismatches as would be the case for SPs, preventing miRISC sequestration. In this regard, a target site with a 3 nt loop, opposite nt 10 and 11, as incorporated into MIMs and STTMs, would be clearly beneficial, leading to a combination of a noncleavable binding site with a strong $\Delta G$ interaction and this is what appears to occur naturally in plants (Franco-Zorrilla et al., 2007; Wu et al., 2013).

Interestingly, the $\Delta G$ interaction between the miR165/166 and the different approaches somewhat correlates with their efficacy; the MIM165/166 has the poorest $\Delta G$ (−26.9 kcal/mol), while both cmsSP165/166 and STTM165/166-48 have stronger $\Delta G$ values (−31.3 kcal/mol and −35.5 kcal/mol, respectively). The fact that the MIM165/166 contains a T:C mismatch at nt position 11 of the miRNA that was changed to a G:C pair in STTM165/166-48 (Figure S3) underlies the difference in $\Delta G$ values and possibly contributes to the poor efficacy of MIM165/166. This is supported by the observation that repairing this mismatch resulted in a slightly stronger efficacy in SP165/166 (15× STTM) compared to SP165/166 (15× MIM) (Figure 6). Continuing this trend, MIM159 (−28.8 kcal/mol) has a stronger $\Delta G$ to miR159 than the cmsSP159 (−26.3 kcal/mol). Such data argue that $\Delta G$ values are a factor worth taking into account when designing such constructs.

However, it is clear that the $\Delta G$ value is not an absolute indicator of efficacy as highlighted by the MIM159 having a much greater efficacy than STTM159 despite both having identical miR159 binding sites. Additionally, in rice, a MIM that had two mismatches against an artificial miRNA (amiRNA) conferred a stronger efficacy than STTM159 despite both having identical miR159 binding sites. Therefore, factors beyond complementarity must be contributing to decoy efficacy. One obvious factor could be the stability of the decoy transcript; for instance, many natural SP transcripts in animals are circular, which may increase their resistance to degradation (Hansen et al., 2013). Although all decoys analysed in this study were in the same vector backbone, meaning that the transcription rate should not be a differential factor, it is possible that the different decoy transcripts have different RNA stabilities.

Another likely factor is RNA secondary structure. In the development of the STTM technology, Yan et al. (2012) have

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**Figure 7** Phenotypic analysis of different decoy constructs targeting miR159. (a) Rosette tissue of 4-week-old wild type (Col-0) and representative examples of 4-week-old primary transformants having a weak, moderate or severe phenotype. Scale bar = 10 mm. (b) Percentage of primary transformants of different decoys falling into the respective phenotypic categories. All plants were grown side-by-side under the same conditions. $n$ = number of primary transformants analysed. (c) qRT-PCR analysis of miR159a levels, normalized to snor101, in different decoys. RNA was extracted from 4-week-old rosette tissue. Measurements are the average of three technical replicates with error bars representing the SEM. Asterisks mark statistically significant changes compared to wild type. NP: No phenotype, W: weak, M: moderate, S: severe phenotype.
shown that the length of the DNA spacer between the two MIM sites is predicted to influence secondary RNA structure, which was hypothesized as a contributing factor to the strong efficacy of STTM165/166. For instance, increasing the spacer length of the STTM165/166 from 48 nt to 88–96 nt increases the frequency of transgenic plants with a severe phenotype from approximately 30% to over 60% (Yan et al., 2012). However, even if the spacer sequence is identical between different STTMs targeting different miRNAs, the sequence of the miRNA binding sites will vary, likely resulting in altered RNA secondary structures as based in silico predictions (Figure S4a). Likewise, changing the miRNA binding site in cmSPs and MIMs changes their predicted RNA secondary structure which may subsequently impact miRNA binding site accessibility and decoy stability (Figure S4b,c). However, target site accessibility did not strongly correlate with decoy efficacy (Figure S4c), where MIM172 had the lowest accessibility, despite having a strong efficacy (Todesco et al., 2010). Similarly, there was no clear correlation between decoy efficacy and RNA secondary structure, where whether a miRNA binding site was located in a stem or a loop was not indicative of decoy efficacy. Therefore, bioinformatic predictions of these parameters are not a reliable indicator of decoy efficacy.

With regards to a SP construct, one possible advantage of having so many miRNA binding sites is that there is more chance of having at least some sites with high accessibility, and this could be the reason why increasing the number of binding sites within a cmSP improves efficacy. However, a higher number of miRNA binding sites does not always correlate with a better efficacy as clearly shown by miRNA binding sites does not always correlate with a better endogenous function phenotype; therefore, we do not advocate it as a superior technology, but more of a complementary technology.

Experimental procedures
Plant material and growth conditions
Arabidopsis thaliana ecotype Columbia (Col-0) was used in all experiments and is referred to as wild type. The mir159a and mir159ab mutant plants are in a Col-0 background and represent T-DNA insertion loss-of-function mutants, which have been described previously (Allen et al., 2007). Plants were grown on soil (Debco Pluggar 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 μmol/m²/sec).

Generation of binary vectors and transgenic plants
All SPs and STTMs sequences were synthesized [GenScript (USA) or IDT (USA)] and cloned into the Gateway donor vector pDONR/Zevo (Invitrogen). Synthesized genes were sequenced to verify their integrity and then subcloned into the Gateway compatible destination vector pMDC32 (Curtis and Grossniklaus, 2003) using the Gateway LR Clonase II enzyme mix (Invitrogen) according to the manufacturer’s instructions. The artificial target mimics MIM159 and MIM165/166 (Todesco et al., 2010) were obtained from the European Arabidopsis Stock Centre (NASC). They were subcloned into pDONR/Zevo using the Gateway BP Clonase II enzyme mix (Invitrogen) according to the manufacturer’s instructions and then recombinated into pMDC32 through Gateway LR reaction as described above.

All expression vectors were 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). WtSP159, cmSP159 and MIM159 were also transformed into mir159a.

For the generation of the binary vector pOp6-cmSP165/166, an LR reaction containing pDONR/Zevo-cmSP165/166, pEN-L4-pOp6M2-R1, pEN-R2-F-L3 and the destination vector pB7m4GW.0 (Karimi et al., 2007) was carried out, making use of the MultiSite Gateway recombination system (Invitrogen). Similarly, pOp6-GUS was created by carrying out an LR reaction with pEN-L1-S-L2, pEN-L4-pOp6M2-R1, pEN-R2-F-L3 and pB7m4GW.0 according to the manufacturer’s instructions. PoP6-cmSP165/166 and pOp6-GUS were then transformed into A. tumefaciens GV3101 and subsequently into the enhancer trap line HET:59a (provided by NASC) by floral dipping as described above.

Free energies (ΔG) of RNA duplexes formed between SP transcripts and miRNAs were calculated using the DINAMelt software (http://mfold.rna.albany.edu/?q=DINAMelt/Two-state-melting; Markham and Zuker, 2005, 2008).

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. 20 μg of total RNA was digested in 80 μL reactions according to the protocol, with the addition of RNaseOUT™ Recombinant RNase Inhibitor (Invitrogen) at a concentration of 1 μL/10 μg RNA. 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). CYPLOPHILIN 5 (At2 g29960) 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-Perala et al., 2010).

**TaqMan assays for mature miRNA analysis**

TaqMan MicroRNA Assays (Applied Biosystems) were used to quantify 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 snrR107 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.

**GUS staining**

In situ GUS staining was performed using the method described by Jefferson (1987) with the modifications described in Li et al., 2014.

**Acknowledgements**

We thank the European Arabidopsis Stock Centre NASC for providing the MIMI159 and MIM165/166 clones, and the HET-59a enhancer trap line, Rob Allen and Ira Deveson for critically reading the manuscript. This research was supported by an Australian Research Council grant DP130103697, and International ANU PhD scholarships to M.R. and Y.L.

**References**


Supporting information
Additional Supporting information may be found in the online version of this article:

Figure S1 Detailed phenotype of severe cmSP165/166 and MIM159 plants.
Figure S2 Analysis of target transcript and miRNA levels in cmSP165/166 (15x) and MIM165/166 plants.
Figure S3 Nucleotide sequences of cmSP165/166, MIM165/166 and STTM165/166.
Figure S4 Prediction of RNA secondary structure and miRNA target site accessibility of different decoy constructs.
Table S1 Free energy calculations of the interaction between cmSPs, MIMs, STTMs and their corresponding miRNAs.