Expression of a microRNA-Resistant Target Transgene Misrepresents the Functional Significance of the Endogenous microRNA: Target Gene Relationship

Dear Editor,

A current major challenge in microRNA (miRNA) biology is the elucidation of their functions. A key first step in this pursuit is the identification of functionally relevant miRNA:target gene relationships. Although the molecular verification of miRNA targets with degradome or 5′-end RACE approaches has been very successful in plants, the detection of miRNA-guided cleavage products may not be reflective of functional importance (Allen et al., 2010), which can only be definitively defined by genetic approaches. This remains challenging, as isolation of loss-of-function mutations in miRNA genes has been rare, due to both their small size and the fact that many exist in multi-gene families. Consequently, much of our current understanding of miRNA function has relied heavily on gain-of-function approaches, such as the transgenic expression of miRNA-resistant target genes, where multiple synonymous mutations are created within the miRNA binding site of a putative target gene, conferring the gene resistant to miRNA regulation (Garcia, 2008). By comparing the phenotypic impact of the expression of this miRNA-resistant target to its wild-type counterpart, functionally significant miRNA-mediated regulation may be elucidated.

Here we have used this miRNA-resistant transgene approach to understand the functional role of miR159 in *Arabidopsis*. MiR159 is bioinformatically predicted to target approximately 20 genes, 10 of which are validated targets (Allen et al., 2010). However, only the regulation of two redundant *GAMYB-like* genes, *MYB33* and *MYB65*, appears to be functionally relevant, as the severe pleiotropic rosette defects displayed by a strong loss-of-function *mir159a/mir159b* double mutant (*mir159ab*) are fully suppressed in a *mir159ab.myb33.myb65* quadruple mutant (Allen et al., 2007). This raises the question of what is the functional significance of miR159 regulation for these other target genes.

One such gene is *MYB120*, another member of the *GAMYB-like* family which has a highly conserved miR159 binding site. It is cleaved by miR159 *in vivo* and overexpression of miR159 results in down-regulation of *MYB120* transcript levels (Schwab et al., 2005; Allen et al., 2010). As determined by online microarray data, *MYB120* is predominantly transcribed in anthers and pollen, but negligibly in rosettes (Winter et al., 2007). To investigate what functional impact miR159 regulation has on *MYB120*, *Arabidopsis* plants expressing either a *MYB120* transgene or its miR159-resistant version, *mMYB120*, were generated. Both transgenes were made from a large genomic clone of *MYB120* containing extensive 5′ and 3′ flanking regions to maximize the chance of faithfully reproducing endogenous expression, with only the seven synonymous mutations introduced into the miR159 target site of *mMYB120* being the distinguishing feature of the two transgenes (Figure 1B).

The phenotype of all 25 *MYB120* transformants generated was indistinguishable from wild-type (Figure 1C and D, and Supplemental Figure 1). Of the 34 *mMYB120* lines obtained, all had flowers, anthers, and pollen that were indistinguishable from wild-type (Supplemental Figure 1), but, paradoxically, 21 *mMYB120* lines had altered rosette development similar to that of *mir159ab*, including dwarfed rosettes with upwardly curled leaves (Figure 1E and F). This result suggests that miR159 regulation of *MYB120* is important for rosette development, despite the *mir159ab.myb33.myb65* mutant demonstrating otherwise (Allen et al., 2007). To investigate this paradox, we first examined *MYB120* mRNA levels in rosettes of wild-type and *mir159ab* plants by qRT–PCR. Consistently with online microarray data, the *MYB120* mRNA level is very low in wild-type rosettes; however, an approximately sevenfold increase was seen in the *mir159ab* mutant implying miR159 regulation (Figure 1A). Similar fold-level changes of *mMYB120* transcripts did not result in any obvious phenotypic impact, as the *mMYB120* line m2 that had a sixfold increase in transcript levels was indistinguishable from wild-type (Figure 1G). Conversely, in *mMYB120* plants displaying morphological abnormalities, the fold-level changes of *mMYB120* mRNA levels were found to be several orders of magnitude higher (Figure 1G). This demonstrates

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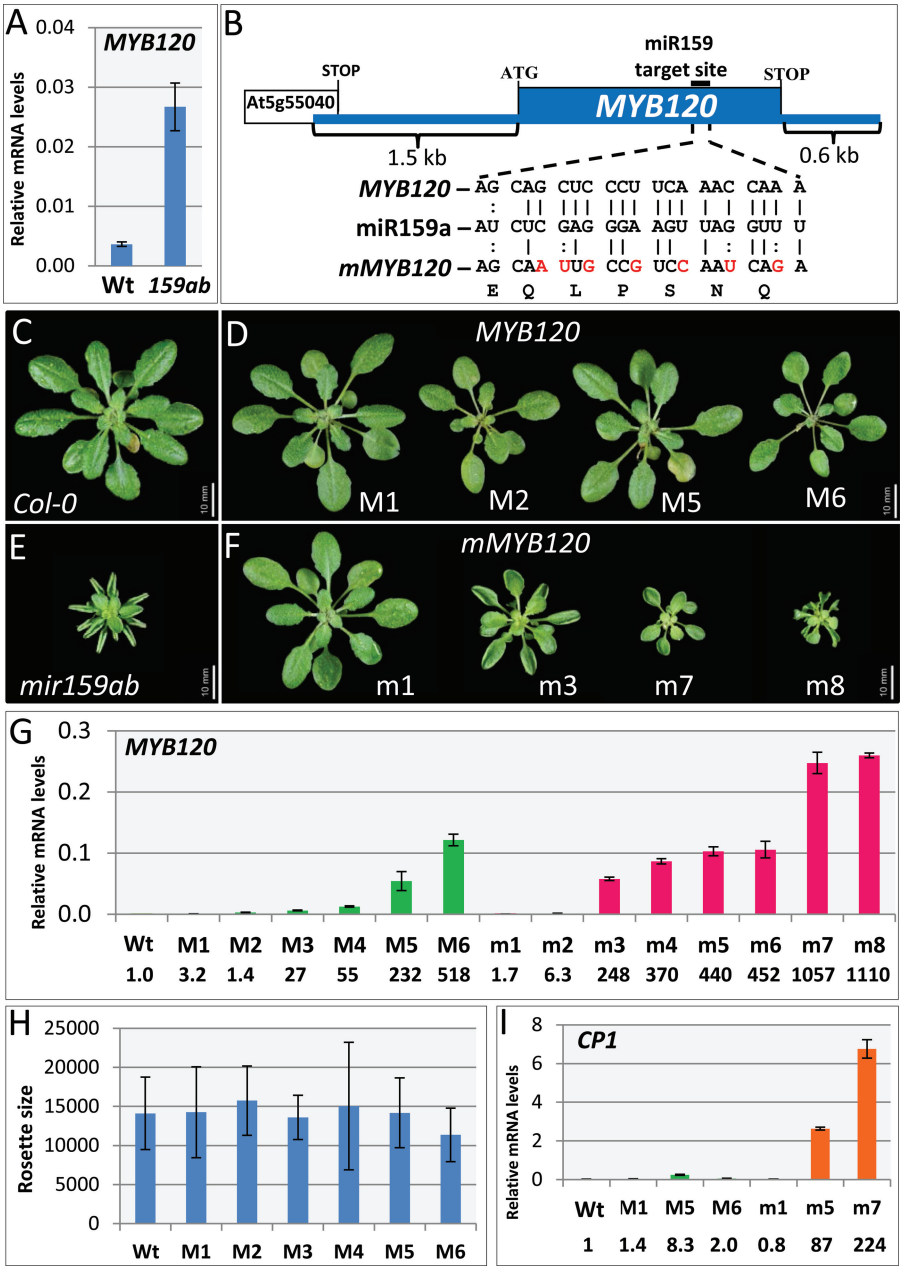

Figure 1. Generation and Analysis of *MYB120* and *mMYB120* Plants.

(A) *MYB120* mRNA levels in rosettes of wild-type (Wt) and *mir159ab* (*159ab*) measured using qRT–PCR with primers that span the miR159 binding site, so that only un-cleaved *MYB120* mRNA will be detected.

(B) The *MYB120* and *mMYB120* transgenes. The entire *MYB120* coding sequence (blue box), a 1487-bp upstream region containing all sequences to the adjacent gene (At5g55040, shown as a white box), and a 636-bp region downstream of the stop codon were amplified and cloned. Seven synonymous nucleotide substitutions were made in the miR159 target site of *MYB120* to generate *mMYB120* as has been previously reported (Allen et al., 2010). The native and mutated miR159 target sites are aligned with the miR159a sequence, with the nucleotide changes indicated in red. *MYB120* and *mMYB120* encode identical proteins. Figure is not to scale.

(C–F) Aerial views of rosettes of 45-day-old plants grown side by side under short-day conditions. (C) Wild-type. (D) Independent *MYB120* transformants. (E) *mir159ab*. (F) Independent *mMYB120* transformants. Scale bar = 10 mm.

(G) *MYB120* mRNA levels in the rosettes of wild-type (Wt), and different T2 transgenic *MYB120* (M1–6) and *mMYB120* (m1–8) lines. Relative fold changes compared to wild-type are shown below the names of the transgenic lines.

(H) Average rosette size of 42-day-old wild-type (Wt) and *MYB120* (M1–M6) plants. Unit of measurement shown is relative pixel. Error bars stand for the SD.

(I) *CP1* mRNA levels in the rosettes of wild-type (Wt), and different *MYB120* (M1, M5, and M6) and *mMYB120* lines (m1, m5, and m7). Relative fold changes compared to wild-type are shown below the names of the transgenic lines. All qRT–PCR measurements were done as described (Allen et al., 2010), and were normalized with *CYCLOPHILIN* and are the average of three technical replicates with error bars representing the SEM.

that the fold-level increase in *MYB120* transcript levels in *mir159ab* must still be too low to confer a phenotypic impact. This is in agreement with the genetic analysis finding that the miR159:*MYB120* relationship is not important for rosette development (Allen et al., 2007). Therefore, this transcript analysis clearly resolves the paradox, where the phenotypic abnormalities seen in *mMYB120* plants are a transgenic artifact, leading to the exaggeration of the importance of miR159-mediated regulation of *MYB120* with regard to rosette development.

Interestingly, the steady-state mRNA levels of both *MYB120* and *mMYB120* transgenes could accumulate to a very high level relative to wild-type, regardless of being regulated by miR159 or not. Although the transcript levels of the miR159-resistant transgene *mMYB120* positively correlated with the severity of phenotypic defects observed in the different *mMYB120* lines, no such correlation was seen with *MYB120* plants, as some *MYB120* lines (M5, M6) had very high un-cleaved *MYB120* mRNA levels, which did not result in any obvious phenotypic consequences (Figure 1G). Confirming this, the average rosette size of plants from six *MYB120* lines was compared to wild-type and, in all instances, no statistically significant differences were found (Figure 1H). Moreover, the transcript level of *CYSTEINE PROTEINASE1* (*CP1*, At4g36880), a marker gene for GAMYB-like activity (Allen et al., 2010), was approximately 80–200-fold higher in some *mMYB120* lines (m5 and m7) showing phenotypic abnormalities (Figure 1I), whereas no such dramatic increases were seen in any *MYB120* transgenic lines. Together, these morphological and molecular phenotypes confirm that very little GAMYB activity is being produced from the high levels of *MYB120* transcripts present in these *MYB120* lines. This demonstrates that miR159 can strongly silence these high *MYB120* transcript levels in the transgenic *MYB120* lines, M5 and M6, and indicates that a potent non-cleavage mechanism(s) is likely involved in this strong miR159-mediated silencing.

Misrepresentation of endogenous miRNA:target gene relationships by a miRNA-resistant transgene has been observed with another *GAMYB*-like gene, *MYB101*, although not to such an extreme (Allen et al., 2010). Like *MYB120*, *MYB101* is predominantly transcribed in anthers and pollen, as determined by online microarray data and by reporter gene studies (Allen et al., 2007; Winter et al., 2007; Allen et al., 2010). Despite this, transgenic expression of either a genomic clone of *MYB101* (9/21 transformants) or its miR159-resistant version *mMYB101* (3/12 transformants) could result in defects in rosettes resembling those of *mir159ab*. Although *mMYB101* transformants displayed *mir159ab*-like phenotypes with a much lower frequency than *mMYB120* (21/34 transformants), these *mir159ab*-like phenotypes in *mMYB101* plants appeared to be derived from transgene overexpression several orders of magnitude higher than the endogenous *MYB101* gene. Together, these experiments demonstrate that expressing a transgene, even under its native promoter with extensive flanking genomic sequences, cannot guarantee faithful replication of endogenous transcriptional patterns.

This transgenic approach, of analyzing phenotypic alterations resulting from the disruption of miRNA target sites in certain putative miRNA targets, has been used widely to identify functionally important miRNA-mediated regulation (Garcia, 2008). In several examples, the expression of the miRNA-resistant target transgene can phenocopy that of the corresponding *mirna* loss-of-function mutant, demonstrating that it can accurately reflect the importance of *in vivo* miRNA regulation. For instance, expression of a miR164-resistant *CUC2* transgene can phenocopy a *mir164a* mutant (Nikovics et al., 2006), and a miR159-resistant *MYB33* transgene can phenocopy *mir159ab* (Allen et al., 2007). However, as miRNAs usually repress multiple target genes, it would be expected that the phenotype derived from a miRNA-resistant transgene would only partially phenocopy that of the *mirna* mutant. Nevertheless, the opposite generally appears to be true, where, in many instances, expression of miRNA-resistant transgenes under their endogenous promoters has a stronger impact on the plant's phenotype compared to a corresponding loss-of-function miRNA phenotype. For example, expression of a *mMYB33* transgene can result in a much more severe phenotype than the *mir159abc* mutant (Allen et al., 2007, 2010); a miR164-resistant *CUC1* gene leads to much more severe phenotypes than a loss-of-function *mir164abc* mutant (Mallory et al., 2004; Sieber et al., 2007); expression of either a miR160-resistant *ARF10*, *ARF16*, or *ARF17* transgene renders much stronger abnormalities than observed in *MIM160* plants (for references, see Garcia, 2008; Todesco et al., 2010). Moreover, a miR172-resistant *APETALA2* gene gives additional floral phenotypes not observed in *MIM172* plants (Zhao et al., 2007; Todesco et al., 2010). As the transgene insertion site (position effect) is a strong contributor to where and at what level the transgene is transcribed, it is likely that a range of phenotypes will be generated in any series of transgenic lines. Therefore, extensive characterization of transgene expression will be required to determine which of these lines are representative of the endogenous miRNA–target gene regulatory relationship.

In summary, this study continues our extensive functional characterization of the miR159 regulatory system in *Arabidopsis*. Through detailed molecular analysis of gain-of-function *mMYB120* transgenic lines, and comparison to previous loss-of-function *mir159ab* mutants, we clearly demonstrate that the phenotype derived from the miRNA-resistant *mMYB120* transgene grossly exaggerates the functional importance of the endogenous miR159–*MYB120* relationship in the rosette due to transgene overexpression. Hence, this provides a clear example that such miRNA-resistant target transgene experiments should be interpreted with great caution, where the likelihood of phenotypes corresponding to transgenic artifacts must be considered. We strongly advocate that investigating miRNA–target relationships using this approach without corresponding molecular analysis and interpretation should be readdressed.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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