

Article Addendum

Regulation of *HD-ZIP III* Genes by MicroRNA 165

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Addendum to:

Overexpression of miR165 Affects Apical Meristem Formation, Organ Polarity Establishment and Vascular Development in Arabidopsis

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ABSTRACT

MicroRNAs (miRNAs) 165 and 166 are able to cleave their target mRNAs of *HD-ZIP III* genes, thus regulating the functions of these genes. Although it is generally accepted that both miR165 and miR166 perform the same functions in the regulation of *HD-ZIP III* genes in Arabidopsis, no experimental data are available to support this notion. Recent work has shown that overexpression of miR166 downregulates the expression of three *HD-ZIP III* genes, *ATHB-9/PHV*, *ATHB-14/PHB* and *ATHB-15*, which in turn recapitulates the phenotypes of simultaneous loss-of-function mutations of these genes. In the March issue of *Plant & Cell Physiology*, we have demonstrated that overexpression of miR165 leads to the down-regulation of all five *HD-ZIP III* genes, and concomitantly recapitulates the phenotypes of loss-of-function mutation of *IFL1/REV* and those of simultaneous loss-of-function mutations of *IFL1/REV*, *ATHB-9/PHV* and *ATHB-14/PHB*. These results indicate that miR165 and miR166 differentially regulate the functions of *HD-ZIP III* genes in Arabidopsis. In this addendum, we show that overexpression of the antisense form of the miR165a gene leads to formation of amphivasal vascular bundles, a phenotype reminiscent of that of the dominant mutation of *IFL1/REV*. This finding provides direct evidence for a role of miR165 in regulation of vascular patterning.

miRNAs have been shown to play important roles in diverse processes in plants, such as growth and development, and stress and disease responses.¹⁻⁴ They regulate the functions of their target genes by binding to the complementary sequences in the target transcripts and subsequently causing translational attenuation or transcript cleavage. In the Arabidopsis genome, a number of miRNAs have been identified by computational prediction and sequencing of small RNAs.⁴ Among them, two miRNAs, miR165 and miR166, differ in only one nucleotide in their mature RNA sequences (Fig. 1).⁵ There exist two copies of miR165 genes and six of miR166 genes in the Arabidopsis genome.⁵ Both miR165 and miR166 are predicted to target the transcripts of *HD-ZIP III* genes, which is supported by biochemical analysis^{6,7,8} and genetic analysis of dominant mutations of *HD-ZIP III* genes.^{7,9,10-15} An intriguing question is whether miR165 and miR166 act redundantly or play differential roles in the regulation of the *HD-ZIP III* genes.

miR165 OVEREXPRESSION RECAPITULATES LOSS-OF-FUNCTION PHENOTYPES OF *HD-ZIP III* MUTANTS

The existence of multiple copies of miR165/166 genes in Arabidopsis provides possible multiple mechanisms in their regulation of the *HD-ZIP III* genes. For example, the miR165/166 genes may have different tissue expression patterns so that they regulate the functions of *HD-ZIP III* genes in a tissue specific manner. Although the transcripts of miR165 and miR166 have been detected in the shoot apical meristem (SAM), leaf primordia and vascular tissues in Arabidopsis,^{16,17,18} the expression pattern of individual miR165/166 genes is not clear. Recently, Jung and Park⁸ used the miR165/166 promoters fused with the glucuronidase (GUS) reporter gene to show that several miR165/166 genes exhibited different tissue-level expression patterns. It should be noted that the expression of the promoter-GUS reporter gene may not represent the endogenous gene expression pattern because the 5' upstream sequence of a gene may not contain all the regulatory elements essential for the endogenous gene expression. Another possible mechanism of regulation by miR165/166 is that miR165 and miR166 may exhibit differential efficiency in the cleavage of *HD-ZIP III* transcripts due to their one-nucleotide difference (Fig. 1). One approach to test this hypothesis is to examine whether overexpression of miR165/166 could lead to downregulation of the *HD-ZIP III* transcripts

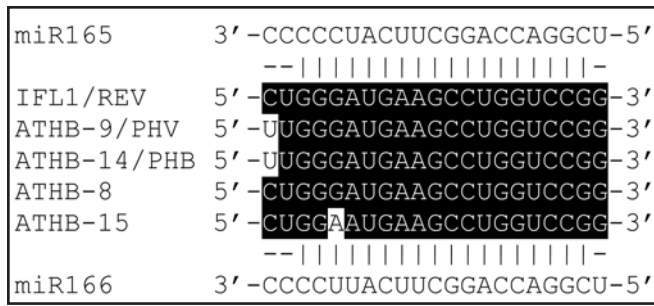


Figure 1. Alignment of mature miR165 and miR166 sequences and their complementary target sequences in the *Arabidopsis HD-ZIP III* mRNAs.

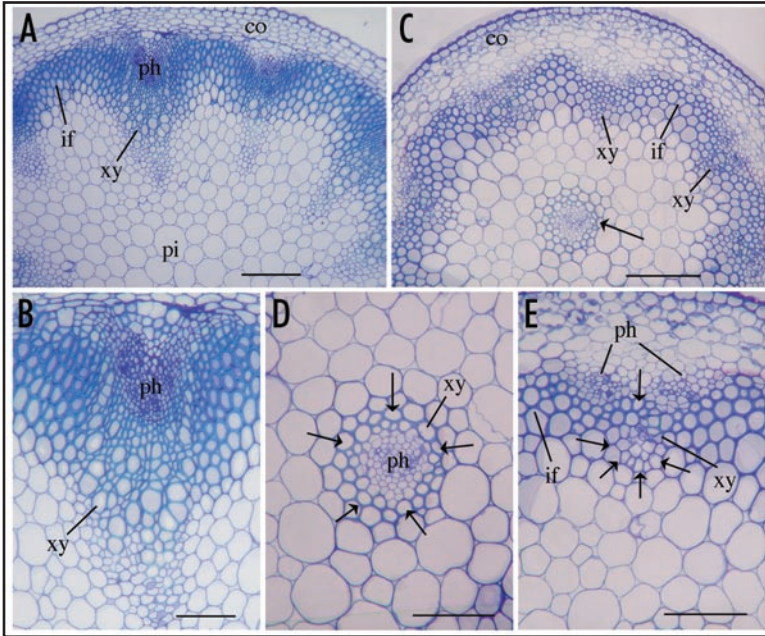


Figure 2. Overexpression of the antisense form of the *miR165a* gene causes an alteration in vascular patterning. The antisense form of the *miR165a* gene was overexpressed under the cauliflower mosaic virus 35S promoter in wild-type *Arabidopsis*. Transgenic plants were selected and their inflorescence stems were sectioned and stained with toluidine blue for visualization of the vascular anatomy. (A) Section of a wild-type stem showing the regular arrangement of vascular bundles and inter-fascicular fibers in the stele. (B) High magnification of a wild-type vascular bundle showing the collateral vascular pattern with the xylem and phloem in parallel. (C) Section of an antisense-*miR165a* stem showing the aberrant formation of a vascular bundle in the middle of the stele (arrow) in addition to the normal vasculature. (D) High magnification of the misplaced vascular bundle in the antisense-*miR165a* stem showing an amphivasal vascular pattern with xylem (arrows) surrounding phloem. (E) High magnification of a normally placed vascular bundle in the antisense-*miR165a* stem showing xylem cells (arrows) arranged in a circular pattern. co, cortex; if, inter-fascicular fiber; ph, phloem; pi, pith; xy, xylem. Bars in (A) and (C), 197 μ m and bars in (B), (D) and (E), 82 μ m.

and concomitantly recapitulate the loss-of-function phenotypes of *HD-ZIP III* mutants.

Overexpression by activation tagging of two *Arabidopsis* miR166 genes, miR166a and miR166g, has recently been shown to cause a significant reduction in the mRNA levels of three *HD-ZIP III* genes, *ATHB-9/PHV*, *ATHB-14/PHB* and *ATHB-15*.^{7,18} The miR166 overexpressors exhibit an enlargement of SAM and an enhancement of vascular development, a phenotype seen in the simultaneous loss-of-function mutant of *ATHB-9/PHV*, *ATHB-14/PHB* and

ATHB-15.¹⁹ The same effects were also observed by overexpression of miR166 under the cauliflower mosaic virus 35S promoter.⁸ Thus it appears that overexpression of miR166 only downregulates the expression of a subset of *HD-ZIP III* genes and recapitulates the phenotypes exhibited by simultaneous loss-of-function mutations of *ATHB-9/PHV*, *ATHB-14/PHB* and *ATHB-15*.

We have recently shown that overexpression of miR165 causes a reduction in the transcript levels of all five *HD-ZIP III* genes in *Arabidopsis*.²⁰ As a result, the miR165 overexpressors exhibit a loss of SAM, an alteration in organ polarity, an inhibition in vascular development and an aberrant differentiation of interfascicular fibers. These phenotypes resemble those exhibited by the loss-of-function mutation of *IFL1/REV*^{21,22,23} and simultaneous loss-of-function mutations of *IFL1/REV*, *ATHB-9/PHV*, *ATHB-14/PHB*.¹⁹ These results demonstrate that overexpression of miR165 and miR166 has differential effects on the downregulation of *HD-ZIP III* genes and concomitantly recapitulates different phenotypes exhibited by loss-of-function mutations of *HD-ZIP III* genes. They suggest that the regulation of *HD-ZIP III* genes by miR165 and miR166 is mediated not only by their tissue specific expression but also by their differential effects on the cleavage of the target transcripts.

OVEREXPRESSION OF THE ANTISENSE FORM OF THE *miR165a* GENE LEADS TO FORMATION OF AMPHIVASAL VASCULAR BUNDLES

It has been shown that a point mutation in the miR165 target sequence in *IFL1/REV* causes an inhibition in the transcript cleavage and a high level accumulation of its full-length transcript,¹³ which leads to dominant phenotypes, including formation of amphivasal vascular bundles and altered organ polarity.^{10,13,24} To further investigate the roles of miR165 in the regulation of *HD-ZIP III* genes, we generated transgenic *Arabidopsis* plants with overexpression of the antisense form of the miR165a gene. We reasoned that antisense inhibition of miR165 should cause similar effects as the *HD-ZIP III* dominant mutations that occurred in the miR165 target sequence, and thus recapitulate the phenotypes exhibited by the dominant mutants. Examination of transgenic plants with antisense inhibition of miR165 revealed ectopic formation of amphivasal vascular bundles in the inflorescence stems (Fig. 2). In addition, the xylem cells in the normally placed vascular bundles also appear to be arranged in a circular pattern (Fig. 2). These results demonstrate that antisense inhibition of miR165 recapitulates the vascular phenotype exhibited by the dominant mutation of *IFL1/REV* and that miR165 is important in regulation of vascular patterning.

To understand the molecular mechanisms by which miR165/166 and *HD-ZIP III* regulate plant development, the next critical step will be to identify and study the functions of their downstream target genes. A recent global gene expression analysis revealed a link between miR165 overexpression and an alteration in the expression of genes involved in auxin signaling,²⁰ which is consistent with the early observation that the *ifl1* mutation alters polar auxin transport.²³ Investigation of the inter-relationship between *HD-ZIP III* and their downstream genes will likely provide valuable insights in the elucidation of the complex pathways controlling vascular pattern formation, organ polarity and interfascicular fiber development in *Arabidopsis*.

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