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MicroRNA control of *PHABULOSA* in leaf development: importance of pairing to the microRNA 5′ region

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MicroRNAs (miRNAs) are ~22-nucleotide noncoding RNAs that can regulate gene expression by directing mRNA degradation or inhibiting productive translation. Dominant mutations in *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*) map to a miR165/166 complementary site and impair miRNA-guided cleavage of these mRNAs *in vitro*. Here, we confirm that disrupted miRNA pairing, not changes in PHB protein sequence, causes the developmental defects in *phb-d* mutants. *In planta*, disrupting miRNA pairing near the center of the miRNA complementary site had far milder developmental consequences than more distal mismatches. These differences correlated with differences in miRNA-directed cleavage efficiency *in vitro*, where mismatch scanning revealed more tolerance for mismatches at the center and 3′ end of the miRNA compared to mismatches to the miRNA 5′ region. In this respect, miR165/166 resembles animal miRNAs in its pairing requirements. Pairing to the 5′ portion of the small silencing RNA appears crucial regardless of the mode of post-transcriptional repression or whether it occurs in plants or animals, supporting a model in which this region of the silencing RNA nucleates pairing to its target.

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Introduction

MicroRNAs (miRNAs) are endogenous ~22-nucleotide (nt) RNAs, some of which are known to play important regulatory roles by serving as guide RNAs for the post-transcriptional repression of protein-coding genes (Lai, 2003; Bartel, 2004). The first to be discovered were the *lin-4* and *let-7* miRNAs, which are required for proper larval development in *Caenorhabditis elegans* (Lee *et al*, 1993; Wightman *et al*, 1993; Moss *et al*, 1997; Reinhart *et al*, 2000). Hundreds of animal miRNAs have since been found, primarily by cloning and computation (Bartel, 2004; Griffiths-Jones, 2004). Regulatory roles for some of these other miRNAs have been demonstrated through functional studies (Brennecke *et al*, 2003; Johnston and Hobert, 2003; Xu *et al*, 2003; Chen *et al*, 2004a) or have been implied by computational predictions accompanied by experimental support for these predicted regulatory relationships (Lewis *et al*, 2003; Stark *et al*, 2003; Yekta *et al*, 2004). Overall, the metazoan miRNAs appear to have diverse and perhaps widespread functions (Lewis *et al*, 2003; Bartel and Chen, 2004).

MicroRNAs are also found in plants (Llave *et al*, 2002a; Park *et al*, 2002; Reinhart *et al*, 2002), where they appear to be predominately involved in directing the repression of genes involved in development (Rhoades *et al*, 2002; Jones-Rhoades and Bartel, 2004). Mutations of genes with known or presumed roles in miRNA biogenesis or function, such as *dcl1, hen1*, *hyl1*, *ago1*, and *hst*, have dramatic developmental anomalies (Park *et al*, 2002; Reinhart *et al*, 2002; Schauer *et al*, 2002; Bollman *et al*, 2003; Han *et al*, 2004; Kidner and Martienssen, 2004; Vaucheret *et al*, 2004; Vazquez *et al*, 2004). Furthermore, specific plant miRNAs have recently been shown to have important functions during embryonic, vegetative, and floral development (Aukerman and Sakai, 2003; Emery *et al*, 2003; Palatnik *et al*, 2003; Chen, 2004; Mallory *et al*, 2004; Vaucheret *et al*, 2004).

MicroRNAs regulate gene expression by guiding mRNA cleavage or by repressing productive translation of their target mRNAs (Wightman *et al*, 1993; Olsen and Ambros, 1999; Llave *et al*, 2002b; Yekta *et al*, 2004). When cleavage of the message occurs, it is near the center of the miRNA complementary site, predominantly between the nucleotides pairing to residues 10 and 11 of the miRNA (Llave *et al*, 2002b; Kasschau *et al*, 2003; Palatnik *et al*, 2003; Xie *et al*, 2003; Chen *et al*, 2004b; Floyd and Bowman, 2004; Jones-Rhoades and Bartel, 2004; Mallory *et al*, 2004; Pfeffer *et al*, 2004; Vazquez *et al*, 2004; Yekta *et al*, 2004), as is seen for cleavage directed by small interfering RNAs (siRNAs) (Elbashir *et al*, 2001a). The mechanism by which miRNAs repress productive translation is essentially unknown, but for two targets of the *C. elegans lin-4* miRNA repression occurs after translation initiation, suggesting that elongation is slowed or that the protein is marked for degradation, perhaps without any effect on the rate of polypeptide synthesis (Olsen and Ambros, 1999; Seggerson *et al*, 2002). The extent of complementarity between metazoan ~22-nt RNAs and their target mRNAs, particularly within the central region of the complementarity, appears to determine whether miRNAs or

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siRNAs will direct RNA cleavage or translational repression (Hutvagner and Zamore, 2002; Zeng *et al*, 2002; Doench *et al*, 2003; Saxena *et al*, 2003; Zeng *et al*, 2003).

The pairing requirements for small RNA function in plants have not been systematically explored, and the degree to which they mirror the requirements seen for animal small RNAs is not known. Most plant miRNAs have extensive complementarity to plant mRNAs (Rhoades *et al*, 2002; Jones-Rhoades and Bartel, 2004) and guide cleavage of their target mRNAs (Llave *et al*, 2002b; Kasschau *et al*, 2003; Tang *et al*, 2003). These targets usually have perfect Watson–Crick complementarity at the six pairs surrounding the cleavage site, supporting the idea that pairing in this central region is important for cleavage. Nonetheless, miR172 also has extensive pairing to the *AP2* mRNA, yet its dominant mode of repression is not cleavage but rather translational repression (Aukerman and Sakai, 2003; Chen, 2004). This suggests that the extent of central-region complementarity between a small RNA and its target is not the only determinant of small RNA function in plants.

The first indication of the biological consequences of disrupting miRNA-mediated regulation in plants came with the discovery that miR165 complementary sites coincide with sites of dominant mutations in two related HD-ZIPIII transcription factor genes, *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*) (Rhoades *et al*, 2002). These mutations decrease the degree of base pairing between the mutant messages and miR165. In *Arabidopsis*, the establishment of leaf polarity requires the generation and perception of positional information along the radial axis of the plant. The adaxial (inner) and abaxial (outer) positions within the leaf primordium become the upper and lower regions of the mature leaf as the leaf tissue grows upward and then outward from the shoot apical meristem. Dominant *phb-d* and *phv-d* mutations cause abaxial to adaxial transformation of leaf fates. The most severely affected organs in *phb-d* and *phv-d* plants develop with radial symmetry and exhibit adaxial traits around their circumference, including the development of ectopic axillary meristems (McConnell and Barton, 1998). A role for PHB in the interpretation of positional information is further supported by the preferential expression of *PHB* transcript in the adaxial domain of the developing leaf in wild-type (WT) plants and the expansion of *PHB* mRNA expression into the abaxial domain in *phb-d* mutants (McConnell *et al*, 2001).

The observation that the miR165 complementary sites, which also have the potential to pair to the nearly identical miR166, map precisely to the loci of the *phb-d* and *phv-d* lesions suggested that disrupting the complementarity between miR165/166 and the *PHB* or *PHV* mRNAs perturbs proper plant development by preventing the miRNA-directed clearing of target messages from abaxial tissues (Rhoades *et al*, 2002). Indeed, studies showing that miR165/166 specifies *PHB* and *PHV* cleavage *in vitro* and that *phv-1d* sequences are cleaved less efficiently than the WT sequence support this proposal (Tang *et al*, 2003). Further support comes from the observation that miR165/166 precursors accumulate preferentially in the abaxial domain of leaf primordia (Juarez *et al*, 2004; Kidner and Martienssen, 2004). Moreover, the biological relevance of miR165/166-directed regulation has been established in studies of *REVOLUTA* (*REV*), an HD-ZIPIII transcription factor gene highly homologous to *PHB* and *PHV*. Expression of *REV* mRNA with silent mutations in the miR165/166 complementary site leads to leaf tissue radialization similar to that observed in *phb-d* mutants, demonstrating the importance of miRNA regulation of *REV* and implying that the same is true for related HD-ZIPIII transcription factors that are predicted miRNA targets (Emery *et al*, 2003).

An alternative hypothesis is that changes in the PHB and PHV proteins cause the mutant phenotypes of the *phb-d* and *phv-d* lesions. A total of 10 *PHB* and *PHV* dominant alleles that cause abaxial to adaxial transformations have been isolated in independent screens (McConnell *et al*, 2001; S Letson and MKB, unpublished observations), but all alleles result from just two types of mutations: a splice site mutation resulting in a 33-nt insertion, isolated twice, and a G-to-A nucleotide point mutation at the 3′ end of the miRNA-binding site resulting in a glycine-to-aspartate substitution, independently isolated eight times. Both types of mutations alter a conserved protein domain proposed to bind a hydrophobic ligand responsible for regulating PHB and PHV protein function. Although the preponderance of alleles altering the same codon could indicate a crucial position within the miRNA165/166 complementary site, it could also reflect a rare amino-acid change that alters the protein's function or even a key position that simultaneously activates and misexpresses the protein.

To distinguish between these possibilities, we compared the phenotypic effects of overexpressing WT *PHB* mRNA, a *phb-3d* mRNA with the glycine-to-aspartate substitution, a *phb-1d* mRNA with the 33-nt insertion, and mRNAs with a series of silent point substitutions in the miR165/166 complementary site. A silent substitution near the 3′ end of the miRNA complementary site is sufficient to confer Phb-d phenotypes of leaf radialization on transgenic plants, proving that the Phb-d phenotype is not the result of changes to the PHB protein. This substitution greatly reduces *PHB* mRNA cleavage rates *in vitro*. In contrast, two independent substitutions at more central positions of the miRNA complementary site that cause only leaf curling in plants reduce to a lesser extent mRNA cleavage rates *in vitro*. Scanning point mutagenesis of the miR165/166 complementary site of *PHB* mapped the portion of the site most sensitive to mismatches, showing the importance of pairing to a heptanucleotide region of the complementary site that corresponds to nucleotides 3–9 of the miRNA. Examining locations of mispaired nucleotides in the 50 other confirmed miRNA:mRNA duplexes revealed that nonpaired nucleotides are most rare at positions 3–10 of the miRNA, suggesting that pairing to this region is important for the function of most plant miRNAs. Our results help explain why only two types of mutations within the miRNA complementary site have been found as *phb-d* mutants in genetic screens. They also further unify the regulatory mechanisms of plant and animal miRNAs, in that pairing to the miRNA 5′ region appears crucial for the functions of both.

Results and discussion

A silent substitution in the miR165/166 complementary site of PHB recapitulates the phb-d phenotype

If the genetic basis of Phb-d mutant phenotypes is that the mutant mRNA is resistant to miRNA-directed cleavage, silent mutations within the miRNA complementary site should

produce the dominant Phb-d phenotype. In contrast, if Phb-d mutant phenotypes result from an activating amino-acid substitution in a ligand-binding domain, then only nucleotide changes that alter the protein sequence will cause the dominant phenotypes. We compared the effects of overexpressing WT *PHB* mRNA, *phb-d* mRNA, and *PHB* mRNA with silent point substitutions in the miRNA complementary site. Leaves of *phb-d* plants have abaxial to adaxial transformations ranging in severity from ectopic outgrowths of dark green, shiny, adaxial tissue on the abaxial leaf surface to completely adaxially radialized, rod-shaped leaves that develop ectopic axillary meristems (McConnell and Barton, 1998). Although a few plants expressing WT *PHB* from the constitutive 35S promoter (*35S:PHB*) have one or two adaxially radialized leaves, the majority of plants have no adaxial transformations and no plants resemble the Phb-d plants overall (McConnell *et al*, 2001; Figures 1 and 2A). In contrast, 57.1% of *35S:phb-1d* T1 plants display unambiguous abaxial to adaxial transformations (McConnell *et al*, 2001; Figure 2B). Although the *phb-1d* dominant mutation is predicted to be most disruptive to potential miRNA binding due to a 33-nt insertion in the middle of the miRNA complementary site (McConnell *et al*, 2001), the *phb-3d* G202D (GGT to GAT) point mutation, which adds a single mismatch near the 3′ end of the miRNA complementary site, is equally effective at conferring Phb-d phenotypes (49.5% of T1s; Figure 1). *35S:PHB G202D* plants display the full spectrum of phenotypes seen in *phb-1d* mutant plants, and transgenic plants can develop identically to the *phb-1d/phb-1d* homozygous mutants (Figure 2B). Like homozygous mutants, these transgenic plants have radialized leaves, leaves with ectopic patches of adaxial tissue on the abaxial surface, and ectopic meristems (Figure 2E, F, and I). Similarly, a silent substitution at the same codon, *35S:PHB G202G* (GGT to GGA), confers the same phenotypes as the *35S:PHB G202D* transgene (Figure 2C and J). Thus, the basis for the *phb-d* mutant phenotype is the disruption of miRNA binding.

Silent substitutions at different positions within the miR165/166 complementary site have distinct effects on leaf development

A miR165/166-programmed RISC guides *PHB* mRNA cleavage *in vitro*, supporting the idea that such a mechanism serves to eliminate *PHB* mRNA from cells in the abaxial leaf primordium (Tang *et al*, 2003). Because some single-nucleotide mismatches at the center of siRNAs severely reduce RISC cleavage of RNAs in animals, we tested two silent substitutions in the center of the miR165/166 complementary site to compare the severity of their effects to those of substitutions at the 3′ end of the site. In contrast to transgenic plants expressing *35S:PHB G202D* and *35S:PHB G202G*, plants with point substitutions at the more central sites had no obvious abaxial to adaxial transformation of the leaf blade (Figure 1). Instead, some *35S:PHB P201P* T1 plants (16/140) and *35S:PHB K200K* T1 plants (5/95) had upwardly curled leaves at frequencies significantly different from that of *35S:PHB* transformants in which none (0/157) had curled leaves ($P<0.01$ Fisher's exact test). Upward curling of the leaf blade also occurred in some leaves of *35S:phb-1d*, *35S:PHB G202D*, and *35S:PHB G202G* transgenic plants that had other leaves with obvious abaxial to adaxial transformation of the leaf blade (Figure 2G and H and data not shown). One possibility is that leaf curling is a weak gain-of-function phenotype of *PHB* reflecting an intermediate level of *PHB* mRNA misexpression.

Axillary meristems normally develop from a population of *SHOOTMERISTEMLESS* (*STM*)-expressing cells at the adaxial leaf base, but radialized leaves of *phb-1d* plants express *STM* RNA around the circumference of the leaf base and produce ectopic meristems (McConnell and Barton, 1998). Similar to *phb-1d* mutants, *35S:phb-1d*, *35S:PHB G202D*, and *35S:PHB G202G* transgenic plants also produce ectopic meristems (Figures 1 and 2I, J). Although the leaf fates were not obviously transformed as judged by surface characteristics, 18% (8/45) of *35S:PHB P202P* T1s had ectopic meristems forming on the

	35S:PHB mRNA paired to miR165	T1s with adaxialized leaves (*n*)	T1s with >20% adaxialized leaves (*n*)	T1s with eptopic meristems (*n*)
WT *PHB*	I G M K P G P D AUU GGG AUG AAG CCU GGU CCG GAU CC CCC UAC UUC GGA CCA GGC U	7.6% (8/105)	0% (0/105)	0% (0/69)
phb-1d	33 nt I G M K ▼ P G P D AUU GGG AUG AAG CCU GGU CCG GAU CC CCC UAC UUC GGA CCA GGC U	57.1% (36/63)	44.4% (28/63)	ND
phb-3d G202D	I G M K P D P D AUU GGG AUG AAG CCU GAU CCG GAU CC CCC UAC UUC GGA CCA GGC U	49.5% (47/95)[a]	29.5% (28/95)[a]	16.7% (12/72)[a]
PHB G202G	I G M K P G P D AUU GGG AUG AAG CCU GGA CCG GAU CC CCC UAC UUC GGA CCA GGC U	50.7% (38/75)[a]	33.3% (25/75)[a]	12.2% (6/49)[a]
PHB P201P	I G M K P G P D AUU GGG AUG AAG CCA GGU CCG GAU CC CCC UAC UUC GGA CCA GGC U	5.0% (5/101)[b]	1.0% (1/101)[b]	17.8% (8/45)[a]
PHB K200K	I G M K P G P D AUU GGG AUG AAA CCU GGU CCG GAU CC CCC UAC UUC GGA CCA GGC U	16.0% (12/75)[b]	1.3% (1/75)[b]	0% (0/36)

Figure 1 Point mutations within the 3′ region of the *PHB* miR165/166 complementary site confer stronger dominant phenotypes than those in central positions. Predicted base pairings of the *PHB* mRNA (top strand) and miR165 (bottom strand) are shown on the left below the amino-acid sequence of the PHB protein. Nucleotide substitutions, as well as any associated changes in protein sequence, are noted in red. Ectopic meristems are only scored on the abaxial base of the first two true leaves to avoid confusion with true axillary meristem formation from the adaxial base of rosette leaves. [a]Significantly different from WT *PHB* ($P<0.01$; Fisher's exact test; www.matforsk.no/ola/fisher.htm). [b]Significantly different from *phb-1d* ($P<0.01$).

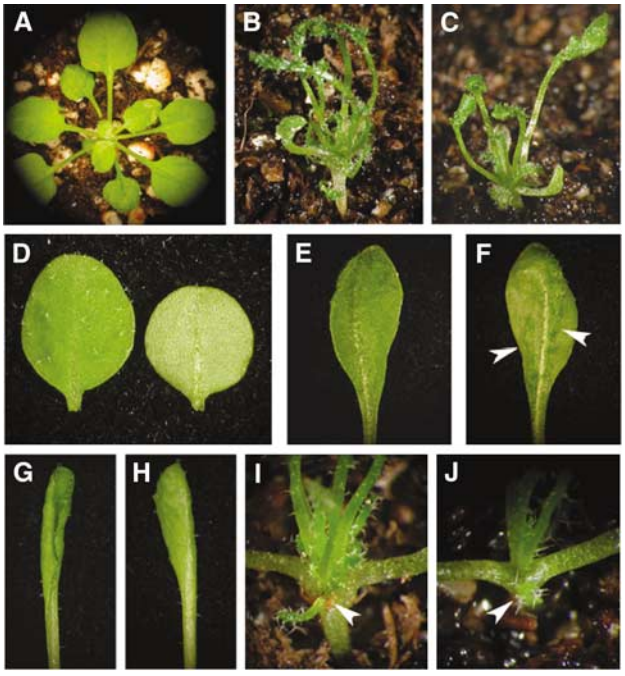

Figure 2 Dominant leaf phenotypes caused by mutations in the miR165/166 complementary site. (**A**) *35S:PHB* plant with WT development. (**B**) *35S:phb-1d* plants have radialized, reduced leaves with adaxial characteristics all around the circumference of the leaf. (**C**) *35S:PHB G202G* plants phenocopy *35S:phb-1d* plants. (**D**) The adaxial (left) and abaxial (right) surfaces of a WT leaf. Leaves from *35S:phb-1d* transgenic plants less severely affected than those in (B) can have normal adaxial surfaces (**E**) but ectopic regions of adaxial tissue (arrowheads) on the abaxial surface (**F**), and they can also be curled but with normal adaxial (**G**) and abaxial (**H**) surfaces. Ectopic meristems form on the abaxial base of the first or second leaves of *35S:phb-1d* (**I**) and *35S:PHB G202G* (**J**) transgenic plants.

underside of at least one of the first two true leaves (Figure 1). The observation that WT *PHB* transcript extends centrally from the adaxial domain into the adjacent meristem has been used to support the idea that the adaxial leaf base and the meristem behave as a unit in axillary meristem development (McConnell *et al*, 2001). However, these data suggest that either axillary meristem formation is sensitive to a lower threshold of PHB activity than leaf surface characteristics or that the population of cells giving rise to the new meristem can be patterned independently of the leaf blade.

Developmental defects observed in PHB mutant plants correspond to a reduction in miR165/166-mediated PHB cleavage efficiency

Our transgenic analysis of the *PHB* miRNA complementary site demonstrates that mutations at different positions have varying developmental consequences and suggests that the 3′ region of the *PHB* miRNA complementary site plays a critical role in the recognition of *PHB* by miR165/166. To interpret the effects of these mismatches, it is useful to know the precise site of miR165/166-directed cleavage. MicroRNA cleavage sites can be mapped by using a modified form of RNA ligase-mediated 5′-RACE (rapid amplification of 5′ cDNA ends) that takes advantage of the monophosphate present at the 5′ terminus of the 3′ cleavage fragment (Llave *et al*, 2002b). miR165/166 is predicted to regulate five members of the HD-ZIPIII transcription factor family, *PHB*, *PHV*, *REV*, *ATHB-8*, and *ATHB-15* (Rhoades *et al*, 2002). When RNA isolated from WT *Arabidopsis* tissues was subjected to 5′-RACE, a distinct PCR band was observed for each of the five targets (Figure 3A). Cloning and sequencing of amplified products mapped the 5′ end of the cleavage products to the nucleotide predicted to pair to the tenth nucleotide of miR165/166 (Figure 3A), a result analogous to those observed for miRNA- and siRNA-directed cleavage of other mRNAs (Elbashir *et al*, 2001a; Llave *et al*, 2002b; Kasschau *et al*, 2003; Xie *et al*, 2003; Jones-Rhoades and Bartel, 2004; Mallory *et al*, 2004; Vazquez *et al*, 2004), including recent reports of miR165/166-directed cleavage of *REV* mRNA (Floyd and Bowman, 2004; Zhong and Ye, 2004). These results demonstrate that miR165/166 directs the cleavage of these five HD-ZIPIII mRNAs *in planta* and establish that the location of these cleavage sites is the same in all five mRNAs.

To determine if the varying developmental defects observed in the transgenic plants reflect changes in mRNA cleavage efficiency, we tested the mutant RNA sequences in wheat germ extracts that were previously shown to efficiently cleave WT *PHB* and *PHV* RNA but not mutant *phv-1d* RNA, in a reaction guided by the wheat miR165/166 endogenously present in the extracts (Tang *et al*, 2003). The cleavage efficiency of the three *PHB* RNAs carrying silent substitutions in their miR165/166 complementary site was reduced compared to WT *PHB* RNA (Figure 3B and C). Interestingly, the cleavage rate of the two *PHB* mutants that exhibit only mild phenotypes in plants (*35S:PHB P201P* and *35S:PHB K200K*) was reduced 13- and 11-fold, respectively, whereas the cleavage rate of the *PHB* mutant that shows a strong phenotype (*35S:PHB G202G*) was 200-fold below that of WT *PHB* RNA (Figure 4A). Since the *phb-3d* RNA also showed a strong reduction in cleavage rate (58-fold), this argues for an inverse correlation between the efficiency of *PHB* cleavage and the severity of developmental abnormalities observed in the transgenic plants and suggests that *Arabidopsis* can tolerate a substantial dampening in miR165/166-directed *PHB* cleavage (about 13-fold) without a dramatic impact on development, whereas a more impaired cleavage has severe developmental consequences. Semiquantitative RT–PCR experiments monitoring uncleaved *PHB* mRNA levels were consistent with the proposal that the leaf phenotypes observed in the *PHB* mutant plants result from a dampening of miR165/166-directed *PHB* cleavage (data not shown).

Substitutions in the 3′ region of the PHB miRNA complementary site are more disruptive to RNA cleavage than those at central or 5′ regions

Because silent mutations in different regions of the *PHB* miRNA complementary site had different impacts on cleavage efficiency and development, we decided to further investigate the contribution of each nucleotide in the miR165/166 complementary site using the wheat germ extract. We extended the original mismatch scheme to create single mismatches that disrupt base pairing between the *PHB* mRNA and miR165/166 throughout the length of the *PHB* miRNA complementary site. Each A (or G) of the mRNA that pairs to a U of miR166 was changed to a C to create a C:U mismatch; similarly, each Watson–Crick-paired C, G, and U of the mRNA was changed to an A, to create A:G, A:C, and A:A mismatches. This set of mismatches was chosen because these four possibilities have comparable frequencies within

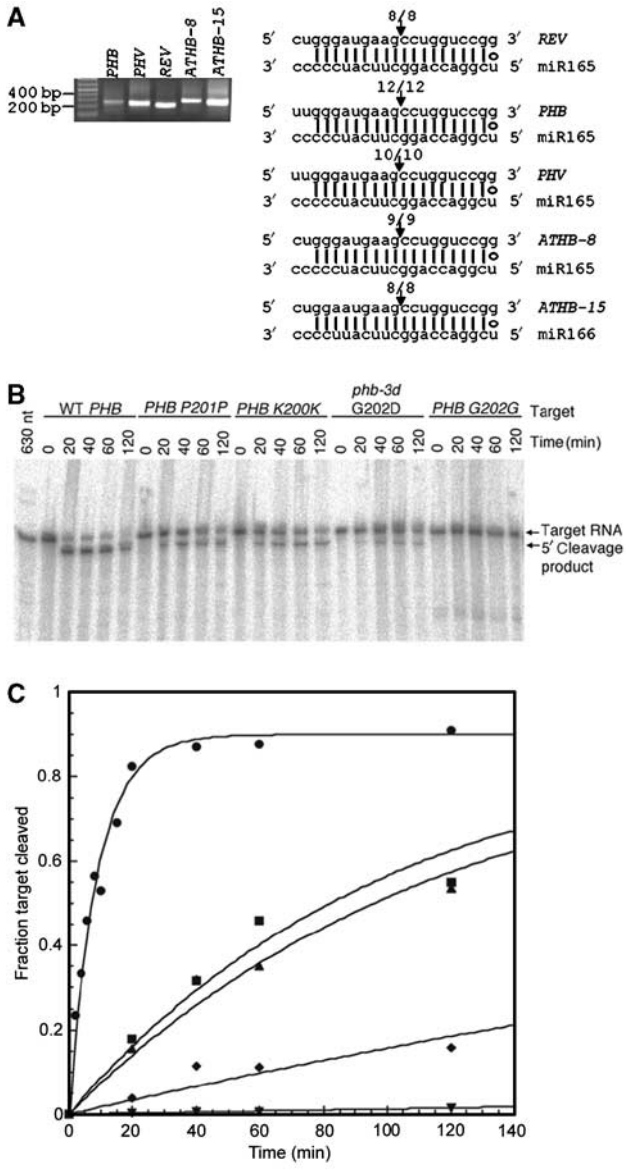

Figure 3 Point substitutions within the *PHB* miR165/166 complementary site have different effects on mRNA cleavage rates. (**A**) miR165/166 cleavage sites in *PHB*, *PHV*, *REV*, *ATHB-8*, and *ATHB-15* mRNAs determined by RNA ligase-mediated 5′-RACE. Agarose gel separation of the nested PCR products that were cloned and sequenced is shown on the left. The frequency of 5′-RACE clones corresponding to each cleavage site (arrows) is indicated as a fraction, with the number of clones matching the target message in the denominator. (**B**) 5′-radiolabeled transcripts prepared from WT *PHB* and four mutant *PHB* constructs described in Figure 1 were introduced into wheat germ extracts, and the time course of cleavage was examined on a sequencing gel. (**C**) Quantification of the data in (B) (circles, WT *PHB*; squares, *PHB K200K*; triangles, *PHB P201P*; diamonds, *phb-3d* G202D; inverted triangles, *PHB* G202G).

phylogenetic RNA secondary structures (Kierzek *et al*, 1999), suggesting that they might have similar effects on helix stability and geometry.

The wheat germ analysis revealed that substitutions that disrupt base pairing in the 5′ half of the *PHB* miRNA complementary site reduce the cleavage efficiency no more than what was observed for *PHB* mutant RNAs that trigger only mild developmental defects when expressed in *Arabidopsis* (Figure 4A). Similarly, substitutions at the extreme 3′ end of the *PHB* complementary site did not dramatically alter the cleavage rate. Conversely, most substitutions between these two regions reduced cleavage rates to a degree equal to or exceeding that demonstrated to perturb WT *Arabidopsis* development substantially (Figure 4A). Detailed interpretation of the differential effects of mismatches with regard to their positions within the miR165/166 complementary site was confounded by the differing and largely unknown effects on helix stability and geometry of each mismatch type within the context of each combination of nearest-neighbor base pairs. Nonetheless, the clustering of the most severe effects at the 3′ region of the complementarity was striking, particularly when compared to the less severe effects of the same mismatches (although in the context of different nearest-neighbor pairs) in the central and 5′ regions of complementarity. We conclude that, on the whole, pairing to the 5′ portion of the miRNA (the 3′ portion of the complementary site) is most important for governing the specificity of miR165/166 regulation.

PHB naturally contains mismatches to miR165/166. To determine if the natural mismatches reduce cleavage efficiency, we compared the cleavage efficiency of a WT *PHB* transcript with that of transcripts containing sites that are perfectly complementary to either miR165 or miR166. There was no significant difference in the cleavage rates of these three transcripts (Figure 4A), indicating that the natural mismatches between miR165/166 and *PHB* do not compromise cleavage efficiency and suggesting that tolerance for mismatches at these positions serves to broaden the range of targets accessible to this miRNA.

The 5′ regions of plant miRNAs are most complementary to target mRNAs

To investigate whether the nucleotide pairing requirements observed for miR165/166 and *PHB* extend to additional miRNA-target pairs in *Arabidopsis*, we inspected the locations of mispaired nucleotides in other known miRNA:mRNA duplexes. For each of 49 confirmed miRNA targets of conserved miRNAs recently compiled (Jones-Rhoades and Bartel, 2004) and the two newly confirmed miRNA targets (Figure 3A), we identified the most complementary miRNA and counted the number of mismatched nucleotides, G:U pairs, and bulged nucleotides at each position relative to the 5′ end of the miRNA, normalizing the frequencies of mispairs such that each target family had equal weight. Nonpaired nucleotides were most common at the ends of the duplexes (positions 1, 2, 20, and 21) as well as at positions 14 and 15. Nonpaired nucleotides were most rare at segments 3–10 of the miRNA (Figure 4B), with segments 3–4 and 7–10 having no mismatches, and segments 3–4 and 9–10 having no mismatches, G:U wobble pairs, or bulges in any confirmed miRNA target. No preference was given for pairing to this region when these targets were predicted (Rhoades *et al*, 2002; Jones-Rhoades and Bartel, 2004). Therefore, analysis of 51 confirmed miRNA:mRNA duplexes indicates that pairing to the 5′ region of the miRNA is more important than pairing to the 3′ region of the miRNA. Whether, pairing to the 5′ region is generally more important than pairing to the central region is difficult to say, but it appears at least as important, implying that the importance of 5′ pairing observed in our mutagenic

studies of the miR165/166 complementary site in *PHB* extends more broadly to the other miRNA-target pairs in plants.

A similarity between animal and plant miRNAs

In animals, studies of the specificity of siRNA-guided mRNA cleavage and repression have primarily focused on the center of the complementarity, near the site of mRNA cleavage. These studies have revealed that in some cases a single purine:purine (A:A, G:G, or A:G), pyrimidine:pyrimidine (U:C), or pyrimidine:purine mismatch (U:G) within the central region of complementarity can severely compromise cleavage (Elbashir *et al*, 2001b; Brummelkamp *et al*, 2002; Ding *et al*, 2003; Miller *et al*, 2003). However, there are cases where a single purine:purine (G:G) or pyrimidine:purine (C:A) mismatch within the central region of complementarity only mildly affects cleavage (Boutla *et al*, 2001; Holen *et al*, 2002).

A study examining the effect of single-nucleotide mutations in all 21 positions of a short-hairpin RNA (shRNA) that targets the gag gene of HIV-1 indicates that nucleotide pairing between the mRNA target and both the central and 5′ regions of the shRNA is important for efficient RNAi (Pusch *et al*, 2003). The importance of pairing to the 5′ region of siRNAs was also suggested by siRNA–siRNA crosslinking experiments. Psoralen crosslinked siRNAs remained RNAi competent, suggesting that complete unwinding of siRNA duplexes was not required to mediate efficient RNAi, leading to the proposal that initial unwinding of the duplex siRNA from the 5′ end, relative to the antisense strand, was sufficient to allow efficient RNAi (Chiu and Rana, 2002, 2003). In addition, recent kenetic analyses indicate that pairing at the 5′ region of siRNAs contributes disproportionately to the binding energy of *Drosophila* RISC and the RNA target (Haley and Zamore, 2004).

Animal miRNA targets often do not pair to the central region of the miRNAs, and this has been used to explain the observation that animal miRNAs appear to more frequently repress productive translation rather than mediate mRNA cleavage. In plants, pairing to targets is often more extensive, and most of the microRNAs examined appear to trigger mRNA cleavage. Our data indicate that mismatches near the 3′ end of the miR165/166 complementary site rather than the 5′ region are the most disruptive to target cleavage. The importance of pairing to the 5′ sequences of metazoan miRNAs has been suspected since the observation that a target of the *lin-4* miRNA has 'core elements' of complementarity to the 5′ region of the miRNA (Wightman *et al*, 1993). Furthermore, the 5′ sequences of metazoan miRNAs are typically the most evolutionarily conserved and have been used to group animal miRNAs into families (Lai, 2002; Ambros *et al*, 2003; Lewis *et al*, 2003; Lim *et al*, 2003). This region of animal miRNAs is perfectly complementary to elements in *Drosophila* 3′ UTRs that are important for post-transcriptional repression of *Drosophila* messages, suggesting

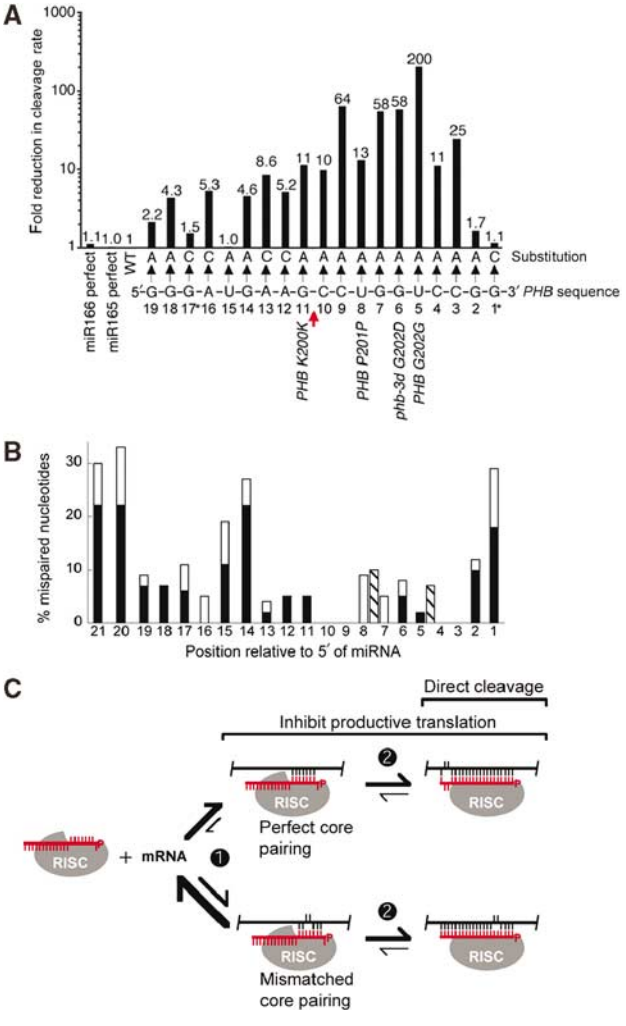

Figure 4 The importance of pairing between the 3′ region of the complementary site and the 5′ region of the miRNA. (**A**) Single-nucleotide substitutions were made throughout the length of the miRNA complementary site in *PHB* RNA and the resulting RNA mutant transcripts were introduced into wheat germ extracts. The sequence of the miR165/166 complementary site is indicated, with the introduced substitutions listed above and the corresponding positions of the miRNA listed below, numbered from the 5′ end of the miRNA. Cleavage rates were normalized to that of the WT *PHB* fragment (0.11 min^{-1}), and the fold reduction in cleavage rate is plotted. The positions where G–U mismatches occur between the WT *PHB* RNA and miR166 (*), the substitutions of the four 35S:*PHB* RNAs expressed in plants, and the site of miRNA-directed cleavage (red arrow) are noted. (**B**) Pairing between miRNAs and their experimentally confirmed targets. Shown are normalized frequencies of mismatched nucleotides (solid bars), G:U pairs (open bar), and bulged nucleotides (hatched bars) in validated miRNA:mRNA duplexes of *Arabidopsis*. Each miRNA complementary site was considered when paired to its most complementary miRNA, and the frequencies of mispairs from each target family were normalized so that each family had equal weight. Bulged mRNA nucleotides are counted as being between the miRNA nucleotides that pair to flanking mRNA nucleotides. No confirmed miRNA:mRNA duplexes are predicted to have bulged nucleotides in the miRNA. (**C**) A minimal two-step scheme that would explain the importance of pairing to the 5′ region of miRNAs and siRNAs in both animals and plants. The silencing RNA, with 5′-phosphate (P), is in red; mRNA segments are in black. In this speculative model, RISC presents only the 5′ region of the silencing RNA for duplex nucleation during initial target recognition (step 1). Once core pairing forms, the central and 3′ portions of the silencing RNA are allowed to pair with the message (step 2). The reduced residence time for mismatched core pairing (lower pathway) would favor sites with perfect core pairing (upper pathway), even if the second step were equally favorable for both classes of sites, and even if the overall stabilities of duplex formation for the naked RNAs were equivalent. In animals, simple association (step 1) is proposed to be sufficient for inhibiting productive translation, particularly if more than one RISC associates with the same message, whereas more extensive pairing (step 2) is proposed to be required for mRNA cleavage.

that these messages are under control of miRNAs and that pairing to the 5′ region of the miRNA is most crucial for miRNA recognition (Lai, 2002). Indeed, when searching for regulatory targets of mammalian miRNAs, demanding perfect pairing to nucleotides 2–8 of the miRNA is more useful than demanding pairing to any other heptanucleotide region of the miRNA (Lewis *et al*, 2003). In addition, cell culture reporter assays show that miRNA-like inhibition of productive translation depends most on pairing to the 5′ region of the small silencing RNA (Doench and Sharp, 2004). Therefore, although animal and plant miRNAs appear to most often regulate their targets using differing mechanisms, our data suggest an unexpected similarity between plant and animal miRNAs: for both, pairing to the 5′ region of the miRNA appears to be critical for function.

Why does complementarity between the 5′ end of the miRNA and the 3′ end of the target appear to be universally important, regardless of the mechanism of gene regulation? One possibility is that this sequence plays a primary role in target recognition by nucleating the pairing between the miRNA and its targeted message (Figure 4; Bartel, 2004). In this scenario, mismatches in this core region may inhibit initial recognition of the target, and thus prevent cleavage or translational repression regardless of the degree of complementarity elsewhere in the mRNA.

A limited number of nucleotide changes would lead to gain-of-function leaf radialization alleles

Phenotypic screens for leaf polarity mutants have been performed on *Arabidopsis* plants generated from seeds subjected to ethylmethane sulfonate (EMS) mutagenesis, which preferentially induces G-to-A (and the corresponding C-to-T) nucleotide transitions (Klungland *et al*, 1995). These mutant screens have identified two types of lesions in *PHB* and *PHV*: (1) a G-to-A splice-site mutation resulting in a 10- or 11-aa insertion, isolated twice in *PHB* and (2) a G-to-A point mutation at the 3′ end of the miRNA complementary site that creates a glycine to glutamate change (G202D), isolated three times in *PHB* and five times in *PHV*. If the phenotype observed in the *phb-d* plants simply reflects a disruption in miR165/166 pairing, the question arises as to why only two types of lesions in the miR165/166 complementary site have been repeatedly isolated in these mutant screens. There can clearly be hot spots of EMS mutagenesis in the genome, but our results suggest another explanation. It appears that mismatches at only four positions, all in the 3′ portion of the complementary site, disrupt miR165/166-mediated cleavage to a degree sufficient to have strong phenotypic consequences in *Arabidopsis* and radialize the leaf blade. Of these four, one causes the G202D mutation. Another (the site of the *PHB* G202G change) can be excluded because the WT nucleotide is a T (U in the mRNA), which would be refractory to EMS mutagenesis. The two remaining possibilities would produce nonconservative substitutions in the PHB protein: one would be a C-to-U transition that changes P201 to a leucine, and the other would be a G-to-A transition that would change G202 to serine. If these two nonconservative substitutions disrupt PHB function, they would not be isolated as *phb-d* alleles, because the dominant phenotypes associated with these alleles appear to involve the misexpression of a functional protein.

Mutations at other positions within the miR165/166 complementary site of *PHB* that are less disruptive to RNA cleavage may simply cause no change in phenotype or weaker phenotypes. Indeed, mutations have been isolated in other HD-ZIPIII proteins, which disrupt pairing to these positions and have less severe polarity defects. For example, a C-to-T transition in the *rev-10d* (*avb1*) substitution allele, which corresponds to a change at the second C of the P203 codon of *PHB*, causes mild adaxial–abaxial polarity defects in a subset of leaves as well as defects in vascular patterning (Emery *et al*, 2003; Zhong and Ye, 2004). Considering that multiple silent substitutions in the miRNA complementary site of *REV* do confer strong leaf radialization, the *rev-10d* vascular patterning defects could be interpreted as a more weakly dominant or less penetrant phenotype (Emery *et al*, 2003; Zhong and Ye, 2004). Consistent with this interpretation, a substitution at this position of *PHB* has an intermediate effect on RNA cleavage (although a different but possibly more disruptive mismatch was created—an A:G for *PHB* rather than a U:G for *REV*; Figure 4A). Mutations at other positions in the miR165/166 complementary site that are predicted to have even lesser effects on mRNA cleavage might also give weaker phenotypes, such as the leaf curling caused by our K200K and P201P silent substitutions. Indeed, a point mutation in the 5′ end of the miR165/166 complementary site (position 18 relative to the 5′ end of miR165/166) of the maize HD-ZIPIII family member *rolled leaf1*, a homolog of *Arabidopsis REV*, causes leaf curling due to adaxial sectors that arise adjacent to the midvein in older leaf primordia, whereas development of early leaf primordia is unaffected. The ability of the *in vitro* cleavage data (Figure 4A) to explain both the lack of diversity among *phb-d* alleles isolated in leaf radialization screens and the more mild phenotypes of the *rev-10d* and *RLD1-O* alleles supports the idea that the relative differences in cleavage rates observed *in vitro* have relevance *in vivo*. Furthermore, the comparative sequence analysis (Figure 4B) suggests that mismatches at analogous positions within the miRNA complementary sites of other miRNA targets could have consequences on cleavage similar to those seen for miR165/166 and *PHB*, implying that altering the position of silent mutations within other miRNA-target pairs could be a useful strategy for tuning the severity of the resulting phenotypes.

Materials and methods

PHB mutant constructs

Point mutations in the microRNA complementary site of the *PHB* cDNA expressed from the CAMV 35S promoter were created in pJM2 with the Quik-Change XL Site-Directed Mutagenesis Kit (Stratagene) and confirmed by sequencing. The 35S:*phb-1d* construct was pMKB2 (McConnell *et al*, 2001).

Growth and phenotypic analysis of transgenic plants

Plants were transformed with *Agrobacterium* strain GV3101 carrying the plasmid of interest using the floral dip method (Clough and Bent, 1998). For selection, seeds were sterilized and plated on MS medium containing 25 μg/ml hygromycin and 100 μg/ml carbenicillin. The exception was *35S:phb-1d* seeds, which were plated to MS medium containing 50 μg/ml kanamycin and 100 μg/ml carbenicillin. Transgenic plants were grown in soil at 20°C with 9 h of daylight for 2–3 weeks before examination for characteristics of abaxial to adaxial transformation: adaxial radialization, dark green patches of epidermis with wax on the lower surface of the leaf, and ectopic meristems forming at the base of the first true

leaves. The first and second leaves are at right angles to the cotyledons and, therefore, have no subtending leaves from which axillary meristems could normally be generated.

PHB transcript preparation and in vitro RNA cleavage assays

Arabidopsis PHB transcripts containing the miR165/166 complementary site were generated by PCR from *PHB* cDNA template (primers: 5′-GGCCGTAATACGACTCACTATAGGGGTCCATTCGATGA GCAGAG and 5′-CGCGAAATAGCGACTATGC) followed by T7 polymerase *in vitro* transcription. The PHB mutant templates were generated using the 5′ PHB primer and modified 3′ primers that introduced the desired nucleotide changes. Cap labeling, *in vitro* cleavage assays, and wheat germ lysate preparations were as described (Tang *et al*, 2003).

Mapping the site of miRNA-directed cleavage

5′-RACE was performed as previously described (Mallory *et al*, 2004) using the following primers:

*PHB*_1	5′-CACCAGTTGCAGAAGTAAGCGACC;
*PHB*_2	5′-GCTAAAGTCGTAGGAGCATACATCTGCG;
*PHV*_1	5′-GTACTATATCTCAGCGTCC;
*PHV*_2	5′-CTGAGTGTTGACAAGCTCGA;
*REV*_1	5′-GCCAGAGTCGTTGGTGCATACG;
*REV*_2	5′-CTCGATTGTGCCACCATTACCAG;
*ATHB-8*_1	5′-GTATTGTTCAGTGATCGTTCGCATATCAC;
*ATHB-8*_2	5′-GATGTGTAACGTAGCATCCAG;
*ATHB-15*_1	5′-CGTAACAGCCAGAAATCGCGTGGTGG;
*ATHB-15*_2	5′-CCAATGTGTTGGTGCATAGAGCTGCA.

RT–PCR

Total RNA was prepared from aerial tissues of plants grown in long-day conditions (16 h light, 8 h dark) as described (Mallory *et al*, 2004). A 4 μg portion of total RNA was used for oligo-dT-primed first-strand cDNA synthesis followed by RNaseH digestion (ThermoScript RT system, Invitrogen). PCR amplification using 50 ng of cDNA as template was performed using primers that flanked the miR165/166 complementary site, to selectively amplify only the uncleaved mRNA (primers 5′-GAACCGCAGATGTCGTGA GAAG and 5′-CACCAGTTGCAGAAGTAAGCGACC). The primers are located in different exons to discriminate between genomic contamination and products amplified from cDNA. PCR amplifications were performed for 26 cycles, which was determined to be in the dynamic range of the assay. Amplification of *ACTIN2* served as the control (primers: 5′-GCACCCTGTTCTTCTTACCG and 5′-AACC CTCGTAGATTGGCACA).

Analysis of positions of miRNA:mRNA mispairs

For a set of 51 experimentally validated miRNA targets, the positions of all mispairs (mismatches, G:U pairs, and insertions) were counted when paired to the best-pairing miRNA. The normalized frequencies of mispairs at each position, relative to the 5′ end of the miRNA, were calculated such that each miRNA family had equal weighting, regardless of the number of validated targets for each family.

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