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Radial Patterning of *Arabidopsis* Shoots by Class III HD-ZIP and KANADI Genes

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Summary

Background: Shoots of all land plants have a radial pattern that can be considered to have an adaxial (central)-abaxial (peripheral) polarity. In *Arabidopsis*, gain-of-function alleles of *PHAVOLUTA* and *PHABULOSA*, members of the class III HD-ZIP gene family, result in adaxialization of lateral organs. Conversely, loss-of-function alleles of the KANADI genes cause an adaxialization of lateral organs. Thus, the class III HD-ZIP and KANADI genes comprise a genetic system that patterns abaxial-adaxial polarity in lateral organs produced from the apical meristem.

Results: We show that gain-of-function alleles of *REVOLUTA*, another member of the class III HD-ZIP gene family, are characterized by adaxialized lateral organs and alterations in the radial patterning of vascular bundles in the stem. The gain-of-function phenotype can be obtained by changing only the *REVOLUTA* mRNA sequence and without changing the protein sequence; this finding indicates that this phenotype is likely mediated through an interference with microRNA binding. Loss of KANADI activity results in similar alterations in vascular patterning as compared to *REVOLUTA* gain-of-function alleles. Simultaneous loss-of-function of *PHABULOSA*, *PHAVOLUTA*, and *REVOLUTA* abaxializes cotyledons, abolishes the formation of the primary apical meristem, and in severe cases, eliminates bilateral symmetry; these phenotypes implicate these three genes in radial patterning of both embryonic and post-embryonic growth.

Conclusions: Based on complementary vascular and leaf phenotypes of class III HD-ZIP and KANADI mutants, we propose that a common genetic program dependent upon miRNAs governs adaxial-abaxial patterning of leaves and radial patterning of stems in the angiosperm shoot. This finding implies that a common patterning mechanism is shared between apical and vascular meristems.

Introduction

Lateral organs of seed plants, such as leaves and floral organs, are usually polar. As lateral organs are derived from the flanks of apical meristems, there exists an inherent positional relationship between them – the adaxial side of the lateral organ primordia is adjacent to the meristem, and the abaxial side is at a distance from it. Initial establishment of polarity in lateral organs requires communication from the apical meristem, with, in a simple scenario, a signal emanating from the apical meristem inducing adaxial fates in cells of lateral organs in closest proximity to the meristem [1]. The class III HD-ZIP genes *REVOLUTA* (*REV*), *PHABULOSA* (*PHB*), and *PHAVOLUTA* (*PHV*) exhibit similar mRNA expression patterns in apical and floral meristems, vasculature, and the adaxial domains of lateral organ primordia [2, 3]. The expression patterns of these genes in the lateral organs are complemented by the abaxial expression patterns of genes of the KANADI and YABBY families [4–7]. It has been hypothesized that complementary regions, perhaps based on mutual antagonism, of action of the class III HD-ZIP genes and KANADI genes leads to the establishment of adaxial and abaxial domains in developing lateral organs [2, 6, 7] and that their juxtaposition leads to lamina expansion [8]. Consistent with this hypothesis, gain-of-function alleles of *PHB* and *PHV* result in an adaxialization of lateral organs [2, 9] and a loss of YABBY gene activity [4]. Conversely, loss-of-function alleles of KANADI lead to a loss of abaxial tissues and an expansion of *REV*, *PHB*, and *PHV* expression [6, 10], and ectopic, uniform expression of KANADI throughout leaves results in an abaxialization of the organs [6, 7].

Five class III HD-ZIP genes exist in the *Arabidopsis* genome: *REV*, *PHB*, and *PHV* comprise a clade, with *PHB* and *PHV* as a sister pair, and *ATHB8* and *ATHB15* form a separate clade (Figure 1). *PHB*, *PHV*, and *REV* are expressed in the adaxial domains of lateral organs, in vascular tissues, and in the apical meristem [2, 3], while *ATHB8* and *ATHB15* appear to be expressed exclusively in the vascular tissues ([11], J.F.E. and J.L.B., unpublished data). Loss-of-function *rev* alleles exhibit aberrant axillary and flower meristem formation and alterations in the position of interfascicular fibers in the stem [3, 12–14], while loss of *ATHB8* activity results in no aberrant phenotype [15]. However, due to the extensive overlap in expression patterns, loss-of-function alleles of single genes are unlikely to reveal the full extent of gene function. The gain-of-function alleles of *PHB* and *PHV*, which exhibit dramatic adaxialization of lateral organs [2, 9], have single-nucleotide substitutions, or small insertions due to altered splicing, near the amino end of the START domain, a domain hypothesized to bind a steroid-like ligand [16]. This has led to speculation that the gain-of-function phenotypes might be due to altered ligand perception, and that PHB/PHV could act as a receptor for an adaxializing signal emanating from the apical meristem [2]. However, the recent identification of miRNAs complementary to this region of the START domain suggests that the dominant gain-of-function phenotypes may be due to altered miRNA binding [17–19].

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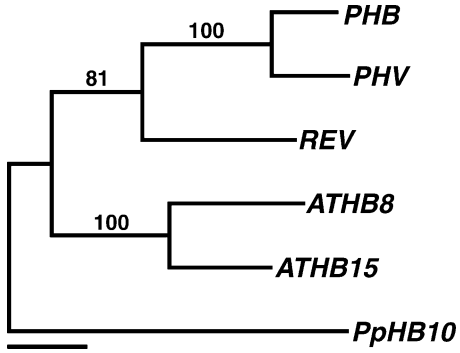

Figure 1. Phylogram of the Class III HD-ZIP Gene Family Members of *Arabidopsis*

The tree was rooted by using a class III HD-ZIP sequence from *Physcomitrella patens*, *PpHB10* [30]. The scale bar represents 50 changes, and the numbers at the nodes represent bootstrap support for the given relationships.

Results

Gain-of-Function *REV* Mutations

A semidominant gain-of-function *REV* allele, *rev-10d*, was identified in a genetic screen based on its enhancement of the *kanadi1-2* mutant phenotype. The most striking phenotype of plants carrying the *rev-10d* allele (in both the heterozyogous and homozygous state) is an alteration in vascular patterning. Stem vascular bundles in *rev-10d* plants display a radialized amphivasal pattern (Figures 2C and 2D), with xylem surrounding phloem, in contrast to the polarized wild-type collateral structure consisting of central (adaxial) xylem and peripheral (abaxial) phloem (Figures 2A and 2B). Additionally, vascular bundles may be located more centrally within the stem as compared to those in the wild-type. However, not all vascular bundles are radialized, with those near a leaf trace tending to be less radialized than those that are located more centrally. In *rev-10d* plants, decurrent strands of leaf tissue that are attached to the stem and are subtending and continuous with cauline (stem) leaves are often observed (Figure 2F). This tissue is usually associated with bending of the stem, apparently due to differential growth rates between the stem and the decurrent leaf tissue. However, the adaxial-abaxial polarity of leaves and floral organs is not noticeably affected in *rev-10d* mutants. Based on phenotype and map position, a previously identified mutant, *amphivasal bundles* [20], is likely a gain-of-function *rev* allele.

KANADI and class III HD-ZIP genes exhibit complementary expression patterns in the vasculature as well as in leaves. KANADI expression is restricted to the developing phloem, positioned abaxially (Figures 2O–2Q), and class III HD-ZIP expression is limited to the developing xylem, positioned adaxially [11, 21, 22]. While *KAN2* and *KAN3* are expressed in developing phloem throughout the plant, *KAN1* expression in the phloem is largely limited to the root. These complementary expression patterns have a functional significance since loss-of-function KANADI mutants exhibit stem vascular patterning defects indistinguishable from the *REV* gain-of-function phenotypes (Figures 2M and 2N). While radialized vascular bundles in *phb-1d* leaves could be viewed as a consequence of radialization of the leaves [2, 8], the altered vascular patterning in the stems of *rev-10d* and *kan1-2 kan2-1 kan3-1* plants indicates a direct role for KANADI and class III HD-ZIP genes in vascular patterning.

microRNA Regulation

The semidominant gain-of-function mutations in *PHB* and *PHV* map to single amino acid changes in a short region near the amino terminus of the START domain. The presence of these mutations led to the hypothesis that they disrupt ligand binding through this domain or abolish the need for such binding [2]. The *rev-10d* allele was also found to contain a single point mutation near the beginning of the fifth exon, a C to T transversion, which causes an amino acid substitution, P190L, just carboxyl to the most common gain-of-function mutations recovered for *PHB* and *PHV* (Figure 3).

In the course of this investigation, elegant work by others [17, 18] demonstrated the existence of two microRNAs, MIR165 and MIR166, with nearly complete complementarity to the START coding region of class III HD-ZIP mRNAs and overlapping the sites of mutations giving rise to gain-of-function phenotypes in *PHB*, *PHV*, and *REV* (Figure 3). This discovery suggests that these genes may be regulated by microRNAs and that the gain-of-function phenotypes described may be due to a disruption of this regulation, rather than to changes in the protein products [18, 19]. Accordingly, we generated a *REV* cDNA, *rev-δmiRNA*, containing two nucleotide substitutions in the region complementary to MIR165/166 (Figure 3). These changed the complementarity to the microRNAs but did not change the amino acid sequence of the protein product produced by translation. This cDNA was placed under the *REV* promoter, which includes a 7 kb region upstream of the *REV* coding sequence. When introduced into wild-type plants, a phenotype similar to that of the *rev-10d* plants was observed (Figures 2G–2L). Control plants transformed with a wild-type *REV* cDNA under the control of the same promoter showed a wild-type phenotype, indicating that the gain-of-function phenotype is due to the change in mRNA sequence, not to an increase in *REV* mRNA or protein levels. In plant systems, cleavage of target mRNAs by miRNA- and DICER-mediated cleavage has been observed for some genes [19, 23]. Consistent with miRNA-mediated regulation of *REV*, in 5′ RACE experiments, a 3′ cleavage product was detected (data not shown). The cleavage site is identical to that identified by in vitro cleavage of *PHAVOLUTA* mRNA [19].

Loss-of-Function Alleles

Homozygous loss-of-function *rev* plants often fail to generate axillary meristems and exhibit a loss of floral organs [12]. In addition, leaves curl under both distally and laterally, but there are no conspicuous effects on adaxial-abaxial leaf polarity [12]. Since *REV*, *PHB*, and *PHV* exhibit overlapping expression patterns in the apical meristem and vasculature in addition to the adaxial

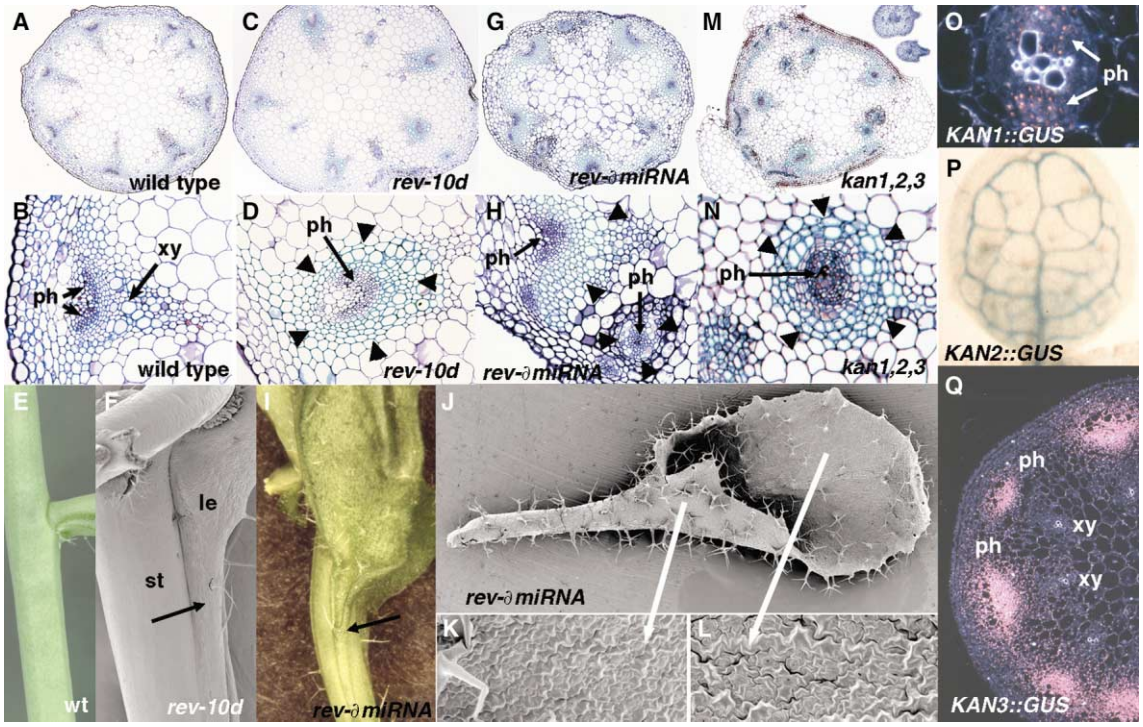

Figure 2. Phenotypic Alterations in Vascular Patterning in *rev-10d*, *rev-δmiRNA*, and *kan1 kan2 kan3* Plants

(A and B) Wild-type vascular bundles in the stem have (A) xylem (xy) located centrally and (B) phloem (ph) strands located peripherally.

(C and D) In *rev-10d* stems, vascular bundles are often radialized and amphivasal, with xylem tissue (arrowheads) surrounding phloem tissue (ph).

(E and F) Ectopic leaf tissue (le, arrow) is fused to the stem (st) in *rev-10d* plants (F); compare with wild-type (E). Phenotypic alterations in *rev-δmiRNA* plants resemble those of *rev-10d* plants.

(G and H) The stem vascular bundles of *rev-δmiRNA* plants are often radialized and amphivasal, with xylem (arrowheads) surrounding phloem (ph). Vascular bundles close to leaf traces are usually not radialized, but are rather more horseshoe shaped.

(I) Ectopic leaf tissue (arrow) is often fused to the stem.

(J–L) In addition, trumpet-shaped leaves may develop (J); adaxial epidermis is present on the (K) outside and abaxial epidermis is present on the (L) inside of the trumpet. This pattern is reminiscent of the trumpet-shaped leaves in *PHB* and *PHV* gain-of-function mutants [2, 9].

(M and N) Vascular bundles in *kan1-2 kan2-1 kan3-1* stems also exhibit an amphivasal pattern, with xylem (arrowheads) surrounding phloem (ph).

(O–Q) (O) *KAN1*, (P) *KAN2*, and (Q) *KAN3* are expressed in the vasculature and localize to the developing phloem. Shown here are *KAN1* in the root, *KAN2* in the vasculature of a leaf, and *KAN3* in the stem.

domain of lateral organs [2] (Figure 4), we examined the phenotypes of plants compromised in the function of all three genes. Plants homozygous for loss-of-function alleles, *phb-6* and *phv-5*, exhibit phenotypes indistinguishable from that of wild-type. However, plants (*phb-6 phv-5 rev-9*) homozygous for loss-of-function alleles of all three genes exhibit a dramatic phenotype. Such plants lack an apical meristem (Figure 5C), and in the most severe manifestation, they produce only a single radial, abaxialized cotyledon with no apparent bilateral symmetry (Figure 5A). Consistent with a role for these genes in apical patterning of the embryo, expression of *REV* commences as early as the 16-cell stage of embryogenesis (Figure 4A), and *PHB* is also expressed at this stage [2]. Initial expression appears throughout the upper half of the embryo proper but subsequently becomes localized to the apical central region by the late globular stage (Figures 4B and 4C). As cotyledons emerge, expression is restricted to their adaxial regions and to the developing provasculature in the hypocotyl (Figures 4C and 4D). Postembryonically, *REV* is expressed in all apical meristems, flower meristems, and developing vasculature, and it is expressed adaxially in lateral organs (Figure 4E) [3]. *PHV* expression commences slightly later than *REV* and *PHB* and is detected in the apical region of globular embryos (Figures 4F and 4G) and is later localized to the adaxial regions of the cotyledons (Figure 4H). Both the expression patterns and loss-of-function phenotype are consistent with these genes acting early in the genetic hierarchy that patterns the apical region of the embryo and includes the establishment of bilateral symmetry [24, 25]. In less severely affected plants, two, usually radialized, cotyledons are produced (Figure 5B), a phenotype similar to that observed when KANADI genes are uniformly ectopically expressed [6, 7]. The vasculature in the radialized cotyledons is also radialized, with phloem surrounding the xylem; this is consistent with an abaxialization of the cotyledons (Figure 5D).

When *KAN1* is expressed throughout flower meristems, development is arrested (Figure 5E). Indeed, ectopic expression of KANADI in any apical or flower meri-

REVOLUTA

wild-type	CCT	GGG	ATG	AAG	CCT	GGT	CCG	GAT	TCG	GTT	GGC	ATC	TTT	GCC...
	P	G	M	K	P	G	P	D	S	V	G	I	F	
rev-10d	CCT	GGG	ATG	AAG	CCT	GGT	CTG	GAT	TCG	GTT	GGC	ATC	TTT	GCC...
	P	G	M	K	P	G	L	D	S	V	G	I	F	A ...
phb-3d,4d,5d	CCT	GGG	ATG	AAG	CCT	GAT	CCG	GAT	TCG	GTT	GGC	ATC	TTT	GCC...
phv-1d,2d,3d,4d	P	G	M	K	P	D	P	D	S	V	G	I	F	A ...
rev-δmiRNA	CCT	GGG	ATG	AAG	CCT	GGA	CCA	GAT	TCG	GTT	GGC	ATC	TTT	GCC...
	P	G	M	K	P	G	P	D	S	V	G	I	F	

Figure 3. Nucleotide and Amino Acid Sequences Spanning the MIR165/166 Binding Site in *REV* Alleles

Blue nucleotides denote those complementary to MIR165; pink nucleotides represent those altered in *rev* (or *phb*/*phv*) mutant alleles. Red amino acids represent those altered in *phb*, *phv*, and *rev* mutant alleles.

stem leads to its arrest (data not shown). These results are consistent with the idea that KANADI activity is antagonistic to meristem function through its interactions with class III HD-ZIP genes.

Discussion

The class III HD-ZIP genes direct the development of at least three tissues, the adaxial domains of lateral organs, the apical meristem, and the vascular bundles, and in each case, the activity of the class III HD-ZIP genes is opposed by the antagonistic activity of the KANADI genes. Based on gain-of-function alleles, *PHB* and *PHV* are most important for patterning in lateral organs, whereas *REV* is more important for vascular pattering. All three genes contribute to the establishment of a functional apical meristem and to adaxial tissues in lateral organs. The other two family members, *ATHB8* and *ATHB15*, also likely direct vascular development, although their precise roles are not yet known. Thus, the five class III HD-ZIP genes in *Arabidopsis* have diversified and have both common and unique functions.

Our results demonstrate that, at least for *REV*, the gain-of-function phenotype can be produced at the level of the mRNA sequence. Presumably, this is due to dis-

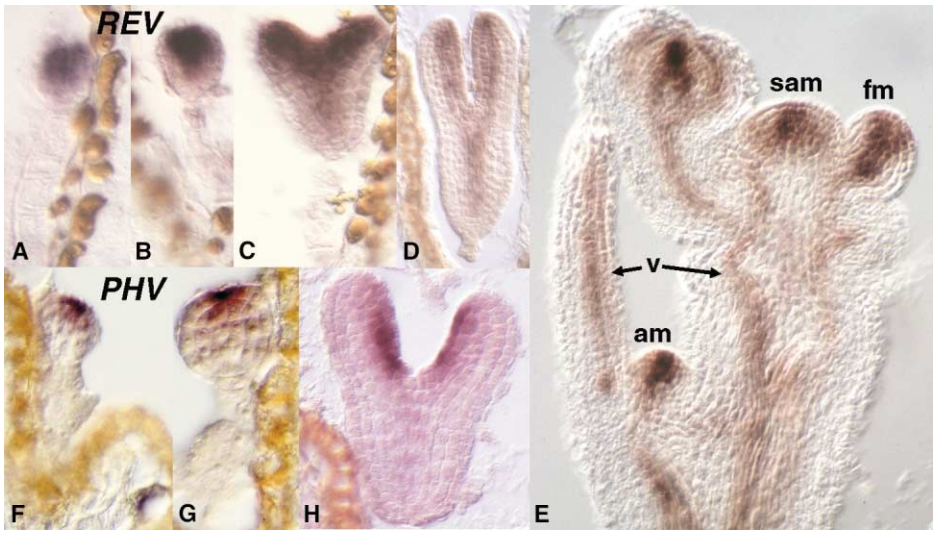

Figure 4. *REV* and *PHV* Expression Patterns

(A and B) *REV* is expressed in the apical region of globular embryos, and expression commences as early as the (A) 16-cell stage and becomes restricted to the central apical region of (B) late globular embryos.

(C and D) As the cotyledons emerge, *REV* is limited to their adaxial regions as well as to the central provascular tissues.

(E) Postgermination, *REV* is expressed in apical (sam), axillary (am), and floral (fm) meristems, adaxial domains of lateral organs, and the vasculature (v).

(F–H) *PHV* expression is similar to, but more spatially restricted than, that of *PHB* and *REV*. *PHV* is detected in the apical central region of globular embryos (F and G) and becomes localized to the adaxial regions of cotyledons, with low levels of expression in the provascular tissues (H).

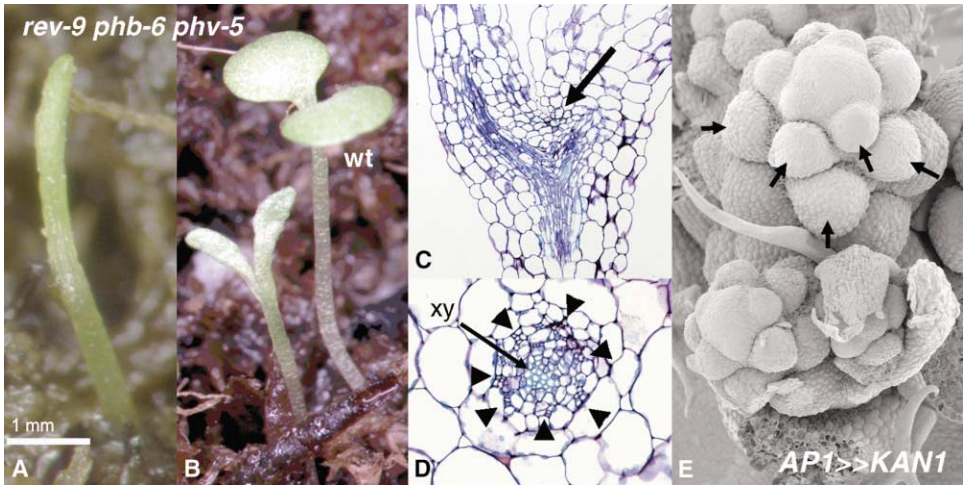

Figure 5. *phb phv rev* Triple Loss-of-Function Phenotypes

(A and B) In the most severe manifestation, the above-ground portion of *phb-6 phv-5 rev-9* plants consists only of a single abaxialized radial cotyledon and the hypocotyl ([A], compare with wild-type seedling in [B]). In less severely affected plants, bilateral symmetry is evident, and cotyledons may display some lamina expansion (B).

(C) All *phb-6 phv-5 rev-9* plants lack evidence of an apical meristem (arrow).

(D) In the radialized cotyledons, the vascular bundles are also radialized, with phloem tissue (arrowheads) surrounding xylem tissue (xy).

(E) Expression of *KAN1* in the flower meristem (arrows) results in the arrest of meristematic activity.

In this case, *KAN1* is being expressed under control of the *AP1* promoter through a transactivation system [31].

ruption of negative regulation by MIR165/166 [19]. In this scenario, the gain-of-function phenotypes are due to both increased levels of transcript and a broader expression domain [2]. A loss of miRNA-mediated negative regulation could account for both the spatial expansion and the increase in expression levels, although positive autoregulation cannot be discounted [2]. However, our data do not preclude a role for a steroid-like ligand acting through the START domain of the protein, as this domain is highly conserved among each of the five *Arabidopsis* class III HD-ZIP genes, and a single relatively conservative amino acid change within this domain in *REV* results in a dramatic loss-of-function phenotype [3, 26].

The role of the class III HD-ZIP/KANADI genetic program is to generate or interpret radial polarity in both the leaves [2, 6, 7] and stems of the shoot. In the case of the stem, class III HD-ZIP activity is required to maintain a central (adaxial) meristem, with KANADI activity promoting differentiation of abaxial (peripheral) tissues, at least with respect to tissues produced from the vascular cambium. We favor a model in which a centrally produced steroid-like ligand serves to activate PHB, PHV, and REV in the central regions of stems and the adaxial regions of lateral organs, with KANADI activity restricting *PHB*, *PHV*, and *REV* expression from abaxial regions of these tissues (Figure 6). Based on both genetic and molecular data, the activities of members of these two genes families may act mutually antagonistically. For example, gain-of-function alleles of KANADI result in an abaxialization of lateral organs and a loss of meristem development [6, 7], similar to the *phb phb rev* loss-of-function phenotype. Conversely, gain-of-function alleles of class III HD-ZIP genes result in an

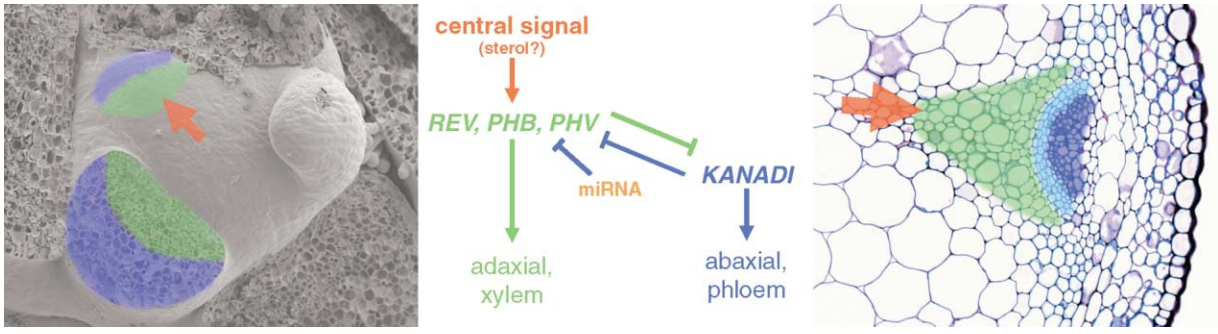

Figure 6. Model of How Class III HD-ZIP and KANADI Activities Pattern Lateral Organs and Vasculature

A centrally derived signal (red) activates class III HD-ZIP genes, whose activity is antagonistic with that of KANADI activity. Both KANADI and MIR165/166 negatively regulate class III HD-ZIP genes, although the relationship between the two is not presently known. In lateral organs, class III HD-ZIP activity promotes adaxial fates and KANADI activity promotes abaxial fates, whereas in the vascular bundles, interactions between the two gene classes pattern the arrangement of xylem and phloem tissues. While the vascular bundle shown is already differentiated, the initial patterning events likely occur just below the apical meristem, where provascular cells are being specified.

adaxialization of lateral organs (*phb-1d*) [9] and a radialization of vascular bundles (*rev-10d*), similar to the phenotype observed in *kan1 kan2 kan3* plants (Figure 2 and A.I. and J.L.B., unpublished data). At the molecular level, loss of KANADI activity results in an expansion of class III HD-ZIP gene expression [6], and, conversely, KANADI expression is greatly reduced in a *phb-1d* background (Eyal Blum and Y.E., unpublished data). While both KANADI and MIR165/166 activities are thought to antagonize class III HD-ZIP activity, their relative relationship is not presently clear. Since the relative positions of lateral organs and vascular bundles with respect to the apical meristem are similar, the source for the putative polarizing signal could be shared. Perhaps most significantly, the same genetic program is used to radially pattern tissues derived from the apical meristem and the vascular procambium (meristem); this finding suggests that these meristems function in a similar manner mechanistically. In this model, the class III HD-ZIP genes are required for meristem, apical and vascular, formation; antagonistic interactions with KANADI activity radially pattern the tissues derived from the respective meristems.

Based on the phenotype of *kan1 kan2 kan3* plants, it is not clear whether the primary role of KANADI in the stem may be to exclude the activity of the class III HD-ZIPs, rather than the specification of cell types per se. In the leaves, KANADI activity is required for the proper specification of abaxial cell types, and loss of KANADI activity leads to loss of abaxial cell types [6, 7]. Conversely, ectopic expression of KANADI results in ectopic differentiation of abaxial cells with a concomitant loss of adaxial cell types in the leaf. The loss of tissues can be dose dependent, with partially adaxialized organs having adaxial tissues surrounding abaxial ones. In contrast, in the stem, the peripheral tissues (e.g., phloem) are not lost in *kan1 kan2 kan3* plants, and the position and pattern of the vascular tissues are the only things that are conspicuously altered. These results suggest that either KANADI activity does not specify peripheral cell types directly, or that it is redundant with other specification activities.

Since *ATHB8/15* are likely still active in a *phb phv rev* background, it is not yet clear what phenotype would result if all Class III HD-ZIP gene activity were eliminated. However, it is tempting to speculate that these genes may specify provascular initials [15], and that subsequent antagonistic interactions with KANADI genes pattern the arrangement of cell types within the bundles. Consistent with this hypothesis is the observation that uniform expression of KANADI throughout lateral organs results in a complete loss of vascular development in these organs [6]. Furthermore, as the evolution of vasculature preceded that of seed plant leaves [27], the ancestral function of the class III HD-ZIP/KANADI genetic program may have been in central/peripheral spatial patterning in the vasculature in early land plants and may have subsequently been co-opted to the role of adaxial/abaxial pattering in leaves.

Experimental Procedures

All plants were Landsberg *erecta* (Ler), except where noted. All plant transformations were accomplished by using the BART binary vector [6] in *Agrobacterium* strain ASE.

The *rev-10d* mutant P190L was isolated in a screen of M2 plants. M1 seeds were subjected to mutagenesis with 0.175% ethylmethane sulfonate for 12 hr. M2 seeds from roughly 4,000 M1 plants were screened. *rev-9*, a loss-of-function *rev* allele (E428), was isolated from a T-DNA enhancer trap screen [10]. *phb-6* (SGT6404) and *phv-5* (SGT11109) were isolated from a Ds transposon insertion mutagenesis [28]. The *phb-6 phv-5 rev-9* triple mutant was identified in a population segregating all three alleles by first identifying plants homozygous for both *phb* and *phv* based on PCR analysis of their respective Ds insertions; then, plants segregating *rev* were identified by Basta resistance. The novel triple mutant phenotype was only observed when all three mutations were segregating. Segregation in the progeny of plants derived from a self of *phb phv rev*/+ plants resulted in 152 plants with two normal cotyledons, 13 monocot-type plants (Figure 5A), and 11 plants with two radialized cotyledons (Figure 5B). The plants with two normal cotyledons segregated approximately 2:1 (104:48) for BASTA resistance, and this ratio is indicative of segregation of the *rev-10* allele. That less than one-quarter of the plants exhibited an aberrant cotyledon phenotype suggests that compromised activity of all three genes results in some lethality. The *kan3-1* allele was isolated from a T-DNA knockout population at the University of Wisconsin Biotechnology Center [29]. The *kan1-2* and *kan2-1* alleles have been described previously [6].

A wild-type *REV* cDNA was isolated from Ler inflorescence cDNA by using primers of sequences 5′-CAGAGACACCTAAACAACAACC-3′ and 5′-AGACTTTTTTTGAGGGTCGAGC-3′. The *rev-G189D* construct was generated from this cDNA clone by using the Stratagene Quikchange technique with two primers of sequence 5′-GGGAT GAAGCCTGATCCGGATTCGGTTGGC-3′ and its exact complement. These primers create an AT pair in place of the wild-type GC pair in the second base of codon 189. They were used in an inverse PCR with Pfu polymerase (Stratagene), followed by DpnI digestion and transformation of *E. coli* to recover a *REV* cDNA containing the mutation in codon 189. The *rev-*δ*miRNA* construct was generated by using the same technique, by using primer 5′-GGGATGAAGCCT GGACCAGATTCGGTTGGC-3′ and its exact complement, which change codons 189 and 190 to alternate codons for the same amino acids. The *REV* promoter was obtained by PCR on Ler genomic DNA by using the primers 5′-CCGGGCCCAAAATATTTGGGTTATTGTAACA-3′ and 5′- CTGGATCCTTTAGCTCGACCCTCAAAAAAAG-3′, which generated a 7.1 kb product from the 5′ flank of the *REV* coding sequence.

The *KAN2::GUS* and *KAN3::GUS* marker lines were generated by fusing a 5.3 (*KAN2*) or 3.5 (*KAN3*) kilobase fragment 5′ to the ATG of *KAN2* or *KAN3*, respectively, (amplified from Colombia DNA) to the GUS gene in pRITAI [10]. The NotI fragment of this plasmid was introduced into the binary vector pMLBART [10]. The *rev-9* allele was generated by a T-DNA insertion with a GUS gene with a minimal promoter such that it acts as an enhancer trap. In *rev-9*, the T-DNA is located in the 5′ untranslated region of the *REV* gene such that GUS gene expression is driven by the endogenous *REV* promoter.

Total mRNA was extracted from vegetative and reproductive shoot tips by using Trizol reagent. 5′ RACE-ready cDNA was generated, and 5′ RACE was performed by using the SMART RACE kit (Clontech). Two gene-specific primers (gsp) were used to do a nested 5′ RACE for *REV*. The first, outer reaction utilized the SMART universal primer mix and the gsp 5′-GCAGGCTGCCTTCCTAATC CATACACT-3′. The second, inner reaction utilized the SMART-nested universal primer and the gsp 5′-CATTAGGCCCAGCTCCA GAGCCAGATA-3′. Two bands were cut from the gel and were TA cloned by using the TOPO TA cloning kit (Invitrogen). One band, about 1.5 kb, corresponded in length to the full 5′ end of the REV cDNA, and the other, just over 300 bases in length, matched the expected size of the 5′ end if it were cleaved within the miRNA binding site.

Basal portions of 10-week-old inflorescence stems of wild-type, *rev-10d*, *rev-*δ*miRNA*, and *kan1 kan2 kan3* plants, as well as whole seedlings of the *phb phv rev* genotype, were fixed in a solution of 1.5% glutaraldehyde, 1% paraformaldehyde, and 4% acrolein in PIPES buffer (84 mM PIPES, 8.4 mM EGTA, and 1.6 mM $MgSO_4$) at pH 6.8. Specimens were left in fixative a minimum of 24 hr, then rinsed in PIPES buffer and dehydrated through an ethanol series to 95% ethanol.

Specimens were then infiltrated with catalyzed monomer A of the JB-4 embedding kit (Polysciences) and were embedded in an

oxygen-free environment following the basic protocol provided with the kit. Blocks were serially sectioned at 4 μm on a Reichert-Jung 2050 (Leica) rotary microtome by using glass knives. Slides were stained in 0.1% toluidine blue, examined, and photographed on a Zeiss Axioskop microscope equipped with a Zeiss Axiocam digital camera by using bright-field microscopy.

Full-length sequences of the five class III HD-ZIP proteins and *PpHB10* from *Physcomitrella* [30] were manually aligned. Heuristic searches were performed by using PAUP4.0b. Of 755 included characters, 175 were phylogenetically informative, yielding a single most parsimonious tree of 793 steps.

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