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Establishment of polarity in lateral organs of plants

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Background: Asymmetric development of plant lateral organs initiates by partitioning of organ primordia into distinct domains along their adaxial/abaxial axis. A recent model proposes that a meristem-born signal, acting in a concentration-dependent manner, differentially activates *PHABULOSA*-like genes, which in turn suppress abaxial-promoting factors. As yet, no abaxial factors have been identified that when compromised give rise to adaxialized organs.

Results: Single mutants in either of the closely related genes *KANADI1* (*KAN1*) or *KANADI2* (*KAN2*) have little or no effect on plant morphology. However, in *kan1 kan2* double mutant plants, there is a replacement of abaxial cell types by adaxial ones in most lateral organs. The alterations in polarity establishment are associated with expansion in the expression domain of the PHB-like genes and reduction in the expression of the previously described abaxial-promoting *YABBY* genes. Ectopic expression of either of the *KANADI* genes throughout leaf primordia results in dramatic transformation of adaxial cell types into abaxial ones, failure of lateral blade expansion, and vascular tissue formation.

Conclusion: The phenotypes of *KANADI* loss- and gain-of-function alleles suggest that fine regulation of these genes is at the core of polarity establishment. As such, they are likely to be targets of the *PHB*-mediated meristem-born signaling that patterns lateral organ primordia. *PHB*-like genes and the abaxial-promoting *KANADI* and *YABBY* genes appear to be expressed throughout primordia anlagen before becoming confined to their corresponding domains as primordia arise. This suggests that the establishment of polarity in plant lateral organs occurs via mutual repression interactions between ab/ad factors after primordium emergence, consistent with the results of classical dissection experiments.

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Background

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 primordium is adjacent to the meristem and the abaxial side is at a distance from it. The fundamental positional relationship of leaves relative to the shoot apical meristem (SAM) was suggested to form the physiological basis for their asymmetric development [1]. This view was further supported by experiments in which the lateral organ primordia were separated from the apical meristem by incision [2, 3]. When young potato leaves were separated from the SAM, a small radial leaf was formed, suggesting that the meristem may act as a source for a signal required for proper polarity establishment in lateral organs [2]. Furthermore, the establishment of polarity is required for proper lamina development, with the juxtaposition of abaxial and adaxial domains responsible for induction of lamina outgrowth [4]. The end result in most plants is a laminar leaf with an adaxial (top) surface specialized for light capture and an abaxial (bottom) surface specialized for gas exchange.

Several key players involved in polarity establishment of lateral organs in plants have been shown to represent plant-specific molecules [5–9]. Semidominant gain-of-function mutations in the presumed lipid binding domain of either *PHABULOSA* (*PHB*) or *PHAVOLUTA* (*PHV*) result in transformation of abaxial tissues of lateral organs into adaxial ones [8, 10]. These adaxial-promoting genes are expressed in the meristem, throughout very young leaf primordia, with their expression becoming restricted to the adaxial sides of primordia as they become visibly distinct from the meristem. On the basis of their molecular structure, these proteins were proposed to translate meristem-born cues into repression of abaxial-promoting factors on the adaxial side of lateral organs [8]. In this scenario, specific missense mutations in the *PHB*/*PHV* sterol/lipid binding domain render the proteins constitutively active, such that abaxial factors are repressed throughout organ primordia irrespective of the signal gradient. Possible candidates for the suppressed abaxial-promoting factors are members of the YABBY gene family. *YABBY* genes are expressed on the abaxial side of all lateral organ primordia and are capable of inducing the differentiation of abaxial cell types when expressed adaxially [5–7]. How-

ever, simultaneous loss-of-function of two redundant *YABBY* genes, *FILAMENTOUS FLOWER* (*FIL*) and *YABBY3* (*YAB3*), does not result in a conspicuous gain of adaxial cell fates [5].

On the basis of the unique genetic interaction in *Arabidopsis* carpels between *CRABS CLAW* (*CRC*), the founding member of the YABBY gene family, and *KANADI1* (*KAN1*), we suggested the presence of a second abaxial-promoting pathway that overlaps with the function of the *YABBY* genes [7]. While numerous morphological differences differentiate the two sides of all lateral organs, carpels (the female floral organs that give rise to the fruit) provide a simple and sensitive organ to assay polarity. Placentae, bearing ovules, develop only internally (adaxially), and, therefore, loss of abaxial tissues and gain of adaxial ones results in formation of ectopic external ovules. Plants mutated for both *crc* and either *kan1* or *pickle* (*pkl* AKA *gymnos*) develop duplications of placentae in the medial regions of the abaxial sides (external) of their carpels. Since neither individual mutant displays these aberrations, such synergism suggests redundancy, either in a single pathway or in independent pathways. These observations led to the formulation of a model suggesting that distinct mechanisms promote polarity establishment of *Arabidopsis* carpels [7]. According to this model, *CRC* promotes abaxial cell fate in the carpels, but its role is masked either by other abaxial-promoting genes such as *KAN1* or by genes, such as *PKL*, that temporally restrict meristematic activities.

To elaborate and test this model, a screen for genetic enhancers of *pkl* and *kan1* was carried out in the *kan1-2 pkl-12* background. The primary goal of the screen was to determine whether *crc* is a unique genetic enhancer of *kan1* and *pkl*. Therefore, the screen for *kan1-2 pkl-12* enhancers focused on mutant loci exhibiting development of external ovules. We describe here two functionally redundant genes that act to promote abaxial cell fate in all cells of the carpels. Compromising the activity of these genes, *KANADI1* and *KANADI2*, leads to ectopic formation of adaxial cell types in abaxial positions of all lateral organs, in a manner reminiscent of *phb-1d* mutants. Conversely, ectopic expression of either gene is sufficient to transform asymmetric lateral organs, such as cotyledons or leaves, into radial, abaxialized structures. We propose that these genes, together with the previously described *YABBY* genes, are the abaxial-promoting factors that are negatively regulated by *PHB*/*PHV*.

Results

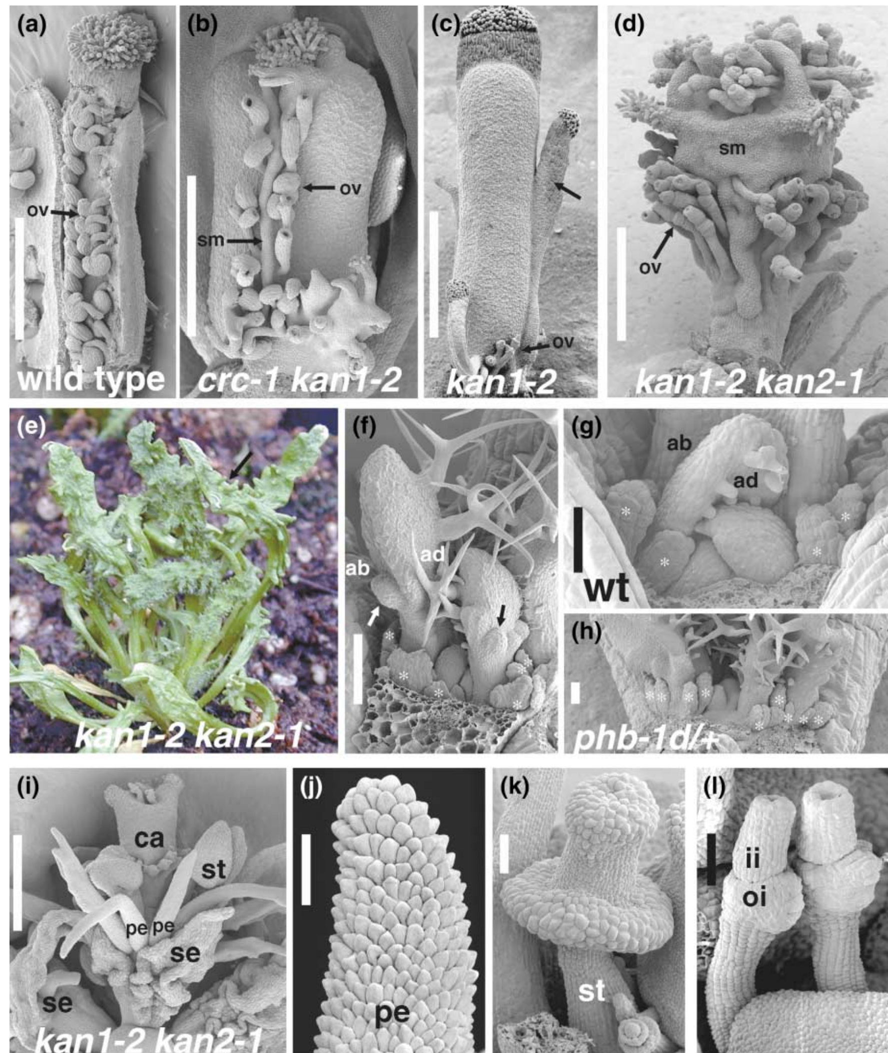
Genetic enhancers of *kanadi1* imply its role in the establishment of tissue polarity

Several enhancers with externally developing ovules were identified in a screen of 1200 M2 EMS mutagenized families in the *kan1-2 gym-12* background. The phenotype of each of the newly isolated enhancers was examined in wild-type (Figure 1a), *crc* (to determine possible allelism), *kan1-2* (Figure 1c), and *pkl-15* backgrounds. Four complementation groups were identified: *crc* (3 new alleles), *hasty* (*hst*, 3 new alleles), *splayed* (*syd*, 2 new alleles) and a new locus, *kanadi2* (*kan2*, 3 new alleles). Of these complementation groups, only *crc* interacted with *pkl* to produce external ovules, whereas the others depended solely on *kan1* being mutated for the production of ectopic ovules. None of the single mutants exhibited such properties, and while *syd* and *hst* have pleiotropic effects as previously described ([11]; Wagner and Meyerowitz, personal communication), plants mutated at the *kan2* locus alone are indistinguishable from wild-type. We find these results to further support the clear distinction between the pathways regulated by *pkl*, regulation of primordial/meristematic genes [12], and by *kan1*, promotion of abaxial cell fate in parallel with other genes (e.g., *CRC*).

kan1 kan2 mutants exhibit altered tissue polarity in all lateral organs

While in *kan1 crc* /*hst* /*syd* double mutants ectopic adaxial tissues are restricted to the medial domain of the carpels, normally occupied by the abaxial replum (Figure 1b), gynoecia of *kan1 kan2* double mutants develop external ovules around their entire circumference (Figure 1d). No traces of abaxial valve tissues are evident in these doubly mutant carpels. Furthermore, unlike *kan1 crc* plants, all lateral organs display gross morphological defects in *kan1 kan2* plants. Cotyledons are narrow, cup-shaped and point upward. Leaves are narrow, dark green and develop ectopic outgrowths on their abaxial side only (Figure 1e). In wild-type plants, two stipules develop in lateral marginal positions, hence flanking the base of each leaf (Figure 1g). In *kan1 kan2* plants, four, and sometimes up to six, stipules are formed at the base of each leaf, surrounding its entire circumference (Figure 1f). Similarly, in moderately adaxialized *phb-1d*/+ plants, stipules surround the entire leaf base (Figure 1h), although in severely adaxialized homozygous *phb-1d* mutants no stipules are formed. On the other hand, ectopic meristems are commonly formed at the base of the abaxial side of the *phb-1d* leaves, a feature that has not been observed in *kan1 kan2*. The stems of *kan1 kan2* fail to elongate upon flowering, and while floral organs retain the correct identity, they are highly abnormal in morphology (Figure 1i). Filamentous organs comprise most of the flower perianth, with filamentous petals having conical epidermal cells, a characteristic of adaxial epidermal cells in wild-type, on all sides (Figure 1j). Stamens are often reversed with the locules facing the perianth rather than the carpels (Figure 1i). Occasionally a single locule-like disc is topped by a presumed radial connective (a tissue normally found on the abaxial side of the stamen and characterized by the presence of stomata) on top of a short filament (Figure 1k). Mature ovules have reduced outer integuments, resembling those of *ino*

Figure 1



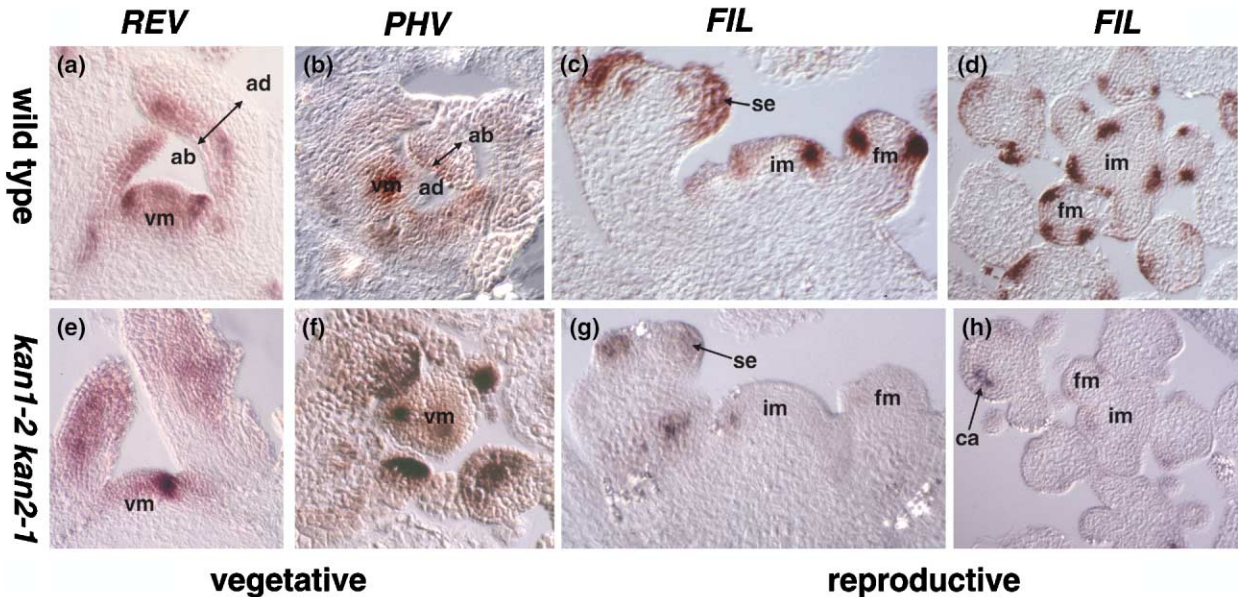
KANADI loss-of-function phenotypes. In the wild-type *Arabidopsis* gynoecium, ovules are restricted to the internal (adaxial) side **(a)**. When both *crc* and *kan1* are mutated, both internal and external ovule-bearing placentae are formed **(b)**. Plants mutant at *kanadi1* alone display only a weak phenotype, primarily in the first formed carpels, where a few external ovules develop at the base of the gynoecium and straps of ectopic style (arrow) form along the abaxial replum **(c)**. In contrast, *kan1 kan2* plants develop external septum and ovules around the entire abaxial circumference of the ovary and on its distal end **(d)**. Unlike the mild or lack of phenotypic alterations of the single mutants *kan1* and *kan2*, respectively, *kan1 kan2* plants exhibit gross morphological aberrations in all lateral organs. Shown here is a six-week-old plant with narrow leaves having outgrowths (arrow) formed on their abaxial side **(e)**. The abaxial outgrowths (arrows) are visible shortly after leaf primordia have expanded, appearing first as a row along the bottom third of the leaf, and later, in a less organized pattern as the leaf elongates **(f)**. At the base of each *kan1 kan2* leaf, several stipules (*), some of which are fused, can be found around the entire leaf circumference (f). In wild-type leaves, only two stipules are formed on the flanks of each leaf, normally associated with the adaxial side **(g)**. Stipules are also found around the entire base of the partially adaxialized leaves of *phb-1d/+* **(h)**. All floral organs of *kan1 kan2* are misshapen **(i)**. Sepals are narrow and sometimes develop outgrowths from their abaxial side. Petals are often radial with conical cells normally found on the adaxial side differentiating on all sides. **(j)**. Stamens are often reversed with the locules facing the perianth rather than the carpels (i). Occasionally a single locule-like disc is topped by a radial connective (a tissue normally found on the abaxial side of the stamen and characterized by the presence of stomata) on top of a short filament **(k)**. Mature ovules have recessed outer integuments, resembling those of *ino* ovules, which have been interpreted to be adaxialized **(l)**. The scale bars represent 500 μm in (a-d) and (i) and 50 μm in (f-h) and (j-l). The asterisk marks stipules. Abbreviations are as follows: ov, ovule; sm, septum; ab, abaxial; ad, adaxial; se, sepal; pe, petal; st, stamen; ca, carpels; ii, inner integument; and oi, outer integument.

ovules, which have been interpreted to be adaxialized (Figure 1l) [13]. Overall, the common theme in the alterations described above is the aberrant positioning of cell types, primarily along the ab/ad lateral organ axis.

Expression pattern features of *kan1 kan2* plants

To characterize the polar nature of *kan1 kan2* lateral organs, adaxial- and abaxial-specific gene expression in the mutant background was compared to wild-type. Various members of the class III HD-zip transcription factors (PHB-like) are found in a complementary expression pattern to the abaxial *YABBY* genes [5, 6, 8, 14]. mRNA of *REV* and *PHV* is localized to the SAM, throughout leaf primordia anlagen, and restricted to the adaxial domain as developing primordia separate from the meristem (Figure 2a,b). Later expression is confined to the provascular and vascular tissues of leaves and stems [15]. The expression of the adaxial genes, *REV* and *PHV*, initiates normally in *kan1 kan2* leaf primordia, but confinement to the adaxial domain is delayed (Figure 2e,f). At least in the case of *PHV*, levels of mRNA are higher as well. In wild-type, mRNA of *FIL* is first detected throughout leaf primordia anlagen and becomes confined to the abaxial side of the leaf [5, 6]. In *kan1 kan2* seedlings, initial *FIL* expression appears normal, albeit the domain is reduced in size. In developing leaf primordia, *FIL* was not detected in more than 2-3 cell layers, even though these primordia have more cell layers than wild-type (not shown). Similarly,

Figure 2



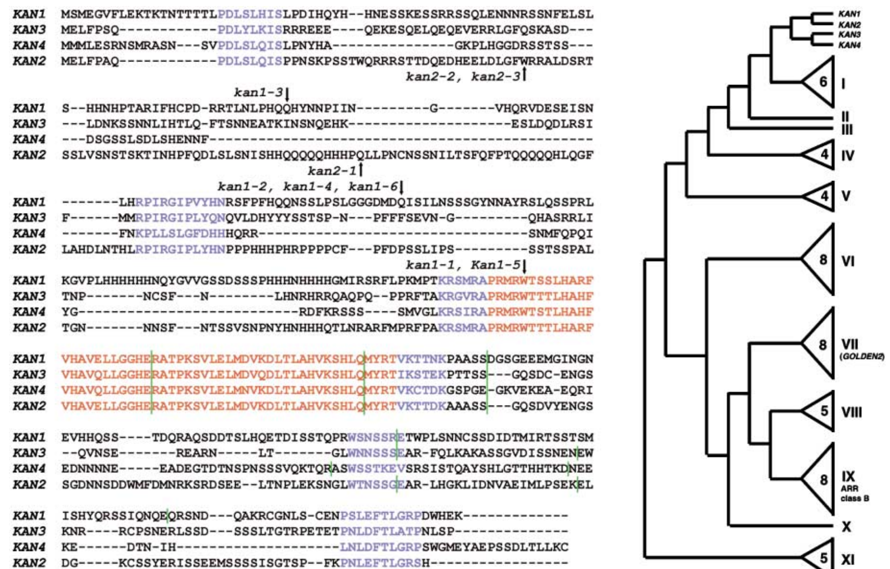
Polar gene expression in *kan1 kan2* plants. Twelve-day-old seedlings of wild-type **(a,b)** and *kan1 kan2* **(e,f)** were probed with antisense DIG labeled RNA for *REV* (a,e) and *PHV* (b,f). Clear expansion of the adaxially expressed *REV* mRNA is found in the *kan1 kan2* leaf primordia, mostly spatially. Using *PHV* as a probe, both quantitative and spatial differences are notable [compare (b) with (f)]. In flower meristems and floral organ primordia, *FIL* mRNA marks the site of primordia initiation and later becomes restricted to the abaxial domain or primordia **(c,d)**. In *kan1 kan2* inflorescences **(g,h)**, weak *FIL* expression is detected in anlagen, but later expression is largely absent except in those organs that still exhibit lateral expansion (e.g., sepals and carpels).
Abbreviations are as follows: vm, vegetative meristem; im, inflorescence meristem; fm, flower meristem; ab, abaxial; ad, adaxial; se, sepal; ca, carpel.

FIL mRNA marks the entire anlagen domain of flower primordia and floral organs before it becomes restricted to the abaxial domains of organ primordia (Figure 2c,d). In the severely radialized floral organs of *kan1 kan2*plants, *FIL* expression is weakly observed in the anlagen, but subsequent to primordia emergence, no abaxially localized expression is seen (Figure 2h,i). Whenever lateral expansion is found, as in sepals and carpels, later *FIL* expression was detected. Taking these data together, we suggest that the *KANADI* genes play a central role in promoting abaxial identity of all lateral organs, and in their absence, the normal balance between the abaxial and adaxial domains is disrupted.

The KANADI genes encode closely related, plant-specific GARP proteins

The two *kanadi* mutants display a strong synergistic interaction: while *kan2* has no visible aberrant phenotype on its own, it has a dramatic effect in *kan1* background, even when heterozygous (not shown). Since neither of the single mutants has a dramatic phenotype, we assumed that the two genes are not only functionally but also structurally redundant. *KAN1* cosegregates (0/392 recombinant gametes) with the SSLP marker nga106 (CHR5, 33cM), while *KAN2* maps to chromosome 1, halfway between the CAPS markers *UFO* (49.5 cM) and 7G6 (8/272 recombinant gametes from each). Comparisons of the BAC sequences spanning these regions (using the BLAST 2 SEQUENCES algorithm) revealed two closely related sequences: MQK4.31, *KAN1* and F27G20.7, *KAN2*. The chromosome 1 sequence matched partial EST sequences, allowing annotation of a putative gene composed of six exons. Using this sequence as a predictive model, primers were designed for both the 5′ and the 3′ ends of *KAN1*, *KAN2*, and their related homologs *KAN3* (BAC FCA9) and *KAN4* (MFO20.5). Several rtPCR products for the four genes were cloned and their sequences determined. Genomic sequence for each of the mutant alleles was determined, verifying that these genes encode *KAN1* and *KAN2*. All six *kan1* and the three *kan2* EMS-induced alleles exhibited G/C to A/T substitutions in their first exons, resulting in premature stop codons 5′ to a highly conserved motif (Figure 3). To verify that the putative cDNA clones represent the entire coding ORF, 5′ RLM-RACE (Ambion) was used to map their transcription start sites. For both *KAN1* and *KAN2*, two alternative sites were identified. *KAN1* transcripts start at –321 and –91 and *KAN2* transcripts start at either –255 or –89 relative to the putative translation start site.

Figure 3



Alignment of the predicted *Arabidopsis* KANADI genes and their phylogenetic relationships. Alignment (left) of the deduced amino acids of *KAN1*, *KAN2*, and their most similar homologs *KAN3* and *KAN4*. The red box represents the highly conserved domain found in members of the GARP gene family. The blue boxes represent the "KANADI-specific" motifs, which are found together only in these four genes. Vertical lines (green) mark the splice sites, and arrows demarcate the premature stop codons identified in the different mutant alleles. GenBank accession numbers are: *KAN1*, AY048688; *KAN2*, AY048689; *KAN3*, AY048690; *KAN4*, AY048691. The phylogenetic relationships amongst *Arabidopsis* GARP gene family members are shown at right; the numbers represent sizes of the clades. *KAN1-4* form a monophyletic clade and are only distantly related to the *GOLDEN2* [17] and ARR class B genes [19]. The ARR class B genes are characterized by a response regulator domain, a domain also found in some genes of clade VII (but not in the two genes most similar to *GOLDEN2*). If the tree is rooted with a gene from Chlamydomonas (the only non-angiosperm member of the gene family presently identified), clade XI is basal.

KAN1-4 belong to the plant-specific GARP gene family whose members encode a novel class of transcription factors containing a highly conserved domain of 54 AA [16]. GARP family members can be subdivided into two classes: those that contain a receiver domain and potentially act as two-component response regulators, and those that lack this domain [9, 17–22]. Among *KAN1-4*, the highly conserved domain is extended to 66 AA and four additional short "KANADI-specific" motifs (7-11 AA) are present. Phylogenetic analysis of 55 *Arabidopsis* GARP family members demonstrates that *KAN1-4* form a monophyletic clade (Figure 3). *GOLDEN2*, which plays a role in cell fate specification in maize leaf development [17], is only distantly related to the *KANADI* genes.

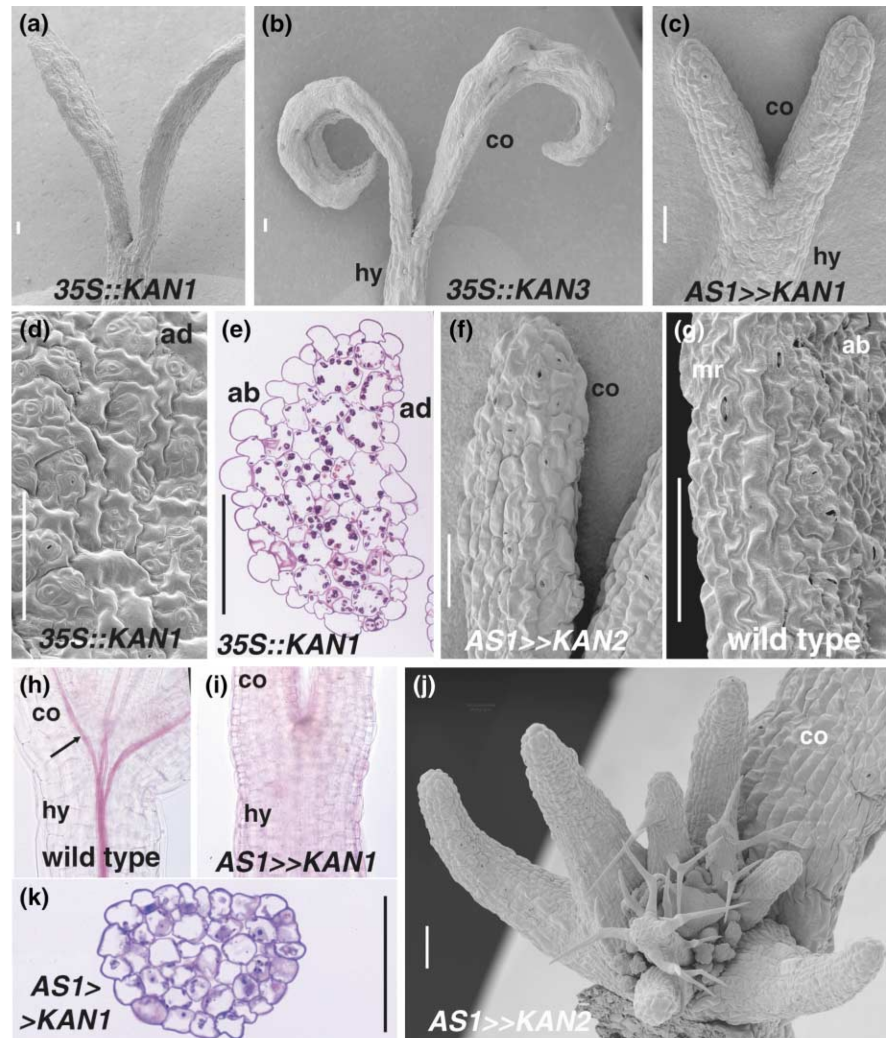
Ectopic expression of the KANADI genes – ectopic abaxial cell fates, meristem arrest, and lack of vasculature formation

The *kan1 kan2* mutant phenotype implies a role for *KAN1* and *KAN2* in promotion of abaxial cell fate. It was shown previously that ectopic expression of factors involved in polarity establishment can convert abaxial cell types into adaxial ones [8] and vise versa [5–7, 9]. Ectopic expression of any of *KAN1*, *KAN2*, or *KAN3* using the constitutive CaMV35S promoter gave rise to similar, albeit more dramatic, phenotypes, as were previously observed for ectopic *YABBY* gene expression [5]. Of 30 plants carrying the *35S::KAN1* transgene, 23 developed only small narrow cotyledons and an arrested meristem (Figure 4a), three produced a few radialized leaves, and four appeared normal. Similar results were obtained by *35S::KAN2* (27, 5, and 3, respectively) and to a lesser extent by *35S::KAN3* (10, 13, and 3; Figure 4b). Both surfaces of the narrow cotyledons were similar in appearance to the abaxial surface of wild-type cotyledons, displaying rough topology and high density of stomata (Figure 4d). Strikingly, no traces of vascular tissues were found within those cotyledons (Figure 4e).

To address the significance of polar *KANADI* gene expression, both *KAN1* and *KAN2* were expressed under the control of the *ASSYMMETRIC LEAVES1* (*AS1*) promoter. This promoter was chosen because *AS1* is normally expressed throughout emerging lateral organ primordia and not in the apical meristem [23–24]. Using a two-component expression system [25], we generated driver lines expressing the chimeric transcription factor LhG4 under the control of the *AS1* promoter. Reporter constructs carrying the *KANADI* coding regions under the control of the p6Op promoter, which is activated by LhG4, were introduced directly into *AS1::LhG4* plants. Plants carrying the reporter or the driver constructs alone did not show any phenotype, indicating no background activity.

When the *AS1* promoter drove either *KAN1* or *KAN2*, most plants grew to be slightly larger than the size of a mature wild-type embryo (Figure 4c). Cotyledons became

Figure 4



Phenotypes resulting from ectopic expression of the KANADIs. Ubiquitous expression of any of three *KANADI* genes using the 35S promoter results in narrow to radial cotyledons **(a,b)**. An even more dramatic effect is generated when *KAN1* is transactivated by the *AS1* promoter [**(c)**; we use *AS1>>KAN* as nomenclature to represent transactivation). In plants with flattened cotyledons, the adaxial epidermises of the cotyledon **(d)** are similar to the abaxial surfaces of wild-type cotyledon **(g)**. No traces of vascular bundles were found in these cotyledons **(e)**. Radial cotyledons had uniform surfaces comprised of stomata and rectangular cells **(f)**, similar to those found on the margins of wild-type cotyledons (g). Vascular bundles (arrow) are clearly evident in 7-day-old cleared wild-type seedlings **(h)** but are missing from 14-day-old cotyledons and most of the hypocotyl of severely radialized *AS1>>KAN1* plants **(i)**. A small proportion of the *AS1>>KANADI2* plants develop nearly normal cotyledons (one was removed), yet completely radialized leaves subsequently **(j)**. These leaves displayed heteroblastic morphology, lacking trichomes on the first formed ones and having trichomes on all sides of the later formed leaves. In addition, these leaves lacked the adaxial palisade mesophyll and any traces of vasculature **(k)**. All plants shown except (h,i) are 21 days old. All scale bars represent 100 μm except for (j), where it represents 50 μm. Abbreviations are as follows: co, cotyledon; hy, hypocotyl; ab, abaxial; ad, adaxial; and mr, margin.

completely radialized 250-400 μm in length, roots were similar in length, and only the hypocotyl displayed some expansion. The surface of the radial cotyledons contained long rectangular cells, similar to those found on wild-type cotyledon margins (Figure 4f,g). Again, no traces of vascular tissues were found in the cotyledons and the upper three-quarters of the hypocotyl (Figure 4h,i). On rare occasions, the cotyledons developed normally, but completely radialized leaves were formed. Although these leaves had epidermal cell types normally found on the margins of wild-type leaves, trichome distribution provides a marker for their polar identity. While the first 5-6 radial leaves had no trichomes at all, the later ones had trichomes around their entire circumference (Figure 4j). As trichomes form adaxially on the first 5-6 leaves and are later found on both leaf surfaces, we interpret these leaves as abaxialized while maintaining their normal heteroblasty. Transverse sections through these radial leaves revealed uniform radial anatomy, with subepidermal cells resembling the abaxial spongy mesophyll. Here too, no vascular elements were found (Figure 4k). Taken together, these results suggest that uniform *KANADI* gene expression throughout cotyledons and leaf primordia can convert adaxial tissues into abaxial ones and inhibit formation of vascular bundles. It is important to note that vascular bundles are also missing from most leaves of severely adaxialized *phb-1d* plants [10], suggesting that polarity per se may be essential for vasculature formation rather than any of these genes specifically.

Discussion

The *KANADI* genes are primary determinants of abaxial cell fate

A model describing leaf development by Waites and Hudson [4] predicted the formation of two separate domains along the ab/ad leaf axis. According to this model, once organ primordia are separated from the apical meristem, the cells adjacent to the meristem acquire different iden-

tity than the cells at a distance from the meristem. Adaxial expression patterns of *PHB*-like genes [8] and abaxial expression of *YABBY* genes [5–6] confirmed the existence of such domains at the biochemical level. The results presented herein suggest that at least three *KANADI* genes, members of the GARP gene family, are primary determinants of the abaxial domain in all lateral organs, and in their absence, adaxial cell types develop in abaxial positions. Furthermore, *KANADI1* expression becomes restricted to the abaxial domain of developing leaf primordia [9] and when ectopically expanded can convert adaxial cell types into abaxial ones.

While plants mutant for either *kan1* or *kan2* alone have very limited or no morphological alterations, respectively, all lateral organs in plants mutated for both genes have gross defects. Most alterations can be viewed as a replacement of abaxial cell types with adaxial ones, particularly in petals or carpels. These alterations in polarity result in narrow leaves, filamentous floral organs, and formation of *ino*-like ovules around the entire gynoecium circumference. These phenotypes stand in contrast to those of loss-of-function mutations in *YABBY* genes, which have been also been proposed to promote abaxial cell fate [5–6]. Plants with null alleles of both *FIL* and its redundant family member *YAB3* have reduced polar distinction between the two sides of their lateral organs, yet no clear gain of adaxial identity (Kumaran and Sundaresan, personal communication; [5]). While the floral organs of *kan1 kan2* plants are almost entirely adaxialized, the leaves still retain some abaxial characters, implying the existence of additional abaxial-promoting genes. Obvious candidates include the remaining *KANADI* genes for which loss-of-function alleles are not available and the residual YABBY activity found in these leaves. Indeed, sequences 5′ to the coding region of *KAN3* drive reporter gene expression in the abaxial regions of developing leaves, but not in flowers (Y.E. and J.L.B., unpublished data).

Further evidence for the pivotal role of the *KANADI* genes in promotion of abaxial cell fate comes from the analysis of their ectopic expression. Uniform expression of any of the three described *KANADI* genes is capable of completely radializing lateral organs. In prior studies, no gene was found to induce such a dramatic effect in its native form [5, 8], even when strong ubiquitous promoters were used. Finally, the epistasis of the expanded *KANADI* expression domain over the endogenous adaxial-promoting factors supports the model that a primary role of adaxial factors is repression of abaxial ones.

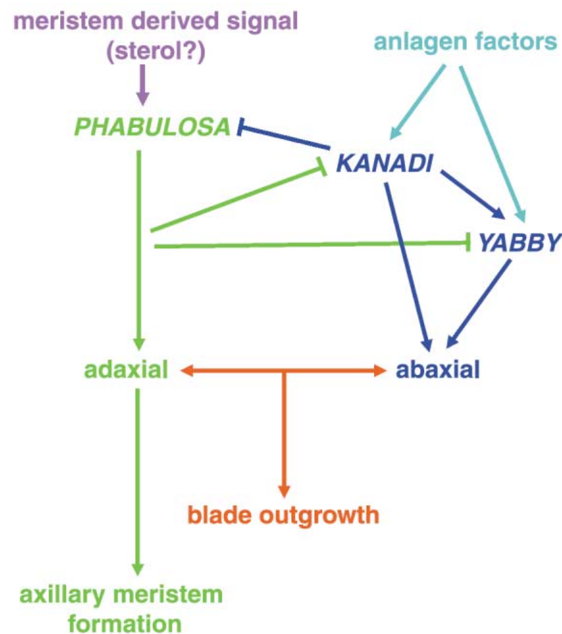
The ab/ad axis formation is a quantitative integration of intrinsic and extrinsic signals

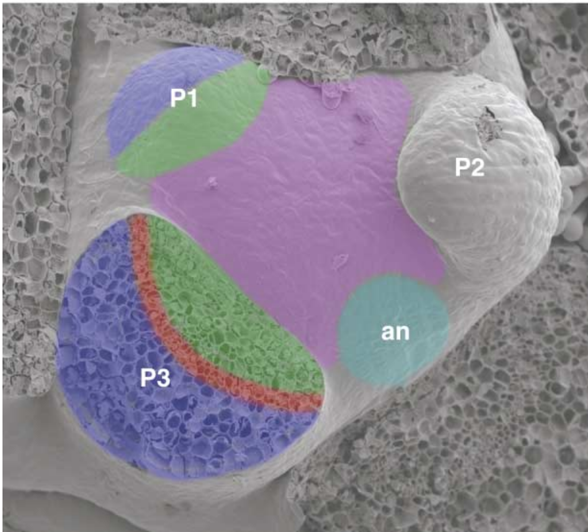
As incipient lateral organ primordia develop from the flanks of the shoot apical meristem, factors both intrinsic and extrinsic to the organ primordia contribute to the specification of cells as adaxial or abaxial. The apical meristem itself is a likely source of signal(s) that promotes adaxial cell fate [2]. Signal perception may be mediated through the PHB/PHV/REV proteins ([8]; Figure 5) in a concentration-dependent manner. Abaxial cell fate is then the "default" in the absence of such signals; for instance, if the lateral organ primordia are surgically separated from the apical meristem [2]. This default state could be the result of the failure to restrict genes promoting abaxial identity: the *YABBY* and the *KANADI* genes, which are initially activated throughout lateral organ anlagen [5–6, 9]. In support of this, the expression of *FIL* is greatly reduced in *phb-1d* plants [5]. At present, it is not possible to tell whether adaxial identity is a consequence of the absence of abaxial factors or presence of adaxial ones. Epistatic relations between gain-of-function alleles of the corresponding factors should help to clarify this point.

The relationship between the two pathways promoting abaxial identity is presently not clear. While YABBY genes are activated in the anlage of *kan1 kan2* plants, KANADI activity is required for their proper abaxial localization, suggesting KANADI function is, in some respects, upstream of YABBY function. In support of this, *35S::YAB3* is epistatic to the *kan1 kan2* phenotype (46/60 wild-type plants showed the described arrested seedling *35S::YAB3* phenotype [5] compared to 66/84 in the progeny of self-fertilized *kan1 kan-2*/+ plants). Conversely, that *35S::KAN1/2/3* is also epistatic to *fil yab3* (Y.E. and J.L.B., unpublished data) implies a more complex relationship between the *YABBY* genes and *KANADI* genes than a linear pathway. However, these epistasis experiments are complicated by extensive redundancy within each of the gene families, and, therefore, the gain-of-function alleles will have to be tested in complete loss-of-function backgrounds. Despite this reservation, the distinct phenotypes of *kan1 kan2* and *fil yab3* double mutants argues for a parallel mode of action with both common and distinct targets for the two gene families.

In the simplest scenario described above, the *KANADI* and *YABBY* genes could be primary targets for PHB-like suppression, and, conversely, clear expansion of *PHV* and *REV* mRNA expression patterns in *kan1 kan2* leaf primordia suggests that KANADI function, at least in part, restricts PHB-like activity. However, extensive overlap exists between the domains of the abaxial- and adaxial-promoting genes in the leaf anlagen (Figure 5). These results indicate that the adaxial suppression of abaxial factors is either indirect or that the repression depends on cellular conditions that differ between anlagen and primordium. For example, the *KANADI* and/or *YABBY* genes themselves could modulate the ligand-receptor adaxial signaling by regulating PHB ligand stability. Regardless of the molecular mechanism, the different mutant phenotypes suggest that repression/activation relation-

Figure 5





Model of polarity establishment in lateral organs. Top upper panel details a genetic model of lateral organ polarity establishment, with the spatial and temporal aspects mapped onto a potato apical meristem in the lower panel. An emerging picture from classical and molecular genetic analyses is that as incipient lateral organ primordia develop from the flanks of the shoot apical meristem, factors both intrinsic and extrinsic to the organ primordia contribute to the specification of cells as adaxial (green) or abaxial (blue). The apical meristem (purple) itself likely provides a signal(s) that promotes adaxial cell fate [2], whose perception may be mediated through *PHB*/*PHV*/*REV* (*PHABULOSA* in figure) [8]. The ultimate source and biochemical nature of the ligand is unknown. *PHB*, *FIL*, and *KAN* are all expressed in the leaf anlagen, but their expression becomes confined to mutually exclusive domains as the primordia form. Abaxial cell fate may be a "default" in the absence of signal, for instance, if the lateral organ primordia are separated from the apical meristem. This default state could be the result of the failure to repress genes promoting abaxial identity (e.g., *YABBY* and *KANADI* genes), which are initially activated throughout the anlagen (aqua) [5,6,9]. Surgical experiments indicate that while polarity is labile in P1, it is irreversibly established by P2 [2]. KANADI activity may mediate between PHABULOSA and YABBY activities, however, the precise relationships between these pathways remain to be elucidated. Subsequent interactions between the juxtaposed adaxial and abaxial domains, perhaps mediated by relative levels of KANADI and YABBY activity, are required for lamina outgrowth (red) [4].

ships at early stages are quantitative rather than qualitative, providing a flexibility that potentially allows various leaf morphologies to develop. This is consistent with results from surgical experiments which demonstrated that while polarity was labile in P0 and P1 (Figure 5), it is irreversibly established by P2 [2].

Why does the meristem cease to function when abaxial factors are missexpressed? Superficially, the meristem arrest observed in both *35S::KANADI* and *35S::YABBY* plants appears similar. Yet, some differences were observed. In *35S::YABBY* the arrest was associated with an enlarged central area and occasional formation of numerous leaf/stipule like structures [5]. In *35S::KANADI*, the region between the radial cotyledons never expanded beyond the level of a cleft, and no filamentous structures were observed. Apparently, an apical meristem was not formed at all in these plants. One possible mechanism for the failure to make a meristem could be the suppression of the PHB-like genes activities. That *rev* mutants often fail to develop axillary meristems [26] is suggestive of such a mechanism, and analysis of loss-of-function alleles for the other PHB-like genes could shed more light on this phenomenon.

Phenotypes of both loss- and gain-of-function mutations of the *KANADI* genes implicate these genes in promoting abaxial cell fates. Loss of KANADI activity leads to ectopic expression of genes promoting adaxial cell fates, loss of proper abaxial localization of *YABBY* gene expression, and development of adaxial tissues in abaxial positions. Conversely, adaxial expression of any of *KAN1*, *KAN2*, or *KAN3* results in the development of abaxial tissues in adaxial positions. These data argue that a primary function of the *KANADI* genes is to promote abaxial cell fates. Based on the loss of the apical meristem and vasculature (tissues derived from the central region of the embryo) in gain-of-function *KAN1* alleles, *KAN1* has also been proposed to specify peripheral cell identity in the embryo [9]. However, lack of vasculature may not be a suitable marker for central tissue identity. For example, in *AS1*>>*KAN* plants, the epidermis of the radialized leaves exhibits abaxial characteristics while retaining proper heteroblasty; *phb-1d* mutants have the complementary phenotype, with the epidermis exhibiting adaxial characteristics [10]. However, vascular tissue is absent in radialized

leaves in both of these genotypes. Furthermore, by promoter analyses, *KAN1*, *KAN2*, and *KAN3* appear to be expressed in the vasculature of wild-type plants (Y.E., S.F.B., and J.L.B., unpublished data), and alterations in the vascular patterning in *kan1 kan2* stems suggest roles for these genes in this tissue. Since the evolution of vasculature predated that of leaves, the PHB-like/KANADI genetic program that patterns polarity in lateral organs may have been derived from an ancient role in vascular patterning.

Materials and methods

Plant growth, mutagenesis, crosses, mapping and transformation

All mutants are in the Landsberg *erecta* (Ler) background. Plants were grown under 18 hr cool white fluorescent light at 20°C. *kan1-2 pkl-12* seeds were mutagenized with 17 mM ethylmethanesulphonate for 12 hr and phenotypic enhancers selected in the M2. The enhancers were backcrossed to either *kan1-2, phl-15*, or wild-type Ler. Multiply mutant plants were generated by cross-fertilizing homozygous mutants and identifying desired mutant combinations among phenotypic categories in the F2 segregants. Genotypes were confirmed by monitoring Mendelian ratios and by progeny testing. The single mutant phenotypes of *kan1* enhancers were determined by analyzing progeny from 6 to 10 non-*kan1* plants in F2 families (derived from double mutants crossed to wild-type) as F3 families. Families in which all *kan1-2* plants had external ovules were further characterized.

Loci were mapped by crossing single or double mutant lines to the Colombia ecotype, and linkage was detected using SSLP and CAPS markers among F2 plants homozygous for the mutation (or the double mutant in the case of *kan2*). All transgenic plants were generated by the floral dipping method and transformants were selected on soil due to resistance to kanamycin or the herbicide BASTA.

Plasmids and cDNA clones

cDNAs of the four *KAN* genes were obtained by rtPCR of Colombia inflorescence RNA (primers correspond to the ends of the sequences deposited in GenBank). For pKAN1/2/3::GUS, a 4.9/5.3/3.5 kb fragment 5′ to the ATG of *KAN1/2/3*, respectively, was amplified from Colombia DNA and subcloned into pRITAI, to generate pKAN::GUS. The NotI fragment of this plasmid was introduced into the binary vector pMLBART (a gift from Kim Richardson). To generate the AS1::LhG4 construct, the coding region for LhG4 was excised from pBIN-PLUS:LhG4 (a gift from Klaus Palme) and inserted into the unique BamHI-site of pBJ36 (a gift from Bart Jannsen). Six thousand base pairs of the 5′ upstream sequence of AS1 (AtPHAN) were amplified by PCR and introduced in front of the LhG4. The resulting AS1::LhG4 fragment was inserted into pMLBART. For the 6Op::KAN1/2/3 reporters, the full-length *KANADI1/2/3* cDNAs were inserted into the HindIII/BamHI sites of p6OP-TATA-BJ36. The resulting Op::KAN1/2/3 fragments were excised and inserted into pART27 [27]. All plasmids were introduced into *Agrobacterium* strain ASE by electroporation and transformed into Ler wild-type plants.

Phylogenetic analysis

Sequences of 55 GARP gene family members from *Arabidopsis* were compiled after searching GenBank with the putative DNA binding domain of *KAN1*. Reiterative searches did not reveal additional genes in the *Arabidopsis* genome. Sequences corresponding to the 66 amino acid conserved domain of the KANADIs were aligned, and sequences were corrected for putative annotation errors. Heuristic searches were performed using PAUP4.0b. Fifty-four characters were phylogenetically informative: 21 most parsimonious trees of 729 steps were obtained, and a consensus tree was computed. GenBank accession numbers of the *Arabidopsis* GARP gene family members are as follows: clade I (AAD20098, AAF63176, AAD25661, AAD29772, AAD21740, AAC28774), clade II (AAD19767), clade III (BAB11197), clade IV (AAD30233, BAB02417, BAB01353, CAB36828), clade V (AAF05867, AAK01148, BAB09482, AAF24605), clade VI (AAD17450, AAG50807, AAD49976, AAG09552, BAA95766, AAK63945, AAB38775, AAG13042), clade VII (AAF69721, CAB87277, AAC77865, AAK20121, AAD21709, AAK60316, CAA17145, CAB83117), clade VIII (AAF19573, BAB11531, AAB86457, CAB62334, BAA97491), clade IX (BAB10999, BAA74527, BAB03073, AAD12696, CAA06431, CAA16597, BAB10342, AAF19224), clade X (BAB01705), and clade XI (CAB81449, AAF63776, AAD21748, AAF18654, BAB09814).

Microscopy

SEM, histological analyses, tissue clearing, GUS staining, and in situ hybridization were carried out according to Eshed et al. [7]. *REV* and *PHV* probes were generated by linearizing the full-length cDNA plasmids and synthesizing DIG-labeled antisense RNA using T7 RNA polymerase.

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