Genetic Interactions Between Leaf Polarity-Controlling Genes and \textit{ASYMMETRIC LEAVES1} and 2 in \textit{Arabidopsis} Leaf Patterning

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During leaf development, establishment of adaxial–abaxial polarity is essential for normal leaf morphogenesis. This process is known to be strictly regulated by several putative transcription factors, microRNA165/166 (miR165/166), a trans-acting short-interfering RNA (tasiR-ARF), as well as proteins involved in RNA silencing. Among the putative transcription factor genes, \textit{ASYMMETRIC LEAVES1} and 2 (\textit{ASI} and 2) facilitate the specification of leaf adaxial identity; however, the mechanism by which \textit{ASI} and \textit{AS2} cooperate with other leaf polarity components remains largely undetermined. In the current study, we characterized the phenotype of mutants by combining \textit{asi} and \textit{as2} with mutations of several key transcription factors. Our data showed that double mutant plants carrying \textit{as1/as2} and \textit{rev}, \textit{phb} or \textit{phv} enhanced \textit{as1/as2} defects by producing more severely abaxialized leaves. Additionally, triple mutants, obtained by combining \textit{as1/as2} with double mutant \textit{filamentous flower yabby3} (\textit{fil yab3}) or \textit{kanadi1 kanadi2} (\textit{kan1 kan2}), exhibited additive phenotypes. These genes, including \textit{PHAVOLUTA} (\textit{PHV}), \textit{PHABULOSA} (\textit{PHB}) and \textit{PHAVOLUTA} (\textit{PHV}) are expressed in the adaxial leaf domain and are required for specifying adaxial identity (McConnell and Barton 1998, McConnell et al. 2001, Emery et al. 2003, Zhong and Ye 2004). Several other genes, including \textit{PHILANTHROUS FLOWER} (\textit{PHIL}), \textit{YABBY3} (\textit{YAB3}), \textit{KANADI1} and 2 (\textit{KAN1} and \textit{KAN2}), and \textit{AUXIN RESPONSE FACTOR4} (\textit{ARF4}), are expressed in the abaxial leaf domain and promote abaxial identity (Chen et al. 1999, Sawa et al. 1999, Siegfried et al. 1999, Eshed et al. 2001, Kerstetter et al. 2001, Pekker et al. 2005, Fahlgren et al. 2006, Garcia et al. 2006). Recent studies also suggested that \textit{ASI} and \textit{AS2} genes mediate the leaf adaxial identity (Xu et al. 2003); \textit{ASI} encodes a protein that contains an R2-R3 MYB-domain (Byrne et al. 2001, Kerstetter et al. 2001, Pekker et al. 2005), \textit{as2} encodes a plant-specific leucine-zipper protein (Iwakawa et al. 2002) that associates with \textit{AS1} (Xu et al. 2003). Given that \textit{ASI} and \textit{AS2} might regulate the same genetic pathways during leaf morphogenesis (Xu et al. 2003), mutations in either the \textit{ASI} or \textit{AS2} gene could result in overall similar plant phenotypes (Ori et al. 2000, Sun et al. 2000, Semiarti et al. 2001). More recent data revealed that genes functioning in the RNA-induced gene silencing and the protein degradation pathways were also required for leaf adaxial identity. These genes, including \textit{RNA DEPENDENT RNA POLYMERASE6} (\textit{RDR6}), \textit{SUPPRESSOR OF GENE SILENCING3} (\textit{SGS3}), \textit{PHB}, \textit{PHAVOLUTA}, \textit{KAN2}, \textit{BREVICOLOMA} (\textit{BRC}), \textit{ARGONAUTE7/ZIP}, \textit{ARGONAUTE7/ZIP5}, \textit{HUB}, \textit{MICRORNA165/166}, \textit{MICRORNA165/166}, \textit{HUB}, \textit{MICRORNA165/166}, \textit{PHB}, \textit{PHAVOLUTA}, \textit{RDR6}, \textit{RNA DEPENDENT RNA POLYMERASE6}, \textit{REV}, \textit{REVOLUTA}, \textit{RT-PCR}, reverse transcription–PCR; SAM, shoot apical meristem; SEM, scanning electron microscopy; \textit{SGS3}, \textit{SUPPRESSOR OF GENE SILENCING3}; \textit{YAB}, \textit{YABBY}.

Keywords: Adaxial–abaxial polarity — \textit{Arabidopsis} — Asymmetric leaves1(2) — Leaf development — MicroRNA165/166.


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To understand better the regulatory mechanisms underlying the establishment of leaf polarity, elucidation of genetic interactions among the regulatory components seems important. However, the genetic interactions between AS1–AS2 and other leaf polarity genes have not, to date, been fully elucidated. In the present study, we demonstrate that loss-of-function as mutants contained altered levels of REV, PHB, PHV and FIL mRNAs. In addition, we report the phenotypic analyses of mutants by combining as1 and as2 with other leaf polarity-defective mutants. Based on the phenotypic analyses of 35S::MIR165a transgenic plants, we discuss possible genetic models by which the leaf ad/abaxial polarity is normally established.

Results

AS1 and AS2 positively regulate REV, PHB and PHV

Previous studies showed that mRNA levels of the class III HD-ZIP gene PHB were elevated in 35S::AS2 transgenic plants (Lin et al. 2003), suggesting that AS2 might positively regulate PHB. To elucidate further the mechanism by which ASI and AS2 genes may affect the accumulation of class III HD-ZIP gene transcripts, we utilized real-time reverse transcription–PCR (RT–PCR) to analyze REV, PHB and PHV expression in as1 and as2 mutants. Our data demonstrated that transcript levels of these three genes were all reduced in as1-101 and as2-101 rosette leaves as compared with those in wild-type plants (Fig. 1A–C), but were much lower than those in both 35S::AS1 transgenic plants and a gain-of-function as2 mutant, iso-2d (Nakazawa et al. 2003). These results indicated that normal AS1 and AS2 functions are required for the proper REV, PHB and PHV expression in leaves.

Characterization of rev-6 as2-101, phb-6 as2-101 and phv-5 as2-101 phenotypes

To investigate further the regulatory network in leaf ad/abaxial polarity formation, we constructed double mutants by combining as1-101 or as2-101 with rev-6, phb-6 and phv-5, and analyzed their phenotypes (see Supplementary Table S1). Compared with wild-type leaves (Fig. 2A), as2-101 (Fig. 2B) produced some rosette

![Fig. 1](https://via.placeholder.com/150)

**Fig. 1** Altered REV, PHB and PHV expression. REV (A), PHB (B) and PHV (C) transcript levels from the first two pairs of rosette blades were analyzed using real-time RT–PCR. Leaves were collected from the 28-day-old plants of wild-type Ler, as1-101 and as2-101 single mutants, a 35S::AS1 transgenic line, and a gain-of-function AS2 mutant iso-2d. Two other independent 35S::AS1 transgenic lines have also been analyzed, and results from these two lines were consistent with those of the line shown in this figure (data not shown). Bars indicate the SD.
leaves with a lotus leaf structure (arrow), with petioles noted underneath leaf blades, as well as a needle-like structure, both of which reflected the loss of leaf adaxial identity (Xu et al. 2003, Qi et al. 2004). The frequency of lotus leaf and needle-like leaves in as2-101 was relatively low (Table 1), and rev-6 leaves (Fig. 2C) did not show such abnormalities. Interestingly, rev-6 as2-101 leaf surfaces became very scabrous (Fig. 2D, G) and the frequency of needle (Fig. 2D, E) and lotus structures (Fig. 2D, F) increased markedly (Table 1). Similar abnormal leaf phenotypes were also observed in the rev-6 as1-101 double mutant plants (see Supplementary Fig. S1B). While needle-like structures typically appeared in the first pair of rosette leaves in the as1-101 and as2-101 single mutant (Xu et al. 2002, Xu et al. 2003, Qi et al. 2004), they were also observed in the second pair of rosette leaves in rev-6 as2-101 (Fig. 2D) and rev-6 as1-101 (data not shown) plants.

Scanning electron microscopy (SEM) analysis of leaf and petal epidermal cells was utilized to investigate the rev-6 as2-101 defects further. The adaxial epidermis of wild-type Ler leaves was characterized by an undulating surface composed of uniformly sized cells (Fig. 2H), while smaller and less uniformly sized cells mixed with some long and narrow cells were noted in the abaxial epidermis (Fig. 2I, arrowhead) (McConnell and Barton 1998). Although abaxial epidermal cells appeared normal (data not shown), the adaxial surface of rev-6 as2-101 leaves was mosaic, with both adaxial and abaxial epidermal patches (Fig. 2J, arrowhead). This aberrant phenotype appeared on leaves of the Antirrhinum mutant phantastica (Waites and Hudson 1995) and the Arabidopsis double mutant rdr6-3 as2-101 (Li et al. 2005), but was not observed on the as2 adaxial leaf surface.

Petals of rev-6 as2-101 plants also displayed severe defects in ad/abaxial identity. Wild-type adaxial petal epidermis showed cone-shaped cells with straight lines (Fig. 2K), while the abaxial petal epidermis showed cobblestone-shaped cells with wavy lines (Fig. 2L) (McConnell and Barton 1998). Although the abaxial petal surface of rev-6 as2-101 was normal (data not shown), wavy lines were noted on all adaxial epidermal cells (Fig. 2M), similar to the petal abaxial epidermal cells of wild-type plants (Fig. 2L). These results indicate that petals in rev-6

Table 1  Frequencies of lotus- and needle-like leaves in mutant plants

<table>
<thead>
<tr>
<th>Mutants</th>
<th>No. of plants</th>
<th>Lotus leaves (%)</th>
<th>Needle-like leaves (%)</th>
</tr>
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<tbody>
<tr>
<td>as2-101</td>
<td>260</td>
<td>12.1 ± 1.6</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>phv-5 as2-101</td>
<td>224</td>
<td>16.5 ± 1.9</td>
<td>0.7 ± 0.5</td>
</tr>
<tr>
<td>phb-6 as2-101</td>
<td>132</td>
<td>20.1 ± 1.6</td>
<td>4.5 ± 1.4</td>
</tr>
<tr>
<td>rev-6 as2-101</td>
<td>328</td>
<td>23.5 ± 1.7</td>
<td>14.6 ± 1.0</td>
</tr>
</tbody>
</table>

Plants were grown at 22°C. Only the first two pairs of the rosette leaves of the 28-day-old plants were scored. Values are mean ± SD.

Total number of plants that were analyzed.
4. Genetic interactions in leaf polarity formation

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as2-101 mutant plants are also abaxialized. We also constructed double mutants by combining as2-101 with another rev allele, rev-9, and found that the leaf phenotypes of rev-9 as2-101 and rev-6 as2-101 were very similar (see Supplementary Fig. S2).

We next analyzed phb-6 as2-101 and phv-5 as2-101 double mutant leaves, and found that phb-6 as2-101 and phv-5 as2-101 also enhanced as2-101 leaf phenotypes, while they were less severe compared with those in rev-6 as2-101. Surfaces of expanded leaves of phb-6 as2-101 and phv-5 as2-101 were similar to those in wild-type plants (data not shown). However, the frequency of lotus- and needle-like leaves in double mutants was enhanced compared with those in the as2-101 single mutant (Table 1). The as1 double mutant exhibited overall similar phenotypes to the as2 double mutant, indicating that REV, PHB and PHV functions are required for a pathway which is controlled by both AS1 and AS2 genes, and REV might play a more critical role among the three in the pathway.

Similarity of rev-6 as2-101 and rdr6-3 as2-101 double mutant leaf phenotypes

In addition to the rippled leaf surface, rev-6 as2-101 leaves often produced outgrowths on the adaxial leaf side (Fig. 3A), some of which grew to form leaflets (see the boxed region in Fig. 3A, and Fig. 3B). Additionally, small leaflets generated additional outgrowths on their adaxial side (Fig. 3B, arrowhead). Although organ-like structures were not observed on cauline leaves, sepals and petals, they were rippled on their adaxial surfaces (Fig. 3C-E). Leaf phenotypes of rev-6 as2-101 resembled those of the rdr6-3 as2-101 double mutant (Li et al. 2005), which produced a high frequency of needle-like (Fig. 3F) and lotus-like leaves (Fig. 3G), and had highly rippled leaf surfaces (Fig. 3H). Moreover, rdr6-3 as2-101 leaves also produced outgrowths on the adaxial leaf side, some of which developed into leaflets (Li et al. 2005).

We reported previously that levels of mature miR165/166 and FIL mRNA were elevated in rdr6-3 as2-101 double mutant leaves (Li et al. 2005). We further analyzed miR165/166 and FIL levels in order to determine whether the similar leaf phenotypes observed in rev-6 as2-101 resulted from molecular mechanisms similar to those in rdr6-3 as2-101. Surprisingly, while miR165/166 levels were much lower than those found in rdr6-3 as2-101 leaves (Fig. 3I), FIL levels were dramatically elevated in rev-6 as2-101 leaves, resembling those in rdr6-3 as2-101 leaves (Fig. 3J). In situ hybridization was utilized to investigate whether the altered transcript levels of FIL were also accompanied by changes in its expression pattern. In wild-type Ler (Fig. 3N), rev-6 (Fig. 3O) and as2-101 (Fig. 3P) plants, FIL mRNAs accumulated in the abaxial side of the leaf primordium, whereas they were either extended towards the adaxial side of the expanded leaves (Fig. 3Q, arrowheads) or were present throughout the entire needle-like leaf primordium of rev-6 as2-101 (Fig. 3R). To examine further the role of FIL in the abnormal rev-6 as2-101 leaf morphology, we constructed fil-1 rev-6 as2-101 and analyzed the triple mutant leaves. Compared with the rev-6 as2-101 double mutant, fil-1 rev-6 as2-101 displayed an additional inflorescence phenotype (see Supplementary Fig. S3), similar to that of the fil rev double mutant (Chen et al. 1999). In addition, most fil-1 rev-6 as2-101 leaves did not show visible outgrowths (Fig. 3K, M), though the adaxial leaf surface was slightly pleated. While a small proportion of the fil-1 rev-6 as2-101 leaves still had ectopic outgrowths (Fig. 3L, M), the severity of this phenotype was greatly suppressed (Fig. 3L, for comparison see Fig. 3A). These results suggest that the ectopic expression and overexpression of the FIL gene may predominantly contribute to the abnormal rev-6 as2-101 leaf phenotypes.

MIR165a overexpression in rev as2, phb as2 and phv as2 double mutants

It is generally thought that miR165 and miR166 are important to leaf ad/abaxial polarity formation by repressing REV, PHB and PHV (Emery et al. 2004, Zhong and Ye 2004). However, our previous results showed that overexpression of MIR165a in wild-type plants did not result in leaf defects in the ad/abaxial polarity (Li et al. 2005). To understand better miR165/166 action in leaf polarity formation, we introduced a 35S::MIR165a fusion into rev-6 as2-101, phb-6 as2-101 and phv-5 as2-101 double mutants, and subsequently analyzed the phenotypes of the transgenic plants. Wild-type Ler and each corresponding single mutant carrying the 35S::MIR165a fusion were used as controls. Our data demonstrated that 35S::MIR165a/Ler plants exhibited slightly down-curved leaves (Fig. 4A), while 35S::MIR165a/as2-101 plants had an increased frequency of lotus- and needle-like leaves (data not shown) with rippled leaf surfaces (Fig. 4B).

Although 35S::MIR165a/rev-6 leaves (Fig. 4C) had leaf phenotypes similar to those of 35S::MIR165a/Ler plants, the morphology of 35S::MIR165a/rev-6 as2-101 transgenic plants was severely affected. A total of 61 independent transgenic lines were grouped into two categories. One category consisted of 17 lines, which showed milder plant phenotypes, with early appearing unexpanded leaves (Fig. 4D, arrowheads) and late appearing very rippled leaves (Fig. 4D, arrow). The second category consisted of 44 lines, which had normal-looking cotyledons, but with all other leaves needle-like in appearance (Fig. 4E). These plants were arrested at the seedling stages, and the needle-like leaves were covered
Fig. 3 Phenotypic comparison between *rev-6 as2-101* and *rdr6-3 as2-101*. (A) The adaxial surface of some *rev-6 as2-101* leaves produced ectopic outgrowths, some of which grew further to form leaflets with the rippled adaxial surface. (B) A *rev-6 as2-101* leaflet that is the close-up of the boxed region in (A), which had additional outgrowths (arrowhead). (C–E) The adaxial surfaces of cauline leaves (C), sepals (D) and petals (E) in *rev-6 as2-101* were also rippled. (F–H) The leaf phenotypes of *rev-6 as2-101* are similar to those in *rdr6-3 as2-101*, which also produces needle-like (F) and lotus leaves (G), and the rippled adaxial surface of the expanded leaves (H). (I, J) The miR165/166 levels were distinct between *rev-6 as2-101* and *rdr6-3 as2-101* leaves (I), while the elevated *FIL* mRNA levels were consistent in both *rev-6 as2-101* and *rdr6-3 as2-101* leaves (J). (K–M) The *rev-6 as2-101* leaf phenotypes were suppressed in the *fil-1* background. Most *fil-1 rev-6 as2-101* leaves had no visible ectopic outgrowths (K, M). Although there were some outgrowth-containing leaves in *fil-1 rev-6 as2-101* (L), the phenotypes appeared much weaker as compared with those in *rev-6 as2-101*. *n*, numbers of leaves analyzed. The first two pairs of rosette leaves from 28-day-old wild-type and mutant plants were used for RT–PCR and miR165/166 level analyses. (N–R) In situ hybridization using an antisense *FIL* probe on transverse sections of primordia in wild-type *Ler* (N), *rev-6* (O), *as2-101* (P), *rev-6 as2-101* flat (Q) and needle-like (R) leaf primordia. Note that some flat primordia showed expanded *FIL* expression towards the adaxial side (Q) (arrowheads). Bars = 1 mm in (A), 3 mm in (C), 500 μm in (B, D), 200 μm in (E, F–H, K, L), and 10 μm in (N–R).
with long and narrow epidermal cells (Fig. 4F), resembling those on leaf petioles (Byrne et al. 2000, Sun et al. 2002). We also investigated 35S::MIR165a/phb-6 as2-101 and 35S::MIR165a/phv-5 as2-101 transgenic plants; however, the phenotypes of these plants were indistinguishable from those of the 35S::MIR165a/as2-101 transgenic plants. These results further supported the idea that among the three functionally redundant class III HD-ZIP members, REV was of premier importance for adaxial leaf identity.

Phenotypes of plants carrying multiple mutations

To elucidate further possible genetic interactions between AS1/2 and YAB or KAN genes, we constructed fil-1 yab3-2 as1-101, fil-1 yab3-2 as2-101, kan1-2 kan2-1 as1-101 and kan1-2 kan2-1 as2-101 triple mutants. Compared with fil-1 yab3-2 (Fig. 5A) (Kumaran et al. 2002), the fil-1 yab3-2 as2-101 plants produced lotus leaves (Fig. 5B, arrowhead), resembling as2-101 single mutant plants. Other leaves of triple mutants (Fig. 5E) appeared to have both fil-1 yab3-2 and as2-101 characteristics, with the leaf blade width between those of as2-101 (Fig. 5C) and fil-1 yab3-2 (Fig. 5D) plants (see Supplementary Table S2). fil-1 yab3-2 as1-101 showed similar plant phenotypes to those of fil-1 yab3-2 as2-101 (see Supplementary Fig. S1D, G). Additionally, kan1-2 kan2-1 double mutant plants showed narrow leaves with ectopic outgrowths emerging on the abaxial side (Fig. 5F, arrowhead, G) (Eshed et al. 2001, Eshed et al. 2004). The early appearing rosette leaves of kan1-2 kan2-1 as2-101 triple mutant plants also contained lotus leaves (Fig. 5H, arrow, I). However, the outer side of the lotus leaf, which is equivalent to the abaxial side of a wild-type leaf, produced ectopic outgrowths (Fig. 5H, arrowhead, I). The similar kan1-2 kan2-1 as2-101 phenotypes were also observed in the kan1-2 kan2-1 as1-101 triple mutant (see Supplementary Fig. S1I). The additive fil-1 yab3-2 as1-101, fil-1 yab3-2 as2-101, kan1-2 kan2-1 as2-101 and kan1-2 kan2-1 as2-101 triple mutant phenotypes suggested that the AS1/AS2 genes might not interact genetically with YAB and KAN genes.

Fig. 4 Phenotypes of plants carrying a 35S::MIR165a construct. (A) A 35S::MIR165a/Ler plant. (B) A 35S::MIR165a/as2-101 plant, showing slightly rippled leaves. (C) A 35S::MIR165a/rev-6 plant. (D) A 35S::MIR165a/rev-6 as2-101 plant, showing that rosette leaves were either very narrow and rippled (arrow) or unexpanded (arrowhead). (E) A more severe 35S::MIR165a/rev-6 as2-101 plant, showing all needle-like rosette leaves. (F) A needle-like leaf of the severe 35S::MIR165a/rev-6 as2-101 plant, showing an insufficiently differentiated epidermal pattern. F is a close-up of the boxed region in (E). c, cotyledon. Bars = 2 mm in (A–E) and 50 μm in (F).
The severe abaxialized leaves in *rev-6 as2-101* or adaxialized leaves in *kan1-2 kan2-1* resulted in outgrowths on the adaxial or abaxial leaf surfaces, respectively. To test whether ectopic outgrowth production was the result of genetic interaction between *AS2–REV* and *KAN1–KAN2* controlled pathways, we constructed *kan1-2 kan2-1 rev-6 as2-101* quadruple mutants. The plant stature of the quadruple mutant was very small (Fig. 5J), and ectopic outgrowths appeared on both adaxial and abaxial sides. Bars = 0.5 cm in (A, B, F, H, J), 1 mm in (C–E, G, I) and 500 μm in (K–M).

**Fig. 5** Phenotypes of *fil-1 yab3-2 as2-101, kan1-2 kan2-1 as2-101* and *kan1-2 kan2-1 rev-6 as2-101*. (A, B) Seedlings of the double mutant *fil-1 yab3-2* (A) and triple mutant *fil-1 yab3-2 as2-101* (B). An arrowhead indicates a lotus leaf which was not observed in *fil-1 yab3-2*. (C–E) One of the first pair of rosette leaves from *as2-101* (C), *fil-1 yab3-2* (D) and *fil-1 yab3-2 as2-101* (E). Note that the leaf of the *fil-1 yab3-2 as2-101* triple mutant is narrower than that of the *as2-101* single mutant but broader than that of the *fil-1 yab3-2* double mutant. (F, G) A seedling (F) and a rosette leaf (G) of the *kan1-2 kan2-1* double mutant, showing that outgrowths (arrowhead) had emerged on the abaxial leaf side. (H, I) A seedling (H) and a rosette leaf (I) of the *kan1-2 kan2-1 as2-101* triple mutant, with outgrowths (arrowhead) appearing on the outside surface of a lotus leaf (arrow). (J–M) A seedling (J) and rosette leaves (K–M) of the *kan1-2 kan2-1 rev-6 as2-101* quadruple mutant, with an adaxial view (K, L) and abaxial view (K, M) of rosette leaves, showing that ectopic outgrowths appeared on both adaxial and abaxial sides. ad, adaxial leaf side; ab, abaxial leaf side.
Discussions

AS1 and AS2 have been demonstrated to play critical roles in conferring leaf adaxial identity (Xu et al. 2003, Garcia et al. 2006). AS1 and AS2 can form protein complexes (Xu et al. 2003), which may regulate different downstream effectors or interact with other components that mediate formation of normal ad/abaxial leaf polarity. In the present study, we show more detailed analyses of as1/2 double, triple and quadruple mutants. We provide more genetic and molecular data to elucidate further the genetic interactions by which AS1 and AS2 cooperate with other leaf polarity components in the regulatory network for leaf polarity. Our data suggest that AS1 and AS2 negatively regulate the miRNA biogenesis. This hypothesis was supported by the dosage-effective HD-ZIP III gene action model. Since PHB and PHV levels were reduced in rev-6 as2-101 leaves (see Supplementary Fig. S4), the rev-6 as1-101 or rev-6 as2-101 double mutant should be equivalent to plants with loss of function in REV and with reduction of PHB and PHV transcript levels. The loss of function in AS1/AS2 resulted in an elevation of miR165/166 levels, which in turn partially repressed PHB and PHV. The same effect may have occurred with phb as1, phb as2, phv as1 and phv as2, in which these double mutants displayed enhanced as1 or as2 abnormalities in leaf polarity. Given that REV plays a major role in leaf adaxial fate (Prigge et al. 2005), the rev as1/2 double mutant exhibited more severe loss of adaxial identity than phb as1/2 and phv as1/2. Consequently, although the single mutants rev, phb and phv did not show leaf ad/abaxial defects, each of these mutations with as1 or as2 resulted in plants with more enhanced abnormalities in leaf adaxial identity.

Other possible mechanisms for the leaf polarity control

Plants with as2 mutation showed a small but detectable increase in the miR165 level (Li et al. 2005, Xu et al. 2006, and this work). However, leaves from wild-type plants carrying 35S::MIR165a contained a markedly elevated level of miR165 but did not show obvious ad/abaxial leaf abnormalities (Li et al. 2005, and this work). It is possible that the miR165 increase in as2 is developmentally more relevant to the leaf ad/abaxial polarity than that of 35S::MIR165 transgenic plants, as the increase in the as2 mutant may occur earlier in leaf development. This hypothesis is supported by a recent observation that PHB::MIR165a/Ler plants could produce the severely abaxialized leaves (Alvarez et al. 2006). Although miR165 content was increased in 35S::MIR165a/Ler transgenic plants, in terms of the spatial- and temporal-based dosages, the miR165 level might not be high enough to repress their target genes. Further reduction in the effective dosage of REV, PHB and PHV transcripts by introducing 35S::MIR165a into the rev as2 background (with reduced PHB and PHV levels in the rev mutant) would result in plants with more severely abaxialized leaves. Although 35S::MIR165a/rev transgenic plants also reflect reduced PHB and PHV levels in the rev background, the reduction of PHB/PHV transcripts by 35S::MIR165a may not be as efficient as that by as2 mutation.

Since AS1/AS2 and REV/PHB/PHV all encode putative transcription factors, our data also support an alternative model where AS1/AS2 independently regulate both MIR165/166 and REV/PHB/PHV expression. Although leaves of the 35S::MIR165a/Ler plants showed a dramatic reduction in transcript levels of class III HD-ZIP genes (Li et al. 2005), expression of these genes in leaf primordia of the transgenic plant is not known. If the 35S::MIR165a construct only produces very low miR165 dosages in the leaf primordium stages while AS1/AS2 genes in the transgenic plants can normally up-regulate the REV/PHB/PHV expression, leaf polarity in the transgenic plants should be less affected.

Our data also suggest that REV/PHB/PHV may also be required for MIR165/166 repression. In the rev single mutant, miR165 levels were slightly elevated, indicating that REV may negatively regulate MIR165. Although the increase of miR165/166 in rev was small, we found that this result was highly reproducible by three independent experiments (data not shown).
If the REV has a function to repress miR165 in planta, it may act slightly later than AS1/AS2 during leaf development, so that as1/as2 mutations show leaf polarity defects but rev does not. In the future, it should be of great interest to test these models further with additional experimental results.

The molecular mechanisms responsible for the similarity of rev as2 and rdr6 as2 leaf phenotypes

Our results revealed that phenotypes of rev-6 as2-101 and the previously reported rdr6-3 as2-101 were very similar. Both produced a high frequency of lotus- and needle-like leaves. Additionally, the adaxial surface of the expanded leaves was rippled and exhibited outgrowths on the adaxial surface, with some outgrowths continuing to form leaflets (Li et al. 2005). Interestingly, miR165/166 levels in rev-6 as2-101 and rdr6-3 as2-101 leaves were markedly different. In rev-6 as2-101, miR165/166 levels were only slightly increased in leaves, similar to those in as2-101 single mutant leaves, whereas in rdr6-3 as2-101, miR165/166 levels were dramatically elevated. Leaf phenotypes in rev-6 as2-101 and rdr6-3 as2-101 may both result from the same substantial reduction of the class III HD-ZIP transcripts. In rdr6 as2, the dramatically elevated endogenous miR165/166 levels strongly repressed all REV, PHB and PHV genes. It was proposed that REV/PHB/PHV and FIL act antagonistically, and the reduced REV/PHB/PHV activity in the rdr6 as2 double mutant may result in the increased FIL expression. In contrast, the rev as2 leaf phenotypes were caused by loss of function in the major adaxial-promoting factor REV, together with a reduction of the PHB and PHV dosages by as2, as mutation in as2 resulted in an increase in miR165/166 levels. This also promoted FIL mRNA levels, which mimicked the molecular environments of the rdr6 as2 double mutant.

Leaves producing outgrowths have been previously reported in the kan1-2 kan2-1 double mutant leaves (Eshed et al. 2001, Eshed et al. 2004). Unlike rev as2 and rdr6 as2 double mutants, from which outgrowths appeared on the adaxial leaf side, kan1-2 kan2-1 produced outgrowths on the abaxial leaf surfaces. It was previously proposed that the juxtaposition of adaxial and abaxial identity promotes outgrowths (Waites and Hudson 1995), and our results are in agreement with this hypothesis. In the abaxial leaf surfaces of rev as2 and rdr6 as2 or in the adaxial surfaces of kan1 kan2, leaf cell identities were normal. Therefore, no outgrowth was formed on the abaxial leaf side of rev-6 as2-101 and rdr6-3 as2-101 or on the adaxial leaf side of kan1-2 kan2-1. We further examined this hypothesis by longitudinal sectioning of the rev-6 as2-101 outgrowths, and found that all five outgrowths analyzed were sandwiched between the adaxial and abaxial cell patches (see Supplementary Fig. S5).

Genetic interaction analyses between AS1/AS2 and YAB and KAN genes

Our results revealed that fil yab3 as1/2 and kan1 kan2 as1/2 triple mutants had the additive leaf phenotypes of fil yab3 and as1/2 or kan1 kan2 and as1/2 mutant plants, indicating that AS1/2 did not interact genetically with FIL-YAB3 and KAN1-KAN2. However, our other data showed that the severe rev-6 as2-101 phenotypes could be partially suppressed by the fil mutation. PHB/PHV/REV and FIL-YAB3 are known to be expressed in the mutually exclusive domains (Eshed et al. 2001) and are adaxial- and abaxial-promoting genes, respectively (McConnell et al. 2001, Eshed et al. 2001, Emery et al. 2004). In the as2 single mutant, PHB/PHV/REV transcripts may be reduced to a certain level, at which the activity balance between PHB/PHV/REV and FIL-YAB3 is not dramatically affected. Thus the plants with fil, yab3 and as1/2 mutations only showed additive phenotypes. The severe leaf polarity phenotypes of rev as2 are caused by loss of function in a major player REV together with transcript reduction of PHB and PHV due to the as2 mutation. In this genetic background, the original balance between PHB/PHV/REV and FIL-YAB3 may be severely disrupted by suppression of PHB/PHV/REV, leading to a dramatic increase of FIL/YAB3 levels. Therefore, in the fil rev as2 triple mutant, fil mutation resulted in a partial rescue of the rev as2-caused phenotypes.

Our results, and those from others (Lin et al. 2003), also demonstrated by RT–PCR analyses that AS1/2 acted upstream of genes in the YAB and KAN families, although these results may reflect a change in tissue identity in loss- or gain-of-function AS1/2 lines. The AS1–AS2 protein complex appeared to be a critical repressor, as it is known to repress several important regulatory genes including BREVIPEDICELLUS, KNAT2, KNAT6, MIR165/166, FIL and KAN1 (Byrne et al. 2000, Semiarti et al. 2001, Lin et al. 2003, Li et al. 2005). A recent study showed that AS1 protein was able to bind HIRA, a homolog of the histone chaperone, that may function in chromatin remodeling for heterochromatic and euchromatic gene silencing ( Phelps-Durr et al. 2005). In the future, biochemical data would be important in further elucidation of the AS1–AS2 molecular mechanisms in the network of leaf ad/abaxial polarity establishment.

Materials and Methods

Plant materials and plant growth

The Arabidopsis as1-101 and as2-101 mutants in the Landsberg erecta (Ler) genetic background were generated as described previously (Sun et al. 2000, Sun et al. 2002, Xu et al. 2002). Seeds of rev-6 (Ler) were provided by S.E. Clark (University of Michigan, Ann Arbor, MI, USA), seeds of yab3-2, phb-6/phb-6 phv-5/phv-5 rev-9/+ and kan1-2/kan1-2 kan2-1/+ (all in the
Construction of double and multiple mutants

Homozygous as2-101 plants were crossed to homozygous rev-6, phb-6 and phv-5, respectively. F1 progeny of those crosses were all phenotypically normal. For the rev-6 and as2-101 cross, double mutants were recognized among the F2 plants as those with the novel phenotypes of leaves, with the F2 segregation of 256 wild-type, 92 as2-101, 95 rev-6 and 30 with novel phenotypes. To define these phenotypically novel plants, we crossed one of them to wild-type Ler. Among a total of 381 F2 plants analyzed, 214 are wild-type plants, 71 and 73 showed as2-101 and rev-6 phenotypes, respectively, and 23 displayed similar novel phenotypes to that used in the cross. These results suggested that these plants are rev-6 as2-101 double mutant. To verify that we had obtained rev-6 as2-101, we crossed five putative rev-6 as2-101 plants to rev-6 and as2-101 single mutants, respectively, and the rev-6 as2-101 × rev-6 and rev-6 as2-101 × as2-101 crosses yielded only rev-6 and as2-101 plants in the F1 progeny, respectively. In addition, we also crossed an additional rev allele, rev-9, to as2-101, and the F2 progeny showed a segregation of wild type: rev-9:as2-101:rev-9 as2-101 like novel plants in a 9:3:3:1 ratio, indicating that the phenotypically novel plants are indeed the rev as2 double mutant (see Supplementary Fig. S2). Homozygous phb-6 and phv-5 had no obvious phenotypic changes (Emery et al. 2003). Since both phb-6 and phv-5 mutations were caused by transposome Ds insertions, the mutant plants could be genotyped by PCR. In the F2 population of crosses phb-6 × as2-101 and phv-5 × as2-101, we PCR-analyzed individual as2-101-like plants, and identified phb-6 as2-101 using primers 5′-GGTTCCCGTCGGATTTCGACT-3′ (for Ds border), 5′-CTCCTCGGTTCTCTCTCTCCT-3′ and 5′-TATCTACACAATGATACG-3′, and phv-5 as2-101 using 5′-ACGGTCCGGAAAACAGCTCAG-3′ (for Ds border), 5′-GGTTCCCGGGCTCTCTCATCT-3′ and 5′-TGCTCTGAAAAACAGGTGGACT-3′ (Emery et al. 2003). The identified phb-6 as2-101 and phv-5 as2-101 double mutants were further PCR-verified in the F3 generation, in which plants showed no further segregation of as2 phenotypes from the Ds insertion.

For the fil-1 yab3-2 as2-101 triple mutant, we first obtained the fil-1 yab3-2 double mutant, which had the previously reported phenotypes (Kumaran et al. 2002). We then pollinated homozygous as2-101 pollen to homozygous fil-1 yab3-2 flowers. Among a total of 782 F2 plants analyzed, 12 showed the consistent novel phenotypes. These phenotypically novel plants, which were close to 1/64 of the total F2 plants, were considered to be the putative fil-1 yab3-2 as2-101 triple mutants. For kan1-2 kan2-1 as2-101, the homozygous as2-101 pollen was used to pollinate kan1-2/kan1-2 kan2-1/+ flowers. Seeds from individual F1 plants were collected, and F2 plants were grown by lines. One F2 segregating population (a total of 343 plants) contained five plants with novel phenotypes, close to a 63:1 ratio. These five plants were considered to be the putative kan1-2 kan2-1 as2-101 triple mutant. For the fil-1 rev-6 as2-101 triple mutant, the homozygous rev-6 as2-101 pollen was used to pollinate fil-1 plants. Seeds from six individual F2 rev as2-like plants were collected, and F3 plants were grown by lines. Two independent F3 populations contained 15 and 32 plants with similar novel leaf phenotypes together with 48 and 98 rev as2-like plants, respectively. The segregation ratio for these two F3 populations was close to 3:1, and therefore these phenotypically novel plants, which displayed less rippled rev as2 leaves and similar fil rev flowers (see Supplementary Fig. S3), were considered to be putative fil-1 rev-6 as2-101 triple mutants. For construction of kan1-2 kan2-1 rev-6 as2-101, the homozygous rev-6 as2-101 pollen was used to pollinate kan1-2/kan1-2 kan2-1/+ plants. Three separate F2 populations containing one, two and 19 plants with similar novel plant phenotypes were identified from a total of 358, 572 and 4,914 plants, respectively. The segregation is consistent with a 255:1 ratio and, therefore, these 22 plants were considered to be the putative kan1-2 kan2-1 rev-6 as2-101 quadruple mutants.

Chi-square analyses were carried out for the double, triple and quadruple mutants as shown in Supplementary Table S1.

35S::MIR165a transgenic plants

The 35S::MIR165a fusion was constructed and transformed into plants as described previously (Li et al. 2005). A total of 139, 82, 41, 10 and 61 transgenic lines were obtained from transforming wild-type, as2-101, rev-6, phb-6 as2-101, phv-5 as2-101 and rev-6 as2-101 plants, respectively. The transgenic plants with the most consistent phenotypes from each transformation experiment were verified by PCR using a 35S-specific primer (5′-GTCCTCTAAATGCCATCA-3′) and a primer matching the MIR165a precursor sequence (5′-AGAGGCATAACA TGTTGG-3′).

Real-time RT–PCR

Transgenic plants carrying the 35S::ASI fusion were generated as described previously (Sun et al. 2002). Total RNA was extracted as described previously (Huang et al. 1995), and reverse transcription was performed with 1 μg of total RNA using a kit (Fermentas, Vilnius, Lithuania). Real-time PCR was performed with Taqman probes (Sangon, Shanghai, China) for REV and FIL or with the double-stranded DNA-specific dye SYBR green (Shenyou, Shanghai, China) for PHV and PHV following the manufacturer’s instructions. Amplification was monitored in real time by a fluorometric thermal cycle machine, Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Taqman probes and other primers for real-time PCR were as follows: 5′-ACACCATCACAGAATCCAGCACAATACCG-3′ (exon2/exon3) for REV (Taqman probe), and 5′-GACCTTTAATCTCTCCCGCTATGT-3′ and 5′-CCTCTGTAAGATGGACAGTGT-3′ for ACTIN; 5′-TCCG AGCTGCCACTCCATTTGCATCTT-3′ (Taqman probe), 5′-AGA TGCTGGAGATGAAGCTCTAACCAGACACAG-3′ (exon4/exon5) for REV; 5′-CCACGTCCGG TCCACATTTATGTCCTGAT-3′ (exon4/exon5) for FIL; 5′-TGGAGAATGGAGACAC-3′ and 5′-TGTGGAGAATGGAGACAC-3′ (exon4/exon5) for PHV and 5′-GGTGAGGAGACACCGTG-3′ and 5′-GATGTTCAAGCTACGAGGAGACAC-3′ (exon4/exon5) for PHV (Williams et al. 2005). Quantifications of each cDNA sample were made in triplicate, and the consistent results from three separately prepared RNA samples were used. For each quantification, conditions were 1 ≥ E ≥ 0.80 and r2 ≥ 0.980, where E is the PCR efficiency and r2 corresponds to the correlation coefficient obtained with the standard curve. For each quantification, a melt curve was realized at the end of the amplification experiment by steps of 1°C from 60 to 99°C. Results were normalized to that of ACTIN.
MicroRNA filter hybridization, in situ hybridization and microscopy

The microRNA hybridization was performed according to our previous conditions (Li et al. 2005). In situ hybridization was performed with the antisense probe for the FIL gene (Li et al. 2005), according to a method described by Long and Barton (1998). Fresh leaves of plants were examined using a SZH10 dissecting microscope (Olympus, Tokyo, Japan), and photos were taken using a Nikon E995 digital camera (Nikon, Tokyo, Japan). Preparations of SEM were described previously (Chen et al. 2000).

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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