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REVIEW

Transcriptional, post-transcriptional and post-translational regulations of gene expression during leaf polarity formation

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Leaf morphogenesis requires the establishment of adaxial-abaxial polarity after primordium initiation from the shoot apical meristem (SAM). Several families of transcription factors are known to play critical roles in promoting adaxial or abaxial leaf fate. Recently, post-transcriptional gene silencing pathways have been shown to regulate the establishment of leaf polarity, providing novel and exciting insights into leaf development. For example, microRNAs (miR165/166) and a *trans*-acting siRNA (*TAS3*-derived tasiR-ARF) have been shown to repress the expression of several key transcription factor genes. In addition, yet another level of regulation, post-translational regulation, has been revealed recently by studies on the role of the 26S proteasome in leaf polarity. Although our understanding regarding the molecular mechanisms underlying establishment of adaxial-abaxial polarity has greatly improved, there is still much that remains elusive. This review aims to discuss recent progress, as well as the remaining questions, regarding the molecular mechanisms underlying leaf polarity formation.

***Keywords*:** *Arabidopsis*, leaf development, polarity formation

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Introduction

Leaves derive from primordia, which form in peripheral zone of the shoot apical meristem (SAM). After emerging from the SAM flanks, the leaf primordia establish the polarity along three major axes: the adaxial-abaxial, proximodistal, and mediolateral axes. Among these three axes, the adaxial-abaxial axis is of primary importance for the subsequent asymmetric growth of the leaf and lamina expansion [1, 2]. In *Arabidopsis*, several leaf characteristics define the normal adaxial-abaxial polarity. Firstly, epidermal pavement cells of the adaxial surface are relatively large and of uniform size, whereas the cells of the abaxial side are smaller and of non-uniform size. Secondly, mesophyll cells between the two epidermal cell layers constitute two distinct parts, the closely arranged adaxial palisade mesophyll cells and the less closely placed abaxial spongy mesophyll cells. Thirdly, the trichome density differs between the two surfaces, with a higher adaxial trichome density on early juvenile leaves. Finally, vascular structures of the leaf show anatomically distinct xylem and phloem distributions, with adaxial xylems and abaxial phloems.

In the 1950s, a series of surgical experiments carried out by Sussex established a conceptual framework for a hypothetical signal, now called the Sussex signal, which moves from the SAM to neighboring leaf anlagen to trigger adaxial leaf specification. Young leaf anlagen that had been surgically isolated from the SAM developed into radially symmetric structures, lacking adaxial differentiation [3, 4]. More recently, experiments using a more sophisticated laser ablation method strongly supported the existence of the Sussex signal, as destruction of the L1 cells between

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the SAM and the young anlagen also resulted in radially symmetric leaves [5].

Recent studies on leaf morphogenesis have led to significant progress toward understanding the genetic and molecular regulation of leaf polarity establishment. To date, five groups of transcription factors, two types of small RNAs and the 26S proteasome protein degradation machinery have been shown to modulate establishment of adaxial-abaxial polarity. Identification of these important regulatory components indicates that at least three levels of regulation help establish normal leaf polarity: transcriptional, post-transcriptional and post-translational. In this review, we describe recent progress in understanding formation of the leaf adaxial-abaxial axis, focusing on the model plant *Arabidopsis*, and discuss questions for future studies in this field.

Roles of putative transcription factors in leaf adaxial-abaxial patterning

A growing number of transcription factors have been shown to participate in the formation of leaf adaxial-abaxial polarity. These factors can be grouped into five functional categories.

Genes in the class III HD-ZIP family

Genes in the class III HD-ZIP family encode proteins containing a homeodomain (HD), a leucine zipper motif (ZIP) and a sterol/lipid-binding domain (START domain). Three members in this family, *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*) and *REVOLUTA* (*REV*), play roles in promoting leaf adaxial fate [6]. *PHB/PHV/REV* are expressed in the central region of the SAM and the entire young leaf primordia before the P2 stage, but their transcripts become adaxially localized in leaf primordia in and after this stage [7-9]. Dominant *phb*, *phv* and *rev* alleles lead to adaxialized trumpet- or rod-like leaves [2, 7, 9-12]. Although plants carrying a recessive mutation in one of these three genes do not result in leaf defects in adaxial-abaxial patterning, plants lacking functional copies of all three genes exhibit a dramatic phenotype, with an abaxialized, needle-like cotyledon distal to the hypocotyl [9, 13]. Since the PHB, PHV and REV proteins are predicted to possess a putative sterol/lipid-binding START domain, it was proposed that PHB/PHV/REV might act as receptors for a SAM-derived sterol/lipid ligand to initiate adaxial leaf differentiation [7]. Unfortunately, such a ligand has not yet been identified. In addition to the potential sterol/lipid binding function, a portion of the START domain-encoding sequence is complementary to that of two miRNAs, miR165 and miR166. These two miRNAs have been found to negatively regulate START-domain proteins by mediating transcript cleavage and degradation (see below).

ASYMMETRIC LEAVES1 and 2 (AS1 and AS2)

AS1 encodes a putative R2-R3 MYB domain transcription factor [14, 15], while *AS2* encodes a LATERAL ORGAN BOUNDARIES (LOB) domain protein containing a leucine-zipper motif [16-18]. AS1 and AS2 are able to bind to each other to form a complex [19], and may thus regulate the same downstream targets in leaf development. Consistent with this prediction, *as1* and *as2* mutants have similar leaf phenotypes. *AS1* transcripts have been detected throughout leaf primordia but not in the SAM [14]. In contrast, *AS2* transcripts have been detected in the adaxial side of cotyledons of embryo and may subsequently accumulate in the adaxial side of leaves [18]. Thus, the AS1-AS2 protein complex may form and function within the adaxial leaf domain. In the *Arabidopsis* Landsberg *erecta* accession, the first pair of rosette leaves in *as1* and *as2* mutants sometimes have petioles growing underneath the lamina, forming a lotus-leaf structure, which indicates a defective adaxial-abaxial polarity. However, this leaf phenotype is sensitive to genetic backgrounds and growth conditions [19, 20]. Interestingly, plants over-expressing *AS2*, either in *35S::AS2* transgenic lines or in the gain-of-function *as2* mutant *iso-2d*, exhibit adaxialized leaves [16, 19, 21], whereas over-expression of *AS1* does not perturb adaxial-abaxial polarity [15, 19, 22]. This observation suggests that the dose of the normally spatially restricted AS2 may be critical in determining the activity of the AS1-AS2 complex, and the role of AS1 in leaf polarity formation is largely dependent on its interaction with AS2.

AS1 orthologs have been found in a variety of plant species such as *Antirrhinum* [1, 23], maize [24, 25], tobacco [26], tomato [27] and pea [28]. The *Antirrhinum AS1* ortholog, *PHANTASTICA* (*PHAN*), was the first adaxial-abaxial polarity gene identified from the plant kingdom [1, 23]. Notably, in the eudicot species listed above, loss of function in the *AS1* orthologs leads to more severe adaxial-abaxial abnormalities than those in *Arabidopsis*, but the reason is unclear.

KANADI1 (KAN1), 2, and 3

KAN1, 2, and *3* encode putative GARP family transcription factors. These three genes are expressed in a domain complementary to that of *PHB*/*PHV*/*REV* in multiple tissues [9, 29-32]. Although *kan1* and *kan2* single mutant phenotypes are mild, *kan1 kan2* double mutants displayed adaxialized leaves with outgrowths appearing on the abaxial leaf surface [30]. Interestingly, *kan1 kan2 kan3* triple mutant showed even stronger phenotypes than *kan1 kan2* double mutant, indicating a redundant function(s) among these three proteins [33]. The abaxial outgrowths were

proposed to be caused by the appearance of ectopic adaxial cell patches on the abaxial leaf surface, leading to juxtaposition of adaxial and abaxial cells on the same leaf surface. These ectopic juxtapositions then proliferate and give rise to outgrowths [33]. Although the molecular mechanism of mutual suppression between *KAN1/2* and *REV*/*PHB*/*PHV* is not known, the complementary expression patterns of these two groups of genes might be very important in leaf adaxial-abaxial polarity establishment [9, 32].

AUXIN RESPONSE FACTOR3 and 4 (ARF3 and ARF4)

ARF is a plant-specific transcription factor family comprised of 23 members in *Arabidopsis* (for a review, see [34]). Functions of *ARF3* (also known as *ETTIN*) and *ARF4* in controlling leaf polarity were first revealed by the ability of *arf3* and *arf4* mutations to suppress the effect of *KAN1* over-expression [35]. In addition, *arf3 arf4* double mutants resemble *kan1 kan2* mutants, with adaxialized leaves and abaxial outgrowths, indicating that *ARF3* and *ARF4* are required for specifying leaf abaxial identity [35]. *ARF4* is expressed in the abaxial leaf domain, while high levels of *ARF3* are found in leaf anlagen, leaf margins, and in vascular bundles of older leaf primordia [35]. *ARF3*/*4* transcripts are targets of a *TAS3*-derived *trans*-acting short interfering RNA, tasiR-ARF [36], which guides the cleavage of *ARF3/4* mRNAs (see below).

FILAMENTOUS FLOWER (FIL) and YABBY3 (YAB3)

FIL and YAB3 belong to the YABBY transcription factor family, and contain a conserved zinc-finger domain and an HMG-like YAB domain [37, 38]. *FIL* and *YAB3* are both expressed in the abaxial leaf domain [38]. Both *fil* and *yab3* single mutants produce normal leaves, whereas *fil yab3* double mutants have narrow leaf laminae [38, 39]. Over-expression of *FIL* resulted in patches of ectopic abaxial cells on the adaxial leaf surface, indicating that *FIL* promotes abaxial cell fate [38]. In addition to the abaxial-promoting function, these *YAB* genes may also be involved in the outgrowths observed in *kan1 kan2* double-mutant leaves, as these outgrowths were accompanied by increased *FIL* expression [33].

Recent characterization of the *Antirrhinum YAB* gene *GRAMINIFOLIA* (*GRAM*) revealed a novel role for this gene in leaf development [40, 41]. *GRAM* is abaxially expressed and promotes lateral leaf growth and abaxial cell fate. However, *GRAM* also functions redundantly with the ubiquitously expressed *PHAN* to promote adaxial identity [40, 41]. In addition, GRAM has been shown to interact with adaxially localized STYLOSA (STY), a homolog of the *Arabidopsis* flowering regulator LEUNIG [41]. Interestingly, the YAB homolog in maize has been detected only in the adaxial leaf domain [42], which is opposite to the localization of *Arabidopsis* and *Antirrhinum* YABs. The divergent *YAB* expression patterns in different species suggest that *YAB* genes may serve as a polar responser, rather than a polar determinant.

miRNA165/166 and tasiR-ARF as endogenous regulators in modulating polarity controlling transcription factors

Recent studies have uncovered that small RNAs, including miR165, miR166 and tasiR-ARF, regulate several key leaf polarity transcription factors described above. miR165 and miR166 differ by only one nucleotide, and have been found to share near-perfect complementarity with a part of the START domain of class III HD-ZIP genes [43, 44]. miR165/166 repress *PHB*/*PHV*/*REV* mainly through mRNA cleavage [9, 45], and also promote DNA methylation of the *PHB* and *PHV* loci [46], likely leading to transcriptional silencing of these genes. In *Arabidopsis*, maize and tobacco, nucleotide changes in the START domain of several class III HD-ZIP genes result in dominant mutations, due to a loss of miR165- or miR166-mediated regulation [2, 7, 9-12]. These dominant alleles all lead to the development of adaxialized leaves with excessive accumulation of the corresponding class III HD-ZIP transcripts throughout the leaf after emergence of leaf primordia [7, 9-12].

Over-expression of *MIR165a* driven by the 35S promoter does not perturb adaxial-abaxial polarity, in spite of reduced class III HD-ZIP transcript levels [47]. It is possible that spatial and temporal regulation of the class III HD-ZIP genes by miR165/166 is very critical, and the expression pattern between the 35S promoter and the native miR165/166 promoters may not be identical [48]. For example, if the *35S::MIR165a* construct yields fewer miRNA at the critical P2 leaf stage but produces more at later leaf developmental stages, the leaf adaxial-abaxial polarity will not be markedly affected. Consistent with this hypothesis, miR165 levels were elevated in *PHB::MIR165* transgenic lines, while miR166 levels were elevated in a miR166g enhancer trap line [48, 49], and both led to perturbations of adaxial-abaxial leaf polarity. It is possible that the additionally increased miR165 and miR166 in these lines are accumulated to the regions where *PHB*/*PHV*/*REV* are expressed, so that the leaf adaxial-abaxial polarity of these lines is severely affected.

Analyses of miRNA165/166 expression by *in situ* hybridization have generated differing reports. In the embryo, miR166 (maybe including miR165) is initially detected in the abaxial domain, and then expands to the adaxial domain and cotyledon tips [49]. In leaf primordia, miR165 (maybe including miR166) has been reported to be detected either only in the abaxial leaf domain [50] or throughout the

entire leaf primordia [47]. Since several groups have successfully utilized the promoter-reporter fusion method and a recently developed locked nucleic acid (LNA) method to characterize miRNA expression patterns [51-53], these two methods together may reveal the true miR165/166 expression patterns in the future.

ta-siRNAs are 21- or 24-nucleotide regulatory RNAs, which were first discovered separately by two groups [54, 55]. In *Arabidopsis*, there are at least five genes (*TAS1a*, *1b*, *1c*, *TAS2* and *TAS3*) that are transcribed to produce ta-siRNAs via stepwise processes. ta-siRNAs act to repress cognate genes by a manner similar to miRNAs [54, 55]. To date, proteins identified to be involved directly in ta-siRNA production and activity include RDR6, DRB4, SGS3, DCL4 and AGO7 (also called ZIPPY) [54-57]. Plants with mutations in these genes all exhibit an accelerated vegetative phase change from juvenile to adult leaf [54, 57].

One ta-siRNA, tasiR-ARF (ta-siR1778 or ta-siR2142), which derives from the *TAS3* transcript, was found to direct the degradation of *ARF3* and *ARF4* mRNA; and in the *rdr6, sgs3, ago7* and *dcl4* mutants, levels of *ARF3* and *ARF4* transcripts were both elevated [56, 58-60]. Although *rdr6* exhibits only mild phenotypes, the mutation greatly enhances *as1* and *as2* phenotypes, producing severely abaxialized leaves [47]. Similarly, mutations of *sgs3, dcl4* or *ago7* also enhance the *as1/2* phenotypes, suggesting that the ta-siRNA biogenesis and action pathways are important for leaf patterning [60, 61], and that *AS1-AS2* and *TAS3* pathways may be partially functionally redundant. Interestingly, *arf3* was able to partially suppress *as1 ago7* phenotypes, as revealed by the analysis of *arf3 as1 ago7* triple mutant [60], indicating that the *as1 ago7* phenotypes are at least partially due to the increased *ARF3* transcript level in the *as1* background. *TAS3* is expressed in the adaxial leaf domain, suggesting that *TAS3* and its product tasiR-ARF regulate *ARF3/4* in this region [60]. Transgenic plants producing *ARF3* transcripts insensitive to tasiR-ARF resembled *as2* mutant [58, 59]; however, whether *ARF3/4* affect *AS1/2* expressions remains to be determined.

26S proteasome may target a regulator that promotes leaf abaxial identity

The involvement of the 26S proteasome in leaf adaxial-abaxial polarity formation identity was first discovered by characterization of an *as1/as2* enhancer mutation, *ae3* (*as1/as2 enhancer3*) [62]. The *ae3* single mutant exhibits weak abnormalities in adaxial-abaxial leaf polarity, with only a few earlier appearing rosette leaves being radially symmetric. *ae3 as2* double mutants exhibit very severe leaf phenotypes with most leaves lacking normal adaxial differentiation. *AE3* was identified to encode a 26S proteasome subunit, RPN8a.

The 26S proteasome is a highly conserved protein degradation complex in eukaryotes, consisting of about 31 subunits arranged into two subcomplexes: the 20S catalytic core particle (CP) and the 19S regulatory particle (RP) [63]. The CP can cleave peptide bonds, while the RP assists in recognizing and unfolding target substrates tagged with poly-ubiquitin chains, removing the chains, and in directing the unfolded polypeptides into the CP for degradation [63]. The RP can be further divided into two parts known as the base and the lid [64], and the RPN8a subunit that is disrupted in the *ae3* mutant is located in the 19S lid.

In addition to the critical role of the 26S holoenzyme in the degradation of ubiquitinated proteins, the 19S RP is also known to have protein degradation-independent roles in regulating gene expression in yeast [65-67]. To examine whether the protein degradation or gene expression regulation function of the 26S proteasome is involved in leaf polarity formation, Huang *et al*. analysed several double mutants by combining *as2* with 26S proteasome subunit mutations from the 19S lid and base, and 20S core complexes. All the double mutants exhibited comparable leaf phenotypes, with abaxialized leaves, although the severity of the phenotypes differed [62]. These results strongly suggest that the proteolytic function of the 26S proteasome is required for specifying leaf adaxial identity, possibly by targeting abaxial-promoting regulators or inhibitors of adaxial-promoting factors during leaf polarity formation. In the future, it will be important to incorporate knowledge of the role of the 26S proteasome into the known regulatory network by identifying targets that may participate in leaf polarity formation.

The Sussex signal

Microsurgical and laser ablation experiments on isolated incipient leaf primordia from the SAM have suggested the existence of the Sussex signal, an adaxializing signal originating from the SAM [3-5]. The nature of the molecule serving as the Sussex signal, however, has long been debated in the field of leaf development. During the past several years, three types of molecules have been proposed to be the Sussex signal candidates: (1) sterol/lipid, (2) miR165/166, and (3) tasiR-ARF. Because the START domain in PHB/PHV/REV appears capable of binding sterol/lipid and these three proteins are expressed and function in the adaxial leaf domain to specify leaf adaxial identity, it was proposed that an as-yet unidentified sterol/lipid may act as the signaling molecule [7]. A specific amino-acid residue substitution in the START domain of PHB/PHV/REV renders these proteins constitutively active, resulting in the formation of adaxialized leaves.

A more recent experiment showed that transgenic plants carrying a miR165-resistant *PHB*, harboring an altered nucleotide but not amino-acid sequence within the START domain, resembled the *phb* dominant mutants [68]. These results indicate that the miRNA binding sequence is crucial for regulating *PHB* function. Since small RNAs are known to be able to move between cells [69, 70], and miR165/166 and tasiR-ARF have been demonstrated to regulate leaf polarity-controlling transcription factors, they have been proposed as SAM-derived Sussex signal candidates [50, 60]. However, miR165/166 and tasiR-ARF are produced by leaf primordia as well. For example, the primary miR165/166 (pri-miR165/166) has been detected in leaves [71, 72], while *TAS3* transcripts are found in the adaxial domain of leaf primordia [60]. It seems unlikely that leaf primordia import small RNAs from the SAM while they themselves are able to produce these small RNAs.

Results from the miR165-resistant *PHB* experiment indicated that the wild-type START domain protein could cause adaxialized leaf phenotypes if the *PHB* mRNA accumulates to a certain level. However, the possibility that sterol/lipid molecules serve as the Sussex signal cannot be ruled out. If the level of the hypothetic sterol/lipid from the SAM is not a limiting factor for PHB binding and its subsequent function, both the constitutively active PHB (in the case of the *phb-d* dominant mutant) and the over-accumulated PHB (in the case of the miR165-resistant transgenic plant) should lead to the same adaxialized leaves. In the future, new ideas and methods will be necessary in identifying the Sussex signal molecule(s).

The regulatory network that modulates adaxial-abaxial leaf polarity

The establishment of adaxial-abaxial leaf polarity is a complex process involving many interactive pathways and regulations at different levels, including the transcriptional, post-transcriptional and post-translational levels. Among all components identified during leaf adaxial-abaxial polarity formation, transcription factors appear to be central to the polarity process. *PHB/PHV/REV* and *KAN1/2/3* act antagonistically, and exhibit complementary expression domains in multiple tissues. Mutual suppression between *PHB/PHV/REV* and *KAN1/2/3* should be very important in determining leaf polarity [9, 30, 32]. Similarly, the *AS1-AS2* and *ARF3-ARF4* pathways likely antagonize each other, as over-expression of tasiR-ARF-insensitive *ARF3* phenocopies the *as2* mutant [58], whereas over-expression of *AS2* partially phenocopies the *arf3 arf4* double mutant [16].

In normal leaf development, post-transcriptional regulation may provide a fine tuning for functions of the adaxial- and abaxial-promoting transcription factors. *RDR6/SGS3/DCL4/AGO7* related post-transcriptional gene silencing pathway is necessary for tasiR-ARF production, and this pathway together with the *AS1/AS2* pathway are important in suppressing miR165/166 [47]. These two classes of small RNAs in turn negatively regulate *PHB/PHV/REV* and *ARF3/ARF4* transcription factors, respectively. The regulation by which the adaxial- or abaxial-promoting transcription factors are controlled by small RNAs is very important, as alterations of these small RNA levels result in aberrant adaxial or abaxial polarity of leaves. The important role of small RNAs in leaf polarity has been highlighted by another layer of evidence. The *Arabidopsis* gene *ARGONAUTE1* is known to participate in both miRNA and ta-siRNA biogenesis, and in the *ago1* mutant both miRNA and ta-siRNA levels were dramatically reduced [55, 73]. Characterization of different *ago1* mutant alleles revealed that both adaxial and abaxial leaf identities are abnormal [50, 73-75], reflecting that both adaxial- and abaxial-promoting pathways are affected in these mutants.

How the 26S proteasome regulates leaf polarity is not yet clear. The 26S proteasome may not be directly involved in small RNA biogenesis and action, because *ae3 as2* double mutant leaves do not have markedly elevated levels of miR165/166, nor do they display a shortened vegetative phase [62]. Thus, a simple hypothesis is that the proteasome targets an abaxial fate-promoting protein. If this is true, the role of post-translational regulation is similar to that of post-transcriptional regulation, by which the spatial and temporal activities as well as the dose of polarity controlling transcription factors may be precisely balanced.

Perspectives

Although considerable progress has been made in elucidating the regulatory network involved in the establishment of adaxial-abaxial leaf polarity, new discoveries are always accompanied by new questions. How do polarity controlling transcription factors regulate their downstream targets and which genes are their targets? What is the true Sussex signal molecule? Numerous mutations have been identified with abaxialized-leaf phenotypes, but none have been shown to disrupt a pathway involved in the synthesis of a particular molecule that may serve as the Sussex signal. The past few years have witnessed the utility of genetic approaches to dissect the complex network of leaf adaxial-abaxial polarity formation. In the future, forward genetics together with new methods will undoubtedly provide exciting new insights into this field.

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