The three-domain model
A new model for the early development of leaves in Arabidopsis thaliana

Miyuki Nakata* and Kiyotaka Okada
National Institute for Basic Biology; Myodaiji-cho; Okazaki Aichi, Japan

Keywords: leaf development, blade outgrowth, cell differentiation, adaxial-abaxial patterning, the adaxial, middle and abaxial domains, WOX transcription factors, PRS, WOX1, the three-domain model, Arabidopsis thaliana

Blade outgrowth and region-specific cell differentiation are crucial events during the early development of plant leaves, and the progression of both of these events requires a normal adaxial-abaxial pattern. In a recent study, we had demonstrated that two WUSCHEL-RELATED HOMEBOX (WOX) family genes, i.e., PRESSED FLOWER (PRS) and WOX1, act redundantly in blade outgrowth and adaxial-abaxial patterning. During leaf development, the two genes are expressed in the domain between the adaxial and abaxial domains, designated “the middle domain.” Together with additional data, we recently proposed “the three-domain model” in which the middle domain is distinct from the adaxial and abaxial domains and plays a key role in blade outgrowth and the pattern formation of the three domains through the function of two WOX genes. In this report, we provide three additional results that reinforce our model: (1) an expanded pattern of abaxial-specific MIR165A expression in prs wox1, (2) a genetic interaction between the two WOX genes and adaxial-specific REVOLUTA gene in adaxial-abaxial patterning and (3) an altered expression pattern of the middle domain-specific marker, consistent with disruption of the adaxial-abaxial pattern.

One of the distinctive features of leaves is their flat and broad shape, enabling the perception of a significant amount of sunlight and efficient photosynthesis. The flat and broad shape of leaves results from blade outgrowth predominantly along the medio-lateral axis, and surgical experiments have demonstrated that blade outgrowth is closely related to the normal differentiation of both adaxial (upper) and abaxial (lower) tissues.1 Genetic analyses of model plants have identified the regulatory genes that determine the adaxial or abaxial cell fate,2 and these genes are also involved in blade outgrowth and margin development.3-6 Based on the leaf phenotype of the phamistatica mutant, which presents severely abaxialized leaves, Waites and Hudson proposed that the cells located at the boundary between the adaxial and abaxial domains proliferate predominantly in the lateral direction and form the flat and broad blade.6 However, it has remained unknown how blade outgrowth and margin development are induced at the adaxial-abaxial boundary or how the adaxial and abaxial cells interact with each other.

Recently, two groups reported that two WUSCHEL-RELATED HOMEBOX (WOX) genes (PRESSED FLOWER [PRS]/WOX3 and WOX1) act redundantly in blade outgrowth and margin development.7,8 Mutations of PRS and WOX1 reduce the width of leaves5-8 (Fig. 1A and B) and the number of dividing cells in the leaf primordia.8 Our results of RNA in situ hybridization analyses revealed that the expression of PRS and of WOX1 are restricted to the two middle mesophyll layers and the margin cells, which are located between the adaxial and abaxial sides.8 We have designated the PRS and WOX1 expression domain as “the middle domain.” The ectopic expression of WOX1 in the abaxial region leads to adventitious outgrowths and the formation of margin-like structures on the abaxial side of the leaves. Based on these results, we conclude that PRS and WOX1 are key regulators of blade outgrowth through the upregulation of cell proliferation and margin cell differentiation in the middle domain.

In our recent report, we had clarified that PRS and WOX1 are required for adaxial-abaxial patterning.8 Our analyses revealed that mutations of PRS and WOX1 induce the formation of tissues harboring mixed characters of both the adaxial and abaxial sides in the marginal region of leaves. In prs wox1 leaf primordia, the adaxial-specific AS2 gene and abaxial-specific FIL gene were coexpressed in the cells neighboring the margin. Furthermore, we examined the pattern of the adaxial-specific 35Spro:miYFP-W marker line9 in which the distribution of the abaxial-specific miR165/166 is visualized as a suppression of the intensity of YFP (yellow fluorescent protein) fluorescence. The YFP signal was limited to the medial region of the adaxial side in prs wox1 more than in the wild-type background, suggesting that PRS and WOX1 are involved in the specification of the domain in which miR165/166 represses the expression of their target genes, the adaxial-specific HD-ZIPIII gene family.8 Of the miR165/166 coding genes, the MIR165A gene is reported to show strong expression in leaf primordia, and...
the expression is detected specifically on the abaxial surface. To investigate whether PRS and WOX1 are required to restrict the MIR165/166 genes to the abaxial epidermis, in this study, we explored a GFP (green fluorescent protein) marker line of MIR165A (MIR165Apro:GFP) in both wild-type and prs wox1 backgrounds (Fig. 1C-F). The GFP fluorescence in wild type was detected on the abaxial domain and contribute to the specification of the middle domain. In this report, to confirm that the middle domain is specified in adaxial-abaxial patterning, we examined whether the severe defect of the adaxial-abaxial pattern is accompanied by the altered pattern of the middle domain. The KE1895 line is an enhancer-trap line in which GUS expression was linked to the WOX1 functional region. Together with our previous data, the middle domain-specific WOX genes would contribute to the adaxial-abaxial patterning of the marginal region of the leaf through the negative regulation of the adaxial and abaxial regulators, including AS2, FIL, and MIR165A.

We have also demonstrated that PRS and WOX1 are involved in the adaxial-abaxial patterning of the entire leaf primordia in coordination with adaxial- and abaxial-specific regulators. Mutations of PRS and WOX1 enhance abnormal patterning along the adaxial-abaxial axis in prs wox1 as2 and fil yab3. Here, we show that mutations of PRS and WOX1 enhance the defects of the revoluta (rev) mutant, which harbors a mutation of the adaxial-specific REV gene belonging to the HD-ZIPIII family. The leaves of the rev single mutant showed an almost normal phenotype (Fig. 2A), consistent with a previous report. In contrast, the prs wox1 rev mutant often forms radialized organs with no or few trichomes in the vegetative phase (Fig. 2B and C). Because trichomes are characteristic of the adaxial side, these results suggest that the radialized organs in prs wox1 rev are abaxialized leaves. This finding offers collateral evidence about our conclusion that PRS and WOX1 coordinate adaxial-abaxial patterning together with both adaxial factors (AS2 and REV) and abaxial factors (FIL/YAB and KAN).

Our data suggest that the middle domain is established within the context of adaxial-abaxial patterning. In P3-P6 leaf primordia, the middle domain, where PRS and WOX1 are expressed, does not overlap with the expression domains of adaxial- and abaxial-specific genes, except for FIL. We found that the abaxial-specific KAN genes repress PRS and WOX1 expression in the abaxial domain and contribute to the specification of the middle domain. In this report, to confirm that the middle domain is specified in adaxial-abaxial patterning, we examined whether the severe defect of the adaxial-abaxial pattern is accompanied by the altered pattern of the middle domain. The KE1895 line is an enhancer-trap line in which GUS expression was linked to the WOX1 functional region of wox1-101. The GUS signal was detected in the middle domain of the leaf primordia in wox1-101 heterozygous (Fig. 3A and D) and homozygous plants (Fig. 3B and E), and the GUS pattern was the same as the WOX1 expression, suggesting that the KE1895 line is a WOX1 enhancer-trap marker; we used this line as a middle-domain marker in the present study. The GUS expression of KE1895 in the prs wox1 background was found broadly in the marginal region of the leaf primordia in addition to the middle mesophyll cells (Fig. 3C and F), indicating that the middle domain is not missing even in the prs wox1 leaf. The GUS signal was faint in the leaf primordia of prs wox1 as2 (Fig. 3H) when compared with the normal pattern in wox1 as2 (Fig. 3G). The leaves of prs wox1 as2 were also severely abaxialized (Fig. 3I), indicating that the shrinking of the middle domain is associated
with severe abaxialization. These results support our idea that the establishment of the middle domain depends on the adaxial-abaxial pattern.

In the previous model based on two domains, including the adaxial and abaxial domains, (1) the antagonistic interactions between the regulators of the two domains are required for establishing the adaxial-abaxial pattern and (2) the juxtaposition of the adaxial and abaxial domains results in blade outgrowth and margin development at the adaxial-abaxial boundary.2,5,6 In our previous study,8 we proposed a new model of early leaf development, named “the three-domain model” based on three domains: the adaxial, abaxial and middle domains. The model contains three new concepts. First, the middle domain of the three-domain model is located between the adaxial and abaxial sides but does not overlap with the adaxial and narrowly defined abaxial domains compared with “the adaxial-abaxial boundary” of the previous model. Second, the adaxial-middle-abaxial pattern is established along the adaxial-abaxial axis via the antagonistic interactions among the adaxial, abaxial and middle domain-specific regulators (Fig. 4A and B). In fact, in our previous report8 and in this study, we showed the negative regulation between the middle-domain regulators (PRS and WOX1) and the adaxial and abaxial regulators (Fig. 4B). We also demonstrated the importance of the middle-domain regulators in adaxial-abaxial patterning through the analyses of genetic interactions. Furthermore, double mutations of PRS and WOX1 with or without the AS2 mutation have an opposite effect on the expression of the middle-domain marker, and these results are consistent with our idea that the middle-domain regulators are involved in the specification of the middle domain through the regulation of the three-domain structure. Third, the middle-domain regulators organize blade outgrowth and margin development (Fig. 4A). The adaxial and abaxial regulators indirectly affect these developmental processes through the arrangement of the middle domain (Fig. 4A). In prs wox1, the leaves lost the marginal feature, and their outgrowth along the medial-lateral axis was inhibited, even though the expression of the middle-domain marker was expanded, indicating that PRS and WOX1 play a central role in blade outgrowth and margin development in the middle domain. Nevertheless, a slow blade outgrowth was observed even in the prs wox1 leaves,8 implying that other regulators act in blade outgrowth from the middle domain redundantly with PRS and WOX1 and that these predicted regulators may compensate in the blade outgrowth of prs wox1 leaves. How the middle-domain regulators, including PRS and WOX1, promote blade outgrowth along the medial-lateral axis remains to be elucidated.

Extant seed plants generally form flat and broad leaves, whereas, based on fossil records, ancestral seed plants are proposed to have only branch-like organs.14 Thus, the acquisition of flat and broad leaves, resulting from blade outgrowth along the medio-lateral axis, is one of the most significant events in the evolution of seed plants. In our model, the middle domain-specific WOX genes induce blade outgrowth, and their expression domain is specified within the context of adaxial-abaxial patterning. Phylogenetic analyses reveal that the WUS subclade of the WOX family, which includes PRS and WOX1, has drastically diverged during the evolution of seed plants,15,16 corresponding to the acquisition of flat and broad leaves. How the interactions among the three domains were established and the middle domain-specific WOX genes acquired the ability to induce blade outgrowth along the medio-lateral axis during the evolution of seed plants would be a key problem to help solve the evolutionary process of flat and broad leaves.

Materials and Methods

The Arabidopsis thaliana accession Columbia (Col) was used as the wild-type material in this study. rev-2 and wox1-101 (KE1895 line) have been described in Nakata et al., 2012.8 rev-6,17 and MIR165Apro::GFP18 have been previously described.

The fluorescent images were captured using an LSM 510 microscope equipped with an META device (Carl Zeiss). The fluorescence of MIR165Apro::GFP was analyzed in cross-section, as previously described.18

The GUS staining was performed according to previously described methods.19 The samples were cleared and mounted using a clearing solution (chloral hydrate:glycerol:water, 8:1:2) and observed using an Axioplan2 microscope equipped with an AxioCam HR digital camera (Carl Zeiss). For sectioning, the stained seedlings were dehydrated in a graded ethanol series. The

Figure 2. Leaf phenotypes of rev and prs wox1. rev. (C) Close-up view of (B). Scale bars, 5 mm (A-B) and 1 mm (C).
infiltration and embedding in Technovit 7100 (Heraus Kulzer GmbH) was performed as instructed by the manufacturer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
We thank the ABRC for providing the *prs*-2 seeds, Satoshi Tabata (Kazusa, Japan) for providing the *wox1-101* seeds, Shunsuke Miyashima (University of Helsinki, Finland) and Keiji Nakajima (NAIST, Japan) for providing the *MIR165Apro:GFP* seeds and Kiyoshi Tatematsu (NIBB, Japan) and members of Kiyotaka Okada’s lab (NIBB) for their technical advice and helpful discussions. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry for Education, Culture, Sports, Science, and Technology (MEXT) of Japan (No. 19060004) and a Grant-in-Aid for Creative Scientific Research from the Japan Society for the Promotion of Science (No. 19GS0315) to K.O. M.N. was supported by a JSPS Fellowship from the Japan Society for the Promotion of Science (No. 20-2203).
Figure 4. Schematic views of the three-domain model and the interactions of regulators. (A) Schematic view of the three-domain model. Antagonistic interactions among the three-domain regulators result in a normal pattern of cell differentiation and blade outgrowth. (B) Schematic view of the reported interactions of the adaxial-, middle- and abaxial-specific regulators. The expressed factors are shown as black characters in each domain, and the repressed factors are shown as white characters.