The **SHOOT ORGANIZATION2** Gene Coordinates Leaf Domain Development Along the Central–Marginal Axis in Rice

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We describe how rice leaves are regionalized and regulated along the central–marginal axis. The **SHOOT ORGANIZATION2** (sho2) mutant, a weak allele of **SHOOTLESS4** that is a ZIPPY/ARGONAUTE7 homolog in rice, shows a variety of leaf abnormalities; filamentous leaves, bi- or trifurcated leaves, separation of the filamentous structure from the leaf blade or deletion of the margin. All of these phenotypes can be interpreted as combinatorial defects in the growth of the blade or deletion of the margin. The leaf founder cells for the lateral and marginal domains are recruited normally in sho2, indicating that sho2 is defective in the growth of leaf domains after the founder cells are recruited. The expression pattern of **SHO2** in the outer layer of the shoot apical meristem and the adaxial surface of the leaf, as well as the altered expression of **HD-ZIP III** and **ETTIN** homologs in the central domain of sho2 leaves, suggest that normal development of the central domain is a prerequisite for the synchronous growth of the three domains. This synchrony is thought to be mediated by a small interfering RNA-dependent process.

**Keywords:** Class III homeodomain leucine zipper — **ETTIN** — Leaf development — **Rice** — shoot organization mutant — trans-acting siRNA.

**Abbreviations:** **DL**, DROOPING LEAF; **ETT**, **ETTIN**; **HD-ZIP III**, class III homeodomain leucine zipper gene; **SAM**, shoot apical meristem; **SEM**, scanning electron microscopy; **SHL4**, **SHOOTLESS4**; **SHO2**, **SHOOT ORGANIZATION2**; siRNA, small interfering RNA; ta-siRNA, trans-acting siRNA.

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**Introduction**

The leaf is the most fundamental lateral organ in plants, although it shows a large morphological diversity among plant species. Leaves are repetitively produced from the shoot apical meristem (SAM), a group of indeterminate and pluripotent cells at the tip of the stem. Leaf shape depends on the activity of several developmental events: determination of leaf founder cells in the SAM, axis formation, establishment of the leaf domain identity and the subsequent growth.

The first event in leaf development is the recruitment of leaf founder cells on the flank of the SAM. This group of cells is referred to as the P0 leaf primordium. Molecular evidence for leaf founder cells is provided by the down-regulation of the class I **KNOTTED1**-type homeobox genes (**KNOX**), **KNOTTED1** in maize (Jackson et al. 1994), **SHOOT MERISTEMLESS** in **Arabidopsis** (Long et al. 1996) and **OSH1** in rice (Sentoku et al. 1999). The process of leaf founder cell recruitment contributes significantly to the final leaf shape in monocotyledons (Scanlon 2000).

The leaf is typically a flattened structure but is polarized along the proximal–distal, central–marginal and adaxial–abaxial axes. In both monocots and dicots, several genes responsible for adaxial–abaxial patterning have been identified. The **PHANTASTICA** (**PHAN**) gene, which encodes a MYB-like transcription factor, is an adaxial determinant in **Antirrhinum**, as revealed by the formation of needle-like abaxialized leaves by a severe **phan** mutant (Waites and Hudson 1995, Waites et al. 1998). Class III homeodomain leucine zipper (**HD-ZIP III**) gene family members regulate the adaxial identity of leaves in both monocots and dicots (Juarez et al. 2004a, Prigge et al. 2005). A triple loss-of-function mutant of three **HD-ZIP III** genes, **REVOLUTA**, **PHABULOSA** (**PHB**) and **PHAVOLUTA** (**PHV**), produces a single radial and abaxialized cotyledon during embryogenesis (Emery et al. 2003, Prigge et al. 2005). In contrast, gain-of-function mutants of **PHB** and **PHV** produce strongly adaxialized leaves (McConnell and Barton 1998, McConnell et al. 2001). Similarly, a dominant gain-of-function allele of the **HD-ZIP III** gene, **rolled leaf1** of maize, displays adaxialized leaf phenotypes (Juarez et al. 2004a). However, members of the **KANADI** (**KAN**) and **YABBY** gene families contribute to the specification of abaxial cell identity in lateral organs of **Arabidopsis** (Kerstetter et al. 2001, Eshed et al. 2004). **ETTIN** (**ETT**) and **AUXIN RESPONSE FACTOR4** (**ARF4**), both of which encode members of the auxin response factor family, are also
regulators of leaf abaxial identity (Pekker et al., 2005). The phenotype of the ett arf4 double mutant is similar to that of the kan1 kan2 double mutant (Pekker et al. 2005).

The above adaxial–abaxial patterning is closely associated with small RNA-mediated processes. The localized expression of HD-ZIP III and ETT/ARF4 is regulated by microRNA165/166 (miR165/166; McConnell et al. 2001, Emery et al. 2003, Juarez et al. 2004a) and the trans-acting small interfering RNA (ta-siRNA) production pathway, is required for the determination of adaxial characteristics in leaves (Nagasaki et al. 2007), indicating that a partial ETT/ARF4 regulates HD-ZIP III expression (Nagasaki et al. 2007). In the course of a cloning experiment, we showed that a partial ETT/ARF4 regulates HD-ZIP III expression and negatively regulates ETT/ARF4 expression (Nagasaki et al. 2007). Therefore, the HD-ZIP III and ETT/ARF4 homologs are possible downstream genes of SHL4/SHO2 in rice.

Here we analyzed sho2 leaf development and elucidated how the rice leaf is genetically and developmentally established. Our results suggest that SHL4/SHO2 plays a crucial role in leaf domain development through miR165/166-dependent gene regulation.

**Results**

**sho2 leaf phenotypes**

During the first 2 weeks after germination, sho2 seedlings rapidly produced filamentous leaves in an irregular phyllotaxy (Itoh et al. 2000). However, the abnormalities disappeared gradually, and apparently normal leaves were produced until about 1 month after germination (Fig. 1A, B). Interestingly, at the transition stage (2–4 weeks after germination), sho2 produced a variety of leaves with defective central–marginal patterning.

In the wild type, the leaf blade and sheath are separated by the ligule and auricle on the adaxial side (Fig. 1C), and the central, lateral and marginal domains grow synchronously (Fig. 1D). Cross-sections of the leaf blade show a large midrib (lacuna) and vascular bundles on the abaxial side in the central domain, and the adaxial–abaxial pattern in the lateral domains is obvious due to the presence of bulliform cells on the adaxial surface (Fig. 1E). In the sheath, the leaf margins are membranous, and vascular bundles are positioned on the abaxial side as in the blade (Fig. 1F).

In sho2, the synchronization of growth among the three domains along the central–marginal axis was frequently disrupted. The most severe phenotype of sho2 was filamentous leaves. Many of these leaves had no obvious blade–sheath boundary, although some filamentous leaves had a small ligule on the adaxial side and showed a sheath-like structure in the proximal region (Fig. 1G). Cross-sections of these leaves with no ligule revealed that they were radially symmetric and showed no obvious adaxial–abaxial polarity, including the vascular bundle (Fig. 1H). Irrespective of the presence of the ligule, the filamentous leaves had no flat structure (leaf lamina). The distal region of the filamentous leaf with a ligule (Fig. 1G) appeared radial, and there was no apparent boundary between the adaxial and abaxial surfaces (Fig. 1I). However, the vascular bundle was located on the abaxial side, and sclerenchymatous cells differentiated between the abaxial epidermal layer and the vascular bundle, as in the wild type (Fig. 1I). In the middle region of the leaf, a lacuna characteristic of the midrib formed in the center of the leaf (Fig. 1J). The adaxial epidermis in the sheath region was smooth and had no hairs (Fig. 1K), as on the adaxial surface of the wild-type leaf sheath (Fig. 1F).

Thus, the filamentous leaf with a ligule showed almost normal adaxial–abaxial polarity. These leaves formed due to the independent growth of the central domain from the lateral and marginal domains, whose growth...
Fig. 1  Phenotypic variation of sho2 leaves in the vegetative phase. (A) Wild-type plant 1 month after germination. (B) sho2 plant 1 month after germination. (C) Wild-type leaf. The ligule has formed at the boundary of the leaf blade and sheath (arrowhead). The inset is a close-up view of the ligular region. (D) Abaxial view of the leaf blade. The midrib is obvious as a central ridge. (E) Transverse section of a
was suppressed. The most frequently observed phenotype was the bifurcation or trifurcation of the leaf blade (Fig. 1L, N). Cross-sections of the bifurcated leaves revealed that one of the leaf fragments had a normal midrib on the edge, whereas the other fragment lacked a midrib (Fig. 1M). This phenotype indicates that one of the lateral (+ marginal) domains began to grow independently of the remaining domains, and synchronous growth resumed during the later stage. In an extreme case, trifurcation occurred throughout one leaf (Fig. 1O). In this leaf, two flap-like structures and a filamentous structure were independently inserted into the stem (Fig. 1P). This phenotype suggests that the central domain (filamentous structure) and two lateral (+ marginal) domains (flap-like structures) grew independently throughout the development of the leaf. Another unique phenotype observed was a separation of the filamentous structure from the center of the abaxial blade (Fig. 1Q). In this leaf, the filamentous structure showed the same anatomy as the filamentous leaf in Fig. 1I, and in the lamina the midrib (lacunae) formed normally in the central region, but there was no midvein (Fig. 1R). This phenotype could be interpreted as asynchronous growth of the tissue containing the midvein with the other domains. A mild manifestation of the sho2 leaf phenotypes was the deletion of one of the leaf margins in the sheath (Fig. 1S and T).

As described above, the sho2 leaf phenotypes were highly variable. All of the phenotypes could be interpreted as the result of asynchronous growth among the three domains or the deletion of one or two domains. However, it is clear that the growth of the central domain is predominant. Thus, the rice leaf comprises three central–marginal domains, the central, lateral and marginal domains, each of which has its own identity but is regulated hierarchically.

**Development of wild-type and sho2 leaf primordia**

To address how the abnormal leaf phenotypes of sho2 were associated with the development of leaf primordia, the early stages of leaf development were observed using scanning electron microscopy (SEM). In the wild type, a new leaf primordium emerges on the flank of the SAM and enlarges to form a crescent structure (Fig. 2A). The central region of the primordium, where a midrib will develop later, protrudes first. Following the protrusion of the central region, the other regions gradually grow and encircle the shoot apex, forming the hood-like structure (Fig. 2B). As the primordium enlarges in both length and width, the margins of the primordium overlap each other and the boundary of the leaf blade and sheath is established (Itoh et al. 2005). The pattern of leaf development in the wild type is stable throughout the vegetative phase.

In contrast, the developmental pattern of the sho2 leaf primordia varied with the developmental stage of the plant. During the first week after germination, when sho2 produced filamentous leaves, none of the primordia observed followed the distichous leaf arrangement and did not encircle the SAM (Itoh et al. 2000). At 2 weeks after germination, although the normal distichous phyllotaxy was restored, the morphology of the leaves remained filamentous and did not form a hood-like structure (Fig. 2C). In these leaves, only the central domain elongated, leaving small bulges surrounding the SAM (Fig. 2D). In leaf primordia that formed about 3 weeks after germination, a pair of bulges encircled the shoot apex and eventually began to grow independently of the filamentous structure (Fig. 2E). Judging from the position and growth manner, the filamentous structure corresponds to the central domain and the pair of bulges corresponds to the lateral and marginal domains of one primordium. In the later stage of leaf development, the lateral domains grew synchronously with the central domain, resulting in a bi- or trifurcated leaf (Fig. 2F). At this stage, filamentous and furcated leaves were sometimes both present. At the base of the filamentous leaf, leaf primordial tissue surrounded younger leaf primordia, indicating that lateral domains whose growth was suppressed were present on the leaf base (Fig. 2F).

The occurrence of filamentous and furcated leaves and the loss of leaf margins support the idea that along the central–marginal axis, the rice leaf is composed of three domains, each with its own identity. The above observations further indicate that all sho2 leaf primordia acquire...
entire domains at their incipient stage, and abnormal leaf phenotypes are derived from asynchronous growth of the three domains or suppressed growth of the lateral and marginal domains, although most of the asynchronous growth is limited to the early stage of leaf development. The results also suggest that the three domains interact hierarchically. Since only the central domain elongated alone, it probably plays a predominant role in leaf development. Isolated growth of the marginal domain was never observed; its growth was invariably coupled with that of the lateral domain. This shows that the marginal domain occupies the lowest position of the hierarchy.

Double mutant of sho2 and drooping leaf

To investigate the developmental interaction between the central and lateral domains, we constructed double mutants of sho2 and drooping leaf (dl) that showed the drooping leaf phenotype due to the lack of a midrib (Fig. 3A; Nagasawa et al. 2003). The DL gene is expressed in the central domain of the early leaf primordium, and is thought to specify midrib identity (Yamaguchi et al. 2004). The dl mutant is defective in proliferation of the specific cells in the central domain of the P3–P4 leaf primordia where a midrib will form, but show no abnormality in the early leaf development (Yamaguchi et al. 2004), indicating that the central domain of dl is normal and shows synchronous growth with the lateral domains. The seedlings of the sho2;dl double mutant showed a retarded growth and much longer plastochron than that of the single sho2 mutant in the early vegetative phase. Subsequently, the sho2;dl double mutants showed a more pronounced dwarf phenotype than the sho2 mutants, due to suppressed leaf elongation. However, with the exception of the midribless phenotype, the leaf shape abnormalities were much weaker in the double mutant than in the sho2 mutant throughout the vegetative phase. Filamentous leaves were not observed (Fig. 3B, C), and the frequencies of bifurcated and trifurcated leaves were considerably lower. Finally, the majority of the leaves of sho2;dl were normally shaped. In spikelets, sho2 produced lemmas with very long awns, but the sho2;dl double mutant produced relatively normally shaped lemmas with no awns (Fig. 3D, F). These results indicate that the dl mutation partially rescues the sho2 phenotype. To understand how DL functions in sho2 leaf development, we observed DL expression in sho2 leaf primordia. DL mRNA was normally observed only in the central domain, indicating that DL specifically acts in the central domain (Fig. 3F, G). These results indicate that DL expression is regulated independently from SHO2, but it enhances sho2 phenotypes. Thus, establishment of midrib identity is required for the distal growth of the central domain and interaction with the lateral domain.
Leaf founder cell recruitment in the sho2 SAM

To investigate how the leaf initiation events occurred in the sho2 SAM, we observed the expression pattern of the OSH1 gene as a marker for indeterminate cells in the SAM. OSH1 is normally expressed throughout the SAM except for the L1 layer, but is down-regulated in leaf founder cells, which form a ring-like domain on the flank of the SAM (Fig. 4A; Sentoku et al. 1999). In sho2 plants at 2–3 weeks after germination, when the plants were producing filamentous or furcated leaves, the OSH1 expression pattern in the SAM was similar to that in the wild type, with OSH1 down-regulated in a ring-like domain (Fig. 4B), indicating that all domains would form in the sho2 leaf primordium. Thus, the abnormal leaf morphology of sho2 is not caused by the abnormal recruitment of founder cells in the SAM.

Relationship of the sho2 leaf phenotype to the expression pattern of the possible downstream gene of SHO2/SHL4

SHO2/SHL4 is expressed in the outer layer of the SAM and the adaxial domain of leaf primordia during embryogenesis (Nagasaki et al. 2007). In the vegetative phase in the wild type, the expression pattern was similar to that in the embryo, although expression was detected in the xylem tissue of vascular bundles (Fig. 5A, B). HD-ZIP III and ETT/ARF4 are thought to be downstream genes of SHO2/SHL4 (Nagasaki et al. 2007). OSHB1, a rice HD-ZIP III gene, was expressed in the adaxial domain of leaf primordia and xylem tissue of the vascular bundle, as was SHL4/SHO2 (Fig. 5C, D). However, the expression patterns of the genes differed in the SAM. OSHB1 was expressed in the center and the leaf initiation site, whereas SHO2/SHL4 expression was observed in the outer layers (Fig. 5C, D, J). The expression of OsETT3, an ETT/ARF4 ortholog, was observed on the abaxial side of the leaf primordia and phloem tissue, but not in the SAM (Fig. 5E, F). These expression patterns suggest that OSHB1 and OsETT3 are competitively regulated along the adaxial–abaxial axis of the leaf, and SHL4/SHO2 may be involved in the adaxial–abaxial polarity of leaves.

In the leaf primordium of sho2, which shows separation of the central and the lateral domains, the OSHB1 expression pattern in the lateral and marginal domains was similar to that in the wild type (Fig. 5G). In the central domain, however, no adaxial expression of OSHB1 was detected, although expression was retained in the vascular bundles (Fig. 5G). The loss of adaxial expression was also observed in the most severely filamentous leaves (Fig. 5H), although some filamentous leaves retained reduced adaxial expression (Fig. 5I). In the shoot apex, OSHB1 expression in the P1 primordium was not significantly affected, but its expression in the center of the SAM was reduced (Fig. 6J, K).

OsETT3 transcripts accumulated abaxially in the lateral and marginal domains of sho2, as in the wild type (Fig. 5L). However, in severely filamentous leaves, OsETT transcript accumulation was detected throughout the
adaxial and abaxial domains of the leaves (Fig. 5M), although a polarized expression pattern was observed in some filamentous leaves (Fig. 5N). This indicates that the lateral and marginal domains retain adaxial-abaxial polarity, and a filamentous morphology of the central domain does not always represent the loss of the polarity. In the shoot apex, the abaxial expression in the central region of the P1 primordium was enhanced (Fig. 6A, B).

These results indicate that the sho2 phenotype is associated with a decreased level of OSHB1 expression in the central domain of the leaf and in the center of the SAM, and an increased level of OsETT3 expression in the central domain of the leaf. Thus, SHO2/SHL4 positively regulates HD-ZIP III expression and/or negatively regulates the expression of OsETTs in the central domain of the leaf.

Discussion

Leaf domains along the central–marginal axis in rice

The sho2 leaf phenotypes support the idea that grass leaves are composed of several semi-independent domains along a central–marginal axis (Freeling 1992, Scanlon et al. 1996). We further propose that the central domain is independent of the lateral domains, as shown by the filamentous and trifurcated leaves of this mutant. In dicots with simple leaf structures, such as Arabidopsis and Antirrhinum, despite the large number of mutants, there is little evidence that the leaves are composed of developmentally distinct domains along a central–marginal axis. In Arabidopsis, the pressed flower mutant shows a loss of the marginal regions in the adaxial and abaxial sepals (Matsumoto and Okada 2001). It has been proposed that tomato compound leaves are subdivided into the central blade and marginal domains along the transverse dimension (Kesseler et al. 2001). However, there is no example that shows a separated and independent growth of each leaf domain. Thus, the three distinct leaf compartments along a central–marginal axis and the developmental regulation among the domains are unique to monocots, or at least grass species.

Based on our observations, we can summarize the sho2 leaf phenotypes as follows. In the wild-type leaf, the three central–marginal domains grow synchronously (Fig. 6A, B). However, sho2 is defective in this synchrony. The filamentous leaves result from the independent growth of only the central domain, leaving the other domains as traces (Fig. 6C). The bi- and trifurcated leaves of sho2 are the result of the independent growth of each domain followed by synchronous growth (Fig. 6D). The leaves with a filamentous structure separated from the middle region are caused by the central domain growing independently of the other domains in the early stage (Fig. 6E). Suppression of the growth of one marginal domain gives rise to a leaf without a margin (Fig. 6F). Developmental independence of the leaf domains convincingly explains the variable sho2 leaf phenotype.

Double mutants of sho2;dl revealed that DL affects the growth of the central domain and coordinates growth between the central and lateral domains. One possible explanation for how the dl mutation affects the growth of leaf domains is that DL, which is expressed in the central domain, is involved not only in midrib formation but also in the growth of the central domain. In fact, sho2;dl showed suppressed formation of filamentous leaves and growth of the awn, which is equivalent to the central domain of the leaf, indicating that DL is a positive regulator of central domain growth. In the single dl mutant, normal lateral domains may compensate the defects in distal growth of the central domain. In sho2;dl, the distal growth of both the central and lateral domains was suppressed, resulting in
shortened leaves with normal central–marginal patterning. Considering that DL is expressed in the central domain, which is growing singly in the sho2 leaves, the central domain may be an inducer of lateral domain growth, and DL and SHO2 interact during leaf development. DL encodes a protein of the YABBY family and is involved in midrib formation by promoting cell proliferation in the central region of the leaf (Yamaguchi et al. 2004). YABBY genes act as abaxial determinants in Arabidopsis, but also probably act as regulators of the outgrowth of lateral organs in Arabidopsis and maize (Eshed et al. 2004, Juarez et al. 2004b, Henderson et al. 2005). Although it is unknown whether genetic interactions exist between YABBY and ETT/ARF4 homologs in rice, it has been reported that the ETT/ARF4 genes positively regulate FIL, another member of the YABBY gene family in Arabidopsis (Garcia et al. 2006). OsETT3 is up-regulated in the central domain of the sho2 leaf, and the domain where DL is expressed partially overlaps that of OsETT3. Thus, it is also possible that the function of DL is enhanced in the central domain of sho2 leaves. Although the three domains of the rice leaf are under genetically distinct developmental control, the central domain plays an important role in leaf development.

sho2 leaf morphology is independent of the distribution of leaf founder cells in the SAM

It is thought that several distinct processes take place during the early development of grass leaves. The first process is the recruitment of leaf founder cells in the SAM, and the second process is the proliferation of leaf founder...
cells to form the P1 primordium. Several mutants associated with the first and second processes have been reported in maize. In the maize ns mutants, which produce narrow leaves, the KNOX protein accumulates in an area of leaf founder cells corresponding to the pre-marginal region of the leaf (Scanlon et al. 1996). Similarly, in the maize rough sheath2 (rs2) mutant, which displays semi-bladeless and bladeless leaves, the leaf founder cells are reduced and incompletely encircle the SAM (Schneeberger et al. 1998). lbi1 and a similar mutant, rgd2, which forms narrow or radial leaves, show incomplete down-regulation of the KNOX protein in the SAM (Timmermans-Marja et al. 1998, Henderson et al. 2005). The abnormal leaf shape in these mutants is primarily caused by defects in the first and/or second processes, although additional developmental defects in each mutant have been proposed.

Although sho2 leaves are phenotypically similar to lbi1 and rgd2 leaves, sho2 leaves are not likely to be caused by a reduction in the leaf founder cells, because the down-regulation of OSH1 occurs normally around the sho2 SAM, and small bulges encircling the SAM were consistently observed in furcated and filamentous sho2 leaves. Thus, the abnormal leaf morphology of sho2 is caused by a defect in the subsequent growth after founder cells are recruited normally. This also means that the distribution pattern of leaf founder (determinate) cells in the SAM does not always predict the final leaf shape. Therefore, SHO2/SHL4 is involved in a third process of leaf development, a growth trigger, which has not been postulated previously.

SHO2 partially regulates leaf polarity

Filamentous sho2 leaves frequently show defects in adaxial–abaxial polarity. In filamentous leaf primordia, the adaxial expression of OSHB1 is lost or reduced from the adaxial side of the central domain. In contrast, that of OsETT3 is greatly up-regulated on the adaxial side of the central domain. Thus, the filamentous leaf (central domain) develops as an abaxialized structure, although some filamentous leaves retain normal adaxial–abaxial polarity. Because the SHL4/SHO2 gene is expressed in a domain complementary to that of OsETT3, the most conceivable explanation for the polarity defect in the central domain is a reduced level of ta-siR-ARF on the adaxial side, causing the overaccumulation of OsETT3 mRNA. Alternatively, an unknown ta-siRNA produced by SHO2-related components positively regulates OSHB1 expression by directing the precursors of miR166 in the adaxial domain of the leaf. In contrast to the central domain, the lateral domain shows an almost normal expression of OSHB1 and OsETT3, and thus retains normal polarity. Accordingly, SHL4/SHO2 regulates adaxial–abaxial polarity only in the central domain, although it is expressed on the adaxial sides of all domains.

SHO2/SHL4 regulates coordination among the leaf domains through siRNA-dependent gene regulation

The abnormal phenotype of sho2 strongly indicates that SHO2/SHL4 is involved in the coordinated growth of leaf domains. However, strong alleles of SHL4 show a complete loss of the SAM during embryogenesis, and variable leaf phenotypes of sho2 were only observed during the transition stage from the early to the late vegetative development. The variation of phenotypic severity with developmental stages and alleles could be explained by quantitative and temporal regulation of endogenous small RNAs in plants. The expression level of some miRNAs (e.g. miR156) varies with phase transitions in the vegetative phase (Chuck et al. 2007, Wu and Poethig 2006). Furthermore, the loss-of-function mutant of ZIP that is the SHO2/SHL4 homolog in Arabidopsis shows a heteroblastic effect, although ZIP is expressed constitutively during the vegetative phase (Hunter et al. 2003). Thus, the phenotypic severity of sho2 may reflect the amount and/or importance of SHO2/SHL4-mediated small RNAs on each developmental stage.

An important question to be addressed regarding leaf development is how the coordination of the three domains is regulated. The expression of HD-ZIP III is lower in sho2 seedlings due to the overproduction of miR166 (Nagasaki et al. 2007). Introduction of an miR166-resistant version of one of the OSHB1 genes into sho1, a mutant of another component of the ta-siRNA production pathway, partially rescued the phenotype (Nagasaki et al. 2007). Accordingly, HD-ZIP III genes are possible downstream genes, and a reduction in their expression is a possible cause of the sho2 leaf phenotype. On the other hand, the ETT/ARF4 genes are post-transcriptionally regulated by a TAS3-derived ta-siRNA in Arabidopsis (Allen et al. 2005, Adenot et al. 2006, Fahlgren et al. 2006, Garcia et al. 2006, Hunter et al. 2006). SHO2/SHL4 is an ortholog of ZIP/AGO7, which acts in the ta-siRNA production pathway (Nagasaki et al. 2007). Thus, orthologs of the ETT/ARF4 genes in rice would be direct targets of SHO2/SHL4 through ta-siR-ARF.

In sho2 filamentous leaves, adaxial–abaxial polarity was frequently impaired. However, the bi- or trifurcated leaves and margin-deleted leaves retained normal adaxial–abaxial polarity. Thus, adaxial–abaxial polarity in the central domain probably does not regulate coordinated growth among the central–marginal domains. Similarly, alteration of gene expression in the SAM is probably not related to the abnormal phenotype in the central–marginal direction, because OSHB1 expression was not altered in the peripheral region of the SAM where the leaf founder cells differentiate, and OsETT3 expression was not detected in the SAM.

Independent growth of the central domain is commonly observed in sho2 leaves. The altered expression of
OSH1 and OsETT3 in the central domain appears to be involved in this phenotype, as well as in adaxial–abaxial polarity. How do the three domains grow asynchronously in sho2? One plausible explanation is that the isolated growth of the central domain at the early stage fails to induce growth of the lateral domains. This is consistent with the fact that the dl mutation partially suppressed the asynchronous growth of leaf domains in sho2, i.e. the growth of the central domain greatly affects that of the lateral domains. In the wild-type young leaf primordia, normal development of the central domain would allow a mobile signal that is necessary for the proper growth of the lateral domains to emanate from the central domain to the lateral domains. Considering that SHO2/SHL4 is involved in the pathway that produces 21 nt siRNAs, which can move as cell-to-cell silencing signals (Dunoyer et al. 2005), reduced production of siRNAs in sho2 may cause the altered expression of target genes such as OSH1 and OsETT3 in the central domain, resulting in defective siRNA-mediated signaling to the lateral domains. This explanation is consistent with a hierarchy in which the central domain is essential for the growth of the lateral domains and most of the asynchronous growth is limited to the early leaf development.

In summary, the rice leaf is composed of three hierarchically regulated domains, of which the central domain has primary importance. Our results also suggest that coordination among the leaf domains is mediated by a novel signaling mechanism that involves siRNA-dependent gene regulation.

Materials and Methods

Plant materials

The sho2 mutant of rice (Oryza sativa L.) (Itoh et al. 2000) was used. For the double mutant analysis, we used the dl-2 allele, a weak allele of dl (Nagasawa et al. 2003). Mutant and wild-type seeds were sterilized in 2% sodium hypochlorite and germinated at 28°C on MS medium (pH 5.8) supplemented with 3% sucrose and 1% agar. For long-term cultivation, mutant plants were transplanted into pots 1 month after germination.

Histology

For paraffin and plastic sectioning, leaves and shoot apices at 2–4 weeks after germination were fixed in FAA (formalin-glacial acetic acid: 70% ethanol = 1:1:18), and dehydrated in a graded ethanol series. For paraffin sectioning, the tissues were embedded in Paraplast Plus (McCormick Scientific, St. Louis, MO, USA) after substitution with xylene and cut in 10 μm sections with a rotary microtome. For plastic sectioning, the tissues were embedded in Technovit 7100 resin ( Heraeus Kulzer, Germany) and cut into 3 μm sections. The sections were stained with 0.05% toluidine blue–O and observed with a light microscope (Olympus AX-80, Tokyo, Japan). For SEM observations, samples dehydrated in 100% ethanol were infiltrated with 3-methyl-butylacetate, critical-point dried, sputter-coated with platinum, and observed under an SEM (S-4000; Hitachi, Japan) at an accelerating voltage of 10 kV.

In situ hybridization

Shoot apices were fixed in FAA for about 20 h at 4°C. Next, they were dehydrated in a graded ethanol series, replaced with xylene, and embedded in Paraplast Plus (McCormick Scientific, St. Louis, MO, USA). Microme sections of 8 μm were placed on slide glasses coated with Vectabond (Vector Laboratories, Burlingame, CA, USA). DIGoxigenin-labeled antisense probe was prepared from the full-length rice OSH1 cDNA. The OSH1 probe was prepared as described (Nagasaki et al. 2007). For the OsETT3 probe, we first searched for rice homologs of ETI1/ARF4 in the public databases of the DNA Data Bank of Japan and the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Four ETI homologs were identified in the rice genome, all of which have two conserved tasiARF-binding sites (data not shown). A full-length cDNA for one of the ETI homologs (AK072330) was obtained from the rice genome resource center (http://www.rgrc.dna.affrc.go.jp/). An antisense RNA probe for OsETT3 was prepared from a vector containing the full-length cDNA for AK072330. The DL probe was prepared as described (Yamaguchi et al. 2004). In situ hybridization and immunological detection of the signals were carried out according to the methods of Kouchi and Hata (1993).

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