Establishment of adaxial-abaxial polarity is essential for lateral organ development. The mechanisms underlying the polarity establishment in the stamen remain unclear, whereas those in the leaf are well understood. Here, we investigated a rod-like lemma [rol] mutant of rice (Oryza sativa), in which the development of the stamen and lemma is severely compromised. We found that the rod-like structure of the lemma and disturbed anther patterning resulted from defects in the regulation of adaxial-abaxial polarity. Gene isolation indicated that the phenotype was caused by a weak mutation in SHOOTLESS2 (SHL2), which encodes an RNA-dependent RNA polymerase and functions in trans-acting small interfering RNA (ta-siRNA) production. Thus, ta-siRNA likely plays an important role in regulating the adaxial-abaxial polarity of floral organs in rice. Furthermore, we found that the spatial expression patterns of marker genes for adaxial-abaxial polarity are rearranged during anther development in the wild type. After this rearrangement, a newly formed polarity is likely to be established in a new developmental unit, the theca primordium. This idea is supported by observations of abnormal stamen development in the shl2-rol mutant. By contrast, the stamen filament is likely formed by abaxialization. Thus, a unique regulatory mechanism may be involved in regulating adaxial-abaxial polarity in stamen development.

INTRODUCTION

Lateral organs, such as leaves and floral organs, develop from the flank of the meristem. Establishment of adaxial-abaxial polarity is essential for the proper development of the lateral organs in plants (Husbands et al., 2009). In Arabidopsis thaliana, HOMEODOMAIN-LEUCINE ZIPPERIII (HD-ZIP III) genes, such as PHABULOSA (PHB), are required for specification of adaxial cell fate, whereas KANADI (KAN) and YABBY genes are involved in that of abaxial cell fate (Siegfried et al., 1999; Bowman, 2000; Kerstetter et al., 2001; McConnell et al., 2001). Small RNAs, such as miR166, act as the abaxial determinant by suppressing HD-ZIPIII function through posttranscriptional regulation (Emery et al., 2003; Kidner and Martienssen, 2004). AUXIN RESPONSE FACTOR3 (ARF3; also known as ETTIN [ETT]) and ARF4 are required for abaxial identity (Pekker et al., 2005; Garcia et al., 2006). In snapdragon (Antirrhinum majus), PHANTASTICA plays a crucial role in adaxial cell fate (Waites and Hudson, 1995; Waites et al., 1998).

Genes encoding proteins that function in the biosynthesis of trans-acting small interfering RNA (ta-siRNA) are likely to be also involved in the control of adaxial-abaxial polarity in lateral organs (reviewed in Chitwood et al., 2007; Busbaker et al., 2007; Voinnet, 2009). ta-siRNAs are derived from TAS transcripts, which are first processed by microRNA-guided cleavage and transcribed by RNA-DEPENDENT RNA POLYMERASE6 (RDR6) together with the zinc-finger protein SUPPRESSOR-OF-GENE-SILENCING3 (GSG3) (Yoshikawa et al., 2005). The resulting double-stranded RNAs are processed into 21-nucleotide ta-siRNAs by DICER-LIKE4 (DCL4). The ta-siRNAs are then loaded into an RNA-induced silencing complex containing ARGO-NAUTE (AGO) protein and function in the cleavage of target mRNA (Allen et al., 2005; Xie et al., 2005; Yoshikawa et al., 2005). The abaxial determinants ETT and ARF4 are targeted by a class of ta-siRNAs, termed ta-siR2141 and ta-siR2142 (so called tasiR-ARF), produced from TAS3 (Allen et al., 2005). The initial step of TAS3 RNA cleavage requires a unique AGO protein, AGO7 (Montgomery et al., 2008).

In grasses, such as rice (Oryza sativa) and maize (Zea mays), genes involved in the ta-siRNA pathway, in addition to genes homologous to Arabidopsis KAN and HD-ZIP III (Juarez et al., 2004; Candela et al., 2008; Zhang et al., 2009), seem to have essential roles in the regulation of adaxial-abaxial polarity in leaf development (reviewed in Chitwood et al., 2007; Busbaker et al., 2009). Mutants of rice shoot organization1 (sho1), sho2, and maize leaf bladeless1 (lbr1) form abnormal shoots, in which the adaxial identities of the leaves are partially compromised (Timmermans et al., 1998; Itoh et al., 2000). Rice SHO1 and SHO2 encode proteins closely related to Arabidopsis DCL4 and AGO7, respectively (Liu et al., 2007; Nagasaka et al., 2007). Maize
**The rol Mutation Is Associated with Loss of Adaxial-Abaxial Polarity in the Lemma**

We isolated a recessive mutant, named *rol*, which showed pleiotropic phenotypes in both the inflorescence (panicle) and spikelet (Figure 1; see Supplemental Figure 1 online). In a *rol* panicle, the branching pattern was disturbed and the number of spikelets was decreased (see Supplemental Figure 1 online). In some cases, a branch terminated without developing spikelets by forming a dome-like structure at its end. In *rol* spikelets, morphological defects were observed in the lemma, palea, and stamen, and a lack of organs or reduction in their number was also observed.

A prominent phenotype of *rol* spikelets was a needle-like structure at the lemma position (Figures 1B and 2A, Table 1). The needle-like structure seemed to be divided into two parts along the distal-proximal axis. In the distal part, the epidermal surface of the needle-like structure resembled those of the awns formed in Kasalath, an *indica* variety (the awn is reduced in most *japonica*) (Figure 2). By contrast, the proximal part showed morphological features resembling those of the abaxial surface, rather than the adaxial surface, of the lemma (Figures 1G to 1I, 2C, and 2F); that is, convex cells (tubercles) and many trichomes were formed in both the lemma and the proximal part of the needle-like structure. Cells similar to those at the adaxial surface in the wild-type lemma were not observed in this needle-like structure. A cross section of the proximal part of the needle-like structure showed radial symmetry. The epidermal cells were arranged in a circular shape, and their morphologies were similar to those of abaxial cells of the wild-type lemma (Figure 1K). These observations suggested that the proximal part of the needle-like structure corresponded to a lemma lacking adaxial identity (i.e., rod-like lemma). In addition to the needle-like structure, the lemmas of *rol* exhibited aborted development and an elongated awn (Figures 1C and 1D, Table 1).
Next, we examined the spatial expression patterns of marker genes for adaxial-abaxial polarity. We used *ETT3* (Os01g0753500), which is an ortholog of Arabidopsis ETT and is known as OsETT3 (Sato et al., 2001; Nagasaki et al., 2007), and *PHB3* (Os03g0640800), which is closely related to Arabidopsis PHB and was temporarily named OSHB3 (Zhong and Ye, 2004). In the wild-type lemma, *ETT3* and *PHB3* were expressed in some parts of the abaxial and adaxial sides, respectively, and in vascular bundles (see Supplemental Figure 2 online). In the *rol* mutant, however, *ETT3* was expressed in all tissues of the rod-like lemma (Figure 1L). On the other hand, *PHB3* expression was not detected in the epidermal and subepidermal tissues but was restricted to the vascular tissue (Figure 1M). Taken altogether, these analyses of the rod-like lemma suggest that the *rol* mutant has a defect in adaxial-abaxial polarity, and the lemma is abaxialized in this mutant.

**rol** Mutation Disturbs Anther Patterning

In the wild type, the anther has a bilaterally symmetrical structure and consists of two thecae, each of which contains two pollen sacs (Figures 3A, 3E, and 4A). The thecae are joined by the connective.

In the *rol* mutant, a reduction in the number of pollen sacs and abnormal positioning of the thecae were observed. The phenotypes of *rol* stamens were classified into three types in addition to the wild-type stamen (Table 2). The first type of *rol* stamen, the most severe phenotype, produced no pollen sacs, and the distal end of this stamen had a pin-like structure (pin-like type; Figures 3B and 3F). The second type of *rol* stamen had only two pollen sacs, which often curled (Figures 3C and 3G). These two pollen sacs seemed to correspond to a theca. A putative connective tissue was observed at the opposite side of the theca. Therefore, it is likely that this type of stamen consists of one theca and a connective (one-theca [OT]-type). The third type of *rol* stamen had an abnormal arrangement of pollen sacs, although four pollen sacs were produced (Figures 3D and 3H). In this type, all pollen sacs (two thecae) were located on the adaxial side of the stamen (adaxially localized two-thecae [ATT]-type). We did not observe a stamen with one or three pollen sacs in *rol* spikelets (Table 2). Therefore, these results suggest that the theca is a developmental unit of the anther.

Next, we compared stamen primordia at an early developmental stage. In the wild type, stamen primordia were found to be round in the early stages of development and subsequently became rectangular. The four corners of the rectangular stamen...
Became rectangular, PHB3 transcripts disappeared from the adaxial domain and were detected in the two lateral domains of the stamen primordia (Figures 4K and 4L, arrows). The PHB3 expression domain corresponded to the region between the pollen sacs within a theca in the mature anther. PHB3 transcripts were also detected in the procambium in the stamen primordia (Figures 4J to 4L). Thus, these results indicated that the spatial expression patterns of two marker genes, ETT1 and PHB3, were markedly changed and rearranged during anther patterning in the stamen primordia.

To confirm this change, we analyzed the spatial expression pattern of the two marker genes by two-color in situ hybridization, which made it possible to detect expression of the two genes simultaneously at a chosen developmental stage. The results revealed that the rearrangement of the expression patterns of the two genes was completed before the appearance of the four corners in the stamen primordium (Figures 4M to 4O). PHB3 transcripts (red) were detected in the two lateral domains and in the procambium. At the same stage, ETT1 transcripts (purple) were detected in both the adaxial and the abaxial domains of the stamen primordium. The results also indicated that the four corners became obvious at the regions between the expression domains of ETT1 and PHB3 (Figures 4M to 4O, arrowheads).

Taken together, these results indicate that rearrangement of the expression patterns of the two marker genes takes place before morphological changes of the stamen primordia, from the round shape to the rectangular shape. Therefore, it seems that the cell fates along the adaxial-abaxial axis are markedly changed during anther patterning. In addition, formation of the protrusions in the anther primordia seems to be closely associated with the boundaries between the expression domains of the two marker genes.

In the presumptive region where the filament would differentiate, ETT1 was expressed in the epidermal and subepidermal cell layers and in the procambium (Figure 4Q), whereas PHB3 expression was restricted to the procambium (Figure 4R). This result suggests that the filament may develop without adaxial identity.

**Spatial Expression Patterns of ETT1 and PHB3 in the rol Stamen**

In the rol mutant, the expression domain ETT1 was expanded widely in the stamen primordia during the early round stage (Figure 5C) compared with the wild type (Figure 4F). This result suggests that rol stamen primordia have a defect in adaxial-adaxial polarity from an early developmental stage.

Three types of expression patterns of marker genes were observed in association with the stamen primordia morphologies. In the rol stamen primordia with a pin-like structure, ETT1 was expressed in the entire region of the epidermal and subepidermal cell layers (Figure 5D). On the other hand, PHB3 transcripts were

| Table 1. Percentage of Abnormal Lemmas in the rol Mutant |
|----------------|----------------|----------------|---------------|
| Rod-Like     | Aborted        | Normal Shape with the Awn | Normal |
| 66.2         | 28.4           | 5.4             | 0             |
| \( n = 500. \) |                |                 |               |
not detected in the outer layers of the stamen primordia and were restricted to the procambium (Figure 5G). In the rol stamen primordia with two protrusions, probably corresponding to an OT-type stamen, ETT1 transcripts were detected in the abaxial side of the protrusion and a putative connective region, which expanded in this type of stamen, and were excluded from the protrusions themselves (Figure 5E; see Discussion). PHB3 transcripts were detected in the domain between the two protrusions and in the procambium (Figure 5H). In the rol stamen primordia with four protrusions, probably corresponding to an ATT-type stamen, ETT1 transcripts were widely distributed in the abaxial domain of the primordium and in the domain between the two thecae (Figure 5F). PHB3 transcripts were detected in the domain between the two protrusions within each theca and in the procambium (Figure 5I).

Despite the aberrant morphologies of the rol stamen in terms of protrusions, the position of the expression domains of two markers is likely to be common to both the wild type and rol: ETT1 was expressed between the thecae, whereas PHB3 was expressed between the pollen sacs in a theca. It should be noted that the protrusion was formed in the region between the expression domains of ETT1 and PHB3 even in the aberrant rol stamen primordia.

The rol Mutant Has a Defect in the Gene Encoding RNA-Dependent RNA Polymerase, Which Is Required for the ta-siRNA Pathway

We tried to isolate the gene responsible for the rol mutant by positional cloning to elucidate its molecular function. The putative ROL locus was mapped to a region between two molecular markers, RM7075 and RM5638, on chromosome 1. Because this region encompasses the centromere, it would be difficult to isolate this gene by a standard positional cloning method. We therefore focused on the fact that the rol plants developed filamentous structures in leaves at a low frequency, a phenotype similar to the leaf phenotype observed in shoot organization (sho) mutants (Itoh et al., 2000, 2008). The SHO genes are involved in the regulation of adaxial-abaxial polarity and encode proteins involved in the ta-siRNA pathway (Itoh et al., 2000, 2008; Nagasaki et al., 2007). A database search revealed that a putative gene encoding an RNA-dependent RNA polymerase (RdRP) involved in the ta-siRNA pathway was located in the region to which the putative ROL locus roughly mapped.

Next, we identified a nucleotide change that caused an amino acid substitution in the gene for this RdRP in the rol mutant (Figures 6A and 6B). Introduction of an 8.6-kb genomic fragment encompassing the RdRP gene rescued the mutant phenotype of the rol spikelet (Figures 1E and 1F). Therefore, we concluded that the rol phenotype was caused by a mutation in the gene encoding RdRP. This gene is closely related to RDR6 in Arabidopsis and has been reported as the SLH2 gene in rice (Nagasaki et al., 2007). Hereafter, we refer to the mutant (allele) as shl2-rol (an allele of the SLH2 locus) instead of rol.

Loss-of-function mutation of SLH2 causes failure in the formation of the SAM in the embryo. Twelve shl2 alleles have been reported to date (Nagasaki et al., 2007). Most alleles have a nonsense mutation, frameshift mutation, or amino acid substitution in the conserved RdRP domain. By contrast,
shl2-rol has an amino acid substitution in the N-terminal region far from the RdRP domain (Figure 6B), suggesting that the effect of this mutation is weak on RdRP activity. The shl2 mutants so far reported are embryonic or seedling lethal. Because of the weak mutation, shl2-rol may grow to the reproductive phase and show the unique spikelet phenotypes described above.

We examined the spatial expression pattern of SHL2 in the spikelet. SHL2 was expressed in all organs, including the stamen and lemma, from the early to the late stages (Figures 6C and 6D). In addition, the SHL2 signal was detected ubiquitously without any specific localization within the organs. This expression pattern suggests that SHL2 is not a primary factor that localizes the ETT transcripts in the patterns described above.
DISCUSSION

The SHL2 Gene Is Involved in the Regulation of Adaxial-Abaxial Polarity in the Floral Organs in Addition to in the Leaves

In this study, we revealed that the regulation of polarity along the adaxial-abaxial axis is disturbed during the development of lateral organs, such as the stamen and the lemma in the shl2-rol mutant. Gene isolation indicated that the shl2-rol mutant is caused by a mutation in the SHL2 gene, which encodes an RDR6-like protein that acts in the ta-siRNA pathway in Arabidopsis. Nagasaki et al. (2007) demonstrated that genes involved in the ta-siRNA pathway are essential for SAM formation in the rice embryo, and one of the shootless mutants (shl2) is caused by a defect in an ortholog of Arabidopsis RDR6. Accordingly, the gene that causes the rol phenotype is a novel allele of SHL2, in which the amino acid substitution (G160V) probably causes a weak defect in RdRP activity. This weak mutation in SHL2 seems to provide a unique opportunity to examine the regulation of polarity of lateral organs in the reproductive phase in rice because most known alleles of shl2 are embryonic lethal.

Analyses of phenotypes and expression patterns of marker genes indicated that the rod-like lemma is likely to be completely abaxialized in the shl2-rol mutant. As discussed below, pleiotropic phenotypes observed in the shl2-rol stamen may be a consequence of the defects in adaxial-abaxial polarity. Polarity defects in the palea were not severe compared with those in the lemma in the shl2-rol mutant. It is possible that this phenotypic difference may be partially due to differences in the levels and expression patterns of four ETT genes between the lemma and palea.

Although most shl2 mutants are embryonic lethal because of a failure in SAM formation, the weakest allele shl2-8 produces leaves that are defective in adaxial identity (Satoh et al., 1999; Satoh et al., 2003). Therefore, SHL2 is likely to be involved in the regulation of adaxial-abaxial polarity in the lateral organs in the vegetative and reproductive phases in rice. This conclusion is consistent with the observations that genes involved in the ta-siRNA pathway, such as SHO1 and SHO2 in rice and lbl1 in maize, are responsible for the regulation of adaxial-abaxial polarity in the leaf (Itoh et al., 2000, 2008; Liu et al., 2007; Nagasaki et al., 2007; Nogueira et al., 2007). Furthermore, a dcl4 mutant corresponding to sho1 causes formation of a rod-like lemma as the shl2-rol does (Liu et al., 2007). Thus, the ta-siRNA pathway seems to be associated with the regulation of polarity of lateral organs throughout the life cycle in rice.

This finding is in contrast with the observation that mutations in genes in the ta-siRNA pathway cause no obvious phenotype of polarity defects in Arabidopsis (Peragine et al., 2004; Adenot et al., 2006). In rice shl2, sho1, and sho2/dcl4 and maize lbl1, the levels of tasiR-ARF and cleavage products of ETT are reduced, in association with upregulation of ETT or spatial expansion of ETT expression domains (Liu et al., 2007; Nagasaki et al., 2007; Nogueira et al., 2007). It is conceivable that the expansion of ETT expression domains observed in the shl2-rol mutant may result from partial failure of the mechanism that produces ta-siRNA.

Rearrangement of the Expression Patterns of ARF and PHB during Stamen Development

During anther development, a marked rearrangement of the spatial expression patterns of ETT1 and PHB3 was observed. ETT1 and PHB3 were initially expressed in the abaxial and adaxial domains of the stamen primordia, respectively. Subsequently, however, the expression patterns of both genes changed dramatically: the genes were expressed in positions

Table 2. Percentage of Abnormal and Wild-Type Stamens in the rol Mutant

<table>
<thead>
<tr>
<th>Anther Type</th>
<th>Pin</th>
<th>OT</th>
<th>ATT</th>
<th>Wild Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of pollen sacs</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Percentage (n = 2238)</td>
<td>2.5</td>
<td>0</td>
<td>20.7</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 5. Scanning Electron Microscopy Images of Stamen Primordia and Spatial Expression Pattern of ETT1 and PHB3 during Stamen Development in rol.

(A) and (B) Scanning electron microscopy images of stamen development. Red, blue, and yellow arrowheads indicate pin-like, OT-type, and ATT-type stamen, respectively. (C) An early stage of stamen development. (D) and (G) Pin-like stamen primordia. (E) and (H) Primordia of OT-type stamen. (F) and (I) Primordia of ATT-type stamen. Brackets indicate a theca ([E], [F], [H], and [I]); and arrows indicate the region where the stomium differentiates later ([H] and [I]). Black arrowheads indicate the procambium ([G], [H], and [I]). Solid circles indicate the direction of the center of the flower. pr, protrusion that differentiates later into the pollen sac; st, stamen. Bars = 50 μm in (A) to (C) and 20 μm in (D) to (I).
that crossed at right angles. On the basis of these observations, we propose a model that explains anther patterning in relation to the regulation of adaxial-abaxial polarity (Figure 7).

In this model, adaxial-abaxial polarity is established in the stamen primordia as it is in other primordia of ordinary lateral organs, such as the leaf. Thus, adaxial identity is established in the domain adjacent to the meristem, and abaxial identity is established in the domain opposite to it. After the adaxial-abaxial rearrangement, a new polarity is established in a new unit, the theca primordium. This notion is consistent with the idea that the theca is a developmental unit, as shown by phenotypic analysis of the shl2-rol mutant. The blue box indicates the conserved RdRP domain.

The polarity change appears to be very rapid. Despite many efforts, we could not detect marker expression demonstrating the transition stage of polarity rearrangement. In addition, the complementary expression pattern of ETT1 and PHB3 was maintained during anther development, except for their expression in vascular tissue, where both genes were expressed, suggesting that cell fate along the adaxial-abaxial axis is strictly regulated. In proper adaxial-abaxial patterning during leaf development, positional information is required. It is assumed that crosstalk may exist between the miR166-mediated regulation of HD-ZIPIII and the tasiR-ARF–mediated regulation of ETT, and small RNA seems to be involved in this crosstalk (Chitwood et al., 2007; Nagasaki et al., 2007; Nogueira et al., 2007). In addition, small RNAs are thought to act as a mobile signal between the adaxial and abaxial domains (Chitwood et al., 2009). Therefore, it is possible that such intercellular communication via small RNAs may be implicated in the rapid and exact rearrangement of adaxial-abaxial polarity in anther patterning.

Figure 6. Schematic Representation of the SHL2 (ROL) Gene and Its Encoded Protein, and Spatial Expression Pattern of SHL2.

(A) The SHL2 gene. Boxes indicate coding regions. Arrowhead indicates the mutation site in the shl2-rol mutant. The red bar shows the 8.6-kb genomic fragment used for the complementation test. This fragment contains a promoter of ~1.5 kb.

(B) Schematic representation of the SHL2 protein. Arrowhead indicates the amino acid substitution in the shl2-rol mutant. The blue box indicates the conserved RdRP domain.

(C) and (D) Spatial expression pattern of SHL2 in the spikelet. Bars = 50 μm. ca, carpel; le, lemma; pa, palea; st, stamen.

Figure 7. Model of Anther Patterning in Rice.

(A) Model of anther patterning in the wild type.

(B) Adaxial-abaxial polarity in a theca. Arrowheads indicate the protrusions at the region between the domains of the adaxial and abaxial identities.

(C) Model of anther patterning in rol. (a) Pin-like stamen, (b) OT-type stamen, and (c) ATT-type stamen. The domains with the adaxial and abaxial identities represented by the expression domains of ETT1 and PHB3 are shown in red and blue, respectively. Each bracket represents a unit of the adaxial-abaxial polarity. Double-headed arrows represent the axis of adaxial-abaxial polarity. ab, abaxial side; ad, adaxial side. Dashed arrow indicates developmental progression.
Reconstitution of Adaxial-Abaxial Polarity during Stamen Development

Close relationships between lateral lamina growth and adaxial-abaxial polarity in leaf development have been widely accepted (Waites and Hudson, 1995; McConnell and Barton, 1998; McHale and Marcotrigiano, 1998; Timmermans et al., 1998). Failure to establish adaxial-abaxial polarity inhibits lateral lamina growth and produces radialized leaves. Likewise, the rod-like lemma may be formed by a failure in the outgrowth of lateral domains in shl2-rol. It seems likely that a similar mechanism may underlie anther development. Stamen primordia emerge initially as spherical structures. After rearrangement of adaxial-abaxial polarity, four protrusions emerge and then differentiate into pollen sacs. The regions where the initial protrusions form lie between the two domains that express the adaxial or abaxial gene markers. Thus, initiation of the protrusions and subsequent pollen sac differentiation in anther development are likely to be analogous to the formation of the outgrowth and lamina expansion in leaf development.

The pleiotropic phenotypes observed in the shl2-rol stamen would result from failure of the proper establishment of adaxial-abaxial polarity (Figures 3 and 7C). If there was a serious defect in the establishment of polarity, then no protrusions would be formed in the stamen primordium, and pin-like stamens would develop. In the case of an intermediate defect, half of the stamen primordia would establish polarity to form one theca, and an OT-type stamen would be produced. In the case of a weak defect, two thecae might develop according to the two polarity units, although the abaxial domain, which corresponds to the region between the thecae, would be expanded (ATT-type stamen). Thus, the phenotype of the stamen and the spatial expression patterns of the marker genes are in agreement with each other in the shl2-rol mutant.

The expression pattern of the marker genes suggested that the filament lacks adaxial identity in the wild-type stamen. It is likely that the anther and the filament develop as independent polarity units. The filament would not be affected by the shl2-rol mutation, probably because the main effect of this mutation is loss of adaxial identity.

Once adaxial-abaxial polarity is established, this polarity is maintained in flattened lateral organs, such as leaves and petals, throughout their development. In the stamen, however, regulation of adaxial-abaxial polarity is likely to differ in the distal and proximal parts. In anther development in the distal part of the stamen, rearrangement of the polarity occurs after the initial establishment of adaxial-abaxial polarity, and the polarity unit seems to change from the stamen primordium to the theca. The anther may develop in accordance with the new polarity. By contrast, polarity may be lost in the proximal part of the stamen; thus, the filament that lacks adaxial identity seems to develop as a filamentous structure.

In Arabidopsis, ETT is initially expressed in the abaxial domain of the stamen primordia in a pattern highly similar to ETT3 in rice (Sessions et al., 1997). Later, ETT expression is detected in four domains, including the region between the thecae (interthecal furrows). PHB is initially expressed on the adaxial side of the stamen primordium and is later expressed in the domain between the pollen sacs within the theca (Dinneny et al., 2006). These expression patterns partially resemble those of ETT1 and PHB3 in rice and suggest that the expression domain of ETT and PHB is rearranged during stamen development in Arabidopsis, as in rice. In addition, kan, phb-c, and fil form radialized pin-like stamens, suggesting that failure to establish adaxial-abaxial polarity is closely related to loss of the anther (Chen et al., 1999; Sawa et al., 1999a; Eshed et al., 2001, 2004). Therefore, the model of stamen development that we propose here might be generally applicable to stamen development in angiosperms. ETT and FIL are initially expressed in the same pattern in the abaxial domains of the stamen primordia, but their expression domains differ at later stages of development: ETT is expressed between the thecae, whereas FIL is expressed in the connective (Sessions et al., 1997; Chen et al., 1999; Sawa et al., 1999b; Siegfried et al., 1999). Thus, the role of these abaxial determinants might be differentiated at the later stages of stamen development in Arabidopsis.

METHODS

Plant Materials

A novel mutant (named rol in this study) was found in M2 plants that had been mutagenized during tissue culture from Nipponbare. Nipponbare was used as a wild-type strain for comparing phenotypes and for in situ expression analysis. Kasalath, a cultivar of Oryza sativa spp indica, was used for observation of the awn.

Scanning Electron Microscopy

Young panicles and flowers were fixed in 4% (w/v) paraformaldehyde and 0.25% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, at 4°C overnight. They were then dehydrated in a graded ethanol series, and critical point-dried at their critical point, sputter-coated with platinum, and observed with a scanning electron microscope (model JSM-820S; JEOL) at an accelerating voltage of 5 kV.

In Situ Hybridization

Rice ETT1 (Os05g0563400) and ETT3 (Os01g0753500) have been previously described by Sato et al. (2001) and Nagasaki et al. (2007), respectively. Rice PHB3 (Os12g0612700) corresponds to OSHB3 (Zhong and Ye, 2004). To make probes for rice ETT1, ETT3, PHB3, and SHL2, partial cDNA fragments were amplified with the primers listed in Supplemental Table 1 online and cloned into the pCRII vector (Invitrogen). Using these plasmids as templates, a region containing the partial cDNA sequences and both T7 and SP6 promoter sequences was amplified with M13 forward and reverse primers. After removal of the primers, the resulting PCR products (100 to 200 ng) were used for RNA transcription. Synthesis of DIG-labeled RNA probes, in situ hybridization, and immunological detection were performed by the methods described by Suzuki et al. (2004).

For two-color in situ hybridization, an RNA probe for PHB3 was labeled using a fluorescein isothiocyanate (FITC) RNA labeling kit (Roche). Double hybridization with this FITC-labeled PHB3 probe and a DIG-labeled ETT1 probe was performed using the method described by Kouchi et al. (1995). The sections were hybridized at 55°C with a mixture of the two probes. After hybridization, the FITC-labeled probe was detected by incubation with an antifluorescein antibody conjugated to alkaline phosphatase.
(Roche) in combination with Fast Red TRInaphtol AS-MX (red) (Sigma-Aldrich) at 37°C for 4 to 6 h. To inactivate the alkaline phosphatase, the slides were incubated twice in 2× SSC at 68°C for 1.5 h each. The second detection was performed using anti-DIG antibody conjugated with alkaline phosphatase (Roche) in combination with NBT/BCIP solution (purple) (Roche). The slides were incubated at 37°C for 12 h. The slides were then mounted with glycerin, and signals were observed under a light microscope (BX-50; Olympus).

Isolation of the Gene Responsible for the rol Mutation

A putative rol locus was mapped using an F2 population derived from the rol mutant and Kasalath. The locus was mapped to a region between the cleaved-amplified polymorphic sequence markers RM7075 and RM5638 on chromosome 1 using 28 rol homozygotes. SHL2, which encodes RdRP (Nagasaki et al., 2007), which is similar to Arabidopsis RDR6 and is involved in the ta-siRNA pathway, was identified in this region by a database search. The genomic sequence of the SHL2 locus in the rol mutant was determined by direct sequencing after PCR amplification. Sequence analysis revealed that a mutation causing an amino acid substitution occurred in this gene, suggesting that the rol mutation is caused by a mutation in SHL2.

For complementation analysis, an 8.6-kb fragment encompassing the RdRP gene was amplified using Nipponbare genomic DNA as a template and cloned into a pENTR 2B vector (Invitrogen). For transformation of rice, a pBI-Hm12-GW plasmid containing the Gateway rfC cassette (Invitrogen) (Yoshida et al., 2009) was used. By an LR recombination reaction, the 8.6-kb fragment was transferred into pBI-Hm12-GW. The recombinant plasmid was introduced into Agrobacterium tumefaciens (Roche) in combination with Fast Red TRInaphtol AS-MX (red) (Sigma-Aldrich) at 37°C for 4 to 6 h. To inactivate the alkaline phosphatase, the slides were incubated twice in 2× SSC at 68°C for 1.5 h each. The second detection was performed using anti-DIG antibody conjugated with alkaline phosphatase (Roche) in combination with NBT/BCIP solution (purple) (Roche). The slides were incubated at 37°C for 12 h. The slides were then mounted with glycerin, and signals were observed under a light microscope (BX-50; Olympus).

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Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: SHL2 (AB359923), ETT1 (AB071290), ETT3 (AK072350), and PHB3 (AK102183).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Phenotypes of the rol Mutant.

Supplemental Figure 2. Spatial Expression Patterns of ETT3 and PHB3 in Wild-Type Spikelet.

Supplemental Table 1. Primers Used to Make in Situ Probes.

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Distinct Regulation of Adaxial-Abaxial Polarity in Anther Patterning in Rice
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