The SHOOTLESS2 and SHOOTLESS1 Genes Are Involved in Both Initiation and Maintenance of the Shoot Apical Meristem Through Regulating the Number of Indeterminate Cells

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ABSTRACT

To characterize the SHL2 and SHL1 genes in detail, we analyzed three strains carrying weak alleles of SHL2, shl2-6, shl2-7, and shl2-8, and one weak allele of SHL1, shl1-3. In contrast to strong alleles, which result in lack of shoot meristem, strains bearing these weak alleles formed shoot meristem frequently during embryogenesis. In shl2-6 and shl2-7 mutants, the meristem was lost during seed development. Only the shl2-8 mutant could survive after germination, but it showed abnormal initiation pattern and morphology of leaves. In strains bearing the weak alleles, the shoot meristem was composed of a small number of indeterminate cells and ultimately converted into leaf primordium. The shl1-3 mutant showed phenotypes similar to those of shl2-8. Thus SHL2 and SHL1 are required for both initiation and maintenance of shoot meristem. In shl2 mutants, there was a positive correlation between the size of the expression domain of OSH1 representing the number of indeterminate cells, the frequency of shoot meristem initiation, and the duration of meristem survival. Thus the shoot meristem will not initiate in an “all-or-nothing” fashion, but is formed in various degrees depending on the strength of the alleles. Double-mutant analyses indicate that SHL2 functions upstream of SHO to establish proper organization of the shoot meristem.

The shoot system is constructed throughout the plant life cycle by the continuous activity of small groups of cells called shoot apical meristems (SAMs). The SAM originates during embryogenesis and is later responsible for generating the aboveground organs of the plant. Thus, understanding the developmental events that determine the aboveground architecture requires detailed examination of the SAM. The SAM can be thought of as having two fundamental functions: self-perpetuation and the formation of lateral organs (for review see Steeves and Sussex 1989). The former is executed by a cluster of infrequently dividing cells that are positioned in the center of the SAM. This region of the SAM is called the central zone (CZ). The latter function is performed in the peripheral zone (PZ) that surrounds the CZ. Alternatively, the SAM is viewed in terms of clonally distinct cell layers (L1, L2, and L3). This stratification reflects the orientation of planes of cell divisions in different layers. Recent studies revealed that these zones and layers form separate symplasmic domains (Rinne and van der Schoot 1998; Gisel et al. 1999). For the SAM to function properly, the establishment and maintenance of these zones and layers are essential.

Several key genes that play important roles in the above functions of the SAM have been studied in Arabidopsis, maize, and rice. As for the maintenance of the Arabidopsis SAM, SHOOT MERISTEMLESS (STM) functions to keep central meristem cells indeterminate (Endrizzi et al. 1996; Clark et al. 1997). In strong stm alleles, the SAM is rarely formed, but, in a weak mutant allele, abnormal plants develop, suggesting that STM is required for proper organization of the SAM (Barton and Poethig 1993; Endrizzi et al. 1996). The clavata (clv) mutations increase the number of cells in CZ, and CLV genes seem to regulate the proliferation of CZ cells (Clark et al. 1993, 1995; Kayes and Clark 1998). Molecular and biochemical analyses have shed light on intracellular events in CLV signaling. The CLV1 gene encodes a serine/threonine receptor kinase (Clark et al. 1997), and CLV3 seems to act as its ligand (Fletcher et al. 1999; Brand et al. 2000). Further, CLV genes interact with WUSCHEL (WUS), a gene that is required for stem cell identity, to establish a negative feedback loop between the stem cells and the underlying organizing center (Brand et al. 2000; Laux et al. 1996; Mayer et al. 1998; Schoof et al. 2000). In addition, the POLTERGEIST gene functions downstream of the CLV genes and redundantly with WUS (Pogany et al. 1998; Yu et al. 2000). ZWILLE/PINHEAD (ZLL/PNH) is also required for maintaining stem cells in undifferentiated state; these mutants form a defective SAM that terminates shortly after germination (McConnell and Barton 1995; Moussian et al. 1998). The ZLL/PNH gene is supposed to prevent STM downregulation during embryogenesis, possibly by providing signal from the pro-
vascular tissue to the overlying meristem cell population (Moussian et al. 1998; Lyne et al. 1999). Thus the regulation of SAM organization is relatively well understood in Arabidopsis. However, loss-of-function mutations rarely cause a complete loss of the SAM in Arabidopsis. So it is not well understood how the SAM is first formed in the embryo.

In monocots, which have distinct organization of embryos and shoots compared to dicots, the SAM development is poorly understood. In rice, recessive mutations in at least four loci cause loss of the SAM without affecting radicle differentiation (Satoh et al. 1999). In maize, several shootless mutants are known (Clark and Sheridan 1991; Pilu et al. 2002), although detailed analysis has not been carried out. Knotted1 (kn1)-type homeobox genes are closely associated with SAM organization. Loss-of-function mutations in kn1 suggest that kn1 is necessary for the maintenance of indeterminate cells in the SAM (Kerstetter et al. 1997; Vollbrecht et al. 2000).

Maintenance of the SAM is balanced with the process of organogenesis. In Arabidopsis, STM and CLV genes competitively regulate the balance between undifferentiated cells and lateral organs (Clark et al. 1996), and MGOUN genes may affect the partitioning of PZ cells into organ primordia (Laufs et al. 1998). In the SAM of narrow sheath mutants of maize, fewer cells are recruited into leaf founder cells than in the wild type (Scanlon et al. 1996). Regular production of leaf primordia that is reflected in stable phyllotaxy (spatial distribution) and plastochron (time interval between two successive primordia) is another interesting function of SAM. The phyllotaxy is altered in clv mutants of Arabidopsis (Clark et al. 1993, 1995) and abphy1 of maize (Jackson and Hake 1999), both of which have enlarged SAMs. The rice plastochron 1 mutant has enlarged SAMs and produces leaf primordia more rapidly (Itoh et al. 1998). The shoot organization (sho) mutants (Itoh et al. 2000) have flat SAMs and aberrant plastochron and phyllotaxy and play important roles in maintaining the proper organization of the SAM. Although these genes are supposed to play distinct roles in the initiation of leaves, it is generally considered that the initiation pattern of leaves is closely associated with the size and shape of the SAM.

In spite of these studies, the mechanisms for SAM maintenance have not been well understood. In rice, at least four SHOOTLESS (SHL) genes are indispensable for SAM initiation (Satoh et al. 1999). However, since these mutants lack the SAM, it is not known if the SHL genes function in the SAM after it is established. To understand the entire function of SHL genes, we have identified three weak alleles of SHL2, shl2-6, shl2-7, and shl2-8, and one weak allele of SHL1, shl1-3, and reveal that SHL2 and SHL1 are required not only for initiation but also for maintenance of the SAM. In addition, SHL2 and SHL1 regulate the pattern of leaf initiation together with the SHO genes.

**MATERIALS AND METHODS**

**Plant materials:** Mutants used in this study are summarized in Table 1. We identified three weak recessive alleles of the SHL2 locus on chromosome 1 in rice (Oryza sativa L.), shl2-6, shl2-7, and shl2-8, from M2 populations mutagenized with N-methyl-N-nitrosourea (MNU). The shl2-6 and shl2-8 were derived from cv. Taichung 65, and shl2-7 was derived from cv. Kinmaze. We also identified a strong recessive allele, shl2-9. Although the strong alleles (shl2-1–shl2-5) thus far reported (Satoh et al. 1999) formed abnormal SAM at a very low frequency, shl2-9 embryos invariably lacked SAM. We identified another weak recessive mutant, shl1-3, of different locus SHL1 derived from MNU-treated cv. Taichung 65. Allelism was examined using heterozygous plants for each mutation and was confirmed by the presence of mutant embryos in F1 seeds at a frequency of ~25% and by the segregation value in F2 progeny.

Since the seedlings of shl2 weak alleles were similar to those of sho mutants (Itoh et al. 2000), we crossed shl2-3/+ and shl2-6/+ plants with pollen of sho2/+ plants to determine the relation between SHL2 and SHO2.

**Growth conditions:** As the mutant seedlings of shl2-6, shl2-7, and shl1-3 became etiolated when grown in soil, plants were grown aseptically on MS medium (Murashige and Skoog 1962) supplemented with 6% sucrose and 0.8% agar (pH 5.8) at 28°C under 12 hr light:12 hr dark.

**Preparation of plastic sections:** Developing seeds and seedlings were fixed in FAA (formalin:glacial acetic acid:70% ethanol; 1:1:18) and dehydrated in a graded ethanol series. They were embedded in a resin, Technovit 7100 (Kurzer, Germany), polymerized at 45°C, and sectioned at 3–5 µm thick. Sections were stained with toluidine blue and observed with a light microscope.

**Scanning electron microscopy:** Shoot apices were fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for about 16 hr at 4°C. After rinsing with 0.1 M sodium phosphate buffer (pH 7.2), they were postfixed in 1% osmium tetroxide for 3 hr at 4°C and rinsed with buffer. Then samples were dehydrated in a graded ethanol series, and 100% ethanol was replaced with 3-methyl-butyl-acetate. Samples were critical-point dried, sputter coated with platinum, and observed with a scanning electron microscope (model S-4000, Hitachi, Tokyo) at an accelerating voltage of 10 kV.

**In situ hybridization:** Embryos and shoot apices were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M sodium phosphate buffer. Then they were dehydrated in a graded ethanol series, replaced with xylene, and embedded in Paraplast Plus (Oxford Labware, St. Louis). Micromtome sections (8 µm thick) were applied onto glass slides coated with Vectabond (Vector Laboratories, Burlingame, CA). Digoxigenin-labeled antisense probes were prepared from the coding regions of histone H4 and OSH1 deprived of their poly(A) ends. In situ hybridization and immunological detection were carried out by the methods of Kouchi and Hata (1993).

To estimate the number of cells expressing OSH1, we stained the nuclei of the embryo sections used for in situ hybridization with 4',6-diamidino-2-phenylindole, and we counted the number of nuclei in the OSH1 expression domain. We used five embryos of the wild type and each mutant just before SAM initiation (3 days after pollination (DAP) in the wild type and 5 DAP in mutants).

**Induction of calli and shoot regeneration:** Detection of ma-
Table 1: Characteristics of mutants used in this study

<table>
<thead>
<tr>
<th>Allele</th>
<th>Background cultivar</th>
<th>Segregation* (wt:mutant)</th>
<th>Frequency of SAM formation (%)†</th>
<th>Phenotype†</th>
</tr>
</thead>
<tbody>
<tr>
<td>shl2-3</td>
<td>Taichung 65</td>
<td>257:74</td>
<td>6.7</td>
<td>Strong</td>
</tr>
<tr>
<td>shl2-6</td>
<td>Taichung 65</td>
<td>136:44</td>
<td>55.3</td>
<td>Weak</td>
</tr>
<tr>
<td>shl2-7</td>
<td>Kinmaze</td>
<td>202:68</td>
<td>77.3</td>
<td>Weak</td>
</tr>
<tr>
<td>shl2-8</td>
<td>Taichung 65</td>
<td>300:69</td>
<td>92.2</td>
<td>Weak</td>
</tr>
<tr>
<td>shl2-9</td>
<td>Taichung 65</td>
<td>153:50</td>
<td>0.0</td>
<td>Strong</td>
</tr>
<tr>
<td>shl1-3</td>
<td>Kinmaze</td>
<td>251:83</td>
<td>83.7</td>
<td>Weak</td>
</tr>
<tr>
<td>sho2</td>
<td>Taichung 65</td>
<td>244:84</td>
<td>100.0</td>
<td>—</td>
</tr>
</tbody>
</table>

* Segregation was examined on M3 embryos set on M2 heterozygous plants.
† In total, 40–55 mutant seeds were examined from 200–300 seeds set on heterozygous plants for each mutant.
‡ Phenotypic severity was determined by the frequency of SAM formation in embryo.

Results

Since the shl2 alleles (shl2-1–shl2-5) thus far reported (Satoh et al. 1999) lack a SAM in most embryos, the function of SHL2 in later stages of SAM development is not clear. To identify weaker alleles of SHL2, we screened the mutants exhibiting weak seedling phenotypes from M2 plants mutagenized with N-methyl-N-nitrosourea and obtained three weak alleles, shl2-6, shl2-7, and shl2-8. These mutants form a SAM in the embryo but arrest soon after germination. The transheterozygotes of any combination among strong and weak alleles (shl2-3/shl2-6, shl2-1/shl2-7, shl2-6/shl2-7, or shl2-7/shl2-8) showed the phenotype of the weaker allele irrespective of the difference in the background cultivars. Thus the effect of different background is small. We also identified a strong allele, shl2-9, which caused complete loss of SAM in all embryos. In addition, we found a weak allele of SHL1, shl1-3. We examined the development of these mutants in detail.

Embryo development in shl2 mutants: Embryo phenotype: The developmental course of embryos was examined in wild type and in strong (shl2-3) and weak (shl2-6, -7, and -8) mutants (Figure 1). In the wild-type embryo, the globular stage lasts until 3 DAP. At 4 DAP, the coleoptile, shoot, and radicle meristems are first observed, and the first leaf primordium is apparent at 5 DAP (Figure 1A). In embryos of shl2 mutants, the protrusion of the coleoptile did not occur, and no organs were formed except for scutellum and radicle (Figure 1, B–E). Up to 5 DAP, embryo phenotypes of strong (shl2-3) and weak (shl2-6–shl2-8) alleles were indistinguishable from each other. At 6–7 DAP, although no further morphological changes occurred in shl2-3 embryos (Figure 1G), shl2-6–shl2-8 mutants frequently initiated a SAM, epiblast, and first leaf primordium, but lacked a coleoptile (Figure 1, H–J). The frequencies of SAM initiation in shl2-6, shl2-7, and shl2-8 were 55, 77, and 93%, respectively. In shl2-6 embryos, however, the SAM disappeared after producing one or two leaf primordia, resulting in a SAM-less mature embryo (Figure 1M; Table 2). Also, in the weaker allele, shl2-7, the SAM was lost in most embryos after forming two or three leaf primordia (Figure 1N). In these two alleles, the SAM seemed to be consumed during the production of leaf primordia. In contrast, a SAM survived in more than half of the mature shl2-8 embryos, which had three leaf primordia (Figure 1O; Table 2). The SAM of shl2-8 was maintained for 1–4 weeks after germination. Thus, shl2-8 is the weakest allele, judging from the embryo phenotype (frequencies of SAM initiation and of mature embryos with SAM). These results show that the SHL2 gene is essential for maintaining the SAM.

Interestingly, the first leaf in shl2-6–shl2-8 mutants was initiated at the normal position, but was thicker than that in the wild type, and its epidermis was composed of palisade-shaped cells, which are characteristic of scutellar epithelium (data not shown).

OSH1 expression: To examine the organization of the SAM in shl2 embryos, we examined the expression of OSH1, a rice counterpart gene of the maize homeobox gene knl, which marks indeterminate cells in the SAM (Matsuoka et al. 1993; Sato et al. 1996). In the wild type, OSH1 is first expressed in a ventral region of the globular embryo where the SAM will develop, and the
expression is maintained in the indeterminate cells of SAM (Figure 2, A, F, and K). The expression of *OSH1* is restricted to an extremely narrow region of 4- to 5-DAP embryos in the strong *shl2*-3 allele (Figure 2B), suggesting that in the strong alleles, the *OSH1* expression region is too small for initiating a SAM. In embryos of *shl2*-6–*shl2*-8 at 5 DAP, the domain of *OSH1* expression was smaller than that in wild-type embryos but larger than that in strong allele embryos (Figure 2, B–E). In *shl2*-3 embryos, the expression of *OSH1* almost disappeared before 7–8 DAP (Figure 2G), while in *shl2*-6–*shl2*-8, the expression was maintained in a smaller region than in the wild type (Figure 2, H–J). *OSH1* expression in embryos of the weak alleles, *shl2*-6 and *shl2*-7, disappeared as SAMs were consumed (Figure 2, M and N). However, in embryos of the weakest allele, *shl2*-8, that frequently maintained the SAM throughout embryogenesis, *OSH1* was still expressed at 10 DAP (Figure 2O). Accordingly we suggest that the *SHL2* gene is necessary for the initiation and maintenance of the SAM through establishing the domain of indeterminate cells in the SAM.

Interestingly, the expression domain of *OSH1* (the number of indeterminate cells) seemed to be positively correlated with the frequency of SAM initiation. Then we counted the number of cells expressing *OSH1* of the above mutants and the strongest allele *shl2*-9 using embryos just before SAM formation (Figure 3). As for *shl2*-3 and *shl2*-9, we used 5-DAP embryos. Among the *shl2* mutant alleles, the number of cells expressing *OSH1* is largest in *shl2*-8, smallest in the strong allele *shl2*-9, and intermediate in *shl2*-6 and *shl2*-7. It is clear that the number of cells is positively correlated with the frequency of SAM initiation (Figure 3). On the basis of this correlation, we can estimate a threshold value of

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Embryogenesis (%)</th>
<th>&lt;1 week after germination (%)</th>
<th>&gt;1 week after germination (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>shl2</em>-6 (n = 200)</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>shl2</em>-7 (n = 109)</td>
<td>94</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><em>shl2</em>-8 (n = 75)</td>
<td>31</td>
<td>50</td>
<td>19</td>
</tr>
</tbody>
</table>

We counted the number of ungerminated mutant seeds and seedlings aborted before producing the fourth leaf, giving the data of the second column, because the mutants differentiated three or fewer leaf primordia in embryo. Then we counted the number of 1-week-old seedlings with SAM divided, giving the data in the fourth column. The data in the third column were residues.
the number of cells expressing OSH1 (indeterminate cells) required for initiating SAM. Since 106 cells expressed OSH1 in the wild-type embryo and 73 cells expressed OSH1 in the shl2-8 embryo, 80 or more indeterminate cells expressing OSH1 are needed for initiating normal SAM. The shl2-6 embryo expressed OSH1 in 38 cells on average and initiates SAM, but the shl2-3 embryo expressed OSH1 in 25 cells and failed to initiate SAM. Thus, SAM is formed but ill organized if $\sim$30–80 cells are recruited for expressing OSH1. If only $\leq$30 cells express OSH1, SAM is not formed. In addition, the duration of SAM maintenance (Table 2) is also correlated with the number of cells expressing OSH1 among weak alleles. Accordingly, the domain size of indeterminate cells regulates the initiation and subsequent maintenance of SAM.

**Plant phenotypes of weak shl2 mutants:** Strains bearing any of the three weak shl2 alleles were able to germinate. However, shl2-6 and shl2-7 seedlings developed a few malformed leaves that were already present in the embryo and then aborted. In these seedlings, a SAM was not observed. In contrast, a considerable number of shl2-8 seedlings survived for a few weeks, but developed into abnormal plants. Some shl2-8 plants grew longer, but none survived $>2$ months. Since shl2-8 seedlings were relatively vigorous and showed a variety of interesting phenotypes, we examined the shoots of shl2-8 in detail.

**Early vegetative phase:** Within 1 week after germination, shl2-8 seedlings were characterized by the rapid production of small narrow leaves with irregular phyllotaxy (Figure 4). The mean plastochron was $<2$ days, about half that of the wild type, and the phyllotaxy was quite irregular (Figure 4, B and C). The leaf morphology

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**Figure 2.—Expression pattern of OSH1 in wild-type and shl2 embryos.** (A, F, and K) Wild type. (B, G, and L) shl2-3. (C, H, and M) shl2-6. (D, I, and N) shl2-7. (E, J, and O) shl2-8. (A–E) Four DAP, (F–J) 7 DAP, and (K–O) 10 DAP. The SAM is indicated by arrowheads. Bar, 50 μm (for A–O).

**Figure 3.—Correlation of the number of cells expressing OSH1 and the frequency of SAM initiation in wild-type and shl2 embryos.**
Variation in leaf morphology between wild-type and shl2-8 seedlings was observed. Thread-like leaves were more frequent in wild-type than in shl2-8 seedlings, indicating that shl2-8 mutants had accelerated cell division followed by the defect of founder cell recruitment (Figure 4C). In many shl2-8 seedlings, a SAM was not visible at the base of the most recent leaf primordium. In some cases, a trichome was formed at the tip of a dome-like structure, which was in the position normally occupied by the SAM. In other cases, a leaf primordium was present at the position of the SAM (Figure 4D). Considering the fact that more than half of shl2-8 seedlings arrested their growth at ~1 week after germination (Table 2), the above observations imply that the SAM of shl2-8 seedlings disappears due to its conversion into a leaf primordium. The SAM of shl2-8 surviving seedlings was abnormally shaped. In general, the SAM was wider in the mutant than in the wild type, but its height was nearly half that of the wild type, resulting in a flat shape (Figure 4, F and G).

To elucidate how cells were organized in the mis-shaped SAM, we examined the expression pattern of OSH1 (Figure 4, E–G). In the SAMs of surviving shl2-8 seedlings, OSH1 was expressed in a slightly smaller region than in the wild-type SAM (Figure 4, F and G), although several SAMs showed apparently normal OSH1 expression. In many cases, the downregulation of OSH1 expression occurred in the peripheral and rib zones (Figure 4, F and G). These results indicate that at the seedling stage, the proportion of indeterminate cells was reduced in the shl2-8 SAM and may be the cause of rapid and irregular initiation of leaf primordia.

To understand the rapid leaf production in shl2-8 mutants, we examined the expression of the histone H4 gene, which is expressed specifically in the S phase of the cell cycle. In the wild-type SAM at 1 week after germination, hybridization signals were detected in no more than two cells of the peripheral zone in the median longitudinal plane (Figure 5A). However, cells with hybridization signals were more frequent in the SAM of shl2-8 seedlings than in the wild type (Figure 5, B and C), indicating that shl2-8 mutants had accelerated cell divisions in the SAM. In addition, the cells expressing histone H4 were not restricted to the peripheral zone (where cell division activity was relatively high in the wild-type SAM), but were also frequently detected in the central zone (Figure 5, B and C). These results indicate that the SAMs of shl2-8 mutants are abnormally organized and are defective in the spatial regulation of cell divisions.

A variety of leaf abnormalities were detected in shl2-8 seedlings (Figure 6). Relatively wide leaves often split into two or three at the tip (Figure 6C). Many leaves showed aberrant histology: the adaxial epidermal cells in the basal region of the leaf underwent extra anticlinal divisions whereas at the tip they became enlarged. Other histological abnormalities were also observed (Figure 6, D and E). The thread-like leaves showed normal histology (Figure 6F). Thus shl2-8 affects both the morphogenesis and the initiation pattern (phyllotaxy and plastochron) of leaves, as well as the leaf histology.

Late vegetative phase: shl2-8 plants that survived past 1 week after germination were different from younger seedlings in several traits (Figure 7). In the surviving plants at 2–3 weeks after germination, all the leaves were thread-like, and the phyllotaxy was mostly distichous (Figure 7, A and B). The leaves at this stage showed normal histology (Figure 6G). Subsequently, the leaf size was progressively reduced, and by 2 months after germination, no more leaves were formed, and growth terminated. As observed in the early phase, a trichome was often formed at the tip of the apical dome, suggesting that the SAM was partially converted into a trichome (Figure 7, C and E). In other cases, a leaf primordium was observed in the position of the SAM (Figure 7D).
7, B and F), suggesting that SAM was converted into a leaf primordium.

In shl2-8 mutants at this stage, OSH1 was expressed in a narrow region of the flat SAM (Figure 7, D and E). In the wild-type SAM, expression was downregulated in the L1 layer and in leaf primordia. In shl2-8, in addition to these regions, downregulation was also observed in a considerably wide region of the SAM (Figure 7D). When the apex showed leaf identity, two patterns of OSH1 expression were observed. When a trichome was formed from the apical L1 layer, OSH1 was expressed in a small internal region of the SAM (Figure 7E). In another case, OSH1 was not expressed, and the whole apex was converted into a leaf (Figure 7F). The former case may reflect a transition stage to the latter case.

From the above results, we can infer that the SAM of shl2-8 mutants disappears during development. The shl2-8 SAM is initiated incompletely due to an insufficient number of indeterminate cells. This initial defective state seriously affects subsequent SAM development. The SAM is gradually consumed by leaf primordia and the number of indeterminate cells is progressively reduced. Finally, SAM identity is lost and development terminates after the SAM is converted into a leaf primordium.

To investigate the general function of the SHL2 gene in the establishment and maintenance of the SAM, we regenerated adventitious shoots from scutellum-derived calli. In contrast to the failure of adventitious shoot induction from calli of strong shl2 alleles (Satoh et al. 1999), shoots could be regenerated from calli of weak alleles, shl2-6, shl2-7, and shl2-8. However, like shl2 seedlings, they rapidly developed narrow leaves, in more or less irregular phyllotaxy, and aborted before maturation (data not shown).

**Development of the weak shl1-3 mutant:** The shl1-3 mutant followed a developmental course similar to that of shl2-8. In shl1-3 strains, SAM initiated in ~84% of embryos, in contrast to the lack of SAM in 94% of strong shl1-1 embryos. However, the SAM was not observed in 70% of mature embryos, indicating that the SAMs formed in the early embryos disappeared after producing 1–3 leaf primordia (Figure 8A). Thus, SHL1 gene as well as SHL2 gene is required not only for initiation but also for maintenance of the SAM. The expression of OSH1 was examined in the embryos just before SAM initiation. The expression domain of OSH1 in shl1-3 embryo was larger than that in the strong allele shl1-2 embryo (Figure 8, B and C), as observed in the weak alleles of shl2. Again, the size of OSH1-expression domain was positively correlated with the frequency of SAM initiation between the weak and strong alleles.

The development of shl1-3 plants was similar to that of shl2-8. After germination, shl1-3 seedlings rapidly pro-

**Figure 5.**—Histone H4 expression in wild-type and shl2-8 shoot meristems. (A) Wild-type SAM 1 week after germination. Histone H4 is expressed in two cells (arrowheads). (B and C) shl2-8 SAM 1 and 3 weeks after germination, respectively. In these meristems, histone H4 is expressed in a large number of cells of SAM. Bar, 50 μm (for A–C).

**Figure 6.**—Leaf phenotypes of shl2-8 seedlings. (A and B) Wild-type leaf blade and sheath, respectively. (C–F) shl2-8 leaves from 1-week-old plant. (C) Bifurcation at the tip. (D) Cross section of basal region of a relatively wide leaf showing over-proliferation of adaxial epidermal cells (arrowheads) and other histological abnormalities. (E) Cross section of apical region of a relatively wide leaf used in D. Adaxial epidermal cells are enlarged (arrowheads). (F) Cross section of thread-like leaf. (G) Narrow leaf from 3-week-old plant. Bars, 1 mm (A and B), 200 μm (D and E), and 100 μm (F and G).
duced thread-like leaves in an irregular phyllotaxy (Figure 8D) and showed frequent expression of histone H4 gene in the SAM (Figure 8E). In the SAM, the OSH1 expression was restricted (Figure 8F). Frequently, a trichome was produced from the tip of the SAM (Figure 8G). Finally, the SAM was converted to leaf primordium, and the plants died <2 months after germination.

shl2 sho2 double mutant: The phenotypes of seedlings and adventitious shoots of weak shl2 and shl1 mutants were very similar to those of shoot organization (sho) mutants (Figure 9; Iront et al. 2000). In embryos, both shl2-8 and sho mutants lack a coleoptile, have an enlarged first leaf whose epidermis resembles scutellar epithelium (Figure 9A), and show OSH1 expression in a narrow region (Figure 9B). In the seedlings, both mutants produce narrow and short leaves rapidly in irregular phyllotaxy (Figure 9, C and D), and the SAMs are flattened. The major difference is that shl2-8 plants terminate before the reproductive phase, while sho plants recover in the late vegetative phase and undergo reproductive development. In addition, the histological abnormalities observed in shl2-8 leaves were not detected in sho leaves. Finally, the expression domain of OSH1 in the sho embryo (Figure 9B) was larger than that in shl2. In general, the sho mutants show less severe phenotypes than the weak shl2-8 mutant does. However, the phenotypic similarities suggest that these genes are functionally related or may operate in the same pathway.

To determine the genetic interaction between SHL2 and SHO, we constructed shl2-3 sho2 and shl2-6 sho2 double mutants. The shl2-3 is a strong allele showing almost no SAM formation in embryo. In F1 progeny, normal, shl2-3, and sho2 embryos segregated in the ratio of 114:50:38, well fitted to the expected 9:4:3 ratio, indicating that SHL2 is epistatic to SHO2. The shl2-6 shows more severe phenotypes than shl2-7 and shl2-8 do, and the sho2 phenotype is weaker than those of the other sho mutants. Therefore, it is relatively easy to discriminate between shl2-6 and sho2 mutants in embryos and seedlings. The F2 progeny of F1 plants heterozygous for both loci segregated, as expected, for shl2-6 and sho2 embryos. The F2 progeny were classified as normal, shl2-6, and sho2 phenotypes. Among the F2 embryos and seedlings, we could not detect a double-mutant phenotype distinct from each single mutant. The segregation value of F2 embryos derived from three F1 plants was normal:shl2-6: sho2 = 47:29:14, not significantly deviating from the expected 9:4:3 ratio. Thus shl2 is epistatic to sho2, suggesting that SHL2 functions upstream of SHO2 in a pathway regulating SAM development.

**DISCUSSION**

This study has demonstrated that weak shl2 and shl1 alleles cause defects in both initiation and maintenance of the SAM. In addition, abnormalities were also detected in the initiation pattern of leaf primordia and in leaf morphogenesis. Accordingly, SHL2 and SHL1 genes play a fundamental role during multiple stages of shoot development.

**Function of SHL2 and SHL1 genes in shoot development:** In a previous report (Satoh et al. 1999), we demonstrated that the SHL2 and SHL1 genes, like other SHL genes, are indispensable for the initiation of the SAM. The results in this study indicate that SHL2 and SHL1 are also involved in the maintenance of the SAM, since in the weak alleles, shl2-6-shl2-8 and shl1-3, the SAM is produced but is sooner or later consumed by leaf primordia. As for the consumption process, seedlings of the weakest alleles, shl2-8 and shl1-3, demonstrate that the indeterminate cells in SAM recognizable through their OSH1 expression are gradually reduced during
Initiation and Maintenance of Meristem

Figure 8.—Phenotypes of shl1-3 mutant. (A) Mature shl1-3 embryo with SAM (arrowhead) and ill-shaped leaf primordium (arrow). (B) OSH1 expression in 5-DAP shl1-2 embryo. (C) OSH1 expression in 5-DAP shl1-3 embryo. (D) shl1-3 seedling 1 week after germination showing irregular phyllotaxy of thread-like leaves. (E) Histone H4 expression in the SAM of shl1-3 seedling 1 week after germination. (F and G) OSH1 expression in the SAM of shl1-3 seedlings. In F, OSH1 expression is downregulated in both sides of the SAM (arrowheads) and in G, a trichome (arrow) is formed at the tip of the SAM. Bars, 200 μm (A) and 50 μm (B, C, E, F, and G).

development. When only a small number of indeterminate cells are remaining in the center of SAM, the apical L1 layer gains leaf identity, because it forms a trichome (Figure 7E). When the indeterminate OSH1-expressing cells are no longer present, the whole apical dome is transformed into a leaf primordium (Figure 7F). If the former situation represents the transition to the latter case, the loss of meristem identity seems to proceed from the outermost cell layer (L1) toward the inside. Thus, the SHL2 gene functions in establishing and maintaining indeterminate cells in the SAM-related region.

To date very few loci have been reported accounting for both initiation and maintenance of the SAM. In Arabidopsis, the STM gene functions in both initiation and maintenance of the SAM (Barton and Poethig 1993; Endrizzi et al. 1996; Clark et al. 1997). However, it is not well understood how the weak stm allele affects SAM maintenance. The STM is a kn1-type homeobox gene orthologous to OSH1. Since shl2 located on chromosome 1 and shl1 on chromosome 12 affect the expression domain of OSH1 (on chromosome 3), OSH1 is estimated to function downstream of SHL2 and SHL1, suggesting that STM would act downstream of SHL2-like gene.

In Arabidopsis, WUS and CLV3 genes are required for SAM maintenance through retaining stem cells (Mayer et al. 1998; Fletcher et al. 1999; Brand et al. 2000; Schoof et al. 2000). They are assumed to negatively interact to maintain the stem cell population (Brand et al. 2000; Schoof et al. 2000). It is relevant to note, in this context, that the wus mutants show a "stop-and-go" mode of seedling development and have very flat SAM when they stop development. These phenotypes are different from those of shl2 and shl1 mutants. Thus SHL2 and SHL1 may differ from WUS and CLV3 genes in their effect on SAM maintenance. The CUC1 and CUC2 genes exhibit a unique function in specifying the SAM boundary and are required for STM expression (Aida et al. 1997, 1999; Takada et al. 2001). Although there is a possibility that SHL2 and/or SHL1 also determine the SAM boundary, cuc genes do not seem to play an important role in SAM maintenance. It might be that SHL2 and SHL1 genes exert different functions from the above genes.
Interestingly, phyllotaxy and plastochron are also affected in \textit{shl2-8} and \textit{shl1-3} mutants. These phenotypes may result from the aberrant SAM organization. It is known that modified phyllotaxy/plastochron is associated with a change in SAM shape and/or size (Itoh et al. 1998; Jackson and Hake 1999). Also in \textit{cv} mutants of Arabidopsis, which have enlarged SAMs, the initiation pattern of lateral organs is modified (Clark et al. 1993). The abnormal expression pattern of \textit{OSH1} and histone \textit{H4} in the SAM would reflect the abnormal organization of the \textit{shl} SAMs. Therefore the initiation pattern of lateral organs depends on the maintenance of proper organization of the SAM, which may be a primary role of \textit{SHL2} and \textit{SHL1}.

Abnormalities are also detected in \textit{shl2} leaves, including abnormal histogenesis at the early seedling stage such as extra cell divisions in the adaxial epidermis and enlarged air space in the proximal region. In \textit{sho} mutants, which show phenotypes similar to those of \textit{shl2-8}, no histological abnormalities in leaves are observed, although the leaf shape is severely affected (Itoh et al. 2000). This suggests that \textit{shl2} affects a wide variety of developmental processes of the shoot and may be a key gene positioned upstream in shoot development.

\textbf{Novel aspect of SAM initiation:} Although several mutants defective in SAM initiation have been identified in Arabidopsis (Barton and Poethig 1993; Endrizzi et al. 1996; Aida et al. 1997), it remains unclear how the SAM identity is established. From the analysis on \textit{shl2} alleles, we are able to infer a novel aspect of SAM initiation.

As seen in Figure 3 and Table 2, it is clear that a positive correlation exists between the size of \textit{OSH1}-expression domain and the frequency of SAM initiation and the duration of SAM maintenance. In other words, the extent of SAM initiation and maintenance depends
on how many (indeterminate) cells are recruited into the SAM. This indicates that SAM formation is not an all-or-nothing process. SAM is constructed in various degrees, depending on how many indeterminate cells are recruited into SAM. As a result, when the SAM domain (the number of indeterminate cells) is small, the SAM is not produced as in strong shl2 alleles, but when it is intermediate, SAM is incompletely and transiently formed as observed in weak shl2 alleles. From the comparison of many shl2 alleles (Figure 3), we could quantitively estimate the threshold values for SAM formation: ~30 cells for the initiation and 80–90 cells for normal construction. At present, it is still unclear whether the number 30 has some general meaning such that an organ primordium needs at least 30 founder cells for initiation. In addition, the initial number of indeterminate cells for SAM is correlated with the duration of SAM maintenance. Thus, incompleteness at the initial step affects the subsequent activity of SAM. Incompletely initiated SAMs of weak shl2 alleles are not recovered, but are transiently maintained. This aspect of SAM formation has not been reported in other plants.

**Functional redundancy between shl2 and sho genes:** The embryo and seedling phenotypes of shl2-8 are very similar to those of sho mutants (Tamura et al. 1992; Itoh et al. 2000) in that both mutants commonly show flat SAMs, irregular phyllotaxy, short plastochron, and a reduction of the OSH1-expression domain. These phenotypes are unique to shl and sho mutants. This suggests that they may function in the same regulatory cascade. In fact, shl2 is epistatic to sho2, as shown by the double mutant phenotype of shl2 sho2. Detailed examination, however, reveals several differences between shl2 and sho mutants. First, the nine mutant alleles of SHO1–SHO3 do not show any of the histological abnormalities of leaves observed in shl2-8. Second, the OSH1-expression domain in embryos of nine sho mutants is larger than that of shl2-8. These differences indicate that shl2 shows more severe phenotypes and affects a larger number of traits than sho does. Thus, SHL2 is considered to be associated with fundamental processes of shoot development upstream of SHO genes.

In this study, we show that the SHL2 and SHL1 genes are required for both initiation and maintenance of the SAM and are also involved in the initiation and morphogenesis of leaf primordia. Whether the other SHL genes function in shoot development after SAM formation remains to be shown.

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