The Arabidopsis OBERON1 and OBERON2 genes encode plant homeodomain finger proteins and are required for apical meristem maintenance

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Maintenance of the stem cell population located at the apical meristems is essential for repetitive organ initiation during the development of higher plants. Here, we have characterized the roles of OBERON1 (OBE1) and its paralog OBERON2 (OBE2), which encode plant homeodomain finger proteins, in the maintenance and/or establishment of the meristems in Arabidopsis. Although the obe1 and obe2 single mutants were indistinguishable from wild-type plants, the obe1 obe2 double mutant displayed premature termination of the shoot meristem, suggesting that OBE1 and OBE2 function redundantly. Further analyses revealed that OBE1 and OBE2 allow the plant cells to acquire meristematic activity via the WUSCHEL-CLAVATA pathway, which is required for the maintenance of the stem cell population, and they function parallel to the SHOOT MERISTEMLESS gene, which is required for preventing cell differentiation in the shoot meristem. In addition, obe1 obe2 mutants failed to establish the root apical meristem, lacking both the initial cells and the quiescent center. In situ hybridization revealed that expression of PLETHORA and SCARECROW, which are required for stem cell specification and maintenance in the root meristem, was lost from obe1 obe2 mutant embryos. Taken together, these data suggest that the OBE1 and OBE2 genes are functionally redundant and crucial for the maintenance and/or establishment of both the shoot and root meristems.

KEY WORDS: Arabidopsis, PHD finger, meristem

INTRODUCTION

In higher plants, the apical-basal axis is established during embryogenesis. Following the determination of the body axis, apical meristems, which contain a pool of pluripotent stem cells, are formed at the apical and basal poles. After embryogenesis, the stem cells in the shoot apical meristem and in the root apical meristem produce the cells that are required for the aerial organs and the root system, respectively (Steeves and Sussex, 1989; Laux et al., 2004).

The shoot apical meristem is maintained through a balance between the proliferation of a group of stem cells residing in the center and the initiation of organ primordia on the flanks of the meristem. This balance is regulated by key genes, the expression of which is strictly regulated spatially and temporally (reviewed by Carles and Fletcher, 2003; Laux et al., 2004).

In Arabidopsis thaliana, a homeobox gene WUSCHEL (WUS) plays a central role in the maintenance of the stem cell pool in the shoot apical meristem. The WUS gene is expressed in a small group of cells proximal to the stem cells (Mayer et al., 1998) and is sufficient to induce the expression of CLAVATA3 (CLV3), which encodes a small peptide believed to be the extracellular ligand for the receptor CLV1 in the CLV pathway (Fletcher et al., 1999; Schoof et al., 2000). The CLV pathway, in turn, negatively regulates the stem cell population by restricting WUS expression. Thus, the feedback loop between WUS and CLV3 is formed and maintains the size of the stem cell pool (Brand et al., 2000; Schoof et al., 2000). Another gene involved in the shoot apical meristem formation, SHOOT MERISTEMLESS (STM), which encodes a KNOX-class homeodomain transcription factor, exerts its function to regulate the size of the stem cell pool by preventing the incorporation of stem cells into organ primordia (Barton and Poethig, 1993; Endrizzi et al., 1996; Long et al., 1996).

In the root apical meristem, four distinct types of initial cells surround the mitotically less active quiescent center (QC), which maintains the stem cell status of the initial cells by inhibiting their differentiation (Dolan et al., 1993; van den Berg et al., 1997). The PLETHORA1 (PLT1) and PLETHORA2 (PLT2) genes, which encode members of AP2-type putative transcription factors, are essential for the stem cell specification and maintenance in the root meristem (Aida et al., 2004). Expression of SCARECROW (SCR) and SHORT-ROOT (SHR) genes, both of which encode GRAS family transcription factors, are required for the QC specification (Di Laurenzio et al., 1996; Helariutta et al., 2000; Sabatini et al., 2003).

In animals, recent studies have demonstrated that the plant homeodomain (PHD) finger can specifically recognize the trimethylation of lysine 4 on histone H3, a hallmark for active genes (Li et al., 2006; Peña et al., 2006; Shi et al., 2006; Wysocka et al., 2006). It has been shown that the Arabidopsis PHD finger proteins function in fertility and flowering (Wilson et al., 2001; Pineiro et al., 2003; Yang et al., 2003; Sung and Amasino, 2004). However, the function of the PHD finger proteins in plants remains largely elusive.

In this study, we have identified two genes, OBERON1 (OBE1) and OBERON2 (OBE2), which are involved in the maintenance and/or establishment of both the shoot and root apical meristems in Arabidopsis. The OBE1 and OBE2 genes encode proteins with a
PHD finger domain and a coiled-coil domain. Although single mutation of either gene exhibits no apparent phenotype, obe1 obe2 double mutants exhibit aberrant development of both the shoot and root apical meristems, resulting in a seedling lethal phenotype. These observations suggest that OBE1 and OBE2 act redundantly and are required for the maintenance and/or establishment of the apical meristems.

MATERIALS AND METHODS

Plant materials

The Arabidopsis thaliana Columbia (Col-0) ecotype was used as the wild type. The obe-1 (SALK_075710), obe-2 (CS94914), clv3 (CS86724), sim-1 (Baron and Poethig, 1993) and was-1 (Laux et al., 1996) mutants were obtained from the Arabidopsis Biological Resource Center (ABRC). obe-2-1 (KG16805) was isolated by screening a total of 74,000 T-DNA insertion lines deposited at the Kasaza DNA Research Institute. The WUS::GUS and QC46 were kindly provided by Thomas Laux (Grol-Hardt et al., 2002) and Ben Scheres (Sabatini et al., 2003), respectively. Plants were grown on MS agar plates containing 1% sucrose or on rockwool bricks surrounded by vermiculite under long-day conditions (16 hours light/8 hours dark) at 22°C.

Isolation of OBE1 by yeast one-hybrid screening

The cis-regulatory region (~228–153) of the ERECTA (ER) promoter (Yokoyama et al., 1998) was used as bait in a yeast one-hybrid screening to search for transcriptional regulators that interact with the ER regulatory region. The Saccharomyces cerevisiae strain YM4271 containing this promoter fragment fused to the β-GAL selection marker was used to screen for specific DNA-binding proteins in the Arabidopsis CoLYEs cDNA library donated by John Mulligan and Ronald Davis (Ellidge et al., 1991). Screening of one million transformants yielded 52 positive clones. OBE1 was identified among the candidates as the gene encoding a PHD finger protein.

Construction of plasmids and transgenic plants

For the OBE1p::OBE1-GFP construct, genomic region corresponding to 4353 bp upstream of the OBE1 stop codon TAG was inserted upstream of the SGFp (S65T) coding region of PHB-GFP, pBI-GFP was constructed by replacing a SalI/EcoRI fragment from the SGFp (S65T) plasmid (kindly provided by Yasuo Niwa) containing the sGFP coding region and the GUS coding region of the 35S::sGFP (S65T) plasmid (Takahashi et al., 1992). For RT-PCR, 28-day-old plants were used, except for the transcriptional start of WUS::GUS (kindly provided by Yasuo Niwa) containing the sGFP coding region and the nopaline synthase gene (4353 bp upstream from the stop codon TAG was inserted upstream of the sGFP (S65T) coding region of pBI-GFP. pBI-GFP was constructed by replacing a BamHI fragment from the 35S::sGFP (S65T) plasmid and a 1.2 kb fragment downstream of the transcriptional start of CLV3 with the sGFP (S65T) coding region. For histological analysis, tissue samples were fixed in FAA (50% ethanol, 3.7% formaldehyde and 5% acetic acid), dehydrated in an ethanol series, and embedded in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany). Sections (8 μm) were stained in an aqueous 0.1% Toluidine blue solution for 2 minutes at room temperature.

RESULTS

OBE1 and OBE2 genes encode nuclear PHD finger proteins

OBE1 (At5g07780) and its paralog OBE2 (At5g48160) encode proteins consisting of 566 and 574 amino acid residues, respectively (Fig. 1A). The OBE1 gene is composed of two introns and three exons, including a 5′ untranslated exon located 0.6 kb upstream of the predicted translation start site. The OBE2 gene has a similar structure, except that the untranslated exon is located 0.4 kb upstream of the predicted translation start site (Fig. 1B). Both OBE1 and OBE2 proteins contain a PHD finger domain in the central region, consisting of a conserved Cys4-His-Cys3 zinc-finger domain, which has been shown to recognize the trimethylated lysine 4 of histone H3 (Aasland et al., 1995; Li et al., 2006; Shi et al., 2006; Peña et al., 2006; Wysocka et al., 2006), and a coiled-coil domain in the C-terminal region, which may possibly be involved in the multimer formation (Fig. 1A). OBE1 as well as OBE2 constitutes a small subfamily of PHD finger proteins in Arabidopsis. OBE1-related sequences have also been identified in pea, tobacco and rice, which we designated PsOBE1, NbOBE1 and OsOBE1, respectively (see Fig. S1A in the supplementary material), PHD finger sequences

For in situ hybridization, samples were fixed overnight in PBS containing 4% paraformaldehyde at 4°C and rinsed twice in PBS. The fixed tissue was dehydrated in an ethanol series and embedded in ParaplastPlus (Sigma, St Louis, MO). Tissue sections (8 μm thick) pretreatment was performed according to Mayer et al. (Mayer et al., 1998), except that protease treatment was performed with proteinase K (1 μg/ml; Sigma) for 30 minutes at 37°C. A probe concentration of 50 ng/ml/kb was used in the hybridization. After incubation at 45°C overnight, slides were washed according to Lincoln et al. (Lincoln et al., 1994), and incubated for 5–16 hours in 0.5 mg/ml NBT and 0.125 mg/ml BCIP in detection buffer [100 mM Tris–HCl (pH 9.5), 100 mM NaCl and 50 mM MgCl2], and after the reaction was stopped in 10 mM Tris, 1 mM EDTA. OBE1 and OBE2 riboprobes were generated from the full-length cDNA clones. The MP, PLT1, PLT2, SCR, STM, WOX5 and WUS riboprobes were generated as described previously (Di Laurenzenzo et al., 1996; Long et al., 1996; Hardtke and Berleth, 1998; Mayer et al., 1998; Aida et al., 2004; Haeker et al., 2004). Antisense and sense probes were synthesized with digoxigenin-11-UTP (Roche Diagnostics, Indianapolis, IN, USA) using T3 RNA polymerase.

For GUS staining, tissues were prefixed at room temperature in 90% acetone for 20 minutes, rinsed in staining buffer without 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc), and infiltrated with staining solution [50 mM sodium phosphate, pH 7.0; 0.2% (w/v) Triton X-100; 2 mM potassium ferrocyanide; 2 mM potassium ferricyanide; 1.9 mM X-Gluc] under vacuum on ice for 15 minutes and then incubated at 37°C for 2-8 hours.

For GFP analysis, embryos were removed from developmental seed coat, mounted on slides with water and observed using a confocal laser scanning microscope (OLYMPUS FV500).

Phenotypic analysis

For analysis of embryo phenotypes, ovules were cleared as described (Willemsen et al., 1998) and embryos were visualized using Nomarski optics on a Nikon ECLIPSE 80i photomicroscope. Starch granules in the columella root cap were visualized as described (Willemsen et al., 1998).

For histological analysis, tissue samples were fixed in FAA (50% ethanol, 3.7% formaldehyde and 5% acetic acid), dehydrated in an ethanol series and embedded in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany). Sections (8 μm) were stained in an aqueous 0.1% Toluidine blue solution for 2 minutes at room temperature.

For scanning electron microscopy, seedlings grown on MS plates were fixed in FAA, dehydrated in a graded ethanol series and critical point-dried using liquid CO2. After coating with gold, samples were viewed using a Hitachi scanning electron microscope.
of the proteins listed in Fig. S1A (see supplementary material) as well as four known PHD finger proteins in Arabidopsis are aligned in Fig. S1B (see supplementary material), which shows that OBE1-related proteins and other PHD finger proteins share the conserved cysteine residues with the characteristic spacing and the conserved aromatic amino acid residue preceding the C-terminal-most cysteine pair (Bienz, 2006).

To examine subcellular localization of OBE1 and OBE2, we generated transgenic Arabidopsis plants expressing either OBE1-GFP or OBE2-GFP fusion protein, or control GFP protein driven by the cauliflower mosaic virus (CaMV) 35S promoter. Five T2 lines for each fusion construct were examined, and we found that both OBE1-GFP and OBE2-GFP were localized in the nucleus (Fig. 1C, data not shown), while only GFP protein is observed over entire cells (Fig. 1D).

Expression patterns of OBE1 and OBE2

We first examined the expression of OBE1 and OBE2 in various tissues by semi-quantitative RT-PCR. OBE1 and OBE2 are expressed at a similar level with all tissues examined (Fig. 2A).

OBE1p::OBE1-GFP expression was confirmed by analyzing OBE1-GFP fusion protein in the torpedo stage embryo from an OBE1p::OBE1-GFP transgenic plant. Scale bars: 25 μm.

OBE2 throughout the embryonic development. OBE1 and OBE2 displayed the same expression pattern at all stages examined. Transcripts of OBE1 or OBE2 are first detectable in the embryo proper but not in the suspensor at the four-cell stage (Fig. 2B, data not shown). From the eight-cell to the bent-cotyledon stages, both genes are expressed throughout the embryo except for the suspensor retained up to the heart stage (Fig. 2C,D, data not shown). This expression pattern was confirmed by analyzing OBE1-GFP expression using transgenic plants introduced with the OBE1p::OBE1-GFP transgene (Fig. 2F). We conclude that OBE1 and OBE2 are expressed uniformly in all tissues except for the suspensor.

OBE1 and OBE2 act redundantly during early plant development

In order to define the role of OBE1 and OBE2 during Arabidopsis development, we analyzed loss-of-function mutants of OBE1 and OBE2. The obe1-1, which harbors a T-DNA insertion in the first exon of the OBE1 gene is a null allele, and OBE1 expression was not detectable in its homozygous plants (Fig. 1B; see Fig. S2 in the supplementary material). obe2-1 has a T-DNA insertion 120 bp upstream of the translational start codon for OBE2 (Fig. 1B), and in homozygous obe2-1 mutants, OBE2 transcripts were not detected, suggesting that obe2-1 also represents a null allele (see Fig. S2 in the supplementary material). obe2-2 is caused by a nonsense mutation within the PHD finger domain and is likely to be a null allele (Fig. 1B). These three mutants exhibited no detectable phenotypic differences from wild-type plants (data not shown). We next generated double mutants in combination of obe1 and obe2. All of the plants homozygous for both obe1-1 and obe2-1 or for obe1-1 and
obe2-2 displayed diminutive phenotype (the name of OBERON is derived from this phenotype) and were lethal during early development as described below. The OBE1p::OBE1-GFP transgene completely rescued the lethal phenotype of obe1 obe2 (data not shown), indicating that deprivation of both OBE1 and OBE2 is responsible for the lethal phenotype. As the phenotypes of the obe1-1 obe2-2 and obe1-1 obe2-1 double mutants were identical, we used obe1-1 obe2-1 as the obe1 obe2 double mutant for all further analyses.

**obe1 obe2 seedling phenotype**

In Arabidopsis, wild-type seedlings have two separated cotyledons, which are arranged symmetrically at the apex (Fig. 3A). In the obe1 obe2 mutants, the number of cotyledons varied from one to four, the size of which was reduced (Fig. 3B,C, Table 1). In addition, some obe1 obe2 seedlings showed a fused cotyledon phenotype (data not shown).

The vegetative shoot meristem forms rosette leaves in wild-type seedlings (Fig. 3D). By contrast, the obe1 obe2 mutant displayed premature termination of the shoot meristem, a defect that was classified into two types depending on the severity of the phenotype (Fig. 3E,F). Type I plants (82%, 109/133) displayed modest defects, in which the first pair of leaves but no additional leaves emerged (Fig. 3E, Table 2). They terminated the emergence of further leaves after the first pairs and eventually died within 10-30 days. Type II plants (18%, 24/133) displayed more severe defects, in which only cotyledons emerged and no rosette leaves occurred even 14 days after germination (Fig. 3F, Table 2). Scanning electron microscopic analysis revealed that the centers of both type I and type II mutant apices (Fig. 3H,I) are smaller than those of wild-type apices (Fig. 3G). In type I mutant plants grown for 14 days after germination, the next pair of leaves arise in the axils of the cotyledons (Fig. 3J), suggesting a loss of the shoot apical meristem. To examine more closely the defects of the obe1 obe2 shoot apical meristem, we analyzed mutant apices at the histological level. In comparison with the normal tunica-corpus structure in the wild-type apex (Fig. 3K), the obe1 obe2 apices were filled with relatively large and vacuolated cells that were not organized into distinctive cell layers (Fig. 3L).

**obe1 obe2 root phenotype**

The obe1 obe2 double mutant is also defective in root development (Fig. 4A). Histological analysis has revealed that the obe1 obe2 root fails to establish the normal radial pattern and contains enlarged and vacuolated cells without any morphological distinctions (Fig. 4B,E). In addition, the columella cells and the QC could not be identified morphologically in longitudinal sections of the distal root region (Fig. 4C,F), indicating that the morphology and arrangement of cells within the root apical meristem are disrupted in the obe1 obe2 mutant. To determine whether the columella cell layers derived from the columella initial cells are present in the obe1 obe2 mutant, we examined starch granule accumulation, a hallmark of mature columella cells (Willemsen et al., 1998). Unlike wild-type plants, obe1 obe2 mutants failed to accumulate starch (Fig. 4D,G), suggesting that columella initial cells are not produced or maintained in the obe1 obe2 mutant. These results suggest that the obe1 obe2 mutant fails to establish or maintain the root apical meristem.

**Developmental defects of obe1 obe2 embryos**

Next, to determine early developmental defects in obe1 obe2 embryos, we examined and searched for embryos with morphological defects at various stages among the population derived from self-fertilized plants heterozygous for obe1 and homozygous for obe2, or from plants heterozygous for obe2 and homozygous for obe1. Embryos with phenotypic deviation from the wild type were first identified at the early globular stage. In a smaller fraction than expected (4%, 5/128) of the early globular embryos from the self-fertilized plants, we detected aberrant oblique cell divisions in the hypophysis. These are not observed in wild-type counterparts (Fig. 5A,E). From the transition stage onwards, putative obe1 obe2 defects seemed to be evident; at this stage, 19% (10/54) of embryos from the self-fertilized plants lacked asymmetric cell divisions, resulting in a single cell layer where the cortex and endodermis precursors would otherwise be formed (Fig. 5B,F). Furthermore, the inner cells of the lower tier, which form the vascular cells of hypocotyl and root, failed to produce elongated

Table 1. Frequency of obe1 obe2 plants with different number of cotyledons

<table>
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<th>Parental genotype</th>
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<tr>
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<td>1</td>
<td>8</td>
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Frequency (%) of indistinguishable from wild type.

Frequency (%) of obe1 obe2 mutant plants with respect to the number of cotyledons.

Fig. 3. Phenotypes of obe1 obe2 mutants. (A-C) Three-day-old seedlings of wild type (A) and obe1 obe2 (B,C). (D-F) Fourteen-day-old seedlings of wild type (D) and obe1 obe2 (E,F). (G-J) Scanning electron micrographs of the shoot apical meristem of 7-day-old wild-type (G), 5-day-old obe1 obe2 (H,I), and 14-day-old obe1 obe2 seedlings (J). (K,L) Histological sections of the shoot apices from 4-day-old seedlings of wild type (K) and obe1 obe2 (L). Scale bars: 1 mm in A-F; 100 μm in G-L.
cell files (Fig. 5B,F). At the early heart stage, some protodermal cells at the apical region of the obe1 obe2 embryo divided periclinally instead of anticlinally, as observed in the wild-type embryos (Fig. 5C,D,G,H). Some of obe1 obe2 embryos initiated three cotyledons (compare Fig. 5L with 5I). In addition to the cotyledon defects, mutant embryos had thickened hypocotyls (compare Fig. 5J with 5M). At the bent-cotyledon stage, the cotyledon defects, mutant embryos had thickened hypocotyls (compare Fig. 5J with 5M). At the bent-cotyledon stage, the cotyledon defects, mutant embryos had thickened hypocotyls (compare Fig. 5J with 5M). At the bent-cotyledon stage, the cotyledon defects, mutant embryos had thickened hypocotyls (compare Fig. 5J with 5M).

In summary, although the specification of the root pole cells in obe1 obe2 embryos may be defective, initial patterning of them still occurs.

Altered expression of the shoot meristem regulators in obe1 obe2

Because obe1 obe2 seedlings result in the premature termination of the shoot apical meristem, we examined the expression of CLV3::GUS and WUS::GUS reporters. CLV3::GUS is expressed in the stem cells (Fig. 6A) and WUS::GUS is expressed in the organizing center (Fig. 6C). In the obe1 obe2 seedlings, neither CLV3::GUS nor WUS::GUS was expressed (Fig. 6B,D). Consistent with the seedling phenotype, these results indicate that the shoot apical meristem of the obe1 obe2 mutant terminates prematurely. The expression of CLV3 and WUS has been established during embryogenesis (Mayer et al., 1998; Fletcher et al., 1999). Thus, we next investigated CLV3 and WUS expression in obe1 obe2 embryos. In wild-type embryos, CLV3 expression starts at the early heart stage (Fig. 6E) and continues throughout embryogenesis (Fig. 6G). By contrast, in obe1 obe2 embryos, CLV3 expression was substantially decreased at the early heart stage (Fig. 6F) and became undetectable afterwards (Fig. 6H). WUS is first expressed in the four subepidermal apical cells of the 16-cell stage embryo. As embryogenesis proceeds, WUS expression becomes restricted to a small central cell group underneath the stem cells (Mayer et al., 1998). The onset of WUS expression was not affected in obe1 obe2 embryos (data not shown). WUS expression continued until the heart stage (Fig. 6J) and, similar to CLV3 expression, its expression was reduced at the heart stage (Fig. 6J) and became undetectable afterwards in obe1 obe2 embryos (Fig. 6L). This was different from WUS expression in wild-type siblings (Fig. 6K). Next, we examined the expression of STM, which is also crucial for proper embryonic shoot apical meristem formation and acts independently of WUS (Barton and Poethig, 1993; Endrizzi et al., 1996). At the torpedo and bent-cotyledon stages, STM was expressed in obe1 obe2 embryos in the same way as in wild-type siblings (Fig. 6M-P), indicating that loss of OBE1 and OBE2 does not affect the STM-mediated maintenance of the undifferentiated stem cell pool. These observations suggest that the stem cell population in the shoot apical meristem has been established by the heart stage but will soon be lost in the obe1 obe2 mutant. The transient presence of the stem cell population is supported by the observation that obe1 obe2 seedlings occasionally develop the first pair of leaves, as shown in the previous section.

Genetic interaction of obe1 obe2 with other mutations

To examine whether OBE1 and OBE2 genetically interact with the WUS-CLV or STM pathway in the maintenance of the shoot apical meristem, we generated triple mutants obe1 obe2 wus, obe1 obe2 clv3 and obe1 obe2 stm. The wild-type seedling successively produces rosette leaves from the shoot apical meristem (Fig. 7A), whereas the wus-1 mutant reiterates the initiation of rosette leaf formation and subsequent arrest of the shoot meristems, producing disorganized groups of leaves and shoots (Fig. 7B) (Laux et al., 1996). obe1 obe2 wus-1 triple mutants were indistinguishable from the obe1 obe2 double mutants (Fig. 7C,D; Table 2), suggesting that the obe1 obe2 mutation suppresses the production of disorganized leaves and shoots attributable to the wus-1 mutation. Similarly, the obe1 obe2 phenotype was unaffected by the introduction of the clv3 mutation (Table 2). These results indicate that obe1 obe2 is epistatic to the wus and clv3 mutations.

The severe stm mutant lacks an embryonic shoot meristem, but some mutant plants give rise to leaves from axils of cotyledons (Fig. 7E) (Barton and Poethig, 1993; Clark et al., 1996). Although the stm-1 mutant displays fusions in cotyledonous petioles (Barton and Poethig, 1993; Clark et al., 1996), the obe1 obe2 stm-1 mutant seedlings formed both fused petioles and partially fused cotyledons (Fig. 7F). Additionally, similar to obe1 obe2 type II seedlings, obe1 obe2 stm-1 plants never formed leaves (Fig. 7F, Fig. 3F; Table 2). Together with the observation that STM expression was not affected in the obe1 obe2 embryos, these results indicate that obe1 obe2 stm exhibit additive effects, and thus OBE1 and OBE2 do not function in the STM pathway. 

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**Table 2. Seedling phenotypes of double and triple mutants**

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**Fig. 4. Root phenotypes of obe1 obe2 seedlings.**

(A) Three-day-old seedlings of wild type (left) and obe1 obe2 (right). (B,C,E,F) Toluidine Blue staining of transverse (B,E) and longitudinal (C,F) sections of wild-type (B,C) and obe1 obe2 (E,F) roots. (D,G) Visualization of starch granules in the central root cap of wild type (D) or obe1 obe2 (G). Scale bars: 1 mm in A, 25 μm in B-G.
OBE1 and OBE2 are required for the onset of the root apical meristem marker expression

To assess how the morphological abnormalities were correlated with alterations in cell fate and tissue patterning, we investigated the expression of the root meristem markers in obe1 obe2 plants. We first addressed whether QC cells are present in the double mutant plant by examining expression of the QC marker QC46. In wild type, QC46 is expressed in the QC of seedlings and embryos (Fig. 8A,B). We found that the expression of QC46 was completely lost in the obe1 obe2 seedling and embryo (Fig. 8F,G). We also analyzed the expression of WUSCHEL-RELATED HOMEOBOX 5 (WOX5), another QC-specific gene, the expression of which initiates in the hypophysis of the early globular stage embryo and, subsequently, becomes restricted in the lens-shaped cell and its derivatives in normal embryogenesis (Haecker et al., 2004; Sarkar et al., 2007). Of the early globular stage embryos from a plant heterozygous for obe1 and homozygous for obe2, 27% (3/11) failed to express WOX5 (Fig. 8H) and the remaining 73% (8/11) expressed WOX5 in the hypophysis (Fig. 8C). At the late globular stage, 26% (8/31) of embryos from an OBE1/obe1 obe2/obe2 mother plant failed to express WOX5 (Fig. 8I) and the remaining 74% (23/31) exhibited wild-type WOX5 expression in the lens-shaped cell (Fig. 8D). These data suggest that QC cells are not present in the obe1 obe2 embryo.

Next, we addressed whether the hypophysis is specified in the double mutant by examining expression of MONOPTEROS (MP) in early globular stage embryos from a plant heterozygous for obe1 and homozygous for obe2. MP is initially expressed in the pro-embryo as well as in the provascular tissue from the early globular stage onwards (Hardtke and Berleth, 1998). MP functions in the induction of the formation of the lens-shaped cell (Weijers et al., 2006), and loss-of-function mutation of MP leads to a rootless phenotype (Berleth and Jürgens, 1993), which is similar to that observed in obe1 obe2 double mutant plants. In the provascular cells adjacent to the hypophysis, MP functions to induce the formation of
the lens-shaped cell (Weijers et al., 2006). Ninety-three percent (25/27) of early globular stage embryos from an OBE1/obe1 obe2/obe2 mother plant showed wild-type MP expression (data not shown). At the heart stage, MP was still expressed in the obe1 obe2 embryo (Fig. 8E,J).

Finally, we examined the expression of the PLT1, PLT2 and SCR genes, which are required for the QC specification in the root meristem (Aida et al., 2004). PLT1 expression is initially observed throughout the lower tier in the eight-cell embryo and then becomes restricted to the distal part of the embryonic root (Aida et al., 2004). In the eight-cell stage embryos from an OBE1/obe1 obe2/obe2 mother plant, 77% (23/30) exhibited PLT1 expression indistinguishable from the wild type (Fig. 8K); 23% (7/30) exhibited no PLT1 expression (Fig. 8O). After the division of the hypophysis, PLT1 expression was not detectable in 28% (10/36) embryos (Fig. 8P), while the remaining 72% (26/36) exhibited wild-type expression of PLT1 in the lens-shaped cell and the provascular cells (Fig. 8L). Similar results were obtained with PLT2 expression (data not shown). In normal embryogenesis, SCR expression is initiated from the globular stage embryo; later, SCR is expressed in the lens-shaped cell descendants, cortex-endodermis initials and the endodermal lineage (Di Laurenzio et al., 1996). In the globular stage embryos from an OBE1/obe1 obe2/obe2 mother plant, 33% (6/18) did not show SCR expression (Fig. 8Q) and the rest of the embryos (12/18) exhibited wild-type SCR expression (Fig. 8M). At the heart stage, SCR expression was not observed in the obe1 obe2 embryos, which were judged from their morphology (Fig. 8R), but was observed in other embryos supposed to possess the wild-type OBE1 allele (Fig. 8N). These data suggest that none of the four genes PLT1, PLT2 SCR and WOX5 is expressed in globular stage obe1 obe2 embryos. Therefore, we concluded that stem cell progenitors are missing from the basal region of obe1 obe2 embryos at the globular stage.

Fig. 8. Expression of the root apical meristem marker genes in the obe1 obe2 double mutant. (A,F) QC46 expression in the central root cap of wild-type (A) and obe1 obe2 (F) seedlings. (B,G) QC46 expression in wild-type (B) and obe1 obe2 (G) heart stage embryos. (C-E,H-R) In situ hybridization with WOX5 (C,D,H,I), MP (E,J), PLT1 (K,L,O,P) and SCR (M,N,Q,R) antisense probes hybridized to embryos from self-fertilized OBE1/obe1 obe2/obe2 plant. Longitudinal sections of in situ hybridization specimens are shown. (C,H) Early globular stage embryos. (D,I,L,P) Late globular stage embryos. (E,J,N,R) Heart stage embryos. (K,O) Eight-cell stage embryos. (M,Q) Globular stage embryos. About one quarter of the embryos from self-fertilized OBE1/obe1 obe2/obe2 plants failed to express WOX5 (H,I), PLT1 (O,P) or SCR (Q,R), while the remaining three-quarters of the embryos exhibited wild-type-like expression of the above genes. By contrast, MP expression was detected in all embryos (J). Scale bars: 25 μm in A-J,L-N,P-R; 10 μm in K,O.
DISCUSSION
We have characterized the role of the two paralogous genes \textit{OBE1} and \textit{OBE2} in \textit{Arabidopsis} development. Both \textit{OBE1} and \textit{OBE2} encode PHD finger proteins, and \textit{obe1 obe2} double mutants displayed severe defects in the shoot and root apical meristems. Our data suggest that \textit{OBE1} and \textit{OBE2} are functionally redundant and play an important role in the maintenance and/or establishment of the shoot and root apical meristems.

\textbf{OBE1 and OBE2 encode PHD finger proteins}

We have shown that \textit{OBE1} and \textit{OBE2} belong to the PHD finger protein family and contain a coiled-coil domain that is thought to be required for homo- or heterodimerization. In agreement, both \textit{OBE1} and \textit{OBE2} have been shown to interact in homo- and heterodimer combinations by yeast two-hybrid analysis (C.F. and Y.K., unpublished). The PHD finger domain is shared by various nuclear proteins and has been proposed to be involved in protein-protein interactions (Aasland et al., 1995). In animals, the PHD finger domain is known among transcriptional co-regulators and proteins in chromatin modifying complexes, such as Jade-1, p300, CBP and ING1 (Aasland et al., 1995; Bordoli et al., 2001; Peng et al., 2002; Panchenko et al., 2004) and constitutes a highly specialized trimethyl-lysine binding domain (Li et al., 2006; Peña et al., 2006; Shi et al., 2006; Wysocka et al., 2006). In plants, the PHD finger domain has been identified in MS1, MMD, EBS and VIN3, which are regarded as putative transcriptional regulators involved in fertility or flowering (Wilson et al., 2001; Pineiro et al., 2003; Yang et al., 2003; Sung and Asamaso, 2004). We have demonstrated that \textit{OBE1} and \textit{OBE2} proteins are localized in the nucleus, suggesting that they may also play a role as important transcriptional regulators.

\textbf{Shoot apical meristem defects in the obe1 obe2 double mutant}

In \textit{Arabidopsis}, the shoot meristem is established during embryonic development, gives rise to rosette leaves and subsequently produces an inflorescence shoot. This continuous organ formation from the shoot meristem requires the maintenance of the undifferentiated stem cell pool. In the \textit{obe1 obe2} mutant, the onset of \textit{CLV3} and \textit{WUS} expression was observed but was not maintained. These findings suggest that the embryonic shoot apical meristem of the \textit{obe1 obe2} mutant is established by transient \textit{CLV3} and \textit{WUS} expression, but is not maintained in later stages of development because of the loss of \textit{CLV3} and \textit{WUS} expression. By contrast, \textit{STM} expression persisted during embryogenesis in the \textit{obe1 obe2} mutant, suggesting that the \textit{obe1 obe2} shoot apex has meristematic cells that are predominantly dependent on the \textit{STM} activity. Consistent with this notion, introduction of the \textit{stm} mutation into \textit{obe1 obe2} prevented the formation of the first pair of leaves, which would otherwise be observed in the \textit{obe1 obe2} seedling. The additive phenotypes of the \textit{obe1 obe2 stm} triple mutant indicate that the \textit{OBE1} and \textit{OBE2} exert their function parallel to the \textit{STM} pathway. By contrast, the \textit{obe1 obe2 clv3} and \textit{obe1 obe2 wus} triple mutants were phenotypically indistinguishable from the \textit{obe1 obe2} double mutant, indicating that the \textit{obe1 obe2} mutation is epistatic to the \textit{clv3} and \textit{wus} mutations. Thus, \textit{OBE1} and \textit{OBE2} activities are required for \textit{WUS-CLV} function and maintenance.

\textbf{Root apical meristem defects in the obe1 obe2 double mutant}

The root apical meristem is established during embryogenesis. The embryonic root apical meristem formation is initiated by the specification of the hypophysis. The asymmetric division of the hypophysis creates the lens-shaped progenitor cell for the QC (reviewed by Jenik et al., 2007). The auxin response transcription factor MP and its negative regulator BODENLOS (BDL) are required for the hypophysis specification (Weijers et al., 2006). The specification of the QC identity in the lens-shaped cell and its progeny is established by co-expression of the SCR, PLT1 and PLT2 genes (Aida et al., 2004). Although the \textit{obe1 obe2} mutant resembles the \textit{mp} mutant, \textit{MP} was expressed in the \textit{obe1 obe2} embryo, suggesting that the defects of the basal pole of the \textit{obe1 obe2} embryo are not due to loss of the MP function. By contrast, in the \textit{obe1 obe2} mutant, the initial expression of \textit{PLT1} and \textit{PLT2} was not detectable, nor was expression at subsequent stages. These observations suggest that \textit{OBE1} and \textit{OBE2} are required for \textit{PLT1} and \textit{PLT2} expression, and may act downstream of \textit{MP/BDL} to mediate the establishment of the embryonic root apical meristem. Similarly, the initiation of \textit{SCR} and \textit{WOX5} expression also requires \textit{OBE1} and \textit{OBE2}. As \textit{WOX5} expression depends on the \textit{SCR} activity (Sarker et al., 2007), the \textit{SCR}-mediated QC specification pathway should be subsequently disrupted in the \textit{obe1 obe2} mutant. Therefore, we propose that \textit{OBE1} and \textit{OBE2} are necessary for the expression of the key regulators involved in the initiation of the root apical meristem.

\textbf{Putative role of OBE1 and OBE2 in the meristematic activity}

We have demonstrated that the \textit{OBE1} and \textit{OBE2} genes exert their function in the maintenance and/or establishment of shoot and root apical meristems by controlling the expression of the meristem genes such as \textit{WUS}, \textit{PLT1} and \textit{PLT2}. Even in severe \textit{wus} mutants, which are defective in shoot apical meristem development during embryogenesis, new leaves are eventually initiated at the flat apices during the later stages of development (Laux et al., 1996), suggesting that cells from the terminated shoot apices of \textit{wus} mutants still retain the competence for meristem activity. However, the initiation of new leaves does not occur when \textit{OBE1} and \textit{OBE2} are mutated under the \textit{wus} background. The ectopic expression of \textit{WUS} and \textit{PLT1/2} genes is able to induce the ectopic shoot and root, respectively (Aida et al., 2004; Gallois et al., 2004), whereas the ectopic expression of \textit{OBE1} and/or \textit{OBE2} was not able to induce ectopic organs (data not shown). These observations suggest that \textit{OBE1} and \textit{OBE2} are required for plant cells to reach an appropriate state for the establishment and/or maintenance of the apical meristems by the action of meristem genes, rather than by the specification of the apical meristems directly.

Taken together, our findings indicate that \textit{OBE1} and \textit{OBE2} may allow plant cells to respond to such meristematic activity and are required for the meristem-inducing genes to function properly. The fact that the \textit{OBE1} and \textit{OBE2} encode proteins with a PHD finger domain that has been shown to be involved in remodeling of the chromatin structure may support the above hypothesis.

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\textbf{Supplementary material}

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/10/1751/DC1
References