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Control of embryonic meristem initiation in *Arabidopsis* by PHD-finger protein complexes

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SUMMARY

Plant growth is directed by the activity of stem cells within meristems. The first meristems are established during early embryogenesis, and this process involves the specification of both stem cells and their organizer cells. One of the earliest events in root meristem initiation is marked by re-specification of the uppermost suspensor cell as hypophysis, the precursor of the organizer. The transcription factor MONOPTEROS (MP) is a key regulator of hypophysis specification, and does so in part by promoting the transport of the plant hormone auxin and by activating the expression of TARGET OF MP (TMO) transcription factors, both of which are required for hypophysis specification. The mechanisms leading to the activation of these genes by MP in a chromatin context are not understood. Here, we show that the PHD-finger proteins OBERON (OBE) and TITANIA (TTA) are essential for MP-dependent embryonic root meristem initiation. TTA1 and TTA2 are functionally redundant and function in the same pathway as OBE1 and OBE2. These PHD-finger proteins interact with each other, and genetic analysis shows that OBE-TTA heterotypic protein complexes promote embryonic root meristem initiation. Furthermore, while *MP* expression is unaffected by mutations in *OBE*/*TTA* genes, expression of MP targets *TMO5* and *TMO7* is locally lost in *obe1 obe2* embryos. PHD-finger proteins have been shown to act in initiation of transcription by interacting with nucleosomes. Indeed, we found that OBE1 binds to chromatin at the *TMO7* locus, suggesting a role in its MP-dependent activation. Our data indicate that PHD-finger protein complexes are crucial for the activation of MP-dependent gene expression during embryonic root meristem initiation, and provide a starting point for studying the mechanisms of developmental gene activation within a chromatin context in plants.

KEY WORDS: *Arabidopsis*, PHD finger, Embryogenesis

INTRODUCTION

Apical meristems, located at the growing tips, are indispensable for plant development because these produce all plant organs post-embryonically (Weigel and Jürgens, 2002). The meristems and the stem cells contained within these are formed during embryogenesis. The first manifestation of embryonic root meristem initiation is marked by the specification of an initially extra-embryonic suspensor cell as hypophysis. The hypophysis divides asymmetrically and its small descendant cell will become the quiescent center (QC), which maintains stem cell identity in adjoining cells of the root meristem (reviewed by Möller and Weijers, 2009). Root meristem initiation has been studied mostly using genetic approaches. Few mutations that specifically affect embryonic root initiation have been identified (Mayer et al., 1991), and most of those that have been described converge on the activity of the auxin-dependent transcription factor MONOPTEROS (MP)/AUXIN RESPONSE FACTOR 5 (Hardtke and Berleth, 1998; Weijers et al., 2006) (reviewed by Möller and Weijers, 2009). MP is inhibited by the interacting BODENLOS (BDL)/IAA12 protein. The plant hormone auxin promotes degradation of BDL, thereby releasing MP from inhibition (Hamann et al., 2002). Knowledge about the network operating downstream of MP in root initiation, and the mechanisms of gene regulation by MP beyond inhibition by BDL is fragmented. Recently, a first set of MP targets was identified. Among these, *TARGET OF MP 5* (*TMO5*) and *TMO7* genes are directly activated by binding of MP to their promoters. In turn, TMO7 is required for MP-dependent embryonic root meristem initiation (Schlereth et al., 2010). MP also promotes the transport of auxin through controlling PIN1 activity, resulting in auxin accumulation in the future hypophysis that is a fundamental event for MP-dependent embryonic root meristem initiation (Friml et al., 2003; Weijers et al., 2006). In addition to MP and its direct target TMO7, several other factors have been shown to contribute to embryonic root formation. PLETHORA (PLT) proteins, which belong to the AP2-type transcription factor family, are essential for the specification and maintenance of the stem cells (Aida et al., 2004; Galinha et al., 2007), while GRAS family transcription factors SCARECROW (SCR) and SHORT-ROOT (SHR) are important for controlling the radial tissue organization of the root (Di Laurenzio et al., 1996; Helariutta et al., 2000; Sabatini et al., 2003). Although all these transcription factors have been shown to be involved in embryonic root meristem formation, their activity appears to be required after initial MP-dependent initiation. Key unanswered questions are what the connections between these components are, and what mechanisms ensure strict spatial control of these genes.

OBERON1 (*OBE1*) and *OBE2* genes encode plant homeodomain (PHD)-finger proteins and these genes act redundantly in MP-dependent embryonic root initiation (Saiga et al., 2008; Thomas et al., 2009). The PHD-finger domain is found in a wide variety of proteins involved in the regulation of chromatin structure (Taverna et al., 2007). PHD-finger domain is constituted of a conserved Cys4-His-Cys3 zinc-finger domain (Aasland et al., 1995). Recent studies demonstrated that the PHD-

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finger domain specifically binds to histone H3 trimethylated at lysine 4 (Li et al., 2006; Peña et al., 2006; Shi et al., 2006; Wysocka et al., 2006; Lee et al., 2009), which is associated with nucleosomes near the promoters and 5′ ends of highly transcribed genes (Zhang et al., 2009), and recruit transcription factors and nucleosome-associated protein complexes to chromatin (Saksouk et al., 2009). Interestingly, although *PLT1*, *PLT2*, *SCR* and *WOX5* are not expressed in *obe1 obe2* double-mutant embryos, *MP* is normally expressed. As expression of *PLT1*, *PLT2* and *WOX5* depends on MP (Aida et al., 2004; Sarker et al., 2007), one possibility is that OBE1 and OBE2 act to control embryonic root meristem formation downstream or at the level of MP (Saiga et al., 2008). However, the function of OBE proteins in the MP pathway is not known.

Here, we demonstrate the role of PHD-finger proteins involved in MP-dependent embryonic root initiation in *Arabidopsis*. *TITANIA1* (*TTA1*) and *TTA2* genes, which are closest homologs of *OBE1* and *OBE2*, are functionally redundant and required for MP-dependent embryonic root initiation. Our data show that OBE1 locally mediates the activation of *TMO5* and *TMO7* genes. Construction of triple and quadruple mutants among *obe1*, *obe2*, *tta1* and *tta2* showed that OBE1/2 and TTA1/2 also act redundantly in embryogenesis. Our findings suggest that activation of transcription factor genes during root initiation requires the activity of a PHD-finger protein complex.

MATERIALS AND METHODS

Plant materials

The *Arabidopsis thaliana* Columbia (Col-0) ecotype was used as the wild type. The *tta1-1* (SALK_042597), *tta2-1* (SALK_082338) and *tta2-2* (SALK_016218) mutants were obtained from the *Arabidopsis* Biological Resource Center (ABRC). *obe1* and *obe2* mutants, *OBE1p::OBE1-GFP*, *TMO5p::3×nGFP* and *TMO7p::3×nGFP* transgenic lines have been described previously (Saiga et al., 2008; Schlereth et al., 2010). Plants were grown on MS agar plates containing 1% sucrose or on rock-wool bricks surrounded by vermiculite under long-day conditions (16 hours light/8 hours dark) at 22°C.

Construction of plasmids and transgenic plants

For the *TTA1p::TTA1-GFP* and *TTA2p::TTA2-GFP* constructs, genomic regions corresponding to 4780 bp upstream from the *TTA1* stop codon TGA and corresponding to 5333 bp upstream from the *TTA2* stop codon TAA, respectively, were cloned into pGEM-T (Promega) then subcloned into pBI-GFP (Saiga et al., 2008).

For the *MPp::OBE1* and *ARF13p::OBE1* constructs, the coding region of *OBE1* was cloned into pGreenII BAR (pGreen-OBE1). *MP* and *ARF13* promoter fragments (Schlereth et al., 2010) were introduced into pGreen-OBE1.

All constructs were transformed into wild-type or *obe1*/+ *obe2* plants by the floral dip method (Clough and Bent, 1998).

Phenotypic analysis

For observation of embryos, histological analysis and microscopy were performed as described previously (Saiga et al., 2008).

Expression analysis

In situ hybridization was performed as described previously (Saiga et al., 2008). The *MP*, *PLT1*, *SCR* and *WOX5* riboprobes were generated as described previously (Saiga et al., 2008).

Chromatin immunoprecipitation (ChIP)

ChIP experiments were performed according to Gendrel et al. (Gendrel et al., 2005) with minor modification. Globular stage embryos from silique of *OBE1p::OBE1-GFP* were used to precipitate OBE1-GFP-bound chromatin. For immunoprecipitation, a polyclonal anti-GFP antibody (ab290, Abcam) was used.

Yeast two-hybrid assays

Yeast two-hybrid interactions were performed using the HybriZAP-2.1 Two-Hybrid Predigested vector kit (Stratagene). The open reading frames of *OBE1*, *OBE2*, *TTA1* and *TTA2* were amplified from wild-type cDNA using gene-specific primers. Amplified DNA fragments were subcloned into pGEM-T and subsequently cloned into pAD-GAL4-2.1 and pBD-GAL4 Cam. The bait and prey constructs were transformed into the yeast strain YRG-2. Mating and selection for interactions were performed according to the manufacture's protocol (Stratagene). All experiments were repeated at least three times.

Protein complex isolation and mass spectrometry

For immunoprecipitation, 1 g of *OBE1p::OBE1-GFP* and Col-0 siliques were ground in a mortar with liquid nitrogen. Protein extraction, immunoprecipitation and mass spectrometry were performed as reported by Zwiewka et al. (Zwiewka et al., 2011) with minor modifications. nLC-MS/MS analysis was carried out using a LTQ-Orbitrap. Data were analyzed using the Bioworks software package version 3.1.1 (Thermo Scientific).

RESULTS

TTA1 and TTA2 are essential for normal pattern formation

We have previously demonstrated that the PHD-finger proteins OBE1 and OBE2 are indispensable for the establishment and maintenance of the both shoot and root apical meristem (SAM and RAM, respectively) (Saiga et al., 2008). In *Arabidopsis*, there are two close homologs of *OBE1* and *OBE2*, and we named these *TITANIA1* (*TTA1*) and *TTA2* (Fig. 1A). TTA1 (At1g14740) and TTA2 (At3g63500) proteins share 55% amino acid similarity, suggesting that TTA1 and TTA2 function redundantly, as is the case of OBE1 and OBE2. To test this possibility, we analyzed loss-of-function mutants of *TTA1* (*tta1-1*) and *TTA2* (*tta2-1* and *tta2-2*) (supplementary material Fig. S1A). As none of single mutants exhibited obvious phenotypes (data not shown), we generated double mutant combinations of these mutants. All *tta1 tta2* double mutants showed seedling lethality (supplementary material Fig. S1B,C) and these phenotypes were completely rescued by introducing *TTA1p::TTA1-GFP* or *TTA2p::TTA2-GFP* (supplementary material Fig. S1D; data not shown). These observations indicate that TTA1 and TTA2 indeed function redundantly. We used *tta2-1* as the *tta2* mutant for all further analyses.

tta1 tta2 double mutants exhibited a rootless phenotype and this defect is probably derived from disruption of normal pattern formation during embryogenesis. To determine how embryonic pattern formation is perturbed in *tta1 tta2*, we examined embryos from self-fertilized plants heterozygous for *tta1* and homozygous for *tta2* as double homozygous plants died before flowering. It is expected that ~25% of embryos from these plants might segregate as *tta1 tta2* double homozygous.

We found additional and abnormal cell divisions at the embryo proper from the two-cell to 16-cell stage (Fig. 1B,F; data not shown). Although it was observed in only a fraction of embryos from *tta1*/+ *tta2* plants (Table 1), both *TTA1p::TTA1-GFP* and *TTA2p::TTA2-GFP* completely rescued those defects (data not shown), indicating that deprivation of both *TTA1* and *TTA2* is responsible for those phenotypes. At the globular stage, during which the hypophysis divides into a smaller apical cell and larger basal cell in the wild-type embryo (size of apical cell, 5.7±0.5 μm; size of basal cell, 11.1±0.3 μm; n=20), the hypophysis of *tta1 tta2* embryos divided abnormally (Fig. 1C,G), resulting in production of two equally size descendants (size of apical cell, 8.1±0.6 μm; size of basal cell, 9.2±0.5 μm; n=20) (Fig. 1D,H). Furthermore, cell

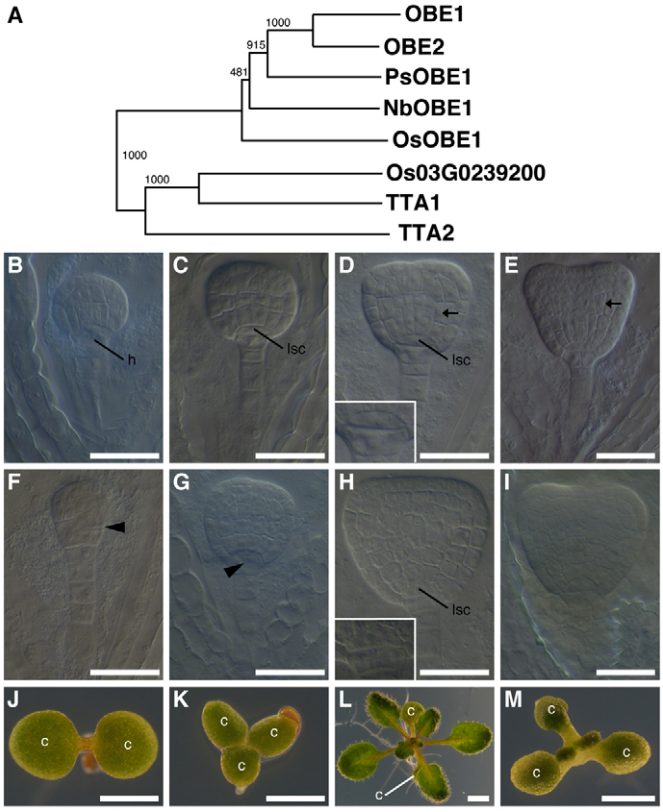


Fig. 1. Phenotypes of *tta1 tta2* double mutants. (**A**) Phylogenetic tree for the OBE family proteins, derived using complete sequences constructed with ClustalX2.0. The tree was made with 1000 bootstrap trials, with correction for gaps in sequences. PsOBE1, NbOBE1 and OsOBE1 are putative orthologs of OBE1 from *Pisum sativum*, *Nicotiana benthamiana* and *Oryza Sativa*, respectively. (**B-I**) Embryonic phenotypes of wild type (B-E) and *tta1 tta2* mutants (F-I) at the early globular (B,F), late globular (C,G), triangular (D,H) and heart (E,I) stages. Insets in D,H represent magnified view of hypophysis descendants. In D, arrows indicate the longitudinal division of ground tissue cells. In F and G, arrowheads indicate aberrant cell divisions. (**J-M**) Seedling phenotypes of wild type (J,L) and *tta1 tta2* mutants (K,M) at 4 days (J,K) and at 14 days (L,M) after germination. h, hypophysis; lsc, lens-shaped cell; c, cotyledon. Scale bars: 30 μm in B-I; 1 mm in J,K; 2 mm in L,M.

division of endodermis/cortex cell files in *tta1 tta2* is missing (Fig. 1E,I). These observations suggest that the rootless phenotype in the *tta1 tta2* double mutant results from an early defect in embryonic root initiation. After germination, *tta1 tta2* seedlings have a variable number of cotyledons (Fig. 1J,K; Table 2) in addition to the rootless phenotype and eventually die after forming the first pair of leaves (Fig. 1L,M).

TTA1 and TTA2 are required for root meristem patterning

To address whether only cell division is affected in *tta1 tta2* embryos, or whether cell identities are incorrectly specified, we examined the expression of marker genes by in situ hybridization or fluorescence microscopy-based expression analysis. In this analysis, we used embryos obtained from self-fertilized plants heterozygous for *tta1* and homozygous for *tta2*, in which it is expected ~25% of embryos might segregate as *tta1 tta2* double homozygous.

Table 1. The frequency of abnormal embryos among the progeny of *tta1*/+ *tta2* plants

Parental genotype	Phenotypes		*n*
	Normal*	Abnormal cell division	
Two- to 16-cell stage			
Wild type	100	0	102
tta1/+ *tta2*	96	4	159
Early globular stage			
Wild type	100	0	130
tta1/+ *tta2*	92	8	110
Late globular stage			
Wild type	100	0	118
tta1/+ *tta2*	78	22	216
Triangular stage			
Wild type	100	0	134
tta1/+ *tta2*	78	22	179

Embryos from segregating double mutant plants were morphologically analyzed at different stage during embryogenesis.
*Frequency (%) of embryos that are indistinguishable from wild type.
n, number of embryos analyzed.

As the hypophysis division defect in *tta1 tta2* embryos strongly resembles the *mp* mutant, we first addressed whether *MP* expression is lost in *tta1 tta2* embryos. Ninety-six percent (23 out of 24) of early globular stage embryos showed wild-type *MP* expression (data not shown). At the heart stage, *MP* was still expressed in the *tta1 tta2* embryos (Fig. 2A,E), suggesting that the phenotype is not due to a loss of *MP* expression. We next investigated the expression of the *PLT1* and *SCR* genes. *PLT1* is required for the QC specification and is expressed in the basal region of embryo proper at the globular stage in wild type (Fig. 2B) (Aida et al., 2004). However, no *PLT1* expression was detectable in 25% (11/44) of embryos (Fig. 2F). *SCR* is also required for the QC specification, in which it acts in parallel with *PLT* genes (Sabatini et al., 2003; Aida et al., 2004). *SCR* is initially expressed in the hypophysis at the early globular stage and is subsequently activated in the ground tissue (Fig. 2C) (Wysocka-Diller et al., 2000). By contrast, of the globular stage embryos, *tta1 tta2* embryos (14/57) failed to express *SCR* (Fig. 2G). These data indicate that specification of the QC is defective in *tta1 tta2* embryos. Interestingly, *SCR* expression was also lost from ground tissue cells (Fig. 2G), which is consistent with the failure of these cells to divide in the double mutant (Fig. 1I). Consistent with a loss of QC identity, *WOX5* expression, which initiates in the hypophysis at the globular stage and subsequently becomes restricted in the lens-shaped cell and its derivatives in the wild-type embryo (Fig. 2D) (Haecker et al., 2004), was completely lost in *tta1 tta2* embryos (8/30) (Fig. 2H). In summary, *MP* was still expressed in *tta1 tta2* embryos, but expression of *PLT1*, *SCR* and *WOX5* was lost, suggesting that cell identity specification in this mutant is compromised downstream of *MP* activity.

TTA1 and TTA2 are expressed ubiquitously during embryogenesis

As TTA1 and TTA2 are redundantly required for specification of the hypophysis and establishment of the embryonic root, we predicted that TTA1 and TTA2 proteins are expressed in the basal region of embryo proper and/or upper-most suspensor cells at the early globular stage, when the hypophysis is specified (Weijers et

Table 2. Frequency of *tta1 tta2* plants with different number of cotyledons

	Distribution of phenotypes				
Parental genotype	Normal*	Two‡	Three‡	Four‡	*n*
Wild type	100	0	0	0	194
tta1/+ tta2	76	3	19	2	273

*Frequency (%) of embryos indistinguishable from wild type.
‡Frequency (%) of *tta1 tta2* mutant plants with respect to the number of cotyledons.
n, number of seedlings analyzed.

al., 2006). To investigate the expression pattern of TTA1 and TTA2 proteins, we generated *TTA1p::TTA1-GFP* and *TTA2p::TTA2-GFP* transgenic lines, and analyzed the expression pattern of these throughout embryonic development. Both constructs complemented the double mutant phenotype (supplementary material Fig. S1D; data not shown), indicating that the fusions encode functional proteins. GFP fluorescence was first detected in the two-cell stage embryos (Fig. 3A). At the early globular stage, TTA1-GFP was found both in the basal region of embryo proper and suspensor cells (Fig. 3B), which is consistent with the finding that TTA1 has a role for hypophysis specification. During embryonic development, TTA1 was expressed not only in the basal region but also in the apical region of the embryo proper (Fig. 3C,D). TTA2 displayed the same expression pattern at all stages examined (Fig. 3E-H). Interestingly, despite the ubiquitous expression of both genes, the phenotype resulting from the loss of both genes is remarkably specific to the hypophysis.

TTA and OBE function in the same pathway through forming a heterotypic protein complex

Given the finding that *tta1 tta2* exhibited similar defects as observed in *obe1 obe2* embryos (compare with Saiga et al., 2008), we generated multiple mutant combinations among these mutants. We found that *obe1 tta1*, *obe1 tta2*, *obe2 tta1* and *obe2 tta2* double mutants exhibited no obvious phenotypes. This result suggests that the OBE1/2 and TTA1/2 proteins are not simply redundant, but rather that one protein from each pair is required for normal development. To determine the consequences of progressively eliminating the entire OBE1/2 TTA1/2 clade, we next analyzed embryos of *obe1 obe2 tta2* triple mutants from an *obe1*/+ *obe2 tta2* mother plant. Among the progeny of such plants, 20% of embryos exhibited embryonic lethality (Table 3). *obe1 obe2 tta2* did not show novel phenotypes in the basal region where the formation of the embryonic root meristem is already disrupted in *obe1 obe2* and *tta1 tta2* embryos. However, development of the apical region where cotyledon primordia and shoot apical meristem are produced was disturbed (Fig. 4A-C,E-G). During transition from triangular to heart stage in wild-type siblings in the same silique, cotyledon primordia had correctly emerged (Fig. 4A,B); however, emergence of cotyledon primordia was not observed in *obe1 obe2 tta2* embryos (Fig. 4E,F). In addition, the apical region of *obe1 obe2 tta2* embryos was abnormally expanded compared with wild-type siblings (Fig. 4B,F). *obe1 obe2 tta2* triple mutant embryos arrested at the triangular stage (Fig. 4C,G). We further investigated other triple mutant combinations and found that all of them showed same phenotypes (data not shown). Finally, we investigated the phenotypes of *obe1 obe2 tta1 tta2* quadruple mutants. We found that ~5% of embryos from *obe1*/+ *obe2 tta1*/+ *tta2* mother plants were swollen when wild-type siblings were at the bent-cotyledon stage (Fig. 4D,H; Table 3). These data indicate that although the OBE1/2 and TTA1/2 pairs are not redundant in root formation, all four proteins function redundantly in development of the apical pole, as well as in progression beyond the triangular stage of embryogenesis.

The genetic interactions seen among OBE1/2 and TTA1/2 proteins are consistent with joint requirement of multiple proteins for biological function, for example, in a protein complex. To determine whether protein complexes can be formed among these proteins, we initially tested all possible pairwise combinations between OBE and TTA proteins, including their homodimers, using a yeast two-hybrid assay. In this assay, all tested interactions were positive (Fig. 5A), suggesting extensive interaction potential among all proteins.

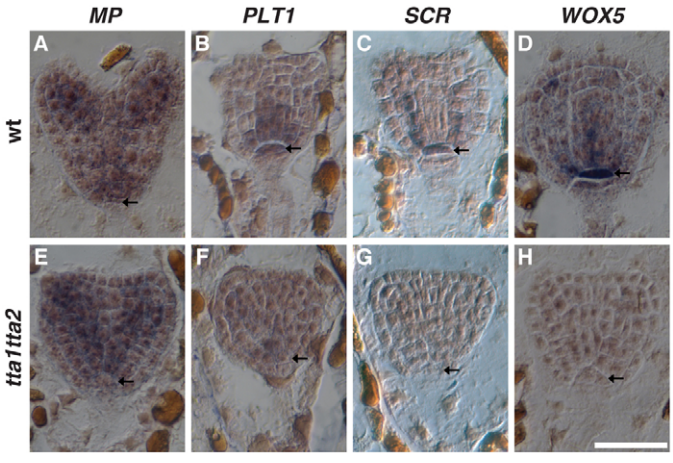


Fig. 2. Expression of the root meristem marker genes in *tta1 tta2* embryos. (**A**,**E**) *MP* mRNA expression in wild-type (A) and *tta1 tta2* (E) embryos. (**B**,**F**) *PLT1* mRNA expression in wild-type (B) and *tta1 tta2* (F) embryos. (**C**,**G**) *SCR* mRNA expression in wild-type (C) and *tta1 tta2* (G) embryos. (**D**,**H**) *WOX5* mRNA expression in wild-type (D) and *tta1 tta2* (H) embryos. Arrows indicate lens-shaped cells. Scale bar: 30 μm.

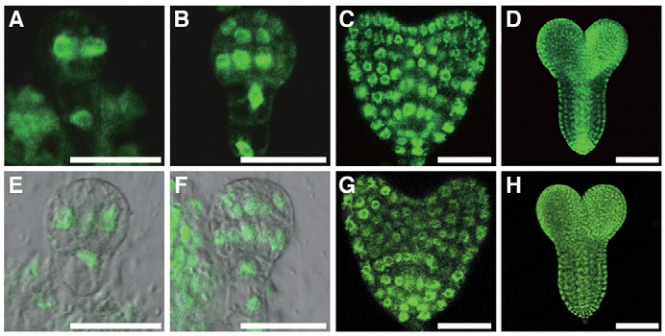


Fig. 3. Expression patterns of TTA1 and TTA2. (**A-D**) TTA1p::TTA1-GFP expression in wild-type embryos at the two-cell (A), early globular (B), heart (C) and torpedo (D) stages. (**E-H**) TTA2p::TTA2-GFP expression in wild-type embryos at the two-cell (E), early globular (F), heart (G) and torpedo (H) stages. Scale bars: 30 μm.

Table 3. Combinations of *obe1*, *obe2*, *tta1* and *tta2* mutants

Parental genotype	Phenotypes			*n*
	Normal*	Triangular arrest	Swollen embryo	
Wild type	100	0	0	190
obe2 tta1/+ tta2	77	23	0	337
obe1/+ obe2 tta1	81	19	0	202
obe1/+ obe2 tta1/+ tta2	60	35	5	446

*Frequency (%) of embryos indistinguishable from wild type.
n, number of embryos analyzed.

To determine whether complexes involving multiple OBE/TTA proteins are found in vivo, we used a translational fusion construct for OBE1-GFP that was previously shown to be functional (Saiga et al., 2008). We isolated the OBE1-GFP protein complex using immunoprecipitation on silique tissue. Next, associated proteins were identified by tandem mass spectrometry. Strikingly, in addition to OBE1, peptides uniquely representing OBE2, TTA1 and TTA2 were identified in pull-down experiments with OBE1-GFP siliques, but not with wild-type siliques (Fig. 5B; Table 4). Given the finding that TTA and OBE proteins act downstream or at the level of MP, we analyzed the mass spectrometry results for MP peptides, but did not find any. Hence, there is no evidence for a direct association between OBE1 and MP. These results demonstrate that OBE1 is found in complex with other OBE/TTA proteins, although this experiment does not resolve the size or topology of such complexes.

OBE1 is required for the expression of direct *MP* target genes

We have shown previously that *obe1 obe2* double mutants are defective in embryonic root initiation, resulting in rootless phenotype similar to *mp* mutants (Saiga et al., 2008). In *obe1 obe2* embryos, the expression of *PLT1* is lost; however, *MP* is still expressed, suggesting that OBE1 and OBE2 act downstream or at the level of MP.

Recently, *TMO5* and *TMO7* genes, both of which encode bHLH type transcription factors, have been identified as direct targets of MP that mediate MP-dependent embryonic root initiation (Schlereth et al., 2010). To determine whether OBE1 is involved in the regulation of these direct MP target genes, we examined *TMO5* and *TMO7* expression in *obe1 obe2* embryos. *TMO5* is expressed in cells adjacent to the hypophysis and in cotyledon primordia in wild type (Fig. 6A). Although the expression in the lower domain of the embryo is abolished in *obe1 obe2* embryos, the apical expression is maintained (Fig. 6C). The expression of *TMO7*, which is expressed in the hypophysis-adjacent cells in wild type (Fig. 6B), was completely abolished in *obe1 obe2* embryos (Fig. 6D). These findings indicate that OBE1 and OBE2 are required for the expression of *TMO5* and *TMO7* genes in cells adjacent to the hypophysis. In addition, the observation that only basal expression of *TMO5* was abolished in *obe1 obe2* embryos suggests different requirements for *OBE/TTA* gene activity in root and cotyledon patterning, as was also suggested by the genetic analysis.

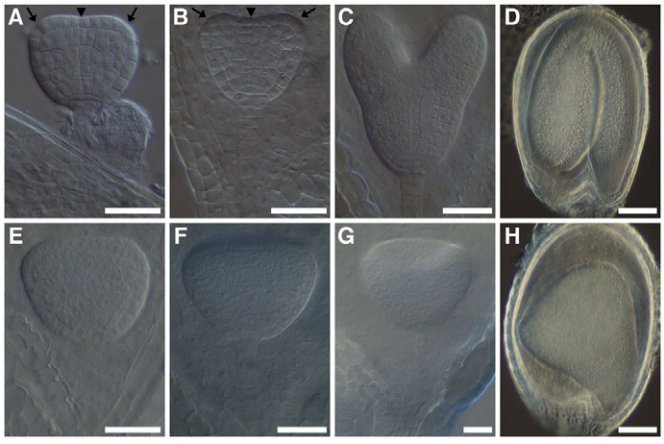
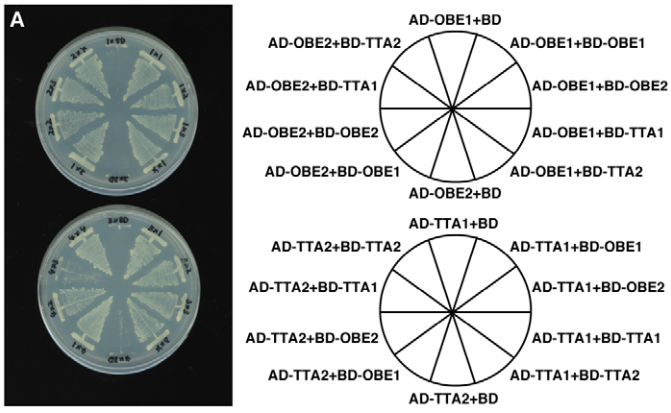


Fig. 4. Phenotypes of triple and quadruple mutants.
(**A-H**) Embryonic phenotypes of wild-type siblings (A-D), *obe1 obe2 tta2* (E-G) and *obe1 obe2 tta1 tta2* (H) at the late globular (A,E), triangular (B,F), heart (C,G) and bent-cotyledon (D,H) stages. Arrows indicate the positions where cotyledon primordia have emerged. Arrowheads indicate the positions where shoot apical meristem are formed. Scale bars: 30 μm in A-C,E-G; 100 μm in D,H.



B

MGTSSGSNLPHQMLPPRQQLQTSLSLVSSDPHLSRSNSGIVRESPAESASSQETWPTSKSIM
GRKTDSGKTGPDSHDQHVIRHVSIADKVSLRDIARERLDIVAERMHRLPEEYLEELKNGLKA
ILEGNGAQPIDEFMFLQKFVQTRSDLTSKTLVRAHRVQLEVLVVINTGIQAFLHPNINLSQS
SLIEIFVYKRCRNIACQNELPADGCPCEICANRKGFCNLCMCVICNKFDFAVNTCRWIGCDV
CSHWTHTDCAIRDGEISMGVSPKSVSGMGEMLFKCRACNHTSELLGWVKDVFQHCAPNWDRE
SLMKELDFVSRIFRGSEDTRGRKLFWKCEELMEKIKGGLAEATAAKLILMFFQEIELDSPKS
LESGEGGGTIAPQDACNRIAEVVKETLRKMEIVGEEKTRMYKKARMGLEECEREVEEKAKQV
AELQMERQKKKQQIEEVERIVRLKQAEAEMFQLKANEAKVEAERLERIVKAKKEKTEEEYAS
NYLKLRLSEAEAEKEYLFEKIKEQESGGNGGEASQAVMYSKIREMLHGYNASSPRVDPRSNQ
RNPFRSNP

Fig. 5. Interactions between OBE1/2 and TTA1/2 proteins.
(**A**) Yeast strain YRG-2 harboring the indicated plasmids were grown on medium lacking tryptophan, leucine and histidine. (**B**) Sequence of OBE1 protein with all highlighted peptides (in grey) that have been identified in the mass spectrometry analysis of at least one of three independent immunoprecipitation experiments. Note that peptides are found from across the entire protein, indicating that intact proteins were precipitated (399/566 amino acids; 70.5%).

Table 4. Identification of OBE1 and interacting proteins by immunoprecipitation

		Experiment 1			Experiment 2		
AGI	Protein name	*n*	%	Sf	*n*	%	Sf
At3g07780	OBE1	40 (37)	62	40	43 (40)	62	39
At5g48160	OBE2	8 (5)	17	4.8	7 (4)	13	5.3
At1g14740	TTA1	17 (17)	24	14	17 (17)	25	14
At3g63500	TTA2	22 (22)	26	17	25 (25)	23	19

Identification of OBE1 and interacting proteins from immunoprecipitations with 1 g of siliques expressing OBE1-GFP under the control of its endogenous promoter. Two independent pull-down experiments have been performed. Control experiments with wild-type siliques were performed at the same time, and, subsequently, all samples were subjected to mass spectrometry analysis. The peptides found with pull-down experiments with wild-type and OBE1-GFP siliques were compared, and all shared peptides were excluded for further analysis.
n, number of different peptides (number of unique peptides); %, percentage coverage of protein; Sf, total score factor calculated using Bioworks v3.3.1.

As *MP* is still expressed, but not *TMO7* (the direct target of MP) in *obe1 obe2* embryos, we hypothesized that OBE1 and OBE2 mediate the *TMO7* expression through modification of, or binding to, chromatin at the *TMO7* locus. To confirm the association of OBE1 with *TMO7* promoter region, we performed chromatin immunoprecipitation (ChIP) analysis with the *OBE1p::OBE1-GFP* transgenic line that could rescue the defects of *obe1 obe2*. Three DNA fragments in the *TMO7* promoter region were enriched using a GFP antibody (Fig. 6E,F; supplementary material Fig. S2), demonstrating in vivo binding. Interestingly, the binding profile of OBE1 along the tiles chosen for the *TMO7* locus closely resembled that of MP binding, as previously demonstrated (Schlereth et al., 2010), suggesting that a functional interaction may exist.

It has been demonstrated that while the *TMO7* transcript is expressed in the cells adjacent to the hypophysis, the TMO7 protein moves to the hypophysis where it acts to mediate root formation (Schlereth et al., 2010). If OBE1 mediates root formation in part by controlling *TMO7*, one would predict a requirement for OBE1 in the cells adjacent to the hypophysis but not in the hypophysis itself. As OBE1 is ubiquitously expressed at this stage (Saiga et al., 2008), it cannot be deduced where its activity is required. To determine the domain of OBE1 activity in root formation, we misexpressed *OBE1* in *obe1 obe2* mutants from two different promoters, and observed embryonic root initiation of those plants. *OBE1* driven by *MP* promoter, which is expressed in the cells adjacent to the hypophysis but not in the hypophysis itself (Schlereth et al., 2010), could rescue the embryonic root initiation defects in *obe1 obe2* (Fig. 6G). By contrast, *OBE1* expression driven by the suspensor-specific *ARF13* promoter (Schlereth et al., 2010) in *ARF13p::OBE1* lines did not rescue the defects in *obe1 obe2* roots (data not shown). These data indicate that OBE1 in the cells to the adjacent to the hypophysis is crucial for the embryonic root initiation, and OBE1 is important for *TMO7* expression but not for its protein function.

DISCUSSION

Our results indicate that TTA1 and TTA2 are redundantly required for embryonic root initiation in *Arabidopsis*. The observations that cell divisions of the hypophysis of *tta1 tta2* are defective, and that *MP* is expressed but *PLT1*, *SCR* and *WOX5* are absent in *tta1 tta2* embryos suggest that the rootless phenotype observed in *tta1 tta2* is mainly derived from disruption of the hypophysis specification.

TTA1 and *TTA2* seem to function in the same pathway in which *OBE1* and *OBE2* act because: (1) phenotypes of *tta1 tta2* double mutants are similar to those of *obe1 obe2*; (2) the expression patterns of cell identity marker genes are identical to those of *obe1 obe2*; (3) expression patterns of all four proteins completely overlap; and (4) OBE1/2 and TTA1/2 proteins could interact with each other *in vivo*.

Because MP is present in the adjacent cells to the future hypophysis but not in the hypophysis itself, it follows that MP promotes the hypophysis specification in a non-cell-autonomous manner (Hardtke and Berleth, 1998). *TMO7* expression is activated by MP in the adjacent cells to the hypophysis and TMO7 protein moves to the hypophysis. Our findings indicate that OBE1 mediates the MP-dependent *TMO7* expression because: (1) the expression of *TMO7* but not *MP* is lost in *obe1 obe2* embryos; (2) OBE1 associates with the *TMO7* promoter region; and (3) OBE1 function in the adjacent cells to the hypophysis but not in the hypophysis is required for embryonic root initiation (Fig. 7). *OBE1*, *OBE2*, *TTA1* and *TTA2* expression seem not to be regulated by MP, and protein complex identification with either OBE1 or MP failed to detect

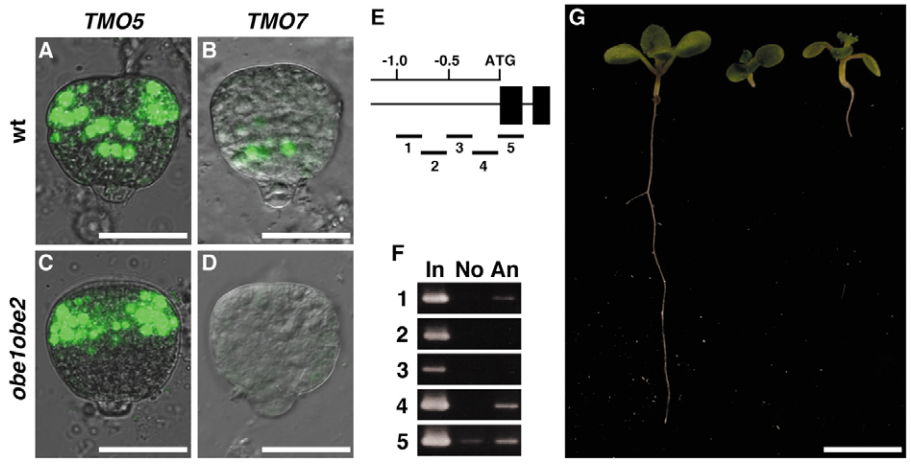


Fig. 6. OBE1 mediates the expression of MP target genes. (**A**,**C**) *TMO5p:: 3×nGFP* expression in wild-type (A) and *obe1 obe2* (C) embryos. (**B**,**D**) *TMO7p::3×nGFP* expression in wild-type (B) and *obe1 obe2* (D) embryos. (**E**) Schematic representation of the *TMO7* promoter region. The thin line and black boxes represent non-coding regions and exons, respectively. Bars with numbers illustrate the DNA fragments amplified in F. (**F**) PCR-amplified *TMO7* promoter fragments from ChIP of *OBE1p::OBE1-GFP* embryos with (An) or without (No) anti-GFP antibody. In, chromatin input. (**G**) Phenotypes of *MPp::OBE1* in *obe1 obe2* plants at 6 days after germination. From left to right: wild type, *obe1 obe2* and *obe1 obe2* harboring *MPp::OBE1*. Scale bars: 30 μm in A-D; 10 mm in G.

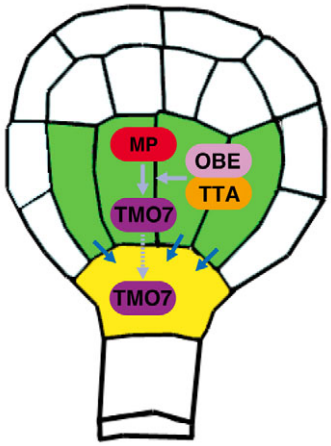


Fig. 7. Model for action of OBE and TTA in embryonic root initiation. At the early globular stage, MP activates the expression of *TMO7* in the basal part of pro-embryo (green). OBE-TTA protein complex, in which chromatin remodeling factors might be included, mediate MP-dependent *TMO7* expression, presumably by alteration of the chromatin property at the *TMO7* locus. TMO7 protein is translated in the basal part of pro-embryo, and then moves (gray dotted arrow) into the uppermost suspensor cell (yellow). MP also activates the auxin transport to the uppermost suspensor cell through PIN1 activity (blue arrows). The accumulation of both TMO7 and auxin in the uppermost suspensor cell specifies it as hypophysis.

interactions between the proteins (B.M. and D.W., unpublished), suggesting that MP does not interact with OBE proteins. Although these findings implicate the function of chromatin regulators in the MP pathway, a key issue is through what molecular mechanisms these PHD finger proteins control *TMO5/7* expression. One possibility is that OBE proteins are contained in a chromatin remodeling complex such as histone acetyltransferase (HAT), and mediate the transcriptional activation of target genes. Decondensation of nucleosome structure mediated by histone acetylation allows transcription factors to access target genes (Jenuwein and Allis, 2001). In *Saccharomyces cerevisiae*, the PHD-finger protein Yng1, which interacts with H3K4me3, is contained in NuA3 HAT complex. Yng1 mediates NuA3-dependent H3K14 acetylation through a specific interaction between the PHD-finger domain and H3K4me3, and promotes gene activation (Taverna et al., 2006). As similar mode of action could underlie OBE/TTA function, and this hypothesis awaits the identification of the chromatin mark that is recognized by OBE-TTA complexes. It can of course not be excluded that OBE and TTA do not act through recognition of histone modifications. We have recently identified non-PHD-finger OBE1-interacting proteins by mass spectrometry analysis of the OBE1 protein complex (S.S., B.M. and D.W., unpublished). Future analysis of these proteins could reveal how OBE proteins control the gene expression downstream of the MP.

Auxin is another signal involved in this signaling but its accumulation alone is not sufficient to promote the hypophysis specification (Weijers et al., 2006). Whereas auxin response is activated in extra-embryonic cells below the future hypophysis, TMO7 protein exists only in upper-most extra-embryonic cell, suggesting that accumulation of both auxin and TMO7 is required for the hypophyisis specification (Schlereth et al., 2010). *TMO7* expression is absent in *obe1 obe2* embryos, whereas the establishment of auxin response maxima in *obe1 obe2* embryos is largely similar to the wild-type pattern (Thomas et al., 2009) (S.S., M.A., D.W. and Y.K., unpublished), suggesting that OBE1 mainly controls the expression of the *TMO7* rather than establishment of the auxin maxima in the hypophysis specification.

The recent identification of *TMO* genes as MP targets provides entry points to connect the upstream regulator MP with its several downstream pathways. A key question is how region-specific MP activity is controlled. The analysis of *TMO5* and *TMO7* expression in *obe1 obe2* mutants provides insight into this problem. Although *TMO7* is eliminated entirely, *TMO5* expression is lost only in the basal embryo domain. This suggests that the requirements for gene activation by MP in the basal and apical embryo domains differ. The precise molecular mechanisms for this regional MP activity remain to be determined, but the OBE proteins should allow dissecting these.

All triple mutant combinations among *obe1*, *obe2*, *tta1* and *tta2* exhibited no additional phenotypes in the formation of embryonic root meristem that are already disrupted in *obe1 obe2* and *tta1 tta2* double mutants, whereas development of apical region in triple mutants displayed more severe phenotypes than those of double mutants, indicating that OBE1/2 and TTA1/2 function in development of embryonic shoot meristem and cotyledons synergistically. Previously. we have demonstrated that the embryonic shoot meristem of *obe1 obe2* might be formed initially but is not maintained because the expression of shoot meristem marker genes *WUSCHEL* and *CLAVATA3* in *obe1 obe2* embryos is initiated but is not maintained. By contrast, the embryonic root meristem of *obe1 obe2* was not formed, as judged by the expression patterns of root meristem marker genes (Saiga et al., 2008). These suggest that a more complex mechanism operates in the embryonic shoot meristem and cotyledon development, as was also suggested by the differential effect of *obe1 obe2* mutations on *TMO5* expression in the two embryo poles.

The observation that all triple mutant combinations exhibited more severe phenotypes than those of double mutants is curious because both TTA1 and TTA2 and OBE1 and OBE2 are functionally redundant. One possible explanation is that there are differences in functionality among dimers containing OBE1/2 or TTA1/2. During embryogenesis, hetero-dimer formation might be important. For example, the *obe1 obe2 tta1* triple mutant should only have TTA2 homo-dimer, and this results in embryo lethality. However, the *obe1 obe2* double mutant, in which TTA1-TTA2 hetero-dimer can exist, can form cotyledons and germinate. However, the *obe1 obe2* and *tta1 tta2* double mutants have no OBE-TTA hetero-dimers and functional embryonic apical meristems were not established. Taken together, our results indicate that OBE-TTA dimer formation might be most important for *Arabidopsis* embryogenesis. More detailed analysis should elucidate how the OBE-TTA protein complex acts in the apical region during embryonic development. Finally, this work opens up avenues for studying the regulation of developmentally important genes through transcription factors and chromatin proteins.

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Competing interests statement
The authors declare no competing financial interests.

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