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The *WOX11–LBD16* Pathway Promotes Pluripotency Acquisition in Callus Cells During De Novo Shoot Regeneration in Tissue Culture

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De novo shoot regeneration in tissue culture undergoes at least two phases. Explants are first cultured on auxin-rich callus-inducing medium (CIM) to produce a group of pluripotent cells termed callus; the callus is then transferred to cytokinin rich shoot-inducing medium (SIM) to promote the formation of shoot progenitor cells, from which adventitious shoots may differentiate. Here, we show that the *Arabidopsis thaliana* transcription factor gene *LATERAL ORGAN BOUNDARIES DOMAIN16* (*LBD16*) is involved in pluripotency acquisition in callus cells. *LBD16*, which is activated by *WUSCHEL RELATED HOMEOBOX11* (*WOX11*), is specifically expressed in the newly formed callus on CIM and its expression decreases quickly when callus is moved to SIM. Blocking the *WOX11–LBD16* pathway results in the loss of pluripotency in callus cultured on CIM, leading to shooting defects on SIM. Further analysis showed that *LBD16* may function in the establishment of the root primordium-like identity in the newly formed callus, indicating that the root primordium-like identity is the cellular nature of pluripotency in callus cells. Additionally, *LBD16* promotes cell division during callus initiation. Our study clarified that the *WOX11–LBD16* pathway promotes pluripotency acquisition in callus cells.

Keywords: *Arabidopsis thaliana* • Callus • De novo shoot regeneration • *LBD16* • Pluripotency • *WOX11*.

Abbreviations: CIM, callus-inducing medium; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein-9; CUC2, CUP-SHAPED COTYLEDON2; ESR1, ENHANCER OF SHOOT REGENERATION1; GUS, β-glucuronidase; LBD16, LATERAL ORGAN BOUNDARIES DOMAIN16; MS, Murashige and Skoog; PLT, PLETHORA; qRT–PCR, quantitative reverse transcription–PCR; RNA-seq, RNA sequencing; SIM, shoot-inducing medium; WIND1, WOUND INDUCED DEDIFFERENTIATION1; WOX, WUSCHEL RELATED HOMEOBOX; WUS, WUSCHEL.

Introduction

De novo shoot regeneration (also called de novo shoot organogenesis) has been exploited in tissue culture for more than half a century (Duclercq et al. 2011, Ikeuchi et al. 2013, Su and Zhang 2014, Xu and Huang 2014, Ikeuchi et al. 2016, Kareem et al. 2016a). Formation of adventitious shoots from callus in tissue culture usually requires two culturing phases on different media. Here we use *Arabidopsis thaliana* leaf and hypocotyl explants in tissue culture as an example (**Fig. 1A**).

In the first phase, explants are cultured on callus-inducing medium (CIM) with high auxin levels and low cytokinin levels in dark conditions to induce callus formation. Callus initiation was shown to follow the rooting pathway (Che et al. 2007, Atta et al. 2009, Sugimoto et al. 2010, Sugimoto et al. 2011, He et al. 2012, Liu et al. 2014), and the newly formed callus resembles a group of root primordium-like cells (Duclercq et al. 2011, Liu et al. 2014). Acquisition of pluripotency, i.e. the competence for shooting or rooting, in callus cells is essential in this phase. At the molecular level, *PLETHORA3* (*PLT3*), *PLT5* and *PLT7* are key genes involved in the acquisition of pluripotency in callus cells via regulation of a group of root primordium-related genes such as *PLT1/2* (Kareem et al. 2015). When the callus is cultured on CIM for a relatively long time, partial differentiation into the root apical meristem-like structure can be observed in the callus mass (Atta et al. 2009, Sugimoto et al. 2010, Kareem et al. 2015). The partially differentiated callus has lower competence for shooting. Therefore, newly formed callus is usually used for shoot or root induction in Arabidopsis tissue culture.

In the second phase, callus is moved to shoot-inducing medium (SIM) with high cytokinin levels and low auxin levels in light conditions; here it becomes green callus from which adventitious shoots may form. Reprogramming of green callus cells into shoot progenitor cells is essential in this phase, and adventitious shoots are differentiated from shoot progenitor cells

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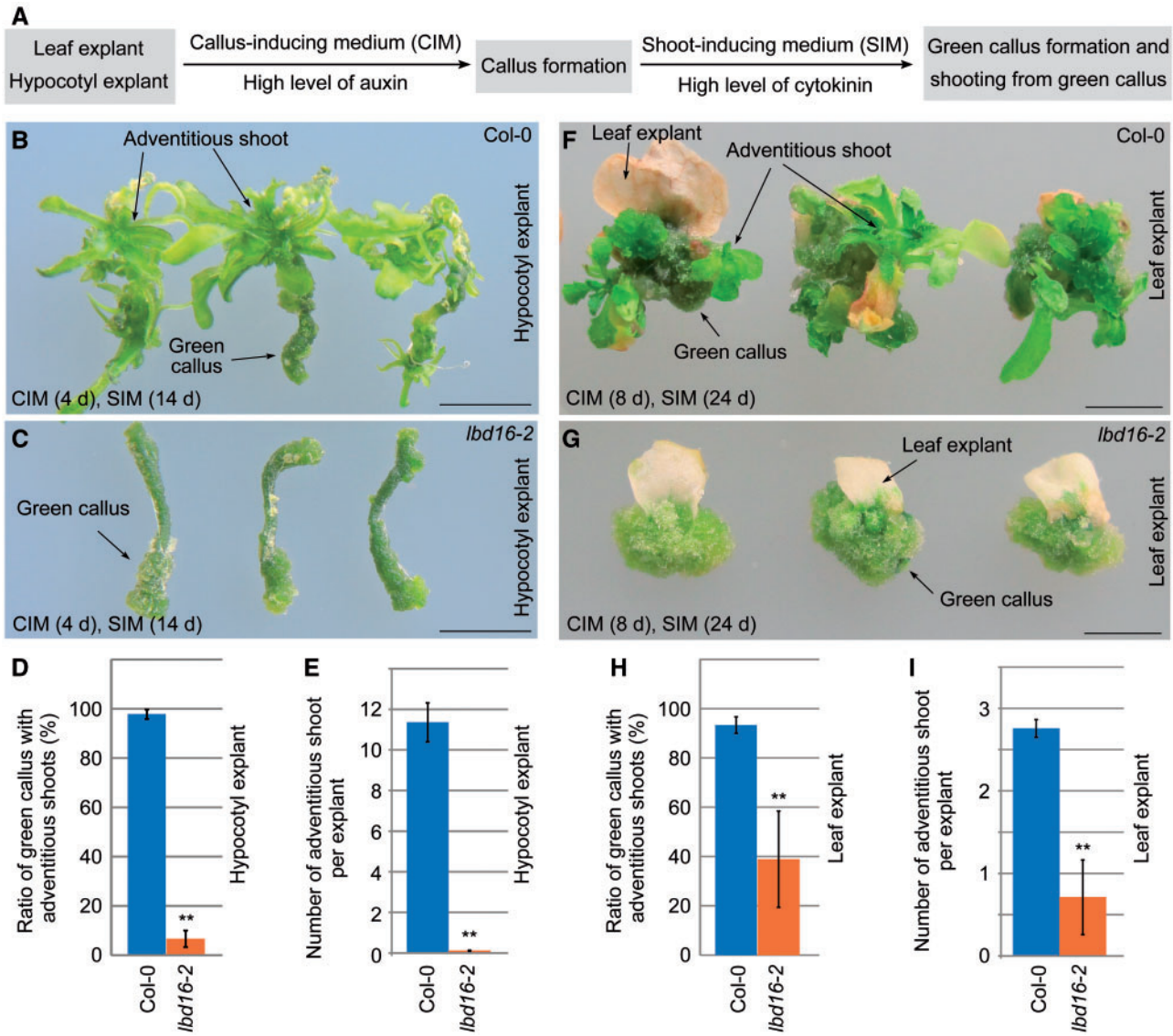

Fig. 1 LBD16 is involved in de novo shoot regeneration. (A) A schematic of the two phases of tissue culture. (B and C) Shoot regeneration on green callus from hypocotyl explants of Col-0 (B) and *lbd16-2* (C) on SIM. (D) Ratio of green callus with adventitious shoots from hypocotyl explants of Col-0 and *lbd16-2*. (E) Number of adventitious shoots per hypocotyl explant of Col-0 and *lbd16-2*. (F and G) Shoot regeneration on green callus from leaf explants of Col-0 (F) and *lbd16-2* (G). (H) Ratio of green callus with adventitious shoots on leaf explants of Col-0 and *lbd16-2*. (I) Number of adventitious shoots per leaf explant of Col-0 and the *lbd16-2* mutant. Hypocotyl explants were cultured on CIM for 4 d and then transferred to SIM for 14 d for phenotypic analysis (B–E). Leaf explants were cultured on CIM for 8 d and then transferred to SIM for 24 d for phenotypic analysis (F–I). Error bars (D, E, H, I) show the SE from three biological replicates. $n = 30$ for each replicate. $^{**}P < 0.01$ (*t*-test). Scale bars = 5 mm (B, C, F, G).

(Gordon et al. 2007). Recent studies suggested that induction of *WUSCHEL* (*WUS*) expression by cytokinin is required for shoot progenitor cell identity formation (Dai et al. 2017, Meng et al. 2017, Zhang et al. 2017). *ENHANCER OF SHOOT REGENERATION1* (*ESR1*), which is regulated by cytokinin and *WOUND INDUCED DEDIFFERENTIATION1* (*WIND1*), promotes shooting (Banno et al. 2001, Iwase et al. 2011, Iwase et al. 2017). *PLT3/5/7* are also involved in the promotion of shooting via regulation of *CUP-SHAPED COTYLEDON2* (*CUC2*) (Kareem et al. 2015).

We previously showed that cell fate transition during callus formation on CIM is similar to that which occurs in the adventitious rooting pathway (Liu et al. 2014, Xu 2018). Auxin activates *WUSCHEL RELATED HOMEOBOX11* (*WOX11*) and *WOX12* for the first step of cell fate transition from the regeneration-competent cell to the founder cell. *WOX11/12* activates the expression of *WOX5/7* and *LATERAL ORGAN BOUNDARIES DOMAIN16* (*LBD16*) for the second step of cell fate transition from the founder cell to the root primordium or the newly formed callus with cell division (Liu et al. 2014, Hu and Xu 2016, Sheng et al. 2017). However, it is not clear how this cell fate transition pathway contributes to the acquisition of pluripotency during callus initiation. LBDs are plant specific LOB-domain transcription factors (Shuai et al. 2002), some of

which have been shown to be involved in callus initiation (Fan et al. 2012). In this study, we show that *LBD16* is specifically expressed in the newly formed callus on CIM but not in green callus on SIM, and that the *WOX11–LBD16* pathway is involved in the acquisition of pluripotency in callus cells.

Results

The *WOX11–LBD16* pathway is required for shooting from green callus on SIM

To reveal the role of the *WOX11–LBD16* pathway in de novo shoot regeneration, we first analyzed the shooting phenotype in tissue culture. We cultured leaf explants and hypocotyl explants of Arabidopsis wild-type Columbia-0 (Col-0) and the *lbd16-2* mutant on CIM and then moved the newly formed callus to SIM for shooting induction. Both leaf explants and hypocotyl explants of the wild type formed adventitious shoots from green callus on SIM (**Fig. 1B, F**). However, shooting was severely defective in the green callus of *lbd16-2* (**Fig. 1C, G**). In our culture conditions, approximately 98% of hypocotyl explants and approximately 93% of leaf explants could form adventitious shoots on SIM in the wild-type background while only about 7% of hypocotyl explants and about 39% of leaf explants of *lbd16-2* formed shoots (**Fig. 1D, H**). Additionally, approximately 11 and approximately three adventitious shoots could, on average, be formed per hypocotyl explant and per leaf explant, respectively, in the wild type; in the *lbd16-2* background, however, only about 0.1 and about 0.7 adventitious shoots could, on average, be formed per hypocotyl explant and per leaf explant, respectively (**Fig. 1E, I**).

To confirm further that *LBD16* is involved in shoot regeneration, we designed the new *lbd16-3* mutant allele using the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein-9) method (**Fig. 2A**) (Yan et al. 2015). The *lbd16-3* allele has a 1 bp deletion in the first exon, causing the frameshift of the whole LBD. The *lbd16-3* mutant showed shooting defect from the green callus on SIM (**Fig. 2B**). In addition, we analyzed the phenotype of the $35S_{pro}$:*WOX11-SRDX* transgenic plants, in which the *WOX11* pathway was suppressed (Liu et al. 2014). The result showed that $35S_{pro}$:*WOX11-SRDX* plants had a defect in shoot regeneration (**Fig. 2C**). Therefore, de novo shoot regeneration in tissue culture requires the *WOX11–LBD16* pathway.

Next, we analyzed the formation of shoot progenitor cells in both wild-type and *lbd16-2* backgrounds. We carried out quantitative reverse transcription–PCR (qRT–PCR) analysis to detect the expression of the shoot progenitor marker gene *WUS* when calli from wild-type and *lbd16-2* leaf explants were moved to SIM (**Fig. 3A**). *WUS* expression could be up-regulated after 2 d and reached high levels at 8 d in the wild-type green callus on SIM. However, *WUS* expression could not be up-regulated in the *lbd16-2* background. Using the WUS_{pro}:*WUS-GUS* marker line, we detected the expression pattern of *WUS* in the green callus of wild-type and *lbd16-2* hypocotyl explants. The β-glucuronidase (GUS) signal could be clearly observed in the wild-type green callus (arrows in **Fig. 3B**) but was barely detectable in the *lbd16-2* background

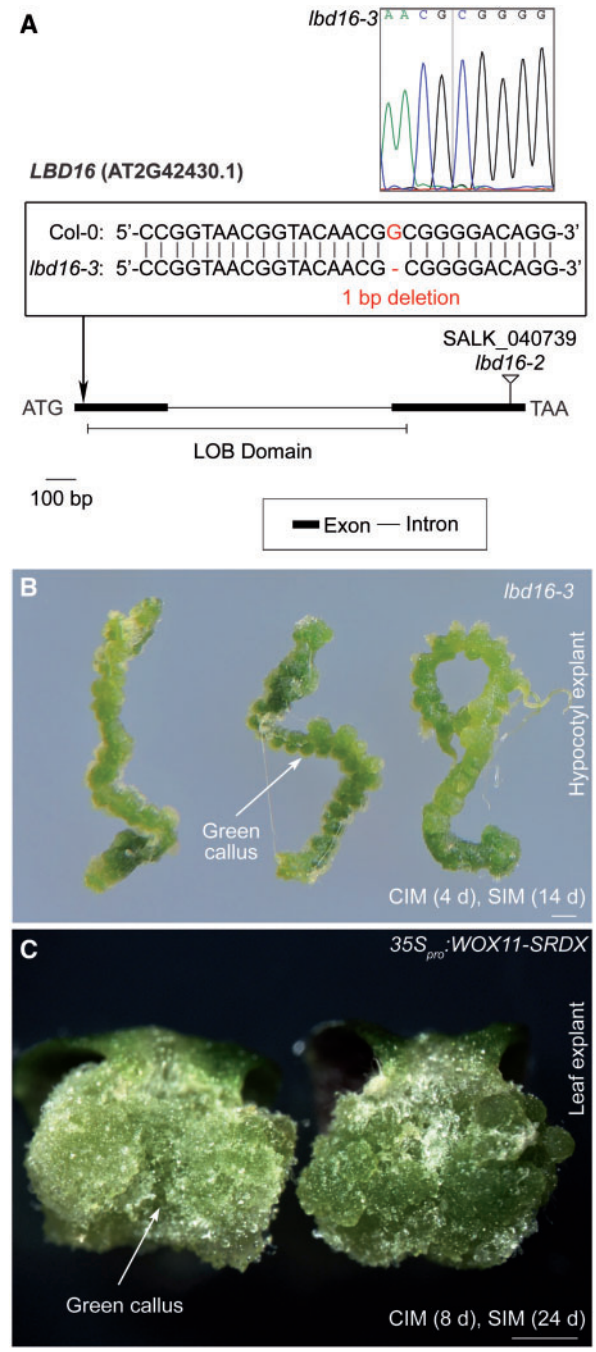

Fig. 2 Analysis of *lbd16-3* and $35S_{pro}$:*WOX11-SRDX* in de novo shoot regeneration. (A) Structural diagram of the *LBD16* gene, showing the T-DNA insertion allele *lbd16-2* and the CRISPR/Cas9 allele *lbd16-3*. The sequencing result is listed in the boxed region. (B and C) Shoot regeneration on green callus from hypocotyl explants of *lbd16-3* (B) and leaf explants of $35S_{pro}$:*WOX11-SRDX* (C) on SIM. A total of 90 *lbd16-3* hypocotyl explants were analyzed and 81 of them showed a shooting defect (B). More than 30 leaf explants of $35S_{pro}$:*WOX11-SRDX* were analyzed and all of them showed a shooting defect (C). For controls and culture conditions, see Fig. 1B and F. Scale bars = 1 mm (B, C).

(**Fig. 3C**). These data suggest that the shoot progenitor cell could not be normally formed from the green callus of *lbd16-2*. Therefore, mutation of *LBD16* results in loss of pluripotency in callus cells.

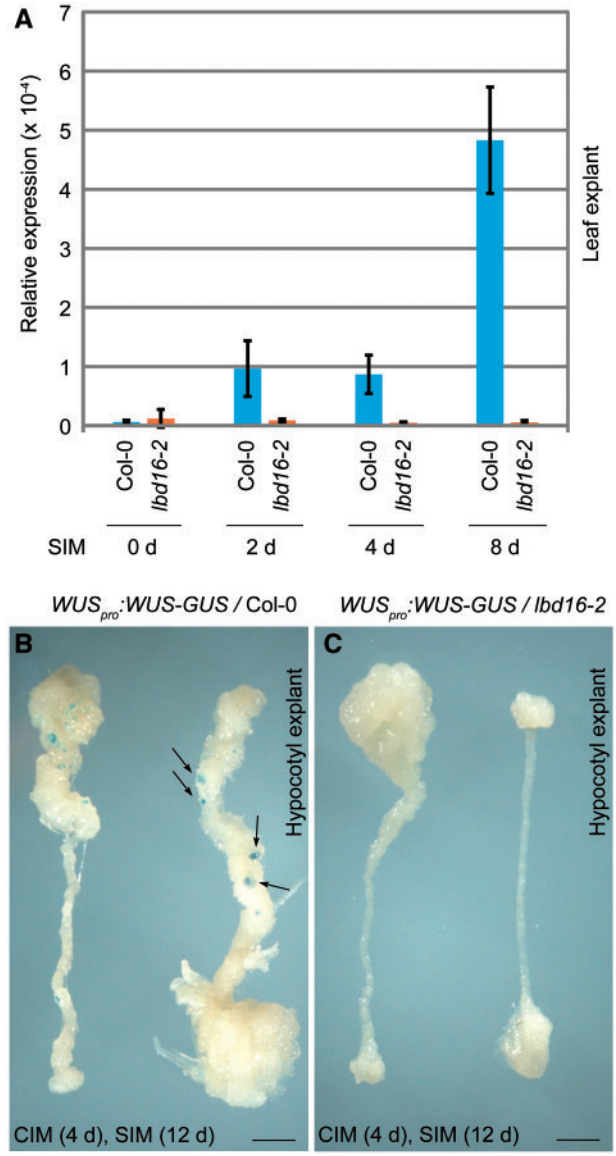

Fig. 3 LBD16 is required for shoot progenitor cell formation. (A) qRT–PCR analysis of *WUS* expression in Col-0 and *lbd16-2* green callus from leaf explants on SIM. Error bars show the SE from three biological replicates. Each biological replicate contains three technical replicates. The leaf explants were cultured on CIM for 8 d and then transferred to SIM for 0, 2, 4 and 8 d. (B and C) GUS staining of WUS_{pro}:*WUS-GUS* in Col-0 (B) and *lbd16-2* (C) backgrounds cultured on SIM. The hypocotyl explants were cultured on CIM for 4 d and then transferred to SIM for 12 d. Scale bars = 1 mm (B, C).

LBD16 is specifically expressed in the newly formed callus on CIM but not in green callus on SIM

We used the $LBD16_{pro}$:*LBD16-GUS* marker line to analyze *LBD16* expression during tissue culture. The GUS signal was barely detectable in the vascular tissue of hypocotyl explants before tissue culture (**Fig. 4A**) and was strongly induced in the newly formed and fast dividing callus cells when the hypocotyl explants were cultured on CIM (**Fig. 4B, C**). When the callus was moved to SIM for 2 d, the GUS signal was not detected in the green callus (**Fig. 4D**).

We carried out qRT–PCR to analyze *LBD16* expression in leaf explants in tissue culture. *LBD16* was barely detected in leaf explants before tissue culture, and highly up-regulated on CIM (**Fig. 4E**). Thin sectioning showed that *LBD16* was specifically expressed in the newly formed callus (**Fig. 4F**). Its expression level reduced rapidly when the callus was moved to SIM at 4 h and after (**Fig. 4E**).

Overall, our data suggest that *LBD16* is specifically expressed in the newly formed callus on CIM but not in green callus on SIM. The expression pattern of *LBD16* indicates that it specifically functions in callus cells on CIM but not on SIM for the acquisition of pluripotency.

LBD16 promotes root primordium-like cell identity in the newly formed callus on CIM

To study the molecular mechanism underlying the role of *LBD16* in the promotion of pluripotency in callus, we performed an RNA sequencing (RNA-seq) experiment to analyze its downstream genes (**Fig. 5A**). Leaf explants from the wild type and *lbd16-2* before culture (0 d) and at 4 d on CIM were used. By comparing genes that were up-regulated in wild-type and *lbd16-2* leaf explants during callus formation on CIM, 749 genes were found with significantly lower expression levels in the *lbd16-2* callus compared with the wild-type background (**Fig. 5B;** Supplementary Table S1). Using the online tool Genevestigator (https://genevestigator.com/gv/) (Hruz et al. 2008), we found that these genes were preferentially expressed in roots (**Fig. 5C**) and that the root primordium identity genes *WOX5*, *PLT1* and *PLT2* were among these genes (**Fig. 5C**) (Aida et al. 2004, Sarkar et al. 2007, Liu et al. 2014, Tian et al. 2014, Lavenus et al. 2015, Hu and Xu 2016, Kong et al. 2016). Furthermore, *PLT1/2* were reported to be involved in pluripotency acquisition in callus (Kareem et al. 2015).

Next, we performed qRT–PCR to analyze the expression levels of *WOX5*, *PLT1* and *PLT2* during callus formation in both wild-type and *lbd16-2* backgrounds (**Fig. 5D–F**). In wild-type and *lbd16-2* leaf explants, expression of the three genes was not detected before culture (0 d). Expression of *WOX5* was up-regulated at 2 d on CIM in the wild-type background but the expression level was not up-regulated in the *lbd16-2* background. After 4 and 8 d culturing on CIM, *WOX5* was highly expressed in the wild-type background and was expressed at a lower level in *lbd16-2*. Expression of *PLT1* and *PLT2* was up-regulated at 4 d on CIM in the wild-type background but was not detected at 4 d in the *lbd16-2* background. After 8 d culturing on CIM, *PLT1* and *PLT2* were highly expressed in the wild-type background, with lower expression levels observed in *lbd16-2*. Overall, up-regulation of *WOX5* and *PLT1/2* was slower in the leaf explants of *lbd16-2* compared with the wild type, suggesting that an inability to gain the root primordium-like identity in the newly formed callus in time might explain the failure in the acquisition of pluripotency in *lbd16-2*. However, currently we do not know whether *PLT1/2* and *WOX5* are directly or indirectly regulated by *LBD16*.

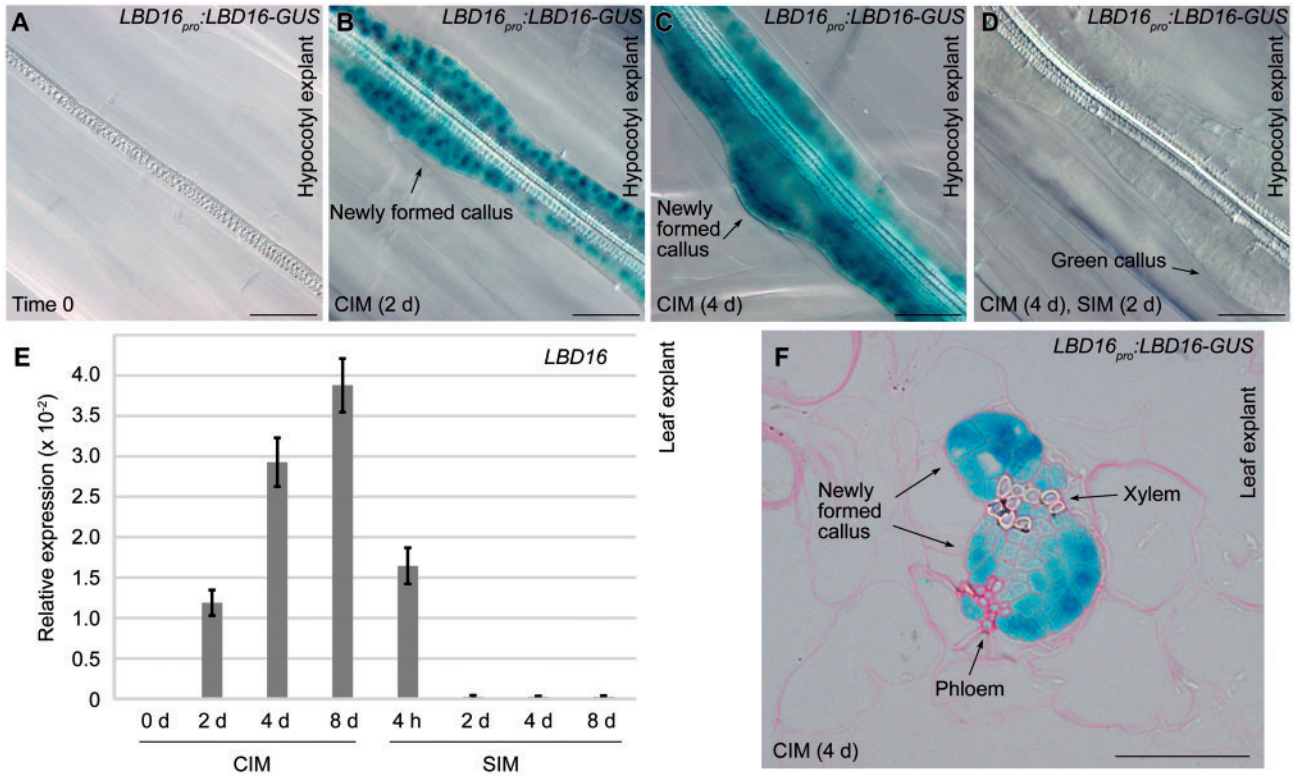

Fig. 4 LBD16 expression pattern on CIM and SIM. (A–D) GUS staining of *LBD16*$_{pro}$*:LBD16-GUS* hypocotyl explants at 0 (A), 2 (B) and 4 d (C) cultured on CIM and 2 d cultured on SIM after 4 d on CIM (D). (E) qRT–PCR analysis of transcription levels of *LBD16* in leaf explants on CIM and after transfer to SIM. Error bars show the SE from three biological replicates. Each biological replicate contains three technical replicates. (F) Transverse section of an *LBD16*$_{pro}$*:LBD16-GUS* leaf explant cultured on CIM for 4 d. Scale bars = 50 μm (A–D, F).

We also analyzed *PLT3* and *PLT7* expression during callus formation on CIM in wild-type and *lbd16-2* leaf explants (**Fig. 5G, H**). Both genes were highly up-regulated in the wild type and *lbd16-2* when leaf explants were cultured on CIM. Therefore, *LBD16* may not be involved in the control of *PLT3/7* activation during callus formation.

LBD16 promotes callus cell division on CIM

As *LBD* genes may control cell division during root formation (Lee et al. 2009, Feng et al. 2012, Lee et al. 2013), we analyzed cell division during callus formation in wild-type and *lbd16-2* hypocotyl explants at 2 and 4 d after culture on CIM by confocal imaging. Cell division in *lbd16-2* callus was slightly slower than that in the wild type (**Fig. 6**) (Fan et al. 2012). Therefore, *LBD16* might also contribute to the promotion of cell division for callus initiation.

Discussion

During de novo organogenesis in tissue culture, the formation of callus on CIM is required for pluripotency acquisition. Pluripotency of callus cells is the competence for initiation of adventitious shoots and roots upon different inductive cues. High levels of cytokinin can initiate shooting when callus is moved to SIM, while callus can produce roots when it is cultured on root-inducing medium (RIM) with low auxin levels. Here, we propose a model for the mechanism underlying pluripotency acquisition and shoot formation at the cellular and molecular levels (**Fig. 7**).

At the cellular level, obtaining the root primordium-like identity is required for pluripotency acquisition in the newly formed callus on CIM. It is known that callus formation follows the rooting pathway controlled by auxin and that the newly formed callus on CIM is similar to that of root primordium cells during adventitious or lateral root formation (Che et al. 2007, Atta et al. 2009, Sugimoto et al. 2010, Duclercq et al. 2011, Sugimoto et al. 2011, He et al. 2012, Liu et al. 2014). The major difference between the callus initiation and rooting is the source of auxin. In the rooting process, finite and self-controlled endogenous auxin promotes root primordium formation and then the root primordium can differentiate into the root meristem. However, on CIM, large amounts of exogenous auxin can dramatically initiate a great number of root primordia-like cells, i.e. the newly formed callus, and maintain their cell identity in the primordium stage. As with the root primordium-like cell identity, callus is competent for organogenesis. Consistent with this hypothesis, the root primordium can directly initiate adventitious shoot formation when stimulated with cytokinin treatment (Chatfield et al. 2013, Kareem et al. 2016b, Rosspopoff et al. 2017).

At the molecular level, pluripotency acquisition in callus involves expressing root primordium identity genes on CIM. In this study, we show that the *WOX11–LBD16* pathway

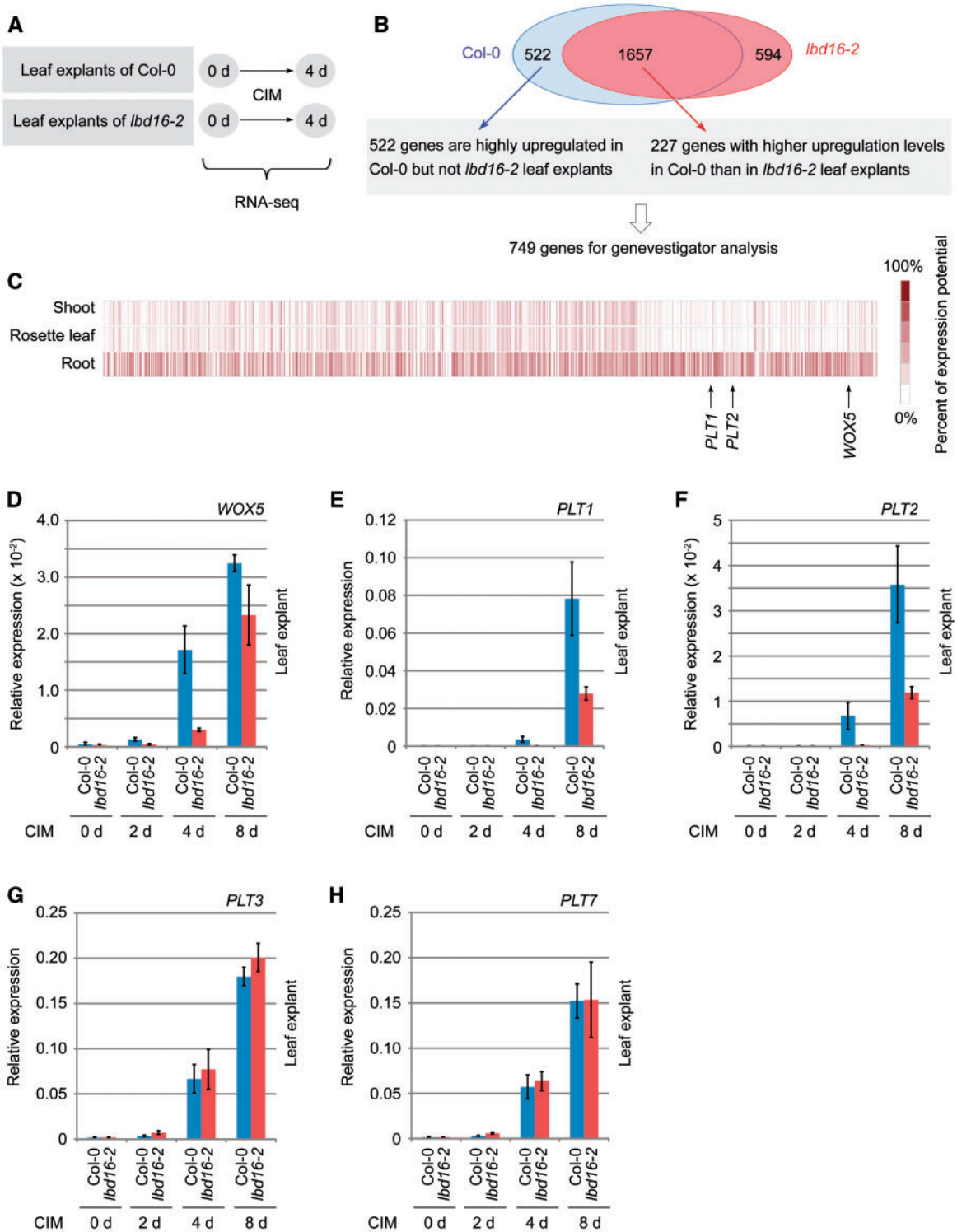

Fig. 5 Analysis of gene expression in Col-0 and *lbd16-2*. (A) RNA-seq analysis using Col-0 and *lbd16-2* leaf explants cultured on CIM at 0 and 4 d. (B) RNA-seq data showed genes that were up-regulated in Col-0 or *lbd16-2* leaf explants on CIM. Among those genes, 522 genes were highly up-regulated ($\log_2$ fold change >3, for 4 d vs. 0 d) in Col-0 leaf explants but not in *lbd16-2* leaf explants. Among the overlapping genes, 227 genes had higher up-regulation levels in Col-0 than in *lbd16-2* leaf explants (>3-fold, Col-0 vs. *lbd16-2*). Therefore, we collected the two groups of 749 genes (522 genes + 227 genes) as candidate genes that might be regulated by *LBD16* during callus formation on CIM (see also **Supplementary Table S1**). (C) Genevestigator analysis of tissue-specific expression patterns of the 749 candidate genes. Note that many of these genes were preferentially expressed in roots. (D–H) qRT–PCR analysis of transcription levels of *WOX5* (D), *PLT1* (E), *PLT2* (F), *PLT3* (G) and *PLT7* (H) during tissue culture of leaf explants on CIM in Col-0 and *lbd16-2*. Error bars show the SE from three biological replicates. Each biological replicate contains three technical replicates.

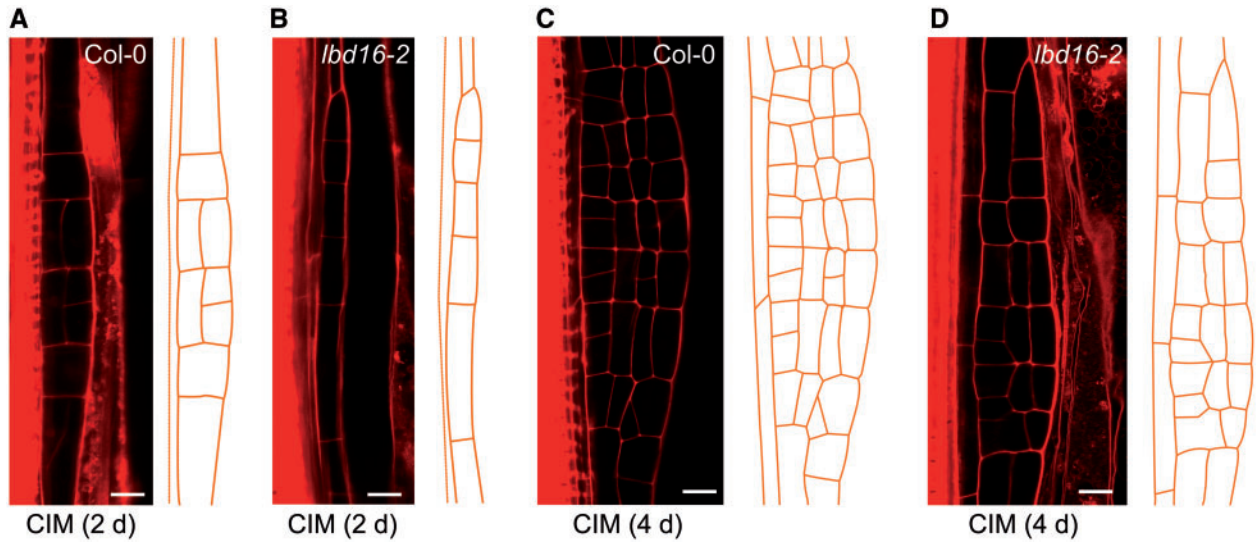

Fig. 6 LBD16 promotes cell division during callus initiation on CIM. (A–D) Callus from hypocotyl explants of Col-0 (A, C) and the *lbd16-2* mutant (B, D) cultured on CIM for 2 (A, B) or 4 d (C, D). Note that callus of the *lbd16-2* mutant consists of fewer cell layers than the wild-type callus. Red staining of confocal images in each left-hand panel indicates cell walls stained with propidium iodide. Orange outlines in each right-hand panel are schematics of callus cells from each confocal image. Scale bars = 10 μm (A–D).

regulates shoot regeneration efficiency by specific promotion of the root primordium-like identity on CIM. Auxin activates *WOX11/12* expression to establish the founder cell, and then *WOX11/12* up-regulate *LBD16* during the transition from founder cell to the newly formed callus (Liu et al. 2014, Sheng et al. 2017). During lateral and adventitious rooting, *LBD16* is expressed in the root primordium (Okushima et al. 2007, Goh et al. 2012, Hu and Xu 2016, Sheng et al. 2017). During tissue culture, *LBD16* is specifically expressed in the newly formed callus on CIM but not in the green callus on SIM, indicating that it has a specific role in callus cells but not in green callus cells and shoot progenitor cells. *PLT3/5/7* are also involved in the promotion of the root primordium identity during callus initiation in tissue culture and during lateral root primordium initiation in lateral rooting (Kareem et al. 2015, Du and Scheres 2017). Cumulative loss of function of *PLT3/5/7* completely abolishes the ability to regenerate the shoot irrespective of the origin of explants (Kareem et al. 2015). However, *PLT3/5/7* are not root primordium-specific genes as they also function on SIM to promote shoot formation from shoot progenitor cells (Kareem et al. 2015). It is not clear whether *PLT3/5/7* may regulate the *WOX11–LBD16* pathway and whether the *WOX11–LBD16* pathway acts dependently or independently of the *PLT3/5/7* pathway to regulate root primordium-like identity. The analysis of the cross-talk of the *WOX11–LBD16*, *PLT3/5/7* and other regeneration pathways involving *PLT1/2*, *WOX5/7*, *WUS*, *ESR1*, *WIND* genes and *CUC* genes will be important for further understanding of how callus cells acquire pluripotency.

LBD16 promotes cell division during callus initiation. Proper cell division might also contribute to pluripotency acquisition in callus. A recent study on the direct shoot regeneration from lateral root primordium suggested that acquiring shoot regeneration capacity is restricted to a developmental step of lateral root primordium formation (Rosspopoff et al. 2017). It is possible that retarded cell division in the *lbd16* callus may impair the developmental steps for pluripotency acquisition.

Overall, gain of the root primordium-like identity is the key event for callus to acquire pluripotency, and *LBD16* is a specific root primordium gene that ensures callus pluripotency. Further analysis of the direct targets of *LBD16* will improve our understanding of pluripotency acquisition in tissue culture.

Materials and Methods

Plant materials

Arabidopsis Col-0 was used as the wild type in this study. The *lbd16-2* mutant was described previously (Fan et al. 2012). The $LBD16_{pro}$:*LBD16-GUS* (Sheng et al. 2017) and $WOX5_{pro}$:*GUS* (He et al. 2012) transgenic plants have been described previously. For the construction of WUS_{pro}:*WUS-GUS*, a 3.3 kb genomic fragment of the *WUS* promoter and the gene body was PCR amplified and inserted into the pBI101 vector, using the following primers: 5′-cccaagcttAGTTAAAAAATGAGTAATCCACCAG-3′ and 5′-cgggatccGTTCAGACGTAGCTCAAGAG-3′. The construct was then introduced into wild-type plants via *Agrobacterium tumefaciens*-mediated transformation.

To generate the *lbd16-3* mutant, an *LBD16*-specific sequence (5′- CCGGTAACGGTACAACGGCG-3′) was used as the target for Cas9 to mutate *LBD16*. Vector construction was performed as previously described (Yan et al. 2015). The CRISPR/Cas9 construct was introduced into Col-0. The genomic fragments covering the mutation sites were amplified from the T_1 and T_2 transgenic plants by PCR and sequenced.

Tissue culture

For tissue culture using leaf explants, seedlings were grown on half-strength Murashige and Skoog (1/2 MS) medium (Murashige and Skoog 1962) at 22°C, with 16 h light and 8 h darkness. The first pair of rosette leaves were cut from 12-day-old seedlings and cultured on CIM (3.21 g l^{-1} Gamborg B5 basal medium, 0.5 g l^{-1} MES, 3% sucrose and 0.8% agar, pH 5.7, supplemented with 0.2 μM kinetin and 2.2 μM 2,4-D) at 22°C under continuous dark for 8 d to induce callus formation. Those leaf explants were then transferred to SIM (4.43 g l^{-1} MS medium, 0.5 g l^{-1} MES, 1% sucrose and 0.8% agar, pH 5.7, supplemented with 2 μg l^{-1} *trans*-zeatin, 1 μg l^{-1} d-biotin and 0.4 μg l^{-1} indole

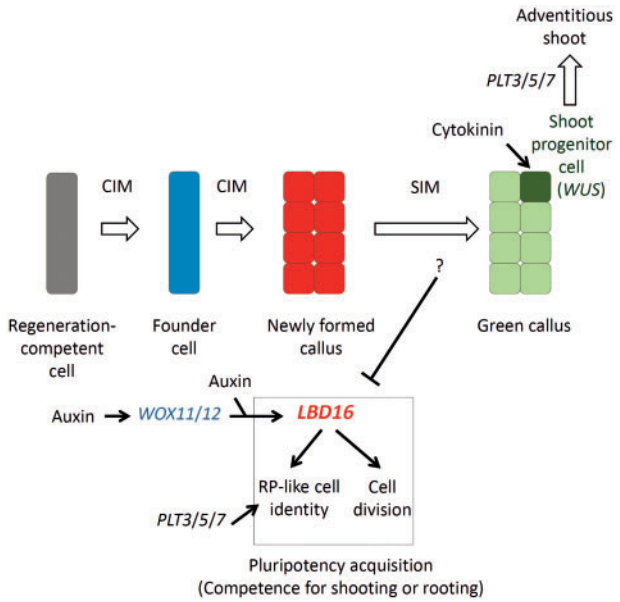

Fig. 7 Model of *LBD16* in pluripotency acquisition in callus cells. On CIM, high auxin levels activate *WOX11/12* expression for fate transition from the regeneration-competent cell to the founder cell (Liu et al. 2014). *WOX11/12*, probably together with auxin (Fan et al. 2012), then activate *LBD16* for the fate transition from the founder cell to callus cells with cell division (Liu et al. 2014). *LBD16* is preferentially expressed in the newly formed callus and is involved in pluripotency acquisition, probably via the establishment of the root primordium-like (RP-like) cell identity and promotion of cell division (Fan et al. 2012, this study). *PLT3/5/7* are also involved in pluripotency acquisition in callus cells via promotion of the root primordium-like cell identity (Kareem et al. 2015). When the callus is moved to SIM, *LBD16* expression quickly decreases, though the underlying mechanism has not been determined (indicated by the question mark). High cytokinin levels in SIM activates *WUS* expression for the fate transition from callus cells to shoot progenitor cells based on the pluripotency of callus cells (Dai et al. 2017, Meng et al. 2017, Zhang et al. 2017); shoot progenitor cells can then differentiate into the adventitious shoots (Gordon et al. 2007). *PLT3/5/7* also function in this process to promote adventitious shoot formation from shoot progenitor cells (Kareem etal. 2015).

butyric acid) at 22°C under continuous light to induce adventitious shoots from the callus (He et al. 2012, Liu et al. 2014, Hu et al. 2017).

For tissue culture of hypocotyl explants, seeds were grown on 1/2 MS medium at 22°C under continuous light for 1 d for germination and then transferred to continuous dark conditions at 22°C for 5 d for hypocotyl elongation. Approximately 1 cm long hypocotyl segments (from the joint point of root and hypocotyl) were excised as explants and cultured on CIM at 22°C under continuous darkness for 4 d to induce callus formation, and then transferred to SIM at 22°C under continuous light to induce adventitious shoots from the callus.

qRT–PCR

RNA extraction, reverse transcription and qRT–PCR were performed as previously described (He et al. 2012). Relative expression levels of genes were normalized against the reference gene *ACTIN*. Gene-specific primers for qRT–PCR are as follows: 5′-CCTGTTTATGGATGTGTCTC-3′ and 5′-ACATAACTTACCAACTTATC-3′ for *LBD16*, 5′-GGCTACATGAGTAGCCATGT-3′ and 5′-GAACTTCCGATTGGCCATAC-3′ for *WUS*, 5′-GCTAGGGAGAGGCAGAAACG-3′ and 5′-TTCCTCTTGACAATCTTCTTCGC-3′ for *WOX5*, 5′-TCGAGGAGTTACAAGGCATCAC-3′ and 5′-TGCTGCTTCTTCCTCAGTGC-3′ for *PLT1*, 5′-ACTTGGGAACATTCAGCACGG-3′ and 5′-AGTGTGTTGCTCTCCAGGATG-3′ for *PLT2*, 5′-TACCGAGGTGTTACAAGGCATC-3′ and 5′-TGCTTCCTCTTCCGTTGCAAAG-3′ for *PLT3*, 5′-AGGTGTCACAAGGCATCATC-3′ and 5′-CTTCCTCTTCGGTTGCAAAG-3′ for *PLT7*, and 5′-TGGCATCA(T/C)ACTTTCTACAA-3′ and 5′-CCACCACT(G/A/T)AGCACAATGTT-3′ for *ACTIN*.

RNA-seq and data analysis

Leaf explants of Col-0 or *lbd16-2* cultured on CIM for 0 or 4 d were collected for RNA-seq. RNA extraction, library construction, paired-end 150 bp sequencing on the HiSeq 3000 sequencing system and raw data analyses were carried out by Genergy Biotechnology Co. Ltd. (Shanghai, China) following the manufacturer's instructions (Illumina). Genes with a $\log_2$ fold change >3 were classified as being significantly up-regulated in wild-type or *lbd16-2* leaf explants on CIM. The RNA-seq data were shown from one experiment. Potential candidate genes regulated by *LBD16* in callus pluripotency acquisition were identified (**Supplementary Table S1**).

Cytological analysis

Leaf or hypocotyl explants were fixed in 80% acetone on ice for 15 min and then incubated at 37°C in GUS staining buffer (He et al. 2012, Chen et al. 2014). Thin sectioning was performed as previously described (Zeng et al. 2016). Microscopy observation was performed as previously described (Chen et al. 2014, Liu et al. 2014). For confocal analysis, the cell wall was stained with 1 mg ml^{-1} propidium iodide for about 1 min, and imaging was performed with a Zeiss LSM 880 confocal microscope (ZEISS, Germany).

Accession numbers

Sequence data can be accessed in the Arabidopsis Genome Initiative: *WOX11* (At3G03660), *LBD16* (At2g42430). The RNA-seq data have been deposited in the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE101321.

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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