WOX13-like genes are required for reprogramming of leaf and protoplast cells into stem cells in the moss Physcomitrella patens

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ABSTRACT

Many differentiated plant cells can dedifferentiate into stem cells, reflecting the remarkable developmental plasticity of plants. In the moss Physcomitrella patens, cells at the wound margin of detached leaves become reprogrammed into stem cells. Here, we report that two paralogous P. patens WUSCHEL-related homeobox 13-like (PpWOX13) genes, homologs of stem cell regulators in flowering plants, are transiently upregulated and required for the initiation of cell growth during stem cell formation. Concordantly, Δppwox13l deletion mutants fail to upregulate genes encoding homologs of cell wall loosening factors during this process. During the moss life cycle, most of the Δppwox13l mutant zygotes fail to expand and initiate an apical stem cell to form the embryo. Our data show that PpWOX13L genes are required for the initiation of cell growth specifically during stem cell formation, in analogy to WOX stem cell functions in seed plants, but using a different cellular mechanism.

KEY WORDS: Stem cell, WOX, Physcomitrella

INTRODUCTION

Dedifferentiated cells can dedifferentiate under both natural and artificial conditions. In mice and humans, artificial expression of transcription factors, for example, can cause transformation of differentiated somatic cells into pluripotent stem cells (reviewed by Masip et al., 2010). Stem cell regeneration from differentiated cells occurs more readily in plants, reflecting the remarkable plasticity of plant cell identity (Birnbaum and Sanchez Alvarado, 2008). It can occur under natural conditions (Steeves and Sussex, 1989) but also after wounding or addition of phytohormones (Skoog and Miller, 1957). In seed plants, experimentally induced regeneration can involve the formation of a mass of disorganized tissue, called the callus, from which a complete plant can subsequently be obtained, but regeneration without an intermediate callus stage has also been reported (Williams and Maheshwaran, 1986; Vogel, 2005; De Smet et al., 2006; Garces et al., 2007; Sena et al., 2009). In non-seed plants, differentiated cells can generate stem cells without the requirement of exogenously supplied hormones and without forming a callus (Chopra and Kumra, 1988; Raghavan, 1989). In the moss Physcomitrella patens (Physcomitrella), differentiated cells at the wound margin of detached gametophore leaves are reprogrammed into apical stem cells, which subsequently produce the filamentous chloronemata by asymmetric divisions (Prigge and Bezanilla, 2010; Ishikawa et al., 2011). Although several transcription factors involved in the induction of reprogramming have been characterized in flowering plants (Banno et al., 2001; Gordon et al., 2007; Sugimoto et al., 2010, 2011; Iwase et al., 2011), no regulators have been reported in non-seed plants.

Seed plants contain three subclades of WUS-related homeobox (WOX) genes (Haecker et al., 2004). First, several members of the WUSCHEL (WUS) subclade are involved in stem cell maintenance: WUS in the shoot meristem (Laux et al., 1996), WOX5 in the root meristem (Kamiya et al., 2003; Sarkar et al., 2007), WOX3 in leaf marginal meristems (Shimizu et al., 2009; Nakata et al., 2012) and WOX4 in the vascular meristem (Hirakawa et al., 2010; Ji et al., 2010). Expression of WUS promotes pluripotent stem cell fate in the shoot apical meristem of Arabidopsis thaliana (Arabidopsis) from a small underlying group of cells, named the organizing center (Mayer et al., 1998; Schoof et al., 2000). In addition, WUS and WOX5 are involved in the de novo formation of meristems and somatic embryos from differentiated cells (Zuo et al., 2002; Gallois et al., 2004; Gordon et al., 2007; Chen et al., 2009; Sugimoto et al., 2010). Second, two members of the WOX9 clade, WOX8 and WOX9, redundantly regulate zygote development and embryo axis formation (Breuninger et al., 2008; Ueda et al., 2011) and WOX9 has an additional role in shoot meristem regulation (Wu et al., 2005). Finally, recent reports show that members of the WOX13 clade affect replum development, and dehiscence, flowering time, lateral root formation, and fertility (Deveaux et al., 2008; Romena-Branchat et al., 2012).

The WOX13 clade appears to contain the evolutionarily basal members, whereas the WUS and WOX9 clades diverged during the seed plant lineage. Concordantly, all WOX genes found in the Physcomitrella genome group into the WOX13 clade (Deveaux et al., 2008; van der Graaff et al., 2009; Nardmann et al., 2009). The
absence of WUS-clade WOX genes in mosses raises the question of how stem cells are regulated. In contrast to the complex meristems of seed plants, growth of mosses depends on single, apical stem cells that undergo asymmetric cell divisions (Kojuji and Hasebe, 2014). In the gametophytic phase of Physcomitrella, chloronema and caulonema stem cells form filaments (Pigge and Bezanilla, 2010) and the gametophore stem cell gives rise to a leafy shoot (Harrison et al., 2009). In the sporophytic phase, the division of the zygote generates an apical stem cell that gives rise to the embryo by several asymmetric divisions, and a basal cell that forms part of the foot structure (Kojuji et al., 2009). Here, we report that loss-of-function mutants of the Physcomitrella WOX13-like genes show aberrations during stem cell formation from the zygote, protoplasts and detached leaves. Phenotypic and molecular analysis implies a crucial role for moss WOX13-like genes in the activation of cellular growth programs.

RESULTS

**PpWOX13-like genes are upregulated during stem cell formation in detached leaves**

Three loci containing WOX-related sequences in the Physcomitrella genome (Nardmann and Werr, 2007; Richardt et al., 2007) cluster with the Arabidopsis WOX13 clade (Deveaux et al., 2008; van der Graaff et al., 2009). Given that Arabidopsis WOX10 and WOX14 in this clade represent Brassicaceae-specific duplications of an ancestral WOX13-like gene (Deveaux et al., 2008), we named the moss homologs PpWOX13-like (PpWOX13L) genes (Fig. 1A). We detected transcripts of PpWOX13L1 [DDBJ/EMBL/GenBank accession number: AB699867, formerly PpWOX4 (Nardmann and Werr, 2007) and PpaWOX02 (Deveaux et al., 2008)] and PpWOX13L3 [accession number: AB699868, formerly PpWOX07 (Nardmann and Werr, 2007) and PpaWOX01 (Deveaux et al., 2008)] by RT-PCR throughout the moss life cycle, whereas no transcript for PpWOX13L2 was detectable at any developmental stage examined (Fig. 1B).

To investigate the spatiotemporal expression patterns of PpWOX13L1 and PpWOX13L3 proteins, we generated several knock-in reporter lines by inserting a fluorescent protein coding sequence just before the stop codon of each gene (supplementary material Fig. S1A-H). Three different plants each of at least two independently generated reporter lines were studied. All different PpWOX13L1 and PpWOX13L3 reporter lines displayed indistinguishable expression patterns (Fig. 1D-M; supplementary material Fig. S1I-R) and no aberrant phenotypes were detected, suggesting that the observed expression patterns are authentic. For brevity, we focus on the PpWOX13L1-Citrine and PpWOX13L3-Citrine reporter lines. Fluorescent signals of both fusion proteins were detected in nuclei of all tissues examined: protonemata, young and mature gametophores, antheridia, archegonia, unfertilized egg cells, zygotes, sporophyte cells, and spores (Fig. 1D-M; supplementary material Fig. S1I-R). Notably, the signal was stronger in the apical stem cells of chloronema and caulonema compared with subapical cells (Fig. 1E; supplementary material Fig. S1I,J, arrows), and in egg cells and zygotes compared with surrounding cells (Fig. 1I,J; supplementary material Fig. S1N,O, red arrows).

In order to investigate PpWOX13L expression during the induced formation of stem cells, we used a detached leaf assay, in which chloronema stem cells are established from reprogrammed differentiated leaf cells (Ishikawa et al., 2011). We observed that whole-leaf PpWOX13L1 and PpWOX13L3 transcript levels transiently increased after detachment (Fig. 1C). To investigate protein levels at a cellular level, we used the reporter lines and found that the expression of both reporters slightly increased in most leaf cells until 12 h after detachment and then specifically in the cells facing the cut from which stem cells were generated (Fig. 1N; supplementary material Fig. S1S and Movie 1). During subsequent chloronema growth, the signal remained stronger in the apical stem cell than in its descendants.

**PpWOX13L-like genes are required for outgrowth and development of the zygote**

To investigate the role of PpWOX13L genes, we generated several independent deletion mutants (supplementary material Fig. S2A-I), replacing each gene via homologous recombination either with a cassette of a GUS reporter gene and a selection marker (Δppwox13l1a and Δppwox13l1b) or with only a selection marker cassette (Δppwox13l1a2 and Δppwox13l1b2). Both single mutants did not display any robust differences compared with the wild type at any stage of the life cycle (data not shown). Although we observed attenuated fertilization, zygote expansion, and subsequent cell divisions in the single deletion lines (Δppwox13l1a2 and Δppwox13l1b2; supplementary material Table S3), they eventually exhibited normal sporangium formation rates and further development compared with wild type (supplementary material Table S2). In the double mutant (Δppwox13l1ab), caulonema, gametophores, and gametophores were also indistinguishable from wild type (supplementary material Fig. S2J-O). Expression of PpWOX13LC was not detected by RT-PCR in the double mutant as in the wild type (supplementary material Fig. S3). In contrast to wild type, we observed fewer sporophytes in two independent double deletion mutant lines (Fig. 2A,B; supplementary material Table S2). A detailed confocal microscopic analysis revealed that the double mutant is able to form sperm and an egg cell, and that it displayed sperm in the archegonium cavity (Fig. 2D), a brownish archegonium neck (supplementary material Table S2) and stronger autofluorescence in the nucleus than in the cytoplasm (Tanahashi et al., 2005), and in these respects was indistinguishable from the wild type (Fig. 2C-F; supplementary material Table S3). The last three criteria are indicative of fertilization in wild type (Tanahashi et al., 2005). Therefore, although we cannot exclude alternative explanations, these findings strongly suggest that fertilization has taken place in the double mutant.

After fertilization, the wild-type zygote expands by dispersed growth, reaching the size of the archegonial cavity before undergoing an asymmetric cell division into an apical stem cell and a basal cell (Fig. 2G,H). Subsequently, ∼60% of brown-necked archegonia contain an embryo with more than two cells 4 weeks after induction of gametangia (Fig. 2H; supplementary material Table S3). By contrast, in the double mutant, we observed only unexpanded zygotes of a size similar to an unfertilized egg (Fig. 2F), but did not find expanded or divided zygotes as in the wild type (Fig. 2G,H). After 2 months of incubation under gametangium-inducing conditions, no normal sporangia were found (supplementary material Table S3). Several gametophores of the double mutant produced malformed sporangia without normal spores, reminiscent of sporangia formed by parthenogenesis (Tanahashi et al., 2005) (Fig. 2B, inset).

To confirm further that the double mutant can form normal, fertile sperm and egg cells, it was crossed with the wild type. To this end, gametophores were submerged with distilled water, which increased the rate of sporophyte formation. Sporangia were formed on 92.5 ±3.9% (mean ± S.D.; n=10) of 100 gametophores of self-crossed wild type, and on 5.6±5.0% of self-crossed double deletion line (n=10).
We observed that 8.9±3.6% of 100 gametophores of the double deletion line that had been crossed with the wild type developed sporangia (n=10). Among the sporangia on the crossed mutant gametophores, we arbitrarily selected three sporangia with normal morphology and sowed the spores on plates. Spores germinated normally and we collected all germinated chloronemata from each line for genotyping for the *PpWOX13L* genes by PCR. In one of the three lines, we identified both the wild type and the mutant alleles of *PpWOX13LA* and *PpWOX13LB*, indicating that the Δppwox13lab egg cell was fertilized with a wild-type sperm. In another two lines,

![fig1](url)

**Fig. 1.** Amino acid sequences and expression of *PpWOX13L*. (A) ClustalW alignment of full-length amino acid sequences of the WOX13-clade proteins in *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Physcomitrella patens* (Pp) and *Ostreococcus tauri* (Ot). The homeodomain and the WOX13-specific N-terminal domain (Deveaux et al., 2008) are highlighted. (B) Semi-quantitative RT-PCR of *PpWOX13LA* and *PpWOX13LB* expression during the life cycle of Physcomitrella. RanD (Phypa55364) is used as RNA control. 7d, 7-day-old tissue containing only protonemata; 14d, 14-day-old tissue containing protonemata and gametophores; 14dai, gametophores 14 days after gametangia induction; 1mai, gametophores with sporophytes one month after gametangia induction. PCR reactions ran for 28 cycles with identical template concentrations. (C) *PpWOX13LA* and *PpWOX13LB* transcript levels (tags per million, TPM) after leaf detachment determined by 5′-DGE (Nishiyama et al., 2012). Abscissae indicate the time after leaf detachment. Results of three independent experiments are shown. (D-N) Localization of *PpWOX13LA*-Citrine fusion protein. (D-G) Bright-field images (upper images in D-F and left-hand image in G) and fluorescent images (lower images in D-F and right-hand image in G) of chloronema (D), caulonema (E), young gametophore (F) and gametophore (G). Arrows in D and E indicate nuclei of apical stem cells. (H-M) Laser scanning microscopy images of antheridia (H), an archegonium with a ventral canal cell (yellow arrow) and an unfertilized egg cell (red arrow) (I), an archegonium with an unfertilized egg cell (red arrow) after degeneration of the ventral canal cell (J), an archegonium containing a young sporophyte (K,L), and a spore (M). (N) Snapshots of Citrine fluorescence at indicated time after leaf excision taken from a time-lapse movie (supplementary material Movie 1). Scale bars: 100 μm (D,E,H,N); 50 μm (F,I-L); 200 μm (G); 25 μm (M).
the analyzed chloronemata contained only mutant alleles. This indicates that the double mutants can fertilize, at least under the submerged crossing conditions. Together, these results indicate that PpWOX13L genes are redundantly required for the outgrowth of the zygote and the initiation of embryogenesis.

**PpWOX13-like genes are required for initiation of cell growth specifically during stem cell formation**

Because of the upregulation of PpWOX13L reporters during stem cell formation from detached leaves, we analyzed whether this process requires PpWOX13 activity. In the wild type, live imaging of detached leaves revealed that the majority of leaf cells at the wound margin that had undergone division subsequently initiated tip growth, giving rise to stem cells that produced chloronema filaments by further divisions (Fig. 3A,C, red arrowheads). A proportion of the divided cells, however, failed to initiate tip growth and to undergo any further divisions within 72 h after detachment (Fig. 3A,C, yellow arrowheads).

In contrast to the wild type, the Δppwox13lab double mutant displays a strongly decreased frequency of divided cells that initiated tip growth (Fig. 3B,C; supplementary material Movie 2).

We observed the same phenotype albeit at low frequency in some Δppwox13la single mutant lines (data not shown), whereas no difference from the wild type was detected for Δppwox13lb. Forty-eight hours after leaf detachment, expression of the protonema-specific reporters RM09 and RM55 (Ishikawa et al., 2011) was present in all cells that underwent divisions in both the wild type and the double deletion mutant line Δppwox13lab#186 (Fig. 3D), regardless of whether tip growth was initiated or not. This suggests that cells at the wound margin of detached leaves, which enter proliferation, acquire at least some aspects of chloronema identity even if they are unable to initiate cellular growth. In summary, during reprogramming of leaf cells into chloronema stem cells, PpWOX13LA and PpWOX13LB are redundantly required for the initiation of cellular growth, but not for entry into cell division or expression of two chloronema identity markers.

As an independent test for PpWOX13L function in stem cell formation, we analyzed the initiation of chloronema stem cells from protoplasts. In the wild type, protoplasts cultivated under high osmolarity conditions to prevent rupture firstly initiate tip growth and secondly divide, producing a primary chloronema stem cell, along with synthesizing a new cell wall (Jenkins and

![Fig. 2. Deletion of PpWOX13L genes blocks zygote outgrowth.](image)
Cellular outgrowth in plants requires the coordination of several activities. First, the cell must have sufficient turgor pressure to overcome the cell wall resistance (Cosgrove, 2005; Taiz and Zeiger, 2010). Second, the cell wall becomes loosened at the site of growth by enzymatic activities that disrupt cross-linkages between cellulose microfibrils (Cosgrove, 2005; Taiz and Zeiger, 2010). In order to investigate whether insufficient turgor might contribute to the impaired outgrowth of reprogrammed leaf cells in the double mutant, we determined the osmolarity required for plasmolysis of the cells at the wound margin. When using 15 (supplementary material Fig. S4B,C) or 45 (data not shown) minutes of incubation, complete plasmolysis of wild-type cells requires 0.55 M mannitol, whereas 0.6 M mannitol is required for the double mutant (n=50), suggesting an even higher turgor in the double-mutant cells. Thus, a reduced turgor is not the reason for the blocked outgrowth during stem cell formation.

To analyze whether cell wall loosening might require PpWOX13L function during stem cell formation, we compared transcriptomes of wild-type and the double-mutant lines after leaf detachment. To this end, we performed digital gene expression profiling with mRNA 5′-end tags (5′-DGE) (Nishiyama et al., 2012). We analyzed the expression profiles in intact gametophore transcriptomes of wild-type and the double-mutant lines after leaf detachment. Comparison of expression between wild-type and the double mutant revealed <60 genes differentially expressed at each time point until 6 h, and 115 and 254 genes were differentially expressed at 12 and 24 h after detachment. Comparison of expression between wild-type and the mutant lines revealed <60 genes differentially expressed at each time point until 6 h, and 115 and 254 genes were differentially expressed at 12 and 24 h, respectively (supplementary material Table S4). Notably, we found that cell wall-related genes are required for upregulation of cell wall loosening genes.
enriched in the set of genes with reduced expression in the mutant (supplementary material Table S5). Among them, we found that 12 mRNAs predicted to encode cell wall loosening enzymes, including expansins, xyloglucan-specific endo-β-1,4-glucanases (XEG), xyloglucan endotransglucosylases/hydrolase (XET) and pectin methyl esterases (PME), are upregulated in the wild type after leaf detachment, whereas this is not the case to the same extent in the double mutant (Fig. 4A-L). For eight of these genes, the lower expression levels in the mutant compared with the wild type were confirmed by quantitative RT-PCR (supplementary material Table S6). Furthermore, we found that during stem cell formation from protoplasts, transcript levels of three β-expansin genes are significantly lower in the double mutant than in the wild type, although those of one α-expansin and XET are not (supplementary material Table S6). By contrast, we did not detect different transcript levels between the wild type and the double mutant for genes associated with cellular tip growth, such as actin cytoskeleton- or polar membrane transport-related functions (supplementary material Table S4). In summary, PpWOX13L activity is required for the upregulation of specific cell wall loosening enzymes in protoplasts and cells of detached leaves that regenerate stem cells.

**Expression of cell wall loosening genes enhances stem cell formation in chloronemata**

We hypothesized that cell wall loosening genes might be involved in stem cell formation. Because transformation of the double mutant was not successful for unknown reasons, we tested this hypothesis in wild-type protoplasts. To this end, we expressed three predicted β-expansin genes (Fig. 4) driven by the rice actin promoter (Zhang et al., 1991), which provides high expression levels in protoplasts and protonemata (Horstmann et al., 2004). We analyzed stem cell formation from protoplasts comparing chloronemata with similar expression intensities based on a co-transformed p35S:GFP reporter. Transformation with each of two different β-expansin genes, denoted as 146965 and 96876, resulted in a significant increase of stem cell formation (Fig. 4N) compared with the untransformed control, whereas transformation with β-expansin 150257 had no significant effect. We conclude that in the wild type, β-expansin activity is a limiting factor for stem cell formation, at least in chloronemata regenerated from protoplasts.

**Arabidopsis WOX13 and WOX14 are not essential during regeneration of root meristem stem cells**

The flowering plant Arabidopsis encodes three members in the WOX13 clade, namely WOX13, WOX14 and the putative pseudo-gene WOX10 (Haecker et al., 2004; Deveaux et al., 2008). We found that WOX13 and WOX14 promoters have transcriptional activity in root meristems (Fig. 5E,F). In order to analyze whether Arabidopsis WOX13 clade genes might play a role in regeneration of stem cells after wounding, in similarity to stem cell regeneration from detached leaves in moss, we performed root regeneration assays using T-DNA insertion mutants (Fig. 5A-D). wox13-2 carries an insertion in exon 1, and transcript could not be detected by RT-PCR (Romera-Branchat et al., 2012), indicating that this mutant is a strong loss-of-function or a null allele. wox14-1 was previously described as pst3645 and has the homeodomain deleted (Deveaux et al., 2008). We found that wox13-2 and wox14-1 single mutants and the wox13-2 wox14-1 double mutant regenerated root meristems at a similar efficiency as the wild type (Fig. 5G). Thus, in contrast to Phycomitrella, we did not detect a regeneration-related function for Arabidopsis WOX13-clade genes.

**DISCUSSION**

Stem cell formation in seed plants is tightly associated with the function of WOX genes. Here, we addressed whether this association might be conserved outside of the seed plants by studying WOX functions in the moss Phycomitrella. We show that both PpWOX13L proteins accumulate in all cells examined (Fig. 1), and that the double knockout mutants had defects in stem cell formation from cut leaves and in protonemata derived from protoplasts as well as in zygotes (Figs 2, 3). In all cases, cell wall expansion was arrested (Figs 2, 3). Expression of cell wall loosening genes, including β-expansin genes, was reduced compared with wild type in the cut leaves of the double mutant (Fig. 4), and β-expansin induction in wild-type protoplasts enhanced the stem cell formation.

Together, these results indicate that the two moss PpWOX13L genes are redundantly required for the formation of stem cells in cut leaves and from protoplasts. The failure to initiate cell growth is the first phenotypic manifestation during stem cell formation in the double mutant, and the upregulation of genes encoding cell wall loosening enzymes is reduced. Because normal growth of chloronema and caulonema is not impaired, we propose that moss WOX13L activity is involved in cellular growth specifically in the process of stem cell formation at least in protoplasts and dissected leaves.

**The role of WOX13L in Phycomitrella**

During stem cell formation from differentiated leaf cells, several cellular changes, including the re-entry into cell cycle and the initiation of tip growth, occur (Ishikawa et al., 2011). Our results indicate that during this process: (1) the expression levels of PpWOX13LA and PpWOX13LB genes are upregulated, (2) both genes are redundantly required to initiate cellular outgrowth but not for entry into the cell cycle, and (3) both genes are required for upregulation of genes predicted to encode cell wall loosening factors. Likewise, stem cell formation from protoplasts was delayed in the double mutant. Furthermore, the initial outgrowth of a lateral bulge during establishment of side-branch forming stem cells from chloronema subapical cells was impaired. Curiously, this defect was seen only on high but not on low osmolarity medium. We reason that owing to the reduced cell wall loosening in the double mutant, a higher turgor pressure is required for cell expansion. Finally, most of the zygotes in the Ppwox13l Δ double mutant neither elongated nor entered cell division to form an apical stem cell, unlike the wild type. Although we cannot exclude that in the zygote, PpWOX13L genes are involved in cell outgrowth and division separately, a plausible hypothesis in analogy to the effects in induced stem cell regeneration is that the initiation of cell growth is primarily regulated by WOX13L function in the zygote, and that the failure to undergo cell division is a consequence thereof.

Is there a common function of PpWOX13L underlying all the observed defects? We propose that the phenotypes observed in the leaves, protoplasts and zygotes of the double mutant can be explained by an impaired activation of cell growth due to insufficient upregulation of cell wall loosening activities. Although zygotes and stem cells employ different growth modes (diffuse cell growth and tip growth, respectively) the requirement of cell wall loosening enzymes is likely to be key in both processes. Further studies on gene regulatory networks in reprogramming leaf cells and protoplasts as well as on zygotes are necessary to examine whether PpWOX13L genes function similarly in these cells.
Notably, all mutant defects are restricted to formation of apical stem cells, whereas growth of the established filaments by their apical stem cells is unaffected. The broad expression of PpWOX13L genes suggest yet unknown functions in non-stem cells.

Alternatively, the global expression of PpWOX13L genes could contribute to the general competence for stem cell formation. Stem cell regulators that are not restricted to the stem cell niche include, for example, WUS and WOX5 in Arabidopsis, which, in addition to their stem cell niches, are expressed in ovules and cotyledons, respectively (Größ-Hardt et al., 2002; Sarkar et al., 2007). In conclusion, we propose that PpWOX13L activity is required for the onset of cell growth programs specifically during stem cell formation, but not for maintaining them.

Fig. 4. PpWOX13L activity regulates cell wall loosening genes. (A-L) Accumulation patterns of transcripts encoding cell wall loosening genes after leaf excision in 5'-DGGE analysis. Protein IDs are shown above each graph. Horizontal axes indicate the time after leaf detachment and vertical axes indicate tags per million (TPM) values. Blue and red lines indicate expression levels in the wild type and the Δppwox13lab#186 double mutant, respectively. Results of three independent experiments are shown.

(M,N) Transient expression of β-expansins (146965 and 96876) increases apical stem cell formation during protoplast regeneration in the wild type, whereas β-expansin 150257 had no significant effect. Primary chloronemal apical cell and secondary chloronemal apical cells derived from subapical cells are indicated by arrows and arrowheads, respectively, and the number of secondary apical cells is shown in the upper right (M). The total number of secondary chloronema apical stem cells was counted at the fifth day after protoplast regeneration on the high osmolarity medium. The total number of observed protoplast regenerations is 60 (divided among three biological replicates) for every construct. Significantly different distributions from the control are shown with P-values (Mann–Whitney U-test). Scale bars: 50 μm.

Comparison with Arabidopsis
The significance of cell growth regulation in stem cells has not been studied yet in seed plants, where the formation of stem cells and zygote development are also regulated by WOX genes. First, several members of the WUS clade in seed plants function in maintaining pluripotent stem cells (Aichinger et al., 2012). In Arabidopsis, these include WUS in the shoot meristem (Laux et al., 1996), WOX5 in the root meristem (Kamiya et al., 2003; Sarkar et al., 2007), WOX4 in the cambium (Hirakawa et al., 2010; Ji et al., 2010) and WOX1 together with PRS (WOX3) in the leaf margins (Shimizu et al., 2009; Nakata et al., 2012). Notably, it is the formation of the primary shoot meristem in the embryo that is most sensitive to reduction of WUS activity, whereas maintenance of stem cells...
during inflorescence meristem growth is less sensitive (Graf et al., 2010). Furthermore, both WUS and WOX5 are essential in regenerating stem cells via tissue culture (Su et al., 2009; Sugimoto et al., 2010). The requirement for WUS and WOX specifically during formation of stem cells but to a weaker extent during further development is similar to that for PpWOX13L in moss stem cell formation reported here. However, there are also notable differences. Whereas in moss, we found that the activation of the cell growth program is an essential part of PpWOX13L function, in Arabidopsis, WUS and WOX5 genes may have a repressive effect on cell enlargement based on their cellular mutant phenotypes (Laux et al., 1996; Sarkar et al., 2007). One interesting possibility to be tested in the future is whether these opposite effects could be explained by the acquisition of the transcriptional repressive WUS box in the WUS-clade genes (Lin et al., 2013).

Second, several WOX genes are required for normal development in the Arabidopsis zygote (Breuninger et al., 2008; Ueda et al., 2011), which superficially parallels the function of PpWOX13L genes in Physcomitrella. However, also here there are marked differences: in Arabidopsis, it is the asymmetry of the zygotic division that is under control of WOX2, 8 and 9 genes, whereas an effect on zygote expansion, as seen in the moss for PpWOX13L genes, has not been reported. Zygote expansion in Arabidopsis is rather under the control of the YODA pathway (Łukowicz et al., 2004), which acts in parallel to WOX genes at later stages of embryo patterning. Expression of a moss YODA ortholog is induced after leaf detachment independently of PpWOX13L (Nishiyama et al., 2012), but its function remains to be investigated.

Our mutant analysis of the only two expressed members of the WOX13-clade in Arabidopsis, WOX13 and WOX14, did not reveal an obvious stem cell regeneration defect. Of course, possible stem cell- or regeneration-related functions of WOX13 and WOX14 could be masked by the existence of yet unknown redundant factors. We consider it unlikely, however, that Arabidopsis WOX13 genes function redundantly with WUS clade genes, given the sequence differences in their recognition helix of the homeodomain that mediates DNA contact (Deveaux et al., 2008; Nardmann and Werr, 2012). Thus, the function of WOX13 clade genes in Arabidopsis appears to be distinct from that of WUS clade genes. WOX13-type genes evolved in an ancestor of green algae and land plants (Deveaux et al., 2008) and in light of the early separation of the two lineages in plant evolution, PpWOX13L function in moss could be either close to the ancestral trait or a derived character. The Appwox13l deficiencies in activating cell outgrowth are consistent with ancestry, as cell expansion is a sine qua non condition for cell divisions in uni- or multicellular organisms, and this view is compatible with the PpWOX13L phylogenetic position in the ancestral branch of the WOX family (Deveaux et al., 2008; van der Graaff et al., 2009; Nardmann et al., 2009). However, the Δppwox13l phenotypes associate the PpWOX13L function with stem cell formation, which is reminiscent of the function of WUS clade members that evolved after the separation of moss and vascular plant lineages (Nardmann and Werr, 2012). The PpWOX13L function in moss thus exemplifies either convergent evolution or recruitment of WOX13 genes to the interface of cell enlargement/division and cellular differentiation in the last common ancestor of mosses and vascular plants.

It is noteworthy in this regard that expression of WOX13-clade genes in Physcomitrella and Arabidopsis is not restricted to...
small signaling centers, and there is no indication of a non-cell-autonomous function. By contrast, \( WUS \) clade function in stem cell regulation appears to involve spatially restricted expression in stem cell organizers and the organization of multicellular niches via non-cell-autonomous mechanisms (Schoof et al., 2000; Sarkar et al., 2007; Hirakawa et al., 2010), which plausibly could have been an acquisition during evolution of increasingly complex stem cell systems typical for angiosperms.

**MATERIALS AND METHODS**

**Plant materials and growth conditions**

*Physcomitrella patens* Cove-NIBB (Nishiyama et al., 2000) or Gransden 2004 (Rensing et al., 2008) lines were used as a wild-type strain and cultured on BCDAT medium supplemented with 1 mM CaCl\(_2\) and 0.8% (w/v) agar (BCDAT agar medium) at 25°C in continuous light or long-day conditions (16 h/8 h) (Nishiyama et al., 2000). Gametophore preparation for transcriptome analysis, quantitative RT-PCR analysis, and plasmolysis experiments (Ishikawa et al., 2011), protoplast preparation (Nishiyama et al., 2000), and gametangia/sporophyte observation (Sakakibara et al., 2008) were previously described.

**Transformation and genetic crosses**

The primer sequences used for plasmid construction are provided in supplementary material Table S1. The constructs used for transformation are shown in supplementary material Fig. S1A-D and Fig. S2A-D. Transformation was performed as described previously (Nishiyama et al., 2000; Knight et al., 2002). Stably transformed lines were screened by DNA gel-blot analyses. To exclude polyclonal lines, flow cytometry analysis was performed (Okano et al., 2009). Crosses were performed as previously described (Tanahashi et al., 2005).

**Microscopy**

Images were captured using SZX16 and BX51 (Olympus). Citrine fluorescent images were obtained using fluorescence microscopes with a U-MNIBA3 filter unit or using confocal laser scanning microscope LSM 510 confocal system (Carl Zeiss) with a detecting fluorescence emission between 420 and 480 nm. Digital gene expression profiling with mRNA 5'-end tags (5'-DGE) analysis

**Digital gene expression profiling with mRNA 5'-end tags (5'-DGE) analysis**

Transcriptome comparisons between the wild-type and the \( \Delta \text{ppwox13lab} \) double deletion lines were performed as previously described (Nishiyama et al., 2012). Gene Ontology enrichment analyses were performed using the BinGO plugin (version 2.4.4) on Cytoscape version 2.8.3 (Maere et al., 2005) with the table (Rensing et al., 2008) available at (ftp://ftp.jgi-psf.org/pub/JGI_data/Physcomitrella_patens/v1.1/Phyap1_1_goinfo_FilteredModels3.tab.gz). For each set of up- or downregulated genes at each time point or the union of such sets for any time point, overrepresented GO terms were searched. Hypergeometric tests with Benjamini and Hochberg FDR were carried out with the significance level of 0.01. Corrected \( P \)-values for significantly overrepresented GO terms were obtained.

**Transient expression assay of \( \beta\)-expansins**

Plasmids for transient expression assay of \( \beta\)-expansins were constructed using pTFH22.4 (accession number: AB758445), which contains a rice actin promoter (McElroy et al., 1990), a multiple-cloning site (SalI-Aecl-Smal-ApaI-SfiI), the pea \( nbc3-3A \) polyadenylation signal, the cauliflower mosaic virus (CaMV) 35S promoter, \( sGF \), and the polyadenylation signal of the nopaline synthase gene. cDNA of each \( \beta\)-expansin coding region was amplified by PCR with primers shown in supplementary material Table S1 and cloned into the AscI-Apal site of pTFH22.4. The original pTFH22.4 was used as a control. Transient transformation was performed using 10 \( \mu \)g of circular plasmid DNA (Nishiyama et al., 2000). The protoplasts were left on high osmolarity plates [6% (w/v) mannitol] after transformation. Twenty protoplasts or regenerating chloronemata with GFP signal were arbitrarily chosen on the second, third, fourth and fifth days after plating using the Olympus BX51 microscope with the GFP filter set. Each regenerating plant was scored for the number of apical cells showing tip growth, and the total number for each class was obtained from three repeat experiments. The difference in the number of the acquired apical stem cells was tested by the non-parametric Mann–Whitney \( U \)-test, because the number of apical stem cells is not considered to follow the normal distribution but is an ordered variable. The calculations were performed using the R program (R Development Core Team, 2012).

**RNA preparation and quantitative RT-PCR analysis**

Excised leaves for quantitative RT-PCR analysis were prepared in the same manner as samples for 5'-DGE analysis and collected 24 h after leaf excision. Regenerated protoplasts for quantitative RT-PCR analysis were prepared following protoplast regeneration. After overnight incubation, protoplasts were collected, suspended in PRM/T medium without agar (BCDAT medium supplemented with 10 mM CaCl\(_2\) and 0.044 M mannitol), and plated on the PPM/B plate (BCDAT medium supplemented with 10 mM CaCl\(_2\), 6% mannitol and 0.8% agar) covered with sterile cellophane. Regenerated protoplasts were collected 4 days after plating.

Total RNA preparation and cDNA synthesis were described previously (Ishikawa et al., 2011). Quantitative RT-PCR (qRT-PCR) was performed using an ABI PRISM 7500 (Life Technologies) with the SYBR GreenER qPCR Super mix Universal (Life Technologies) and appropriate primers shown in supplementary material Table S1. Results were analyzed using the comparative critical threshold method (Livak and Schmittgen, 2001). The transcript levels were normalized against \( \alpha\)-tubulin gene (TU14) transcript levels using \( TU14 \) primers (Ishikawa et al., 2011). The quantification of each sample was performed in triplicate. Three biological replicates were analyzed for transcript accumulation.

**Generation of Arabidopsis reporters**

Promoter fragments including 4.4 kb (\( pWOX13 \)) and 1.4 kb (\( pWOX14 \)) were amplified with primers containing adapter sequences for ligation independent cloning (LIC) (Li and Elledge, 2007; Eschenfeldt et al., 2009),
Arabidopsis root regeneration assay

The QC184 marker (Sabatini et al., 1999) was introduced by crossing with the wox13-2 mutant, and the selected wox13-2 QC184 plants were subsequently crossed to wox14-1. Because these crosses generated a mixture of three ectotypes (QC184: WS; wox13-2: Ler; wox14-1: N0), wild-type controls were selected from the progeny of these crosses. The regeneration assay was carried out as previously described (Sena et al., 2009) and regeneration was scored two days after cutting (four biological replicates for each background, n=22-48 for each replicate).

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RESEARCH ARTICLE


