A single homeobox gene triggers phase transition, embryogenesis and asexual reproduction

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Plants characteristically alternate between haploid gametophytic and diploid sporophytic stages. Meiosis and fertilization respectively initiate these two different ontogenies. Genes triggering ectopic embryo development on vegetative sporophytic tissues are well described; however, a genetic control of embryo development from gametophytic tissues remains elusive. Here, in the moss *Physcomitrella patens* we show that ectopic overexpression of the homeobox gene *BELL1* induces embryo formation and subsequently reproductive diploid sporophytes from specific gametophytic cells without fertilization. In line with this, *BELL1* loss-of-function mutants have a wild-type phenotype, except that their egg cells are bigger and unable to form embryos. Our results identify *BELL1* as a master regulator for the gametophyte-to-sporophyte transition in *P. patens* and provide mechanistic insights into the evolution of embryos that can generate multicellular diploid sporophytes. This developmental innovation facilitated the colonization of land by plants about 500 million years ago and thus shaped our current ecosystems.

An alternation of generations is a common characteristic of all land plants: they alternate between a haploid (1n, gametophyte) and a diploid (2n, sporophyte) phase (called ‘generations’ in botany) characterized by distinct body plans. It is assumed that the formation of different body plans is based on distinct developmental programmes, so that in plants two ontogenies are encoded by one genome. During evolution from algae to flowering plants, the sporophyte gained dominance over the gametophyte. Mosses like *P. patens* are intermediate between algae and flowering plants (1) in terms of evolutionary distance, (2) in that both phases (generations) are multicellular (sympomorphous character) and (3) fertilization takes place by means of motile sperm cells as in algae and animals (symplesiomorphic character). During regular development, checkpoints between the phases are fertilization of the egg cell and meiosis in the spore mother cell. However, ploidy itself is not sufficient to determine phase-specific development because irregular haploid sporophytes can be induced in flowering plants and diploid gametophytes in mosses. Moreover, sexual reproduction can be replaced by reproduction without fertilization. For a century, such irregular phase transitions have been described in different plant systems as parthenogenesis, apogamy or apomixis. Nevertheless, a single gene triggering the complete transition from gametophyte to sporophyte has hitherto not been identified.

Numerous developmental programmes in animals and plants are also expressed in the gametophyte14,23. We analysed *PpBELL1*, *PpBELL2*, *PpBELL3* and *PpBELL4* are not only shorter than *PpBELL1* and *PpBELL2* but lack the regulatory *ZIBEL* domains (Supplementary Fig. 1). Further, *PpBELL2* differs from the other *PpBELL* proteins and from the majority of land plant *BELL* proteins in the fourth position of the SKY domain; usually this is leucine, but in *PpBELL2* it is glutamic acid. The SKY domain is predicted to form an amphipathic α-helix, possibly mediating protein–protein interactions. Because of the glutamic acid residue the hydrophilic face of the predicted amphipathic α-helix in *PpBELL2* is increased compared with that in *PpBELL1*. This difference may result in different specificities in protein–protein interactions of the different *PpBELL* proteins (Supplementary Fig. 2).

It was reported that mRNAs of all *PpBELL* genes accumulate in reproductive organs or in sporophytes and *PpBELL3* and *PpBELL4* are also expressed in the gametophyte15,23. We analysed *PpBELL* mRNA accumulation in protonemata of WT and of a PpFIE knockout mutant using quantitative polymerase chain reaction with reverse transcription (qRT–PCR). Under our conditions *PpBELL3* and *PpBELL4* expression is not detected in gametophytic protonema of WT or mutant. In contrast, *PpBELL1* and *PpBELL2* are
silent in WT protonemata, but their transcripts accumulate in the *PpBELL* mutant (Supplementary Fig. 3). ChIP-seq experiments provided evidence that *PpBELL1* and *PpBELL2* are epigenetically controlled by histone methylation. Together with our expression analysis, we conclude that PRC2 represses the expression of *PpBELL1* and *PpBELL2* in protonemata of *P. patens*.

To observe protein accumulation patterns of *PpBELL1* and *PpBELL2* at cellular resolution, reporter lines were created by targeted knock-in of the coding sequence (CDS) for the beta-glucuronidase (GUS) protein downstream of the respective *PpBELL CDS*. The resulting *PpBELL*–GUS plants develop indistinguishably from WT, indicating that the addition of the GUS reporter to the endogenous BELL proteins does not interfere with BELL function.

Before fertilization *PpBELL1–GUS* proteins accumulate to low amounts, specifically in the egg cell and in the ventral canal cell of the female sexual organ (archegonium), whereas *PpBELL1–GUS* is not detectable in male sexual organs (antheridia) or in sperm cells (Fig. 1a,b and Supplementary Fig. 5). After fertilization, this protein accumulates to high amounts in the entire developing sperm masses emerged from antheridia of the WT and the mutant lines *ΔBELL1-2, -4 and -9* (Supplementary Fig. 7). In contrast, and in accordance with the restricted expression pattern of *PpBELL1*, we found differences between egg cells of WT and *PpBELL1* knockout mutants. The egg cells of mutants are more variable in size and had up to four times the volume of WT egg cells (Fig. 1n,p,q, Supplementary Table 1 and Supplementary Note 1). These morphological differences are in line with the expression pattern of the *PpBELL1* protein and imply a crucial role of *PpBELL1* in egg cell division or early embryo formation.

To gain further insight into *PpBELL1* function, we generated three independent transgenic lines expressing its CDS under the control of a constitutive and strong promoter (Supplementary Fig. 8). All three overexpressor (oe) lines (Supplementary Fig. 9 and Supplementary Table 2) develop WT-like protonema and leafy shoots (Supplementary Fig. 10a–l). Unlike the WT, apogamous sporophytes develop on *PpBELL1oe* protonemata, specifically on caulonemal cells in lieu of side branches (Fig. 2a,g and Supplementary Fig. 10n–p and Supplementary Fig. 11). During WT development buds (Fig. 2b) with a single three-faced apical cell (Fig. 2c) are initiated on caulonemal cells and differentiate into leafy shoots (Fig. 2f). Although *PpBELL1oe* plants also follow this path, they additionally form structures (Fig. 2h) with two-faced apical cells (Fig. 2i) that differentiate into multicellular bodies (Fig. 2i) resembling WT embryos (Fig. 2d,e). The WT embryos and the *PpBELL1oe* apogamous embryos develop with two-faced apical cells (Fig. 2c,k). Moss protonemata comprise two successive cell types: chloronema and caulonemal. Notably, in the *PpBELL1oe* lines the apogamous sporophytes appear only on caulonemal cells (Fig. 2j).

In the WT, this cell type normally terminates differentiation by endoreduplication, resulting in diploid or even tetraploid gametophytic cells. These apogamous sporophytes further
differentiate into spore capsules (Fig. 2l) almost identical to the WT (Fig. 2f). Moreover, they produce spores with the typical coat ornamentation, albeit the spores are smaller than in the WT, do not complete their development beyond the tetrad state and are less viable than WT spores (Fig. 2f,l inserts, Supplementary Note 2). This defect in spore ripening may be a result of BELL1 overexpression, because in the WT PpBELL1 is not expressed after meiosis (Fig. 1i).

Compared with WT embryos, PpBELL1oe embryos have a more round shape (Fig. 3a,b), probably because of relaxed mechanical constraints, as they do not grow within an archegonium. Consequently, WT sporophytes but not apogamous sporophytes are covered by the remainder of the archegonium, the calyptra (Fig. 3c,d). Artificial removal of the calyptra from developing WT sporophytes confirmed its influence on sporophyte shape (Supplementary Fig. 12). This maternal structure increases offspring fitness in mosses29. Nevertheless, WT and apogamous sporophytes possess stomata (Fig. 3g,h), which are distinctive features of sporophytes in land plants, including mosses.

Relative DNA amounts of nuclei were measured from whole mounts of WT and apogamous sporophytes and from WT leaves as a control. Collectively, these data provide evidence that the apogamous sporophytes are diploid (Supplementary Note 3, Supplementary Figs 13, 14 and Supplementary Table 3). This was further supported by the finding that protonemata that regenerated from injured apogamous sporophytes also are diploid, as is the WT sporophyte (Supplementary Fig. 15).

Overexpression of PpBELL1 had no discernible effect in chloronemal cells but induced apogamous sporophytes on caulonemal cells. These caulonemal cells are known to terminate differentiation with endoreduplication in the WT, resulting in diploid or even tetraploid gametophytic cells27,28. Thus, these endoreduplicated gametophytic cells mimic fertilized egg cells by DNA content. It is, however, evident that a change in ploidy itself is not sufficient to initiate gametophytic or sporophytic developmental programmes.

Because BELL and KNOX proteins are described to act as heterodimers31, we analysed the expression of the five moss KNOX (MKN) genes in chloronemata and caulonemata of the WT and of PpBELL1oe (Supplementary Fig. 16). In the WT, no MKN transcripts were detectable in chloronemata, whereas low amounts of MKN2 transcripts accumulated in caulonemata. In PpBELL1oe, transcripts from three MKNs (1, 2, 6) accumulated in caulonemata that had not yet formed apogamous sporophytes. From this we infer that MKN2 proteins are present in WT caulonemata and that they may form heterodimers with PpBELL1 when it is expressed ectopically. Subsequently, a MKN–BELL1 complex may induce the expression of additional MKN genes. This hypothesis is supported by the finding that PpBELL1 can form heterodimers with each of the MKN proteins in planta (Supplementary Fig. 17).

To challenge our conclusion that PpBELL1 is a main genetic trigger for the developmental switch from gametophyte to sporophyte, we generated ΔFIE ΔBELL1 double-knockout mutants. As shown earlier by us13 and others14, null mutants of the PRC2 protein FIE develop irregular sporophyte-like structures on protonemal cells, implying that PRC2 represses the expression of sporophytic genes. We deleted the PpBELL1 gene in the genetic background of a ΔFIE mutant plant and obtained three independent lines (Supplementary Fig. 18). In all three lines the irregular sporophyte-like structures of the ΔFIE mother plant reverted to rudimentary leafy shoots (Fig. 3i–k and Supplementary Fig. 19). We interpret this intergenic suppression as further proof for the function of PpBELL1 as a central molecular trigger for the gametophyte-to-sporophyte transition in P. patens downstream of the PRC2. Interestingly, whereas the ΔFIE mutant sporophyte-like structures develop in far greater numbers than the PpBELL1oe
apogamous sporophytes, we never observed the formation of complete sporophytes in ΔFIE mutants. As a possible explanation, we suggest that additional transcription factors controlled by the PRC2 are constitutively expressed in ΔFIE but are regulated independently of BELL1 in the sporophyte. It is likely to be that these transcription factors are transiently induced during sporophyte development but their repression is necessary for sporophyte maturation. So the ΔFIE mutant sporophyte-like structures could represent embryos unable to complete development.

Land plants evolved from a freshwater alga about 500 million years ago. To conquer land, the unicellular diploid zygote had to divide mitotically before meiosis to develop a multicellular diploid sporophyte attached to the maternal gametophyte, a hypothesis known as 'zygote retention'. Until now, the molecular mechanism establishing zygote retention has not been known. Our results suggest that the altered expression of an algal BELL gene has facilitated mitotic proliferation of the zygote, leading to embryogenesis and a multicellular sporophyte that is attached to the maternal gametophyte, similar to the formation of apomorous embryos and sporophytes from an endoreduplicated vegetative gametophytic cell observed by us in *PpBELL1oe* moss plants. Subsequently, during land plant evolution multiple duplications of BELL and KNOX genes (*Chlamydomonas* 1/1, *Physcomitrella* 4/5, *Arabidopsis* 13/8, poplar 15/19, rice 12/14) and their divergence into gametophyte- and sporophyte-specific families occurred. This set of homeodomain transcription factors then appears to be the key for distinctive gametophytic and sporophytic developmental programmes that are characteristic for plants.

Our findings may serve as an entry point for research with flowering plants where the sporophyte vastly dominates the diminutive gametophyte. If the regulation of asexual reproduction described here has been conserved in evolution, the induction of apomixis in crop plants will facilitate breeding programmes using homozygous progeny of solely maternal origin and thus contribute to increased food production.

**Methods**

**Plant material and culture conditions.** The strain of *P. patens* (Hedw.) Bruch & Schimp. that was used for genome sequencing was propagated in liquid or on solid Knop medium supplemented with 50 µmol l⁻¹ H₂BO₃, 50 µmol l⁻¹ MnSO₄ × H₂O, 15 µmol l⁻¹ ZnSO₄ × 7H₂O, 2.5 µmol l⁻¹ KI, 0.5 µmol l⁻¹ Na₂MoO₄ × 2H₂O, 0.05 µmol l⁻¹ CuSO₄ × 5H₂O and 0.05 µmol l⁻¹ CoCl₂ × 6H₂O. To solid medium 12 g l⁻¹ purified agar (OXOID, Thermo Scientific) and 200 mg l⁻¹ glucose was added. Cultures on solid medium and in liquid medium were inoculated with individual gametophores. The plants were cultivated at 23 °C under a 16-hour light and 8-hour dark cycle.

Regular sporophyte development was induced according to, whereas apomorous sporophytes spontaneously developed under standard growth conditions.
Generation of transgenic lines. For the generation of chimaeric PpBELL-GUS proteins, the region upstream to the stop codon (designated the 5′ region, 1,130 bp) and the 3′ untranslated region (designated the 3′ UTR region, 798 bp) of PpBELL1 were amplified using the primer sets 185 5′-GGATCCGGAGTAGATTAGTTAGCTTGC-3′ (5′ region) and 103 5′-GGATCCGCTTATATTACGCATCTGAAGTT-3′ (3′ UTR region), respectively. The 3′ region (859 bp) and the 3′ UTR (771 bp) of PpBELL2 were amplified using the primer sets 101 5′-GGATCCAGGTTGCAGCTTCAAGGTGTC-3′, 102 5′-GGATCCGCTTATATTACGCATCTGAAGTT-3′ (5′ region) and 103 5′-GGATCCGCTTATATTACGCATCTGAAGTT-3′ (3′ UTR region), respectively. The four ampli- 
ed using the primer sets 185 5′-GGATCCGGAGTAGATTAGTTAGCTTGC-3′ (5′ region) and 103 5′-GGATCCGCTTATATTACGCATCTGAAGTT-3′ (3′ UTR region), respectively. The two ampli- 
ed with the primer sets 327 5′-AACCAGATGCAAGCTTGTACCATGCATTGTCAAAGTTGGCTTC-3′ (5′ UTR region), respectively. The four amplified fragments were sub-cloned into the pET1.2 vector (Thermo Scientific). The 5′ genomic fragment of PpBELL1 was cloned in-frame to the uidA reporter gene followed by the nos terminator in the PpBELL1–hpt locus was validated with the primers 269Fw 5′-CGTGGATACGGCTGTAGTTTAGTAAGG-3′ and 35S_Rv 5′-AACGCGCTTTCCCACCAACG-3′. This construct was used for transformation of A. thaliana. 

For the generation of PpBELL overexpressor lines, the coding sequence of PpBELL1 (3,195 bp) was amplified using the primers BELL1_XhoI_fw 5′-TGGGACCACTGTCGGCAGAG-3′ and BELL1_XhoI_rev 5′-AAAGCATGCAAGCTTGTACCATGCATTGTCAAAGTTGGCTTC-3′, respectively. The two amplified fragments were sub-cloned into the pET1.2 vector. The 5′ UTR genomic fragment was cloned in the PMB8–hpt vector using the HindIII and SalI sites. The resulting construct was used for transformation of A. thaliana.

For the generation of PpBELL deletion construct at the 5′ region was validated with the primer sets 269Fw 5′-CGTGATACGGCTGTAGTTTAGTAAGG-3′ and 35S_Rv 5′-TGGGACCACTGTCGGCAGAG-3′. Proper integration at the 3′ region was validated with the primers 355_Ter_Fw and 237 (1,083 bp). Validation of the presence of PpBELL1 was performed with the primers 184Fw 5′-GGAGGGAGAGGTTACACACAGG-3′ and 268Rev 5′-GGATCCGCTTATATTACGCATCTGAAGTT-3′ (1,648 bp). Absence of PpBELL1 was carried out with the primers 185Fw 5′-GGATCCGGAGTAGATTAGTTAGCTTGC-3′ and 237Rev 5′-TGGGACCACTGTCGGCAGAG-3′ (2,043 bp).

Images were captured using an AxioCam MRC5 coloured CCD camera (Zeiss).

**Data display.** The box plot depicting the egg cell diameter was created with BoxPlotR (http://boxplot.yerselflab.com/). Centre lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; the notches represent the 95% confidence intervals.

**Availability.** All transgenic lines described in this study are deposited in the International Moss Stock Center (http://www.moss-stock-center.org/) with the accession numbers IMSC 40720, 40724, 40725 (BELL1–hpt), 40715, 40761 (BELL1–GUS-16, 21), 40731–40735 (BELL2–GUS-12, -16, -37, -38, -44, 40717–40719 and 40741–40744 (BELL1–Δ, -4, -9, -13, -21, -25, 34) and 40755–40757 (AFeBBEL1–10, -18, -167).

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**References**


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29. Budke, J. M., Gof...


36. Egener, T.


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Author contributions

N.A.H. generated and analysed the *PyBELL* lines and analysed the *FIE/BELL1* double knockouts and *MKN* expression. A.K. generated and I.P. analysed the *BELL1* knockouts; N.A.H. analysed sperms and egg cells of the *BELL1* knockouts. A.K. generated and I.P., N.A.H. and N.O. analysed the *BELL–GUS* lines. A.K., N.A.H. and I.P. analysed *BELL* gene expression. A.K. and N.O. performed the BiFC experiments. N.A.H. performed additional analyses documented in Supplementary Figs 1, 2, 8 and 12. A.K., E.L.D., N.O. and R.R. planned and supervised the study. R.R., N.A.H. and N.O. wrote the manuscript. All authors discussed the results and commented on the manuscript.

Additional information

Supplementary information is available online. Reprints and permissions information is available online at *www.nature.com/reprints*. Correspondence and requests for materials should be addressed to N.O. and R.R.

Competing interests

The authors declare no competing financial interests.