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The function of TONNEAU1 in moss reveals ancient mechanisms of division plane specification and cell elongation in land plants

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SUMMARY

The preprophase band (PPB) is a transient ring of microtubules that forms before mitosis in land plants, and delineates the cytokinetic division plane established at telophase. It is one of the few derived traits specific to embryophytes, in which it is involved in the spatial control of cell division. Here we show that loss of function of *Physcomitrella patens PpTON1* strongly affects development of the moss gametophore, phenocopying the developmental syndrome observed in *Arabidopsis ton1* mutants: mutant leafy shoots display random orientation of cell division and severe defects in cell elongation, which are correlated with absence of PPB formation and disorganization of the cortical microtubule array in interphase cells. In hypomorphic *Ppton1* alleles, PPB are still formed, whereas elongation defects are observed, showing the dual function of TON1 in organizing cortical arrays of microtubules during both interphase and premitosis. *Ppton1* mutation has no impact on development of the protonema, which is consistent with the documented absence of PPB formation at this stage, apart from alteration of the gravitropic response, uncovering a new function of TON1 proteins in plants. Successful reciprocal cross-complementation between *Physcomitrella* and *Arabidopsis* shows conservation of TON1 function during land plant evolution. These results establish the essential role of the PPB in division plane specification in a basal land plant lineage, and provide new information on the function of TON1. They point to an ancient mechanism of cytoskeletal control of division plane positioning and cell elongation in land plants.

KEY WORDS: Land plants, *Physcomitrella patens*, Division plane, Preprophase band, Cytoskeleton, TONNEAU1

INTRODUCTION

Colonization of land by green plants took place around 470 million years ago, and is associated with a number of physiological and developmental innovations to adapt to terrestrial environments (Graham, 1996). These innovations included acquisition of networks of genes controlling multicellular growth and morphogenesis, as well as functions associated with the ability to withstand abiotic constraints such as temperature, water availability, light or gravity. Cells of land plants (embryophytes) are embedded in a pecto-cellulosic wall, and do not have the ability to migrate during development. Consequently, the location of cells is set up in an irreversible fashion by oriented cell divisions occurring in specialized apical histogenetic tissues called meristems. Therefore, fine-tuning of the spatial positioning of mitotic division planes is a key process in the coordination of multicellular development in plants, together with oriented cell expansion.

The microtubule cytoskeleton plays a major role in both processes. Four spatially distinct microtubule arrays form sequentially during the plant cell cycle: the interphase cortical array, the preprophase band (PPB), the mitotic spindle and the phragmoplast. The PPB is a structure specific to embryophytes, which outlines the cortical division site at the onset of mitosis, and plays an essential role in division plane specification (Mineyuki, 1999). The PPB is formed in G2 by progressive narrowing of cortical microtubules to form a ring encircling the nucleus at the cell cortex. It then disappears during prophase, concomitantly with spindle formation. This transient microtubule array topologically defines the final cortical division site of the cell. Recent results point to a role in the spatial positioning of spindle poles as well (Ambrose and Cyr, 2008). In telophase, the new cell plate is deposited through vesicle transport guided by the phragmoplast, a double-ring-shaped structure composed of actin filaments and antiparallel microtubules. It is formed at the center of the cell from remnants of the spindle and grows centrifugally to reach the cortical site previously occupied by the PPB, eventually connecting to the membrane at this very position (Van Damme et al., 2007). It is believed that the PPB leaves a positional cue at the cell cortex, and several proteins that positively or negatively mark this zone have been described (Vanstraelen et al., 2006; Walker et al., 2007; Xu et al., 2008). However, their function remains unclear, and the nature of the signal is unknown. Beyond cell division, microtubules organize into cortical interphase arrays that are involved in oriented cell expansion through guiding deposition of cellulose microfibrils on the outer side of the cell membrane (Paredez et al., 2006). In expanding cells, microtubules and cellulose fibrils are organized in parallel arrays, transverse to the elongation axis.

Two proteins directly and specifically involved in PPB formation have been identified: TON1 (Azimzadeh et al., 2008; Traas et al., 1995) and FASS/TON2/DCD1 (Camilleri et al., 2002;

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Torres-Ruiz and Jürgens, 1994; Traas et al., 1995; Wright et al., 2009). In *Arabidopsis thaliana*, both *ton1* and *fass* loss-of-function mutants are characterized by the same strong developmental phenotype: mutants are extremely dwarf and display chaotic orientation of cell divisions and severe defects in cell expansion. Yet the general architecture of the plant, including phyllotaxis as well as cell differentiation, remains surprisingly correct (Traas et al., 1995). At the cellular level, this phenotype is associated with the absence of PPB, and disorganization of the interphase cortical microtubule arrays (Azimzadeh et al., 2008). The FASS/TON2 protein is a type 2A protein phosphatase regulatory subunit (Camilleri et al., 2002) that is thought to play a regulatory function in PPB formation (Van Damme, 2009). The maize homolog of FASS, DCD1, colocalizes with the PPB and remains at the cortical division site through metaphase (Wright et al., 2009). In *Arabidopsis*, the TON1 protein localizes to the PPB in mitotic cells and to cortical MT during interphase. AtTON1 was shown to interact with centrin, a key component of eucaryotic microtubule organizing centers (Azimzadeh et al., 2008) and with CDKA;1 (Van Leene et al., 2007). Homologs of the *AtTON1* gene have been identified in genome and EST databases from land plants, ranging from mosses to angiosperms, but are absent in algae (Azimzadeh et al., 2008). The predicted protein appears highly conserved among land plants, suggesting a functional conservation in embryophytes.

The model moss *Physcomitrella patens* provides unique facilities to study plant biology and undertake comparative developmental studies (Cove et al., 2006; Lang et al., 2008; Schaefer, 2002). In contrast to that of angiosperms, the bryophyte life cycle is dominated by the haploid gametophyte, which displays two distinct developmental stages: the protonema, a branched network of tip-growing cells displaying filamentous growth (Menand et al., 2007a), followed by the gametophore, a leafy shoot that differentiates from a single meristematic cell emerging from the protonema (Harrison et al., 2009). This transition is associated with the establishment of a three-faced apical meristematic cell and a specific organization of division planes. Remarkably, early studies of microtubule structures in *P. patens* have shown that division takes place without PPB formation in tip-growing protonemal cells (Doonan et al., 1985), whereas formation of a typical PPB precedes cell division in gametophore cells very early after bud differentiation and throughout their development (Doonan et al., 1987), suggesting that reorganization of microtubule arrays correlates with the transition from a tip-growing filament to a meristematic type of growth and development.

To characterize the function of TON1 in a basal land plant and evaluate the extent of functional conservation of TON1 in the embryophyte lineage, we analyzed *Physcomitrella PpTON1* null and hypomorphic mutants generated by targeted mutagenesis and performed cross-complementation experiments between *Arabidopsis* and *Physcomitrella*. We show here that PpTON1 loss of function generates a leafy shoot phenotype that closely mimics the developmental syndrome previously reported in *Arabidopsis*: chaotic cellular organization associated with absence of PPB formation. We further demonstrate successful and reciprocal cross complementation of *ton1* mutations between these two species, which supports the argument for functional conservation of TON1 function during land plant evolution. In addition, our analyses reveal that PpTON1 is involved in the gravitropic response of moss protonemal cells, and characterization of hypomorphic mutants strongly suggests that the involvement of PpTON1 in cell expansion and in the formation of PPB are two distinct processes. Thus our data point to an ancient and conserved mechanism of division plane positioning and cytokinesis in land plants, and bring new insights into the function of TON1.

MATERIALS AND METHODS

Plant material, growth conditions and treatments

The Gransden wild-type strain of *P. patens* was used in this study (Ashton and Cove, 1977). Wild-type and mutant strains were grown on PP-NO_3 medium for phenotypic analyses or on PP-NH_4 (PP-NO_3 + 2.7 mM NH_4-tartrate) for propagation and protoplast isolation (Ashton et al., 1979). Cultures were grown at 22°C with a 16 hours light/8 hours dark regime and a quantum irradiance of 80 μE m^{-2} s^{-1}. Protoplast isolation and polyethylene glycol-mediated transformation were performed as described (Schaefer and Zryd, 1997). Protonema was grown in darkness on PP-NH_4 supplemented with 5g/l glucose for gravitropic experiments. Cytokinin treatment was performed as described (Brun et al., 2003).

Arabidopsis plants were grown in vitro or in greenhouse according to Nacry et al. (Nacry et al., 1998).

Primers

The primers used in this study are described in Table S1 in the supplementary material.

Gene isolation

Two full-length cDNAs homologous to *AtTON1* were identified in moss ESTs (Nishiyama et al., 2003) (clones pphb35l03 and pphn17d20, gift from M. Hasebe, NIBB, Okasaki) and sequenced (GenBank: GQ853587). The predicted *PpTon1* protein sequence (GenBank: ACX32219) only partially matches the predicted protein of *P. patens* genome release V1.1 (jgi|Phypa1_1|167541|). A *PpTon1* genomic fragment of 3956 bp was obtained by PCR using primers PpTon-F2 and PpTon-RB, cloned in a pGEM-T easy and sequenced (plasmid PpTon1-g): this matches with scaffold 150:586881-590836 (http://genome.jgi-psf.org/physcomitrella/physcomitrella.home.html).

Reverse transcription-polymerase chain reaction

Total RNA was extracted from protonemal tissue using the Qiagen RNeasy Plant Mini Kit (Qiagen). One microgram of total RNA was used to obtain doubled-stranded cDNAs using a modified protocol of the SMART PCR cDNA Synthesis Kit (Clontech) without performing the last amplification step. Five microliters of the 1/10 diluted cDNA were used for PCR reactions performed for 24 cycles.

Targeting vector construction, transformation of *P. patens* protoplasts and molecular analysis of moss *Ppton1* mutants

A 2603 bp *Mun*I fragment was excised from plasmid PpTon1-g and replaced by an *Eco*RI fragment of plasmid pBSKS35S-NPTII-lox that carries a G-418 resistant cassette flanked by *loxP* sites (Trouiller et al., 2006). The resulting plasmid, pPpTON1-KO, was linearized with *Bsm*BI and *Sna*BI and used for protoplast transformation. Selection of transgenic strains for resistance to G-418 (50 mg/l) was performed as described by Trouiller et al. (Trouiller et al., 2006). PCR genotyping was performed using primers specific for the resistance cassette (35SProRev and 35STerFwd) and primers specific for the PpTON1 genomic locus (P11/P12) according to Trouiller et al. (Trouiller et al., 2006). Transient expression of the Cre recombinase was performed in *Ppton1-1* strain to generate the deletion mutant *Ppton1Δ-1* strain (Trouiller et al., 2006). The recombinant locus of the *Ppton1Δ-1* strain was analyzed by PCR using primers P4 and P6.

Generation of *Ppton1ΔAt* complemented strains

The moss complementation vector was designed to integrate the *Arabidopsis TON1* cDNA at the endogenous *PpTON1* locus. *Arabidopsis TON1a* cDNA (AV539220, Kazuka DNA Research Institute, Japan) was amplified using AtTON1-1 and AtTON1-2 primers and cloned in pTOPOblunt. The 5′ (873 bp) and 3′ (900 bp) UTR regions of the *PpTON1* genomic locus were amplified by P7/P8 and P9/P10 primer pairs, respectively, and cloned in pTOPO-TA. *AtTON1a* cDNA was then subcloned in the pTOPO-TA carrying the 5′ UTR region of *PpTON1* using

DEVELOPMENT

*Nde*I and *Nsi*I restriction sites. The 5′ UTR of *PpTON1* in frame with the *AtTON1a* cDNA was excised using *Bst*BI and *Xho*I and cloned upstream of the 35S Hygro^R cassette in vector pBHRF (Thelander et al., 2007). The 3′ UTR region of *PpTON1* genomic locus was then excised from pTOPO using *Bbe*I and *Nsi*I restriction sites and cloned downstream of the 35S Hygro^R cassette of the previous plasmid. The final vector, pPpAtTON1 was linearized by *Mss*I and *Nsi*I digestion for *Ppton1Δ-1* transformation. *Ppton1Δ-1* protoplasts were transformed with pPpAtTON1 and selected for resistance to Hygromycin B (25 mg/l). PCR genotyping was achieved with vector-specific primers (AtTON1-2/35SProRev) and genomic-specific primers (P4/P6).

Generation of *Atton1* complemented lines

PpTON1 open reading frame (ORF) flanked by AttB1 and AttB2 sites was amplified from a *P. patens TON1* cDNA (NIBB clone pphn17d20), cloned into Gateway vector pDONR207 using BP recombination (Invitrogen), and sequenced. To obtain the plasmid carrying *PpTON1* ORF under the control of the 35S promoter, the pDONR207:PpTON1 plasmid was then used in a LR reaction with the destination vector pGWB2 (Nakagawa et al., 2007). Transformation of heterozygote *Atton1* mutants (Azimzadeh et al., 2008) with the pGWB2-PpTON1 plasmid was performed as described (Clough and Bent, 1998). *Arabidopsis* DNA was extracted from leaves as described (Pastuglia et al., 2006).

Western blot analysis

Moss colonies grown on PP-NO_3 medium or in-vitro-grown *Arabidopsis* seedlings were grinded in Laemmli buffer 2× (24 mM Tris, 5% SDS, 18% glycerol, 80 mM DTT, 0.1% Bromophenol Blue). Immunoblots were performed with a 1:1000 dilution of an anti-AtTON1 antibody as described (Azimzadeh et al., 2008).

Microscopy

Young buds were processed for immunofluorescence using B-5-1-2 monoclonal anti-α-tubulin (Sigma-Aldrich) as described previously (Pastuglia et al., 2006).

For division plane imaging in young buds, filaments with developing young buds were incubated in propidium iodide 10 μg/ml for 10 minutes then transferred in water onto a slide. For gametophore leaf cell imaging, gametophores were fixed for 1 hour in a 3:1 ethanol and acetone mixture then transferred for storage in 50% methanol and 10% acetic acid at 4°C. Starch digestion was performed overnight at 37°C using 0.1 mg/ml α-amylase (Sigma A4551) in 20 mM phosphate buffer pH7, NaCl 2 mM, $CaCl_2$ 25 mM. The tissue was incubated in Schiff reagent with propidium iodide (100 mM sodium metabisulfite and 0.15 N HCl; propidium iodide to a final concentration of 100 μg/ml was freshly added) for 1 hour. The following mounting steps were as described (Truernit et al., 2008). Imaging of propidium-iodide-stained tissue was performed with a Zeiss LSM 710 confocal microscope. The excitation wavelength were 488 and 561 nm, and emission was collected at 565 to 720 nm for young buds and 565 to 615 nm for leaf cell imaging. Leaf cell measurement data were obtained with ImageJ software (National Institutes of Health, USA, http://rsb.info.nih.gov/ij/).

Scanning electron micrographs were obtained using the Hirox SH-1500 microscope with fresh moss samples.

RESULTS

Characterization of the *PpTON1* gene

The moss genome contains a single TON1 ortholog (*PpTon1*) [Phypa-draft V1.1; (Rensing et al., 2008)]. We established by cDNA sequencing that it encodes a predicted protein of 415 amino acids, significantly larger than angiosperm TON1s (*Arabidopsis* TON1a: 260 residues). Multiple alignment of TON1 protein sequences showed that all land plant TON1s share sequence identity along their entire length (from 35 to 76% similarity to *Arabidopsis* TON1a) (see Fig. S1A in the supplementary material). The PpTON1 protein was the most distant from *Arabidopsis* TON1 (35% similarity overall), but sequence conservation with AtTON1 was striking at the N-terminus (74% similarity), where sequence motifs have been identified (Azimzadeh et al., 2008). Interestingly, the positions of introns were also similar in the moss and the *Arabidopsis* genomic sequences encoding this part of the protein (see Fig. S1A in the supplementary material). The C-terminal part of PpTON1 was less conserved with other plant TON1, and regions of significant similarity were separated by several insertions of up to 50 residues. This is also reflected by a different exon-intron structure of PpTON1 compared with AtTON1. Interestingly, putative TON1 orthologs from gymnosperms had a predicted molecular weight of 35 kDa (315-316 residues), halfway between moss and angiosperm, which suggests that the C-terminal half of *TON1* genes underwent sequence contraction during land plant evolution (see Fig. S1 in the supplementary material). Despite significant size variation of the protein, these observations are consistent with conservation of TON1 function within the embryophyte lineage.

PpTON1 is expressed during both filamentous and meristematic growth

Semi-quantitative RT-PCR performed on cDNA isolated from protonemal tissue and from tissue carrying differentiated gametophore revealed that *PpTON1* gene was expressed at comparable levels in both tissues (Fig. 1). Consistently, a search in *Physcomitrella* EST databases identified five independent cDNAs isolated from both protonema and gametophore libraries. To further define *PpTON1* expression profile during bud and gametophore differentiation, we followed its expression after bud induction by cytokinin, which induces a massive overproduction of buds in *P. patens* (Brun et al., 2003). *PpTON1* transcript levels did not vary markedly during cytokinin-induced bud formation, whereas *PpBIP2* gene, which is upregulated during this process (Brun et al., 2003), became detectable after 48 hours of treatment, and kept increasing up to 96 hours (Fig. 1). Taken together, these data indicate: (1) that *PpTON1* is expressed at similar levels during protonema and gametophore development; (2) that its expression

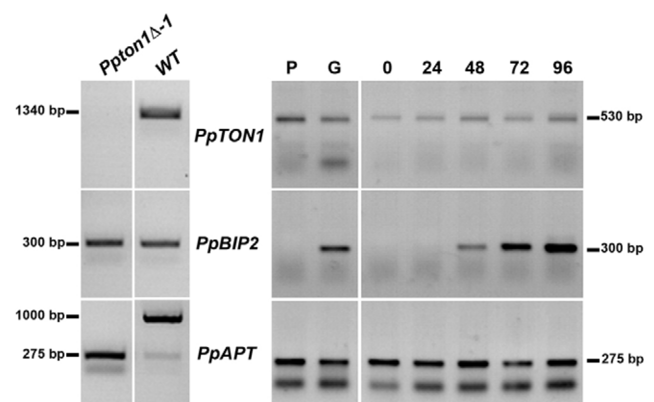

Fig. 1. *PpTON1* is expressed in both protonema and gametophore of *Physcomitrella patens*. Semi-quantitative RT-PCR was performed on total cDNA obtained from young protonemal culture (P), tissues enriched in gametophore (G), and young protonema induced by 100 nM of benzylaminopurine (BAP) for 0 to 96 hours. Controls are PCR amplification from total RNA extracted from *Ppton1Δ-1* tissues (*Ppton1Δ-1*) and wild-type genomic DNA (WT). P1/P19, BIP2-40/BIP2-41 and APT-16/APT-19 primer pairs were used for amplification of *PpTON1*, *PpBIP2* and *PpAPT* sequences, respectively.

is not triggered by BAP, which induces bud formation; and (3) that the absence of PPB during filamentous growth does not correlate with lack of *PpTON1* expression in protonemal cells, suggesting that *PpTON1* may have another function at this developmental stage. These data are also compatible with a housekeeping expression profile of *PpTON1*, as observed in *Arabidopsis*.

Generation of null and hypomorphic *Ppton1* mutants

To investigate the role of *PpTON1* in moss development, wild-type protoplasts were transformed with a replacement cassette designed to delete a fragment encompassing exons 4 to 10 of *PpTON1*, including the highly conserved N-terminal region (see Fig. S2A in the supplementary material). During the selection process, colonies showing severe gametophore alterations could be visually identified within more than 300 G-418 resistant clones. Half these clones formed highly compressed shoots with multilayered leaflets (Fig. 2B,E,H,K,N). Molecular analyses by Southern blot analysis (not shown) and PCR genotyping (see Fig. S2B in the supplementary material) established that this class corresponds to bona fide *Ppton1* null alleles (hereafter named *Ppton1-1*), in which *PpTON1* exons 4 to 10 were replaced by the selectable marker. Remaining clones displayed less severe defects, which essentially affected gametophore length and leaf morphology. Among these, two distinct hypomorphic alleles named *Ppton1-2* and *Ppton1-3* were identified based on phenotypic (Fig. 2C,F,I,L,O) and molecular criteria (see Fig. S2B in the supplementary material and Southern blot analysis not shown). At the molecular level, *Ppton1-2* alleles resulted from 5′ insertion of the vector, and carried a promoterless but complete *PpTON1* coding sequence, whereas *Ppton1-3* were generated upon 3′ insertion, and carried a *PpTON1* gene lacking the two C-terminal exons, corresponding to deletion of residues 355-415 in the PpTON1 protein. In order to obtain a clean deletion mutant, transient expression of the Cre recombinase was performed in *Ppton1-1*, and several G-418 sensitive clones were identified by PCR for the presence of the expected deletion (see Fig. S2C in the supplementary material). None of them could be phenotypically distinguished from *Ppton1-1* alleles, and two independent *Ppton1Δ-1* strains were used for subsequent analyses.

PpTON1 disruption does not affect protonema development, but impairs gravity perception of protonema filaments

Protonema development was not significantly affected in any *Ppton1* mutant alleles. Growth rate and branching pattern of the filamentous network were similar to that observed in wild type. The orientation of division planes was not affected in the two cell types forming the protonema, i.e. perpendicular to the axis of elongation in chloronemata, and oblique in caulonemata (not shown). Moreover, we did not observe any difference in the timing and frequency of caulonema and bud differentiation under standard conditions (not shown). Protoplast isolation and regeneration rates, and phototropic and polarotropic responses of *Ppton1* protonemal filaments were also comparable to those observed in wild type (not shown). These data demonstrate that *PpTON1* is dispensable for filamentous growth of *P. patens* protonema. They are also consistent with previous observations in *Arabidopsis* showing that tip-growing cells such as root hairs and pollen tube are not affected in *Atton1* mutants (Traas et al., 1995).

However, we identified a significant defect in the gravitropic response of *Ppton1* protonemal filaments. Gravistimulated dark-grown wild-type filaments displayed a strong negative gravitropic phenotype (Fig. 3A,E). By contrast, *Ppton1-1* filaments were impaired in gravity perception, and grew more or less in random directions (Fig. 3D,H). In addition, whereas gravitopic response of *Ppton1-2* filaments was identical to wild type (Fig. 3B,F), the *Ppton1-3* allele showed an intermediate gravitropic phenotype between the wild-type and agravitropic *Ppton1-1* filaments (Fig. 3C,G).

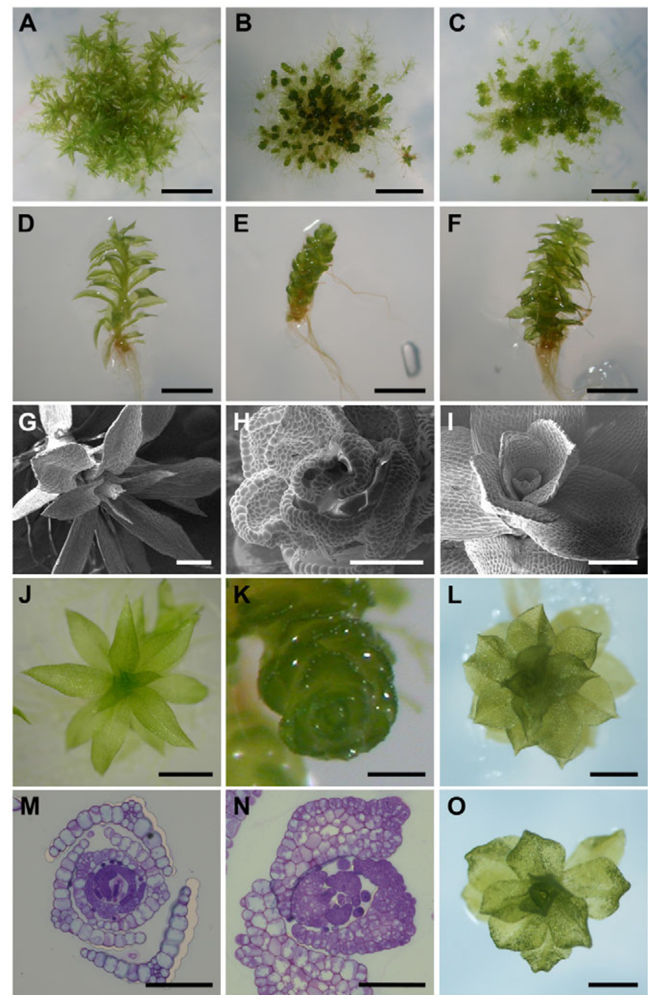

Fig. 2. Comparison of gametophore morphology between wild type and *Ppton1* mutants. (A,D,G,J,M) Wild-type *P. patens*; (B,E,H,K,N) *Ppton1-1* null allele; (C,F,I,L) *Ppton1-2* weak allele; (0) *Ppton1-3* weak allele. (**A-C**) Morphology of one-month-old colonies propagated on $PpNO_3$ medium. (**D-F**) Longitudinal view of isolated gametophores at the same stage. (**G-I**) Scanning electron micrographs of gametophore apex. (**J-L**,**O**) Top view of isolated gametophores. (**M**,**N**) Transverse section of leafy gametophore. Scale bar: 1 cm in A-C; 2.5 mm in D-F; 250 μm in G-I; 1 mm in J,L,O; 500 μm in K; 300 μm in M,N.

Division plane positioning and cell elongation defects in *Ppton1* mutants

In contrast to protonema, development of the gametophore was extremely affected in *Ppton1Δ-1* strains, leading to the formation of longitudinally and radially compressed shoots carrying short and thick leaves (Fig. 2B,E). Yet the helical phyllotaxis of leaves along the stem axis was unaffected (Fig. 2). At the cellular level, microscopic analyses showed that mutant leaves were formed by

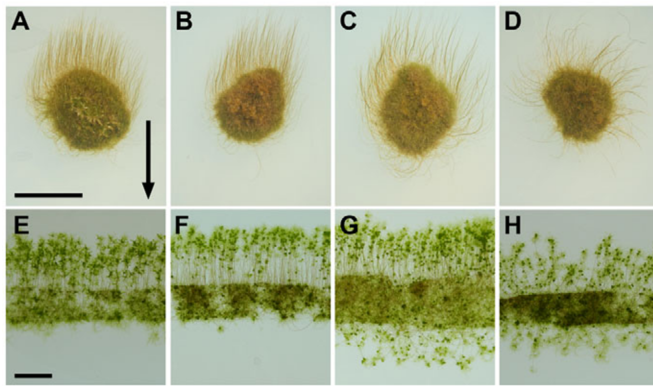

Fig. 3. Comparison of gravitropic responses of wild-type and *Ppton1* mutants protonemal filaments. Wild-type (A,E), *Ppton1-2* (B,F), *Ppton1-3* (C,G) *and Ppton1-1* (D,H) dark-grown protonema filaments. (**A-D**) Protonemal filaments were grown horizontally in light for 15 days and transferred on vertical plate for 15 days in darkness. (**E-H**) Protonemal filaments were grown horizontally in light for 7 days. Bands of protonema were then cut and transferred on vertical plate for 10 days in darkness, and then finally transferred on $PpNO_3$ medium in light for 7 days. This induces gametophore formation, thus enabling visualization of dark-grown filaments. The arrow indicates the direction of gravity in darkness. Scale bar: 1 cm in A-D; 5 mm in E-H.

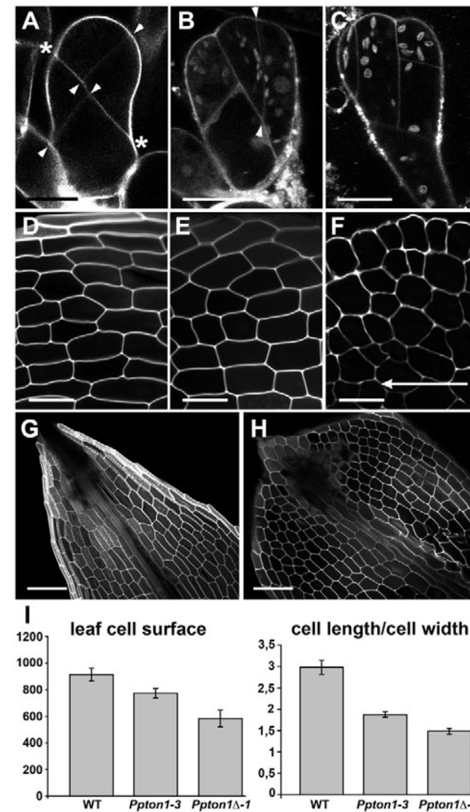

Fig. 4. Division and elongation defects in *Ppton1* mutant gametophore. (**A-C**) Bud initials of wild type (A) and *Ppton1Δ-1* mutant (B,C). In wild type, the first division (asterisks in A) occurs obliquely, producing an apical and a basal cell. The second round of division (arrowheads in A) cleaves these two cells in a perpendicular plane to the first division (Harrison et al., 2009). The *Ppton1Δ-1* bud shown in B displays abnormal division plane of the apical cell (arrowheads). Abnormal division plane positioning is also exemplified in C, where the typical wild-type cell pattern is lost. (**D-F**) Adaxial view of wild-type (D), *Ppton1-3* (E) and *Ppton1Δ-1* (F) leaf cells. Arrow in F indicates the leaf tip (**G**,**H**) Adaxial view of wild-type and *Ppton1-3* leaves. (**I**) Leaf cell surface (in μm^2) and ratio between cell length and cell width in wild type (n=217 cells), *Ppton1-3* (n=439 cells) and *Ppton1Δ-1* (n=141 cells) mutants. Error bars represent the 95% confidence interval of the mean. Scale bar: 20 μm in A-C; 40 μm in D-F; 100 μm in G,H.

multiple layers of roughly isodiametric cells, whereas wild-type leaves were composed of a single layer of elongated cells (Fig. 2M,N). These observations suggest a default in both cell expansion and cytokinetic plane positioning. Defective cell expansion was confirmed by morphometric analyses showing that the length/width ratio of the leaves' cells was reduced approximately twofold in *Ppton1Δ-1* compared with wild type (Fig. 4D,F,I). Defects in mitosis positioning were strongly suggested by the differentiation of multilayered leaves, as wild-type leaves differentiate from a single leaf initial that alternatively divides in two planes to initiate planar growth of a single layered sheet (Harrison et al., 2009). To clearly demonstrate an alteration of division plane positioning, we analyzed the orientation of cell division during the initial stage of bud formation, which is characterized by a precise sequence of cell divisions (Harrison et al., 2009). In the wild type, the first oblique division of shoot initials defines an apical and basal cell that subsequently divides in a plane perpendicular to the first division. By contrast, in *Ppton1Δ-1* buds, abnormal division plane positioning was observed as soon as these early stages (Fig. 4A,C), revealing that *PpTON1* is involved in cell division orientation. Thus, as previously observed in *Arabidopsis ton1* mutants, a combination of misoriented cell division planes together with cell elongation defects account for the developmental syndrome observed in *Ppton1* gametophytes.

Gametophores of both *Ppton1-2* and *Ppton1-3* mutations were less severely affected than in the *Ppton1Δ-1* allele. Compared with the wild type, mature gametophores carried shorter and wider leaves displaying normal phyllotaxis. Significantly, in both lines leaves were formed by a single cell layer, and did not display the chaotic cellular organization displayed in *Ppton1Δ-1* leaves (Fig. 2 and Fig. 4D-H). Leaf cell imaging and measurement demonstrated that *Ppton1-3* leaf cells display both a slight decrease in leaf cell surface and a strong diminution in leaf cell length/width ratio (Fig. 4I). The same tendency was monitored in leaf cells of the *Ppton1-2* allele (data not shown). Cell elongation defects were all the more striking at the leaf tip (compare Fig. 4G with 4H). Hence, the major defect in hypomorphic *Ppton1* alleles appears to be a global defect in cell elongation.

PpTON1 is required for formation of cortical microtubule arrays

Arabidopsis ton1 mutant cells exhibit drastic defects in the formation of cortical microtubule arrays (Azimzadeh et al., 2008; Traas et al., 1995). The striking resemblance between *P. patens* and *Arabidopsis ton1* mutant phenotypes points to similar molecular and cellular defects.

Microtubule organization was assessed in apices of *Ppton1* and wild-type gametophores using α-tubulin immunostaining on cross sections of young buds (Table 1 and Fig. 5). PPB were completely absent in *Ppton1Δ-1*, whereas in the wild type, we observed PPBs in 33% of dividing cells (Fig. 5A,B and Table 1). Remarkably, PPB

Table 1. Comparison of the frequency of microtubule patterns in wild-type and *Ppton1* mutant gametophore cells

Stage	Wild type	*Ppton1-2*	*Ppton1-3*	*Ppton1Δ-1*
Preprophase (%)	33	21	15	0
Perinuclear microtubules (%)	20	27	29	56
Spindle (%)	19	18	17	18
Phragmoplast (%)	28	34	39	25
Total cells (*n*)	69	122	72	71

α-Tubulin immunolocalization on longitudinal sections of *P. patens* gametophores allowed comparison of mitotic microtubule patterns between wild-type and *Ppton1* mutant cells. In addition, PPBs were never seen in more than 300 *Ppton1Δ-1* dividing cells observed.

were clearly seen in *Ppton1-2* (21%) and *Ppton1-3* (15%) weak alleles, although often less frequently, and frayed in appearance compared with wild-type PPB (compare Fig. 5C with 5A,B). Perinuclear accumulation of microtubules was more frequent in *Ppton1Δ-1* (56%) than in wild type (20%), and *Ppton1-2* (27%) and *Ppton1-3* (29%) weak alleles (Table 1 and Fig. 5D,F). This pattern is suspected to correspond to the preprophase stage of mutant cells in *Arabidopsis* (Azimzadeh et al., 2008) and is possibly involved in setting up the prophase spindle before nuclear envelope breakdown. Typical mitotic spindles and phragmoplasts were observed in all *Ppton1* mutant alleles, including *Ppton1Δ-1* loss-of-function allele (Fig. 5G,H). Interphase cortical microtubule array organization was analyzed in young leaflets. Microtubules formed transverse arrays perpendicular to the axis of cell elongation in wild-type interphasic cells, whereas interphasic cortical microtubules were randomly oriented in *Ppton1Δ-1* cells, which concomitantly displayed elongation defects (Fig. 5K,N).

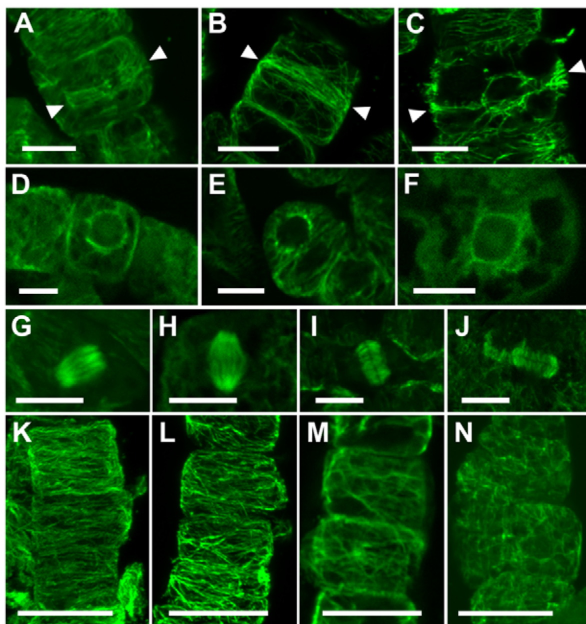

Fig. 5. Microtubule organization in *Ppton1* mutant gametophore. (**A-C**) Preprophase band of microtubules in wild-type (A-B) and *Ppton1-2* (C) gametophore (arrowheads). (**D-F**) Accumulation of perinuclear microtubules in wild-type (D), *Ppton1-2* (E) and *Ppton1Δ-1* mutant (F) cells. (**G**,**H**) Spindle microtubules in wild-type (G) and *Ppton1Δ-1* mutant (H) cells. (**I**,**J**) Phragmoplast of wild-type (I) and *Ppton1Δ-1* mutant (J) cells. (**K-N**) Interphase cortical microtubule organization in wild type (K), *Ppton1-2* (L), *Ppton1-3* (M) and *Ppton1Δ-1* (N) mutants. All images are α-tubulin immunolocalization on longitudinal sections of *P. patens* gametophores. Scale bar: 10 μm in A-F; 20 μm in G-N.

In hypomorphic *Ppton1-2* and *Ppton1-3* mutants, cortical microtubules retained a general transverse orientation, although a higher number of discordant microtubules were observed in the *Ppton1-3* allele (Fig. 5M). Thus, we establish here that the phenotypic similarities observed between *Arabidopsis* and *Physcomitrella ton1* mutants result from similar defects in their ability to properly establish two specific microtubule structures, the PPB and the interphase cortical array. The occurrence of PPBs in hypomorphic alleles establish a dual role for PpTON1 in setting up both the PPB and the interphase array, involved in division plane specification and cell elongation, respectively.

Arabidopsis TON1a complements the *Physcomitrella Ppton1Δ-1* mutant

To assess the ability of *AtTON1* gene to complement the *Ppton1* mutant phenotype, we transformed *Ppton1Δ-1* protoplasts with a cassette containing the *Arabidopsis TON1a* cDNA sequence, flanked by the 5′ and 3′ UTR sequences of the *PpTON1* gene (see Fig. S2A in the supplementary material). Upon targeted replacement, this cassette integrates the endogenous coding sequence by the *AtTON1* coding sequence under the control of the endogenous *PpTON1* 5′ promoter region, at the original *PpTON1* chromosomal locus (see Fig. S2D in the supplementary material). After transformation, five resistant clones carrying the expected targeted replacement were selected for further analysis (called *Ppton1ΔAt*). All five *Ppton1ΔAt* complemented strains displayed a normal developmental pattern compared with the *Ppton1* alleles (Fig. 6). Buds differentiated into elongated leaves with cell files correctly disposed along the leaf axis (Fig. 6F,G). However, we occasionally observed in some leaves local cell pattern defects that ultimately affected the shape of individual leaves (Fig. 6E). We postulate that these local defects result from the presence of the 35S-hygro cassette next to *AtTON1*, which may generate an *AtTON1* antisense transcript by read through (see Fig. S2D in the supplementary material). To ascertain *AtTON1* expression in complemented lines, we used an anti-TON1 antibody raised against *Arabidopsis* TON1 proteins (Azimzadeh et al., 2008). Western blot analysis showed that this antibody is unable to detect the PpTON1 protein (Fig. 6H). All complemented lines expressed the AtTON1 protein (Fig. 6H), demonstrating that the *Arabidopsis TON1* gene functionally complements the *Ppton1* mutation in *P. patens*.

Physcomitrella TON1 complements the *Arabidopsis ton1* mutant

To determine whether the *PpTON1* gene is able to complement the *ton1* mutant in *Arabidopsis*, we transformed heterozygous *Arabidopsis ton1* plants with a construct carrying the *PpTON1* cDNA under the control of the 35S promoter. T1 progeny was selected in vitro for the presence of both the *ton1* mutation and the *PpTON1* construct. Two lines segregating for both were analyzed

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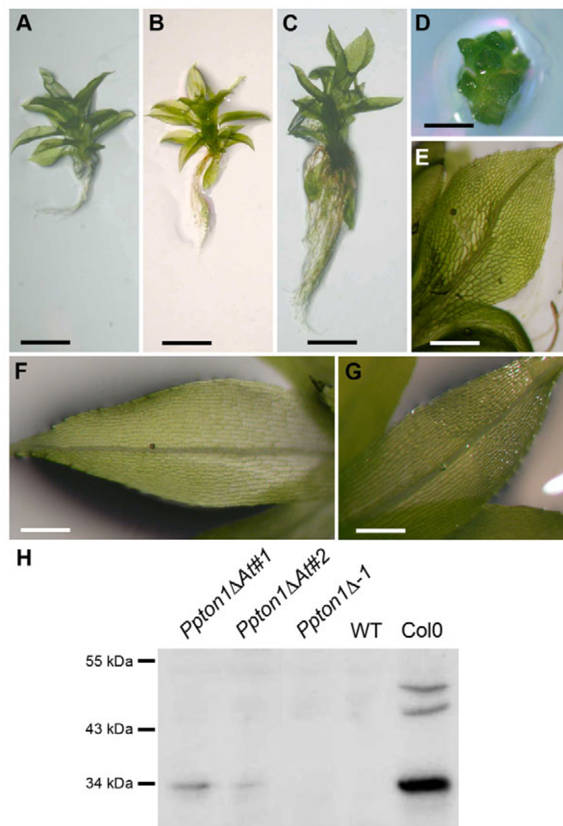

Fig. 6. *AtTON1a* complements the *Ppton1Δ-1* mutant phenotype. (**A-D**) Gametophore morphology of two independent *Ppton1Δ-1* clones expressing *AtTON1* cDNA (B,C) compared with wild type (A) and *Ppton1Δ-1* mutant (D). (**E-G**) Close-up of wild-type (F) and *Ppton1Δ-1* clones expressing *AtTON1* cDNA (E,G). Most leaves display normal shape and cellular organization (G), although slight defects leading to locally disorganized tissue are visible in some lines (E). (**H**) Western blot analysis of protein extracts from *P. patens* colonies and *Arabidopsis* plantlets using an anti-AtTON1 antibody (Azimzadeh et al., 2008). The AtTON1 protein is detected in *Arabidopsis* extract (Col0) and in the two *Ppton1Δ-1* clones expressing the *AtTON1* cDNA. The antibody does not cross react with the moss 45 kDa predicted PpTON1 protein. Scale bar: 2 mm in A-C; 1 mm in D; 20 μm in E-G.

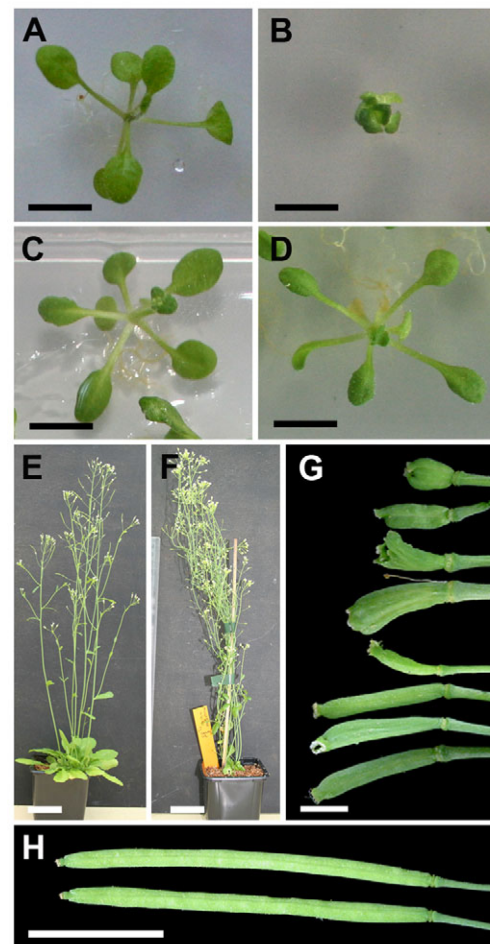

Fig. 7. *PpTON1* cDNA expression complements *Atton1* mutant phenotype. (**A-D**) Wild-type (A) and *Atton1* (B) 12-day-old seedlings grown in vitro compared with two homozygous *Atton1* transgenic lines expressing *PpTON1* cDNA (*Atton1*Pp#1 in C and *Atton1*Pp#2 in D). (**E**,**F**) Seven-week-old wild type (E) compared with *Atton1* plant expressing *PpTON1* cDNA (F). (**G**,**H**) Fully expanded Siliques from wild type (H) and *Atton1* plant expressing *PpTON1* cDNA (G). Four siliques are from the *Atton1*Pp#1 line (G, top) and four from the *Atton1*Pp#2 line (G, bottom). Scale bar: 0.5 cm in A-D; 3 cm in E,F; 2 mm in G; 5 mm in H.

in detail (see Fig. S3 in the supplementary material). In the progeny of these lines, no plant presenting both hygromycin resistance carried by the PpTON1 T-DNA and the *Atton1* phenotype were recovered. Moreover, several homozygous *ton1* mutants displayed a nearly wild-type phenotype. In-vitro-grown plantlets had fully expanded leaves as well as normal roots (Fig. 7A,D). Once transplanted in greenhouse conditions, such complemented plants grew to the same height as wild-type plants (Fig. 7E,F). However, flowers of complemented plants displayed malformed carpels and stigmata, leading to misshaped and empty siliques (Fig. 7G,H). Sepal and petal morphology was also affected (see Fig. S4 in the supplementary material). These slight defects could be a result of expression problems associated with the 35S promoter present in the complementing construct. However, the full complementation of the *ton1* phenotype observed at all other stages of development in *Arabidopsis* demonstrates the functionality of PpTON1 in *Arabidopsis*.

DISCUSSION

PpTON1 is involved in gravitropic response of the moss protonema

To study the function of TON1 in *Physcomitrella* development, we generated loss-of-function and hypomorphic *Ppton1* mutants in *P. patens*. In all alleles, development of the protonoma filaments appeared essentially unaffected. Cell elongation and differentiation were normal, and mitoses were correctly positioned, giving rise to protonemal colonies that could not be distinguished from the wild type. Moreover, we did not detect any abnormalities in microtubule organization of *Ppton1* protonema cells (not shown). Hence, our analysis of *PpTON1* function in these tip-growing cells shows that *PpTON1*, although expressed at this stage, is not required for filamentous growth, which is consistent with the previously reported absence of PPB at this developmental stage in the wild type (Doonan et al., 1987). This corroborates previous observations showing that PPB formation is developmentally

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restricted to the gametophore stage in *Physcomitrella* (Doonan et al., 1987), and further supports the idea that premitotic determination of the cytokinetic plane through PPB formation in embryophytes is coupled to meristematic growth and 3D organization of tissues.

However, we identified an agravitropic phenotype in dark-grown filaments of *Ppton1Δ-1* and *Ppton-1-3* mutants. In gravistimulated filaments of *Physcomitrella* and *Ceratodon purpureus*, cytoplasmic microtubules are rapidly redistributed, and are thought to play an important role in the gravitropic signal-transduction pathway (Allen et al., 2003; Schwuchow et al., 1990). The gravitropic defect of *PpTON1* mutants suggests that PpTON1 is involved in the microtubule-mediated regulation of growth in response to environmental signals such as gravistimulation. The *Ppton1-1* phenotype described here is strikingly similar to that of a moss agravitropic mutant isolated 25 years ago (Jenkins et al., 1986), which displayed similar alterations in gametophore morphology [see figure 6c and 6d in Jenkins et al. (Jenkins et al., 1986)]. This finding uncovers a new function of PpTON1 that would deserve further investigations in other land plants.

PpTON1 controls both the interphase cortical microtubule array and the PPB in the moss gametophore

By contrast, *PpTON1* loss-of-function lines exhibit strong morphological defects at the gametophore stage. Furthermore, the phenotypic syndrome is strikingly similar to the one observed in *Arabidopsis ton1* mutants, in which cells show drastic defects in cell elongation, combined with chaotic cellular organization and orientation of division planes (Azimzadeh et al., 2008; Traas et al., 1995). In *Ppton1Δ-1* gametophores, a general defect in the orientation of cell division was observed from early stages of bud formation, leading to formation of abnormal, multilayered leaves. This provides the first experimental demonstration of the role of the PPB in division plane specification in a basal land plant. In mutant plants, the presence of isodiametric cells revealed a default in cell elongation as well, which correlates with disorganized interphasic cortical microtubule arrays. As in *Arabidopsis ton1* and *fass* mutants, these combined features led to the formation of misshapen and dwarf gametophores. Yet the body plan of the gametophore was not significantly affected, as demonstrated by the conserved phyllotaxy of the leaves and the normal localization of rhizoids. Hence, the phenotype of *Ppton1Δ-1* mutant astonishingly reproduces that observed in *Arabidopsis ton1* mutant. In both organisms, despite drastic defects in division and elongation at the cellular level, organogenesis and morphogenesis is only partly impaired at the whole plant level (Azimzadeh et al., 2008; Traas et al., 1995), exemplifying the importance of a supra-cellular level of control of plant development.

The *Ppton1-2* and *Ppton1-3* hypomorphic alleles provide interesting clues as to the function of PpTON1, as they allow partial uncoupling of the role of PpTON1 in cell elongation from its role in cell division. In contrast to the null allele, cellular division was much less affected in hypomorphic alleles, and leaves were formed of a single layer of cells, as in the wild type. Interestingly, PPBs did form, although less frequently than in wild type. In these alleles, the major cellular defect appears to be perturbation of cell elongation, *Ppton1-2* or *Ppton1-3* leaf cells being more isodiametric than in wild type. Comparison of interphase cortical microtubule organization between the wild-type and *Ppton1* hypomorphic alleles revealed that although globally transverse, more discordant microtubules were seen at the cortex in mutant cells, this being all the more prominent in *Ppton1-3* cells. The *Ppton1Δ-1* mutation led to random organization of interphase cortical arrays. Thus, our analysis of *Ppton1* mutations in moss reveals that PpTON1 function is necessary: (1) for proper organization of cortical microtubules and oriented elongation during intercalary growth of cells; and (2) for PPB formation at the onset of mitosis, and cell division plane positioning during cytokinesis.

TON1 proteins in land plants

Bona fide TON1 sequences are found only in land plants, and are not present in the genomes of green algae currently available. EST sequences corresponding to TON1 were found in all land plants for which a substantial set of data is available. Angiosperm sequences are highly similar and display no significant size variation. However, the few non-angiosperm sequences available show that the size of TON1 proteins varies significantly in moss (416 residues) and gymnosperms (*Pinus*: 315 residues), where the angiosperm TON1 sequence seems to be 'diluted' by intervening stretches of residues. The functional significance, if any, of what seems to be a sequence contraction over evolutionary time, is not clear. However, our cross-complementation experiments do not support the argument in favor of loss of some functions associated with loss of these intervening regions, as the *Arabidopsis* TON1 fully rescues the *PpTON1* loss-of-function phenotype.

The N-terminus of all TON1 proteins shows the highest degree of sequence conservation. It is also a region of high sequence similarity to human centrosomal proteins (Azimzadeh et al., 2008). A LisH-PLL dimerization motif is found in this region, which suggests that all TON1 proteins are able of dimerization. The C-terminus of the protein displays much more variation and insertions. Contrary to the N-terminal region, the C-terminus is predicted to be unstructured (disordered) by most prediction algorithms, and structural constraints are presumably much less on this part of the protein.

Cross-complementation between *Arabidopsis* and *Physcomitrella*

Despite some divergence in protein sequence, especially a significant change of protein size between *Arabidopsis* and *Physcomitrella* (respectively 260 and 415 residues), we observed a remarkable cross-complementation of *TON1* genes between these two distant species. In addition to sequence similarity, and resemblance of mutant phenotype, this shows a clear homology relationship between AtTON1 and PpTON1. Although functional studies in other embryophyte taxons would be needed to definitely establish this point, this strongly suggests conservation of TON1 function among the whole land plant lineage.

The partial complementation observed in *Arabidopsis ton1* flowers is likely to be due to expression of the complementing PpTON1 in floral tissues. This construct is driven by the 35S promoter, which may not provide proper expression of the *PpTON1* transcript in floral tissues. By contrast, the complementation experiment in *Physcomitrella* is based on insertion of the *Arabidopsis* coding sequence at the endogenous locus, presumably providing correct expression pattern to the complementing sequence leading to full complementation. Alternatively, AtTON1 may have a derived function in floral tissue in angiosperms. Further experiments will be needed to assess this point, for example by using the endogenous *AtTON1* promoter to drive the *Physcomitrella* cDNA in *Arabidopsis*.

The *Arabidopsis fass/ton2* mutant displays a phenotype similar to the *ton1* mutation in all respects, with severe developmental (dwarfism, sterility) and cellular (elongation and division) defects (Camilleri et al., 2002; Traas et al., 1995). A *FASS* gene is present in the *Physcomitrella* genome, 83% similar to the *Arabidopsis* FASS at the amino acid level. TON1 partners previously identified in *Arabidopsis* are also present: centrin (Azimzadeh et al., 2008) and CDK (Van Leene et al., 2007). Beyond conservation of TON1 sequence and function, this suggests conservation of the whole pathway in which this protein is involved. Our complementation data finally suggest that this developmental network was already established when bryophytes appeared around 450 million years ago and was recruited from the gametophytic moss generation to the sporophytic generation of present plants during land plant evolution, as previously proposed for the transcription factors required for the differentiation of cells with rooting functions (Menand et al., 2007b).

Overall our results show that genetic disruption of PPB formation in moss leads to severe cellular and developmental defects, and strongly impedes division plane specification. Although a number of experimental evidences exist in angiosperms that link PPB function to division plane determination (Mineyuki, 1999), this is the first demonstration of this functional relationship in a basal land plant lineage, suggesting that the role of the PPB in division plane specification is an ancestral and shared character of land plants. The PPB presumably represents a key innovation of land plants relevant to multicellular meristematic development, cell division within semi-rigid cell walls, and terrestrial colonization (Graham, 1996). In this respect, the availability of gene targeting based on homologous recombination makes *Physcomitrella* a unique model system among plants (Schaefer and Zryd, 1997). The recent release of the complete genome sequence (Rensing et al., 2008) allows the initiation of comparative studies between basal embryophytes and angiosperms. This offers new opportunities to gain information about key changes in developmental and cellular processes during land plant evolution.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.043810/-/DC1

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