Unique Tissue-Specific Cell Cycle in *Physcomitrella*

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Abstract: The moss *Physcomitrella patens* (Hedw.) B.S.G. is a novel tool in plant functional genomics as it has an inimitable high gene targeting efficiency facilitating the establishment of gene/function relationships. Here we report, based on flow cytometric (FCM) data, that the basic nuclear DNA content per cell of *Physcomitrella* is 0.53 pg, equating to a genome size of 1 C = 511 Mbp. Furthermore, we describe a unique tissue-specific cell cycle change in this plant. Young plants consisting of only one cell type (chloronema) displayed one single peak of fluorescence in FCM analyses. As soon as the second cell type (caulonema) developed from chloronema, a second peak of fluorescence at half the intensity of the previous one became detectable, indicating that caulonema cells were predominantly at the G1/S transition, whereas chloronema cells were mainly accumulating at the G2/M transition. This conclusion was validated by further evidence: i) The addition of ammonium tartrate arrested *Physcomitrella* in the chloronema state and in G2/M. ii) Two different developmental mutants, known to be arrested in the chloronema/caulonema transition, remained in G2/M, regardless of age and treatment. iii) The addition of auxin or cytokinin induced the formation of caulonema, as well as decreasing the amount of cells in G2/M phase. Additionally, plant growth regulators promoted endopolyploidisation. Thus, cell cycle and cell differentiation are closely linked in *Physcomitrella* and effects of plant hormones and environmental factors on both processes can be analysed in a straight forward way. We speculate that this unique tissue-specific cell cycle arrest may be the reason for the uniquely high rate of homologous recombination found in the *Physcomitrella* nuclear DNA.

Key words: Cell cycle, differentiation, endopolyploidisation, genome size, homologous recombination, moss, *Physcomitrella patens*.

Abbreviations:

2iP: 6-(γ,γ-Dimethylallylamino)purine
DAP I: 4′-6-diamidino-2-phenylindole
FCM: flow cytometry
HU: Hydroxyurea
IAA: Indole acetic acid
NAA: Naphtalene acetic acid
Mbp: Mega base pairs
PI: Propidium iodide

Introduction

Growth and development of any living organism are based on two fundamental cell activities: cell growth and cell division. Many approaches have been made to elucidate how cells divide, and molecular analysis of the cell cycle started with the isolation of cell cycle mutants in yeast over 30 years ago (Hartwell et al., 1970). In principal, cell division in plants is similar to that of other eukaryotes and homologues of various key cell cycle regulators, like cyclins and cyclin-dependent kinases (cdks), have been isolated (den Boer and Murray, 2000; Lorenz et al., 2002). However, the way cell division is integrated into development is plant-specific (Fowler et al., 1998). Here, continuing organogenesis, as well as flexible growth responses to environmental changes, require precise spatial and temporal regulation of cell division activity in meristems. Also, plant tissues undergo alternate periods of growth (and cell division) and dormancy. Regulatory pathways that communicate environmental constraints, such as nutrient availability, and signals, such as growth factors or hormones control in which cell division occurs. However, such regulatory pathways are still poorly understood (den Boer and Murray, 2000).

Haploid moss plants are widely used in the study of plant development as they are easy to mutagenise and display a more simple developmental pattern. Moss spores germinate by forming the tip-growing chloronema, which contains cells with about 50 chloroplasts and cross walls perpendicular to the growth axis (Fig. **1 a**). Depending upon age, nutrients, light and hormones (for reviews: Bopp, 1990; Cove, 1992; Reski, 1998), tip cells differentiate into caulonema cells, which are longer and more slender, contain fewer chloroplasts and have oblique cross walls (Fig. **1 b**).

The moss *Physcomitrella patens* is unique amongst all other land plants so far studied with regard to the highly efficient homologous recombination in its nuclear DNA (Schaefer and Zryd, 1997; Reski, 1998; Strepp et al., 1998; Nishiyama et al., 2000; Schaefer, 2001). Therefore, *Physcomitrella patens* can be used as a novel system to fascilitate gene/function analyses by targeted gene knockout and subsequent analysis of the k.o.

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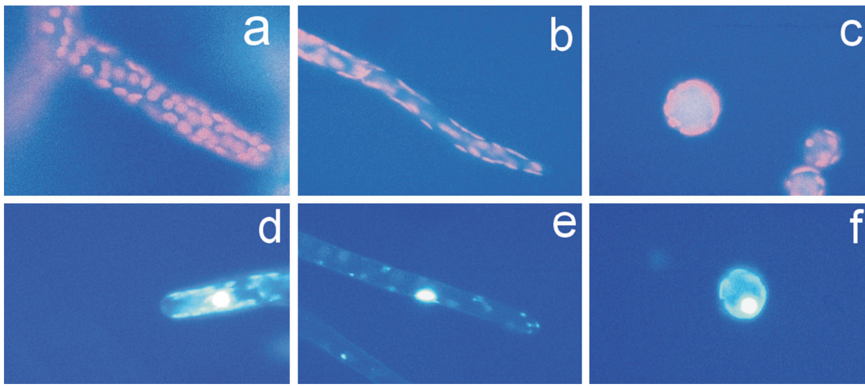

Fig. 1 Tissues of *Physcomitrella*. UV microscopy pictures of chloronema (**a**), caulonema (**b**) and protoplasts (**c**) and of DAPI stained chloronema (**d**), caulonema (**e**) and protoplasts (**f**).

mutants (e.g., Strepp et al., 1998; Girke et al., 1998). This approach is especially straightforward as the dominating phase in mosses is the haploid gametophyte (review Reski, 1998), making loss-of-function mutations readily screenable. Analysis of the moss transcriptome identified on one hand, a large set of novel genes, and on the other hand, a high degree of conservation between *Physcomitrella* and seed plants (Rensing et al., 2002). Despite the huge effort put into *Physcomitrella* functional genomics (Holtorf et al., 2002), some basic genomic data are still missing. One of these is the basic genome size. In a previous report (Reski et al., 1994) we attempted to calculate the basic DNA content via FCM analyses. However, although fast-growing cultures were used, we were only able to identify one peak of fluorescence, making it impossible to allocate this to either G1/G0 or G2/M.

Here, we report the genome size of *Physcomitrella*, describe the change of cell cycle parameters during a 22-day culture period and specify the influence of plant growth regulators (auxin and cytokinin) and a nutrient (ammonium tartrate) on cell cycle and cell differentiation. Furthermore, we speculate that this unique tissue-specific cell cycle arrest may be correlated with homologous recombination orders of magnitudes higher than in any other plant analysed so far.

Materials and Methods

Plant material and growth conditions

All moss strains were grown axenically in 500 ml Erlenmeyer flasks containing 200 ml liquid medium. The Hamburg wild-type strain was grown in modified Knop medium (250 mg l^{-1} KH_2PO_4, 250 mg l^{-1} $MgSO_4 \cdot 7H_2O$, 250 mg l^{-1} KCl, 1000 mg l^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 12.5 mg l^{-1} $FeSO_4 \cdot 7H_2O$, pH 5.8). The flasks were covered with silicone sponges (Bellco, Vineland, N.J., USA) and were cultured on a shaker set at 120 rpm in a growth chamber under controlled conditions (25 ± 1 °C; light was provided from above by two fluorescent tubes, Philips TLD 25; light flux of 30 µmol m^{-2} s^{-1} outside the flasks, light–dark regime of 16 : 8 h).

Inoculations were started from stock cultures which had been sub-cultured at 7-day intervals. After mincing the moss, the culture was transferred (inoculation density 300 mg l^{-1} dry weight) to Knop medium, Knop medium with 5 mM ammonium tartrate, Knop medium with 5 µM NAA, or Knop medium with 5 µM 2iP (pH = 5.8). Two flasks per treatment were prepared, samples were taken twice a week and measured by flow cytometry and photographed. Additionally, the pH of the medium was determined once a week. The experiment lasted three weeks and was repeated once. At the beginning and the end, the dry weight of the plant material was determined.

For further experiments, Knop medium supplemented with a final concentration of 5 µM IAA, 5 µM αNAA, 5 µM fusicoccin, 300 µM tryptophane, 5 mM ammonium tartrate, 5 mM ammonium sulphate, or 5 mM ammonium nitrate, were used, and inoculations were started from stock cultures which had been subcultured at 10-day intervals. Cambridge wild type, and the two mutants *cal*112 and *cal*113 derived from it, were grown in modified Knop medium supplemented with 1.8 µM p-aminobenzoic acid, 1.5 µM thiamine-HCl, and trace elements, according to Ashton et al. (1977).

For treatment with hydroxyurea, protoplasts were isolated from filtered chloronema cultures by agitated incubation for 1 h in the dark in 2% Driselase (Sigma Aldrich, Deisenhofen, Germany) in 0.5 M mannitol. Suspensions were passed through sieves of 100 µm and 45 µm pore size, centrifuged for 10 min at 45 · g and washed with 0.5 M mannitol. Protoplasts were cultured at a density of $1 \cdot 10^5$ protoplasts per ml in modified Knop medium supplemented with 50 g l^{-1} glucose and 30 g l^{-1} mannitol adjusted to an osmolarity of 540 mOs, pH 5.6. After three days, protoplasts were transferred to medium containing 10 mM HU. After 24 h, protoplasts were isolated and measured by flow cytometry.

Seeds of *Phaseolus vulgaris* L. (cv. Saxa) and *Pisum sativum* (cv. Kleine Rheinländerin) were germinated under axenic conditions on Murashige and Skoog medium (Murashige and Skoog, 1962). Young leaves of 21 – 28-day old seedlings were analysed.

Flow cytometric analysis

Flow cytometric (FCM) analysis was carried out essentially according to Ulrich and Ulrich (1991). Suspensions of intact nuclei were prepared from about 30 mg of fresh plant material by chopping the material with a razor blade in a glass petri dish with 2.5 ml of a commercial DAPI-containing buffer (#0-X-5-4001, Partec, Münster, Germany). The solution was filtered through a sieve of 30 µm pore size and kept for 15 – 60 min in the dark.

Plant biol. "366"
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Protoplasts were isolated as desribed above. After passing the protoplasts through sieves of 100 µm and 45 µm pore size and centrifugation for 10 min at 45 · g, the pellet was resuspended in DAPI solution.

For determination of the DNA content, the pellet was resuspended and subjected to centrifugation in a sucrose gradient, as described previously (Rother et al., 1994). Floated protoplasts were stained with 50 ppm PI in buffer, consisting of 4.26 g l^{-1} $MgCl_2$, 8.84 g l^{-1} sodium citrate, 4.2 g l^{-1} 3-(N-morpholino)propane sulfonic acid, 1 ml l^{-1} Triton X-100, 1 mg l^{-1} boiled ribonuclease A, pH 7.0 (Johnston et al., 1999). Young leaves of *P. sativum* and *P. vulgaris* were chopped as described above and stained with PI solution. Nuclei were kept for 60 min in the dark at 4 °C, afterwards, experimental samples were mixed and measured. Three independent measurements were made with *P. sativum* as a reference standard and six replications using *P. vulgaris.* Only histograms with a coefficient of variation < 5 % were used to calculate the DNA content.

Fluorescence of stained nuclei was determined with a Partec cell analyser using either a 100 W high-pressure mercury lamp (for DAPI stained nuclei) or a 25 W argon ion laser (for PI stained nuclei) at flow rates between 40 and 100 s^{-1}. At least 5000 signals were calculated per measurement and analysed with the DAPC software (Partec). In the developmental mutants, figures were prepared with the help of the Excel programme (Microsoft).

Results and Discussion

Genome size determination

Although there is a wealth of molecular data for *Physcomitrella,* e.g., > 95 % of the expressed protein coding genes have been identified as expressed sequence tags (Rensing et al., 2002), the genome size of this plant is still uncertain. To address this question, we used FCM analyses of PI stained nuclei, and included *Pisum sativum* and *Phaseolus vulgaris* as reference plants. *Pisum sativum* is a widely used standard in such experiments due to ready availability of plant material, ease of preparation and stability within and among runs (Johnston et al., 1999). The DNA content of *P. sativum* cv. Kleine Rheinländerin was taken as 4.42 pg (Greilhuber and Ebert, 1994) and only slight variations exist between different *P. sativum* genotypes. Furthermore, Price and Johnston (1996) recommended that the standard should have DNA values close to the target species. Therefore, *Phaseolus vulgaris* was chosen as a second standard. DNA contents between 1.2 pg (Bennett and Leitch, 1995) and 1.44 pg (Marie and Brown, 1993) per 2C nucleus have been reported and do not differ significantly between different genotypes (Nagl and Treviranus, 1995).

Assuming *Phaseolus* to have 0.7 pg/1C (Marie and Brown, 1993) and *Pisum* to have 4.42 pg/1C (Greilhuber and Ebert, 1994), *Physcomitrella* nuclei were calculated to have a DNA content of 0.53 ± 0.01 pg/1C (data not shown). Taking into account that mosses are haploid, and using the estimation that 1 pg DNA equals about 965 million base pairs (Arumuganathan and Earle, 1991), the genome size of *Physcomitrella patens* (Hedw.) B. S. G. was determined to be 511 Mbp.

In a previous report (Reski et al., 1994) the genome size of *Physcomitrella* was calculated to be about 600 Mbp, based on a comparison with *Arabidopsis* and *Brassica napus* in FCM analyses of DAPI stained nuclei. We here revise our estimation to 511 Mbp, based upon three improvements: i) Using propidium iodide instead of DAPI. Intercalators, such as PI, do not show base preference, in contrast to DAPI which preferentially stains AT-rich DNA (Dolezel et al., 1992). ii) Using different plant species as reference plants. Whereas we had previously used the widely cited estimation of 0.14 pg/2C for *Arabidopsis thaliana* (Leutwiler et al., 1984), a revised DNA content of 0.3 pg/2C has now been calculated (Arumuganathan and Earle, 1991; Bennett and Leitch, 1995). iii) Previously, moss nuclei were released from isolated protoplasts generated from chloronemal tissue and thereby only one peak of fluorescence was obtained, which was erroneously taken as a G1/S peak.

In past decades there have been conflicting reports on the chromosome number in *Physcomitrella,* suggesting the existence of at least two chromosomal races (see Reski et al., 1994, for a discussion). To evaluate the possibility that the two *Physcomitrella* laboratory strains used today in molecular biology may differ in genome size, we obtained the Cambridge strain from David Cove (Leeds, England) and compared it to our Hamburg strain in terms of genome size. Both strains were identical in nuclear DNA content (data not shown).

Accumulation of cells at the G2/M transition

Juvenile *Physcomitrella* protonema, containing only chloronema cells (Fig. **2a**), yielded one major peak of fluorescence. After three more days in culture a second, minor peak became visible at half the intensity of the major one (Fig. **3a**), indicating that these were predominantly in G1, while the majority of cells accumulated at the G2/M transition.

To validate this conclusion, moss protoplasts were treated, three days after isolation, with 10 mM hydroxyurea (HU) for 24 h, a treatment well-known to prevent DNA replication and block cells in G1 (e.g., Planchais et al., 2000). Similarly, in our hands, this treatment drastically enhanced numbers of nuclei in the G1 and S phase, with a maximum of 30% cells in G1/S (Figs. **3e** [control] and **3f**). This was taken as evidence that the major peak at channel 200 represented cells at the G2/M transition, whereas the smaller peak at channel 100 represented cells at the G1/S boundary. After removal of the HU, cells reenter the cell cycle. In both controls as well as HU treated cells, a third peak occurred around channel 400, most probably representing diploid nuclei (2C) due to fusion processes during the protoplast isolation procedure. This would be in agreement with FCM analysis of regenerated plants after protoplast isolation revealing that 19.9 ± 6.6 % of the plants were diploid (n = 735 plants) after the isolation procedure.

From this, we conclude that chloronema cells are accumulated at the G2/M boundary, whereas caulonema cells are mainly at the G1/S transition. Interestingly, this finding might be reflected by the morphology of the different types of nuclei: After DAPI staining and inspection in a UV microscope, nuclei in caulonema tip cells appeared to be less intensely stained and spindle-shaped (Fig. **1e**), whereas nuclei in chloronema cells and in protoplasts were more intensely stained and round (Figs. **1d,f**).

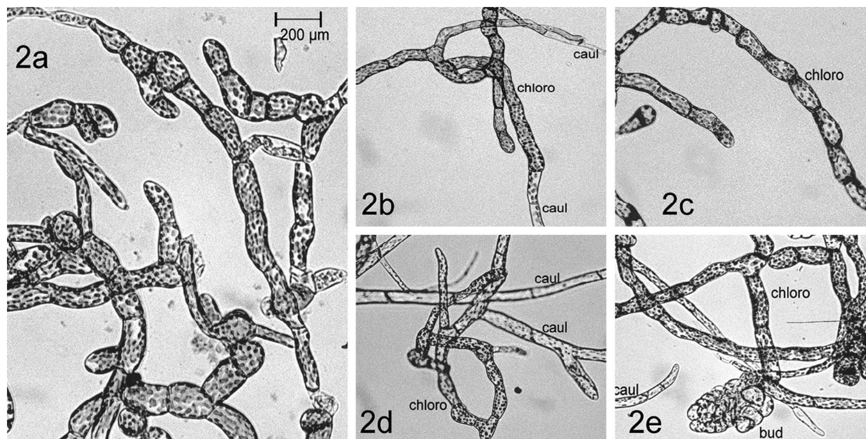


Fig. 2 Differentiation. Photograph of the inoculation material of *Physcomitrella patens* (t = 0 days; **a**), and after culture in Knop medium (t = 15 days; **b**), Knop medium supplemented with 5mM ammonium tartrate (t = 15 days; **c**), Knop medium supplemented with 5 µg l^{-1} auxin (t = 15 days; **d**) and Knop medium supplemented with 5 µg l^{-1} 2iP (t = 7 days; **e**). Amplification × 50. Chloronema (= chloro), caulonema (= caul) and bud formation (= bud).

Ammonium tartrate affects development and cell cycle

A possible correlation between moss development and cell cycle arrest was analysed during a 22-day culture period. Basically, *Physcomitrella* is grown in two different media: either in modified Knop medium with nitrate as the only N source (Reski and Abel, 1985), or in a more complex medium containing ammonium tartrate as an additional N source (Ashton and Cove, 1977). Both media were compared in our recent experiments.

In modified Knop medium *Physcomitrella* developed chloronema cells, characterized by cross walls perpendicular to the growth axis, as well as caulonema cells with oblique cross walls (Fig. **2 b**). The number of caulonema cells increased (with increased age of the culture) and, according to FCM analyses, the number of cells in G1 likewise increased during the course of development up to a maximum of 7.6 ± 1.7 % at the end of the experiment (Figs. **3 a, 4**). When such tissue was treated with cell wall digesting enzymes, however, resulting protoplasts contained only a few G1 nuclei (0.2 %; data not shown).

When *Physcomitrella* was grown in Knop medium supplemented with ammonium tartrate, however, no caulonema cells were formed during the 22-d culture period (Fig. **2 c**). Similarly, no cells in G1 were detectable in FCM analyses under these growth conditions (Figs. **3 b, 4**). Jenkins and Cove (1983) described how ammonium tartrate, as an additional nitrogen source, inhibited caulonema formation in *Physcomitrella.* This was confirmed by Hohe et al. (2002), as well as by our recent experiments. Additionally, we have now found that ammonium tartrate inhibits the occurrence of *Physcomitrella* nuclei in G1, further supporting the conclusion that chloronema cells are arrested at the G2/M boundary and that caulonema nuclei are predominantly in 1C G1.

In an attempt to better pinpoint this effect, we ascertained that ammonium sulphate and ammonium nitrate had similar effects to ammonium tartrate on cell differentiation, as well as on cell cycle arrest. Ammonium uptake resulted in decreased pH values in unbuffered Knop medium (pH 3.84 in Knop medium supplemented with ammonium tartrate compared to 6.13 – 6.38 in Knop medium at the end of the experiment, t = 22 days). The effects observed by supplementation with ammonium could not be mimicked by alterations in the pH of the media (data not shown), indicating that the ammonium ion itself might influence cell differentiation in the *Physcomitrella* protonema.

So far, there are few reports on specific effects on plant development by ammonium. A negative effect on cell division and increase of biomass was observed in asparagus mesophyll cells (Matsubayashi and Sakagami, 1998), and this was not mediated by changes in the pH of the medium due to ammonium uptake. In *Physcomitrella,* however, dry weight did not differ significantly in Knop medium with or without ammonium tartrate (570 mg l^{-1} in Knop medium compared to 620 mg l^{-1} in Knop medium supplemented with ammonium tartrate). Therefore, it seems unlikely that ammonium affects proliferation in this system. Cao et al. (1993) proposed an ammonium-induced inhibition of root development in *Arabidopsis,* probably due to interactions with hormone synthesis, transport, or signalling pathways. Given the effects on cell differentiation observed in our recent and in preceding reports (Hohe et al., 2002), such a mechanism could also be effective in the moss.

Auxin and cytokinin

Both phytohormones, auxin as well as cytokinin, induce specific cell differentiation in mosses (Reski, 1998). Auxin is known to induce caulonema cells (Johri and Desai, 1973; Ashton et al., 1979) and cytokinins are known to induce buds, three-faced apical cells which give rise to the leafy shoots (Reski and Abel, 1985).

In our recent experiments, the number of caulonema cells, as well as the proportion of nuclei in G1 (Figs. **3 c, 4**), were greatly enhanced by addition of auxin (Fig. **2 d**), again confirming the linkage between cell differentiation and cell cycle in *Physcomitrella.* Although, at the end of the experiment, protoplast isolation failed for auxin-treated cells, a time course experiment using chopped samples showed the highest number of cells in G1 at day 9, and this percentage remained constant until the end of the experiment at day 22 (data not shown).

Addition of cytokinin led to enhanced production of buds within 7 days in our recent experiments. These buds were formed on chloronema, as well as on caulonema cells (Fig. **2 e**).

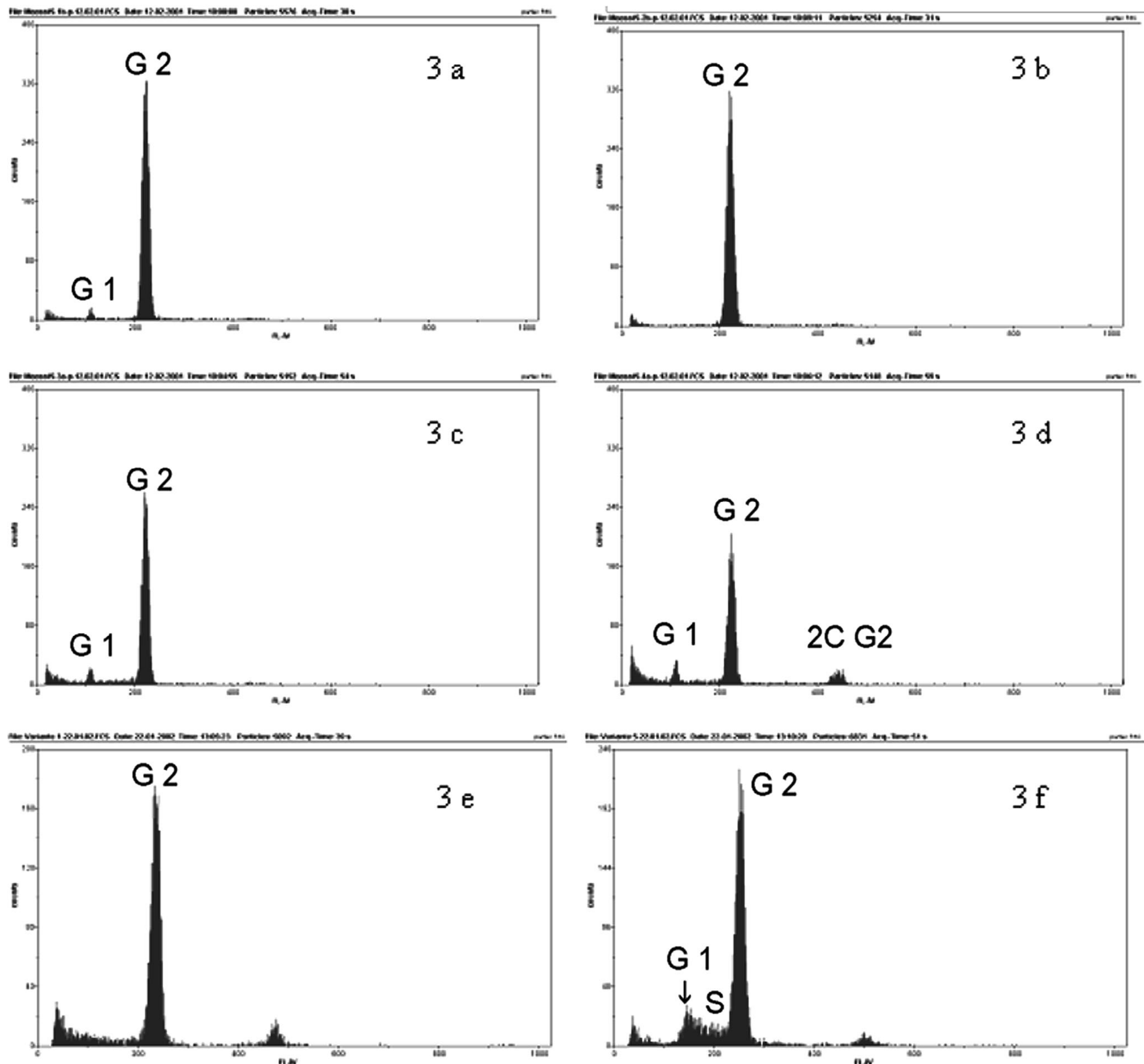


Fig. 3 Flow cytometric analysis. Histograms of isolated protoplasts of *Physcomitrella patens* after culture in Knop medium (t = 12 days; **a**), Knop medium with 5 mM ammonium tartrate (t = 12 days; **b**), Knop medium with 5 µM NAA (t = 12 days; **c**), Knop medium with 5 µM 2iP (t = 12 days; **d**), after culture in Knop regeneration medium (control, t = 96 h; **e**) and after treatment with 10 mM hydroxyurea for 24 h (**f**). Histograms summarizing representative flow cytometric analyses of DAPI stained isolated nuclei. The abscissa represents the channel numbers corresponding to the relative fluorescence intensities of analysed particles (linear mode), while the ordinate indicates the number of events counted.

In time course experiments, the percentage of cells in 1C G1 increased from day 9 onwards, to a level already found in auxin-treated cultures (Figs. **3 d, 4**), indicating that not only caulonema cells but also bud cells were predominantly in the G1 phase of the cell cycle. To substantiate this interpretation, we performed several control experiments. The auxin effects on cell differentiation and on cell cycle could be mimicked by tryptophan, an auxin precursor, but not by fusicoccin, a fungal metabolite that is known to have physiological effects similar to those induced by phytohormones (Aducci et al., 1995).

Moreover, reduction of the inoculation density to 30 mg l^{-1} (compared to standardized inoculum of 300 mg l^{-1}) resulted in an increase in the percentage of cells in G1, up to a maximum of 18 % at day 22, as well as increased caulonema formation. Thus confirming the inverse correlation between cell density and caulonema formation in mosses already described by Johri and Desai (1973).

In an additional control experiment, the moss tissue was transferred daily to new liquid medium and subsequently analysed. We have described earlier (Reutter et al., 1998) that, in

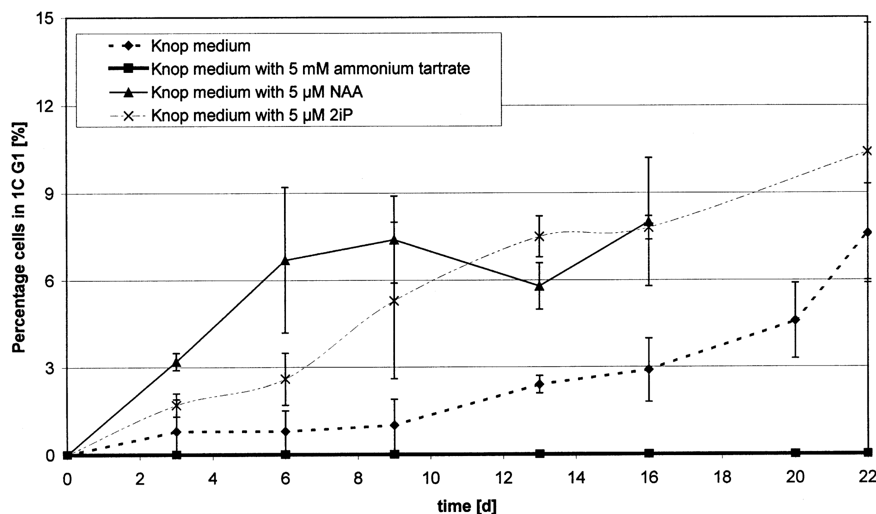
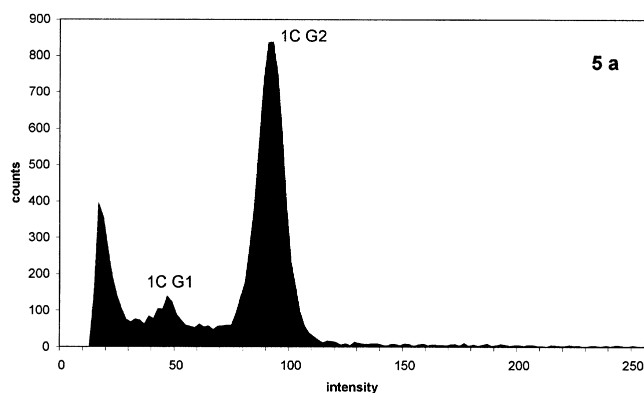


Fig. 4 Cells in G1. Percentage of cells in 1C G1, dependent on the culture medium of *Physcomitrella patens,* based on evaluation of flow cytometric measurements: addition of ammonium tartrate resulted in total inhibition of G1 cells, while addition of either auxin or cytokinin enhanced the occurrence of these cells (n = 4; two flasks, two repetitions).



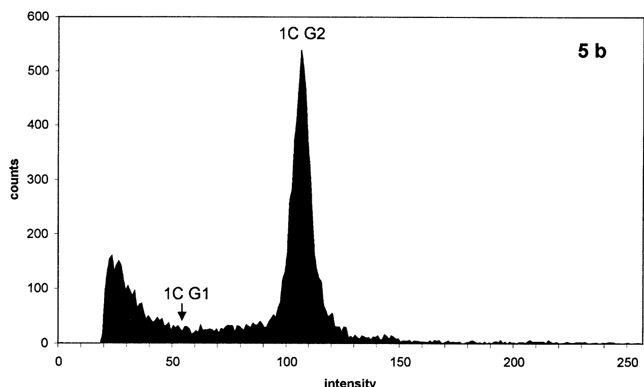


Fig. 5 Developmental mutant. Flow cytometric analysis of 10-day-old cultures of *Physcomitrella* wild type and the dominant auxin-insensitive developmental mutant *cal*113 (Ashton et al., 1979): no accumulation of cells in G1 in the mutant.

liquid cultures, more than 80% of auxin and cytokinin were secreted into the medium. Consequently, daily medium replacement should result in a depletion of these two hormones. In our recent experiments, we found that such cultures remained in the chloronema phase and did not develop caulonema or buds. Concomitantly, in none of these cultures could cells in G1 be detected (data not shown), additionally confirming the close correlation between cell differentiation and cell cycle arrest in *Physcomitrella.*

Developmental mutants

Further validation came from the analysis of two developmental mutants, *cal*112 and *cal*113. These are non-allelic, dominant auxin-resistant mutants which are arrested in chloronema differentiation and consequently do not form caulonema or buds (Ashton et al., 1979). This was confirmed under our laboratory conditions and, moreover, we did not observe more than marginal proportions of 1C nuclei even after protracted cultivation, whereas the G2 peak was always clearly visible in FCM analyses (Fig. **5**).

Endopolyploidisation

Occasionally, a third peak of fluorescence became visible, depending on culture techniques and sample preparation (Fig. **6**). After protracted cultivation, 2 – 4% of the cells were in 2C G2 (or 4C G1) when grown in basic Knop medium and in cytokinin supplemented medium, respectively. Addition of auxin not only enhanced caulonema formation but also led to a steep increase in polyploid cells. No such cells could be detected in ammonium-treated cultures, arrested in chloronema, and in protoplast preparations isolated from any *Physcomitrella* sample, indicating that i) endopolyploidisation is a feature of caulonema cells, and ii) few protoplasts can be obtained from caulonema tissue.

This supports earlier findings with the moss *Funaria hygrometrica,* where older caulonema cells become brownish, lose their regeneration capability and show enhanced nuclear DNA contents, when compared to chloronemal or juvenile caulonemal tissue (Knoop, 1978). In line with this, tissue-specific endoreduplication of nuclear DNA occurs in higher plants as well and is influenced by plant age, hormones and environmental conditions (Joubès and Chevalier, 2000).

Furthermore, a correlation between cell differentiation and endoreduplication has been observed during pod wall development in *Lupinus* (Lagunes-Espinoza et al., 2000) and nodule

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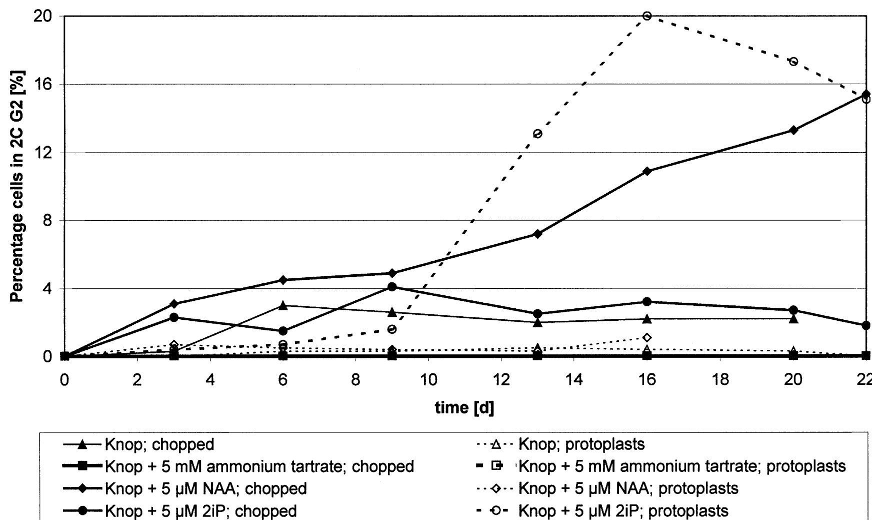

Fig. 6 Polyploid cells. Effect of the culture medium supplements and the preparation method of samples for flow cytometric analysis on the percentage of endopolyploid cells in cultures of *Physcomitrella patens:* addition of ammonium tartrate resulted in a total inhibition of endopolyploidy, while addition of either auxin or cytokinin enhanced the occurrence of these cells (n = 2; two flasks, one repetition).

organogenesis in *Medicago* (Foucher and Kondorosi, 2000). The importance of a cell cycle regulator (ccs52) for cell division, cell differentiation, endoreduplication and subsequent cell size enlargement was demonstrated for the first time in *M. truncatula* (Cebolla et al., 1999).

Cell cycle and cell differentiation

In seed plants, it is known that spatially and temporarily restricted cell cycling correlates well with specific morphogenetic events, e.g., in sunflower leaves (Graniert and Tardieu, 1998) and in developing leaves and pericycle cells of *Arabidopsis thaliana* (Donnelly et al., 1999; Beeckmann et al., 2001). Such root cells are one of the few examples where plant cells mainly rest in the G2, instead of the G1 phase of the cell cycle (Stals and Inzé, 2001).

Here, we have shown with several lines of evidence, for the first time in mosses, a unique tissue-specific cell cycle accumulation. The major juvenile gametophytic tissue, the chloronema, is strictly arrested in the G2/M transition, generating one single peak of fluorescence in flow cytometric analyses. The other two tissues, caulonema and buds, in contrast, are mainly in G1. Additionally, we observed endoreduplication of the nuclear DNA in aging caulonema cells.

This specific and strict correlation was not due to synchronization of the cultures, as we did not supply synchronizing drugs and did not observe synchronous cell division in extensive 24-h experiments. Instead, we postulate that dividing chloronema cells progress rapidly through mitosis and S phase. This might explain the experimental problems in obtaining metaphase chromosomes from *Physcomitrella* (see Reski et al., 1994, for discussion).

To the best of our knowledge, this is the first description of a photoautotrophic, fast-growing plant cell culture predominantly arrested at the G2/M transition. As this can be influenced by environmental factors, like ammonium, and endogenous factors, like auxin and cytokinin, *Physcomitrella* appears as a powerful tool in the analysis of the plant cell cycle. Such analyses will additionally benefit from the unique possibility to study gene function by targeted gene disruption. Taking this route, we have functionally recently identified for the first time in plants a conserved role of D-type cyclins in sugar sensing (Lorenz et al., submitted).

Does a unique cell cycle result in uniquely high homologous recombination?

Physcomitrella is unique among land plants analysed so far due to its highly efficient homologous recombination, facilitating reverse genetic approaches (e.g., Schaefer, 2001). Despite occasional contrary claims (e.g., Terada et al., 2002), such a strategy is not yet feasible in seed plants. So far, protoplasts are the prime target for genetic transformation in *Physcomitrella* (see Hohe and Reski, 2002, and discussion therein) and, with no other method, targeted gene disruption has been achieved in this species (see Egener et al., 2002, and discussion therein). Here, we have shown, at least under various laboratory conditions, that these protoplasts are exclusively arrested at the G2/M boundary. Thus, it is tempting to speculate that factors promoting homologous recombination in plant nuclear DNA are restricted in their occurrence, and/or activity, to the G2/M transition point when homologue chromatids may be associated. In line with this argumentation, Takata et al. (1998) found a relation between repair pathways and cell cycle phase in vertebrate cells. During G1–early S phase, non-homologous end joining played a dominant role in repairing γ radiation-induced double strand breaks, while repair via homologous recombination was preferentially used in late S/G2 phase.

Thus, *Physcomitrella* cultures, like those described here, may be the source of choice to unravel the molecular mechanisms of homologous recombination in plant genomes which are as yet poorly understood (e.g., Ray and Langer, 2002). Application of such knowledge to crop plants would have an enormous effect on public perception of genetically modified plants.

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