The Bryologist 108(1), pp. 27–35

Effect of Ploidy Level on Growth, Differentiation, and Morphology in *Physcomitrella patens*

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Abstract. *Due to its high rate of homologous recombination, the moss* Physcomitrella patens *(Hedw.) B.S.G. is used as a novel system to facilitate gene/function-analyses. Loss-of-function mutations are easy to identify in the moss, as the dominant phase is the haploid gametophyte. Regenerating protoplasts were used to establish different moss lines with ploidy levels of 1C, 2C, and 4C. Flowcytometric analysis of three haploid, three diploid, and two tetraploid* Physcomitrella *lines revealed that haploid and diploid lines were cytologically stable, whereas nuclei of tetraploid lines exhibited varying DNA-contents. The effect of polyploidization on the phenotype, growth, and differentiation of* Physcomitrella *was investigated in vitro. The growth of three haploid, three diploid, and two tetraploid genotypes was evaluated after four weeks of axenic culture. The effect of the genotype on the growth rate of plants cultured on minimal medium was statistically significant; in contrast the ploidy level had no effect. On full medium the effect of the line, as well as the ploidy level, were statistically significant. The dry weight per petri-dish after a culture period of four weeks, was 103–106 mg in haploid lines, 64–78 mg in diploid, and 19–32 mg in tetraploid ones. The effect of the moss line and the ploidy level on differentiation of buds and gametophores was highly significant. Tetraploid moss rarely developed buds and/or gametophores. Sporophyte induction was observed in haploid and diploid genotypes, however not in tetraploid moss. Twelve percent of the germinated spores increased the ploidy level in comparison with the parent plant. A reduction of the ploidy level from 2C to 1C was observed in 7%. Evaluation of older plants (11 weeks after protoplast isolation) showed that polyploidization often resulted in a reduced number of gametophores and a reduced colony diameter on minimal medium compared to haploid plants. More than 70% of the diploid plants had gametophores with phenotypic alterations. Only 1.4% of the diploid plants were indistinguishable from wildtype. Changes in leaf shape and multiple phenotypic deviations from wildtype are most likely indications of polyploidization; however, the unequivocal identification of diploid plants is only possible using flowcytometric analysis, because a multitude of different phenotypic changes were observed in polyploid plants.*

Keywords. Differentiation, flowcytometry, phenotypic deviation, *Physcomitrella patens*, ploidy level.

The moss *Physcomitrella patens* (Hedw.) B.S.G. is unique among all other land plants so far studied with regard to the highly efficient homologous recombination in its nuclear DNA (Holtorf et al. 2004; Reski 1998*a*; Schaefer & Zryd 1997). Therefore *Physcomitrella patens* can be used as a novel experimental system to facilitate gene/function-analyses. This approach is especially straightforward as the dominant phase in mosses is the haploid gametophyte (Reski 1998*a*), making loss-of-function mutations readily screenable. Our aim is the production of a saturated mutant collection of approximately 75,000 *Physcomitrella* plants and their phenotypical characterization to identify interesting mutants (Egener et al. 2002; Holtorf et al. 2002). Phenotypic alterations of *Physcomitrella*-transformants might be the result of either polyploidization or gene knock out. The occurrence of polyploid plants after protoplast isolation and transformation is therefore monitored by flow-cytometry. In this study we investigated whether polyploidization in *Physcomitrella patens* results in specific phenotypic deviations, and has an effect on growth and differentiation in vitro.

0007-2745/05/$1.05/0

METHODS

Cell culture.—The different lines were derived from the Hamburg strain of *Physcomitrella patens*. The moss was grown in petri dishes with modified Knop medium (250 mg l^{-1} KH_2PO_4, 250 mg l^{-1} $MgSO_4 \times 7H_2O$, 250 mg l^{-1} KCl, 1000 mg l^{-1} $Ca(NO_3)_2 \times 4H_2O$, 12.5 mg l^{-1} $FeSO_4 \times 7H_2O$, pH 5.8, solidified with 8 g/liter agar, Oxoid Ltd., Basingstoke, Hampshire, England) overlaid with cellophane discs. Full medium consisted of macroelements and the following supplements: 50 μM H_3BO_3, 50 μM $MnSO_4 \times H_2O$, 15 μM $ZnSO_4 \times 7H_2O$, 2.5 μM KJ, 0.5 μM $Na_2MoO_4 \times 2H_2O$, 0.05 μM $CuSO_4 \times 5H_2O$, 0.05 μM $CoCl_2 \times 6H_2O$, 50 μM adenine, 22 μM myo-inositol, 20 μM choline chloride, 13.8 μM Na-palmitic acid, 8 μM nicotinic acid, 4 μM Ca-D-pantothenate, 1.8 μM p-aminobenzoic acid, 1.5 μM thiamine-HCl, 1.2 μM pyridoxine-HCl, 0.04 μM biotin, 0.04 μM riboflavine, 0.25 g/liter peptone, 2.5 mM ammonium tartrate, and 50 mgL^{-1} glucose. To evaluate the effect of plant growth regulators on growth and development, either one mg l^{-1} NAA (naphtalene acetic acid), one mg l^{-1} 2iP [6-(γ-γ-dimethylallylamino) purine riboside], or one mg l^{-1} NAA and one mg l^{-1} 2iP was added to the medium.

The Knop-medium of suspension cultures was changed every week and the moss was cut to small pieces using an Ultra-Turrax. Every two weeks the inoculation density of the moss was adjusted to a dry weight of 100 mg l^{-1}. Cultures were kept in a climate chamber with 25°C, light flux of 70 μmol $m^{-2}s^{-1}$ (fluorescent tubes Philips TLD 25) and a day-night rhythm of 16:8.

To determine the dry weight a 10 ml sample of suspension culture was taken and the moss was dried for two hours at 105°C.

To evaluate the differentiation of the moss lines, the number of buds and gametophores were counted at four defined one cm^2 spots per petri dish (n = four petri dishes) after four weeks. To document the plants, a stereomicroscope was used with a magnification between six and 66.

Mock-transformants and transformants.—After transformation of *Physcomtrella patens* with a mutagenized *Physcomitrella* cDNA-library (Egener et al. 2002) two haploid (H1 and H2), three diploid (D1, D2, and D3), and two tetraploid (T1 and T2) plants were chosen. Whereas the haploid and diploid plants were unstable transformants, the tetraploids were stable and survived several rounds of selection on antibiotic-containing medium (Ashton et al. 2000).

To produce mock transformants, the transformation procedure was followed as described in Hohe et al. (2003) but without DNA, and the selection steps were followed without adding antibiotics to the medium.

Flow cytometric analysis.—Flow cytometric (FCM) analysis was carried out according to Schween et al. (2003). Moss lines were inoculated in liquid culture with an inoculation density of 100 mg l^{-1} and after protoplast isolation samples were measured twice a week. Experiments lasted three weeks without further subculture. Fluorescence of nuclei or protoplasts stained with DAPI-solution (0.01 mg l^{-1} DAPI, 1.07 g/liter $MgCl_2 \cdot 6H_2O$, 5 g/liter NaCl, 21.11 g/liter TRIS and 1 ml^{-1} Triton) was determined with a PAS cell analyzer (Partec, Münster, Germany) using a 100 W high-pressure mercury lamp.

Sporophyte induction.—Wt, H1, D1, D2, D3, T1, and T2 were treated accordingly to Hohe et al. (2002*b*) to induce sporophytes. For sporophyte induction, gametophores were transferred to Knop medium supplemented additionally with 30 mg/l Fetrilon (Compo, Muenster, Germany). After two weeks growth on this medium the cultures were transferred to 15°C, 20 μmol $m^{-2}s^{-1}$, and short day condition with eight hours light/day for gametangia induction. Seven weeks later, the dishes were flooded with approximately 10 ml autoclaved tap water. After at least six weeks sporophyte induction was evaluated. Spore capsules were harvested, spores were diluted in three ml liquid Knop medium, and afterwards one ml was transferred to three nine-cm petri-dishes each containing full medium covered with a cellophane disk. Ploidy level of the germinated plants was monitored by flow-cytometry approximately four weeks later.

Statistical analysis.—Analysis of variance (MANOVA) was calculated for statistical analysis (Snedecor & Cochran 1968).

RESULTS

Flowcytometric analysis.—All three haploid genotypes showed one peak of fluorescence, which, based on previous results (Schween et al. 2003), represents cells in the cell cycle stage G2 (Figs. 1A–C). Compared to the haploid lines (normalized to 100%) the diploid lines had DNA-contents of 198 ± 4% (D2), 204 ± 1.1% (D1), and 207 ± 1.7% (D3), indicating that in diploid lines small deviations in DNA-content occurred (Figs. 1D–F).

The tetraploid line T1 showed two distinct peaks representing DNA-contents of 198 ± 2.7% and 398 ± 6.0% (Fig. 1G). Flowcytometric analysis of the regenerated plants 11 weeks after protoplast isolation showed only one peak representing tetraploid cells (with a DNA-content of approximately 400%) and no diploid cells. Thus cells with a DNA-content of 2C developed later on during the establishment of the line T1 in liquid culture and/or the following subculture.

After inoculation of T2 into fresh liquid medium, the DNA-content was 368 ± 5.3%. During the culture period of three weeks the culture of line T2 developed two distinct cell populations with slight differences in their amount of DNA (Fig. 1h). Compared to wildtype these peaks represented cells with a DNA-content of 332 ± 4.4% and 369 ± 4.9%. These features of line T2 were already visible shortly after protoplast isolation (after 11 weeks), showing two cell populations and loss of DNA in tetraploid cells.

Differentiation and growth.—Moss gametophytes exist in two morphological forms. First a filament grows by apical cell division, then a three-faced apical cell, the so-called bud, is formed, which develops into a leafy moss plant, the gametophore (Reski 1998*b*). To evaluate the effect of ploidy on the differentiation process, buds and gametophores were counted four weeks after inoculation of one ml moss suspension (adjusted to 100 mg l^{-1} dry weight) on a nine cm-petri dish with either Knop medium or full medium. On full medium the formation of buds and gametophores was almost completely inhibited, irrespective of the

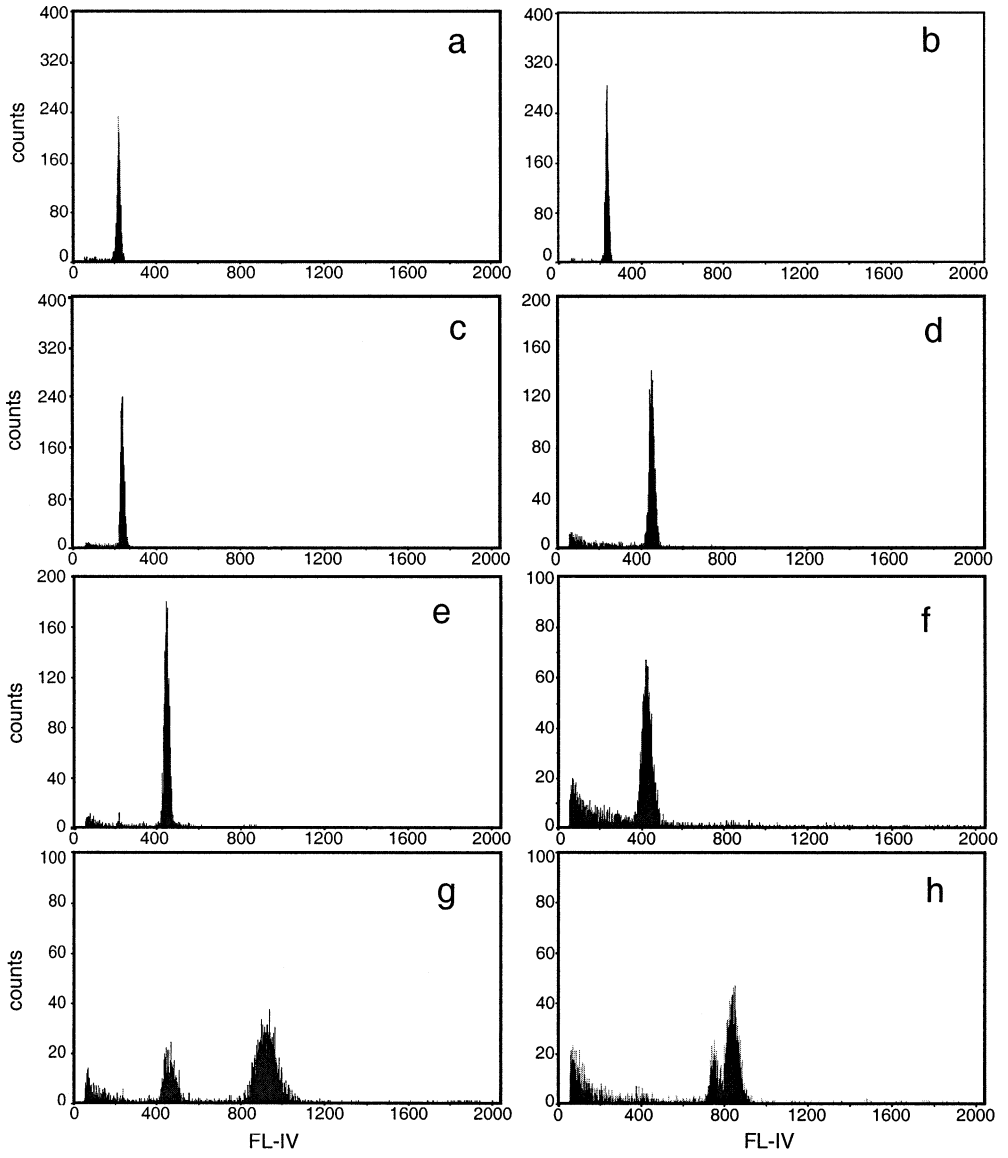

FIGURE 1. Flowcytometric analysis of different *Physcomitrella* lines. — a. Wt (bioreactor-material, haploid). — b. Moss line H1 (haploid). — c. Moss line H2 (haploid). — d. Moss line D1 (diploid). — e. Moss line D2 (diploid). — f. Moss line D3 (diploid). — g. Moss line T1 (tetraploid). — h. Moss line T2 (tetraploid).

TABLE 1. Number of gametophores/cm^2 of different moss lines, counted four weeks after inoculation of freshly minced *Physcomitrella* plants on Knop medium (n = four petri-dishes, four spots per petri-dish).

Moss line	Ploidy	Mean ± S.D.
Wt	haploid (1C)	17.6 ± 4.1
H1	haploid (1C)	7.6 ± 4.2
H2	haploid (1C)	2.8 ± 3.1
D1	diploid (2C)	15.6 ± 5.6
D2	diploid (2C)	7.8 ± 2.5
D3	diploid (2C)	34.4 ± 10.6
T1	tetraploid (4C)	0.1 ± 0.1
T2	tetraploid (4C)	0

ploidy level. In contrast, on Knop medium haploid and diploid genotypes formed between 2.8 and 34.4 gametophores/cm^2 in four weeks (Table 1, Figs. 4A–B). Tetraploid moss only rarely developed buds and/or gametophores; line T1 formed some buds and one gametophore, in line T2 no buds or gametophores were observed (Table 1, Fig. 4C). The effect of the moss genotype and the ploidy level on bud production was highly significant ($p < 0.001$). To test whether differences in the level of phytohormones might cause the inhibition of differentiation in tetraploid plants, either auxin (1 mg l^{-1} NAA), cytokinin (1 mg l^{-1} 2iP), or auxin plus cy-

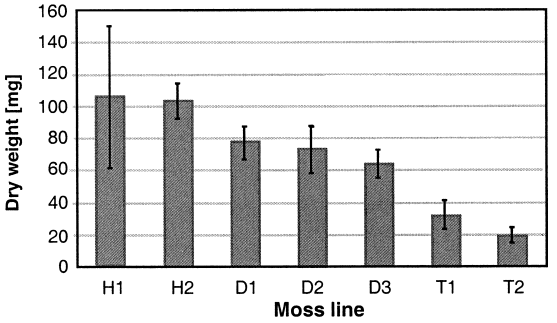

FIGURE 2. Growth of two haploid (H1 and H2), three diploid (D1, D2, and D3) and two tetraploid (T1 and T2) genotypes of *Physcomitrella patens* in vitro: dry weight per petri-dish four weeks after inoculation with 0.1 mg (dry weight) moss culture on medium containing macroelements, ammonium tartrate, microelements, vitamins, glucose, and further supplements. (Mean and standard deviation of 10 petri dishes. Effect of moss line and ploidy level is significant with $p < 0.001$).

tokinin (1 mg l^{-1} NAA and 1 mg l^{-1} 2iP) was added to the medium. This resulted in an increase in bud formation (up to 400 buds per cm^2) in both haploid and diploid lines; however, no reaction could be observed in both tetraploid lines (results not shown).

On Knop medium the dry weight varied between different genotypes (2–12 mg per petri-dish dry weight, t = four weeks, mean of 10 petri-dishes). The effect of the ploidy level on growth was not significant (results not shown). On full medium, browning of polyploid moss was observed in some petri dishes at the end of the culture period. Growth was inhibited in lines with ploidy levels of 2C and 4C. The dry weight after four weeks was 105 and 103 mg per petri-dish for H1 and H2, between 64 and 78 mg for the diploid lines (D1, D2, and D3) and only 32 and 19 mg for T1 and T2 (Fig. 2). The effect of the moss line as well as the ploidy level on growth rate was highly significant ($p < 0.001$).

Sporophyte induction and determination of ploidy level of germinated spores.—The formation of gametangia was observed in both haploid genotypes tested (Wt and H1) and the three diploid lines, but not in the tetraploid ones. Whereas wildtype *Physcomitrella* developed only one mature sporophyte per apex, the diploid line D3 formed numerous capsules rather frequently. However, the capsules were empty and did not contain any spores.

From the other plants (Wt, H1, D1, and D2), spores were harvested and after germination the ploidy level of the plants was determined. No changes in DNA-content were observed in the progeny of the wildtype; parent and all F_1-plants had DNA-contents of 1C. In line H1, D2, and D3 some F_1-plants exhibited an increased ploidy level (compared to the parent). In the diploid line D2 13 (= 23%) germinated plants exhibited a reduction of the DNA-content to a ploidy level of 1C. The results are summarized in Table 2. Figure 3 shows the histograms of the parent plant D2 and four of its progenies displaying different ploidy levels from 1C to 8C. The main peak in the histograms represents cells in the cell cycle phase G2, whereas the second, smaller peak represents endopolyploid cells, which develop in older caulonema cells of *Physcomitrella patens* (Schween et al. 2003).

TABLE 2. Ploidy level of the progenies after sporophyte induction of different moss lines and germination of *Physcomitrella*-plants (the expected ploidy levels of the progeny are bracketed).

		Ploidy level of progeny [%]			
Moss line	*n*	1C	2C	4C	8C
Wt (1C)	39	[100]	0	0	0
H1 (1C)	41	[83]	17	0	0
D1 (2C)	53	0	[74]	26	0
D2 (2C)	56	23	[73]	2	2

Phenotype of diploid Physcomitrella *plants.*—After mock-transformation, the ploidy level of almost 500 plants, resulting from two independent experiments, was tested by flow-cytometry and 11–14 weeks after protoplast isolation the phenotype of 419 haploid and 80 diploid plants was evaluated. Growth on Knop medium was analyzed by visual inspection of the colony diameter and was normal in almost all haploid mock-transformands (more than 90%), but reduced in about 80% of the diploid plants (data not shown). The coverage of the colonies with gametophores was significantly reduced in diploid plants; whereas 74.9% of all haploid plants were fully covered with gametophores, normal coverage was observed in only 7.6% of all diploid moss colonies (Fig. 4D–F).

93.1% of the haploid plants had gametophores similar to the wildtype (Figs. 4D–E) and 6.9% had deviations. In 4% of these cases the phenotypic alteration was slight, for example one or two gametophores of different size or some leaves developing necrotic spots. In contrast, 59 of the 80 diploid plants tested had gametophore morphology clearly deviating from the wildtype (73.7%, Fig. 4G) and only 26.3% looked like the wildtype. In Table 3, the most frequently observed alterations in gametophore morphology are summarized. More than 20% of the diploid plants had double tips or multiple phenotype deviations when compared to the wildtype gametophores, whereas haploid plants rarely showed these features. In most cases, the observed differences in gametophore morphology were not always exhibited by all gametophores of one colony. Some of the different phenotypes of

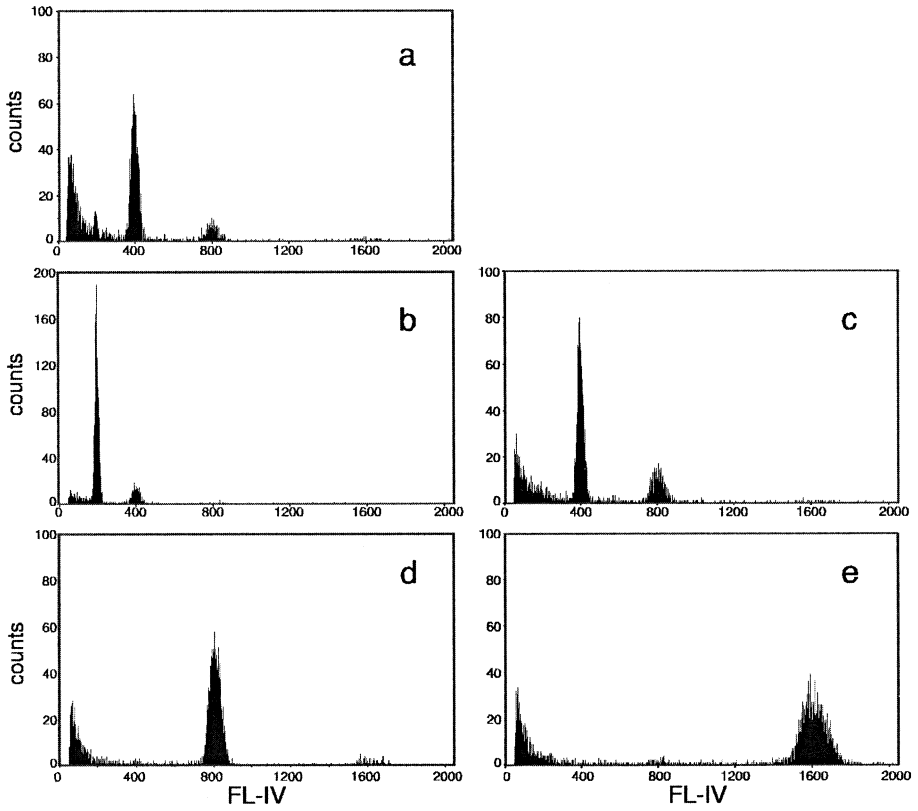

FIGURE 3. Flowcytometric histograms of a diploid *Physcomitrella patens* parent plant and four of its progenies. The main peak in the histograms represents cells in the cell cycle phase G2; the second, smaller peak represents endopolyploid cells that develop in older caulonema cells of *Physcomitrella patens* (for comparison see Schween et al. 2003). Histograms summarizing representative flow cytometric analyses of DAPI-stained isolated protoplasts. The abscissa represents the channel numbers corresponding to the relative fluorescence intensities of analyzed particles (linear mode) while the ordinate indicates the number of events counted. — A. The diploid (= 2C) parent plant D2. — B. Progeny with ploidy level of 1C. — C. Progeny with ploidy level of 2C. — D. Progeny with ploidy level of 4C. — E. Progeny with ploidy level of 8C.

diploid *Physcomitrella* plants are shown in Figures 4F–I.

The correlation between ploidy level and some phenotypic deviations from wildtype are given in Table 4. Changes in leaf shape and multiple phenotypic deviations from wildtype are most likely indications of polyploidization.

DISCUSSION

Flowcytometric analysis.—For flowcytometric analysis different moss lines with ploidy levels of 1C, 2C, and 4C were established using regenerated protoplasts. It is likely that polyploid lines are the result of protoplast fusion during protoplast isolation. Grimsley et al. (1977*a*) reported the production of somatic *Physcomitrella*-hybrids by protoplast fusion, however cytological proof was missing. Reski et al. (1994) analyzed a somatic hybrid and counted 54 ± 2 chromosomes after electrofusion of two moss protoplasts with 27 chromosomes. DAPI-staining demonstrated that the plant was not chimeric, because only one nucleus per cell was observed (Ye et al. 1989). Fusion of two mutants resulted in a somatic hybrid with a different isoenzyme pattern, indicating that parts of the mutant genomes had been eliminated (Ye et al. 1989).

Compared to the haploid lines, the DNA-content of the polyploid lines was not exactly twofold and fourfold. While the diploid lines exhibited only very small deviations, the two tetraploid lines were genetically not stable and displayed cells with different DNA-contents. The tetraploid line T1 developed two distinct peaks during subculture. While one peak represents tetraploid cells in G2, the first peak around channel 400 might represent either tetraploid cells in G1 or diploid cells in G2. In the first case, the percentage of cells in G1 would be much higher (23.1%) than in all other cultures of this experiment. It seems unlikely that cell proliferation and cell division were enhanced in line T1,

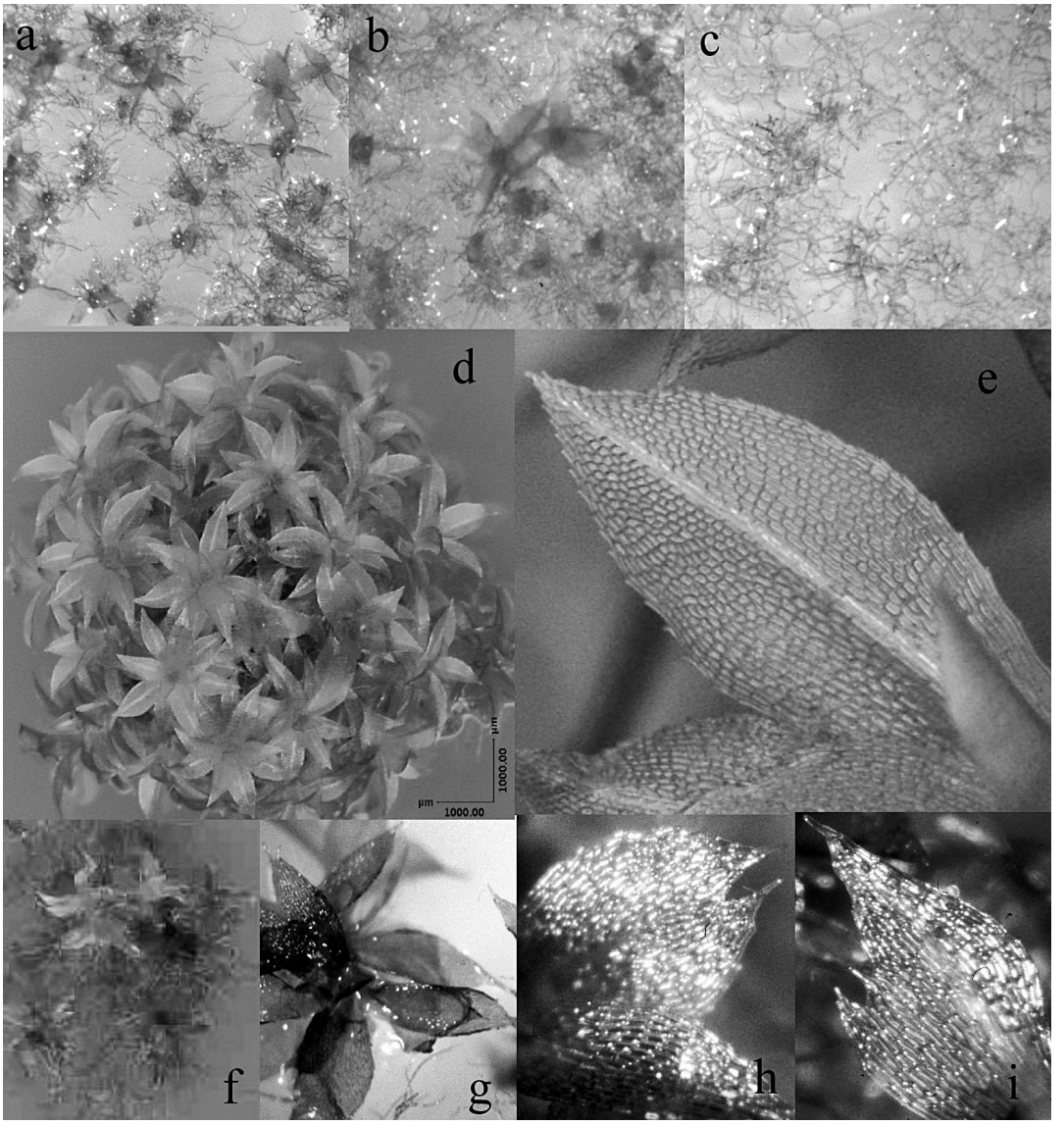

FIGURE 4. Habit of *Physcomitrella patens*. — A. Haploid *Physcomitrella*-line H1 28 days after inoculation of one ml moss suspension (adjusted to 100 mg l^{-1} dry weight) on Knop medium. — B. Diploid line D1. — C. Tetraploid line T1. — D. Moss colony of haploid wildtype plants 11 weeks after protoplast isolation. — E. Normal leaf morphology of haploid wildtype plants. — F. Phenotypical deviation in diploid plants: reduced gametophore coverage. — G. Phenotypical deviation in diploid plants: different leaf shape and asymmetric gametophores. — H. Phenotypical deviation in diploid plants: double tips and missing midrib. — I. Phenotypical deviation in diploid plants: cell outgrowth.

because the dry weight at the end of the culture was even lower compared to the other lines (results not shown). Furthermore, previous experiments with *Physcomitrella* wildtype under similar culture conditions revealed a maximum of 7.6 ± 1.7% of cells in G1 after a culture period of 22 days (Schween et al. 2003). Thus it is more likely that the histogram shows tetraploid and diploid cells within moss line T1. The histograms of line T2 reveals the occurrence of aneuploid cells with different DNA-contents. These aneuploid cells developed shortly after protoplast isolation.

The occurrence of cells with different DNA-content could be due to amitosis followed by mitosis. Nuclear fragmentation (amitosis) leading to chromosome number reduction has been observed in callus cultures of *Nicotiana tabacum* and *Vicia faba* and during protoplast regeneration of *Solanum tuberosum* (d'Amato 1985). In *Vicia faba*, intact nuclei with diploid and polyploid chromosome numbers,

TABLE 3. Phenotypic deviation of gametophore morphology in haploid and diploid plants of *Physcomitrella patens*.

Deviation	Haploid plants [%] (n = 419)	Diploid plants [%] (n = 80)
Necrosis	1.2	3.8
Splitting of leaf tip	0.7	23.8
Twisted leaf-tip	0.2	1.3
Cell outgrowth	0	7.6
Other deviations	4.8	12.2
Multiple deviations	0	25.0

TABLE 4. Correlation coefficients between different phenotypic features and ploidy level in *Physcomitrella patens* (n = 489 plants).

Phenotypic deviation from wildtype	Correlation coefficient
Plant color	0
Coverage of colony with gametophores	0.58
Leaf shape	0.69
Cell shape	0.32
Uniformity of leaves	0.41
Growth on Knop medium	0.55
Multiple phenotypic deviations	0.69

as well as nuclei with a wide range of aneuploid chromosome numbers, developed independently by the hormonal composition of the medium (d'Amato 1985). Our results indicate that haploid and diploid moss lines of *Physcomitrella patens* are cytological stable, but plants with a higher number of chromosomes are not.

Differentiation and growth.—Moss cells start to grow by apical division and form chloronema filaments (for a detailed description of moss development see Reski 1998*b*). Depending on culture conditions division of the tip cells leads to the formation of chloronema cells. Afterwards caulonema cells develop that show regular branching. The next step is bud production, resulting in the formation of the leafy gametophyte.

Differentiation was evaluated four weeks after plating freshly minced moss on petri dishes with either full medium or Knop-medium. On full medium, differentiation was inhibited in all lines, irrespective of ploidy level. The inhibiting effect of ammonium tartrate on gametophore development has already been described by Ashton and Cove (1977). Previous results showed that ammonium tartrate blocked caulonema formation in *Physcomitrella patens* (Hohe et al. 2002*a*; Schween et al. 2003). Addition of sugar has the same inhibiting effect on differentiation (Lorenz et al. 2003). Due to the blockage in differentiation, moss proliferation on full medium is restricted to apical cell division of chloronema cells.

The number of gametophores on Knop medium varied between 7.8 and 34.4 gametophores/cm^2 in diploid lines and between 2.8 and 17.6 gametophores/cm^2 in haploid *Physcomitrella*-lines (Table 1), indicating that differentiation was not inhibited or slowed down in diploid plants during the first four weeks. Ye et al. (1989) observed bud differentiation in wildtype *Physcomitrella* and in a diploid somatic hybrid after the same period of time. This is also in agreement with Engel (1968), who counted the same number of buds in haploid and diploid lines after three weeks. In the tetraploid line T1 some buds and one gametophore were observed. Flowcytometric analysis showed 23% diploid cells (Fig. 1g), so possibly the differentiation was restricted to these cells within line T1. Thus in tetraploid lines the differentiation was profoundly inhibited and might be completely blocked.

Older diploid plants (11 weeks after protoplast isolation) developed fewer gametophores compared to haploid plants; 74.9% of all haploid plants were fully covered with gametophores, but only 7.6% of all diploid moss colonies. This is in agreement with previous results from Courtice and Cove (1983), who obtained hybrids by protoplast fusion; around 60% developed less gametophores compared to wildtype.

Culture on full medium enhanced the growth in all *Physcomitrella* genotypes compared to Knop medium containing only macroelements (19–105 mg dry weight per petri-dish and 2–12 mg per petri dish, respectively; Fig. 2). Whereas on Knop medium the moss is restricted to photoautotrophic growth, addition of sugar to the medium allows light-independent cell proliferation. Four weeks after culture of cut moss on full medium, the dry weight of diploid and tetraploid plants was drastically reduced compared to haploid lines (Fig. 2). In contrast, the effect of the ploidy level of *Physcomitrella patens* growing on Knop medium was statistically not significant in cultures of this stage, however the growth rate of older polyploid moss colonies slowed down. Eleven–14 weeks after protoplast isolation less than 10% of haploid plants, but 80% of diploid plants showed reduced growth on Knop-medium (results not shown). The differences between the two media and between haploid and polyploid plants might be due to accelerated aging processes. On Knop medium, haploid moss colonies remained green for several weeks. In contrast, browning of moss colonies on full medium started after about six weeks (unpublished results), indicating that aging processes already took place. Premature browning was enhanced in polyploid cultures. It has been shown that aging processes in *Physcomitrella* is closely correlated to endopolyploidization (Schween et al. 2003), and the ob-

served inhibition of cell growth and differentiation at later stages might be the result of a high percentage of cells with DNA-contents of 4C and more.

In conclusion, growth and development in diploid *Physcomitrella* is inhibited or slowed down at later stages, probably due to endopolyploidization, but not during the first weeks of development.

Sporophyte induction and determination of ploidy level of germinated spores.—The sex organs (= gametangia) of mosses were formed after transfer of the cultures to 15°C and short-day conditions. In haploid *Physcomitrella* plants there might be numerous female sex organs per apex, but normally only one of these develops into a mature, diploid sporophyte (Hohe et al. 2002*b*). The sporophyte induction of haploid and diploid *Physcomitrella* plants required the same time (around four months). Engels (1968) observed differences in the duration of the life cycle of haploid and diploid plants (seven to eight weeks and four to six months, respectively); however, a comparison of the subsequent differentiation steps of *Physcomitrella* was missing in that study (Engels 1968).

In the diploid line D3, two to six capsules per gametophore were observed rather frequently; however, no spores were formed. *Splachnum luteum* normally develops only one sporophyte, but increases its number of archegonia when fertilization fails (Bauer 1959), thus the developing sporophyte suppresses further development. A similar mechanism might suppress the development of more than one mature sporophyte per gametophore, when fertilization takes place in *Physcomitrella*.

Within the diploid sporophyte, haploid spores develop from the spore mother cells by meiosis. All the germinated spores of the haploid wildtype resulted in plants with the expected DNA-content of 1C (Table 2). In all the other lines (H1, D1, and D2), some progeny exhibited an increase of ploidy level (from 1C to 2C and from 2C to 4C and 8C). Engel (1968) described the aposporous formation of diploid gametophytes in *Physcomitrella patens*, where fragments of the capsule cell wall began to grow protonemata and gave rise to a diploid gametophyte. Although this cannot be excluded, it could be expected that all lines are affected in this case; however, in haploid Wt only haploid colonies developed after germination of spores and in the other lines the percentage of polyploidization in the progenies varies from only 2% in D2 to 26% in D1. Furthermore, this does not explain the occurrence of a progeny with a quadruplication of the DNA-content from 2C in the parent plant to 8C (Fig. 3E). The occurrence of progenies with a doubled DNA-content compared to the parent plant is probably the result of a disturbed meiosis after fertilization. The fact of either apogamous sporophyte induction, apospory, and parthenogenesis in different moss species makes it evident, that sporophyte induction of mosses is a flexible process (Lal 1984).

In the offspring of the diploid line D2, a reduction of the ploidy level back to 1C (Table 2, Fig. 3) was observed. Further analyses have to show whether the reduction in DNA-content is the result of a random process of chromosome elimination or whether duplication of the chromosome set resulted in targeted removal of the redundant duplex chromosomes. Apparently meiosis of *Physcomitrella patens* is a very flexible process, and might result in genetic variability in the progeny.

Phenotype.—Grimsley et al. (1977*b*) described three different types of hybrids after protoplast fusion, and speculated that so-called class II hybrids with more caulonema and less gametophores are diploids. Around 60% of the plants were class II hybrids and around 40% of the plants had very few or no gametophores and more caulonema. Furthermore, less than 1% of the hybrids were otherwise morphological abnormal (Grimsley et al. 1977*b*). Courtice and Cove (1983) described leaves of class II hybrids, which showed discontinuity of leaf boundaries, or splitting of the leaf tip and sometimes with a bifurcated midrib. However, in both studies the clear correlation of ploidy and phenotype was impossible due to missing cytological analysis of the plants. Ye et al. (1989) characterized a diploid somatic hybrid with wildtype-like cells and chloroplast morphology. In *Funaria hygrometrica*, diploid plants had larger leaves and leaf cells, a darker brown protonema pigmentation, and developed less gametophores (Mejia et al. 1988). Examination of ~500 regenerated *Physcomitrella*-plants confirmed that diploid plants displayed phenotypic deviations such as a reduced number of gametophores and/or altered leaf morphology (splitting of the leaf tip, cell outgrowth, Figs. 4F–I) more frequently than haploid plants (Tables 3–4). Only 1.4 % of the diploid plants looked like wildtype and this figure is in good agreement with the results of Grimsley et al. (1977*b*) who reported less than 1%. In a mutant collection of more than 16,000 *Physcomitrella* plants 0.7% of the diploid transformants had a wildtype-like morphology and showed normal growth on Knop medium (Egener et al. 2002). In addition to the previously described phenotypic deviations, characterization of the mock-transformants pinpointed further phenotypical aberrations (reduced growth on Knop medium, no uniformity of leaves on a moss colony, occurrence of multiple phenotypic deviations) that might indicate polyploidization.

Phenotypic alterations of *Physcomitrella*-transformants might be the result of either polyploidi-

zation or gene knock out. Because the identification of the ploidy level by visual inspection is impossible due to the flexibility of the morphological response to polyploidization (Table 4), cytological analysis of the plants is necessary. Flowcytometry is a simple and reliable method to determine the ploidy level of moss transformants.

Acknowledgments

This work was performed in a joint project between Freiburg University and BASF Plant Science GmbH. We would like to thank Mary Buchta, Venera Gashaj, Sabine Glatzel, Hans-Juergen Schwarz, and Rosalinde Zimmermann for their excellent technical assistance.

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ms. received July 21, 2004; accepted Nov. 8, 2004.