Day Length and Temperature Strongly Influence Sexual Reproduction and Expression of a Novel MADS-Box Gene in the Moss Physcomitrella patens

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Abstract: The effect of temperature and light conditions on sexual reproduction (sporophyte formation) of in vitro cultures of the moss Physcomitrella patens was analysed. All parameters tested, i.e., temperature, light intensity and day length had a strong impact on the number of sporophytes formed. The highest number of sporophytes, 559 g fresh weight, developed at 15 °C, 8 h light/day with an intensity of 20 μmol/m²/s. In contrast, at 25 °C, as well as with a day length of 16 h per day, the number of sporophytes was drastically reduced. Vegetative growth, determined as fresh weight per petri dish, was impeded under conditions favourable to sporophyte formation, probably due to nutrient transfer to the sporophytes. Microscopic documentation of the developing sporophytes revealed that, although archegonia were arranged in bundles at the gametophore apices, usually only one archegonium per gametophore apex developed into a mature sporophyte. From an EST database six novel MADS-box genes were identified which, in phylogenetic analyses, did not cluster with the known groups of highly plant MADS-box genes. One of these genes was represented only as a singleton in a cDNA library specifically derived from gametophore apices and developing sporophytes, and, therefore, designated PpMADS-S. RNA amounts of PpMADS-S were two to three times higher under conditions that stimulate sporophyte development (15 °C, 8 h light/day) when compared to conditions favouring vegetative growth (25 °C, 16 h light/day), indicating a possible function in sexual reproduction of this moss. Thus, an efficient experimental system was established to study sex organ formation, fertilization and embryo development in Physcomitrella.

Key words: Long day, MADS-box, photoperiodism, short day, sporophyte development.

Introduction

Extant land plants (embryophyta) are thought to have one single evolutionary ancestor and comprise two major groups: bryophytes (liverworts, hornworts and mosses) and vascular plants (Kerick and Crane, 1997[15]). All land plants are characterized by an alternation of two generations: the haploid gametophyte and the diploid sporophyte. Whilst the gametophyte is reduced to a few cells in seed plants, it is the dominant generation in bryophytes.

Here, sex organ formation, fertilization and embryo development can be studied at exposed sites. The moss Physcomitrella patens would be especially suited for such studies as it is, so far, the only land plant where gene function can be determined by targeted knockout via efficient homologous recombination (Reski, 1998[23]). Due to this unique ability, Physcomitrella has gained special interest as a novel model system for plant functional genomics (Nishiyama et al., 2000[21]; Egner et al., 2002[20]; Holtorf et al., 2002[24]). Such an approach could nicely complement the intensive work on Arabidopsis embryo development (Mayer et al., 1991[18]; Laux and Jürgens, 1997[17]) and add an evolutionary point of view to the analysis of body plan development in land plants.

However, knowledge about sexual processes and sporophyte development in Physcomitrella is scarce and in sharp contrast to the wealth of data on gametophyte development and its control by environmental, hormonal and molecular factors (e.g., reviews of Cove and Knight, 1993[17]; Reski, 1999[24]), leading to poor yields of sporophytes under most laboratory conditions described so far.

Few studies deal with the impact of environmental conditions, such as temperature and light, on sexual processes in Physcomitrella. Engel (1968[11]) reported that sexual reproduction occurs only at temperatures lower than 19 °C and Nakosteen and Hughes (1978[20]) classified Physcomitrella as a day neutral plant regarding gametangia formation. Ashton and Raju (2000[3]) described distribution patterns of gametangia, however, they noticed many immature sporophyte stages which failed to develop into mature sporophytes. Thus, Cove and Knight (1993[17]) pointed out a lack of knowledge on the physiology of sexual reproduction and its control by environmental factors in Physcomitrella. For mosses other than Physcomitrella...
patens, light intensity, photoperiod and temperature appear to control sporophyte formation (Bopp and Bhatla, 1990[23]).

Even less is known about the molecular factors governing sex organ formation, fertilization and embryo development in mosses, although such knowledge would provide significant new insight into the evolution of land plant Bauplâné (e.g., Theißen et al., 2001[28]). Prime candidates to analyse would be genes whose products govern developmental pattern formation in plants, as well as in animals. It was only recently, that sets of homologues of such homeotic genes were found in Physcomitrella, namely KNOX genes (Champagne and Ashton, 2001[19]) and MADS-box genes (Krogan and Ashton, 2000[16]). These findings provided insight into the evolution of these gene sets, but were to date not correlated with moss development.

We here describe the significant influence of temperature, light intensity and day length on sexual reproduction in Physcomitrella and document in detail sporophyte development in this moss. From an EST database assembled from different stage-specific cDNA libraries (Rensing et al., 2002[22]), six novel MADS-box genes were identified. The expression of at least one of these is correlated with sexual reproduction in Physcomitrella.

Materials and Methods

Culture conditions

In vitro cultures of Physcomitrella patens (Hedw.) B.S.G. were propagated in 9 cm petri dishes on modified Knop medium containing 1000 mg/l Ca(NO₃)₂ × 4 H₂O, 250 mg/l KCl, 250 mg/l KH₂PO₄, 250 mg/l MgSO₄ × 7 H₂O and 12.5 mg/l FeSO₄ × 7 H₂O (Reski and Abel, 1985[23]) at 25 C and a photon flux of 70 µmol/m²/s (Philips TLD 25), 16 h light/day. For sporophyte induction, gametophores were transferred to medium as described above, supplemented additionally with 200 mg/l glucose and 30 mg/l Fertilon (Compo, Münster, Germany). After two weeks growth on this culture, the cultures were transferred to 15 °C, 20 µmol/m²/s, 16 h light/day for gametangia induction. Seven weeks later, the dishes were flooded with approximately 10 ml autoclaved tap water and distributed to the temperature and light conditions given in Fig. 1 (n = 8). After another six weeks, the number of sporophytes per dish, as well as the fresh weight of the plants, were determined.

Software

The GCG package 10.2 (Accelrys, U.S.A.) was used for sequence analysis, as well as CLUSTAL W 1.81 (Thompson et al., 1994[20]) for multiple sequence alignment. Homology searches were conducted with BLAST 2 (Altschul et al., 1997[11]). Phylogenetic trees were created with TREЕCON (Van de Peer and De Wachter, 1994[30]) using neighbour-joining with Tajima and Nei parameters and bootstrap resampling. For image analysis, ImageMaster 1D (Amersham-Pharmacia Biotech, Freiburg, Germany) was used.

Primers and internal control

Specific primers binding 5’ (pos. 39–58), as well as 3’ (pos. 373–392) of the MADS domain were called MADS1F (5’-GGTCTGCTGTGCTGGATG-3’) and MADS1R (5’-ACGAACATC-TACACTAGGGG-3’), respectively. Both primers are 20 bp in length and have a G/C content of 55%. Primers for 18S rRNA (internal control) usage were called 18SF2 (5’-TGACTCAA-CACGGGGAAAC-3’) and 18SR (5’-AGCCTGATGACTCACCCTTGTC-3’), respectively. Both primers are 20 bp in length and have a G/C content of 50/55%, facilitating equal efficiencies during PCR amplification. All oligo-nucleotides were purchased from Genset Europe AG (Freiburg, Germany).

Semi-quantitative RT-PCR

Whole RNA was prepared from protonema and gametophore tissue (see below) by grinding under liquid nitrogen and use of a plant RNA extraction kit (Plant RNAeasy, Qiagen, Hilden, Germany). First strand cDNA synthesis (reverse transcription) was carried out using the specific reverse primers mentioned above, both primers (18SR and MADS1R) were used in the same RT reaction, facilitating comparable results for PCR efficiencies of the two transcripts analysed. 500 ng of RNA were transcribed using 20 pmol of each primer, 1 mM dNTPs and 40 U of M-MuLV reverse transcriptase (MBI-Fermentas, St. Leon-Rot, Germany) in 1 × RT buffer for 60 min at 37 °C. Afterwards, RNase digestion (1 ng, 10 min at 65 °C) and sodium acetate/2-propanol precipitation was performed and the cDNA dissolved in 20 µl of water.

The PCR was performed in separated reactions using the same master mix. For MADS, 50 ng of RNA equivalent were used, for 18S, 25 ng. Conditions used were 1 × PCR buffer, as provided, 1 U Taq Polymerase (MBI-Fermentas), 1.5 mM MgCl₂, 200 µM dNTPs, 200 pmol of each primer, 30 cycles of 0: 30/93 °C, 1: 00/54 °C and 0: 45/72 °C.

DNA (10/30 µl PCR volume) was separated on 1.4% agarose and stained with ethidium bromide. Digital images were acquired using a video documentation system and a frame grabber (Amersham-Pharmacia Biotech). Quantification was carried out by colour inverting the image, subsequently followed by lane detection, background removal and band detection using lane edge subtraction. The overall measured band intensity was used for analysis of the transcript level. For each sample, the percentage of PpMADS-S band intensity was calculated relative to the 18S band intensity, which was set to 100% (18S intensity was corrected by a factor of two before, because twice the amount of RNA equivalent was used to produce the MADS bands than the 18S bands). To ensure reproducibility of the data, three RT reactions were performed and two PCR reactions analysed from each of those (n = 6). The six percentage values of PpMADS-S transcript level were used to calculate mean and standard deviation.

Material for RNA preparation

For protonema RNA, 200 ml of a semi-continuous bioreactor culture grown in modified Knop medium (see above), pH 4.5, as described by Hohe and Reski (2002[13]), yielding 180 mg fresh weight and 33 µg RNA were used. Gametophores grown on solid Knop medium (see above) in 9 cm diameter petri dishes were harvested for the isolation of gametophore RNA. Long day gametophores were grown for 4 weeks with 16 h light/day (70 µmol/m²/s) at 25 °C. For short day treatment, gametophores were grown for 6 weeks (short day 1) or 8 weeks (short day 2), with 8 h light/day (20 µmol/m²/s) at 15 °C, (water added after 3 weeks). Long day yielded 160 mg fresh weight/
18 μg RNA, short day 1600 mg/9.6 μg RNA and short day 2380 mg/9.2 μg RNA. On short day 1 gametophores with archegonia were visible, on the short day 2 gametophores, archegonia were visible, as well as green and light-brown sporophytes.

Results

Induction of sporophytes

After identical preculture (15°C, 16 h light, 20 μmol/m²/s) the effect of the three environmental parameters, temperature, day length and light intensity, on sporophyte formation was tested in ten different combinations. The number of sporophytes, as well as the fresh weight, were determined in order to calculate the number of sporophytes per g fresh weight. The number of petri dishes per variant (n) was 8, all results were confirmed in an independent second experiment (data not shown).

All three parameters tested (temperature, light intensity, day length) showed distinct effects on the amount of sporophytes formed (Fig. 1a): The number of sporophytes was highest at 15°C, where an average of 559 sporophytes/g fresh weight developed, whereas only 238 capsules were formed under the same light conditions at 18°C and nearly no sporophytes developed at 25°C. Under long day conditions (16 h day length) this number was dramatically lower in comparison to short day conditions (8 h day length), both at 15°C (18 sporophytes/g fresh weight) and at 18°C (11 sporophytes/g fresh weight). Regarding the photon fluence, the higher value of 70 μmol/m²/s reduced the number of sporophytes, especially at a temperature of 15°C, where only 177 sporophytes/g fresh weight developed under short day conditions when the fluence was 70 μmol/m²/s (in comparison to 559 sporophytes/g fresh weight at 20 μmol/m²/s). Under these conditions, an increased number of white gametophore apices and sporophytes was observed. The highest absolute number of sporophytes per petri dish was 125, found at 15°C, 8 h day length and 20 μmol/m²/s.
Plant growth was markedly reduced under conditions of good sporophyte formation (15 and 18 °C, short day). However, at 25 °C we observed no difference in plant biomass production between plants grown under short day or long day conditions, respectively (Fig. 1b).

Gametangia and sporophyte formation

The sex organs (= gametangia) of mosses are called archegonia (female) and antheridia (male). These were formed on the apices of the leafy gametophores, the adult gametophyte, approximately 6 weeks after transfer of the cultures to 15 °C. Under higher cultivation temperatures gametangia were found only occasionally.

The sex organs were found in bundles on the apices. Most contained several archegonia and some antheridia (Fig. 2A), confirming the monoecious nature of this moss species. Furthermore, this species is self-fertile, as single clones were able to produce sporophytes.

Although there are numerous female sex organs per apex, normally only one of these developed into a mature sporophyte (Fig. 2D). The first visible alteration was browning and twist-
ing of the archegonia neck (e.g., Fig. 2D). After numerous cell divisions, zygotes developed into sporophytes, which were at first oblong (Fig. 2B), later spheroidal (Fig. 2C).

At first sporophyte development was within the archegonium. However, at a certain age the female sex organ was penetrated and disrupted by the growing sporophyte: At its basal end, archegonia were penetrated by cells which connected the sporophyte with the gametophore apex. This connection, the so-called seta, was very short in comparison to many other mosses, e.g., *Funaria hygrometrica*. Very early in sporophyte development a dark ring appeared at the transition from gametophore to sporophyte tissue (Figs. 2B–H), probably marking the zone at which the archegonium was disrupted by the growing sporophyte. This basal gametophytic zone is called the vagina. The apical part of the archegonium stuck to the top of the growing sporophyte and formed the calyptra, still bearing the dried archegonium neck (Figs. 2B–D, F–H).

However, the connection between the calyptra, representing the archegonium residue, and the enlarging sporophyte was relatively loose, thus the calyptra was easily removed by mechanical stress (Fig. 2E). Sporophytes with or without calyptra were found in all developmental stages.

Within the sporophyte, spores develop from the spore mother cells by meiosis. In the beginning they appear light green (Fig. 2D). With increasing maturity they turned yellow (Fig. 2E), later becoming light to dark brown (Figs. 2F, G).

In contrast to other mosses, *Physcomitrella* sporophytes do not possess specialized structures, like peristomata, for the release of spores. The sporophyte wall either dries (Fig. 2G) or bursts (Fig. 2H), releasing the spores.

**Identification of novel MADS-box genes**

Thirty-seven publicly available MADS domain peptide sequences, covering all known MADS box subfamilies, were aligned (accession numbers and alignment are available upon request), and from this alignment a 55 amino acid consensus sequence was created using the GCG program, ProfileMake. BLAST searches with this query against a *Physcomitrella patens* clustered EST database (Rensing et al., 2002[22]) yielded seven significant hits. Of those clusters, two were singletons and five contigs (i.e., built from more than one EST). From the two singletons, one originated from a cDNA library derived from gametophore tissue carrying developing gametangia and sporophytes (Rensing et al., 2002[22]). The EST sequence (S_PP020013057R) is 670 bases long and contains the full MADS domain (pos. 105–276). We termed the deduced *Physcomitrella* MADS domain protein PpMADS-S (for sporophyte). All seven ESTs have been deposited in the international databases and are available under the accession numbers (A) 487536–AJ 487542.

The MADS domain of PpMADS-S is around 82–83% identical at the nucleic acid level to the recently published *Physcomitrella* MADS proteins, PpMADS1 and PpMADS2 (Krogan and Ashton, 2000[16]). In a phylogenetic analysis using the peptide sequence of the MADS domains, PpMADS-S clusters next to CRM4 and CRM5 from the fern *Ceratopteris pteroides* (Münster et al., 1997[19]). Therefore, it does not seem to belong to one of the well characterized MADS subfamilies from vascular plants, like def, glo, agl or squa (e.g., Krogan and Ashton, 2000[16]). Likewise, the other six clusters found (S_PP01026099R, C_PP02003947R, C_PP020056317R, C_PP01024021F, C_PP020028338R, C_PP01019060R) did not confidently group within one of the well-established MADS subfamilies of flowering plants and thus represent novel MADS-box subfamilies. The MADS domain of one of our ESTs (020056317) is identical at the amino acid level to those of the already published PpMADS1–2. The other five sequences represent, together with PpMADS-S, six novel MADS-box sequences. These seven sequences cluster in three distinct clades. Interestingly, one of these clades, consisting of three different MADS domain proteins, is clearly separated from the other two, which cluster next to within the CRM4–5 sequences (Fig. 3). This might be an indication of at least two different functions of MADS domain proteins in *Physcomitrella*.

In vascular, flowering plants, MADS-box genes are mainly involved in flower development. In a non-flowering plant, their function still has to be determined. The most probable function, however, also would be in sexual reproduction.

From all novel sequences, only one was solely represented as a singleton in a cDNA library derived from gametophore apices, sexual organs and developing sporophytes. We named this gene PpMADS-S (for sporophyte). To obtain additional evidence that PpMADS-S is correlated with sexual reproduction in *Physcomitrella*, we analysed its transcript accumulation under different treatments identified to affect sporophyte induction.

A semi-quantitative, but highly standardized, RT-PCR protocol was developed using 18S RNA as constitutive internal control. In all different tissues used in the present analysis, the intensity of the 18S band exhibited a standard deviation of only 7% (n = 23), using specifically transcribed cDNA as a template for the PCR reactions. Therefore, 18S RNA was regarded as being unaffected by the experimental conditions of this study. When using 25 ng of RNA equivalent for the 18S RT-PCR, as done here, the PCR reaction was still in the exponential range, as was checked by doing one PCR cycle less and comparing band intensities. Thus, reliability of this quantification was further enhanced.

PCR reactions with the MADS-specific primer pair yielded one specific band of 354 bp with genomic as well as with cDNA as template, while the 18S rDNA-specific primer pair yielded one specific band of 405 bp with genomic as well as with cDNA as template, indicating specificity of the PCR reaction and the primers used in this study. With these specific primer pairs, expression of the novel MADS domain was quantified under different growth conditions identified to affect sexual reproduction of this moss (see methods for details). To enhance reliability of this quantification, three independent reverse transcriptions (RT) were performed and, additionally, two independent PCR reactions were performed from each RT, accumulating to six different samples (n = 6). The relative expression levels were 13.96 ± 3.50% for protonema, 8.96 ± 2.28% for long day gametophores, 31.82 ± 7.84% for short day 1 gametophores and 26.35 ± 7.98% for short day 2 gametophores (Fig. 4), indicating a 3–3.5-fold increase in transcripts of PpMADS-S under environmental conditions favouring sexual reproduction of *Physcomitrella*, as compared to the gametophore control.
Discussion

Sporophyte induction

In a systematic approach, we analysed the effects of temperature, day length and light intensity on sexual reproduction of Physcomitrella patens in order to establish an efficient protocol for sporophyte formation in this moss.

Engel (1968) already reported on a marked effect of temperature on sexual reproduction of Physcomitrella. He observed that sporophyte formation occurred at 15–19°C, whereas no sporophytes developed at 20–23°C. These findings were confirmed by our recent data showing a drastically reduced number of sporophytes at 25°C compared to cultures at 15°C or 18°C. Such reduced numbers of sporophytes may be a consequence of a reduced number of sex organs, a block in fertilization or a block in embryo and subsequent sporophyte development. Compiling a large data set, Bopp and Bhatia (1990) suggested that elevated temperatures inhibit the induction of sex organs in many moss species. Therefore, pre-culture of all masses in our recent experiments was performed under the known induction parameters at 15°C, long day. However, subsequent exposure to elevated temperatures drastically reduced sporophyte formation when compared to cultures in 15°C, indicating that not only sex organ formation but also fertilization and/or early embryo development is a temperature-sensitive process in Physcomitrella.

Engel (1968) used different light/dark regimes when culturing Physcomitrella but did not report any effect of this parameter on sexual reproduction of this moss. However, he only used a minimum day length of 12 h but did not include pronounced short day conditions. Subsequently, Nakosteen and Hughes (1978) classified Physcomitrella to be a day neutral plant regarding gametangia (= sex organ) formation. This is consistent with our recent data, as antheridia and archegonia (= male and female sex organs) were induced under long day conditions for all subsequent treatments. Most surprisingly, however, high numbers of sporophytes were found only under short day conditions whereas at a day length of 16 h these numbers decreased to near zero even under optimal temperature conditions of 15°C. Thus, fertilization and/or early embryo development in Physcomitrella is not only strictly temperature sensitive, but also strictly regulated by the photoperiod, classifying this moss as a typical short-day plant.

Ashton and Raju (2000) analysed sexual reproduction of Physcomitrella with respect to distribution patterns of sexual organs and observed high numbers of archegonia and immature sporophytic stages which failed to develop into mature sporophytes. These authors assumed that somatic mutations of an old laboratory strain were the reason for this block in sporophyte development, as Ashton and Cove (1977) had induced sporophytes on this specific strain. Both these experiments, however, were performed under continuous illumination, a condition which, at least in our hands, represses sporophyte formation strongly. Thus, the interaction of light and age...
of a culture in sexual reproduction of *Physcomitrella* still needs more detailed analyses.

Comparing two different light intensities, we found a reduced number of sporophytes under medium light conditions (70 μmol/m²/s) when compared to low light intensities (20 μmol/m²/s). This effect was especially pronounced at low temperature (15°C), where several white gametophores and sporophytes were also found, indicating the involvement of photo-oxidative stress in these results.

Thus, with regard to sexual reproduction (fertilization and/or early embryo development), *Physcomitrella* is a short-day plant depending on low light and low temperature. Optimal conditions for sporophyte formation were 15°C, a day length of 8 hours and a fluence of 20 μmol/m²/s. These conditions mimic natural growth conditions of *Physcomitrella* in the northern hemisphere in autumn (Dierssen, 2001[8]).

**Costs of reproduction**

After counting sporophytes at the end of the individual treatments, fresh weight of the plants was determined and an inverse correlation between vegetative growth and sexual reproduction was found. Biomass production was reduced under conditions that favoured sporophyte formation (short day, 15°C and 18°C).

Since vegetative growth was not markedly reduced in short days at 25°C, this effect was presumably not due to reduced carbon assimilation (reduced period of photosynthesis), but probably due to enhanced carbon allocation to sexual reproduction. This phenomenon is known from other mosses as well and discussed in the context of the costs of reproduction (e.g., Ehrlen et al., 2000[10]; Stark et al., 2000[27]).

As we calculated sporophytes per fresh weight, varying fresh weights in the different treatments may have been a major reason for the observed effects.

However, biomass varied between different treatments at most by a factor of 2, whereas the number of sporophytes per gram fresh weight varied between different treatments at most by a factor of 3. Therefore, data adjusted to the differences in fresh weight also showed the effects based on day length, temperature and light intensity, as described and discussed above.

**Sporophyte development**

Based on the optimized culture conditions, we were able to access a huge number of sporophytes at different developmental stages for a more detailed analysis. As Cove and Knight (1993[17]) stated, hard data about sporophyte development in this moss species is scarce. To the best of our knowledge, we describe several morphological features, like twisting of the archegonia neck, its brown colouring as well as a brown ring, probably marking the basal end of the archegonia disruption zone (vaginula), here for the first time for *Physcomitrella* petals. In a detailed study Bopp (1957[19]) found an effect of the calyptra on sporophyte development in some moss species. However, in our hands the calyptra of *Physcomitrella* was only loosely attached to the sporophyte and seemed to have no such effect.

Our analysis was not detailed enough to discriminate whether the marked effects of temperature, photoperiod and light intensity on sporophyte numbers were due to effects on fertilization itself or due to effects on early embryo development. Similarly, we are not yet able to unequivocally state why only one embryo per archegonia bundle was able to develop into a mature sporophyte, although the rest of the archegonia persisted during this process. As we did not see any block in later embryo development in these archegonia, we speculate that this may be due to a block in fertilization or very early embryo development itself. Thus, besides the identified environmental parameters, signals from the developing embryo in this bundle may affect these early processes as well.

Identification of such molecular signals needs highly standardized and efficient protocols to induce large numbers of differentiation stages simultaneously. These protocols do now exist and we have started, as a first step, the expression analysis of a novel MADS-box gene.

**Novel MADS-box genes**

According to our EST analyses, *Physcomitrella* possesses at least seven different expressed MADS-box genes. The MADS domain of one of our ESTs was identical at the amino acid level to that of the already published PpMADS1-2 (Krogan and Ashton, 2000[18]), the others comprise six novel sequences. In phylogenetic analyses, these sequences clustered in three different families of plant MADS domain proteins, indicating different functions of these proteins in *Physcomitrella*. All these families did not belong to the well established families known from higher plants and thus may be ancient. This may simply reflect the fact that mosses and vascular plants diverged approximately 450 million years ago (e.g., Theissen, 2001[28]) and that mosses have a more simple body plan than vascular, flowering plants. This interpretation is supported by an independent paper utilizing genomic data of seven different *Physcomitrella* MADS-box genes which appeared while this article was in proof (Henschel et al., 2002[12]).
However, a functional analysis of these genes in nonvascular plants is missing. *Physcomitrella* is the prime candidate for such studies as its efficient homologous recombination allows the establishment of gene/function relationships in a straightforward way (e.g., Strepp et al., 1998[26]). As a first step into such research, we identified a novel MADS-box gene, PpMADS-S, which may be involved in sexual reproduction of *Physcomitrella*. Using a highly standardized RT-PCR procedure, we found that this gene is transcribed at low levels in protonema and gametophores that do not develop sexual organs. However, its mRNA accumulation is enhanced approximately 3 fold in gametophores that develop gametangia and sporophytes (Fig. 4). We take this as first evidence that this specific, novel MADS-box gene (PpMADS-S) is involved in sexual reproduction of *Physcomitrella*. Functional analysis by targeted gene knock-out will follow.

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