

RESEARCH PAPER

Genome-wide transcriptomic analysis of the sporophyte of the moss *Physcomitrella patens*

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Received 22 February 2013; Revised 26 April 2013; Accepted 28 May 2013

Abstract

Bryophytes, the most basal of the extant land plants, diverged at least 450 million years ago. A major feature of these plants is the biphasic alternation of generations between a dominant haploid gametophyte and a minor diploid sporophyte phase. These dramatic differences in form and function occur in a constant genetic background, raising the question of whether the switch from gametophyte-to-sporophyte development reflects major changes in the spectrum of genes being expressed or alternatively whether only limited changes in gene expression occur and the differences in plant form are due to differences in how the gene products are put together. This study performed replicated microarray analyses of RNA from several thousand dissected and developmentally staged sporophytes of the moss *Physcomitrella patens*, allowing analysis of the transcriptomes of the sporophyte and early gametophyte, as well as the early stages of moss sporophyte development. The data indicate that more significant changes in transcript profile occur during the switch from gametophyte to sporophyte than recently reported, with over 12% of the entire transcriptome of *P. patens* being altered during this major developmental transition. Analysis of the types of genes contributing to these differences supports the view of the early sporophyte being energetically and nutritionally dependent on the gametophyte, provides a profile of homologues to genes involved in angiosperm stomatal development and physiology which suggests a deeply conserved mechanism of stomatal control, and identifies a novel series of transcription factors associated with moss sporophyte development.

Key words: Alternation of generations, gametophyte, microarray, moss, *Physcomitrella*, sporophyte, stomata.

Introduction

Alternation of generations is a fundamental concept in plant biology. Each plant species undergoes two distinct stages in its life cycle: a haploid stage (gametophyte) capable of generating gametes via mitosis and a diploid stage (sporophyte) distinguished by the ability of the plant to generate spores via meiosis. Plant evolution has been characterized by the increasing dominance of the sporophyte stage at the expense of a free-living gametophyte. This has led to the point where, in present-day angiosperms, the sporophytic form dominates and the gametophyte is reduced to a non-free-living stage

enclosed within the pollen or embryo sac (Haig and Wilczek, 2006). The drivers for this evolutionary progression have been proposed to be the environmental stresses associated with the colonization of a terrestrial environment compared with an aquatic one. The acquisition of a few key traits has been proposed to underpin the success of land plants, with subsequent consequences for the evolution of life and climate on the planet (Kenrick and Peter, 1997; Kenrick *et al.*, 1997; Hughes and Otto, 1999; Graham and Wilcox, 2000; Renzaglia *et al.*, 2000; Bennici, 2008; Langdale, 2008; Niklas

and Kutschera, 2009). Despite a plethora of descriptive studies on traits proposed to be required for the adaptation of the sporophyte generation for survival on land, the molecular evolution underpinning these traits has only recently begun to be unravelled. A focus of these studies has been on the comparison of two model plant systems with fully sequenced genomes: *Arabidopsis thaliana*, a representative angiosperm, and *Physcomitrella patens*, a representative bryophyte. Moreover, various genomic tools have been developed for these plants which facilitate gene swap and mutation experiments, allowing the rigorous testing of gene function in an evolutionary context.

The *Physcomitrella* gametophyte consists of protonemal filaments which give rise to gametophores upon which the gametes are generated (Fig. 1). Following gamete fusion, the sporophyte develops as a proto-shoot structure which remains relatively small and attached to the gametophyte (Glime, 2007a). The main bulk of the sporophyte is devoted to the formation of spores which are eventually released for dispersal. Thus, the two forms of the plant (gametophyte and sporophyte) are distinguished not only by their nuclear chromosome content and ontogeny, but also by form and function. These two distinct plant forms are encoded by the same genome (Szovenyi *et al.*, 2011). This raises fundamental questions relating to the biology of the alternation of generations. For example, is there a distinct difference in the spectrum of gene products utilized by the gametophyte and sporophyte plant forms, or are essentially the same gene products used in both but in a different constellation? The advent of genomic sequencing, along with tools for the genome-wide analysis of gene expression data, has opened the door to the analysis of these types of questions. A recent study of *Funaria hygrometrica* (a close relative of *Physcomitrella*) exploited advances in high throughput sequencing to compare RNA extracted from the gametophore and sporophyte tissue (Szovenyi *et al.* 2011). The similarity of the *Funaria* sequences to the fully sequenced genome of *Physcomitrella* allowed the identification of 13 000 gene models whose expression could be compared between the two life history stages. These data indicated that the transition from gametophyte-to-sporophyte was marked by a change in transcription profile; however, the number of genes showing differential expression was moderate, representing only about 4% of the genes that could be unequivocally identified. In addition, the gametophyte-stage tissue used was gametophore-enriched but did not include the earlier protonemal stage of gametophyte development.

One of the reasons for focusing on *Funaria* was that the sporophyte of this moss is relatively large, certainly in comparison to *Physcomitrella*. However, the high-throughput sequencing approach relies on the ability to unequivocally relate novel sequences to an anchor genome, and this leads to a limitation in the approach because of the lack of a *Funaria* genome sequence. An alternative strategy (reported here) is to adopt a more direct approach to comparing transcription profiles of the moss gametophyte and sporophyte by dissecting the minute *Physcomitrella* sporophytes and using the available genomic tools for this plant. This work reports on the analysis of RNA extracted from several thousand *Physcomitrella* sporophytes

dissected at two different stages, thus providing an insight into the transcriptome during early sporophyte development. For comparison, gametophyte tissue at the protonemal stage was used. This is relatively homogeneous tissue compared with the gametophore and is unequivocally free of any potentially contaminating sporophyte tissue. It also has a distinct tissue organization (filamentous cells) so that transcriptome analysis provides an insight into this particular morphology. The RNA analysis was performed using a custom-built microarray based on the sequenced genome of *Physcomitrella* which allowed the expression analysis of over 30 000 genes and was validated by reverse-transcription PCR (RT-PCR) of selected lead genes. The data indicate a much larger difference in transcript profile than previously reported for the gametophore to sporophyte



Fig. 1. The lifecycle of moss alternates between the haploid gametophyte stage and the diploid sporophyte stage. A single haploid *Physcomitrella patens* spore gives rise a gametophore (central image). Fertilization of gametes on the gametophore apex produces a structure called a sporophyte (capsule on top of gametophore in central image). This sporophyte is the diploid phase in the lifecycle of the moss. As the sporophyte matures, haploid spores form within the capsule. Eventually the sporophyte bursts, releasing spores to repeat the lifecycle. Early sporophytes (bottom left) and mid-sporophytes (bottom right) were harvested for RNA extraction and microarray analysis. Bars, 100 μ m (early sporophyte), 200 μ m (mid-sporophyte) (this figure is available in colour at *JXB* online).

comparison in *Funaria* (Szovenyi *et al.*, 2011). In addition, this analysis of the transcriptome during early sporophyte development provides a first insight into the genes that putatively control moss stomatal development and function, as well as gene expression changes that occur as the capsule proceeds towards meiosis and spore generation.

Materials and methods

Plant material

P. patens subspecies *patens* (Hedwig) wildtype ecotype 'Villersexel'. 'Villersexel' was found to produce sufficient sporophytes for analysis, unlike the 'Gransden' ecotype (the strain from which microarray probes were constructed). However, there is little polymorphic difference between the two strains with 829bp/SNP and therefore little effect on hybridization to the microarray (Kasahara, 2011). Plants were grown in Sanyo MLR incubators with a 16/8 light/dark cycle, 140 $\mu\text{mol m}^{-2}$, and 22/16 °C. Protonema and gametophores were grown on organic-nitrogen-supplemented BCD medium in sterile Petri dishes overlaid with sterile cellophane discs (AA Packaging, UK) and sealed with micropore tape, as described (Knight *et al.*, 2002). Protonemal cultures were maintained by subculturing weekly in a sterile laminar flow hood. Mechanical disruption of protonemata was performed using a Polytron homogenizer (Kinematica, Switzerland). Gametophore production was initiated by subculturing protonemal fragments onto agar lacking cellophane discs and culturing these for longer than 14 days. Gametogenesis was initiated by transferring cultures to 15 °C growth cabinet for 3 weeks with a 16/8 light/dark cycle, (140 $\mu\text{mol m}^{-2}$), after which they were flooded with sterile deionized water to promote fertilization of archegonia for about 2 hours. Subsequently, the cultures were returned to gametophyte growth conditions for further development of the sporophytes.

RNA extraction

RNA was extracted from all plant tissues using the Spectrum Total Plant RNA extraction kit (Sigma, STRN250) following manufacturer's recommendations. Approximately 100 mg of tissue was harvested directly into liquid nitrogen in microcentrifuge tubes and ground to a powder. RNA integrity and concentration were analysed by agarose gel electrophoresis and an Agilent Bioanalyzer. Gametophyte RNA (G) was extracted from 7-day-old protonemal tissue. RNA was extracted from early stage sporophytes (ES, defined as pre-expanded columnar capsules with the foot, calyptras, and calyptral remnants removed) and mid-stage sporophytes (MS, defined as ovoid to near-spherical translucent green capsules with the foot, calyptras, and calyptral remnants removed). The ES stage is prior to meiosis and spore formation. The MS stage contains tissue in which the initial stages of meiosis and spore formation are occurring. To meet the quality requirements for the microarray experiment, at least 400 sporophytes were used per extraction. Three or four RNA extracts were then pooled for further precipitation to maximize purity and concentration. Up to 1600 sporophytes were harvested to prepare sufficient RNA for each microarray replicate.

Microarray hybridization

A triplicated transcriptomic microarray experiment was designed to compare gametophyte gene expression against the gene expression of both early and mid stage sporophytes. One of three independent replicate RNA samples of each sporophyte stage was co-hybridized with one of six independent replicate gametophyte samples. Samples were co-hybridized as total RNA with cyanine (Cy-3/Cy-5) fluorescent ULS labels onto an Agilent custom array with anti-sense probes (synthesized as 60-mer length oligonucleotide probes on glass slides using Agilent Technologies' 'SurePrint' inkjet technology (Reski

and Frank, 2005) in collaboration with MOgene LC, an Agilent-certified facility based in St Louis, MO, USA (www.mogene.com, MOGene LC, St Louis, consulted October 2004). Microarrays were performed essentially as described in the manufacturer's instructions with hybridization overnight at 65 °C followed by washing to a stringency of $0.2 \times \text{SSC}$ followed by brief drying by centrifugation. The microarray dataset has been deposited on GEO online database (GSE32928; www.ncbi.nlm.gov/geo).

Microarray analysis

Microarrays were analysed using the limma bioconductor package in the R environment (Smyth, 2005). The probes were background corrected using normexp with an offset of 50 (Ritchie *et al.*, 2007). They were then normalized within arrays using loess and normalized between arrays using Aquantile (Smyth and Speed, 2003). The arrays were split into individual channels. This allowed the comparison of gametophyte vs. early sporophyte, gametophyte vs. mid-sporophyte, and early sporophyte vs. mid-sporophyte. Analysis showed that the three technical and three biological replicates used for each sample comparison were robust (Supplementary Figs. S1 and S2, available at *JXB* online). The limma empirical Bayes method was used to identify differentially expressed genes (Smyth, 2004). A *P*-value of 0.01 and a fold change of 1.5 (0.585 on a log2 scale) were used as a cut-off for the comparisons described below. This fold cut-off was chosen in order to include genes with a low change in expression and, consequently, a low *p*-value was used in order to reduce the likelihood of false positives.

Gene annotation and ontology analysis

Gene sequences associated with each identifier were downloaded (transcripts.Phypha1_1.FilteredModels3.fasta.gz, available at http://genome.jgi-psf.org/Phypha1_1/Phypha1_1.download.ftp.html) (Rensing *et al.*, 2008). These sequences were annotated from the NCBI nr database using BLAST2GO (Conesa *et al.*, 2005; Conesa and Gotz, 2008). Gene ontology terms associated with each gene ID were downloaded (Phypha1_1_goinfo_FilteredModels3.tab.gz) (Rensing *et al.*, 2008). These GO annotations were uploaded to the program BLAST2GO in order to visualize the relative distribution of the GO terms in each category (Conesa *et al.*, 2005; Conesa and Gotz, 2008; Gotz *et al.*, 2008). GO enrichment analysis of functionally significant terms in the GO database using BLAST2GO was applied (Fisher's test, *P* < 0.01).

RT-PCR

RT-PCR for microarray analysis verification was set up as previously described (Malinowski *et al.*, 2011). Reactions paused at defined intervals during the program at the lowest temperature (60 °C) of cycles 20, 24, 28, and 32. At these times, individual aliquots of 8 μl were removed from the tubes and placed on ice to stop the reaction. At the end of the program, aliquots were loaded into wells for agarose gel (1%, w/v) electrophoresis in time order and visualized by UVtech Digital Camera. Primers were selected using Primer3 (<http://frodo.wi.mit.edu/primer3/>). Primer sequences are given in Supplementary Table S1.

Results and discussion

Early sporophyte development involves altered expression of a large fraction of the moss transcriptome relative to the protonemal-gametophyte

Comparison of gametophyte (G) and early sporophyte (ES) data indicate that a substantial number of genes are either upregulated (2042) or downregulated (1919) (Fig. 2 and Supplementary Tables S12 and S15). Since the

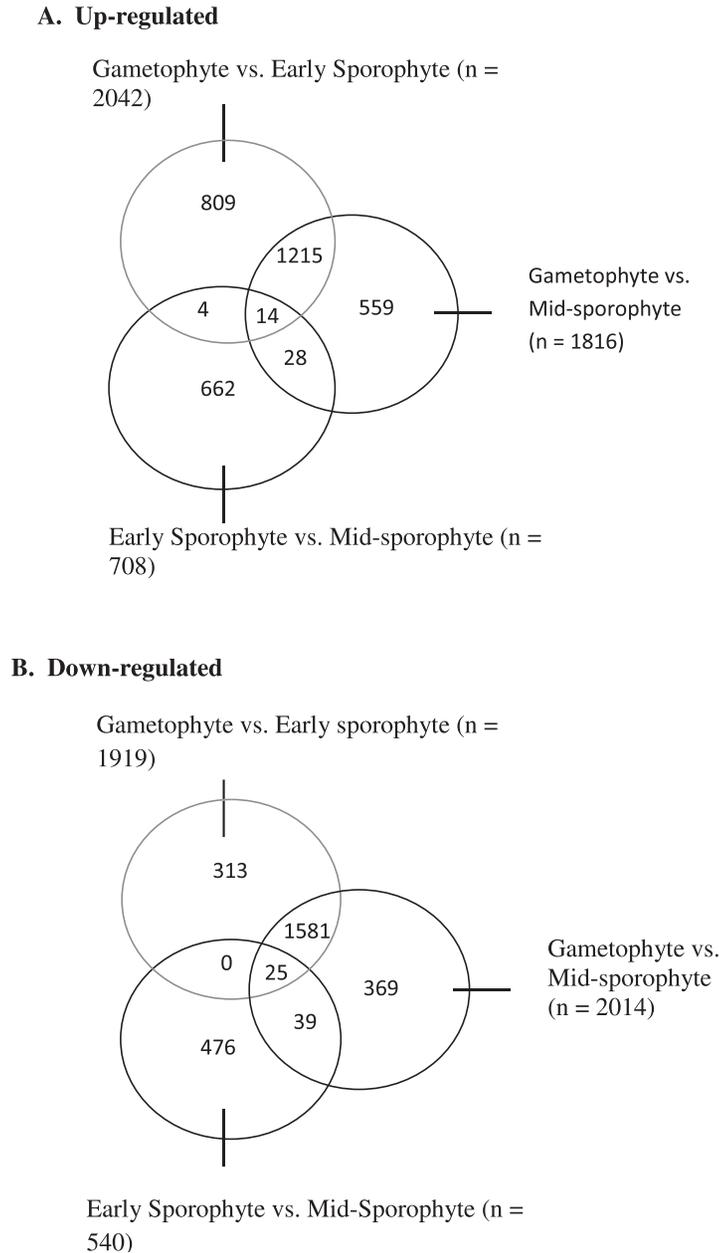


Fig. 2. Global analysis of differences in transcripts between the gametophyte and sporophyte of *Physcomitrella*. Pairwise microarray comparison of RNA extracted from gametophyte, early sporophyte, and mid-sporophyte samples were performed. The overlap between the genes identified as significantly upregulated (A) or downregulated (B) when gene expression in gametophyte tissue was compared either to early sporophyte tissue or mid-sporophyte tissue, or when early sporophyte tissue was compared with mid-sporophyte tissue is shown. The total number of genes identified as being differentially expressed in each comparison is indicated. Significance was set at $P < 0.01$, with a fold-change of 1.5 (log₂ scale).

microarray represents 31 000 independent transcripts within the *Physcomitrella* genome, the combined number of up- and downregulated genes (3961) equates to 12.8% of the entire transcriptome on the array. When mid-sporophyte (MS) and G data are compared a similar picture emerges. As shown in Fig. 2, transcript levels for 1816 genes are upregulated in the MS sample relative to the G sample, with 2014 genes showing a significantly decreased transcript level (Supplementary Tables S13 and S16). This represents a 12.4% shift in transcriptome expression between the G and MS phase, comparable to the value (12.8%) calculated for the G to ES transition.

In contrast, comparison of the early and mid-stage sporophyte transcriptomes reveals a much smaller shift in gene expression. Thus, as shown in Fig. 2, transcript levels for 708 genes are upregulated and 540 genes downregulated during the ES to MS transition. The total number of genes showing a significant change in level during the ES to MS transition (1248) represents only 4% of the transcriptome represented on the microarray (Supplementary Tables S14 and S17). Moreover, only a few (8.5%) of the genes identified as changing transcript level in the comparison of early and mid-stage sporophyte development were detected in the comparison of

gametophyte and sporophyte tissue, whereas a large percentage (>70%) of the genes detected as changing transcript level at the gametophyte to early sporophyte transition were also identified as showing a significant difference in transcript level when gametophyte and mid-stage sporophyte tissue were compared. The genes identified as upregulated or down-regulated in the comparisons of the G, ES, and MS tissues and their gene ontology (as defined by molecular function, biological process, and cell component) are summarized in [Supplementary Table S2](#) and shown in [Supplementary Tables S3–S8](#).

To validate the microarray data a number of analyses were performed. Firstly, RT-PCR experiments were performed on lead genes resulting from the analysis shown in [Fig. 2](#). These genes were chosen because they showed either high predicted levels of differential gene expression or were expected to be expressed similarly in both tissues. RNA was extracted from either protonemal gametophyte or dissected sporophyte tissue, cDNA synthesized, and then PCR performed using primers for genes shown in [Supplementary Table 1](#). As shown in [Fig. 3](#), the majority of genes tested showed an expression pattern consistent with that predicted by the microarray analysis, including both sporophyte- and gametophyte-specific expression. As a second validation step for the microarray data, previous analyses of *Physcomitrella* gene expression were compared with this microarray-based analysis of gametophyte and sporophyte tissue. For example, the microarray data showed that both *PpLFY1* and *PpLFY2* are expressed in gametophyte and sporophyte tissue and did not reveal any significant difference in transcript levels between the two tissue types, which is consistent with published data ([Tanahashi et al., 2005](#)). It should be noted that although *PpLFY1* and *PpLFY2* show no difference in transcript levels between gametophyte and sporophyte tissue, altered expression of these genes leads to a clear phenotype specifically in the sporophyte, highlighting the potential limitation of analyses restricted to transcriptomic data. Previous analysis has identified three KNOX-related genes (*MKN2*, *MKN4*, and *MKN5*) as being specifically sporophyte expressed ([Sakakibara et al., 2008](#)). These genes were not identified in the microarray analysis as being differentially expressed. However, it should be noted that the RT-PCR analysis used in the previously published work required 40 amplification cycles to detect these transcripts and it is highly possible that such relatively low transcript levels are below the sensitivity of the microarrays used. Again, although microarray approaches are useful in providing global views on transcript expression pattern, care must be taken in their interpretation since it is highly unlikely that all transcripts will be detected. The definition of differential expression is to a large extent determined by the statistical analysis performed and this study took a rather conservative approach in the analysis to minimize the identification of potential false positives.

Overall, the RT-PCR results indicate that the microarray data can be used with confidence to indicate gene expression levels in the relevant tissue samples but that, as with all such data, some caution needs to be used in their functional interpretation. It should also be noted that the dissection approach

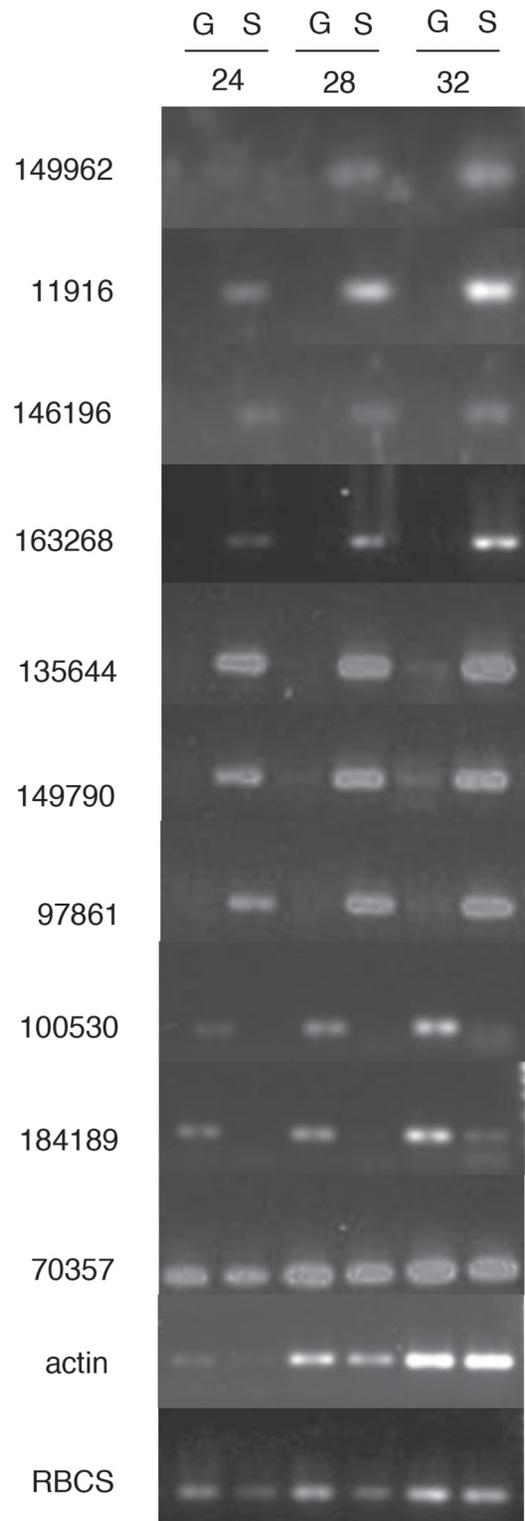


Fig. 3. RT-PCR validation of microarray data. RNA was extracted from gametophyte or sporophyte tissue and reverse transcribed, and then PCR was performed using primers designed to amplify a spectrum of lead genes identified as being differentially expressed in the microarray comparison of gametophyte and sporophyte tissue ([Fig. 2](#)). Amplified DNA was removed at different cycle numbers (24, 28, 32) and run on 1% (w/v) agarose gel before visualization. The *Physcomitrella* gene id/names are indicated on the left.

prevented analysis of the very earliest stages of sporophyte formation (i.e. clearly there are stages prior to the defined ES stage). An analysis of such tissue would require a laser micro-dissection approach coupled with RNA amplification, which was beyond the present investigation.

Taken together, these data suggest that the transition from gametophyte to sporophyte in mosses is accompanied by a relatively large shift in the transcriptome (>12% as represented on the microarray), whereas early to mid-sporophyte development involves much fewer changes in transcript level. It is striking that these data indicate far greater transcriptomic changes than reported in the recent analysis of the gametophyte-to-sporophyte transition in the closely related moss, *F. hygrometrica* (Szovenyi *et al.*, 2011), who used next generation sequencing to analyse RNA extracted from gametophores (the leaf-like organs on which the antheridia and archegonia develop) and growing sporophytes. They then used the *P. patens* genome to tether *Funaria* sequences, which allowed them to map 34% of the *Funaria* transcripts. Of these unambiguously identified *Funaria* sequences, a relatively small percentage (4.2%, 558 genes) was differentially expressed between the gametophore tissue and the sporophyte tissue. Of these, 277 were upregulated and 281 were downregulated. These values compare with those reported here for the microarray comparison of *Physcomitrella* gametophyte and early sporophyte tissue in which transcripts for 2042 genes were upregulated and 1919 genes were downregulated, representing 12.8% of the 31 000 gene sequences on the array. Clearly the two analyses give quantitatively distinct results.

The analysis by Szovenyi *et al.* depended on the ability to map *Funaria* RNA sequences onto the *Physcomitrella* genome. Although partially successful, the total number of unambiguous sequences that were identified by this approach was limited, decreasing the total pool of genes analysed from over 30 000 on the array to fewer than 14 000 via the sequencing route. Both methods used relatively stringent criteria for calling expression as being significantly different, with the reliability of the data depending on duplicate biological samples in the sequencing analysis approach and triplicate biological samples with triplicate technical replicates per biological sample with the microarray analysis reported here.

One further major difference between the two analyses was the developmental stage of the gametophyte. The study by Szovenyi *et al.* used gametophores consisting of leaves and rhizoids whereas this analysis used the earlier protonemal stage as the source of gametophyte RNA. The protonemal stage is characterized by mainly filamentous growth, whereas the gametophore involves a switch of cellular organization into leaves, stems, and rhizoids. Therefore, one possibility is that it is this switch in cellular organization during gametophyte development which underpins the major change in transcriptome expression during moss development, with the switch from gametophyte to sporophyte reflecting a more limited change in gene expression pattern. However, an analysis of the types of genes underpinning the altered expression patterns in the two studies suggests that this is not the case, as described below.

Transition to the sporophyte involves major changes in gene expression that are linked with carbohydrate and lipid/fatty acid metabolism

Carbohydrate metabolism Comparison of the biological processes represented by the genes whose expression are up- or downregulated on the transition from protonemal gametophyte to early sporophyte development and during the development from early to mid-stage sporophyte provides an insight into the types of changes occurring (Fig. 4). The GO terms with the largest absolute number of changes comparing both G vs. ES and G vs. MS samples are involved in carbohydrate metabolism. Thus, the G vs. ES comparison reveals changes in 25% of all genes annotated in the *Physcomitrella* genome linked with this process (significant at $P = 5.9E-09$) (Fig. 4A and Supplementary Tables S3, S4, and S9) and the G vs. MS comparison indicates a switch in expression of 22.7% of all genes annotated as being involved in carbohydrate metabolic processes ($P = 2.5E-06$) (Fig. 4B and Supplementary Tables S5, S6, and S10). In contrast, comparison of ES vs. MS samples indicates changes in only 4.7% of all of the genes associated with this GO term and this is not significant ($P = 0.35$) (Fig. 4C and Supplementary Tables S7, S8, and S11). Carbohydrates are involved in many processes, including the production of structural components, cell signalling, metabolism for immediate energy or conversion to starch or lipid storage, and the development of spores (Koch, 1996; Graham and Wilcox, 2000; Glime, 2007a). Interestingly, although the total number of gene expression changes identified in the *Funaria* gametophyte/sporophyte transition was much lower than reported here, as already discussed, genes linked with carbohydrate metabolism also represented the most common GO term distinguishing the two tissue types in that study. Linked with this, it is noticeable that both studies identified GO terms 'photosynthesis' and 'electron transport' as being good discriminators of gametophyte and sporophyte tissue. Thus, in this study, the ES and MS stages were distinguished by virtually all genes linked with photosynthesis being downregulated relative to the G stage tissue (Fig. 4A, B and Supplementary Tables S2, S9, and S10). These genes represent 39% (ES; $P = 1.95E-08$) and 41.8% (MS; $P = 8.11E-11$), respectively, of all the *Physcomitrella* genes annotated linked with photosynthesis. In contrast, comparison of the transcriptome of ES and MS tissue revealed that only a small level fraction (0.9%) of genes associated with the GO term photosynthesis were differentially expressed and this was not statistically significant (Supplementary Tables S2 and S11). Finally, changes in the GO term glycolysis appeared in all three comparisons (G vs. ES, G vs. MS, and ES vs. MS) but the differences were only marginally significant in the G vs. MS comparison ($P = 0.06$). However, it is noticeable that in the ES vs. MS comparison, all genes of this class were recorded as showing an increase in transcript level. Overall, both this study and that of Szovenyi *et al.* (2011) indicate that the gametophyte-to-sporophyte transition is marked by major changes in carbohydrate and energy metabolism.

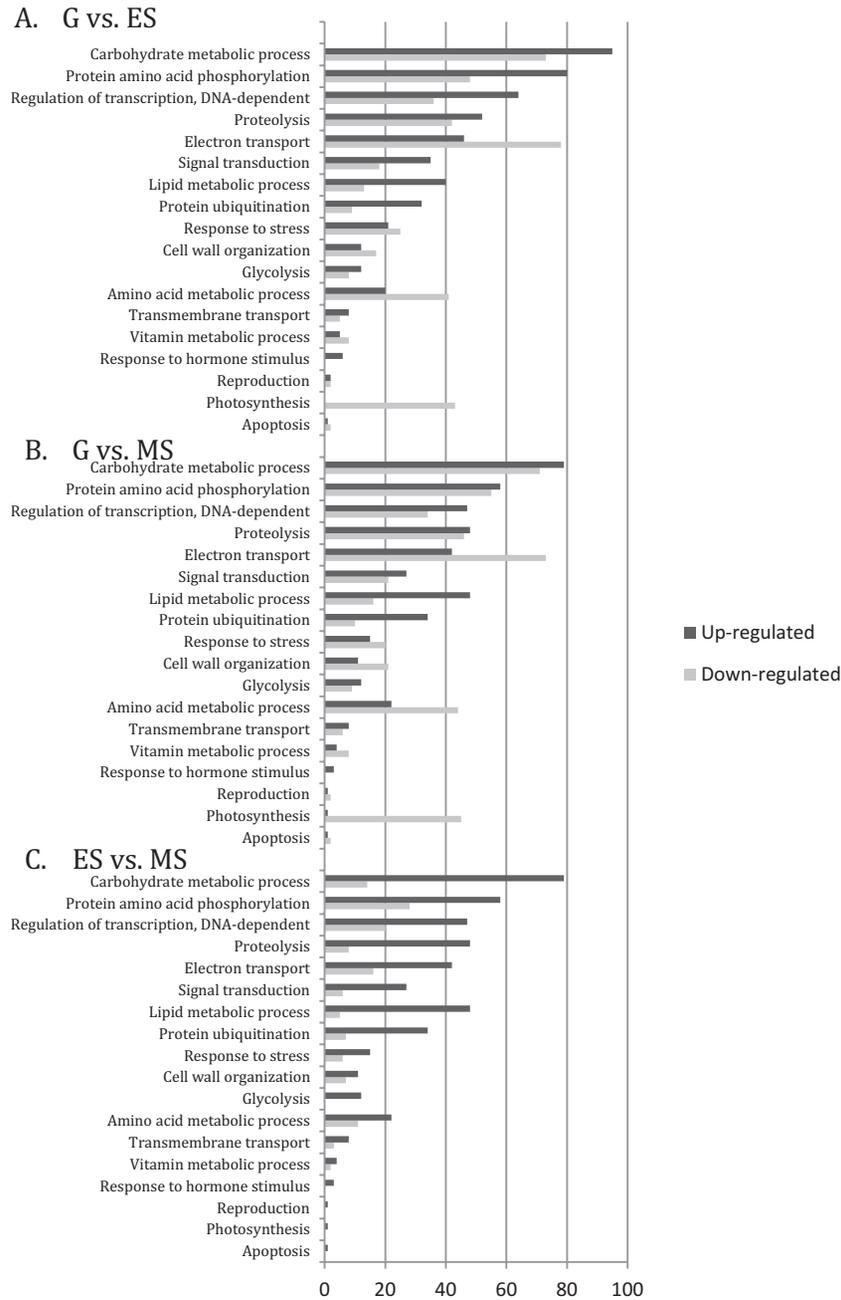


Fig. 4. Comparative functional gene ontology analysis for *Physcomitrella* gametophyte and sporophyte tissue: (A) gametophytic tissue compared to early sporophyte tissue (G vs. ES); (B) gametophyte tissues compared to mid-sporophyte tissue (G vs. MS); (C) early sporophyte tissues compared with mid-sporophyte tissue (ES vs. MS). The charts show the number of genes that fall into a particular gene ontological (GO) term for biological process category in the comparison of gene expression derived from Fig. 2. Upregulated genes are represented by dark grey bars and downregulated genes are represented by light grey bars. GO terms are organized in decreasing order of the number of upregulated genes associated with each category.

Genes affected included those which encode for cellulase (involved in cell-wall construction during cell expansion and fruit ripening; Rose and Bennett, 1999) and citrate synthase (involved in the electron transport chain and amino acid biosynthesis; Wiegand and Remington, 1986). This is to be expected if one considers the structural and reproductive changes that take place between the gametophyte and sporophyte. UDP-Glucose 4-epimerase/UDP-sulfoquinovose synthase was more strongly

represented in all three of the upregulated sporophyte datasets than was the case in the downregulated ones (i.e. has a lower expression in the gametophyte). Various isoforms of the UDP-glucose 4-epimerase gene influence growth and cell-wall biosynthesis, act in stress situations, or influence pollen development in *Arabidopsis* (Rosti *et al.*, 2007). UDP-sulfoquinovose synthase on the other hand is involved in maintaining balance in the thylakoid membrane (Benning, 1998; Sanda *et al.*, 2001). Another

gene, xyloglucan:xyloglucosyl transferase, is involved in the cell wall and this enzyme's substrate, xyloglucan, is involved in developmental processes such as pollen formation in *Arabidopsis* (Otegui and Staehelin, 2000; Freshour *et al.*, 2003) and *Capsicum annuum* (Barany *et al.*, 2010). Xyloglucans have been identified in the *Physcomitrella* cell wall (Pena *et al.*, 2008), but no data are available documenting their function in the moss sporophyte.

Both developmental stages contain green tissue and are, thus, presumably photosynthetic. However, the sporophyte stage represents a commitment of the plant to spore differentiation and eventual death of the sporophyte (Glime, 2007b). To what extent the gametophyte nourishes the sporophyte remains a point of debate, but the combined microarray data from the two studies are consistent with the gametophyte (protonema and gametophore) having a primary role in photosynthesis, resource acquisition, and long-term maintenance of the plant, with the sporophyte (which has high energy costs linked with spore formation and dispersal) being predominantly reliant on the gametophyte for both carbon and energy. A decrease in transcript level of genes linked with electron transport and an increase in transcripts related to glycolysis in the sporophyte also fits with an organ whose energy requirements cannot be met by oxidative respiration alone.

A potential complication of interpreting these data is that a 15 °C temperature shift was required to elicit sporophyte development in *Physcomitrella*, which is likely to influence metabolic-linked gene expression. However, subsequent to this low temperature elicitation, sporophyte development progressed in identical conditions to gametophyte growth, thus any temperature-related difference would need to be maintained despite a return to normal growth conditions. Moreover, the similarity of the types of carbohydrate metabolism-linked genes identified in the two independent studies suggests that this temperature shift is not a major confounding issue.

Lipid and fatty acid metabolism An analysis of the transcript levels for genes associated with lipid metabolism also supports the inference that the sporophyte is distinguished from the gametophyte by a distinct biochemical state, in particular one which is dependent on the import of carbon and energy. Gene expression linked with lipid metabolism tended to be upregulated in the gametophyte/sporophyte comparison (Fig. 4). For example, in the G vs. MS comparison, 23.2% of genes with the GO term 'lipid metabolic processes' showed altered transcript level, and this was significant at the $P = 0.001$ confidence level (Supplementary Tables S2 and S10). Analysis of this GO term indicates a shift of 19.2% of these genes on the G to ES transition ($P = 0.16$) (Supplementary Table S2). Such major shifts were not identified during the ES to MS transition, with only 3.6% of the genes with this GO term being differentially expressed.

As with the recorded shift in genes linked with carbohydrate metabolism, changes in lipid metabolism-associated gene expression are indicative of a major shift in metabolism occurring during sporophyte formation. Lipid and fatty acid metabolism are required for pollen development in *Arabidopsis*, as well as wound response, biotic defence, storage, and signalling (Murphy, 1993; Porta and Rocha-Sosa,

2002; Ryu, 2004; Senger, 2005; Rensing *et al.*, 2007; de Azevedo Souza *et al.*, 2009). If photosynthesis in the sporophyte is insufficient to provide the carbon requirements of the developing sporophyte, then import and potentially local storage and use of alternative carbon sources would be required. Lipids could fulfil this function. It is interesting to note that no major changes in the expression pattern of these genes were noted during later sporophyte development (ES to MS). This is the developmental stage when spore formation begins and one might expect this process to be linked with intense lipid metabolism (Huang *et al.*, 2009). It is possible that many of the metabolic pathways required for spore formation are established early in sporophyte development, laying down the foundation for a later, rapid process of spore differentiation. Finally, the potential role of fatty acid metabolism in pollen development has also been noted (Wang *et al.*, 2008) and it will be interesting to explore the types of lipid metabolism that are involved in moss spore formation.

Other metabolic changes

In the G vs. MS comparison, 21.5% of all *Physcomitrella* genes with the GO term 'cellular amino acid metabolic process' showed a change in transcript level with the majority showing a downregulation in transcript level, which was highly significant ($P = 0.009$) (Supplementary Tables S2 and S10). Comparison of G vs. ES samples showed that 19.5% of the genes displayed an altered transcript level, but this was not significant ($P = 0.12$) (Supplementary Tables S2 and S9). However, when considering only those genes with this GO term which were downregulated, the differences were significant ($P = 1.46E-04$). The functional reason behind potential changes in amino acid metabolism is unclear but it is a further indication that the metabolism of the early gametophyte and sporophyte are distinct.

Cell walls

As noted already, altered carbohydrate metabolism appears as a major signature of the differences that distinguish the gametophyte and the sporophyte. This was also reflected in altered transcript levels of genes implicated in carbon storage and cell-wall formation. Thus, 39% of all genes linked with 'cell-wall organization' in the *Physcomitrella* genome were identified as being significantly differentially expressed in the G vs. ES comparison ($P = 5.0E-06$), with 43% being identified in the G vs. MS comparison ($P = 3.38E-08$). In the ES vs. MS comparison it was 12% ($P = 0.005$) (Supplementary Tables S2, S9, S10, and S11). The sporophyte generation has a distinct morphology and undergoes a specific pathway of differentiation. This includes the generation of spores which are characterized by a highly resistant cell wall. At the same time, the protonemal stage has a distinct cellular organization which might be reflected in specific cell-wall structure.

This analysis supports the hypothesis that changes in cell-wall structure and function distinguish the protonemal and sporophyte stages of moss development and identifies a swathe of genes whose products are potentially involved in this process. For example, genes implicated in xyloglucan

processing were identified as being upregulated in sporophyte tissue. These cell-wall polymers have not been detected in three charophycean green algae species (Manhart and Palmer, 1990; Kranz *et al.*, 1995) but have been identified in moss gametophytes, leading to the suggestion that xyloglucans might have played an important role in the transition to terrestrial life. This hypothesis is further supported by the reports that xyloglucan:xyloglucosyl transferases are involved in pollen development in both *Arabidopsis* (Otegui and Staehelin, 2000; Freshour *et al.*, 2003) and *Capsicum annuum* (Barany *et al.*, 2010). Similarly, genes encoding UDP-glucose 4-epimerase were identified as being upregulated in sporophyte tissue and this enzyme has also been implicated in pollen development in *Arabidopsis* (Rosti *et al.*, 2007).

Changes in transcription factor gene expression

Major changes in the transcriptome suggest that altered transcription factor activity is likely to underpin the dramatic developmental changes involved in the alternation of generations. The data do indeed indicate that there is a change in the expression of genes linked with the regulation of transcription on this transition (Fig. 4A, B and Supplementary Tables S2, S9, and S10). Szovenyi *et al.* (2011) also identified GO terms linked with transcription factors as being a good discriminator of gametophyte and sporophyte tissue. Strikingly, the identical 64 transcription factors were identified as being upregulated in both studies (*Physcomitrella* G vs. ES and *Funaria* gametophore vs. sporophyte), despite the different gametophyte tissue used in the two studies (Fig. 5). This exact correlation from two independent studies using different species suggests that these transcription factors play a crucial role in the gametophyte to sporophyte transition. Interestingly, however, none of the 36 transcription factors identified as being downregulated in the G to ES transition in this study were identified in the *Funaria* experiment, nor were any of the 34 downregulated transcription factors identified in the G vs. MS comparison. These results suggest that the gametophyte-to-sporophyte transition is linked with both up- and downregulation of transcription factor gene expression; however, the *Funaria* study may have missed a large number of the genes whose expression is repressed during this shift. The reasons for this are unclear but may reflect the overall lower number of genes that can be unequivocally identified using a high throughput sequencing approach and an indirect anchor genome.

These data provide a first glimpse of the types of transcription factors associated with sporophyte development. Thus, 12 transcription factors were identified as being differentially expressed during the transition from early to mid sporophyte. These include a MADS box domain protein, GATA transcription factors, and pathogenesis-related transcription factors. The development of the moss sporophyte is distinguished by the acquisition of a number of traits which are thought to be linked to the ability of the sporophyte to withstand abiotic stresses associated with a terrestrial environment. For example, the moss sporophyte develops stomata (Chater *et al.*, 2011) which are thought to allow better control

of water balance, and the differentiation of spores involves the development of novel cell-wall characteristics which allow them to cope with both a dry and UV-rich environment (Wallace *et al.*, 2011). The transcription factors identified in this study will help to identify the key players involved in the acquisition of these developmental novelties which enabled the gradual dominance of the sporophyte generation on land.

Examination of the types of transcription factor showing differential expression between gametophyte and sporophyte tissues revealed some interesting correlations. Two MADS-box transcription factor genes (an unspecified MADS box transcription factor and *PpMAI2*) were found in both the G vs. ES and G vs. MS comparisons and downregulated in the sporophyte with respect to the gametophyte. A third MADS box gene (*PpMADS2*) noted in the G vs. ES comparison was also downregulated in the sporophyte with respect to the gametophyte. *PpMADS2* and *PpMAI2* are MIKC* MADS domain proteins and the MIKC* group genes have previously been identified to be mainly expressed in the *Arabidopsis* male gametophyte (Kofuji *et al.*, 2003; Verelst *et al.*, 2007), with one member of the group being expressed in the female gametophyte (Lehti-Shiu *et al.*, 2005). According to PlantTFDB 2.0 (Zhang *et al.*, 2011) *PpMADS2* is orthologous to *AGAMOUS-LIKE66* and *AGAMOUS-LIKE104*, both of which are involved in pollen development in *Arabidopsis* (Adamczyk and Fernandez, 2009). Previous investigations on MADS box genes in the related species *Funaria* found that orthologues of *PpMADS2* and *PpMAI2* were more strongly expressed in gametophyte tissue relative to sporophyte tissue (Zobell *et al.*, 2010), suggesting that it is conserved between the two bryophytes. Only one MADS-box gene (*PpTIM1*) was noted to be upregulated on the transition from ES to MS stage. This gene was not noted as being significantly differentially expressed in the comparisons with gametophyte tissue, suggesting that it plays a specific role in the developing sporophyte. Further genetic analysis of these transcription factors may reveal evolutionary conserved roles in sporophyte formation and development.

The role of post-transcriptional changes and hormone signalling in the gametophyte to sporophyte transition

Although major changes in transcription factor gene expression occur on the gametophyte-to-sporophyte transition, this analysis indicated that post-transcriptional regulation is also likely to play a role in this developmental switch (Fig. 4). MAPKs were identified as being differentially expressed and similar gene products have been implicated in various aspects of cytoskeleton organization and signalling in higher plants (Reiland *et al.*, 2009; Bartels *et al.*, 2010; Rodriguez *et al.*, 2010), including pollen development (Chaiwongsar *et al.*, 2006) and stomatal development (Lampard *et al.*, 2009). The study on *Funaria* gametophore to sporophyte transition also identified genes associated with the GO term 'protein amino acid phosphorylation' as distinguishing the two samples, again suggesting that, despite the distinct stages of gametophyte tissue used, the two studies identify genes which play a role in the sporophyte transition. This current study

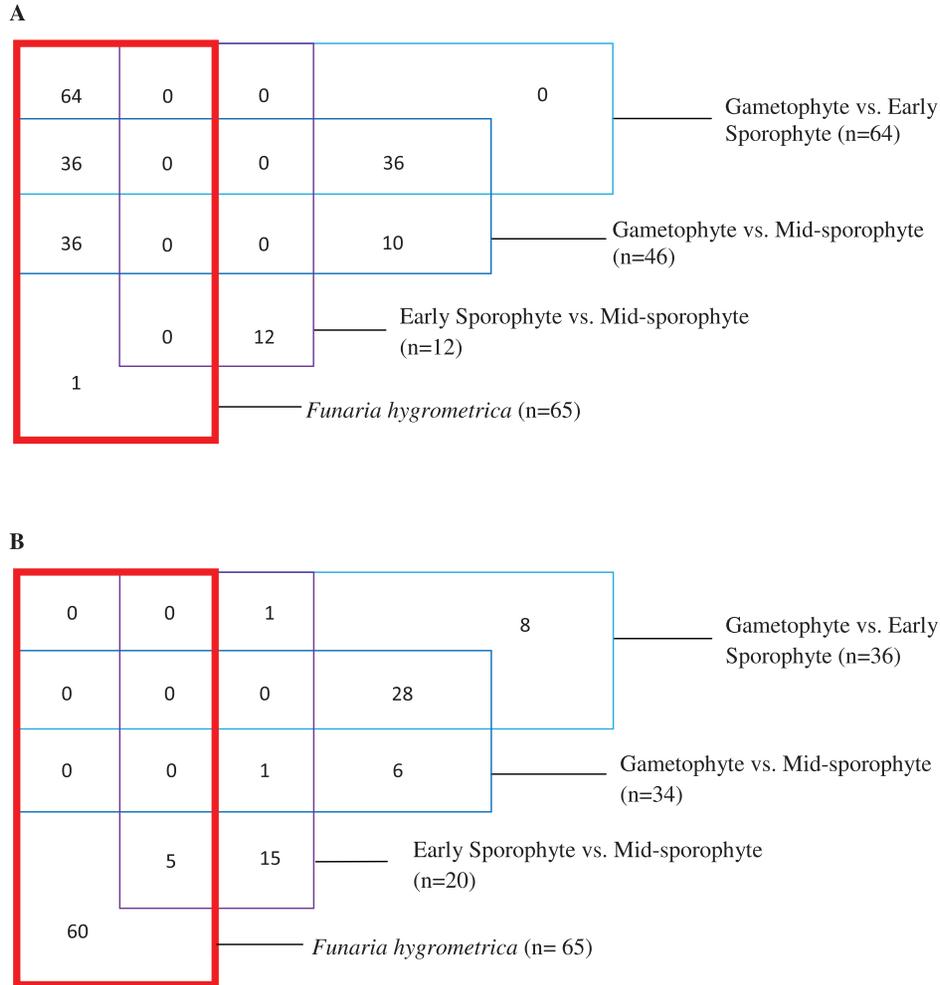


Fig. 5. The pattern of transcription factor gene expression during the gametophyte-to-sporophyte transition in moss: (A) upregulated genes (B) downregulated genes. The distribution of genes encoding transcription factors whose expression is differentially regulated between gametophyte vs. early sporophyte, gametophyte vs. mid-sporophyte, and early sporophyte vs. mid-sporophyte in *Physcomitrella* and the overlap of these transcription factor gene identities with those characterized as being differentially expressed on the gametophyte vs. sporophyte transition in *Funaria* (bold box), described in Szovenyi *et al.* (2011) (this figure is available in colour at *JXB* online).

also analysed the early and mid-sporophyte stages, with the majority of genes linked with protein phosphorylation being upregulated in the G vs. ES comparison, whereas in the G vs. MS comparison the number of genes showing up- and down-regulation of transcript level was very similar. This suggests that early sporophyte development might show a distinct pattern of post-transcriptional regulation compared with later stages of sporophyte development. As suggested by the analysis of carbohydrate and lipid metabolism, the data are consistent with the idea that the fates of many tissues of the moss sporophyte are determined early in sporophyte development.

Hormone signalling is closely linked with post-transcriptional protein modifications and is associated with numerous developmental transitions and responses in higher plant. For example, the expression of some auxin response genes is altered on the transition from gametophyte to sporophyte including an auxin response factor (ARF) (Sessions *et al.*, 1997; Guilfoyle and Hagen, 2007) and Nup160 which has been implicated in cold tolerance, flowering time regulation,

and seedling growth in *Arabidopsis* (Dong *et al.*, 2006; Parry *et al.*, 2006). Ethylene-responsive transcription factors were also differentially expressed (Licausi *et al.*, 2010; Liu *et al.*, 2011). Finally, the expression of *GLP2/GID2* (implicated in gibberellin signalling) was upregulated in the early and mid-sporophyte stages relative to the gametophyte, but was not differentially expressed in the ES vs. MS comparison. The role of gibberellins in moss is unclear (Hirano *et al.*, 2007; Yasumura *et al.*, 2007), but there does appear to be a role for this class of growth factor in *Physcomitrella* spore germination (Anterola *et al.*, 2009).

Changes in stomata-associated gene expression in the moss sporophyte

Physcomitrella, like most mosses, produces stomata only on its sporophyte and not on the gametophyte. Transcripts homologous to many *Arabidopsis* genes controlling stomatal development were detected in the microarray, and several of

these were significantly differentially expressed in this analysis between the protonemal tissue and the early and mid-sporophytic tissues (Supplementary Table S18A). Of particular interest is the sporophyte upregulation of Phypha_209063, a homologue to the *Arabidopsis* bHLH transcription factor ICE1/SCREAM which forms a heterodimer with bHLHs SPCH, MUTE, and FAMA to induce stomatal fate transitions (Kanaoka *et al.*, 2008). MUTE- and FAMA-like genes *PpSMF1* (Phypha_184221) and *PpSMF2* (Phypha_156270) are present in *Physcomitrella* (MacAlister and Bergmann, 2011). Interestingly, *PpSMF1* is significantly upregulated in ES vs. MS. A bHLH heterodimer may therefore be implicated in the initiation and proliferation of stomatal precursors in the moss spore capsule as it is in angiosperms.

Negative regulation of *Arabidopsis* stomata occurs via mitogen-activated protein kinases (MAPKs) in a phosphorylation cascade involving YODA, MKKs 4/5, and MPKs 3/6 (Bergmann *et al.*, 2004; Wang *et al.*, 2007; Lampard *et al.*, 2009). Homologues to all these MAPKs are expressed in the developing sporophyte, although not differentially. Homologues to components upstream of these cascades, the EPIDERMAL PATTERNING FACTOR (EPF) family peptides (Hara *et al.*, 2007, 2009; Hunt and Gray, 2009; Abrash and Bergmann, 2010; Abrash *et al.*, 2011), the leucine-rich repeat receptor kinases ERECTA (ER), ERECTA-LIKE 1 (ERL1), and ERL2 (Shpak *et al.*, 2005), and a leucine-rich repeat-receptor-like protein, TOO MANY MOUTHS (TMM) (Yang and Sack, 1995; Nadeau and Sack, 2002) are also expressed in the sporophyte. Intriguingly, *PpTMM* (Phypha_51089), a homologue of *AtTMM* (Peterson *et al.*, 2010), is not differentially expressed between gametophyte and early sporophyte, but is significantly downregulated in MS vs. G. Furthermore, several ERECTA-like kinase transcripts (Villagarcia *et al.*, 2012) are differentially expressed; *PpERL1a* (Phypha_152090) is significantly downregulated across analyses, whereas *PpERL2b* (Phypha_136017) is significantly upregulated in the sporophyte. Phypha_117216 and Phypha_205498, two homologues of *STOMATAL DENSITY AND DISTRIBUTION 1 (SDD1)*, a subtilase which interacts with TMM (Berger and Altmann, 2000; von Groll *et al.*, 2002), are significantly upregulated in the sporophyte. These expression patterns strongly suggest a specialized role for subtilases in the young capsule and warrant further investigation.

The expanding *Physcomitrella* sporophyte possesses mature and active stomata (Supplementary Fig. S3; Chater *et al.*, 2011) and expression of many homologues to *Arabidopsis* genes present in developed guard cells can be detected in this analysis (Supplementary Table S18B). Key genes that are differentially expressed in this experiment include *OPEN STOMATA 1 (OST1)*-like homologues *PpOST1-3* (Phypha_195464) and *PpOST1-2* (Phypha_106968) (Thelander *et al.*, 2009). These are significantly upregulated in both the early and the mid-stage sporophyte, and their expression may contribute to the partial redundancy observed in *PpOST1-1* (Phypha_194508) function in *Physcomitrella* stomatal closure (Chater *et al.*, 2011). *AtOST1* is a Snf-related serine/threonine protein kinase (SnRK2) which positively regulates ABA-induced stomatal closure in *Arabidopsis* (Mustilli *et al.*, 2002).

PpHA5 (Phypha_234515), a plasma membrane H⁺ ATPase/proton transporter (Garcia-deblas *et al.*, 2007; Okumura *et al.*, 2012), is upregulated in the mid-sporophyte, but not early sporophyte. *PpHA5* has 84.7% similarity to guard cell-associated *Arabidopsis OPEN STOMATA 2 (OST2)* (Merlot *et al.*, 2007) and 80% similarity to *AtHA5*, which is upregulated in guard cells in response to ABA and relative humidity change (Bauer *et al.*, 2013). Phypha_136150, the only *Physcomitrella* homologue to *Arabidopsis ENHANCED RESPONSE TO ABSCISIC ACID 1 (ERA1)*, a farnesyltransferase β subunit that negatively regulates guard cell ABA signalling (Pei *et al.*, 1998; Allen *et al.*, 2002), is significantly upregulated in both early and mid-sporophyte tissue. *PpECA5* (Phypha_223041), a homologue to PIIA-type Ca²⁺-ATPases *AtECA1* and *AtECA3* (Pedersen *et al.*, 2012), is also significantly expressed in the capsule. *AtECA1* and *AtECA3* both show high expression in guard cells (Li *et al.*, 2008; Mills *et al.*, 2008) and are likely to be involved in stomatal calcium-dependent ABA signalling. *PpSUT4C* (Phypha_194787) and *PpSUT4A* (Phypha_194644), moss sucrose transporters (Reinders *et al.*, 2012), are both significantly upregulated in the sporophyte. *Arabidopsis* sucrose transporters *AtSUC2* and *AtSUC3* are expressed in guard cells (Meyer *et al.*, 2004) and are putatively involved in the convergence of osmoregulatory and CO₂ sensing pathways through carbohydrate metabolism (Vavasseur and Raghavendra, 2005). *Physcomitrella* has five β carbonic anhydrases (Tanz *et al.*, 2009), of which three are strongly downregulated in the sporophyte, while one, *Pp β CAII* (Phypha_130288), is significantly upregulated in both the early and mid-sporophyte. β -Carbonic anhydrases are implicated in guard cell CO₂ sensing and signal transduction in *Arabidopsis* (Hu *et al.*, 2010). The sporophyte expression of *Pp β CAII* may indicate a guard cell specific role for this β carbonic anhydrase.

Taken together, the sporophyte expression profile of these *Physcomitrella* genes strongly suggests that both moss stomatal development and guard cell physiology share core genetic mechanisms with *Arabidopsis*. The possibility that these signalling pathways are highly conserved supports the theory that stomata are monophyletic (Raven, 2002; Ligrone *et al.*, 2012) and reinforces the pivotal role stomata have played in the evolution of land plants and terrestrial life (Beerling, 2007).

In conclusion, these data indicate that the transition from the gametophyte to sporophyte stage in a basal non-vascular land plant with a gametophyte dominant lifecycle (*P. patens*, moss) is accompanied by major changes in gene expression profile. They also provide an analysis of the gene expression changes that occur during the early stages of moss sporophyte development. The data support the idea that the moss sporophyte is highly dependent on the gametophyte for carbon and energy to fuel the process of sporogenesis. The sporophyte regulation of genes implicated in stomatal development and physiology, however, suggests that stomagenesis and the regulators of stomatal aperture are deeply conserved across land plants. These data provide a resource for the future identification and characterization of further genes involved in the acquisition of key traits linked with the evolutionary success of the sporophyte in the colonization of the land.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Plot densities of raw and normalized microarray data.

Supplementary Fig. S2. MA plots of raw and normalized microarray data.

Supplementary Fig. S3. Stomatal development in the expanding *Physcomitrella* sporophyte.

Supplementary Table S1. List of primers used in semi-quantitative RT-PCR.

Supplementary Table S2. Number of genes in GO categories.

Supplementary Table S3. List of significantly upregulated gene transcripts (G vs. ES comparison).

Supplementary Table S4. List of significantly downregulated gene transcripts (G vs. ES comparison).

Supplementary Table S5. List of significantly upregulated gene transcripts (G vs. MS comparison).

Supplementary Table S6. List of significantly downregulated gene transcripts (G vs. MS comparison).

Supplementary Table S7. List of significantly upregulated gene transcripts (ES vs. MS comparison).

Supplementary Table S8. List of significantly downregulated gene transcripts (ES vs. MS comparison).

Supplementary Table S9. Genes showing significantly altered transcript level (G vs. ES comparison) listed by metabolic process.

Supplementary Table S10. Genes showing significantly altered transcript level (G vs. MS comparison) listed by metabolic process.

Supplementary Table S11. Genes showing significantly altered transcript level (ES vs. MS comparison) listed by metabolic process.

Supplementary Table S12. The fold change of genes (G vs. ES comparison).

Supplementary Table S13. The fold change of genes (G vs. MS comparison).

Supplementary Table S14. The fold change of genes (ES vs. MS comparison).

Supplementary Table S15. Annotation of genes (G vs. ES comparison).

Supplementary Table S16. Annotation of genes (G vs. MS comparison).

Supplementary Table S17. Annotation of genes (ES vs. MS comparison).

Supplementary Table S18. Expression of moss homologues to stomata-associated *Arabidopsis* genes.

Acknowledgements

The authors thank Cheryl Fleming at the University of Sheffield for assistance with the microarray analysis and members of the Fleming, Gray, and Beerling groups for useful discussions. They are extremely grateful to the Clay Foundation for a donation which enabled the microarray analysis. M-T O'D was supported by an EU grant (GIPS network), SW by a NERC studentship, and CC by a BBSRC studentship.

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