REVIEW ARTICLE

The Moss *Physcomitrium (Physcomitrella) patens*: A Model Organism for Non-Seed Plants

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Short title: The Moss *P. patens*

One-sentence summary: Studies of *P. patens*, a powerful non-seed plant model system, have made fundamental contributions to diverse fields, including evolutionary developmental and cell biology.

ABSTRACT

Since the discovery two decades ago that transgenes are efficiently integrated into the genome of *Physcomitrella patens* by homologous recombination, this moss has been a premier model system to study evolutionary developmental biology (evo-devo) questions, stem cell reprogramming, and the biology of non-vascular plants. *P. patens* was the first non-seed plant to have its genome sequenced. With this level of genomic information together with increasing molecular genetic tools, a large number of reverse genetic studies have propelled the use of this model system. A number of technological advances have recently opened the door to forward genetics as well as extremely efficient and precise genome editing in *P. patens*. Additionally, careful phylogenetic studies with increased resolution have suggested that *P. patens* emerged from within *Physcomitrium*. Thus, rather than *Physcomitrella patens*, the species should be named *Physcomitrium patens*. Here we review these advances and describe the areas where *P. patens* has had the most impact on plant biology.

Introduction: Development of *P. patens* as a model species

*Physcomitrella patens* ‘Gransden’ was established as a model species based on cultures derived from a single spore of a sample collected by H.L.K. Whitehouse from a site in Gransden Wood (Huntingdonshire, UK) in 1962. From the 1960s to the 1980s, this moss was primarily used as a genetic system to isolate and study mutants in a variety of processes including plant morphology, hormone biology, nutrition, and responses to gravity (Engel, 1968; Ashton and Cove, 1977; Ashton et al., 1979; Cove, 1983). Early studies on the effects of phytohormones (Reski and Abel, 1985; Knight et al., 1995; Machuka et al., 1999) revealed that the action of abscisic acid (ABA) was conserved throughout the evolutionary history of land plants (Khandelwal et al., 2010; Richardt et al., 2010). In 1997 (Schaefer and Zryd, 1997)
1997) reported that gene targeting via homologous recombination was feasible in *P. patens*, triggering an immediate generation of mutants (Girke et al., 1998), which, following the development of a method to induce sexual reproduction (Hohe et al., 2002) and initial transcriptomic characterization (Rensing et al., 2002), raised this moss to functional genomics model status (Reski and Reg, 2004; Cove, 2005; Frank et al., 2008). Due to its phylogenetic position as a member of the sister lineage to vascular plants and thus other established model species, *P. patens* was soon targeted for genome sequencing (see Genetics and Genomics of *P. patens*) and destined to become a model for evolutionary developmental and cell biological studies (see Hot Topics in *P. patens* Research).

**Systematics, ecology and geography of *P. patens***

*Physcomitrella* belongs to the Funariaceae within the Funariales, a diverse lineage of small, typically once subapically branched plants with terminal sex organs, possessing a sporophyte with a capsule dehiscing via the loss of a lid, and two opposite sets of peristome teeth lining the capsule mouth (Vitt, 1984). *Physcomitrella* is, however, somewhat atypical for the family, as it lacks both a peristome and a differentiated line of sporangial dehiscence. *Physcomitrella* is a rather derived member of the family (Figure 1), having arisen approximately 10 million years ago (Beike et al., 2014; Medina et al., 2019), and may be sister to a clade comprising *Aphanorrhegma serratum* (now *Physcomitrium serratum*), *P. immersum* (both with immersed capsules), and *P. collenchymatum* and *P. sphaericum* (both with capsules emerging on a stalk from among the leaves; (Medina et al., 2019)).

*Physcomitrella patens* was originally recognized by (Hedwig, 1801) in the genus *Phascum* Hedw. (now *Tortula* Hedw., Pottiaceae) due to its small and immersed capsules lacking a peristome. Subsequently, the species was transferred to the genus *Ephemera* (Hampe, 1837), a phenotypically similar genus long considered to be allied to the Funariaceae (Vitt, 1984) but subsequently recognized as resulting from convergent evolution in the Pottiaceae (Goffinet and Cox, 2000). In 1849, *Physcomitrella* was established as a single species, *P. patens* (Bruch and Schimper, 1849). Subsequently, in 1864, the species was moved to *Aphanorrhegma* (Lindberg, 1864), a then monospecific genus established by (Sullivant, 1848) as an eastern North American endemic species resembling *P. patens* except for its equatorial line of dehiscence of the sporangium. In 1851, an argument was made for accommodating *P. patens* in *Physcomitrium* (Mitten, 1851), a hypothesis congruent with phylogenetic (Liu et al., 2012; Beike et al., 2014) and phylogenomic inference (Medina et al., 2018, 2019), whereby the species (Beike et al., 2014) or subspecies of *Physcomitrella*...
sensu (Tan, 1979) do not share a unique common ancestor (Liu et al., 2012) but arose independently from within *Physcomitrium* (Beike et al., 2014; Medina et al., 2018, 2019) (Figure 1). Consequently, all taxa of *Physcomitrella* were transferred to *Physcomitrium*, and the correct name for *Physcomitrella patens* is thus *Physcomitrium patens* (Hedw.) Mitten (Medina et al., 2019). The species is also known by the common name, the spreading earthmoss (Edwards, Sean R.).

**Distribution**

*Physcomitrium* (previously *Physcomitrella*) *patens* occurs in Europe, North America and East Asia (Medina et al., 2015; Higuchi and Takahashi, 2012), whereas *P. readeri* (including *P. californica*) is disjunct between Southern Australia, Western North America, Japan and Western Europe (Medina et al., 2015) (Figure 2). Such ranges are likely the result of a rather recent expansion (Beike et al., 2014). By contrast, *P. magdalenae* is endemic to Africa where it is known to occur in three localities at equatorial latitudes (Figure 2) (Beike et al., 2014). *Physcomitrium serratum* (formerly *Aphanorrhegma serratum*) is endemic to Eastern North America (Goffinet, 2007a). All these species grow in moist open soil along paths or in fields, or in seasonally wet areas such as flood plains of lakes and edges of rice fields, at low to moderate elevations.

**Anatomy and morphology of *P. patens***

Like all land plants, mosses possess a haplodiplontic life cycle in which both generations, the haploid gametophyte and the diploid sporophyte, are multicellular (reviewed in (Rensing, 2016)). However, unlike all extant polysporangiophytes (e.g., seed plants), the dominant moss generation is the haploid phase, and the sporophytic phase is monosporangiate (i.e., the sporophyte bears a single terminal capsule). Starting from the germination of the spore, i.e., the juvenile phase of the gametophyte, the protonema develops (Figures 3, 4). Protonemata are tip-growing filaments that emerge and spread by branching and apical extension and are composed of two major cell types: chloronema and caulonema. Chloronema, the most basal cell type to emerge from the germinating spore, are rich in chloroplasts and have cell plates that are transverse to the long axis of the cell. The apical cell continues to divide and several days after germination transitions into a caulonemal cell, which has obliquely positioned cell plates, initially has fewer chloroplasts, is typically thinner and grows faster than the chloronema. In the absence of light but in the presence of a carbon
source, only caulonemal cells grow against the gravity vector (Cove et al., 1978) and are then also referred to as skotonema. Caulonemata are reminiscent of fungal runner hyphae and may serve to cover long distances. Under the action of ABA, vegetative diaspores (brachycytes or brood cells) develop (Arif et al., 2019), while the formation of specialized side branch initials (so-called buds) represents the transition to the three-dimensional growth phase leading to the hormone-controlled development of gametophores, the leafy shoots of the moss plant (Reski and Abel, 1985; Harrison et al., 2009; Coudert et al., 2015).

Buds develop into erect, foliate gametophores. Gametophores are structured into a stem and phyllids (also called leaflets; non-vascular leaves). Both structures may possess specialized cell types for water and nutrient transport whose formation is controlled by NAC transcription factors, a process similar to sporophytic vasculature development in vascular plants (Xu et al., 2014). The stem is short and considered to be unbranched (see (Coudert et al., 2017)), and its inner cells are differentiated in an axial or central strand of long, narrow, thin-walled cells. The leaves are few, spirally inserted, and spreading when moist. They are typically ovate lanceolate to obovate, with a short, slender apex. The blade is unistratose (comprising a single cell layer) except for the median region, which is differentiated into a costa or midrib extending 2/3 up the leaf. The laminal cells vary from rectangular to rhombic in shape and are chlorophylllose throughout.

Under short day conditions (Hohe et al., 2002), gametangiogenesis occurs, yielding the male and female gametangia mixed in a single cluster at the apex of now adult gametophores (Landberg et al., 2013; Hiss et al., 2017). Individuals are bisexual, with male (antheridia) and female (archegonia) gametangia split between apical clusters (Goffinet, 2007b). In culture, mixed sex organs have rarely been observed (Nakosteen and Hughes, 1978). Instead, antheridia initially develop at the apex of the stem, which subsequently resume growth to develop archegonia, relegating the antheridia to an axillary position (Figure 4). Thus, under optimal growth conditions, male and female sex organs are terminal, but when vegetative growth resumes after male gametangiogenesis, female sex organs may appear to be present in a distinct cluster. Moss male gametes are biflagellate and require liquid water to swim into the archegonia (Renzaglia and Garbary, 2001). After passing the archegonial neck canal cells and reaching the archegonial venter (base), the sperm cells (also known as spermatozoids or antherozoids) fertilize the egg cell to form the diploid zygote. The zygote develops into an embryo and then into a sporophyte, which possesses a single apical capsule in which meiosis eventually occurs, yielding haploid spores (Landberg et al., 2013; Hiss et al., 2017). A single sporophyte typically develops on a gametophore (Figure 4). The sporophyte is
composed of a short stalk terminated by a spherical capsule covered by a small fleeting calyptra (thin hood) and bearing stomata defined by a single guard cell in its lower portion. A differentiated line of dehiscence is lacking, and the mature capsule opens irregularly to free the small unicellular spores. The sporophyte is anchored to the gametophore by a foot, with transfer cells at the intersection of the two generations insuring the movement of nutrients (Regmi et al., 2017). The spores are covered in part by sporopollenin, a compound also found in male gametophytic pollen of seed plants (Daku et al., 2016).

**Genetics and genomics of P. patens**

As outlined above, the phylogenetic position of the mosses between the (then already sequenced) genomes of Arabidopsis thaliana/rice (Oryza sativa)/poplar (Populus trichocarpa) and Chlamydomonas reinhardtii prompted the idea to sequence the P. patens genome. At the Seventh Annual Moss International Conference, MOSS 2004, in Freiburg, Germany, a genome consortium was formed, and the P. patens genome was subsequently sequenced by the United States Department of Energy (US DOE) Joint Genome Institute. The nuclear genome was published in 2008 (Rensing et al., 2008) and lived up to the expectations that its comparison with the other available plant genomes would allow key events in land plant evolution to be traced. The plastid genome was published by (Sugiura et al., 2003) and the mitochondrial genome by (Terasawa et al., 2007), completing the draft genomic resources.

Since 2010, P. patens has been one of the US DOE flagship genomes (https://jgi.doe.gov/our-science/science-programs/plant-genomics/plant-flagship-genomes/), and more genomic and transcriptomic resources continue to be developed. The genome assembly, previously consisting of approximately 2,000 scaffolds and containing circa 5% gap regions, was recently brought to the pseudo-chromosomal level, with most of the sequence data represented in 27 pseudochromosomes (with 1% remaining gaps), as well as updated plastid and mitochondrial genomes (Lang et al., 2018). Moreover, substantial transcriptomic evidence was generated using RNA-seq (Perroud et al., 2018; Meyberg et al., 2020), complementing microarray-based expression profiling data sets (Hiss et al., 2014; Ortiz-Ramirez et al., 2016) and epigenetic data (Lang et al., 2018; Widiez et al., 2014). The most recent gene annotation (v3.3) used large scale RNA-seq data from the P. patens gene atlas project as expression evidence. This release contains 32,458 gene models and 86,669 protein-coding transcripts and is available from different sources (Table 1), with CoGe and Phytozome being the primary repositories.
The analysis of the most recent genome assembly (Figure 5) revealed that, contrary to most flowering plants, the distribution of genes and transposable elements (TE) is homogeneous along the *P. patens* chromosomes (Lang et al., 2018). This is mirrored by a homogeneous distribution of recombination rates, which is potentially rooted in the observation that *P. patens* is predominantly selfing. Interestingly however, the purging of deleterious mutations works efficiently in this moss (Szövényi et al., 2013). An analysis of the pseudochromosomal assembly also suggested that giant viruses (NCLDV) embedded into the genome become transcriptionally active during gametogenesis and protect the gametes from viral infection via siRNA-mediated silencing. In terms of epigenetics, an analysis of histone modifications suggested that protonemal tissue is epigenetically primed for the drought stress response (a condition that likely affects the gametophore stage), potentially representing an adaptation to the *P. patens* life style (Widiez et al., 2014). In contrast to flowering plants, where genes carrying methylation in the gene body are expressed, most genes showing DNA methylation within their gene bodies in *P. patens* are not expressed (Lang et al., 2018).

**Resources and databases**

**Plant material**

For a long time, the *P. patens* Gransden accession going back to the 1962 Whitehouse isolate was exclusively used for analysis. This accession was mainly distributed vegetatively and spread to laboratories around the world. However, many laboratories observed a severe loss of fertility in their Gransden strains over time, whereas others are still fertile. To overcome the problem of low fertility, a new ecotype, Reute, was recently introduced (Hiss et al., 2017). The use of Reute allows sexual reproduction to be analyzed with ease. A multi-omic comparison of Reute vs. Gransden revealed the accumulation of (epi-)mutations that affect the fertility of the male germline in Gransden (Meyberg et al., 2020). Moreover, fluorescent mutant strains are available (Perroud et al., 2011, 2019) that allow for routine crossing and make it easy to analyze 1:1 segregation of the F1 generation. Another ecotype, Villersexel, is genetically more divergent and has been used to generate the genetic map used for the V3 genome assembly (Kamisugi et al., 2008; McDaniel et al., 2010; Lang et al., 2018). The genetic variability of Gransden, Reute, Villersexel and another accession, Kaskaskia, was revealed using deep sequencing. Many more accessions have also been used in several studies (Medina et al., 2015; Liu et al., 2012; McDaniel et al., 2010; Beike et al., 2014; von Stackelberg et al., 2006; Medina et al., 2018, 2019; Szövényi et al., 2013). These accessions
are available from the authors, as well as the International Moss Stock Center (IMSC),
http://www.moss-stock-center.org/.

Databases

The main repository for the most recent *P. patens* genome assembly is CoGe (Table 2). In addition to the genome sequence (nuclear, plastid, and mitochondrion), this assembly contains annotation (genes, TEs), genetic variability (SNPs), and processed evidence from deep sequencing (transcript evidence, DNA methylation, chromatin modification). In terms of comparative genomics, Phytozome allows cross-species gene families to be identified, PLAZA allows synteny (gene order) analysis to be performed, and TAPscan lists transcription factors and transcriptional regulators. Several tools make use of expression profiling data, namely Genevestigator (Hiss et al., 2014), the *P. patens* eFP browser (Ortiz-Ramírez et al., 2016) and most recently Phytozome (Perroud et al., 2018). The data present in these tools, as well as novel data, were recently integrated into a common web-based tool, PEATmoss (https://peatmoss.online.uni-marburg.de/ppatens_db/contact.php), which includes a gene model lookup database that allows different versions of gene annotations to be compared (Fernandez-Pozo et al., 2019).

Available tools and technologies

Transformation

To perform genetic manipulation, it is critical to be able to transfer DNA molecules of choice into the organism. *P. patens* protonemata can be transformed with DNA by particle bombardment. This method allows for the stable incorporation of DNA (Cho et al., 1999) as well as transient expression assays (Bezanilla et al., 2003; Marella et al., 2006). However, early on in the development of molecular genetics tools for *P. patens*, gene targeting events were achieved using poly-ethylene-glycol (PEG)-mediated transformation of protoplasts (Kammerer and Cove, 1996; Schaefer and Zryd, 1997; Strepp et al., 1998). Given the proper osmoticum, *P. patens* protoplasts can be efficiently regenerated into whole plants, allowing plants derived from a single protoplast harboring DNA molecules of interest to be isolated. Thus, PEG-mediated transformation has become the most widely used transformation method.

Reverse Genetic Approaches
For over two decades, researchers have taken advantage of the innately high frequencies of homologous recombination in *P. patens* to insert DNA molecules into specific sites in the genome. The insertion of sequences encoding fluorescent proteins has opened the door to analyzing the localizations of fusion proteins expressed from the native genomic context, which is critically important, as the overexpression of fusion proteins often leads to aberrant localization. Furthermore, the generation of gene deletions in *P. patens* has allowed gene functional analysis to be performed throughout development.

However, because of gene family expansion due to recent genome duplications (Lang et al., 2018), which is common in plants, the functions of medium to large gene families have been difficult to study using traditional knock-out approaches due to a large degree of functional redundancy. Furthermore, due to the predominant haploid state of *P. patens*, it is impossible to isolate knock-outs of essential genes by transforming haploid protoplasts. To overcome these limitations, researchers have employed gene silencing mediated by RNA interference (RNAi) (Bezanilla et al., 2003, 2005; Nakaoka et al., 2012). The most common approach is to transiently express a DNA construct containing sequences targeting specific genes arranged in inverted repeats. Using this approach, multiple genes can be targeted simultaneously (Vidal et al., 2007). Transient expression of RNAi constructs in seven-day-old protonemata is a powerful approach to studying members of gene families (Vidal et al., 2007, 2009a) and essential genes (Augustine et al., 2008) involved in protonemal tissue development, particularly genes required for polarized growth. However, the stable integration of inverted repeat sequences does not occur at high frequency and thus, it can be challenging to obtain stable transformants containing functional silencing constructs. Thus, to analyze later stages of development, the expression of artificial microRNA constructs (Khraiwesh et al., 2010) and inducible expression using the estradiol inducible system (Zuo et al., 2000; Kubo et al., 2013) for stably integrated RNAi constructs (Nakaoka et al., 2012) have been employed. However, while gene silencing approaches are often very effective, they can suffer from variable levels of silencing.

Recent advances in genome editing technologies (Table 1) capitalizing on CRISPR-Cas9-mediated double-stranded break formation are quickly overcoming the limitations of traditional homologous recombination and gene silencing approaches. The transient expression of a guide RNA and the Cas9 enzyme readily produces double-stranded breaks in *P. patens*, which are repaired by non-homologous end-joining, invariably incorporating mutations that generate frame-shift mutations (Collonnier et al., 2017). Thus, by transforming *P. patens* with multiple guide RNAs and Cas9, multiple genes can be targeted simultaneously
to generate higher-order knockouts in a single transformation (Lopez-Obando et al., 2016). To ensure that regenerating plants have been transformed with the plasmid containing Cas9 and/or the guide RNA, the plants can be subjected to selection for approximately seven days (Mallett et al., 2019). The selection pressure is then removed and the plasmid is lost. After the plants have grown to a sufficient size, DNA is isolated from the plants to identify plants that have been edited. Editing efficiencies can be as high as 90% depending on the efficiency of the protospacer and the expression levels of the guide RNA and Cas9 (Mallett et al., 2019). Various techniques, such as T7 endonuclease assays (Mashal et al., 1995) and competition PCR (Harayama and Riezman, 2017), can be used to rapidly identify the edited sites.

When a homologous template is provided in addition to the guide RNA and Cas9, the double-stranded breaks are repaired from the homologous template, a process known as homology directed repair (HDR), allowing precise modifications to be incorporated at the cut site. In *P. patens*, the homologous template can be delivered as super-coiled plasmid (Collonnier et al., 2017; Mallett et al., 2019) or as double-stranded oligos (Yi and Goshima, 2019). Using HDR, it is possible to seamlessly integrate any number of modifications, from single base pair changes (Yi and Goshima, 2019) to the integration of sequences encoding fluorescent proteins (Mallett et al., 2019). The overexpression or inducible expression of a particular gene could be accomplished easily by changing the promoter sequence.

CRISPR-Cas9-mediated genome manipulation adds a powerful set of genome editing tools to an already genetically tractable system. In particular, CRISPR-Cas9 genome editing occurs without incorporating a selectable marker into the genome. The selection is only used to ensure that the plants have taken up the super-coiled plasmid enabling expression of the guide RNA and Cas9. After a week on selection, untransformed plants have died and the selection pressure is no longer needed. Using HDR coupled to CRISPR-Cas9, the genome can be altered seamlessly, making tagging a gene with a fluorescent protein at the 5’ end trivial. In addition, super-coiled plasmids, which are easier to generate than linear DNA templates, act as efficient HDR templates. Finally, CRISPR-Cas9 editing provides an unbiased approach to uncovering hypomorphs of essential genes. With high rates of editing, null mutations in essential genes will result in very few transformants. However, by targeting the gene at multiple sites with several protospacers, it may be possible to identify viable edited plants with lesions that reduce the function of the protein. In addition to Cas9, a recent study demonstrated that CRISPR using the programmable DNA endonuclease LbCas12a efficiently edited the genome, expanding the tools available for gene editing in *P. patens* (Pu et al., 2019).
Forward genetic approaches

In 1968, Engel used treatment with X-rays, as well as chemical mutagens such as EMS, to isolate auxotrophic and morphological mutants in *P. patens* (Engel, 1968). Subsequent studies identified *P. patens* auxotrophs (Ashton and Cove, 1977), hormone-resistant mutants (Ashton et al., 1979), and mutants with altered responses to gravity (Cove et al., 1978). However, due to a lack of genetic mapping resources and the inability to clone by complementation, the causal lesion for many of these mutants were never identified. In 2010, Prigge and coworkers (Prigge et al., 2010) took a candidate gene approach and identified the causal lesions in a number of mutants resistant to the synthetic auxin, NAA. More recently, the availability of a well annotated genome coupled with marker lines in ecotypes that enable rapid identification of crossed sporophytes (Perroud et al., 2011, 2019) (Figure 4) has paved the way for new genetic screens to identify causal mutations (Stevenson et al., 2016) (Table 3). In one such screen, Ding and coworkers (Ding et al., 2018) mutagenized protoplasts with UV and isolated conditional loss of growth mutants. Moody and coworkers (Moody et al., 2018b) also used UV mutagenesis to identify mutants no longer able to form gametophores. Without gametophores, however, it would not be possible to cross the mutant strain to a different ecotype to obtain a mapping population. To overcome this problem, Moody and coworkers (Moody et al., 2018b) took advantage of somatic diploidization and performed a cross between two somatic diploids. Both of these groups then successfully identified the causal mutations using whole-genome sequencing approaches on plants derived from their mapping population (Ding et al., 2018; Moody et al., 2018b). In addition to mutagenic agents, the tobacco Tnt1 retrotransposon can be introduced into *P. patens* using PEG-mediated protoplast transformation (Vives et al., 2016) or *Agrobacterium*-mediated mutagenesis (Mohanasundaram, 2019), which produced insertional mutations with a preference for genes and GC rich regions in the genome (Vives et al., 2016; Mohanasundaram, 2019). These studies have laid the foundation for robust forward genetic approaches by greatly expanding the available tools and resources, making it possible to readily perform a variety of screens, such as enhancer and suppressor screens.

Imaging

Due to its small stature and anatomical simplicity, most *P. patens* tissues are amenable to microscopic observation (Figures 4, 6). Protonemal tissue is a two-dimensional network of...
filaments comprising single cells arranged in linear branching arrays. The developing gametophore emerges from a protonemal filament as a single cell that undergoes a series of stereotypic cell divisions switching from polarized expansion to three-dimensional diffuse expansion (Harrison et al., 2009). Expanded gametophore phyllids (or leaflets) are only a single cell layer thick, except for their midribs. Given the simplicity of these tissues, microscopic observation readily occurs without the need for dissection or sectioning. In fact, with the recent advent of microfluidic imaging devices, it is now possible to continuously observe development from juvenile to adult tissues under a microscope at high temporal and spatial resolution (Bascom et al., 2016). These microfluidic imaging devices are constructed from polydimethylsiloxane (PDMS), which is highly permeable to gases, allowing for continuous plant growth in a small dish filled with liquid medium in the presence of light. With designs that allow for protoplast trapping (Sakai et al., 2019) in addition to careful control of osmolarity (Bascom et al., 2016), it is also possible to observe protoplast regeneration.

Using these systems, coupled with versatile molecular manipulation, numerous subcellular processes can be observed at high resolution. For example, by imaging the P. patens cytoskeleton using a variety of imaging modalities, detailed studies of polarized growth and cell division have been performed (Figure 6, (Vidali et al., 2009b; Wu and Bezanilla, 2014; Yamada and Goshima, 2018; Kosetsu et al., 2017; Kozgunova et al., 2019; Vidali et al., 2010; Furt et al., 2013; van Gisbergen et al., 2018; Wu and Bezanilla, 2018)). Imaging fluorescent proteins in subcellular compartments has provided insights into their dynamics (Figure 6, (Furt et al., 2012)). Imaging using a recently developed P. patens ratiometric auxin-sensing reporter revealed subtle differences between individual cells (Thelander et al., 2019). A ratio-metric calcium reporter was used to monitor subcellular calcium levels during polarized growth (Bascom et al., 2018) and plant-wide responses to dehydration in gametophore tissue (Storti et al., 2018). With the numerous approaches available to image P. patens, this model system is poised to yield fundamental advances in our understanding of detailed cellular and subcellular processes at high temporal and spatial resolution in a phylogenetically informative lineage of plants.

**Missing tools and resources**

Furthering our understanding of genetic processes in P. patens would benefit from a larger collection of accessions/ecotypes encompassing broader genetic variation. Currently, we are considering Gransden, Reute and Villersexel to be ecotypes, since they show different
phenotypes and genetic variability and can be crossed (Lang et al., 2018). However, studies of
genetic variability within populations are underway and will be needed to answer questions
about genetic and epigenetic variability. Also, while \textit{P. patens} is comparatively well
represented in terms of available accessions, this is not the case for \textit{P. magdalenae} and \textit{P. readeri}, for which additional accessions would be helpful in order to study convergent trait
evolution (see Hot Topics in \textit{P. patens} Research). While the above-mentioned IMSC makes it
possible to store mutants and to make them available to the community, there is no resource
yet like the \textit{A. thaliana} Salk lines, i.e., no collection of mutants that the community can draw
upon.

In terms of databases, more unity of web-based tools and more cross-links would aid
in finding and comparing information. It would be useful to develop a unified expression
profile interface and gene model lookup database, as outlined above.

\textbf{Hot topics in \textit{P. patens} research}

\textit{Polyploidy and hybridization}

Polyplody and hybridization have long been studied in mosses, including \textit{Physcomitrella/Physcomitrium} (Wettstein, 1924; Pettet, 1964; Fritsch, 1991), reviewed by
(Rensing et al., 2012). Engel described 14 chromosomes for \textit{P. patens} Gransden (Engel, 1968). Approximately 26 chromosomes were subsequently identified in this moss (Fritsch, 1991), while most recently, 27 chromosomes were identified (Reski et al., 1994). Due to their
small size, it is difficult to observe mitotic chromosomes in \textit{P. patens}. Even before the
genome was sequenced, an ancestral whole genome duplication (WGD) was evident in this
species (Rensing et al., 2007). The genetic map used for the recent V3 assembly (Lang et al.,
2018) is ordered into 27 linkage groups thought to represent the chromosomes. Indeed, the
most recent genome assembly suggests that at least two rounds of genome duplication might
have brought the ancestral chromosome number of seven to 14 and then to 27 (potentially by
the fusion of two chromosomes).

Inferences from transcriptomic data reveal signatures of past whole-genome
duplications across the phylogeny of mosses (Lang et al., 2018; Devos et al., 2016; Johnson et
al., 2016) unlike the other two bryophyte groups (Lang et al., 2018), and karyological data
from extant populations suggest the widespread occurrence of neopolyploidization within
many species (Fritsch, 1991). In mosses, as in other plants, polyploidy results from either
hybridization or autoploidy. The former mechanism is not uncommon in mosses
(Natcheva and Cronberg, 2004; Shaw, 2009), although its significance during the
diversification of mosses remains ambiguous. Hybridization may account for the origin of several species in the Funariaceae, which might have involved a particular species of *Physcomitrium* as one of the parents (Beike et al., 2014; McDaniel et al., 2010; Medina et al., 2018). *Physcomitrium patens* itself served as one parent for hybridization with species of *Physcomitrium* (Britton, 1895) and even with the more distantly related *Funaria hygrometrica* (Wettstein, 1924). The ability to trigger somatic hybridization (Moody et al., 2018a) and to visualize hybridization events involving *P. patens* (Perroud et al., 2011) provide unique tools to explore the genomic consequences of hybridization in the Funariaceae. Autopolyploidy may be more common than allopolyploidy considering the large number of species, such as *P. patens*, known to harbor more than one karyotype (Fritsch, 1991). How autopolyploidy triggers speciation in plants is a wide-open area of research and remains completely unexplored in bryophytes. Yet, artificial autopolyploids are easily created in mosses through apospory, i.e., the development of a diploid gametophyte from (injured) sporophytic tissue. Furthermore, intragametophytic selfing, which may be preponderant in the Funariaceae, would result in a perfectly homozygous sporophyte. Consequently, the aposporous, diploid gametophyte would in theory bear two identical copies of the genome of the original haploid gametophyte, providing a unique system among land plants for investigating the genomic or epigenetic changes and their transcriptomic consequences in the first generation following autopolyploidy. Ongoing studies in *F. hygrometrica*, for which aposporous gametophyte are more readily developed, reveal that autopolyploidy is immediately characterized by significant shifts in gene expression (Rahmatpour, 2019).

**Evo-devo approaches**

As outlined above, *P. patens* is an excellent moss for evolutionary developmental biology (evo-devo) studies due to its phylogenetic position, its propensity for gene targeting via homologous recombination, its relatively simple morphology, and the dominant haploid phase that is the hallmark of all bryophytes. The latter allows for the analysis of various mutants such as those affected in sexual reproduction: in flowering plants, such mutants are often embryo lethal. In such cases, the *P. patens* gametophytic generation can nevertheless be grown and propagated. On top of that, both generations can easily be tracked and accessed. Prominent evo-devo studies over the past decade include studies revealing the conservation of the polycomb group complex in repressing the sporophytic body plan (Mosquina et al., 2009; Okano et al., 2009) and of HD-TALE transcription factors in repressing the gametophytic or promoting the sporophytic developmental program (Sakakibara et al., 2003; Horst et al., 2018).
Another hot topic is identifying and analyzing the conservation of regulators of plant 3D and tip growth (Spinner et al., 2010; Pires et al., 2013; Perroud et al., 2014; Proust et al., 2016; Moody et al., 2018b; Tang et al., 2020; Perroud et al., 2020). The relatively simple body plan of protonema and the observation that they undergo tip growth and cell division enable them to be analyzed using approaches not feasible in many other plants. These and other approaches have greatly increased our understanding of early land plant evolution. *P. patens* is in an informative phylogenetic position, facilitating trait inference during plant terrestrialization (Puttick et al., 2018; Rensing, 2018; Delaux et al., 2019). The analysis of *P. patens*, together with other model non-seed plants and streptophyte algae (Bowman et al., 2017; Rensing, 2017; Nishiyama et al., 2018), furthers our understanding of how plants conquered land via the analysis of gene presence (comparative genomics) and conservation (evo-devo).

**Reprogramming**

Early on, *P. patens* was recognized as a unique system for studying the molecular basis of stem cell reprogramming due to the ability of differentiated cells to re-enter the cell cycle (Kofuji and Hasebe, 2014), de-differentiate, and form new protonemal cells in response to wounding and in the absence of any exogenously applied phytohormones. In fact, *P. patens* protoplasts can regenerate into whole plants without exogenous hormones, which is an extreme example of reprogramming in response to wounding. These attributes have been used to study the regulatory networks involved in cellular reprogramming. An analysis of gene expression during reprogramming revealed that initially, genes involved in stress responses and proteolysis were upregulated while genes involved in metabolic processes, particularly photosynthesis, were downregulated. As reprogramming progressed, metabolic gene expression including genes involved in photosynthesis and biosynthetic processes recovered. Auxin and cytokinin signaling pathways were also activated (Nishiyama et al., 2012). Quantitative proteomic analyses of protoplast regeneration yielded similar results (Wang et al., 2017). Stemming from gene expression studies (Nishiyama et al., 2012), cyclin dependent kinase A was found to be essential for coordinating cell cycle re-entry and protonemal gene expression required to establish a new stem cell (Ishikawa et al., 2011). In addition, two WUSCHEL-related homeobox 13-like genes were shown to be required for cell growth during reprogramming and the establishment of stem cell fate, as genes for cell wall loosening factors were no longer upregulated in WUSCHEL-related homeobox 13-like gene knockout lines (Sakakibara et al., 2014). Intriguingly, *Cold-Shock Domain Protein 1 (PpCsp1)*, which
shows sequence similarity and shared domains with Lin28, a gene required for induced pluripotent stem cell generation in humans, is upregulated in P. patens cells during reprogramming. The upregulation of PpCsp1 promoted reprogramming, while deleting PpCsp1 and its closely related genes inhibited reprogramming. This study identified a potentially conserved stem cell factor functioning in both plants and animals (Li et al., 2017). Finally, by screening for factors involved in stem cell reprogramming, Ishikawa et al. discovered a transcription factor from the AP2/ERF subgroup that, when overexpressed, altered the epigenetic landscape, decreasing repressive chromatin marks to a level sufficient to induce reprogramming in undamaged differentiated cells (Ishikawa et al., 2019). By isolating and examining the reprogramming ability of one, two, or three cells from gametophores, Sato et al. discovered that there is an inhibitory signal that diffuses from the cell that alters the stem cell fate of neighboring cells, such that if two cells are isolated, 80% of the time, only one of the cells successfully reprograms (Sato et al., 2017). These studies have provided unprecedented insights into cellular reprogramming. Future studies are sure to mechanistically dissect the signaling pathways involved in stem cell fate specification and reprogramming.

**Cell Biology**

In the past ten years, P. patens has become a key model system for studying plant cell biology. Due to the ease of genetically transforming plants and the ability to tag genes at their loci with DNA encoding fluorescent proteins, it has become standard to analyze the localization of proteins expressed from their native genomic context. It is also routine to demonstrate that the tagged protein is functional. With these tools in hand, a number of actin-associated proteins have been identified that are required to carry out tip growth (Vidali et al., 2007; Augustine et al., 2008, 2011; Vidali et al., 2010, 2009a; van Gisbergen et al., 2018, 2012; Wu et al., 2011). A tour-de-force study tagged every single kinesin gene (encoding microtubule-based motors) in the moss genome and analyzed their localization, discovering that some kinesins have conserved functions, while others have surprisingly divergent functions (Miki et al., 2014). Careful quantitative analysis of organellar dynamics in moss protonemata demonstrated that most organelles in P. patens move significantly more slowly in protonemata than in pollen tubes or root hairs (Furt et al., 2012). Analysis of organellar dynamics also demonstrated that moss protonemata do not exhibit cytoplasmic streaming (Furt et al., 2012). Thus, protonemata can be used to analyze the intracellular motility of organelles without the complexity of actively streaming cytoplasm. Taking advantage of this feature, cell biological observations coupled with loss-of-function studies have identified
cargos for kinesin motors, such as the nucleus and chloroplasts (Suetsugu et al., 2012; Miki et al., 2015; Yamada et al., 2017; Yamada and Goshima, 2018). These studies suggest that microtubules might play important roles in organelle transport in plants, potentially shifting the long-standing paradigm that plants predominantly use actin, not microtubules, for long-distance intracellular transport.

Protonemal cells are an excellent system for studying both cell division and polarized growth. Using these cells, the actin-based molecular motor myosin VIII was found to couple the actin cytoskeleton to microtubules, effectively guiding phragmoplast expansion. This study answered a long-standing question regarding the role of actin in cell division (Wu and Bezanilla, 2014). Subsequently, myosin VIII was also shown to link actin to microtubules at the apex of protonemal cells, serving to ensure persistent polarized growth (Wu and Bezanilla, 2018). A number of studies have begun to piece together the role of interdigitating microtubules at the phragmoplast equator in *P. patens*. The overlap zone, which requires the function of the microtubule crosslinking protein Map65, is spatially confined by the microtubule motor Kinesin-4 and recruits components of the exocyst complex, thereby defining the region where vesicles are delivered to build the new cell plate (Kosetsu et al., 2013; de Keijzer et al., 2017; Tang et al., 2019). Relatively few studies have focused on mitosis in *P. patens*. However, a recent study (Kozgunova et al., 2019) demonstrated that the depletion of kinetochore proteins leads to cytokinesis failures, resulting in polyploidy, which is in contrast to animal cells, where depletion leads to aneuploidy. This study raises the intriguing possibility that cytokinesis failure may be a route to polyploidy in plants.

With respect to polarized growth, key factors essential for cell polarity have been identified in *P. patens*, including the small GTPase ROP (Burkart et al., 2015) and actin-associated proteins such as actin depolymerizing factor (Augustine et al., 2008), profilin (Vidali et al., 2007), and myosin XI (Vidali et al., 2010). Using rapid RNAi approaches, the functions of whole gene families in polarized growth have been analyzed. The actin filament-promoting factors formins have class-specific functions, with class II rather than class I formins essential for polarity (Vidali et al., 2009a). The regulators of ROP function comprise a network of GTPase activating proteins, exchange factors, and dissociation inhibitors, all of which are encoded by small gene families. A systematic RNAi approach was used to identify regulators involved in tip growth (Bascom Jr et al., 2018). Together, these studies have elucidated the framework for the molecular regulation of the polarized secretion of cell wall materials in *P. patens*, with many factors conserved across plant lineages.
Outlook

*P. patens* represents only one lineage out of the many in mosses (e.g., orders in (Liu et al., 2019)). While more flowering plant models are being developed, the non-seed (or flagellated) plants are lagging behind. In order to determine such things as whether the unusual genome structure of *P. patens* (Lang et al., 2018) is peculiar or typical for mosses or bryophytes, we need more model organisms (Kenrick, 2017; Rensing, 2017). The liverwort *Marchantia polymorpha* has been established as a model system for similar types of analysis as *P. patens* (Bowman et al., 2017). Together, they represent the monophyletic Setaphyta (Renzaglia et al., 2018), and it is highly probable that while the *Marchantia* lineage subsequently lost genes and regulatory complexity, the *Physcomitrium* lineage gained them (Puttick et al., 2018). Hence, we need to study both organisms, as well as additional species. The hornwort *Anthoceros* is currently being established as a model system, representing the third lineage of bryophytes (Szövényi et al., 2015). Finally, a recent analysis of the genome of *Chara*, representing one of the sister lineages of land plants, revealed many typical land plant genes that were evolutionarily gained before the water-to-land-transition (Nishiyama et al., 2018).

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Table 1. Advances in Genome Editing

| Technique | Consequences | References |
|------------|--------------|------------|
| Transient expression of Cas9 and guide RNA | Non homologous end joining (NHEJ) repair leads to indels and frame shifts | (Collonnier et al., 2017) |
| Transient expression of Cas9 and guide RNAs targeting multiple genomic sites | Non homologous end joining (NHEJ) repair leads to indels and frame shifts | (Lopez-Obando et al., 2016; Mallett et al., 2019) |
| Transient expression of Cas9 and guide RNA together with a homology plasmid | Homology directed repair (HDR) enables the insertion of sequences encoding fluorescent proteins and small cassette containing stop codons in all possible frames to ensure that the stop codon is close to the protospacer site | (Mallett et al., 2019) |
| Transient expression of Cas9 and guide RNA together with homology oligos | HDR enables the insertion of stop codons from the oligos to ensure that the stop codon is close to the protospacer site | (Yi and Goshima, 2019) |
Transient expression of LbCas12A and multiple CRISPR RNAs (crRNAs)

Efficient method to multiplex target sites use the crRNAs. NHEJ results in diverse deletions

(Pu et al., 2019)

Transient expression of SpCas9-NG and guide RNA

Makes it possible to perform base editing by relaxing the stringency of the PAM sequence requirement

(Veillet et al., 2020)

| Table 2: Key features of *P. patens* and online resources |
|---------------------------------------------------------|
| **Life style**                                           |
| Haploid, gametophyte-dominant                           |
| Annual                                                  |
| Grows axenically in simple mineral medium               |
| Self-fertile (predominantly selfing)                    |
| Stress tolerant                                         |
| **Genetics and genomics**                               |
| Can be crossed for genetic studies                      |
| Gene targeting by homologous recombination              |
| Genome editing by CRISPR/Cas9                           |
| Transfection of protoplasts and Agrobacterium-mediated transformation |
| Complete genomes, relatively small nuclear genome (500 Mbp, 27 chromosomes) |
| Transcriptome (expression profiling) data               |
| Many mutants published                                  |
| **Genomes and primary annotation**                      |
| Nuclear genome assembly V3 for browsing at CoGe (including gene models v3.3, SNPs, RNA-seq expression evidence, TE and non-coding RNA annotation, microarray probes, DNA methylation, histone modification) |
| [https://genomevolution.org/coge/GenomeInfo.pl?gid=33928](https://genomevolution.org/coge/GenomeInfo.pl?gid=33928) |
| Nuclear, plastid, and mitochondrial genomes at CoGe     |
| [https://genomevolution.org/coge/OrganismView.pl?gid=33928](https://genomevolution.org/coge/OrganismView.pl?gid=33928) |
| Nuclear genome and gene models at Phytozome (including gene family assignment and RNA-seq expression evidence) |
| [https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Ppatens](https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Ppatens) |
| *P. patens* gene model lookup database (for converting between different annotation versions) |
| [https://peatmoss.online.uni-marburg.de/ppatens_db/pp_search_input.php](https://peatmoss.online.uni-marburg.de/ppatens_db/pp_search_input.php) |
| **Online tools**                                        |
| *P. patens* transcription factors and transcriptional regulators in TAPscan |
| [https://plantcode.online.uni-marburg.de/tapscan/TAPs_for_species.php?species=Physcomitrella%20patens&id=98](https://plantcode.online.uni-marburg.de/tapscan/TAPs_for_species.php?species=Physcomitrella%20patens&id=98) |
| *P. patens* expression atlas PEATmoss (including microarray and RNA-seq dataset) |
| [https://peatmoss.online.uni-marburg.de/expression_viewer/input](https://peatmoss.online.uni-marburg.de/expression_viewer/input) |
| Older expression atlas tools include Genevestigator (https://genevestigator.com/gv/) and an eFP browser |
P. patens in the PLAZA comparative genomics environment
https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v4_dicots/organism/view/Physcomitrella+patens
https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v4_monocots/organism/view/Physcomitrella+patens

P. patens in ENSEMBL Plants
http://plants.ensembl.org/Physcomitrella_patens/Info/Index

Protocols and stocks
Cold Spring Harbor protocols on cultivation of P. patens
http://cshprotocols.cshlp.org/content/2009/2/pdb.prot5136.abstract

Protonema cultivation at Bio-protocol
https://bio-protocol.org/e1556

PHYSCObase protocols
http://moss.nibb.ac.jp/protocol.html

International Moss Stock Center
http://www.moss-stock-center.org/

Protocols on the Bezanilla Laboratory website
https://sites.dartmouth.edu/bezanillalab/moss-methods/

| Table 3. Forward Genetic Approaches |
|-------------------------------------|

### Using UV as a mutagen

| Screen | Identification of lesion | References |
|--------|--------------------------|------------|
| Isolated ABA mutants | Crossed mutant plants to a distinct ecotype and performed whole-genome sequencing on pooled mutants derived from the cross | (Stevenson et al., 2016) |
| Temperature-sensitive mutant screen identifying mutants that are unable to grow protonemata at the restrictive temperature. | Crossed mutant plants to a distinct ecotype and performed whole-genome sequencing on pooled mutants derived from the cross | (Ding et al., 2018) |
| Isolated mutants that were unable to form gametophores in an effort to find genes involved in the transition from 2D to 3D growth | Took advantage of the ability of moss to form somatic hybrids. Crossed two somatic hybrids to generate a mapping population and performed whole-genome sequencing on mutants derived from the cross | (Moody et al., 2018a) |

### Using the Tobacco Tnt1 retrotransposon as a mutagen

| Transformation method | Genomic regions showing insertions | References |
|-----------------------|-----------------------------------|------------|
| PEG-mediated transformation of protoplasts | Generated mutations in genic regions | (Vives et al., 2016) |
| Agrobacterium-mediated | Generated mutations in genic regions and GC | (Mohanasundaram, |
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Figure 1: Summarized phylogeny of the *Funariaceae*. The phylogeny was inferred from variation in 648 target capture nuclear exons, highlighting the polyphyletic distribution of *Physcomitrella* s. lat. (including *Aphanorrhegma*) within *Physcomitrium* (Medina et al., 2019). All relationships between main lineages represented (i.e., genera), except *Entosthodon* (i.e., bootstrap between 90 and 100%) are maximally supported under maximum likelihood analyses of a concatenated alignment of the nuclear loci, concordance among a majority of gene trees inferred from exons and their flanking regions, and a coalescence-based gene tree summary analysis (see Medina et al. 2019 for details).
Figure 2: Physcomitrium patens.
(A) Geographic distribution (black triangle), contrasted to the distribution of P. readeri including P. californica (blue triangles), P. magdalenae (pink circles) and P. serratum (orange area). Map adapted from (Medina et al., 2015) based on specimens examined by Goffinet (Goffinet, 2007a, 2007b) complemented by reports by Faubert (Faubert, 2013) and Higuchi and Takahashi (Higuchi and Takahashi, 2012). (B) Example of an ecological habitat of Physcomitrium patens (i.e., lake flood plain in Yunnan China). (C) A gametophore with a single terminal mature sporophyte. Scale bar, 1 mm.
Figure 3: Life cycle of *P. patens*. Modified and reproduced from (Strotbek et al., 2013), dx.doi.org/10.1387/ijdb.130189wf with the permission of UPV/EHU Press.
**Figure 4**: Anatomy of *P. patens* during different developmental stages.

(A) Tip growing filamentous protonemata eventually develops buds, which grow into (B) leafy gametophores. 

(C, D) Under cold and short day conditions sexual reproductive organs (gametangia) develop on the top (apex) of the gametophore. The male antheridia (C), which release motile biflagellated spermatozoids upon maturity, emerge in bundles comprising different developmental stages of antheridia. (D) The female archegonia arise next to the male organs and are flask shaped. The egg is located in the archegonial venter and after fertilization a sporophyte develops (E-H). 

(E) Upon maturity, the brown sporophyte releases spores of the next generation. (F-H) A hybrid sporophyte of the fluorescent Reute-mCherry and the non-fluorescent Gransden strain. (G) mCherry-fluorescent sporophyte is shown on top of the non-fluorescent gametophore indicating a successful cross of both strains. (H) Chloroplast autofluorescence is visible in the sporophyte as well as in the leaflets. 

Scale bars in (A, C) 50 µm, (B, F, G, H) 200 µm, (D) 100 µm, and (E) 500 µm.
Figure 5: *P. patens* chromosomes.
Visualization of the V3 pseudochromosomal nuclear genome assembly and v3.3 annotation (Lang et al., 2018). Each chromosome is represented by a grey circle, the area of which corresponds to the length of the chromosome. Concentric circles reflect the relative amount of transposable elements (TEs, in yellow) and genes (green) with the areas of each circle corresponding to the total length. The 330 unordered scaffolds are represented as an extra circle. Figure courtesy of Fabian Haas.
Figure 6: Fluorescence imaging of *P. patens.*

(A) Confocal images of cells expressing fluorescent proteins targeted to different organelles. From left to right: endoplasmic reticulum labeled with signal peptide-mEGFP-KDEL, Golgi bodies labeled with the first 49 amino acids of the soybean α-1,2-mannosidase fused to YFP, vacuolar membrane (tonoplast) labeled with mEGFP fused to PpVam3 (Pp3c2_35310), and the mitochondria labeled with the first 76 amino acids of the protein encoded by Pp3c18_16000V3.1 fused to mCherry. (B) Time-lapse confocal images of a cell expressing a mitochondria protein fused to mCherry. Mitochondria became fragmented when the cell enters cell division (12 min). Scale bar, 10 µm. (C) Time-lapse confocal images of the endoplasmic reticulum (ER) and microtubules during cell division. The ER is labeled with signal peptide-mCherry-KDEL and microtubules are labeled with mEGFP-tubulin. Images in (A-C) are maximum projections of Z-stacks. Scale bars, 10 µm. (D) Images of lifeact-mEGFP labeling the actin cytoskeleton, mEGFP-tubulin labeling microtubules, and endoplasmic reticulum acquired with variable angle epifluorescence microscopy (VAEM), which produces images of low background and high contrast, allowing the observation of fine structures with high temporal resolution. Scale bars, 2 µm.
The Moss *Physcomitrium (Physcomitrella) patens*: A Model Organism for Non-Seed Plants
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