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Natural Products from Bryophytes: From Basic Biology to Biotechnological Applications

Armin Horn^{a,b} , Arnaud Pascal^{c*} , Isidora Lončarević^{d,e*} , Raíssa Volpatto Marques^{f*} ,
Yi Lu^{e,f*} , Sissi Miguel^g , Frederic Bourgaud^g , Margrét Thorsteinsdóttir^{e,h} , Nils Cronberg^d ,
Jörg D. Becker^{a,b} , Ralf Reski^{c,i} , and Henrik T. Simonsen^f 

^aInstituto Gulbenkian de Ciência, Oeiras, Portugal; ^bITQB NOVA–Instituto de Tecnologia Química e Biológica António Xavier, Oeiras, Portugal; ^cPlant Biotechnology, Faculty of Biology, University of Freiburg, Freiburg, Germany; ^dBiodiversity, Department of Biology, Lund University, Lund, Sweden; ^eArcticMass, Reykjavik, Iceland; ^fDepartment of Biotechnology and Biomedicine, Technical University of Denmark, Kongens Lyngby, Denmark; ^gPlant Advanced Technologies, Vandoeuvre, France; ^hFaculty of Pharmaceutical Sciences, University of Iceland, Reykjavik, Iceland; ⁱSignalling Research Centres BIOSS and CIBSS, University of Freiburg, Freiburg, Germany

ABSTRACT

Natural products from plants have served mankind in a wide range of applications, such as medicines, perfumes, or flavoring agents. For this reason, synthesis, regulation and function of plant-derived chemicals, as well as the evolution of metabolic diversity, has attracted researchers all around the world. In particular, vascular plants have been subject to such analyses due to prevalent characteristics such as appearance, fragrance, and ecological settings. In contrast, bryophytes, constituting the second largest group of plants in terms of species number, have been mostly overlooked in this regard, potentially due to their seemingly tiny, simple and obscure nature. However, the identification of highly interesting chemicals from bryophytes with potential for biotechnological exploitation is changing this perception. Bryophytes offer a high degree of biochemical complexity, as a consequence of their ecological and genetic diversification, which enable them to prosper in various, often very harsh habitats. The number of bioactive compounds isolated from bryophytes is growing rapidly. The rapidly increasing wealth of bryophyte genetics opens doors to functional and comparative genomics approaches, including disentangling of the biosynthesis of potentially interesting chemicals, mining for novel gene families and tracing the evolutionary history of metabolic pathways. Throughout the last decades, the moss *Physcomitrella* (*Physcomitrium patens*) has moved from being a model plant together with *Marchantia polymorpha* in fundamental biology into an attractive host for the production of biotechnologically relevant compounds such as biopharmaceuticals. In the future, bryophytes like the moss *P. patens* might also be attractive candidates for the production of novel bryophyte-derived chemicals of commercial interest. This review provides a comprehensive overview of natural product research in bryophytes from different perspectives together with biotechnological advances throughout the last decade.

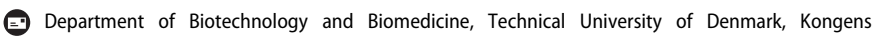
KEYWORDS

Bryophytes; industrial biotechnology; natural products; *Physcomitrella patens*; plant biotechnology

1. Introduction

Bryophytes are the closest modern relatives to the ancestors of the first plants that succeeded to adapt to life on land approximately 470 to 515 million years ago (Morris *et al.*, 2018). They have diversified early into the following three distinct extant phyla: Marchantiophyta (liverworts), Bryophyta (mosses) and Anthocerotophyta (hornworts). The most recent phylogenomic analyses provides evidence for monophyly of bryophytes, (Harris *et al.*, 2020), with mosses and

liverworts as sister groups (setaphyte hypothesis), separate from hornworts (which lack seta) (Renzaglia *et al.*, 2018). Like all land plants (embryophytes), bryophytes have a life cycle with alternating generations. In contrast to other embryophytes, whose diploid sporophyte generation is dominant, in bryophytes, the haploid gametophyte generation is the dominant and persevering stage, whereas the unbranched sporophyte generation is diploid and short-lived (Horst and Reski, 2016). Bryophytes can

CONTACT Henrik T. Simonsen  hets@dtu.dk 

*These authors contributed equally.

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be found in almost all climatic regions on all continents, where they are important components of many terrestrial ecosystems. At regional level, bryophytes are often most species-rich in cool and humid habitats (Prendergast *et al.*, 1993; Ignatov, 2004). This is probably a consequence of the poikilohydric nature of bryophytes, meaning that they have a poor capacity to regulate internal water content and thus are passively dependent on ambient water availability. They also need water for reproduction, because water enables the motile sperm to swim to the egg cell.

Bryophytes are small-sized and morphologically simple but chemically complex (Asakawa *et al.*, 2013). They are rarely consumed by animals (Gerson, 1982), which is likely due to specific chemical constituents that exhibit protective effects. Ricciocarpin, a sesquiterpenoid isolated from the liverwort *Ricciocarpos natans* has molluscicidal activity against the freshwater snail *Biomphalaria glabrata* (Asakawa and Ludwiczuk, 2018). Acetylenic oxylipins extracted from the moss *Dicranum scoparium* showed antifeeding activity against herbivorous slugs (Rempt and Pohnert, 2010). Crude extracts of some bryophytes have already been utilized by ancient tribes as medicine due to their beneficial chemical profile (Flowers, 1957; Sabovljević *et al.*, 2016). Several bryophyte species have been used in Chinese traditional medicine, *Marchantia polymorpha* (DiFuPing) is used for external ailments such as burns and cuts. *Sphagnum teres* is used for eye diseases and skin irritation. *Rhodobryum giganteum* (HuiXinCao) is used for minor heart problems (Harris, 2008). Different biologically active chemicals have been described with antimicrobial (Neomarchantins A and B, and Marchantin C), antifungal (Plagiochin E, Viridiflorol), anticancer (Marchantin A, Porellacetals A-D), antibacterial (mastigophorene C, herbertene-1,2-diol and Sacculatal), and/or antiviral (Marchantin A, B and E) properties (Beike *et al.*, 2010; Asakawa *et al.*, 2013; Klavina *et al.*, 2015; Vollár *et al.*, 2018; Ludwiczuk and Asakawa, 2019; Commisso *et al.*, 2021).

In parallel, an increasing availability of genomic resources has paved the way for gene mining approaches to identify genes involved in specialized metabolism that are absent in seed plants. For example, microbial terpene synthase-like (MTPLs), a novel group of metabolic genes exclusive to nonseed plants, has been identified (Jia *et al.*, 2016). All these traits make bryophytes a fascinating group of plants to study, with a high potential for the discovery of desirable natural products amenable by biotechnological tools.

The moss *P. patens* has already been shown to have a high biotechnological potential as an

alternative green cell factory. Foremost, for the production of valuable proteins such as biopharmaceuticals (Reski *et al.*, 2015, 2018; Campos *et al.*, 2020; Decker and Reski, 2020). Recently, the first moss-made drug candidate (moss-aGal) successfully passed stage 1 clinical trials (“First moss-made drug,” 2015; Hennermann *et al.*, 2019). During recent years, metabolic engineering has successfully evolved from synthesis of biopharmaceuticals to heterologous production of natural compounds such as commercially relevant terpenoids, e.g., artemisinin, patchoulol and santalene (Zhan *et al.*, 2014; Khairul Ikram *et al.*, 2017). Some of the key features of this sustainable cell factory platform are relatively fast axenic growth in simple mineral media, an established photobioreactor system, progressively up-scaled, currently to 500 L, and a well-established procedure for cryopreservation in cell culture banks (Schulte and Reski, 2004). In particular the haploid condition of the gametophyte phase and the relative ease of transformation via homologous recombination with yeast-like efficiency have made this system attractive for transgenic approaches (Hohe *et al.*, 2004; Schween *et al.*, 2005).

In this review, we show how the ecological and genetic diversity of bryophytes is reflected by chemical diversity. We summarize how this knowledge can lead to the discovery of novel bioactive products of commercial interest and how past decades of research focusing on the moss *P. patens* have qualified it not just as a model system in evolutionary developmental and cell biology (Rensing *et al.*, 2020), but also as a prime cell factory for heterologous production of valuable natural products.

II. Ecological diversity of bryophytes

Bryophytes qualify as the most diverse group of plants after angiosperms with regards to their numbers of species, geographical distribution, and habitat diversification (Tuba *et al.*, 2010). They include around 20,000 species (Shaw *et al.*, 2011), thereof mosses around 13,000 (Magill, 2014), liverworts 6,000 and hornworts 200 (Söderström *et al.*, 2016), whereas angiosperms encompass ca. 295,000 species (Christenhusz and Byng, 2016). Even though bryophytes possess lower species diversity and less complex morphology than the more recently diverged angiosperms, they exhibit as much genomic diversity as tracheophytes (including angiosperms), expressed in a broad assemblage of physiological and biochemical adaptations, which are still poorly explored (Glime, 2013). Some of the biochemical adaptations in

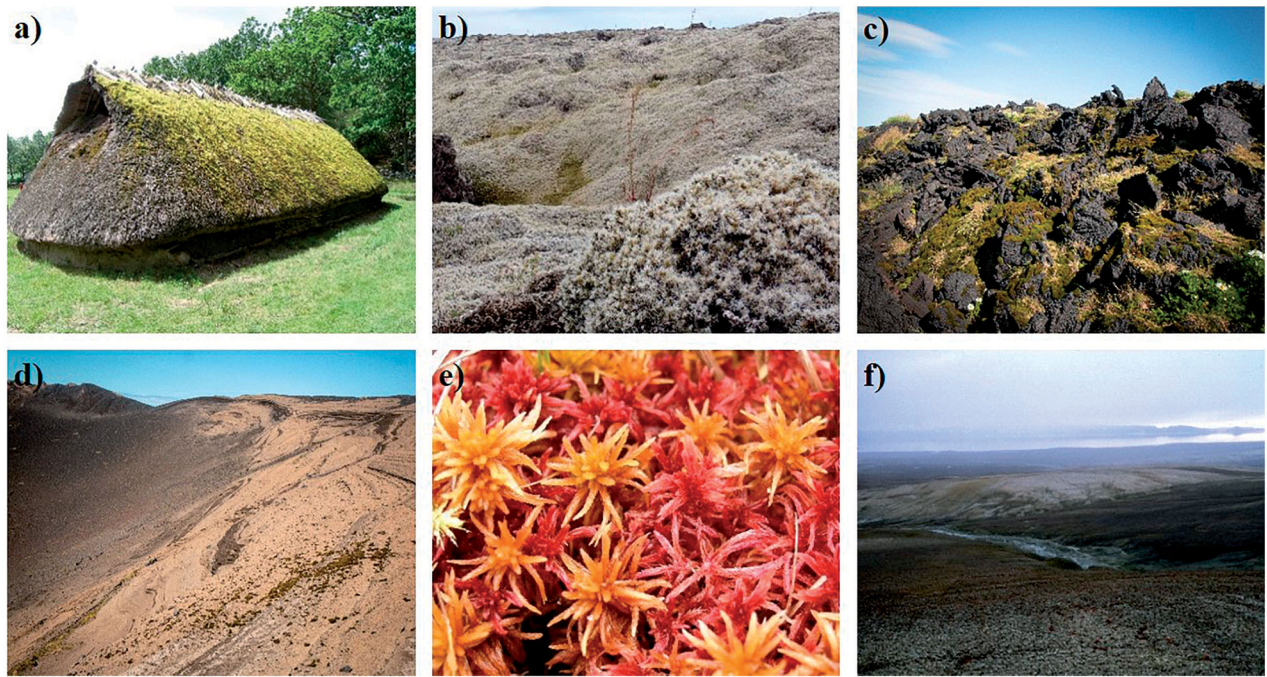


Figure 1. Some of the more extreme bryophyte habitats: (a) Moss cover on thatched roof. Reconstructed bronze age building, Tanums Hede, Sweden 2007; (b) Lava field with *Racomitrium lanuginosum* as dominant component in the vegetation, Ölfus, Iceland, 2018; (c,d) Mosses on the volcanic rocks and palagonite tuff, as part of pioneering vegetation, Surtsey volcanic island, UNESCO world heritage site, Iceland, 2018; (e) Peat mosses, *Sphagnum warnstorffii* and *S. teres*, rich fen, Björnekullakärret, Sweden, 2005; (f) Polar desert with the barren vegetation almost completely composed by bryophytes, Ellef Ringnes Island, Canadian Arctic Archipelago, 1999. (a,b,e,f) Photos by Nils Cronberg; (c,d) photos by Gróa Valgerður Ingimundardóttir.

bryophytes appear to have evolved as a consequence of their often slow growth and small size, protecting them from herbivory (Glime, 2013) and modulating interactions with microbiota and other plants. Chemical interaction, for example by allelopathic substances, may be especially important during early successional stages of bryophyte development. Bryophytes can cope with environments across all climatic regions on the planet, where water is present, from the Antarctic and Arctic permafrost areas to the warm and humid tropical forests, including regions and substrates which are uninhabitable for vascular plants (Tuba *et al.*, 2010).

Thus, the apparent simplicity of bryophyte vegetative bodies (gametophytes) contrast to their complex genomic architecture. This is exemplified in a recent study of vegetative (gametophytic) transcriptomes from two morphologically similar species (*P. patens* and *Funaria hygrometrica*) (Rahmatpour *et al.*, 2021). These closely related species display quite high genomic divergence, with most innovations being in metabolic genes of *F. hygrometrica* (encoding for copper chaperone, copper ion binding, universal stress protein, heat stress transcription factor, riboflavin biosynthesis protein, sulfur compound metabolic process, inorganic cofactors, some defensive mechanisms, etc.),

supporting the hypothesis that moss evolution is driven by metabolic and physiological adaptations to different environments.

A. Ecological roles

All ecosystems on earth, except marine and permanently frozen ecosystems (Vanderpoorten and Goffinet, 2009) are occupied by bryophytes. Their ecosystem functions include primary production, nutrient cycling (including mycorrhizal relationships and nitrogen fixation as hosts for cyanobacteria), water retention, primary and secondary colonization and animal interactions (Tuba *et al.*, 2010). By hosting nitrogen fixing cyanobacteria feather mosses *Pleurozium schreberi* and *Hylocomium splendens* are an important source of nitrogen input to natural boreal forests and this association could be an asset in forest management (Stuiver *et al.*, 2015). Bryophytes have an essential role in global biogeochemical cycles, by sequestering substantial quantities of carbon as peat, notably in wetlands and mires dominated by peat mosses, *Sphagnum* spp. (Figure 1), thus influencing the global climate. In tropical forests, especially montane cloud rain forests, epiphytic bryophytes have a major role in controlling water and nutrient flow, having an overall water

holding capacity equivalent to as much as a 20 mm precipitation event (Ah-Peng *et al.*, 2017).

Among natural environments, they have the largest standing biomass and productivity in peatlands, fens, bogs, Arctic and Antarctic tundra, alpine ecosystems, especially above tree line and moist forests (Vanderpoorten and Goffinet, 2009; Tuba *et al.*, 2010). Despite occupying only 3% of the global land area, peatlands contain about 25% (600 GtC) of the global soil C stock, which is equivalent to twice the amount in the world's forests (Yu *et al.*, 2010; Loisel *et al.*, 2021). Bryophytes are also able to inhabit cities, where some species may serve as indirect or even direct (in situ) bioindicators of air pollution, because of their ability to adsorb and accumulate high concentrations of heavy metals (Rühling *et al.*, 1970; Stanković *et al.*, 2018).

B. Habitat diversity

Bryophytes can grow on a wide range of natural substrates (soil, rock, bark, tree trunks, rotting wood, dung, animal cadavers or leaf cuticles) forming diverse microhabitats, and many bryophytes are actually reliable indicators for specific sets of substratum-related conditions (Townsend, 1964). They can also colonize somewhat more specialized substrates, such as ashes after forest fire, lava and tephra after volcano eruptions, some saline environments (but few are true halophytes) or heavy-metal rich soils (metallophytes) (Townsend, 1964; Ingimundardóttir *et al.*, 2014). They interact with other plants and can promote soil formation and development. Some species even occur on bare volcanic soils and rocks (Figure 1), thus generating environments habitable for vascular plants and facilitating their development (Ingimundardóttir *et al.*, 2014). Bryophytes have been classified according to life strategy (During, 1979), ranging from short-lived fugitives and shuttle species to long-lived perennial stayers. Many species are ecological pioneers or fugitives appearing on substrates with little competition on roofs, soils, rocks and trees (Figure 1). The fugitive life strategy occurs in spatially highly unpredictable environments that exists for short time, where species have fast life span, frequent sexual reproduction and long-lived small spores, such as in *F. hygrometrica*. Shuttle species occur in habitats with regular disturbance regimes, selecting for fast reproduction and large dispersal agents. Perennial stayers are competitive together with vascular plants in habitats such as forest floor, wetlands and various types of heathland, including arctic tundra (Figure 1). Such species (e.g.,

Sphagnum spp. and *Hylocomium splendens*) live in persistent, late successional environments and have long life span, low level of sexual reproduction and dominant vegetative proliferation.

Like tracheophytes, bryophytes possess endophytic fungi (Yu *et al.*, 2014; Chen *et al.*, 2018; Nelson *et al.*, 2018; Nelson and Shaw, 2019), but their functional role in bryophyte ecology is yet to be investigated (Davey and Currah, 2006). Fungal endophytes may provide bryophyte hosts with greater tolerance to extreme pH or promote vegetative growth or adaptation to the extreme environment, as it is found in Antarctic bryophytes (Pressel *et al.*, 2014).

C. Biogeographic distribution

Despite the considerable differences in ecophysiology, distribution patterns and dispersal between bryophytes and vascular plants, biogeographic distributions of bryophytes are largely consistent with those reported in other taxonomic groups (Patiño and Vanderpoorten, 2018). Bryophytes are present in all five major phytobiogeographic regions of the world.

1. Endemism

Spatial analyses of genetic structure in bryophytes suggest higher long-distance dispersal capacity than for angiosperms due to smaller diaspores (wind-dispersed spores), resulting in lower speciation and endemism (Shaw *et al.*, 2015). From a biogeographic point of view, bryophytes are characterized by low rates of endemism, with clearly different regional endemism patterns compared to angiosperms. Several temperate areas, including Patagonia, the Pacific Northwest American region, and Tasmania exhibit high levels of bryophyte endemism, differing from the most important hotspots for angiosperms located in tropic or subtropical climates such as the Mediterranean and Central American regions (Patiño and Vanderpoorten, 2018).

2. Ecotypes/cryptic species

It has sometimes been advocated that bryophyte species, in contrast to the majority of seed plants, do not tend to develop ecotypes, geographic populations genotypically adapted to specific environmental conditions. They rather display an intrinsic broad ability to cope with environmental variation (Patiño and Vanderpoorten, 2018), i.e., individuals display wide physiological and morphological plasticity (Reynolds and McLetchie, 2011) which would then overrule any tendency for local adaptation. Furthermore, many species display low genetic differentiation across large

distribution areas, suggesting efficient gene flow through wind-dispersed spores (summarized in Patiño and Vanderpoorten, 2018), which may counteract local differentiation. However, a high gene flow does not necessarily prevent local adaptation as a response to strong selection pressures and few studies have really tested presence of adaptive local differentiation in a rigorous way. Several genomic studies (Shaw, 2001; Myszczyński *et al.*, 2017; Yousefi *et al.*, 2017) have shown that broadly defined morphological species can be separated into “cryptic species,” lineages with distinctly differentiated genomes but obscure or overlapping morphological differentiation. Although these cryptic lineages show geographical or ecological separation at varying degree, most of the genomic differentiation appear to be manifested at the biochemical level and these lineages, therefore, passed un-noticed in earlier taxonomic revisions based on morphology. However, new discriminating morphological characters are often revealed that enable separation of such cryptic species (Shaw, 2001).

3. Diversity gradient

World tropical regions were for a long time considered poorer in bryophyte species compared to temperate areas, suggesting unclear relationship between latitude and diversity in bryophytes (Vanderpoorten and Goffinet, 2009). There was even some evidence for inverse latitudinal diversity gradient at narrower spatial scales, e.g., in Europe (Mateo *et al.*, 2016). However, recent analyses of the distribution of liverworts and hornworts (Söderström *et al.*, 2016), showed that global species richness of tropical areas is markedly higher than that of the extra-tropical ones, indicating a positive latitudinal diversity gradient (LDG) in hornworts and liverworts (Wang *et al.*, 2017). Equally diverse temperate and tropical regions are sometimes reported for mosses (Geffert *et al.*, 2013), which seems to be in conflict with the paradigm of low moss diversity in the tropics and the presence of inverse LDG in moss species. In general, tropical regions are less investigated than temperate regions and taxonomic revisions of many tropical taxa are missing, so the estimation of the species richness and distribution pattern in mosses needs to be re-investigated, requiring a new critical world checklist of mosses (Geffert *et al.*, 2013; Patiño and Vanderpoorten, 2018).

An interesting feature unique for mosses among bryophytes, is that they possess high levels of endopolyploid nuclei, which occur in specialized tissues, suggesting an increase in gene copy number and ability

to produce an assortment of cell sizes, which in turn could affect other morphological and physiological factors influencing ecology and distribution of mosses (Bainard *et al.*, 2020). The worldwide diversification of bryophytes is paralleled by a huge chemical diversity of specialized metabolites (Asakawa *et al.*, 2013), which provide protection from abiotic and biotic stresses (Xie and Lou, 2009), shaped during their long evolutionary history.

III. Chemical diversity of bryophytes

More than 2,200 chemical constituents have been described from bryophytes and the number is growing rapidly. The natural products isolated from bryophytes are mainly terpenoids (including mono-, sesqui- and diterpenoids), flavonoids, (bis)bibenzyls (exclusively produced by liverworts), and lipids (Sabovljević *et al.*, 2016). Selected natural product structures from different chemical groups isolated from bryophytes are shown in Figure 2. Several hundreds of these isolated compounds exhibit antimicrobial, antifungal, anti-cancer, antibacterial and/or antiviral bioactivity. The majority of these compounds have been extensively described before (Asakawa *et al.*, 2013; Jia *et al.*, 2018; Ludwiczuk and Asakawa, 2019). Thus, only recently discovered compounds or novel bioactivities from already known compounds reported in the last ten years are summarized in this section (Table 1).

A. Terpenoids

Terpenoids, the largest group of natural products, present in all living species, mediate diverse biochemical and ecological processes in bryophytes (Chen *et al.*, 2018). Like in other plants, they contribute to the physiological regulation, as shown for the diterpenoids *ent*-kaurene and derivatives in *P. patens* (Hayashi *et al.*, 2010). These are involved in protonemal differentiation and spore development (Hayashi *et al.*, 2010; Vesty *et al.*, 2016; Chen *et al.*, 2018). Some terpenoids also act as UV-B absorbers and enhance desiccation tolerance by modulating cytoplasmic osmotic potential (Chen *et al.*, 2018).

Liverworts produce a larger variety of terpenoids than mosses and hornworts. Over the past 40 years, more than 1,600 terpenoids have been isolated and identified from liverworts (including lipophilic mono-, sesqui- and diterpenoids), whereas only around 100 sesquiterpenoids, and a few mono- and diterpenoids, have been identified in mosses (Ludwiczuk and Asakawa, 2019). This may be because of the presence

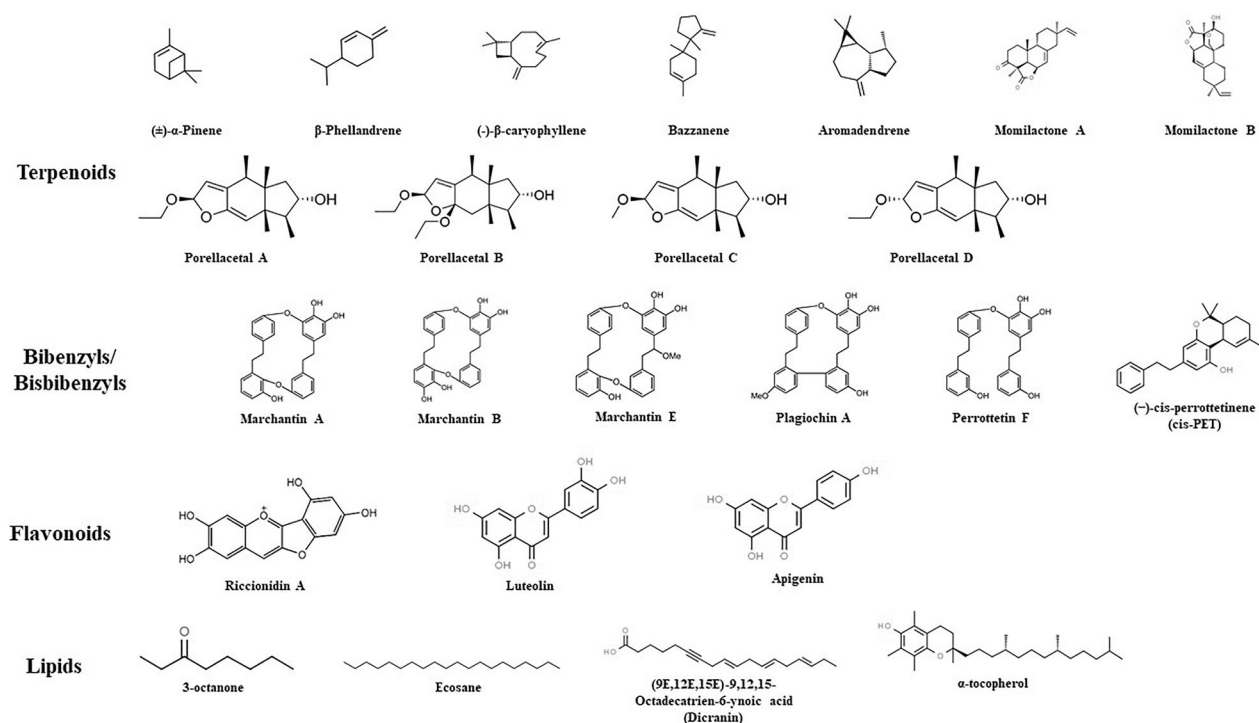


Figure 2. Selected chemical structures of natural products found in bryophytes.

of the oil bodies exclusively in liverworts where terpenoids are stored.

Terpenoids from bryophytes have versatile bioactivities such as anti-bacterial, anti-inflammatory, antifungal, phytotoxicity, and insect antifeedant activities (Asakawa *et al.*, 2013; Chen *et al.*, 2018). Asakawa *et al.* (2013) demonstrated that terpenoids and other aromatic compounds are responsible for the antibiotic and antifungal properties in liverworts, although Chen *et al.* (2018) expressed some uncertainty about to what degree these compounds really repress infestation. Tosun *et al.* (2015) tested the essential oils of three moss species, *Pseudoscleropodium purum*, *Eurhynchium striatum*, and *Eurhynchium angustirete* for antimicrobial activity. Their minimum inhibitory concentrations (MIC) ranged from 278.2 to 2,225 $\mu\text{g/mL}$, with α -pinene (16.1%), 3-octanone (48.1%), and eicosane (28.6%) as main components, respectively. Bacterial and fungal infections do occur in mosses, whereas this is very rare in liverworts due to the contributions of their large pool of anti-bacterial and anti-fungal terpenoids (Chen *et al.*, 2018).

There is evidence of allelopathic effects of terpenoids extracted from liverworts and mosses (reviewed by Whitehead *et al.*, 2018). Momilactones B is a diterpenoid phytoalexin first isolated from the moss *Hypnum plumaeforme* (Figure 2) (Nozaki *et al.*, 2007), which showed allelopathic activity against angiosperms, mosses, and liverworts. Interestingly,

momilactones have only been found in rice before and have shown cytotoxic and antitumor activity against human colon cancer cells (Kim *et al.*, 2007).

Repellent odor and bitter taste of bryophyte terpenoids, and sometimes cytotoxicity may serve an anti-herbivore function as well, which may explain why relatively few animals feed on bryophytes, especially liverworts (Asakawa *et al.*, 2013).

Compared with mosses and liverworts, hornworts are chemically scarcely studied. Previous studies concluded that the chemical constituents of hornworts are very distinct from liverworts and mosses (Asakawa *et al.*, 2013). Several terpenes have been characterized in hornworts (Xiong *et al.*, 2018), however, bioactive chemicals unique to hornworts have not been reported.

B. Phenylpropanoids

1. Flavonoids

The flavonoid pathway (starting from the larger phenylpropanoid pathway) is one of the best characterized among plants, with significant biological and ecological functions (Davies *et al.*, 2020). Flavonoids are widely distributed in mosses, liverworts, and vascular plants (Yonekura-Sakakibara *et al.*, 2019) and flavonoid biosynthetic ability was also reported in divergent evolutionary lineages of microalgae and bacteria (Goiris *et al.*, 2014; Jiao *et al.*, 2020), suggesting

Table 1. Some selected chemical compounds characterized and isolated from bryophytes with different bioactivities and their actions.

Compounds	Type	Species	Bioactivities	Actions	References
β -Phellandrene, β -caryophyllene	Terpenoid	<i>Porella cordaeana</i>	Antimicrobial	MIC 0.5–2 mg/mL for yeast, 1–3 mg/mL for bacteria	(Bukvicki et al., 2012)
b-Bazzanene isobazzanene aromadendrene	Sesquiterpene	<i>Scapania nemorea</i>	Antimicrobial	MIC 0.5–3 mg/mL for bacteria 0.2–1 mg/mL for yeasts	(Bukvicki et al., 2014)
Main components: α -pinene (16.1%), 3-octanone (48.1%), and eicosane (28.6%)	Terpenoid	<i>Pseudoscleropodium purum</i> , <i>Eurhynchium striatum</i> and <i>Eurhynchium angustirete</i>	Antioxidative antimicrobial	MIC ranging from 278.2 to 2,225 μ g/mL	(Tosun et al., 2015)
Porellacetals A-D	Pinguisane (Terpenoid)	<i>Porella cordaeana</i>	Anti-cancer	50–80% maximum inhibition rate	(Tan et al., 2017)
Jamesoniellides Q – S	Diterpenoid	<i>Jamesoniella autumnalis</i>	Anti-inflammatory	Suppress nitric oxide (NO) production	(Y. Li et al., 2018)
(1S,3E,7E,11S,12S)-12-hydroxy-dolabella-3,7-dien-6-one, (1S,3E,7Z,11S,12S)-12-hydroxy- dolabella-3,7- dien-6-one, (3S,5S,8R,10R,13S,16R)-3,13-dihydroxy-ent-kauran-15-one, (4R,5R,7S)-7-hydroxy-7-isopropyl-1,4-dimethylspiro [4.4] non-1-ene-2-carbaldehyde, (6R,7S,10R)-6,7-dihydroxy-3-oxo-eudesma-4E-ene	Terpenoid	<i>Lepidozia reptans</i>	Anti-inflammatory		(S. Li et al., 2018)
Scapanacins A–D	Terpenoid	<i>Scapania carinthiaca</i>	Antihypertensive and antitumor Antiproliferative		(Qiao et al., 2018)
(–)-Cis-perrottetinene (cis-PET)	Bibenzyl	<i>Radula</i>		Structurally resembles (–)- Δ^9 -trans-tetrahydrocannabinol (Δ^9 -trans-THC) from <i>Cannabis sativa</i>	(Chicca et al., 2018)
Marchantin A	Bisbibenzyl	<i>Marchantia polymorpha</i>	Anti-plasmodial	NF54 (IC ₅₀ = 3.41 μ M) and K1 (IC ₅₀ = 2.02 μ M) against <i>Plasmodium falciparum</i>	(Jensen et al., 2012)
Marchantin A	Bisbibenzyl	<i>M. emarginata</i> subsp. <i>tosana</i>	Anti-cancer	IC ₅₀ of 4.0 μ g/mL on human MCF-7 breast cancer cells	(Huang et al., 2010)
Marchantin A, B and E	Bisbibenzyl		Antioxidant	free radical-scavenging activity (EC ₅₀ = 20 μ g/mL)	(Huang et al., 2010)
Plagiochin A	Bisbibenzyl	<i>M. polymorpha</i> and <i>M. paleacea</i> var. <i>diptera</i>	Anti-influenza	Inhibition of PA endonuclease activity, inhibitory properties toward the growth of influenza A and B	(Iwai et al., 2011)
Perrottetin F	Bisbibenzyl	<i>Radula koljana</i>			
Phenanthrene compound	Bisbibenzyl	<i>Plagiochila sciophila</i> <i>Lunularia cruciata</i> <i>Lunularia cruciata</i>	Anti-cancer	Cytotoxic activity against A549 lung cancer cell line with IC ₅₀ values of 5.0 and 5.0 μ M	(Novakovic et al., 2019)
($\pm$)-Rasumatranin A – D, M and N	Bibenzyl-based meroterpenoid	<i>Radula sumatrana</i>	Cytotoxicity	Against human cancer cells	(X. Wang et al., 2017)
Dicranone	Acetylenic Oxylipin	<i>Dicranum scoparium</i>	Antifeeding		(Rempt and Pohnert, 2010)
Hexane extract		<i>Polytrichastrum formosum</i>	Anti-insect	70.33% against <i>Sitophilus granarius</i>	(Abay et al., 2013)

that the ability for flavonoid production originated earlier during evolution than previously thought (Yonekura-Sakakibara *et al.*, 2019). To our knowledge, no flavonoids have been reported from hornworts. Either because the divergence of hornworts occurred before flavonoid pathway evolved or the hornwort ancestor acquired mutations that caused loss of the flavonoid biosynthetic ability and subsequently caused flavonoid loss in this lineage (Davies *et al.*, 2020). The derivatives of the cinnamic acid, which is the central intermediate in the biosynthesis of flavonoids and other phenylpropanoids, are reported in the hornwort *Anthoceros agrestis* (Soriano *et al.*, 2018; Wohl and Petersen, 2020).

Major classes of flavonoids in bryophytes are flavones, flavonols, isoflavonoids, auronones, 3-deoxyanthocyanins, anthocyanins, and recently discovered auronidins exclusive for liverworts (Berland *et al.*, 2019). Flavonoids play diverse roles in bryophyte life-cycle, such as UV-B radiation protection (Waterman *et al.*, 2017; Li *et al.*, 2019), protection against desiccation and extreme temperature fluctuations (mostly due to anthocyanins), and defense against pathogens (sesquiterpenoids have the same function) (Peters *et al.*, 2019). Flavonoids also support the growth of hydroids and leptoids of mosses (which have similar functions as tracheids and sieve cells in vascular plants) by the activity of a few methoxyphenols and cinnamic acids as part of proto-lignin constituents (Townsend, 1964; Peters *et al.*, 2019).

Common flavonoids in liverworts and mosses are luteolin and apigenin and their derivatives (Asakawa *et al.*, 2013), and these flavonoids and their derivatives are present in vascular plants as well. Bi- and tri-flavonoids are more common in Bryophyta, and bioflavonoids are thought to be chemotaxonomic marker of mosses.

Pigments such as cell-wall bound red flavonoids riccionidin (an auronidin) (Berland *et al.*, 2019) and sphagnorubin (Vowinkel, 1975), have been reported from liverworts and peat mosses (Figure 1e), respectively. Auronidins constitute an unreported flavonoid class thus far (Berland *et al.*, 2019) and are unrelated to anthocyanins, which are the main red pigments present in angiosperms. Carella *et al.* (2019) reported that *M. polymorpha* accumulated red pigmented Riccionidin A into the thallus cell walls during biotic stress (upon oomycete pathogen infection), mediated by R2R3-MYB transcription factor, which led to largely increased liverwort resistance. R2R3-MYB activation of flavonoid production in the same species during abiotic stress has also been delineated (Albert

et al., 2018). Some species of other thalloid liverworts roll their thalli over the dorsal surface when dried out, so that dark pigmented ventral side is left exposed (Reeb *et al.*, 2018; Davies *et al.*, 2020), whereas some desiccation-tolerant leafy liverworts also tend to be dark pigmented (Vitt *et al.*, 2014). The adaptive value of these pigmentations of assumed auronidin type is still subject for debate, light screening, ROS scavenging, strengthening of the cell wall and biotic stress defense are mentioned as possible functions (Davies *et al.*, 2020).

Like terpenoids, allelopathic activity has been reported for flavonoid compounds in bryophytes (Whitehead *et al.*, 2018).

2. Bibenzyls/bisbibenzyls

Liverworts (Marchantiophyta) are copious producers of bibenzyls and bisbibenzyls with 103 characterized compounds so far (Yoshida *et al.*, 2016). Their physiological and ecological roles are not fully understood.

Marchantin A is one of the well-studied bisbibenzyls, isolated from *Marchantia* species, whose antibacterial and antifungal activities have been confirmed (Niu *et al.*, 2006). Subsequently, it was reported that Marchantin A inhibited proliferation of protozoan species such as *Plasmodium falciparum* NF54 with $IC_{50} = 3.41 \mu M$ and K1 with $IC_{50} = 2.02 \mu M$; and showed cytotoxic activity against *Trypanosoma brucei rhodesiense*, *T. cruzi* and *Leishmania donovani* with IC_{50} values 2.09, 14.90 and 1.59 μM , respectively (Jensen *et al.*, 2012). Marchantin A also showed malaria prophylactic potential with moderate inhibitory activity against enzymes of *P. falciparum* (Jensen *et al.*, 2012). Marchantin A, as well as marchantin B and E, and other marchantin-related phytochemicals from liverworts, inhibit influenza PA endonuclease activity *in vitro* and exert anti-influenza activity in culture cells (Iwai *et al.*, 2011). *Radula* is another interesting liverwort genus because it produces not only bibenzyls and bis-bibenzyls but also bibenzyl cannabinoids cis-perrottetinene (cis-PET) (Asakawa *et al.*, 2013), which structurally resembles (–)- Δ^9 -trans-tetrahydrocannabinol (Δ^9 -trans-THC) from *Cannabis sativa* (Toyota *et al.*, 2002). The precursor of THC in *C. sativa* is olivetolic acid, whereas stilbene acid is the precursor of PET in *R. marginata* (Hussain *et al.*, 2019, 2018). This natural product cis-PET was proven to be psychoactive by mimicking the action of the endocannabinoid 2-arachidonoyl glycerol and provoked a significant decrease of brain prostaglandin levels in a CB1 receptor-dependent manner in mice (Chicca *et al.*, 2018). So far, *R. chinensis*, *R.*

campanigera, *R. laxiramea*, *R. marginata*, *R. perrottetii* and a Peruvian unidentified species have been proven to contain cis-PET (Asakawa *et al.*, 2020).

3. Lipids and lipid-derivatives

High amounts of arachidonic acid (AA, C20:4) and eicosapentaenoic acid (EPA, C20:5) were detected in bryophytes (Beike *et al.*, 2014). These very long chain unsaturated fatty acids are uncommon in higher plants but abundant in bryophytes because of the presence of $\Delta 6$ -desaturase, $\Delta 5$ -desaturase (first identified by Girke *et al.*, 1998) and $\Delta 6$ -elongase. AA is synthesized from linoleic acid (C18:2) via ω - pathway and EPA from α -linolenic acid (C18:3) via ω -3 pathway (Kaewsuwan *et al.*, 2006). The presence of AA and EPA appear to be an ancestral chemical trait that links bryophytes to charophyte algae, since very long chain unsaturated fatty acids are rarely found in tracheophytes but are commonly produced in algae (Resemann *et al.*, 2019). Lu *et al.* (2019) summarized the fatty acid compositions and contents in several moss and liverwort species from previous studies. Recently, it was reported that the lipidome of *P. patens* protonema comprising 733 molecular species derived from glycerolipids, sterol lipids and sphingolipids, whereas Arabidopsis plants harbor only about 54% of this diversity (Resemann *et al.*, 2021). Moreover, a sphingolipid-modifying enzyme was identified that contributes to pathogen defence and cold tolerance, but has no homolog in seed plants (Resemann *et al.*, 2021). Large amounts of polyunsaturated C20 fatty acids in bryophytes imply that they can produce a broad range of oxylipins (Scholz *et al.*, 2012). *P. patens* has an enzyme lipooxygenase with fatty acid chain-cleaving lyase activity, which uses C18-fatty acids and C20-fatty acids as substrates for producing more oxylipins than angiosperms (Senger *et al.*, 2005; de León *et al.*, 2015) whose pathway is activated during bacterial infection (Alvarez *et al.*, 2016). High amounts of long unsaturated fatty acids and existence of oxylipins represent a metabolic difference between mosses and angiosperms, that might have been advantageous for mosses in terms of tolerance to abiotic stress (Mikami and Hartmann, 2004) and protection from pathogens (Ponce de León and Montesano, 2017). Oxylipins are also involved in plant signaling. Common oxylipins, such as phytohormone jasmonic acid (JA), have been found in all vascular plants but not in bryophytes, in which the JA biosynthesis pathway stops at its precursor 12-oxophytodienoic acid (OPDA) (Stumpe *et al.*, 2010; Wasternack and Feussner, 2018). Acetylenic fatty acid derived oxylipin

may serve as a putative precursor of volatile oxylipin and can be triggered by mechanical wounding (Abay *et al.*, 2015). Dicranin, an acetylenic fatty acid, which is found almost exclusively in the Dicranaceae family, has slug anti-feeding activity (Rempt and Pohnert, 2010). In addition, acetylenic acids sometimes appear as part of triacylglycerol to maximize energy conservation when growth space is limited (Dembitsky, 1993).

Tocopherol plays an important role as antioxidants for long chain unsaturated fatty acids and terpenoids. Two hundred and sixty-six (36.3%) liverwort species accumulated α -tocopherol (Asakawa *et al.*, 2013). In *Porella* and *Pellia* this percentage is even higher (64% and 60%, respectively) (Asakawa *et al.*, 2020).

IV. Genodiversity of bryophytes

Whereas the metabolic diversity of bryophytes is increasingly recognized, the genetics underlying this chemical diversity largely remain to be described. To date, a small number of (draft) genomes (including *P. patens*, *Ceratodon purpureus*, *Fontinalis antipyretica*, *Pleurozium schreberi*, *M. polymorpha*, *Marchantia inflexa*, *Sphagnum fallax*, *Sphagnum magellanicum*, *A. agrestis*, *Anthoceros punctatus*, *Anthoceros angustus*, *H. plumaeforme*) are available (<http://phytozome.jgi.doe.gov/>) (Rensing *et al.*, 2008; Bowman *et al.*, 2017; Lang *et al.*, 2018; Weston *et al.*, 2018; Marks *et al.*, 2019; Pederson *et al.*, 2019; Li *et al.*, 2020; Mao *et al.*, 2020; Zhang *et al.*, 2020). However, within the next 5 years this number is expected to increase significantly. The OneKP database encompasses transcriptome resources of 74 species (7 hornworts, 41 mosses, 26 liverworts) (<https://sites.google.com/a/ualberta.ca/onekp/>), which are mostly obtained from gametophores (2019). The NCBI database currently lists a total of 443 transcriptome datasets, some of which also capture the *P. patens* transcriptome under abiotic stress (<https://www.ncbi.nlm.nih.gov>, Richardt *et al.*, 2010; Beike, Lang, *et al.*, 2015). For *P. patens*, a comprehensive gene atlas encompassing all developmental stages and the impact of some abiotic stresses, is available (Ortiz-Ramírez *et al.*, 2016; Perroud *et al.*, 2018). Generally, the advancement of next generation sequencing techniques, especially the long-read sequencing technology, has resulted in an accelerating accumulation of genomic data for bryophytes the last few years, thus paving the road for functional approaches and comparative genomics.

A. Genome sizes and transcriptome complexities of bryophytes

The genome size in bryophytes ranges between 122 and 20,006 Mbp, which coincidences with the lower spectrum of Angiosperms (Michael, 2014). Despite their small stature, some bryophytes like *P. patens* surpass angiosperms such as the model plant *A. thaliana* in genome size. Liverworts exhibit a higher degree of genome size diversity compared to mosses and hornworts (see Figure 3a). Compared to other plant lineages, bryophytes carry a remarkably high number of protein-encoding genes relative to their size. This transcriptome complexity can be utilized as a valuable source for mining of novel genes. For example, the moss *P. patens* contains more genes than the flowering plant *Catharanthus roseus*, which is known for its specialized metabolite characteristics, at comparable genome size (see Table 2).

Liverworts generally have 8 to 9 chromosomes with little variation, thus genome duplications are unlikely unless very ancient. A small number of (allo)polyploids are known to occur in some genera (e.g., *Porella baueri*) (Boisselier-Dubayle et al., 1998). Despite the sister relationships between liverworts and mosses, it seems that the larger genomic size range has evolved independently in liverworts and is not a

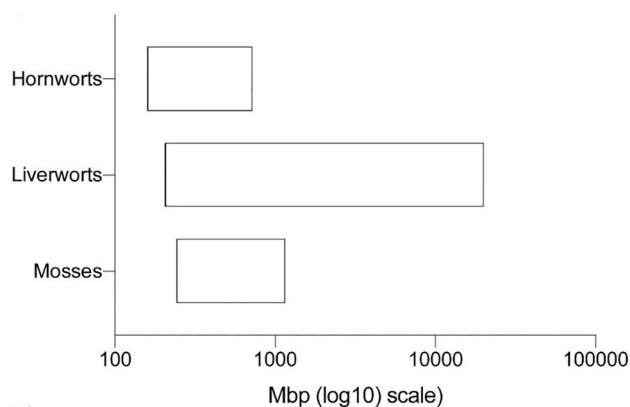


Figure 3. Genome size of different classes of bryophytes (Bainard et al., 2020).

trait shared with mosses (Bainard et al., 2020). Genome duplications have not been found in hornworts yet (Li et al., 2020). By contrast, the moss *P. patens* underwent two whole genome duplication events about 40–48 million years ago and 27–35 million years ago (Lang et al., 2018). An excess of duplicate metabolic genes has been retained after these events, which may explain abundance of such genes in the *P. patens* genome (Lang et al., 2005; Rensing et al., 2007). Interestingly, an abundance of some gene families encoding specialized metabolites could also be observed in the genome of *M. polymorpha* and *A. angustus* (Bowman et al., 2017; Zhang et al., 2020).

B. Conservation of precursor routes of specialized metabolism

In general, the biosynthesis of specialized metabolites is scarcely studied in bryophytes. Throughout the last years, initial insights into the terpenoid and phenylpropanoid pathway have been obtained. It has been suggested that both the terpenoid precursor pathways, mevalonate (MVA) and methylerythritol 4-phosphate pathway (MEP), are conserved throughout land plants based on similar copy number of pathway genes in *M. polymorpha*, *P. patens*, *A. thaliana* and *Oryza sativa* (Chen et al., 2018). This pattern can also be found in the recently annotated genomes from the *Anthoceros* genus (Li et al., 2020; Zhang et al., 2020). However, neither genes associated with major bottlenecks in the pathways, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) and 1-deoxy-D-xylulose-5-phosphate synthase (DXS) nor isopentenyl diphosphate isomerase (IDI), that predominantly controls the DMAPP:IPP flux, which plays a significant role in the synthesis of different isoprenoid classes, have been functionally studied yet. The few described terpenoid synthases (see Table 3) indicate a partial terpenoid pathway conservation across land plants but also the absence of downstream enzymes catalyzing the synthesis of eminently important products for

Table 2. Genome size in comparison to protein-encoding genes in selected plants.

Organism	Genome size	Protein-encoding genes	Gene density (per Mbp)	Reference
<i>P. patens</i>	500	35,000	70	(Lang et al., 2018)
<i>H. plumaeforme</i>	434	32,195	74.18	(Mao et al., 2020)
<i>M. polymorpha</i>	220	19,138	87	(Bowman et al., 2017)
<i>A. punctatus</i>	132.8	25,800	194.3	(Li et al., 2020)
<i>A. agrestis</i>	122.9	24,700	201	(Li et al., 2020)
<i>A. angustus</i>	119	14,629	122.9	(Zhang et al., 2020)
<i>Selaginella moellendorfi</i>	100	27,793	277.9	(Banks et al., 2011)
<i>A. thaliana</i>	135	27,655	204.9	(Zimmer et al., 2013)
<i>Nicotiana benthamiana</i>	3136	50,516	14.4	(Schiavinato et al., 2019)
<i>Picea abies</i>	19,600	30,000	1.5	(Nystedt et al., 2013)
<i>Catharanthus roseus</i>	500	33,258	66.5	(She et al., 2019)

Table 3. Catalytic functions of TPSs from bryophytes that have been characterized.

Species	Enzyme	Substrate	Products	Reference
<i>M. polymorpha</i>	MpDTPS1 MpDTPS3 MpDTPS4	GGPP GGPP Ent-copalyl diphosphate	<i>ent</i> -Atisanerol <i>ent</i> -copalyl diphosphate <i>ent</i> -Kaurene	(Kumar <i>et al.</i> , 2016)
<i>C. conicum</i> *	CcSS CcBPPS	GPP GPP	Sabinene Bornyl Acetate	(Adam and Croteau, 1998)
<i>H. plumaeforme</i>	HpDTC1	GGPP	<i>syn</i> -Pimara-7,15-diene	(Okada <i>et al.</i> , 2016)
<i>P. patens</i>	PpCPS/KS	GGPP	<i>ent</i> -Beyerene <i>ent</i> -Sandaracopimaradiene <i>ent</i> -Kaur-16-ene 16-hydroxy- <i>ent</i> -kaurene	(Zhan <i>et al.</i> , 2015; Hayashi <i>et al.</i> , 2006)
<i>R. natans</i>		GPP	4S-(-)-Limonene	(Adam <i>et al.</i> , 1996)

*The Monoterpene content varies amongst species. *CcSS was identified from a European species, CcBPPS from a North American species.

tracheophytes (e.g., gibberellic acid; see Table 3). Most recently, this perception has been challenged *via* a comparative computational approach, that revealed the presence of gibberellin biosynthesis and inactivation genes in bryophytes (Cannell *et al.*, 2020).

Such as the terpenoid precursor pathway, a conservation of the early phenylpropanoid pathway genes (PAL, C4H, 4CL, CHS, CHIL) is indicated across all land plants (Tohge *et al.*, 2013; Davies *et al.*, 2020). Downstream enzymes in the flavonoid pathway occur in accordance with the respective flavonoid profile in mosses and liverworts, but have not been detected in hornworts yet. Nevertheless, 21 copies of flavonoid 3' monooxygenase genes and 11 of flavonoid,3',5', hydroxylases have been identified in *A. angustus* most recently (Zhang *et al.*, 2020). To date, their functional roles have not been investigated. In contrast to seed plants and liverworts, mosses produce flavonoids, although a chalcone isomerase (*CHI*) gene is missing (Cheng *et al.*, 2018; Clayton *et al.*, 2018). The exact metabolic pathway remains unknown so far.

Chalcone synthase (CHS), belonging to the class III Polyketide synthase (PKS) superfamily, is considered as metabolic gatekeeper of flavonoid biosynthesis in plants. Functional characterization of a CHS from *Plagiochasma appendiculatum* confirmed for the first time this key role in the flavonoid biosynthesis of thalloid liverworts (Yu *et al.*, 2015). Eight *CHS* gene classes have been reported from the moss *P. patens*, but they remain to be functionally characterized (Koduri *et al.*, 2010).

C. Partial conservation of regulatory mechanisms targeting specialized metabolism

Studies of metabolic regulation mostly target developmental processes or transcriptomic dynamics under various stressors. Comparative expression profiling have revealed evolutionary conservation of transcriptional regulation under stress conditions in bryophytes (Richardt *et al.*, 2010; Beike, Lang, *et al.*, 2015).

Genomic data suggests that members of the *Anthoceros* genus and *M. polymorpha* have a small transcription factor repertoire (Bowman *et al.*, 2017; Li *et al.*, 2020). Considering the high genome size range in liverworts, this repertoire might fluctuate significantly and might be linked to specialized metabolism. For example, the liverwort *Radula marginata* carries a significantly larger number of transcription factors compared to *M. polymorpha* and to some mosses (Hussain *et al.*, 2018). Comparative studies revealed six transcription factor (TF) families unique to this organism; possibly related to its cannabinoid metabolism (Hussain *et al.*, 2018; Hussain and Kayser, 2019). In the moss *P. patens*, this transcription factor repertoire is even larger due to ancient genome duplications, although the TF response under salt stress appeared rather limited compared to *A. thaliana* (Rensing *et al.*, 2007; Richardt *et al.*, 2010). It was hypothesized that one of the reasons may be the partial absence of biosynthetic routes, e.g., parts of the jasmonic acid signaling pathway. Predominant co- and post-transcriptional regulation, which has been suggested on the basis of distinct 5'UTR-intron characteristics, is also a possible explanation (Richardt *et al.*, 2010; Zimmer *et al.*, 2013). Interestingly, the first genes to be transcribed upon cold stress in *P. patens* are predominantly moss- or even species-specific and of yet unknown function (Beike, Lang, *et al.*, 2015).

Conservation of the terpenoid precursor pathway has been anticipated in bryophytes, but functional studies targeting its regulation are lacking.

Phytochrome interacting factors (PIFs) have been reported as regulators in the MEP pathway by regulating genes encoding the key limiting enzymes DXS and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) as well as phytoene synthase (PSY), the gatekeeper of carotenoid biosynthesis (Chenge-Espinosa *et al.*, 2018). Functional conservation of PIFs across seed plants, mosses and liverworts has been reported (Lee and Choi, 2017; Possart *et al.*, 2017).

Initial insights into the regulation of chemicals derived from the phenylpropanoid pathway in

Table 4. Transcription factors that have been linked to pathway regulation of aromatic chemicals.

TF	Pathway	Species	Reference
MpMYB02	Anthocyanidins, Bisbenzyls	<i>M. polymorpha</i>	(Kubo <i>et al.</i> , 2018)
MpMYB14	Anthocyanidins, Phenylpropanoids	<i>M. polymorpha</i>	(Albert <i>et al.</i> , 2018)
MpMYB14	Phenylpropanoids	<i>M. polymorpha</i>	(Kubo <i>et al.</i> , 2018)
MpHY5	Phenylpropanoids	<i>M. polymorpha</i>	(Clayton <i>et al.</i> , 2018)
MpRUP1	Phenylpropanoids	<i>M. polymorpha</i>	(Clayton <i>et al.</i> , 2018)
PaBHLH	Bisbibenzyls, Phenylpropanoids	<i>P. appendiculatum</i>	(Wu <i>et al.</i> , 2018)
PaBHLH1	Phenylpropanoids	<i>P. appendiculatum</i>	(Zhao <i>et al.</i> , 2019)
MpBHLH12	Phenylpropanoids	<i>M. polymorpha</i>	(Arai <i>et al.</i> , 2019)

liverworts reveal similarities to seed plants (Table 4). R2R3-MYB transcription factors have a key role in the stress-related regulation of flavonoid biosynthesis. Recently, two R2R3-MYB analogs (MpMYB02, MpMYB14) in *M. polymorpha* have been characterized, indicating a conservation of this regulatory feature across land plants (Albert *et al.*, 2018; Kubo *et al.*, 2018). On the other hand, central parts of the UV-mediated response in flavonoid biosynthesis like the central activator ELONGATED HYPOCOTYL5 (HY5), and negative feedback regulation by REPRESSOR OF UV-B PHOTOMORPHOGENESIS1 (RUP1) are conserved between *A. thaliana* and *M. polymorpha* (Clayton *et al.*, 2018). In addition, three BHLH transcription factors from *P. appendiculatum* (PaBHLH, PaBHLH1) and *M. polymorpha* (MpBHLH12) with regulatory roles in bisbibenzyl and phenylpropanoid biosynthesis have been identified (Wu *et al.*, 2018; Arai *et al.*, 2019; Zhao *et al.*, 2019). Most interestingly, the overexpression of PaBHLH and PaBHLH as well as MpMYB02 and MpMYB14 lead to the accumulation of significantly higher levels of flavonoids, bisbibenzyls and anthocyanidins, respectively (Kubo *et al.*, 2018; Wu *et al.*, 2018; Zhao *et al.*, 2019).

All in all, the small repertoire of transcriptional factors makes bryophytes prime candidates to study the basic mechanisms of pathway regulation as well as pathway evolution in comparative genomic approaches. The identification and functional elucidation of the regulatory steps of specialized metabolites will not only provide insights into the evolutionary machinery, but also reveal key knowledge for a successful biotechnological exploitation.

D. Gene duplication events cause expansion of gene families encoding specialized metabolites

The increasing availability of genomic resources allows new insights into the genomic complexity of bryophytes, including expansion of different gene families in the major bryophyte lineages (Linde *et al.*, 2017). Comparative approaches suggest that genes encoding specialized metabolites are particularly abundant in

liverworts (Davies *et al.*, 2020), possibly reflecting a more pronounced chemical diversity compared to mosses and hornworts. Besides, some gene families encoding specialized metabolites are more expanded in bryophytes than in other land plants. A good example is the polyphenol oxidase (PPO) gene family, which occurs in low copy number in seed plants, and is even absent in some species such as *A. thaliana*. PPOs cause a typical browning reaction in damaged tissues, and there is evidence supporting its role in plant defense (Constabel and Barbehenn, 2008), but in general, the physiological role of PPOs is not well studied. They occur in high copy numbers in mosses and in even higher numbers in liverworts (Tran *et al.*, 2012; Davies *et al.*, 2020). Interestingly, the recent genome assembly of *A. angustus* revealed a high number of protein-encoding PPOs in hornworts as well (Zhang *et al.*, 2020), indicating that all bryophyte phyla have expanded the PPO genes (see Figure 4a). The presence of PPO genes in *M. polymorpha* is linked to tandem repeats and gene clusters (TAGs) and notably, 66% of the PPO genes were associated with TAGs, in contrast to 5.9% TAG presence throughout the rest of the *Marchantia* genome. Functional studies are lacking, but it has been speculated that expansion of PPOs is related to ecological diversification and specialized metabolism (Davies *et al.*, 2020).

Beside TAGs, a role of gene clustering in specialized metabolism has been described most recently in momilactone biosynthesis of the moss *H. plumaeforme*. This is the first evidence of gene clustering of biosynthetic pathway genes of specialized metabolites in bryophytes and emphasizes the significance of the genomic architecture in the synthesis of specialized metabolites not only in vascular plants, but also in bryophytes (Mao *et al.*, 2020).

Other examples of large gene families involved in the synthesis of specialized metabolites are Dirigent proteins or PKS. Particularly type III PKS may be a significant driver of metabolic diversity in plants (Yonekura-Sakakibara *et al.*, 2019). 24 PKS-like genes have been found in *M. polymorpha*, which is a substantially higher number compared to seed plants like

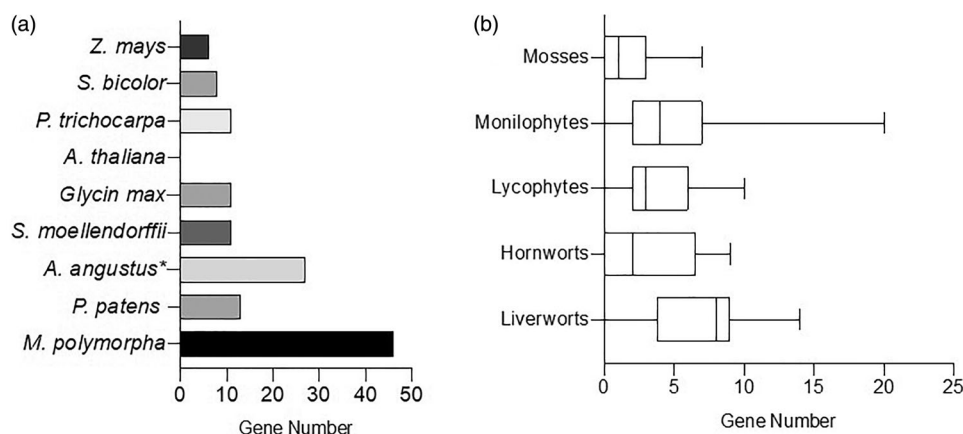


Figure 4. (a) Number of transcribed polyphenol oxidases per genome *number of gene copies on genome (Bowman *et al.*, 2017; Zhang *et al.*, 2020). (b) Average of expressed MTPSL genes per genome on basis of transcriptomic analysis across plant groups known to express MTPSLs (adopted from Jia *et al.* 2016).

A. thaliana (4), *Malus domestica* (10), *Vitis vinifera* (13) and *Populus tremula* (14) (Su *et al.*, 2017). Most of the copies seem to be a result of an ancient *CHS*/*PAL* gene pair duplication. Besides *CHS*, the functional role of most *PKS* remains unknown (Fischer *et al.*, 1995; Bowman *et al.*, 2017; Davies *et al.*, 2020). In *R. marginata*, stilbene synthase, which evolved from a *CHS* gene (Tropf *et al.*, 1994), has been recently identified as one of the precursors involved in the biosynthesis of the psychoactive cannabinoid (-)-*cis*-perrottetinene (*cis*-PET) (Hussain *et al.*, 2018).

E. Mining of novel gene families

The progressive availability of genomic resources allows for mining of genes that are absent in seed plants, which is particularly interesting with regard to the synthesis of specialized metabolites. For example, transcriptome-mining in *M. polymorpha* revealed a novel group of mono- and sesquiterpene-like synthases, most of which resemble microbial terpene synthases, motivating the name microbial terpene synthase-like genes (MTPSLs) (Kumar *et al.*, 2016). MTPSLs contain a single α -domain, in contrast to a typical plant TPS which is comprised of either two ($\alpha\beta$ -type) or three structural domains ($\alpha\beta\gamma$ -type) (Jia *et al.*, 2018). Comparative transcriptome-mining across the plant kingdom have confirmed the exclusive occurrence of this novel class in nonseed plants (see Figure 4b). Liverworts showed by far the highest MTPSL-richness (Jia *et al.*, 2016), although MTPSLs are wide-spread amongst all groups of bryophytes; more than two-thirds of transcriptomes include MTPSLs and members of all four MTPSL clades occurs (Jia *et al.*, 2016). Some of the MTPSL products are identical or similar to terpenoids previously shown

to be products of classical TPS and assumed to take part in the protection against abiotic and biotic stresses (Jia *et al.*, 2016; 2018; Kumar *et al.*, 2016; Xiong *et al.*, 2018). At present, a small number of MTPSLs have been functionally characterized and there is a high potential for future discovery (Jia *et al.*, 2016).

V. Biotechnology

In order to utilize bryophytes for the commercial production of natural compounds, sophisticated ways are needed to cultivate and generate large amounts of biomass rapidly. Another crucial factor concerns development of tools that allow fast and reliable metabolic engineering. Therefore, this section summarizes different approaches that have emerged in the last decade to support the transformation of bryophytes and foremost the moss *P. patens*, into an alternative production platform for natural compounds.

A. Cultivation and scale-up production

A range of species have already been established as axenic cultures, generally by being grown photoautotrophically in simple low-cost inorganic media without the supplementation of microelements, vitamins and phytohormones (Hohe and Reski, 2005; Beike *et al.*, 2010). As part of the Mosstech.eu project around 50 species have been brought into axenic culture, which shows that many bryophytes can be cultivated in cell cultures (www.mosstech.eu). Out of these, 15 species are at present deposited at the International Moss Stock Center (IMSC, <https://www.moss-stock-center.org/>) with the following accession numbers: 40096, 40097,



Figure 5. Cultivation of bryophytes in photobioreactors. (a) Photobioreactor for small-scale production of *P. patens*. (b) Photobioreactor for small-scale production of peat moss (*Sphagnum palustre*). (a,b) Source: ReskiLab, University of Freiburg, <http://www.plant-biotech.net>, CC BY-SA 3.0. (c) Photobioreactor100L wave-bag photobioreactor for industrial applications (source: eleva GmbH).

40098, 40099, 40100, 41101, 41102, 41103, 41104, 41248, 41254, 41255, 41212, 41249, 41246.

In vitro cultures of bryophytes can be initiated from surface-sterilized spores, gemmae or vegetative fragments (Beike *et al.*, 2010). Compared to seed plants, bryophytes possess simple body plans and unique regeneration capacity from fragments and even from single cells. Bryophytes can be axenically cultured on solid agar-based media or in agitated flask liquid cultures from a few milliliters up to several hundreds of liters (Figure 5a,b) (Decker *et al.*, 2014). Efficient protocols for protoplast cultures and growth of whole plants have been established for several bryophytes (Hohe and Reski, 2002; Li *et al.*, 2005; Bach *et al.*, 2014). Growth characteristics and conditions of cultivation for some bryophyte species are presented in Table 5. Among the different developmental stages, the suspension-cultured protonemal tissue is the most suitable for biotechnological approaches because of its genetic stability, reduced somaclonal variation and high homologous recombination rate during genetic transformation (see Figure 5a,b) (Decker and Reski, 2008, 2012; Reski, 1998; Decker *et al.*, 2014). For high-throughput production of moss biopharmaceuticals, disposable 100 L and 500 L wave-bag bioreactors are applied (Niederkrüger *et al.*, 2019) (<https://www.elevabiologics.com>).

A protocol for cryopreservation of bryophytes (more than 140,000 specimens) was developed by Schulte and Reski (2004) and subsequently used to establish the International Moss Stock Center (IMSC <https://www.moss-stock-center.org/>), which ensures longevity and stability of a bryophyte collection (Rowntree *et al.*, 2011).

B. Elicitation of production of natural products in bryophytes

Enhancing the yield of compounds is a major step for large-scale production, which is either done through abiotic and biotic elicitation or through genetic manipulation (see Section V.C.). Elicitation is mainly performed by abiotic (light, temperature, salt, etc.) and biotic (bacteria, fungus, proteins, etc.) stimuli that induce biosynthesis of specialized metabolites (Thakur *et al.*, 2019). In the moss *P. patens*, genes encoding enzymes involved in important defense pathways such as phenylpropanoids, were induced by infection with *Pectobacterium carotovorum* bacteria (Alvarez *et al.*, 2016). The chemical response to the induction was not analyzed. In *P. patens* ultraviolet (UV)-B irradiation induced genes that encode for enzymes for flavonoid biosynthesis (Wolf *et al.*, 2010), and in the liverwort *M. polymorpha*, UV-C induced the synthesis of the bisbibenzyls isoriccardin C, marchantin C, and riccardin F, through the abscisic acid (ABA) signaling pathway (Kageyama *et al.*, 2015). Production of phytoalexins momilactone A and B were also induced by UV and, jasmonic acid- and cantharidin-treatments in the moss *H. plumaeforme* (Kato-Noguchi, 2009). Moreover, it was shown that intracellular flavonoid level in *M. linearis* were induced by the application of methyl jasmonate, 2-(2-fluoro-6-nitrobenzylsulfa-nyl) pyridine-4-carbothioamide and 2,4-Dichlorophenoxyacetic acid (Krishnan and Murugan, 2014). Wounding stress induces the production of the compounds luteolin, apigenin and isoriccardin C in *M. polymorpha*, biosynthesized through the phenylpropanoid pathway (Yoshikawa *et al.*, 2018), which is interesting since blending is often applied during cultivation.

Table 5. Established cultivation systems for different bryophyte species.

Bryophytes	Cultivation system	Relevant features	References
Mosses			
<i>Polytrichum juniperinum</i>	Protonema-derived suspension cultures at shake-flask scale	Growth rate in a mixotrophic condition was ($\mu\text{max } 0.27 \text{ d}^{-1}$) three times greater than in autotrophic and heterotrophic conditions. Protonema cultures tolerated a wide initial medium pH range (4.5–8).	(Ruiz-Molina <i>et al.</i> , 2016)
<i>Sphagnum palustre</i>	Gametophores cultivation in flasks and photobioreactor	Sucrose and ammonium nitrate, added in the media, were able to increase the biomass by around 10- to 30-fold within 4 weeks.	(Beike, Spagnuolo, <i>et al.</i> , 2015)
<i>Sphagnum squarrosum</i>	Protonema-proliferation liquid culture	Detailed time schedule of the thallose protonema regeneration and subsequent developmental processes was established.	(W. Zhao <i>et al.</i> , 2019)
<i>Peat mosses (Sphagnum L.)</i>	Solid and suspension cultures	Establishment of axenic <i>in-vitro</i> cultures of 19 <i>Sphagnum</i> species.	(Heck <i>et al.</i> , 2021)
Liverworts			
<i>Marchantia linearis</i> Lehm. and Lindenb.	Temporarily immersed cultures using Rita® bioreactor	Time required for thallus regeneration, and development was reduced substantially using liquid culture media in Rita®.	(Krishnan and Murugan, 2014)
<i>Conocephalum conicum</i> (L.) Lindenb., <i>Reboulia hemispherica</i> (L.) Raddi and <i>Marchantia paleacea</i> Bertol.	Solid agar-based cultivation	All the three species grew well in half strength Knop's macronutrients + Nitsch's trace elements with 10 ppm freshly prepared ferric citrate under the continuous illumination of 4,500–5,000 lux at $20 \pm 2^\circ \text{C}$ temperature	(Awasthi <i>et al.</i> , 2012)

C. Metabolic engineering in bryophytes

Bryophytes display many features that make them attractive biotechnological platforms for the production of specialized compounds, lipids and recombinant biopharmaceutical proteins. These advantages include standardized cultivation methods under sterile conditions in bioreactors (see section above) and efficient transformation methods for genetic engineering.

The methods for genetic engineering have been developed since 1991 when the first method was published for *P. patens* (Schaefer *et al.*, 1991). It has been followed with methods for engineering of *Ceratodon purpureus* (Thümmel *et al.*, 1992) and *Marchantia polymorpha* (Nasu *et al.*, 1997). The methods for engineering are mainly polyethylene glycol-mediated or involve the use of *Agrobacterium tumefaciens* (see Table 6). The methods employ all the modern transformation technologies, *in-vivo* DNA assembly (King *et al.*, 2016), CRISPR (Collonnier *et al.*, 2017), which now also include multiplexing (Mallett *et al.*, 2019), and TALEN (Kopischke *et al.*, 2017). It has also been shown that using contemporary synthetic biological parts is also possible in bryophytes (Peramuna *et al.*, 2018). The use of mosses and liverworts has recently been reviewed in several papers (Ishizaki *et al.*, 2016; Yongabi Anchang and Simonsen, 2019; Decker and Reski, 2020; Patron, 2020).

The moss *P. patens* has a potential to be a production host for commercially valuable metabolites and proteins. The precursors for the anticancer diterpene, paclitaxel (TaxolTM), were obtained by expressing *taxadiene synthase* gene from *Taxus brevifolia* in *P. patens*. Taxa-4(5),11(12)-diene could be produced at a yield of up to 0.05% of the plant fresh weight (Anterola *et al.*, 2009). The anti-malarial drug artemisinin (a sesquiterpene lactone), was obtained with a yield of 0.21 mg/g dry weight after only 3 days of cultivation by engineering of five artemisinin biosynthetic pathway genes into *P. patens*, which is equivalent to the levels in the original plant *A. annua* (Khairul Ikram *et al.*, 2017).

Physcomitrella patens is also used for the production of valuable ingredients for the perfume industry, such as the sesquiterpenoids patchoulol and β -santalene. The yield of patchoulol achieved was 1.34 mg/g dry weight (Zhan *et al.*, 2014). The diterpenoid sclareol, another valuable component in fragrances, was obtained in *P. patens* at the yield of 2.84 mg/g dry weight (2.28 mg/L culture) in liquid media after 18 days of cultivation (Pan *et al.*, 2015). The heterologous production was enhanced using traditional terpenoid metabolic engineering steps like heterologous

Table 6. Overview of bryophytes transformation achievements.

	Marker gene	Description	Effect	Goal	Transfection	Reference
<i>P. patens</i>	<i>apt</i>	Adeninephosphoribosyl transferase	Kanamycin, Hygromycin, G418 resistance	Random integration	PEG	(Schaefer <i>et al.</i> , 1991)
<i>P. patens</i>	<i>npII</i> , <i>aphIV</i>	Neomycin phospho-transferase, Aminoglycoside O-transferase	G418 resistance; hygromycin resistance	Targeted integration into loci 108, 420, 213	PEG	(Schaefer and Zryd, 1997)
<i>P. patens</i>	<i>GFP</i>	GFP expression	Green fluorescence	Expression plasmid, stability unknown	M.I.	(Brücker <i>et al.</i> , 2000)
<i>P. patens</i>	<i>npII</i>	Neomycin phospho-transferase	G418 resistance	Random integration; integration targeted into <i>lea2</i>	Biolistic	(Šmídková <i>et al.</i> , 2010)
<i>P. patens</i>	<i>human β(1,4)-galactosyltransferase</i>	Human $\beta(1,4)$ -galactosyltransferase	Humanised N-glycosylation pattern	<i>In vivo</i> assembly knock in, in $\alpha(1,3)$ -fucosyltransferase and $\beta(1,2)$ -xylosyltransferase loci	PEG	(Huether <i>et al.</i> , 2005)
<i>P. patens</i>	<i>PpAPT</i> ; <i>HPH</i>	Adenine Phosphoribosyl transferase; Hygromycin-B-40-kinase	2-fluoroadenine resistance; Hygromycin resistance	CRISPR-Cas9 mediated PpATP knock-out and knock-in (HPH introduction)	PEG	(Collonnier <i>et al.</i> , 2017)
<i>P. patens</i>	6 genomic sites	<i>Loc</i> i: Pp3c8_18830V3.1, Pp3c18_4770V3, Pp3c22_15110V3, Pp3c4_16430V3, Pp3c8_18850V3, Pp3c23_15670V3	Cas9 mediated mutation, causing detectable locus size variation	CRISPR-Cas9 mediated multiplex targeted mutation of 6 loci	PEG	(Mallett <i>et al.</i> , 2019)
<i>M. polymorpha</i>	<i>npII</i>	Neomycin phospho-transferase	G418 resistance	Random integration	A.T.	(Nasu <i>et al.</i> , 1997)
<i>M. polymorpha</i>	<i>hpt</i> , <i>GUS</i>	Hygromycin phospho-transferase; β -glucuronidase	Hygromycin resistance; X-Gluc marker	Random integration	A.T.	(Ishizaki <i>et al.</i> , 2008)
<i>M. polymorpha</i>	<i>hpt</i> ; <i>aadA</i>	Hygromycin phospho-transferase; aminoglycoside-3'-adenylyltransferase	Hygromycin resistance; Spectinomycin resistance	Random integration	Biolistic	(Chiyoda <i>et al.</i> , 2008)
<i>M. polymorpha</i>	<i>hpt</i> ; <i>NOP1</i>	Hygromycin phosphotransferase; not mentioned	Hygromycin resistance; impaired air chamber formation	Targeted integration (NOP1 knock-out)	PEG	(Ishizaki <i>et al.</i> , 2013)
<i>M. polymorpha</i>	<i>NOP1</i> ;	not mentioned	Impaired air chamber formation	Talen mediated mutation of NOP1	A.T.	(Kopischke <i>et al.</i> , 2017)
<i>C. purpureus</i>	<i>npII</i>	Neomycin phospho-transferase	Kanamycin resistance	Expression of oat PhyA	PEG	(Thümmler <i>et al.</i> , 1992)
<i>C. purpureus</i>	<i>GFP</i>	GFP expression	Green fluorescence	Expression plasmid, stability unknown	M.I.	(Brücker <i>et al.</i> , 2000)
<i>C. purpureus</i>	<i>Heme oxygenase</i>	Heme oxygenase expression	restores phototropic response	Expression plasmid, stability unknown	M.I.	(Brücker <i>et al.</i> , 2000)
<i>C. purpureus</i>	<i>APT</i>	adeninephosphoribosyl transferase	Kanamycin, G418 Hygromycin, G418	Gene targeting	PEG	(Trouiller <i>et al.</i> , 2007)



Figure 6. Air freshener moss-based product (<https://www.mosspirationbiotech.com>).

expression of HMGR using the truncated version of yeast HMGR and enhancing the storage compartments (Zhan *et al.*, 2014). A modular approach for the production of a range of diterpenes was reported in *P. patens*. Three class II and two class I diterpene synthases (diTPS) enzymes were combined to generate industrially important diterpenes (Banerjee *et al.*, 2019). *P. patens* has shown promising results as cell factory for the production of terpenoids. As a result, a fragrant moss-based product was developed by Mosspiration Biotech (see Figure 6) (<https://www.mossebelles.com>).

Bryophytes contain high amounts of polyunsaturated fatty acids (see Section III.B.3), where most of them are very long-chain polyunsaturated fatty acids (LC-PUFAs). LC-PUFAs are important components of human diet and are mainly obtained from fish and algal oils of limited availability, which stresses the need of a sustainable source of these compounds for human utilization (Lu *et al.*, 2019). Metabolically engineered *P. patens* producing important very long-chain polyunsaturated fatty acids were obtained by encoding $\Delta 5$ -elongase from the marine algae *Pavlova* sp associated with vegetable oil supplementation to enhance biomass and LC-PUFAs production (Chodok *et al.*, 2012). The biosynthesis of docosatetraenoic acid or adrenic acid (ADA) and n-3 docosapentaenoic acid (DPA) was obtained from AA and EPA, produced by *P. patens*. The transgenic moss produces DPA that is a new source of docosahexaenoic acid (DHA) precursors for human diet (Chodok *et al.*, 2012). Likewise, the liverwort *M. polymorpha* accumulates AA, from which prostaglandin F2a, prostaglandin E2 and prostaglandin D2 were generated through heterologous expression of a cyclooxygenase gene from the red alga

Gracilaria vermiculophylla (Takemura *et al.*, 2013). In addition, the bioproduction of prostaglandins was increased using an *in vitro* reaction system and transgenic *M. polymorpha* offers the first bioproduction of PGs in plant species (Takemura *et al.*, 2013).

1. Perspectives

In the past three decades, biotechnologies around bryophytes have gone from being simple and research laboratory scale only to become an industrial used technology. To our knowledge, only few companies currently use bryophytes in biotech production, but several other bryophyte-based products are marketed based on harvest from wild populations, which show that chemical wealth of bryophytes is slowly but surely being exploited. In the future, it is expected that many new products will arise from the ongoing bryophyte research including the use of contemporary synthetic biology technologies. From ongoing research, it can be expected that products within cosmetics, herbal remedies, perfumes and pharmaceuticals will come to market within the next ten years, all based on bryophytes. The establishment of certified good-manufacturing-practice for production in bioreactors (up to 500 L) ensure that lucrative products can also be made within the lucrative market of pharmaceuticals. Thus, it is foreseen that use of bryophytes as new production platforms for plant-derived and environmentally friendly products will increase in the next decade and allow for development of novel technologies that can also be applied to vascular plants.

Authors' contributions

A.H. provided the conceptualization and outline under the supervision of H.T.S. A.H., A.P., I.L., Y.L., R.V.M and H.T.S. designed and wrote the manuscript. All authors reviewed and added corrections to the manuscript. Y.L. contributed the graphical visualization and N.C. provided the photos for Figure 1.

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ORCID

Armin Horn  <http://orcid.org/0000-0001-9583-569X>
 Arnaud Pascal  <http://orcid.org/0000-0001-7655-9650>
 Isidora Lončarević  <http://orcid.org/0000-0001-7936-3047>

Raíssa Volpatto Marques  <http://orcid.org/0000-0002-6959-640X>
 Yi Lu  <http://orcid.org/0000-0001-5095-7124>
 Sissi Miguel  <http://orcid.org/0000-0002-2450-6079>
 Frederic Bourgaud  <http://orcid.org/0000-0002-9898-2625>
 Margrét Thorsteinsdóttir  <http://orcid.org/0000-0002-0649-8503>
 Nils Cronberg  <http://orcid.org/0000-0002-3369-8420>
 Jörg D. Becker  <http://orcid.org/0000-0002-6845-6122>
 Ralf Reski  <http://orcid.org/0000-0002-5496-6711>
 Henrik T. Simonsen  <http://orcid.org/0000-0003-3070-807X>

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