Liverwort oil bodies: diversity, biochemistry, and molecular cell biology of the earliest secretory structure of land plants.

Facundo Romani*[1], Jorge R. Flores*[2], Juan Ignacio Tolopka[3], Guillermo Suarez[4], Xiaolan He[5], Javier E. Moreno[3]

1. Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, U.K. fr391@cam.ac.uk
2. Finnish Museum of Natural History (Botany), P.O. Box 7, FI-00014, University of Helsinki, Finland. jorge.flores@helsinki.fi
3. Instituto de Agrobiotecnología del Litoral, Universidad Nacional del Litoral – CONICET, Facultad de Bioquímica y Ciencias Biológicas, Centro Científico Tecnológico CONICET Santa Fe, Colectora Ruta Nacional No. 168 km. 0, Paraje El Pozo, Santa Fe 3000, Argentina. juanitolopka@gmail.com
4. Unidad Ejecutora Lillo (CONICET –Fundación Miguel Lillo), Miguel Lillo 251, San Miguel de Tucumán, Tucumán, 4000, Argentina; Facultad de Ciencias Naturales, Instituto Miguel Lillo, Universidad Nacional de Tucumán, Miguel Lillo 205, San Miguel de Tucumán, 4000, Argentina. suarezgui@gmail.com
5. Botany Unit, Finnish Museum of Natural History, University of Helsinki, PO Box 7, FI-00014 Helsinki, Finland. xiao-lan.he@helsinki.fi

*equal contribution

Corresponding author: javier.moreno@santafe-conicet.gov.ar

Highlight

Evolutionary history of oil bodies in liverworts and their relationship with the chemical diversity of the lineage.

Abstract

Liverworts are known for their large chemical diversity. Much of this diversity is synthesized and enclosed within the oil bodies (OB), a synapomorphy of the lineage. OB contain the biosynthetic enzymes to produce and store large quantities of sesquiterpenoids and other compounds while limiting their cytotoxicity. Recently, there were important biochemical and molecular discoveries related to OB formation, diversity and biochemistry that allows the comparison with other secretory structures of land plants in an evo-devo perspective. In this review, we address and discuss the most recent advances in OB origin, development and function to understand the importance of these organelles in liverwort physiology and adaptation to the changing environment. Our mapping of OB-types and chemical compounds to the current liverwort phylogeny suggests that OB were already present in the most recent common ancestor of liverworts, supporting OB evolved as the first secretory structure in land plants. Still, we require a better sampling to define the macroevolutionary pattern governing the ancestral type of OB. We conclude that current efforts in finding molecular mechanisms responsible for this morphological and chemical diversity of secretory structures will help understand the evolution of each major group of land plants, and open new avenues in biochemical research of bioactive compounds for both bryophytes and vascular plants.

Keywords

Chemical diversity, evolution, oil bodies, liverworts, sesquiterpenoids, transcription factor genes, terpene synthases genes, evo-devo, trichomes.

Introduction

Plants produce an extraordinary diversity of specialized metabolites that play important roles in responses to the environment. Humans use these compounds for multiple purposes, from medicinal use to food applications. However, many of these compounds are cytotoxic turning their accumulation into a potential risk for their own cells. During evolution, land plants have evolved strategies to accumulate high concentration of specialized metabolites while avoiding autotoxicity. In addition to chemical modifications to regulate the biological activity of specialized metabolites (Li *et al.*, 2021), the main adaptive strategy of land plants is their accumulation and confinement within secretory structures. The different secretory structures were proposed to have independently evolved across multiple land plant lineages (Lange, 2015). Recent works on the differentiation of secretory structures, especially those that had remained poorly known in the liverwort lineage, have provided new insights on how land plants with different evolutionary histories may have repurposed their molecular mechanisms to solve a similar physiological challenge: the accumulation of toxic chemicals.

Bryophytes and vascular plants diverged from a land plant common ancestor around ~430 million years ago (Delwiche and Cooper, 2015). Based on morphological and molecular traits, bryophytes are classified into Marchantiophyta (liverworts), Bryophyta (mosses), and Anthocerophyta (hornworts), and they together have been largely supported as a monophyletic group in recent phylogenomic analyses (Harris *et al.*, 2020). Among bryophytes, only liverworts accumulate cytotoxic compounds in intracellular oil bodies (hereinafter referred to as OB), which accounts for the most ancient fossil record of a secretory structure (Labandeira *et al.*, 2014). OB are present in the cells of both the gametophyte (haploid phase) and the sporophyte (diploid phase) (He *et al.*, 2013; Pihakaski, 1968). On the contrary, most of other plant secretory structures are limited to the sporophyte.

Although OB were described almost two centuries ago, their biological function remained elusive. Structurally, OB are bounded by a distinctive membrane and are easily recognized by their high refractive index under the light microscope. The first report of OB was in the leafy liverwort *Jungermannia taylorii* (now known as *Mylia taylorii*) (Hübener, 1834). OB later received their current name on the account of their fatty nature (Pfeffer, 1874), and subsequent chemical analyses identified terpenoids as their primary constituents (Lohmann, 1903; Müller, 1905). A century later appeared the first direct evidence showing OB as active sites of synthesis of specialized metabolites and containing their biosynthetic enzymes, supporting that the OB compartment is an intracellular secretory structure in liverworts (Suire *et al.*, 2000). Up to now, more than 1600 lipophilic compounds have been extracted from liverworts (Ludwiczuk and Asakawa, 2019). Some compounds can be found in vascular plants, while several are unique to liverworts. So far it is known that OB are important sites of synthesis and storage of mono- and sesqui-terpenoids, and bisbibenzyls, but new discoveries are expected to expand their role in contributing to chemical diversity. In this review we will focus on recent advances in the liverwort OB function as a secretory structure, their evolution in a phylogenetic context, the genes involved in their formation, as well as the role of OB in biochemical signatures of each major group of the lineage. We also compare OB with other land plant secretory structures in an attempt of tracking down the origin of secretory structures in an evo-devo perspective.

Specialized cells are essential for storage and biosynthesis of cytotoxic compounds

OB function was initially related to nutrient reserve, accumulating polysaccharide such as starch (Mirbel, 1835) but it was later debunked given that OB were negative to lugol staining (Gottsche, 1843). On the contrary, OB were dissolved in alcohol and they were positive to Nile Red staining, suggesting energy reserve could be accumulated but in the form of lipids as the lipid droplets (hereinafter referred to as LDs) in other plant species. Early studies growing liverworts under different environmental conditions, such as nutrient starvation, described that both the number and the morphology of OB remain largely unaffected (Garjeanne, 1903; Slavík, 1950). However, it is now clear that the number of OB is greatly induced upon nutrient starvation and non-axenic growth in *Marchantia* (Tanaka *et al.*, 2016), underscoring the inducible nature of OB and their poorly studied role in the ecology of liverworts.

Another hypothesis points to abiotic stress protection, especially against UV light stress (Hieronymus, 1892), cold, drought and osmotic stress (Schuster, 1966). The latest motivated by chemical properties of carbohydrates present in OB and the fact that OB remained turgent after dehydration (Pressel *et al.*, 2009). However, *Marchantia* plants defective in OB formation showed similar growth parameters to those of the wild-type in abiotic stress conditions (Romani *et al.*, 2020). At the same time, essential oils extracted from liverworts OB worked as antibiotic against a wide range of organisms (Asakawa and Ludwiczuk, 2018). Accordingly, recent experiments showed that extracts from plants reduced in OB specific compounds have reduced antibiotic activity against bacteria and fungi (Romani *et al.*, 2020; Wu *et al.*, 2018). These results support the hypothesis that OB can be important for defence against pathogenic fungi and bacteria, but it is unclear how OB interact with microorganisms in nature. The most convincing hypothesis is their protective role against herbivory. Early experiments showed a preference of snails for certain liverwort species with less OB than others (Stahl, 1888). The same liverwort species were subsequently tested after leaching the OB with alcohol, and the snails again preferred the one leached. This fascinating experiment was followed by fossil observations of samples from the Middle Devonian, where damage made by arthropod herbivores to *Metzgeriothallus sharonae* seems to avoid OB cells (Labandeira *et al.*, 2014).

Only recently, studies using *Marchantia* as a model organism for molecular biology allowed testing this hypothesis using controlled genetic experiments. Mutant plants defective in OB cell formation were found to be more susceptible to arthropod herbivores than their wild-type counterparts (Kanazawa *et al.*, 2020; Romani *et al.*, 2020). The opposite effect was observed in plants that constitutively produce more OB cells (Kanazawa *et al.*, 2020). In *Marchantia*, this phenotypic effect correlates with a strong loss of OB-specific compounds such as sesquiterpenes (Romani *et al.*, 2020). Also, Marchantia mutant plants with a deficient defence response, including reduced induction of sesquiterpenes, resulted in enhanced foraging based on the growth of *Spodoptera littoralis* caterpillars (Peñuelas *et al.*, 2019).

In addition, there is now clear evidence that compounds directly isolated from *Marchantia* OB are even cytotoxic for the plant itself (Tanaka *et al.*, 2016). Whereas, the abundance of these compounds in parenchymatic cells or in mutant plants reduced in OB cells is nearly undetectable (Romani *et al.*, 2020; Tanaka *et al.*, 2016), ectopic accumulation of these compounds could be also harmful for the plant. This is exemplified in MpERF13 overexpressing plants, where the increment of OB cell number is detrimental for plant growth (Kanazawa *et al.*, 2020). Altogether, these results suggest that OB specific compounds cannot be accumulated in other subcellular compartment or cell-types likely due to autotoxicity. In that sense, OB are analogous to the glandular trichomes of vascular plants playing a similar function.

As other secretory structures, OB are active sites of synthesis of specialized metabolites and they enclose the enzymes of the pathway (Suire *et al.*, 2000; Tissier, 2018). A contrasting example are myrosin cells that accumulate an enzyme (myrosinase) that activates the cytotoxic activity of glucosinolates in Brassicaceae (Shirakawa and Hara-Nishimura, 2018). In addition to toxicity, the confinement within secretory structures prevents evaporation of highly volatile compounds. Interestingly, the evolution of secretory structures is often associated with the expansion of terpenoid metabolism, as they could have facilitated the accumulation of toxic compounds and their intermediates (Lange, 2015).

Trichomes are common multicellular epidermal features in angiosperms but they are also observed in some lycophytes, ferns, and gymnosperms (Lange, 2015). However, only glandular trichomes work as a proper secretory structure (Tian *et al.*, 2017). Early fern fossil records (~290 Mya) possess glandular trichomes that apparently played a similar role compared to angiosperms` glandular trichomes (Krings *et al.*, 2003). Secretory ducts and cavities from ferns and gymnosperms, salt glands, laticifers and myrosin cells from flowering plants complete the known diversity of plant secretory structures (Lange, 2015). Similar to other plant traits, these structures may have emerged independently multiple times during evolution. However, the diversity of secretory structures and their uneven distribution among species undermine the understanding of their evolutionary origin and trajectories.

Evolution of oil bodies throughout liverworts

Liverworts are classified into three major clades: Haplomitriopsida, Marchantiopsida and Jungermanniopsida (Figure 1). Whereas OB present in Marchantiopsida species are restricted to specialized idioblast cells, in leafy liverworts they are present in all cells from one to many per cell (He *et al.*, 2013) (Figure 1).

To consider OB origin and development under an explicit evolutionary framework, it is necessary to reconstruct the ancestral character states for the OB across the phylogeny of liverworts. Here, we mapped OB across a synthetic phylogeny of liverworts representing the relationships among the main lineages according to the most supported hypotheses (Flores *et al.*, 2021; He-Nygren *et al.*, 2006; Heinrichs *et al.*, 2007; Leebens-Mack *et al.*, 2019; Wickett *et al.*, 2014). Based on our own observations and literature (Bischler-Causse *et al.*, 2005; Gradstein *et al.*, 2001), we scored 71 taxa for the presence/absence of OB into the following types: (0) many, in all cells; (1) one, in all cells; and (2), only in idioblastic cells (see matrix in Flores et al. 2018). In phylogenetics, ontogenetically dependent characters (such as “presence/absence of OB” and “OB type”) may lead to reconstructing unobserved character states at internal nodes when inapplicable character states are scored (Maddison, 1993). To avoid this problem these characters were treated as a step-matrix complex (see details in (Goloboff *et al.*, 2021)). Additionally, liverworts were also scored and mapped for the type of gametophyte: (0) leafy, (1) complex thalloid and (2) simple thalloid.

Our reconstructions reveal that OB were already present in the most recent common ancestor (MRCA) of liverworts (Figure 1) – a hypothesis that has been long speculated, yet scarcely assessed quantitatively (Crandall-Stotler *et al.*, 2005; He *et al.*, 2013; Schuster, 1966, 1992). Likewise, we observed three principal macroevolutionary patterns: (i) the ancestral type of OB in liverworts is ambiguous; (ii) at least one instance of parallelism (*i.e.*, independent origin of a trait in multiple clades of the phylogeny) in the evolution of OB occurred liverworts; and (iii) losses of OB are relatively rare (Figure 1). While these outcomes indicate that the presence of OB in liverworts is explained by common ancestry, the ambiguous condition at the MRCA indicates that further taxa should be sampled to clarify the ancestral condition. Previously, Crandall-Stotler et al. (2005) have inferred many OB in all cells as the ancestral character state in liverworts. However, such an inference was carried out without sampling either mosses or hornworts, thus is biased by limited taxon sampling. Although the bryophyte fossil record has been deemed scarce and poorly preserved (Tomescu *et al.*, 2018), fossil evidence suggests that OB evolved already by the Middle Devonian and were involved in the defence against herbivory (Labandeira *et al.*, 2014). According to recent phylogenetic studies (Bechteler *et al.*, 2021; Flores *et al.*, 2021), the (putative) OB-bearing Devonian fossil *M. sharonae* is either sister to Jungermanniopsida or nested within the group. If the "dark cells" in *M. sharonae* are interpreted as idioblasts (Tremblay, 2016), the phyletic affinities of the fossil imply that OB in idioblasts was a condition that evolved early in the history of the group and suggest that this could be the ancestral state in liverworts MRCA.

The parallelism in the appearance of either many OB per cell (in *Haplomitrium* and Jungermanniopsida) or in idioblastic cells (in *Treubiales* and Marchantiidae [i.e., Marchantiopsida excluding Blasiidae]) raises questions about the mechanisms shaping OB evolution (ii). In comparative biology, parallelism is often attributed to external factors [either biotic (e.g., herbivory) or abiotic (e.g., hydric stress)] with strong selective pressure rather than common ancestry or stochastic causes – although population size and mutation rates might promote parallel evolution (Bailey *et al.*, 2017). Even though the role that phylogenetic constraints play in shaping the evolution of different morphological traits in vascular plants remains contentious (Haak *et al.*, 2014; Karban, 2020; Moreira *et al.*, 2018), liverworts were shown to be phylogenetically structured regarding life-strategy traits (Bischler, 1998; Crandall-Stotler *et al.*, 2005; Flores *et al.*, 2018). For the current taxon sampling, our character mapping suggests that both common ancestry and external factors influenced OB evolution. That is, common ancestry explains the presence of OB in idioblasts in Marchantiopsida and one OB per cell in Jungermanniopsida while external factors account for the parallel appearance of OB in either Haplomitriales or Treubiales. This pattern is enhanced by the few losses of OB in liverwort phylogeny, which indicates a positive selection of such a feature (iii) – i.e., a trait that tends to be favoured in a given population at the expense of its alternative phenotype(s) (e.g., (Yang, 1998)). From a macroevolutionary standpoint, morphological features can be either highly reversible or preserved throughout phylogenies. In the former, characters are often lost numerous times upon being gained within a clade. In Bryophyta, for instance, stomata have been lost over 60 times in moss phylogeny after being acquired at deeper nodes (Renzaglia *et al.*, 2020). Conversely, preserved characters are hardly lost in the evolution of a group. This is the case of OB, which were lost nine times throughout liverwort phylogeny (Figure 1).

Regardless of the OB function, the limited number of losses points to the fact that this trait offers advantages over non-OB bearing plants.

The evolution of morphological characters in bryophytes has often been explained in the light of (a), character correlation (dependence) (e.g., (Crawford *et al.*, 2009)) or (b), response to external factors (e.g., (Bischler and Jovet-Ast, 1981)). While both explanations involve selective pressures, character correlation implies that a dependent character correlates with another evolving trait since they share a common developmental machinery (Barabé *et al.*, 2002) or ecological function (Vamosi *et al.*, 2003). In previous phylogenetic studies, OB were not clearly correlated with other vegetative characters (Bischler, 1998; Crandall-Stotler *et al.*, 2005). However, in our mapping, the type of OB is partially correlated to the type of gametophyte: OB in idioblasts are present in both complex thalloid forms and the leafy-like family Treubiaceae (Figure 1) whereas many OB in all cells are found in leafy forms (including *Haplomitrium*) and simple thalloid forms (Figure 1). Therefore, an exact correlated evolution between the type of gametophytes and OB is not supported by our ancestral character state reconstruction. Nonetheless, we infer that the idioblast differentiation correlates with a more complex morphology of the plant thalloid organization, probably associated to asymmetric cell divisions, a trait that has been lost in the Jungermanniopsida species.

Among external factors, herbivory has been seen to induce responses in vascular land plants and has a proven selective pressure (Agrawal and Weber, 2015; Karban, 2020; Turcotte *et al.*, 2014). Records of herbivory on bryophytes, despite not being widely known, entail mostly dipteran larvae grazing on leaves and gastropods consuming immature capsules (e.g., (Davison *et al.*, 1990)). Although this hypothesis explains the appearance of OB in liverworts and their loss in certain ephemeral taxa (e.g., *Sphaerocarpos* sp.), it does not account for the evolution of different types of OB across liverworts. Alternatively, OB evolution could have been driven by environmental requirements reflected in habitat occupation. In liverworts phylogeny, idioblasts tend to be present in species that are exclusively terricolous (Marchantiopsida and Treubiaceae) while many OB per cell are present in liverworts with broader habitat occurrences (Jungermanniopsida and Haplomitriaceae).

Oil body diversity and their use in taxonomy

OB occur in most liverwort species (Figure 1). As OB are a conspicuous cellular feature, this trait was used in taxonomy according to their appearance, size, number per cell and form

(Müller, 1905). However, the taxonomic value of OB varies in different groups and/or at different taxonomic levels (Schuster, 1966). The limitation of using OB as a taxonomic tool is that their morphological characterization can only be reliable by using fresh material. OB of dried herbarium material disintegrate with time, although idioblast cells may still be recognized.

In Haplomitriopsida, *Haplomitrium* develops numerous tiny OB in each cell, whereas *Treubia* has one large OB within scattered specialized cells usually larger than other surrounding cells. In Jungermanniopsida, OB are present in all cells, similar to *Haplomitrium*, but with great diversity in size, shape and colour (Schuster, 1992; Schuster and Hattori, 1954). In Marchantiopsida, similar to *Treubia*, OB only occur in the gametophyte as scattered specialized cells. Interestingly, several genera of the three major classes have been reported to be devoid of OB, such as *Anthelia*, *Cephalozia*, *Blasia*, and *Riccia*.

The size of OB varies considerably. Small sized homogeneous OB (2-5 x 3-9 μm in average) (Schuster, 1992) usually occur in high numbers (Schuster, 1992). The large sized segmented OB can reach to over 30 μm in diameter from one to a few in each cell (Schuster, 1992). The OB in idioblastic cells can reach 70 μm in diameter such as in *Abotreubia hortonae* (Schuster and Konstantinova, 1995). A major distinction is between homogeneous or segmented OB (Gradstein *et al.*, 1977). For example, the OB types were used as a primary criterion to separate genera of Ptychanthoideae of the family Lejeuneaceae (Schuster and Hattori, 1954). OB were also phylogenetically relevant for the sectional classification of Australasian *Plagiochila* (Plagiochilaceae: Jungermanniopsida) (Renner *et al.*, 2017).

Subcellular features of oil bodies and their secretory pathway

Ultrastructural evidence largely supports the origin of OB from the dilation of the endoplasmic reticulum during cell enlargement and maturation (Duckett and Ligrone, 1995; Pihakaski, 1972b; Suire, 1970, 2000), therefore rejected early hypotheses on their origin from the chloroplast (Kozlowski, 1921; Wakker, 1888), the mitochondrion (Bergdolt, 1926), or the vacuole (Dombray, 1926; Garjeanne, 1903; Zirkle, 1932). The OB are absent in the meristematic cells of the thallus, but rather short lengths of paired membranes enclosing some electron-dense droplets can be seen in older cells close to the meristem area. This was considered by the author as the earliest stage of OB development (Pihakaski, 1972b). Later histochemical studies on *Pellia epiphylla* and *Bazzania trilobata* showed that the OB matrix contains proteins (Pihakaski, 1972a), as suggested before (Garjeanne, 1903).

It is important to distinguish between liverwort OB from subcellular structures often referred as OB or LDs (Lundquist *et al.*, 2020). LDs are present in all green plant lineages and are functionally related to tolerance response to abiotic stress (de Vries and Ischebeck, 2020), commonly observed in seeds. LDs display only a lipid monolayer membrane strongly associated with proteins that could be confounded with OB even at the microscope scale. LDs can often play defence roles and accumulate compounds that are not soluble in the aqueous phase but are not necessarily associated with cytotoxic compounds. LDs coexist with OB in liverworts, and one of the early evidence suggesting they are essentially different subcellular features is the fact that they do not fuse with each other (Duckett and Ligrone, 1995).

The endoplasmic reticulum is a highly dynamic and pleomorphic organelle at the core of the secretory pathway responsible for the transport of *de novo* synthesized cargoes from the endoplasmic reticulum to the plasma membrane or extracellular space. Comparative genomic analysis on the evolutionary history of membrane-trafficking components across diverse eukaryotic organisms suggests that the molecular framework of membrane trafficking is highly conserved across eukaryotic lineages, such as the paralogous protein families GTPase-activating proteins of Arf GAP family, Rab proteins, and syntaxins (SYP), a family of membrane integrated SNARE proteins (Schlacht *et al.*, 2014). Lineage-specific proteins have also been identified to be associated with paralogous expansions, such as the SYP1 group (SNARE), and the RAB11/RABA group (RAB GTPase) in the secretory pathway in tracheophytes (Fujimoto and Ueda, 2012; Kanazawa and Ueda, 2017; Sanderfoot, 2007). In liverworts, the OB organelle is acquired through paralogous expansion followed by neofunctionalization of the SYP1 group, and the OB formation involves a modified secretory pathway representing the cell-phase specific redirection of the secretory pathway (Kanazawa *et al.*, 2020). This conclusion was supported by the finding that two paralogous SYP-1 homologs in *Marchantia* are distinctly targeted to forming cell plates and the OB, with MpSYP12A required for cell plate formation during cytokinesis and MpSYP12B needed for OB cell formation. Furthermore, the promoter of MpSYP12B is enough to drive the localization of a SYP-1 that is normally expressed in the plasma membrane. A similar pattern was shown when a secretory tag was expressed under MpSYP12B regulatory region (Kanazawa *et al.*, 2020). Thus, the OB targeting capability is encoded in the promoter region and not in the protein, suggesting that the redirection of the secretory pathway between the OB and the cell plate is regulated transcriptionally in *Marchantia* in a cyclic fashion (Kanazawa *et al.*, 2020). In contrast to epidermal cells, OB cells does not divide after cell differentiation. The authors also propose that the luminal space of the OB should be topologically equivalent to the extracellular space. It could be plausible that OB and the cell plate share some developmental features, being different ways to redirect the secretory pathway. This has been shown to be a common strategy for the acquisition of organelles with distinct functions. However, this hypothesis remains to be tested more deeply. Intriguingly, Mp*syp12b* mutant did not showed defects in OB cell formation.

From unicellular to multicellular, different secretory structures display diverse cellular features. One common feature is that they accumulate metabolites in a storage space. Glandular trichomes accumulate metabolites in a subcuticular cavity (e.g. peltate trichomes) or this cavity can be enclosed by secretory cells ('schizogenous' oil glands, Figure 2) (Schuurink and Tissier, 2020). Laticifers are cells with a modified cytoplasm filled with latex and other polymers that can usually elongate and/or fuse multiple cells (Ramos *et al.*, 2019). Finally, ducts and cavities are cells forming a storage lumen separated by a cell wall and surrounded by secretory cells. In contrast, OB are intracellular structures surrounded by a lipid bilayer membrane (Kanazawa *et al.*, 2020).

The mechanism of cell differentiation of both glandular and non-glandular trichomes require a strict control of the cell cycle. The endoreduplication is the first step of trichome differentiation as in many other epidermal features. In *Arabidopsis thaliana* (herafter Arabidopsis) single cell trichomes, the SIAMESE complex arrests the cell cycle into endoreduplication and avoid cell division (Fambrini and Pugliesi, 2019). Whereas, in tomato multicellular trichomes the cyclin *CycB2* promotes mitosis (Chalvin *et al.*, 2020; Gao *et al.*, 2017). Also, a thick cuticle layer is essential for trichome formation, suggesting that they are originated by a common mechanism (Lange, 2015). Less is known about other secretory structures. But, endoreduplication is absent in liverworts (Bainard *et al.*, 2019; Edgar *et al.*, 2014) and OB cells lack a cuticle, indicating they do not share these key cellular features found in other plant lineages.

Gene regulation of land plant secretory structures

From an evo-devo perspective, tracing the origin of secretory structures it is necessary to identify the genes responsible for their regulation (Delaux *et al.*, 2019). Like in other epidermal features, transcription factors (TFs) are essential to trigger cell differentiation. For example, myrosin cells have convergent regulators with guard cell differentiation, sharing key transcription factors such as the bHLH FAMA and SCREAM (Shirakawa and Hara-Nishimura, 2018). This suggest myrosin cells evolved from these specialized cells within the *Brassica* lineage.

Trichomes are the most studied of all secretory structures and several genes were associated to their differentiation. A common core component are the Class IV homeodomain-leucine zipper (C4HDZ or GLABRA2/GL2) TFs, a family of TFs involved in cuticle and epidermal regulation, required for the formation of different trichomes in multiple species (Chalvin *et al.*, 2020). A major regulator of non-glandular trichome initiation in Arabidopsis are the R2R3-MYB GLABRA1 (GL1). GL1 share homology with MIXTA genes that regulate glandular trichome, however, they are not interchangeable (Payne and Wagner, 2015). The regulation of non-glandular trichomes is accompanied by TFs belonging to MYB family, bHLH (referred as GL3), WD40 (TRANSPARENT TESTA GLABRA1/TTG1), that form a complex with GL1 named MBW (MYB-bHLH-WD40), and the WRKY (TTG2) (Chalvin *et al.*, 2020). None of them is clearly involved in glandular trichome differentiation suggesting that additional TFs remain to be characterized. Other TFs from the C2H2 family (GLABROUS INFLORESCENCE STEMS3 in Arabidopsis and HAIR from tomato) and R3-MYB (TRYPTICHON/TRY) families were identified as conserved signalling hubs controlling glandular as well as non-glandular trichome initiation. These similarities should not be taken lightly and interpreted as conserved function, since not all are true orthologs and the phenotypes observed in different species are not always the same. However, it is suggestive that despite the differences between angiosperms` trichomes, the molecular mechanisms support the hypothesis that trichomes have a common origin as a modified hair or ancestral trichome (Chalvin *et al.*, 2020).

Marchantia has become an outstanding model for molecular functional evolution and several TFs were already characterized in the last few years (Romani and Moreno, 2021). It was recently shown that in *Marchantia*, two transcription factors (MpC1HDZ and MpERF13) were required for OB cell differentiation (Kanazawa *et al.*, 2020; Romani *et al.*, 2020). So far, none of the orthologs in angiosperms were associated with glandular trichome differentiation, suggesting that an independent set of genes were recruited in liverworts to control this process. Intriguingly, cucumber glandular and non-glandular trichomes are affected in a C1HDZ mutant (Liu *et al.*, 2016), suggesting they might be recruited convergently for different steps of the regulation of secretory structures.

Other TFs are important in the steps following the differentiation of secretory structures. MpMYB02, a R2R3-MYB TF of *Marchantia* that shares homology with Arabidopsis PAP1, is required for the biosynthesis of OB-localized compounds (Kubo *et al.*, 2018). Furthermore, an ortholog to the bHLH MYC1 that controls the synthesis of specialised metabolites in response to pests in Arabidopsis, also plays a conserved role in the regulation of terpenoid biosynthesis in the liverwort *Plagiochasma appendiculatum* (Wu *et al.*, 2018). This relationship between defence-related TF and biosynthesis of compounds produced in secretory structures as a part of defence response against pathogens and herbivores seems to be present in both land plant lineages (Peñuelas *et al.*, 2019). However, the precise role of other defence-related elements, such as MYC1, JAZ and MYCX/Y, in OB formation and OB-related compounds is still unclear.

With these records, it is plausible to assume some degree of functional conservation of the MBW complex in the regulation of specialized metabolism in embryophytes (Figure 2). In that respect, it was recently shown that the *Marchantia* ortholog to TTG1 (MpWDR1 and MpWDR2) can rescue the absence of trichomes and anthocyanin production phenotypes when ectopically expressed in the Arabidopsis *ttg1-1* background (Airoldi *et al.*, 2019), suggesting that some features of this protein in the context of the MWB complex could be ancestral. However, experiments in *Marchantia* using the TTG1 orthologs are still missing to fully understand how this regulatory module could have functionally evolved in land plants. Several other transcriptional regulators associated with trichome formation remain to be studied in bryophytes. So far, the MIXTA homolog in *Marchantia* was found to have a conserved role in cuticle formation (Xu *et al.*, 2021), but no OB-associated phenotypes associated were reported.

Chemical diversity: a hallmark of liverworts

Liverworts have long been recognized to possess a wide array of terpenoids and other aromatic compounds, with sesquiterpenoids being the most diverse. More than 3000 specialized metabolites were described for the lineage, including more than 1600 terpenoids, and with bibenzyls and bisbibenzyls representing the most prominent aromatic compounds (Ludwiczuk and Asakawa, 2019). In contrast to liverworts, mosses and hornworts produce much less specialized compounds, with only about 100 sesquiterpenoids reported in mosses and limited data on the occurrence of terpenoids in hornworts (Asakawa, 1982; Asakawa, 1995; Asakawa *et al.*, 2013; Chen *et al.*, 2018; Ludwiczuk and Asakawa, 2019).

There are now multiple studies supporting that terpenoids and aromatic compounds found in the OB are responsible for the biological activity of liverworts. Microcapillary extraction of OB content followed by mass spectrometry analysis delivered direct evidence of sesquiterpenoids and marchantin A (a macrocyclic bisbibenzyl) specifically accumulated in OB cells of *Marchantia* (Tanaka *et al.*, 2016). Accordingly, the biosynthetic enzymes of the isoprenoid pathway were found in chloroplasts and cytosol in different plant lineages, and additionally immunolocalized within OB of *Marchantia* (Suire *et al.*, 2000). This result supports the OB, not only as terpenoids-storage organelles, but active sites of their biosynthesis (Suire *et al.*, 2000).

In coincidence with the chemical profiles of liverworts, recent reports using the Mp*c1hdz* mutant of *Marchantia*, deficient in OB formation, support a strong depletion of monoterpene (d-limonene), and sesquiterpenes (including cis-thujopsene, β-chamigrene, β-himachalene, 5-hydroxygurjunene) (Romani *et al.*, 2020). On the contrary, diterpenoids levels of the mutant Mp*c1hdz* were similar to the ones found in *wild-type* plants, suggesting that OB are not the main compartment for the synthesis of diterpenoids (Romani *et al.*, 2020), in the same way other compounds such as fatty acids and phytosterols. Thus, at least in *Marchantia*, it is possible to distinguish between compounds that are specifically located within the OB compartment to those accumulated somewhere else. As discussed before, this might be related to the autotoxicity of the compounds; however, it is not clear how this will work in other species, particularly in leafy liverworts.

An interesting case to study the contribution of OB to the chemical diversity of liverworts are OB-lacking species (Figure 1). So far, sesquiterpenoids were not reported for OB-lacking species. Consistently, the genomes of OB-lacking species encode a lower number of genes of the sesquiterpenoid biosynthetic pathway compared to OB-containing species (Jia *et al.*, 2016), suggesting that the OB environment is somehow required for sesquiterpenes biosynthesis in these plants. On the other side, OB-lacking species still produce different bibenzyls and flavone glycosides. This is the case of the liverwort *Blasia pusilla* that accumulates abundant macrocyclic bisbibenzyl dimers known as pusilatins A–D (Hashimoto *et al.*, 1994). The same occurs in *Sphaerocarpos texanus* that accumulates flavonoid glycosides and glucoronide such as luteolin (Markham *et al.*, 1976) and in *Riccia sp.* that accumulates phytosterols, saturated fatty acids and acetylenic fatty acids (Asakawa, 1982; Kohn *et al.*, 1988). *Metzgeria furcata*, an OB-lacking species belonging to Jungermanniopsida, produces flavone C-glycosides and in general, is a rich source of flavonoid compounds (Theodor *et al.*, 1983; Theodor *et al.*, 1981). Interestingly, *Metzgeria* species lacking OB do not accumulate terpenoids (Asakawa *et al.*, 2013). On the contrary, *Ricciocarpos natans*, a species with OB phylogenetically related to *Riccia*, produces an important diversity of terpenoid compounds (Wurzel and Becker, 1990).

Chemical evidence in early studies showed that most of the liverworts elaborate enantiomeric sesqui- and di-terpenoids of those found in seed plants (Hayashi and Matsuo, 1975a, b, 1976; Toyota *et al.*, 1997). This data together with the observation that liverworts possess specific compounds only found in fungi, led to the proposal that liverworts' sesqui- and di-terpenoids were more related to fungi rather than green algae and seed plants (Matsuo, 1982). Evidence supporting this assumption can also be found in other studies, for example, the gymnomitrane-type sesquiterpenes of liverworts, ß-barbatene, was found for the first time in fungi from the fruiting bodies of the polypores *Fomitopsis pinicola* and *Fomesfomentarius* (Fäldt *et al.*, 1999) and later in the fruiting bodies of *Ganoderma lucidum* (Binh *et al.*, 2015). Recent studies suggest that both the structure and the biosynthetic pathway of ß-barbatene from the (R)-bisabolyl to the (S)-cuprenyl cation are identical in the two domains (Dickschat *et al.*, 2011; Métoyer *et al.*, 2018; Wawrzyn *et al.*, 2012). Liverworts also contain chiral isomers only found in brown algae, however these compounds are not necessarily accumulated within the OB.

The dynamic assembly of the biosynthetic enzymes partially explains the diverse production of phytochemicals (Knudsen *et al.*, 2018). Terpene synthases (TPSs) are the key enzymes for terpenoid biosynthesis, and two major ancient pathways produce the precursors of all terpenoids: the mevalonate (MVA) pathway and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Adam *et al.*, 1998; Pichersky and Raguso, 2018). Genome-wide studies have shown that two groups of *TPS* genes are widely present in non-seed plant genomes (Jia *et al.*, 2016). A group of plant diterpene synthases (DTPS) quite similar to the ones found in vascular plants and another similar to microbial TPS called microbial terpene synthase-like (MTPSL) (Jia *et al.*, 2018; Kumar *et al.*, 2016). The MTPSLs, that are present in non-seed plants, have been shown to be absent from seed plant genomes (Jia *et al.*, 2016). *In vitro* experiments showed that DTPSs produce diterpenes and MTPSLs make monoterpenes, sesquiterpenes and diterpenes (Jia *et al.*, 2018). A sequence similarity study found that MTPSLs can be further classified into fungal MTPSL (MpFTPSLs) and bacterial MTPSL (MpBTPSLs) (Kumar *et al.*, 2016). Individual MTPSL members of *Marchantia* were characterized *in vitro* demonstrating their ability to synthesize multiple terpenoids, likely due to different preferences for the same substrate (Jia *et al.*, 2016; Kumar *et al.*, 2016). In addition, the promoter region of Mp*FTPSL2* was strongly active in OB cells while the suppression of several Mp*FTPSL*s genes in transgenic *Marchantia* plants resulted in low accumulation of sesquiterpenes compared to wild type thalli (Takizawa *et al.*, 2021). MTPSL proteins are also involved in sesquiterpene biosynthesis and diversity in the leafy liverworts *Mylia taylorii* (Yan *et al.*, 2021) and *Radula lindenbergiana* (Fan *et al.*, 2021). This specific group of *MTPSL* genes, likely acquired through horizontal gene transfer from bacteria and fungi, might explain the singular chemical compounds found in bryophytes, and more specifically in liverworts (Chen *et al.*, 2018).

Liverworts belonging to the Haplomitriopsida accumulate diterpenoids and glucoside flavonoids. *Haplomitrium gibbsiae* contains a rare group of acylated flavonoids that can be also found in related species (Markham, 1977). It is suggestive that similar compounds were also detected in species lacking OB, including *Riccia crystallina* (Ricciaceae) (Markham *et al.*, 1978) and *Metzgeria conjugata* (Theodor *et al.*, 1981). In addition, *Haplomitrium mnioides* showed the ability to synthesize particular labdane diterpenoids called haplomitrenolides (Asakawa *et al.*, 1990; Zhou *et al.*, 2016). Also, *Treubia isignensis* that belongs to another representative group of the Haplomitriopsida class, was shown to accumulate cyclic sesquiterpenes such as maaliane-, eudesmane-, aristolane and gorgonane, but did not produce aromatic neither oxygenated terpenoids (Coulerie *et al.*, 2014). In sum, while it is now clear that OB are important sites of synthesis and storage of bisbibenzyls, mono- and sesqui-terpenoids, we expect new discoveries that will expand their role in the chemical diversity to this plant lineage.

Outlook

Recent advances in the molecular and cell biology of liverworts have clarified key aspects of OB development and function. Independent recruitments of TF during plant evolution also supports the hypothesis of their independent origin (Lange, 2015). Nonetheless, convergent recruitment of TF families involved in the regulation of secretory structures in other plants is also likely (R2R3-MYBs and MYC1) in downstream steps. These phenomena of convergence, complex regulatory rewiring, and functional innovations are becoming important for reconciling from isolated cases of functional conservation towards understanding main evolutionary forces in land plant evolution.

The ancestral state of OB in liverworts is still an outstanding question. Despite there is not unequivocal evidence, the most likely scenario is OB as specialized idioblastic cells. Trait reconstruction suggests, that leafy liverworts lost this cell differentiation capacity, while OB appeared to be lost multiple times in Jungermanniopsida and Marchantiopsida species. Genetics and genomic studies in a more diverse array of liverwort species will be relevant to shed new light of the evolutionary history of this fascinating organelle.

Secretory structures are important for biosynthesis and diversity of terpenoid compounds and plant interaction with the environment. The fact that different secretory structures such as OB and glandular trichomes evolved independently also implies that their biosynthetic pathways could have diverged as well. Eventually, the evolution of specialized metabolism led to independent biosynthetic pathways but producing similar compounds. For example, the liverwort *Radula marginata* and other *Radula* species produce perrottetinene (Toyota *et al.*, 2002) and perrottetinene acid (Asakawa *et al.*, 2020) an analogue to *Cannabis sativa* Δ9-tetrahydrocannabinol with similar pharmacological properties (Chicca *et al.*, 2018; Gülck and Moller, 2020). These compounds of liverworts are in fact bisbenzyls accumulated in OB (Hussain *et al.*, 2019) and their biosynthesis evolved in a completely different fashion to cannabinoids in vascular plants.

OB cell differentiation is an important trait for plant defences and could be a complementary target for metabolic engineering. This could allow the accumulation of higher concentration of valuable chemical products or the production of novel compound (natural and unnatural) in a scalable chassis. In contrast to yeast or other chassis, the repertoire of compounds that can be accumulated in secretory structures is not limited by autotoxicity (Zhang *et al.*, 2017). This idea has already been tested in glandular-trichomes using specific promoter elements (Huchelmann *et al.*, 2017). *Marchantia* is a powerful chassis for synthetic biology that features an increasing quantity of genetic tools (Sauret-Gueto *et al.*, 2020) and *M. paleacea* was recently used as a chassis for terpene production (Zhang *et al.*, 2020).

With the rise of *Marchantia* as a model organism for plant molecular biology research, and the potential incorporation of more liverworts to the repertoire of genetic tractable species, OB will become a more important trait to study. The findings presented here provide a framework to understand the developmental programs of OB in the context of secretory structures and their chemical diversity, as well as the seemingly widespread pattern of convergent evolution in land plants.

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Conflicts of interest

The authors declare no competing interests.

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Figure legends

Figure 1. Ancestral character state reconstruction of OB across a synthetic phylogeny of liverworts. Branch colour indicates the inferred ancestral state for OB: red (absent), blue (present, one in idioblasts), light green (present, many in all cells), and dark green (present, one per cell) and black (polymorphic genus, some species have and others do not). Grey branches are ambiguously reconstructed as either OB in idioblasts or many in all cells. Tree is rooted using *Takakia* sp., a representative moss species. The three main classes of liverworts are depicted in the tree as coloured circles: Haplomitriopsida (red), Marchantiopsida (blue) and Jungermanniopsida (green). The tree includes a sketch of a representative species of each class. The first column indicates the organization of gametophyte species as complex thalloid (light orange), simple thalloid (brown) and leafy (orange). Then, the chemical classes found in these representative species are mapped in the columns at the right. A black circle represents the presence of the chemical class, a white circle denotes its absence and a blur circle denotes no reported data on that species.

Figure 2. Genetic control of secretory structure development of land plants. Cell differentiation trajectories of OB cells and trichomes present common and distinct features. Schematic illustrations show the main steps of cell differentiation for secretory structure classes studied

in model species. Known transcription factors and cell-cycle regulators involved in cell development are outlined. Essentially, expression of transcription factor in the nuclei (magenta circles) trigger differentiation of epidermal cells. In trichomes, endoreduplication (big nuclei) is required for completing cell elongation and division steps. In order to highlight similarities, genes are coloured by families.