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The Evolutionary Journey of the Sterol Synthesis Pathways in Eukaryotes

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The Evolutionary Journey of the Sterol Synthesis Pathways in Eukaryotes

Evolution of sterol synthesis in eukaryotes

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Abstract

Sterols play key roles in eukaryotic cell membrane stability and dynamics, as signaling molecules, and as precursors in sterol-based metabolic pathways, including the production of steroid hormones. The sterol biosynthetic pathway, based on a common set of core reactions, has been subject to intense diversification in each major eukaryotic clade. As a result, these organisms display a bewildering panoply of sterol-derived compounds that correlate with their multiple lifestyles and adaptations.

In this work, we provide new insights on sterol synthesis evolution and diversification in extant eukaryotes, with a special focus on algae and plants. In particular, we provide detailed information on the sterol synthesis pathway in bryophytes. A comprehensive phylogenetic analysis of bryophyte sterol biosynthetic enzymes suggests that duplications and divergence of the final enzymes of the canonical sterol pathway are taking place in this group.

Keywords: Brassinosteroids, *Marchantia polymorpha*, metabolic evolution, steroids, sterols, synthesis evolution.

Introduction

Sterols are essential molecules in eukaryotes, regulating membrane dynamics, fluidity, and permeability. The formation of mixed lipid rafts with sphingolipids allows cell communication, adhesion, and recognition. Importantly, in multicellular eukaryotes, sterols also act as a precursor for steroid hormones and fat-soluble vitamins (Du et al. 2022). The sterol composition varies across eukaryotic kingdoms: animals primarily produce cholesterol, fungi mainly synthesize ergosterol, brown and red algae generate fucosterol and dermatosterol respectively, and plants produce phytosterols, such as sitosterol, stigmasterol, and campesterol (Benveniste 2004, Schaller 2004, Belcour et al. 2020, Girard et al. 2021). Nevertheless, photosynthetic eukaryotes can also synthesize other sterols, such as cholesterol, which is the major sterol in members of the angiosperm *Solanaceae* family and in many red algae (Milner et al. 2011, Girard et al. 2021), and the endogenous synthesis of sitosterol has been recently reported in animals (Michellod et al. 2023). Hence, the steroid biosynthetic pathway across eukaryotic kingdoms reflects both a deep conservation and a complex evolutionary history of diversification.

Sterol synthesis in Eukarya begins with the mevalonate pathway (MVA) and proceeds with squalene synthesis. Squalene epoxide is cyclized by OXIDOSQUALENE CYCLASES (OSCs) enzymes, forming protosterols (lanosterol and cycloartenol). Post-cyclization steps involve modifications of the sterol core through demethylation, desaturation, reduction and isomerization steps and methylation of the side chain, resulting in the varied repertoire found in eukaryotes (cholesterol, phytosterols and ergosterol) (Desmond and Gribaldo 2009, De Vriese et al. 2020). The acquisition and refinement of these pathways during the evolution of different eukaryotic lineages probably contributed to the diversification of the group.

Here, we provide an updated overview of the evolutionary history of the sterol synthesis in Eukarya, from pre-LECA (last eukaryotic common ancestor) to the modern animal, fungi, and plant kingdoms, with particular focus on the plant lineage, and specially on the bryophyte branch.

Knowledge about sterol synthesis and its signaling derivatives, such as brassinosteroids (BRs), in bryophytes has been limited. However, recent research using models like *Marchantia polymorpha* (*Marchantia*) has shed light on this area. Hatada et al. (2023) reveal that *Marchantia* conserves a sterol synthesis pathway similar to that reported in vascular plants and suggest the potential synthesis of BR-like compounds.

In this work, we provide a comprehensive phylogenetic analysis of the final enzymes in the plant sterol synthesis pathway, including extensive sampling of bryophyte proteomes. Our findings reveal that gene duplications, with concomitant divergence at the sequence and expression levels, have taken place in *Marchantia* and other liverworts. In particular, some of the duplicates retain a broad expression pattern, while others show a coincidental peak in reproductive tissues. Since gene duplication and divergence are known to promote metabolic diversity (Sonawane et al. 2016, Akiyama et al. 2019), our findings suggest a branch point for sterol diversification and may indicate a differential accumulation of sterol molecules in reproductive structures.

The ancient origin of eukaryotic steroid synthesis pathway

All living eukaryotes, derived from LECA between 1200 and 1800 million years ago (Ma), are known as crown eukaryotes. However, these are likely not the sole evolutionary lineages. Besides LECA, other eukaryotic lineages closely related to crown eukaryotes may have arisen and become extinct, referred to as stem eukaryotes (Donoghue 2005, Porter and Riedman 2023). Organisms that lived on Earth left traces of their existence in the fossil record, allowing us to infer the evolutionary process they may have experienced. Sterol molecules' backbones are relatively stable against degradation, so they have been preserved in sedimentary rocks as saturated steranes. These steroid molecules are used as biomarkers to track the evolutionary history of different lineages and the variation in the steroid content in the membranes over time (Brocks et al. 2017, Brocks et al. 2023).

An initial increase in steranes, primarily cholestane, detected in the Tonian (1000 Ma-720 Ma) reflects the appearance of crown eukaryotic forms. This was followed by a burst in the abundance of cholestane, ergostane, and stigmasterane (crown steranes) in Ediacaran sediments (630 Ma-540 Ma), indicating significant diversification and prevalence of crown eukaryotes at that time (Brocks et al. 2023). However, the ability to synthesise these refined steroids was presumably present in LECA. Intriguingly, crown steranes were nearly absent from sediments of earlier periods (Mesoproterozoic 1600-1000 Ma). Brock and colleagues (2023) demonstrated that these sediments contain molecular fossils of protosterols (such as lanostane and cyclosterane) and side-chain methylated derivatives called ursterols. These protosteroids may have been produced by stem eukaryotes, which lacked the complete sterol biosynthetic pathway described in crown eukaryotes and possibly used protosterols as their end-products. This protosterol-producing biota likely coexisted with, or encompassed, the ancestors of the eukaryotic crown

group and dominated during the Mesoproterozoic period. Although the factors driving the rise of crown eukaryotes during the Tonian remains under intensive debate (Brocks et al. 2023, Mills et al. 2024), the presence of a membrane with a variety of steroids and the ability to change their proportions may have conferred advantages in adapting to changing environmental conditions and act as cell-to-cell signals (Du et al. 2022, Sokolov et al. 2022).

This molecular fossil record reflects the time of acquisition of different steroid biosynthetic genes in Eukarya. The protosterol biota already had the capacity to synthesise protosterols and side-chain methylated ursterols, implying that in these eukaryotic lineages related to LECA, enzymatic steps including the MVA pathway, squalene synthesis, squalene epoxidation, oxidosqualene cyclization and methyl group transfer at C24 should have already been present (Brocks et al. 2023) (Fig. 1). Many of the genes involved in the synthesis of crown steroids are inferred to be present in LECA, consistent with its presumed ability to synthesise sterol with a cholestane, ergostane, or even stigmastane backbone (Desmond and Gribaldo 2009).

The evolutionary link between sterol biosynthesis in bacteria and eukaryotes has long been recognized (Wei et al. 2016, Gold et al. 2017). While an earlier hypothesis proposed a eukaryotic origin for sterol biosynthetic genes, recent phylogenetic analyses suggest otherwise. These studies indicate that bacterial homologs of sterol biosynthetic and modification genes occupy deeper-branching positions in phylogenetic trees compared to their eukaryotic counterparts, supporting a bacterial origin and horizontal gene transfer to eukaryotes (Santana-Molina et al. 2020, Hoshino and Gaucher 2021, 2024). Additionally, structural analyses of CYTOCHROME P450 STEROL 14 α -DEMETHYLASE (CYP51) in sterol-producing bacteria, such as *Methylococcus capsulatus*, reveal significant similarities to eukaryotic CYP51 enzymes. Together with the widespread presence of CYP51 in bacteria, this evidence further supports a bacterial origin (Lamb et al. 2021).

Some steroid modification genes may have been acquired from Myxococcota or closely related bacterial lineages, which possess sterol biosynthetic pathways highly homologous to those of eukaryotes (Hoshino and Gaucher 2021, 2024). For instance, *Enhygromyxa salina* is capable of synthesizing cholesterol *de novo* using intermediates that are remarkably similar to those in eukaryotic pathways (Lee et al. 2023). These similarities likely result from inter-domain gene transfer events, either from Myxococcota or extinct bacterial lineages to eukaryotes, or possibly in the reverse direction (Hoshino and Gaucher 2024).

Collectively, these findings highlight the role of horizontal gene transfer and shared selective pressures in the evolution of sterol biosynthesis, rather than vertical inheritance from archaea, which lack the necessary sterol synthesis pathways (Gold et al. 2017, Hoshino and Gaucher 2021).

Emergence of modern eukaryotic kingdoms and the shaping of sterol biosynthetic pathways

The emergence of the different crown eukaryotic kingdoms entailed a reorganization of the catalytic steps, subsequent enzyme losses, specialization, and acquisition of novel functions (Desmond and Gribaldo 2009). These changes led to the diversification of the sterol biosynthetic pathway, accounting for the variety of sterols produced by the different clades.

The canonical sterol synthesis pathways have been extensively studied in plants, animals and fungi using model organisms (Fig. 1, Supplementary Table 1 and 2). Upstream reactions are highly conserved across these kingdoms. Besides the MVA pathway, the methylerythritol phosphate pathway (MEP) in plastids also feeds the sterol synthesis pathway in photosynthetic eukaryotes (Lombard and Moreira 2011). Oxidosqualene cyclization by OSCs constitute one of the initial distinctive steps. Animals and fungi possess LANOSTEROL SYNTHASE (LAS), which produces lanosterol as an intermediate for cholesterol and ergosterol synthesis (Nes 2011). In contrast, plants mainly generate cycloartenol through CYCLOARTENOL SYNTHASE 1 (CAS1), which is involved in synthesizing both phytosterols and cholesterol (Corey et al. 1993). Interestingly, LAS enzyme functionality has also been observed in plants (Gas-Pascual et al. 2014). Organisms which proceed through the cycloartenol pathway also possess CYCLOPROPIL STEROL ISOMERASE (CPI), which catalyses the opening of the cyclopropane ring in cycloeucalenol (Lovato et al. 2000, Men et al. 2008, Darnet et al. 2021). Modifications of the side-chain contribute to the variability of sterol molecules among lineages. The enzyme C-24 STEROL METHYLTRANSFERASE (SMT) transfers a methyl group to the C-24 position of the sterol molecule (Diener et al. 2000). Fungi possess an SMT1 enzyme required for the ergosterol synthesis (Jensen-Pergakes et al. 1998). In *Arabidopsis* (*Arabidopsis thaliana*), alongside SMT1, there are other types of SMT (SMT2 and SMT3) which catalyse a second addition of a methyl group, forming an ethyl side chain at the C-24 position. This bifurcates phytosterol synthesis into 24-methylsterols (campesterol) and 24-ethylsterols (β -sitosterol and stigmasterol) (Schaeffer et al. 2001, Carland et al. 2010). Cholesterol, the primary end product in animals, does not require major lateral chain modifications. Nonetheless, the production of side chain methylated sterols through SMT enzymes has been shown in sponges, rotifers

and annelids (Gold et al. 2016, Michellod et al. 2023). Recently, it has been suggested that the *SMT* gene may have been vertically inherited from an Eumetazoan ancestor and subsequently lost multiple times in various animal lineages (Brooks and Bobrovskiy 2023, Brunoir et al. 2023). Further modifications of the sterol structure include demethylation at the C-14 and C-4 position. C-14 demethylation is catalysed by the conserved CYP51, distributed across eukaryotic kingdoms (Lepesheva and Waterman 2007). Demethylation at C-4 is performed by a highly conserved multienzyme complex found in animals, plants and fungi. This complex consists of STEROL C4 METHYL OXIDASE (SMO), 3 β -HYDROXYSTEROID DEHYDROGENASE/C4-DESCARBOXYLASE (β HSD) and STERONE KETOREDUCTASE (SR) tethered by ERGOSTEROL BIOSYNTHESIS PROTEIN 28 (ERG28). In animal and fungi, a single C-4 demethylation complex sequentially removes the two methyl groups from the C-4 position after C-14 demethylation (Darnet and Schaller 2019). In Arabidopsis, two distinct families of STEROL METHYL OXIDASES (SMO1 and SMO2) have been identified. In this case, demethylation steps occur separately: SMO1 acts on the first C-4 demethylation step preceding C-14 demethylation, while SMO2 acts subsequent to C-14 demethylation (Darnet and Rahier 2004). Subsequent reactions such as Δ^8 - Δ^7 isomerization or reduction of the double bond at C-24 (C24R) are catalysed by non-orthologous enzymes in different kingdoms which were presumably inherited from LECA (Desmond and Gribaldo 2009). In yeasts, the $\Delta^{24(28)}$ -sterol reduction is catalysed by C-24(28) REDUCTASE (ERG4) (Zweytick et al. 2000). C-24 sterol reduction in animals and plants is mediated by two homologous enzymes: 24-DEHYDROCHOLESTEROL REDUCTASE (DHCR4), which proceed through a $\Delta^{24(25)}$ reduction, and SIDE CHAIN REDUCTASE (SSR1), called AtDWF1 in Arabidopsis, which proceed through an isomerization of $\Delta^{24(28)}$ to $\Delta^{24(25)}$ followed by reduction (Choe et al. 1999a, Nes 2011, Sawai et al. 2014). The final steps of sterol processing also involve conserved Δ^7 -STEROL-C5-DESATURASE (C5-SD, AtDWF7 in Arabidopsis) and STEROL Δ^7 -REDUCTASE (7DR, AtDWF5 in Arabidopsis), with the latter absent in fungi (Choe et al. 1999b, Choe et al. 2000, Desmond and Gribaldo 2009). Lastly, C-22 DESATURASES (C22Ds) are found in plants and fungi. These P450 cytochromes may have evolved from an ancestral duplication of CYP51. In plants, it resulted in the CYP710 clan, while in fungi, it led to the CY61 clan. In animals, the CYP710/CYP61 genes were probably lost (Nelson and Werck-Reichhart 2011, Chen et al. 2014).

Variations of these canonical pathways and a mosaic of features have been detected in many eukaryotic species. One example is *Capsaspora owczarzaki*, a single-celled organism related to animals, which is

capable of producing cholesterol, 7-dehydrocholesterol and ergosterol, among other sterols. It possesses a complete canonical ergosterol synthesis pathway but includes enzymatic activities related to metazoans. For instance, it contains both fungi ERG2 and animal EMOPAMIL-BINDING PROTEIN (EBP) to catalyse $\Delta^8\Delta^7$ isomerizations, as well as fungal ERG4 and animal DHCR24, which remove the C-24 double bond from different substrates (Najle et al. 2016) (Supplementary Table 2).

Many gene losses from the canonical biosynthetic pathway have occurred in different lineages. During the Ediacaran period, the rapid expansion of crown eukaryotes, particularly algae and plankton, significantly increased the availability of sterols in aquatic environments (Brocks et al. 2017, Brock et al. 2023). Simultaneously, periods of anoxia occurred during the oxygenation of the oceans (Yuan et al. 2023). Given that sterol biosynthesis is energy- and oxygen-dependent, some animal groups may have evolved to rely on the exogenous uptake of cholesterol rather than synthesizing it internally. This shift likely contributed to the loss of most sterol biosynthetic genes in certain protostomes. In contrast, other taxa, where exogenous sterol uptake was insufficient, retained the complete sterol biosynthesis pathway, as observed in the vertebrate lineage (Zhang et al. 2019).

Diversification of the sterol biosynthetic pathway in Archaeplastida

The Archaeplastida supergroup arose as a result of primary endosymbiosis between a cyanobacteria and a heterotrophic eukaryote. From the Archaeplastida crown-ancestor, three major lineages emerged: Glaucophyta, Rhodophyta (Red algae) and Viridiplantae (i.e. the Chlorophyta and Streptophyta lineages, with the latter including land plants) (Mackiewicz and Gagat 2014) (Supplementary Fig. 1).

Outside of Archaeplastida, other photosynthetic or ancestrally photosynthetic groups, such as Stramenopiles (including diatoms or brown algae), Alveolates or some Rhizaria originated from secondary or even tertiary endosymbiosis event, whereby heterotrophic eukaryotes acquired either red or green alga endosymbionts. Many endosymbiosis algae genes were transferred to the host during these events (Morozov and Galachyants 2019, Falciatori et al. 2020). Both endosymbiotic gene transfers and multiple horizontal gene transfers have occurred in these lineages, shaping their genomic content (Vancaester et al. 2020) and consequently diversifying their metabolic plasticity. As a consequence, significant differences in sterol composition and distinct features of the sterol biosynthetic pathway have been reported in these groups (Darnet et al. 2021).

Stramenopiles and red algae

Diatoms are conspicuous microalgae capable of producing both animal and plant-like sterols (e.g. desmosterol, cholesterol, dihydrobrassicasterol, 24-methylene cholesterol, fucosterol and even clionasterol). In line with their evolutionary history, the proposed sterol synthesis route in diatoms resembles the plant pathway and may proceed through cyclization of cycloartenol, suggesting this may be a Diaphoretikes-specific feature. However, notable differences exist: diatoms possess an alternative SQE (AltSEQ), a specific redSTM found in diatoms and some red algae, a fungal-like $\Delta^{24(28)}$ -sterol reductase responsible for the final steps of C-24 reduction to produce dihydrobrassicasterol or clionasterol, and an enzyme with animal-like $\Delta^{24(25)}$ -sterol reductase activity that converts desmosterol to cholesterol (Gallo et al. 2020, Zonnequin et al. 2024). These findings reflect the significant influx of genes from diverse origins that diatoms seem to have acquired (Morozov and Galachyants 2019 Gallo et al. 2020, Vancaester et al. 2020).

In red algae, major sterols are cholesterol and desmosterol, although other sterols such as campesterol or brassicasterol have also been detected (Belcour et al. 2020, Girard et al. 2021). Two alternative pathways from cycloartenol to cholesterol synthesis have been inferred in *Chondrus crispus*. The first pathway shares similar metabolic intermediates with the cholesterol synthesis route in plants and involves a promiscuous sterol C-24 reductase enzyme acting immediately after cycloartenol production (early SSR pathway) (Sonawane et al. 2016). The second pathway involves a late occurrence of C24-reduction, allowing the production of cholesterol from desmosterol. This late SSR pathway has also been reported in diatoms and brown algae (Belcour et al. 2020, Girard et al. 2021, Zonnequin et al. 2024).

Viridiplantae

Viridiplantae is an ancient, monophyletic group (Supplementary Fig. 1) (McCourt et al. 2023) composed of land plants and green algae (Chlorophyta and Charophyta). Indeed, zygmatophyceae algae (Charophyta) are considered the closest relatives to land plants (Turmel et al. 2006, Buschmann 2020).

The sterol content was analysed in different chlorophytes. *Chlamydomonas reinhardtii*, *Chlorella variabilis* and *Chlorella sorokiniana* contain ergosterol as the major sterol, while *Coccomyxa subellipsoidea* contains phytosterols. Both ergosterol and phytosterols production are inferred to proceed through the land-plant-type branch of sterol synthesis, involving cycloartenol as an intermediate, and the enzymes CAS1, CPI1, and HYDRA 1 (HYD1) (Miller et al. 2012, Brumfield et al. 2017, Voshall et al. 2021) (Supplementary Table 2). A plant-like pathway is presumably present in charophytes, although

studies of sterol content and synthesis in this evolutionary lineage are very limited. The primary sterols reported in this algae group are 28-isofucosterol and a mixture of 24-ethylcholesterol (mainly clionasterol, but also sitosterol) (Patterson and McKenna 1991).

Land plants contain phytosterols (campesterol, stigmasterol and β -sitosterol) as their major sterols, but significant amounts of cholesterol and its derivatives have also been found (Milner et al. 2011, Darnet et al. 2021). Extant land plants comprise two main evolutionary lineages: bryophytes (hornworts, mosses and liverworts) and tracheophytes (lycophytes, monilophytes and spermatophytes), both constituting monophyletic groups (Harris et al. 2020, Harris et al. 2022). The sterol synthesis pathway has been extensively studied in flowering plants, particularly in the model species *Arabidopsis* (*Brassicaceae*), from which the aforementioned canonical pathway is derived. Distinguishing features of the plant pathway include the presence of the cycloartenol intermediates produced by CAS1 enzymes, CIP1 to open the cyclopropane ring, and SMT1 and SMT2 enzymes for the double methylation at C-24, resulting in 24-methylsterols and 24-ethylsterols (Supplementary Table 1 and 2).

Cholesterol is a minor sterol in *Arabidopsis* and is produced from cycloartenol following a pathway similar to that of phytosterol synthesis (Fig. 1). In fact, the same enzymes may act promiscuously on precursors from both pathways. The only bifurcation step occurs after cycloartenol synthesis. While SMT1 produces 24-methylenecycloartenol from cycloartenol to initiate phytosterol synthesis, SSR1 removes the double bond at C-24 from cycloartenol, thus creating a cholesterol-like lateral chain that will not be further modified (Choe et al. 1999a, Sonawane et al. 2016, De vries et al. 2021). In some plant lineages, new cholesterologenesis enzymes have evolved from phytosterol enzymes. In tomato (*Solanaceae*), SSR1 catalyses a two-step reaction: the $\Delta^{24(28)}$ to $\Delta^{24(25)}$ isomerization of 24-methylencholesterol to produce 24-methyldemosterol, followed by $\Delta^{24(25)}$ reduction of the double bond, resulting in campesterol. The divergent SSR2 enzyme has a specific $\Delta^{24(25)}$ reductase activity and acts at the bifurcation of the cholesterol pathway, performing a reaction similar to that catalysed by human DHCR24 (Choe et al. 1999a, Sawai et al. 2014, Sonawane et al. 2016). A similar situation is found for different enzymes: C5-SD1 homologous to AtDWARF7 and C5-SD2; 7-DR1 homologous to AtDWARF5 and 7-DR2, and the pairs of sterol C4 methyloxidases SMO1/SMO3 and SMO2/SMO4 (Fig. 1). This illustrates a scenario in which a new, specific cholesterol pathway evolved from the ancestral phytosterol pathway, with some enzymes specializing while others remained common to both pathways (Sonawane et al. 2016) (Supplementary Table 1).

In angiosperms, besides the significant structural role of sterols in membrane stability and dynamics, and consequently influencing the localization of various membrane components such as signaling elements and ion channels (Men et al. 2008, Du et al. 2022), cholesterol, campesterol and β -sitosterol also serve as precursors of the steroid hormones, brassinosteroids (BRs) (Bajguz and Piotrowska-Niczyporuk 2023). BRs are key regulators of growth, development, and reproduction. Indeed, deficiencies on the BR synthesis or signaling pathway result in pleiotropic defects such as severe dwarfed phenotypes and sterility (Chory et al. 1991, Clouse et al. 1996, Li et al. 1996).

Interestingly, mutants of the sterol synthesis pathway upstream of 24-methylenelophenol, such as *fk*, *cpi1-1*, *cyp1*, *smt3* and *cyp51A2*, exhibit unique phenotypes including defects in embryogenesis, meristematic programs, root growth and development, gravitropism, hormone signaling, vascular patterning, and cellular elongation, which cannot be recovered by application of BRs (Jang et al. 2000, Kim et al. 2005, Men et al. 2008, Carland et al. 2010). This demonstrates the independent and essential roles of these upstream sterols beyond serving as BR precursors. In contrast, mutants of the enzymes catalysing the final steps of the pathway, *dwarf7*, *dwf5* and *dwf1*, display the characteristic BR-deficient phenotype, which can be rescued by BR application (Choe et al. 1999a, Choe et al. 1999, Choe et al. 2000).

Insights on the sterol biosynthetic pathway in the bryophyte branch

The analysis of the sterol synthesis pathway and sterol profiles in seed plants and algae has been the focus of numerous studies due to the potential applications of these compounds as pharmaceuticals and nutraceuticals (Barbalace et al. 2019, Barkas et al. 2023). However, less attention has been paid to sterol production in other evolutionary lineages on land plants, specifically bryophytes. The promotion of the liverwort *Marchantia polymorpha* and the moss *Physcomitrium patens* as plant models allowed a comprehensive view of how biosynthetic pathways have been shaped during evolution of land plants (Shimamura 2016, Bowman et al. 2017, Rensing et al. 2020).

Sterol composition varies among plant species. *Arabidopsis* is characterised by a high content of sitosterol, lower levels of 24-methylcholesterol (campesterol and 22-dihydrobrassicasterol), isofucosterol, stigmasterol, brassicasterol, and very low cholesterol (Benveniste 2004). Other angiosperms, such as *Nicotiana tabacum*, accumulate higher levels of stigmasterol, along with sitosterol, 24-methylcholesterol, and lower levels of cholesterol (Darnet et al. 2021). In contrast, cholesterol levels are high in other *Solenaceae* species, as mentioned (Milner et al. 2011, Sonawane et al. 2016).

In bryophytes, both *Physcomitrium* and *Marchantia* exhibit similar sterol profiles, with high content of stigmasterol and campesterol, but low levels of β -sitosterol and very low cholesterol (Morikawa et al. 2009, Hatada et al. 2023). In *Physcomitrium*, homologous sequences to sterol biosynthetic enzymes found in other streptophytes have been identified. The final enzymes in the pathway, the C-22 desaturases PpCYP710A13 and PpCYP710A14, are capable of converting β -sitosterol into stigmasterol. Thus, the presence of typical land-plant compounds alongside the presumed conservation of the pathway suggests that both angiosperms and bryophytes may share a common sterol biosynthetic pathway (Morikawa et al. 2009).

Recently, Hatada and colleagues (2023) investigated the conservation of the sterol synthesis pathway in *Marchantia* (Supplementary Table 2). They identified homologs of all genes from the *Arabidopsis* sterol synthetic pathway and focused on the function of MpDWF5A, the closest homolog to AtDWF5. This enzyme is expressed throughout *Marchantia* and in its entire life cycle. They demonstrated that MpDWF5A catalyses the reduction of the double bond at the C7 position of precursors from both cholesterol and phytosterols (campesterol and β -sitosterol). Loss of function of MpDWF5A in *Marchantia* results in abnormal phenotypes, including dwarfism, multiple meristem formation, reduced gemma cup number, and inhibition of ventral scale formation. Interestingly, some of these defects, such as dwarfism, were partially alleviated by the exogenous application of phytosterols (campesterol or stigmasterol) and BRs (castasterone and 6-deoxocastasterone). This suggests that at least part of the phenotypes observed in *Mpdwarf5a* mutants are due to a decrease of BR-like compounds, as was shown for *dwf5* mutants in flowering plants (Choe et al. 2000), implying not only the conservation of the sterol pathway but also the potential use of these sterols as precursors of BR-like compounds in bryophytes.

Intriguingly, BR-like compounds have been widely identified among land plants and green algae (Cheon et al. 2013, Stirk et al. 2013, Yokota et al. 2017). However, their role as hormones, as well as their synthesis and signaling pathways, have not yet been clarified outside the vascular clade. Efforts to find homologs of BR biosynthetic enzymes in bryophyte genomes have yielded limited results. Thus far, homologs to STEROID 5 α -REDUCTASE (DET2) have been identified, but BR-committed P450 cytochromes from the CYP90 family are limited, and those from the CYP85A family, responsible for the final steps of the pathway, have only been identified in seed plants (Yokota et al. 2017, Cannell et al. 2020, Liu et al. 2024). This suggests the existence of alternative BR-like biosynthetic pathways in bryophytes, potentially involving DET2 and a few CYP90 homologs.

Homologous sequences to BR signaling elements have been identified in bryophytes. In *Marchantia*, MpBES1 is reported as the ortholog to the BR-responsive transcription factor BRI1-EMS SUPPRESSOR1 (BES1) / BRASSINAZOLE RESISTANT1 (BZR1) of *Arabidopsis* (Wang et al. 2002, Yin et al. 2002). It has been demonstrated that MpBES1 plays a conserved role in regulating plant growth and development (Mecchia et al. 2021). Together, these findings suggest that in *Marchantia* (and possibly other bryophytes), sterols may also serve as precursors for an unknown BR biosynthesis pathway, similar to what has been observed in vascular plants (Hatada et al. 2023), and these BR-like compounds may play a role in growth and development.

Interestingly, although BR receptors of the BRL2-type emerged in land plants, they were lost in liverworts and other bryophyte groups (Ferreira-Guerra et al. 2020, Furumizu and Sawa 2021, Liu et al. 2024). Thus, the mechanism by which these BR-like compounds may be sensed in these organisms remains enigmatic.

Diversification on the sterol synthesis in bryophytes

Remarkably, two copies of the enzymes involved in the final steps of the sterol synthesis pathway, 7DR, C24R and C22D, have been identified in *Marchantia* (Hatada et al. 2023) (Supplementary Table 2).

The *Marchantia* 7DR copies are designated as MpDWF5A and MpDWF5B. MpDWF5A clusters with vascular plant homologs and has been demonstrated to fulfil its role in the synthesis of phytosterol and cholesterol. Conversely, MpDWF5B and its homologs are exclusively found within the bryophyte lineage. MpDWF5B is unable to reduce the 7 position of dehydrocholesterol to cholesterol (Hatada et al. 2023). This suggests the potential involvement of MpDWF5B in an alternative sterol-producing pathway. Notably, its expression pattern is distinct from that of MpDWF5A, appearing to be confined to specific structures such as sexual organs (particularly in antheridia) and sporangia (Supplementary Fig. 2).

The presence of extra copies of the two subsequent enzymes in the sterol synthesis pathway (C24R and C22D) in the *Marchantia* proteome caught our attention. To shed light on the origin of these proteins and infer the possible differences among them, we performed a phylogenetic analysis using different species from Archaeplastida.

Identified SSR1, SSR2 and 24-ISO sequences (Sawai et al. 2014, Sonawane et al. 2016, Knoch et al. 2018) were used as queries to interrogate seed plant proteomes using BLASTP. Hits on basal

angiosperms (e.g., *Amborella trichopoda*) and gymnosperms were employed to refine BLASTP searches on ferns and lycophytes. Furthermore, once C24R sequences were found in a specific group, they were also used to search against the rest of the group members. A similar approach was applied to bryophytes and algae. Additionally, putative C24R enzymes from algae and animals were retrieved from published data (Supplementary Data S1).

Our initial phylogenetic analysis showed a clear separation among sequences of chlorophytes and those of other plant and animal C24 sterol reductases (Supplementary Fig. 3). Intriguingly, chlorophyte C24R share more sequence similarity with diatom fungal-like $\Delta^{24(28)}$ -sterol reductases and ERG4 from yeasts, even though ergosterol synthesis is known to proceed through different sterol pathway in green algae and fungi (Miller et al. 2012). Given the different evolutionary trajectories of chlorophytes and streptophytes, we removed chlorophytes sequences from the subsequent phylogenetic analysis. A new tree was constructed using DHCR24 and animal-like C24R cluster as the true root to decipher the evolution of its homologs in plants (Fig. 2).

Our analysis indicates that C24R duplication may have occurred before the diversification of extant land plants (Fig. 2). We found just one protein-encoding gene for the C24R enzyme in the charophyte proteomes examined. However, two clearly separate clades of land-plant C24R enzymes can be identified. One clade consists of closer homologs to the known SSR1/SSR2 or 24-ISO enzymes, and the other contains a distinct cluster of C24R enzymes (hereafter named C24R-like) (Fig. 2). Specific losses of C24R-like were identified in certain plant lineages: angiosperms and ferns lack these sequences. Furthermore, C24R-like enzymes are present in early diverging branches of mosses and hornworts such as *Leiosporoceros*, *Sphagnum* or *Takakia*, but have been lost in more derived lineages such as *Paraphymatoceros* and *Megaceros*, Dicranidae (*Physcomitrium* or *Ceratodon purpureus*) and Hypnales (*Rynchostegium serrulatum*) (Liu et al. 2019, Frangedakis et al. 2021).

In agreement with the observed separation of C24R enzymes in land plants, Mp5g20140 (MpDWF1A), which clusters with SSR1/SSR2/24-ISO sequences, shares a 72% identity with AtDWF1. Conversely, Mp1g14660 (MpDWF1B), shares a 54% identity with AtDWF1. Alignment of protein sequences reveals a general conservation of the core residues and structure among SSR1/SSR2/24-ISO (C24R), C24R-like and vertebrate DCHCR24 (Supplementary Fig. 5). The N-terminal transmembrane segment followed by a FAD-binding domain and a catalytic domain can be traced in all C24R land-plant enzymes (Klahre et al.

1998, Tsukagoshi et al. 2016). Unfortunately, the crystal structure of DWF1 or its homologs has not been determined yet, hence essential residues involved in substrate recognition, specification, and catalysis among different plant C24R enzymes remain unknown (Knoch et al. 2018, Jiang et al. 2023). Nevertheless, modeling of human DHCR24 and its catalytic sites have revealed critical positions for C24R action (Pedretti et al. 2008). Sequence alignment reveals that the nature of these residues is highly conserved across all land plants, suggesting that C24R-like enzymes may indeed retain Δ^{24} reductase activity (Supplementary Fig. 4, Supplementary Fig. 5).

Land-plant C24R and C24R-like and the homologous charophyte sequences (Zygnemophyceae, Coleochaetophyceae and Klebsormidiophyceae) form a monophyletic group. Charophyte sequences share common characteristics with both C24R groups; however, C24R-like sequences also exhibit distinguishing features that may indicate a faster divergence rate. The conserved C-terminal region found in C24R proteins from all land plants and sister lineages of charophytes is absent in C24R-like proteins (Supplementary Fig. 4, Supplementary Fig. 5). This resembles DCHR24 from animals (Klahre et al. 1998, Pedretti et al. 2008) or proteins with a predicted DCHR24 function present in diatoms and other algae (Gallo et al. 2020, Belcour et al. 2020), which also lack this C-terminal segment. Interestingly, it has been suggested that the presence or absence of this C-terminal tail may influence enzyme specificity (Klahre et al. 1998). Therefore, the absence of this structure may affect the substrate preference of C24R-like proteins.

The analysis of *MpDWF1A* and *MpDWF1B* expression using the MarpolBase Expression tool (Kawamura et al. 2022) revealed distinct patterns. *MpDWF1A* is highly expressed throughout the entire life cycle of *Marchantia*, similarly to *MpDWF5A*. Although *MpDWF1B* is also expressed at various stages, its overall expression level is lower. Notably, the expression of *MpDWF1B* peaks in the male sexual structures, particularly in the antheridium, and during sporeling development (Supplementary Fig. 2).

To identify the origin of the two copies of C22D gene in *Marchantia*, we employed the same approach as for C24R analysis. Specifically, for the initial search against seed plants proteomes, we used AtCYP710A1-A2 (from *Arabidopsis*) and CYP710A11 (from tomato), enzymes known to function as C-22D desaturase in plants (Morikawa et al. 2006) (Supplementary Data S1).

Our analyses revealed that the additional copy of C22D previously identified in *Marchantia* originated from a specific duplication event of this enzyme within the evolutionary branch of Marchantiales (Fig. 3). This duplication event likely occurred at the bases of the diversification of this order, as members of early divergent families (as Lunulariaceae or Marchantiaceae) possess these sequences (Bowman et al. 2022, Wang et al. 2022). One of the clades resulting from the duplication contains sequences from Sphaerocarpaceae, a closely related evolutionary lineage to Marchantiales. The *Marchantia* C22D enzyme found in this cluster (Mp1g00490), referred as MpCYP710A1, shares 55% identity with AtCYP710A1 (*Arabidopsis*) and 64% identity with PpCYP710A13 (*Physcomitrium*). In the other clade, only copies of C22D of Marchantiales are present; Mp6g19120, referred as MpCYP710A2, shares 52% identity with AtCYP710A1, 58% with PpCYP710A13 and 66% with MpCYP710A1.

Alignment of C22D sequences shows that the conserved MCR1 region, which plays a role in anchoring the enzyme to the endoplasmic reticulum (Gutiérrez-García et al. 2021), is clearly present in both copies of the enzyme (Supplementary Fig. 6). Recently, the detailed structure and mechanism of the reaction catalysed by C-22 desaturases was published (Raju et al. 2022). The sequence alignment reveals conservation in the residues lining the substrate entry channel (including the bottleneck region), as well as in the active site and interacting with the substrate (Supplementary Fig. 6). Importantly, the substrate recognition site motif [FLFA(A/S)QDAS(T/S)S] is highly conserved in all land plants and in the two copies of the enzymes. Thus, C22D desaturase activity is likely maintained in both enzymes. Nevertheless, a few variable positions in residues interacting with the substrate or within the tunnel can be observed.

Notably, analysis of the expression of MpCYP710A1 and MpCYP710A2 (Kawamura et al. 2022) shows that MpCYP710A1, which shares higher identity with reported C22D desaturases, is expressed widely throughout the life cycle, especially during sporeling and thallus development. In contrast, MpCYP710A2 expression is more restricted, with a peak in sexual organs (particularly antheridia and archegonia), resembling the expression pattern of MpDWF5B (Supplementary Fig. 2).

MpDWF5A, MpDWF1A, and MpCYP710A1 exhibit a widespread expression pattern, whereas the duplicated copies (MpDWF5B, MpDWF1B, and MpCYP710A2) display a coincidental peak or a more restricted pattern of expression in sexual organs. Clear differences in the amino acid sequences are evident, particularly for MpDWF1B, which may result in changes in substrate preference and even

functionality. Thus, potential divergence in both sequence and gene expression patterns of the duplicated enzymes from the sterol synthesis pathway may be occurring in *Marchantia*, and likely in other land plants, judging by the widespread presence of MpDWF1B and MpDWARF5B homologs.

The sterol content in *Marchantia* has been analysed during the vegetative gametophyte development, a phase when MpDWF5A, MpDWF1A, and MpCYP710A1 are highly expressed (Supplementary Fig. 2). This analysis revealed the typical phytosterol composition of plants including campesterol, stigmasterol, and β -sitosterol (Hatada et al. 2023). However, such analysis has not been conducted during the sexual phase. It remains to be determined whether the spatial co-expression of these duplicated genes in sexual tissues leads to the synthesis of similar or different sterol compounds.

Interestingly, the duplication of the enzymes from the phytosterol pathway has provided a foundation for the emergence and diversification of new metabolite pathways. In fact, in *Solanaceae* species, specific cholesterologenesis enzymes arose from duplications along the phytosterol pathway (Sawai et al. 2014, Sonawane et al. 2016). Interestingly, a third homolog for the sterol Δ^{24} reductase, named 24ISO/SSR-like, has been identified in members of Solanoideae and Petunieae subfamilies. SSR-like likely emerged from two duplication events in *Solanaceae*: the first event gave rise to SSR1 and SSR2, while a second event led to the divergence between SSR2 and SSR-like. The primary function of the SSR-like enzyme is the isomerization of $\Delta^{24(28)}$ to $\Delta^{24(25)}$ sterols, thereby producing 24-methyldemosterol from 24-methylenecholesterol, although it also retains a weak activity in $\Delta^{24(25)}$ reduction. This is the initial committed step in the biosynthesis of withanolides, which are crucial secondary metabolites in *Solanaceae* as defense compounds (Knoch et al. 2018).

Another example within *Solanaceae* involves duplications of the steroid 5α -reductase enzyme that functions in BR synthesis. In tomato, the genes S5 α R1 and S5 α R2 encode functional steroid 5α -reductases. However, S5 α R1 likely retains its role in BR synthesis, while S5 α R2 has specialized in producing α -tomatine, a steroidal glycoalkaloid with a function in defense (Akiyama et al. 2019). Similarly, it has been reported that in the monocot *Paris polyphylla*, enzymes involved in the diosgenin synthesis in plants have evolved from duplications of ancestral BR-biosynthetic CYP90Bs (Christ et al. 2019).

Hence, the functional divergence and specialization of enzymes involved in sterol synthesis constitute one of the driving forces promoting metabolic innovation. Further research into the roles of the duplicated

enzymes in *Marchantia* will clarify their activities and contributions to specific pathways, shedding light on the evolutionary trends these enzymes are following.

Conclusion

- (1) Eukaryote lineages exhibit a conserved framework for sterol biosynthesis inherited from LECA. Specific modifications in the pathway, including enzyme acquisitions, losses, and step reorganizations, have arisen across diverse evolutionary lineages within the Eukarya domain. These adaptations reflect particular niche adaptations and co-evolutionary dynamics with other traits.
- (2) The plant sterol biosynthetic pathway typically involves the production of cycloartenol intermediates by CAS1 enzymes, the opening of the cyclopropane ring by CIP1, and double methylation at C-24 by SMT1 and SMT2 enzymes. Algal groups within the Archaeplastida, as well as other photosynthetic groups such as the Stramenopiles, maintain a plant-like pathway consistent with their evolutionary history. Nevertheless, notable differences in specific enzymes and reactions contribute to the variability observed among these groups.
- (3) Duplication and specialization of various phytosterol enzymes have expanded and modified sterol compositions, also serving as starting points for new sterol metabolic pathways (Knoch et al. 2018, Sonawane et al. 2016).
- (4) Bryophytes possess a sterol synthesis pathway and sterol content similar to those reported in vascular plants. Recent research on *Marchantia* also indicates that these sterols may be used as a precursor for the synthesis of BR-like compounds, which may ultimately regulate plant growth and development.
- (5) Our detailed phylogenetic analysis of the duplicated sterol synthesis enzymes in *Marchantia* reveals:
 - 5.1 The duplication events occurred at various points during the evolutionary history of land plants. DWF1 duplication took place at the base of land plant phylogeny, and CYP710A1 within the Marchantiales branch.
 - 5.2 The duplicated enzyme copies exhibit a high conservation of critical amino acid residues at the catalytic and substrate-binding sites, suggesting that at least part of their original activity has been retained. However, some sequence divergence has also been detected, particularly in the DWF1 copies, which may indicate a potential shift in substrate preference.

- 5.3 The duplicated enzyme copies exhibit distinct expression patterns. Enzymes clustering with vascular plant sterol enzymes show general expression throughout the *Marchantia* life cycle. In contrast, the more divergent copies display either a coincidental peak or a more restricted expression pattern in sexual organs.
- 5.4 Thus, investigating whether the spatial co-expression of these duplicated enzymes in sexual tissues result in the synthesis of similar or different sterol compounds could enhance our understanding of sterol diversity in bryophytes and help leverage their metabolic potential.

Data Availability

Supplementary figures, Supplementary Tables and Supplementary Data S1 are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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Figure Legends

Figure 1. Canonical sterol synthesis pathway in vertebrates and plants. Upstream reactions are highly conserved in both pathways. Isoprenoid precursors are produced by the mevalonate (MVA) pathway. Additionally, the methylerythritol phosphate (MEP) pathway contributes to the isoprenoid precursor pool in photosynthetic eukaryotes. Subsequent squalene synthesis is performed by farnesyl pyrophosphate synthase (FPPS) and squalene synthase (SQS). Oxidosqualene cyclization is carried out by lanosterol synthase (LAS) in animals and cycloartenol synthase (CAS) in plants. Posterior modifications of the sterol molecule involve a set of conserved and divergent enzymes: Sterol 14 α -demethylase (CYP51), sterol C4 methyloxidase (SMO), sterol C14 reductases (TM7SF2/FK), sterol Δ^8 - Δ^7 isomerases (EBP/HYD1); sterol-C5-desaturases (SC5DL/DWF7); sterol Δ^7 reductases (DHCR7/DWF5); sterol C24 reductase (DHCR24/DWF1), C-24 sterol methyltransferase (SMT), cyclopropyl sterol isomerase (CPI), C-22 desaturase (CYP70A1). The specific cholesterol synthesis pathway in tomato is also shown. Divergent enzymes from the canonical phytosterol pathway are sterol C24 reductase (SSR2), sterol C4 methyloxidases (SMO3 and SMO4), sterol-C5-desaturase (C5-SD2) and sterol Δ^7 reductase (7-DR2).

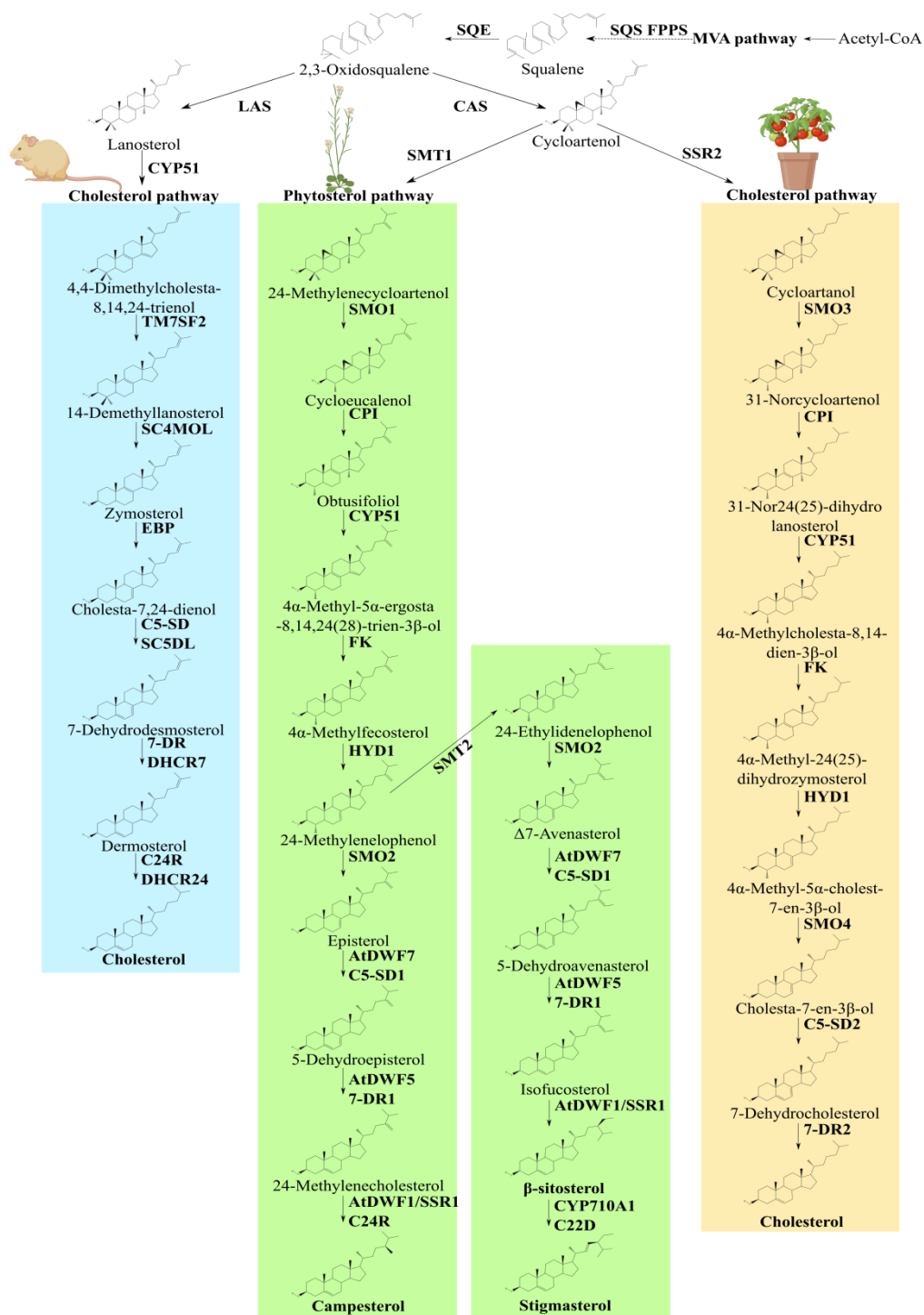
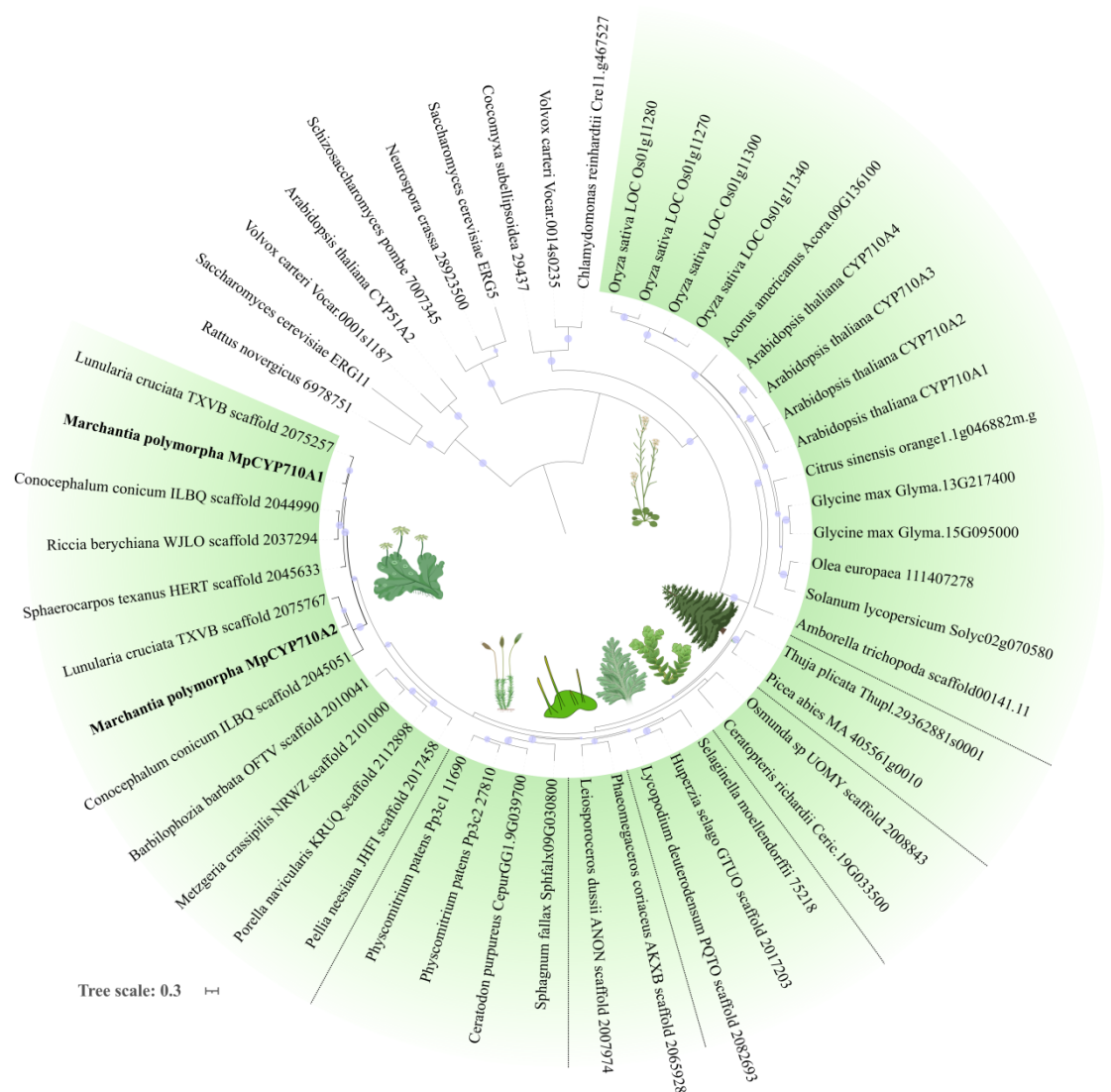


Figure 3. Phylogenetic analysis of C22D sterol enzymes. Phylogenetic tree for C22D enzymes. Sequences were aligned with Muscle in MEGA 11 (Tamura et al. 2021) (Supplementary Fig 6). Phylogenetic analysis was performed using Maximum likelihood method, LG + I + G4 model with 1000 replicates for the bootstrap (BS) test. BS values from 60%-100% are represented as blue dots on branches. Sequences are provided in Supplementary Data S1.



Supplementary Figure Legends

Supplementary Figure 1. Evolutionary relationships among Archaeplastida.

Supplementary Figure 2. Expression patterns of MpDWF7, MpDWF5A/B, MpDWF1A/B and MpCYP710A1/A2 during Marchantia life cycle. Raw TPM data were downloaded from MarpolBase Expression tool (Kawamura et al. 2022, <https://marchantia.info/mbex/clustergram>). Colour scale

represents the median of log2 TPM (Transcripts Per Million) from replicates of the same condition. Grey cells indicate no expression for a specific condition.

Supplementary Figure 3. Different evolutionary trajectory of chlorophyte C24R enzymes.

Phylogenetic tree was constructed as follows. Sequences were aligned with Muscle in MEGA 11 (Tamura et al. 2021). The phylogenetic analysis was performed using Maximum likelihood method, JTT + G2 model with 1000 replicates for the bootstrap test. BS values from 90%-100% are represented as blue dots on branches.

Supplementary Figure 4. Conservation model of Δ^{24} -sterol reductase comparing C24R and C24R-like land plant. A subset of amino acid sequences from land plants C24R and C24R-like enzymes aligned with Muscle were used (Supplementary Fig. 5, black dots). Conservation scores obtained with AL2CO (Pei and Grishin 2001) were mapped onto the predicted structure of AtDWF1 by AlphaFold2 using UCSF Chimera X (Pettersen et al. 2021). Highly conserved regions are coloured in red, more variable residues are coloured in blue. The lack of the C-terminal region of C24R-like is shown in orange.

Supplementary Figure 5. Multi-sequence alignment of C24R and C24R-like enzymes. Amino acid sequence alignment of CR24 and CR24 like enzymes from representative species used in the phylogenetic tree (Fig. 2). Sequences were aligned with Muscle in MEGA 11 (Tamura et al. 2021), and manually trimmed to remove gaps. Predicted transmembrane fragment, FAD domain and substrate binding domain are highlighted in blue, black and green respectively. Blue squares are residues presumably involved in cofactor binding and pink squares in substrate binding (Pedretti et al. 2008). The C-terminal region is absent in C24R-like enzymes. Black dots show the subset of sequences used for the conservation model of Δ^{24} -sterol reductase shown in Supplementary Fig. 4. PNPBR: Potential nucleotide phosphate-binding region.

Supplementary Figure 6. Multi-sequence alignment of C22D enzymes. Amino acid sequence alignment of C22D from representative species used in the phylogenetic tree (Fig. 3). Sequences were aligned with Muscle in MEGA 11 (Tamura et al. 2021). TMH (transmembrane-helix domain) and membrane contact region MCR1 are lined in black over the alignment (Gutiérrez-García et al. 2021). Blue and green squares correspond to residues lining the tunnel and the bottleneck region of the tunnel. Pink squares are residues from the active site, specifically purple squares represent positions potentially interacting with the substrate. Red squares identified the conserved substrate recognition site motif

[FLFA(A/S)QDAS(T/S)S] (Raju et al. 2022). Black open squares highlight the duplicated C22D sequences in Marchantiales.

Supplementary Table Legends

Supplementary Table 1. Detailed description of sterol biosynthesis pathway in plants, including phytosterol and cholesterol pathways.

Supplementary Table 2. Comparison of sterol biosynthetic enzymes across different organisms.

ACCEPTED MANUSCRIPT