Dissecting cholesterol and phytosterol biosynthesis via mutants and inhibitors

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ABSTRACT

Plants stand out among eukaryotes by the large variety of sterols and sterol derivatives that they can produce. These metabolites not only serve as critical determinants of membrane structures, but also act as signaling molecules, as growth regulating hormones, or as modulators of enzymatic activities. Therefore, it is critical to understand the wiring of the biosynthetic pathways by which plants generate these distinct sterols, to allow manipulating them and dissect their precise physiological roles. Here, we review the complexity and variation of the biosynthetic routes of the most abundant phytosterols and cholesterol in the green lineage and how different enzymes in these pathways are conserved and diverged from humans, yeast and even bacteria. Many enzymatic steps show a deep evolutionary conservation, while others are executed by completely different enzymes. This has important implications for the use and specificity of available human and yeast sterol biosynthesis inhibitors in plants, and argues for the development of plant-tailored inhibitors of sterol biosynthesis.

Highlight statement:

The complexity and diversification of sterol biosynthesis in the green lineage make it difficult to manipulate plant sterol biosynthesis via currently available small molecule inhibitors.

Introduction

Sterols are a class of triterpenoid lipids that consist of a hydrated phenanthrene group and a cyclopentane ring. These molecules have a crucial impact on membrane fluidity and transmembrane export and import processes, and some sterols can even act as second messengers or signaling molecules during developmental and cellular signaling processes (Boutté and Grebe, 2009; Boutté and Jaillais, 2020; Valitova *et al.*, 2016; Vriet *et al.*, 2013).

The ancient rise in atmospheric O_2 levels enabled the evolution of the oxygen-dependent sterol biosynthesis pathways found in all eukaryotes (Galea and Brown, 2009; Mouritsen, 2005), and is often used as an indicator of eukaryotic life. This is contrasted by the occurrence of hopanoids in prokaryotes, which are ring-structured molecules that look similar to sterols and exert analogous functions in the membranes as cholesterol, but do not require O_2 for their biosynthesis and lack a 3β-hydroxyl group (Saenz *et al.*, 2015). Notably, some bacteria also produce sterols, a feature that was presumably gained via horizontal gene transfer (Bode *et al.*, 2003; Rivas-Marin *et al.*, 2019).

While sterols occur in all eukaryotic organisms, the types and amounts of the main sterols vary considerably between the different kingdoms. In example, the major sterol produced in animals is cholesterol, whereas fungi mainly produce ergosterol. Plants, on the other hand, produce a wide variety of sterols (or phytosterols), with over 200 variants known to date (Benveniste, 2004; Guo *et al.*, 1995; Schaller, 2004). Among the phytosterols, campesterol, stigmasterol and β-sitosterol make up the predominant molecules of the sterol profile in plants (Benveniste, 2004; Schaeffer *et al.*, 2001). Cholesterol and derivatives are typically 100- to 1000-fold less abundant in plants than in humans, but in some species they make up a large fraction of the total sterol content, as seen in canola, *Solanaceae*, *Liliaceae* and *Scrophulariaceae* (Behrman and Gopalan, 2005). In addition to free sterols, plants also accumulate a variety of sterol conjugates such as fatty acid acyl sterol esters (SE), steryl glucosides (SG) and acyl steryl glucosides (ASG) (Ferrer *et al.*, 2017; Zhang *et al.*, 2020). A survey of different diatoms also revealed a very complex sterol landscape, again with a completely different set of specific sterols being the most abundant, even varying between different diatom lineages (Rampen *et al.*, 2010). Also the green algae display a markedly distinct sterol profile compared to land plants, being mainly dominated by cholesterol, 24-methylene cholesterol and 28-isofucosterol (Li *et al.*, 2017; Lopes *et al.*, 2011). This diversity in sterol composition is sufficient to allow for chemotaxonomic classification of algae (Taipale *et al.*, 2016).

The common evolutionary origin makes that many conserved enzymatic activities of sterol biosynthesis across the eukaryotic kingdoms are sensitive to the same pharmacology.

This allows to use a part of the vast sterol pharmacology, which was originally designed to inhibit human or fungal sterol biosynthesis, to interfere with corresponding enzymes in plants. Here, we review our current understanding of phytosterol and cholesterol biosynthesis in plants, and highlight available pharmacology and mutants, mainly using Arabidopsis (*Arabidopsis thaliana*) as an example.

Early sterol biosynthesis – building the universal terpene precursors

Squalene is the universal precursor of triterpenes; in prokaryotes for hopanoids and in eukaryotes of steroids (including phytosterols, lanosterol and cholesterol). For details about its biochemistry, we refer the reader to specialized literature (Henry *et al.*, 2018; Vranova *et al.*, 2013). In brief, squalene is formed by the condensation of two farnesyl pyrophosphate (FPP) units by SQUALENE SYNTHASE (SQS), and FPP is an assembly of two IsoPentenyl Pyrophosphates (IPP) and one molecule DiMethylAllyl PyroPhosphate (DMAPP), catalyzed by FPP SYNTHASE (FPPS) (Fig. 1). The cytoplasmic IPP and DMAPP levels are subject to a highly complex metabolic regulation in plants, possibly involving at least two distinct mechanisms. The best characterized, and most important pathway for cytosolic IPP production is the mevalonate (MVA) pathway, which is largely conserved across eukaryotes and archaea. Central to this pathway is the activity of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) that produces MVA a precursor of IPP and DMAPP (Fig. 1). An alternative MVA pathway generates isopentenyl phosphate (IP) and dimethylallyl phosphate (DMAP), which can be phosphorylated to form IPP and DMAPP (Henry *et al.*, 2015; Henry *et al.*, 2018). The methylerythritol phosphate (MEP) pathway produces IPP and DMAPP in plastids (Banerjee and Sharkey, 2014), of which a fraction feeds into the cytoplasm (Kasahara *et al.*, 2002). The contribution of the MEP pathway to the cytoplasmic IPP pool varies greatly between species, which in some green algae led to the complete loss of the MVA pathway (Lohr *et al.*, 2012).

Despite the complex regulation of cytoplasmic IPP and DMAPP levels, HMGR activity has been shown to be rate-limiting for sterol biosynthesis in plants (Shimada *et al.*, 2019). Also in humans, HMGR activity is rate-limiting for cholesterol biosynthesis making it a major therapeutic target for treating hypercholesterolaemia (Davies *et al.*, 2016). This medicinal application has led to the development of statins that compete with the endogenous substrate for the HMG-CoA-binding site in HMGR (Istvan and Deisenhofer, 2001). Given the deep evolutionary functional conservation of HMGR activity, statins, such as lovastatin and mevastatin, are also potent inhibitors of plant HMGRs. Consistently, the Arabidopsis *hmgr* mutants (*hmg1* and *hmg2*) are hypersensitive to lovastatin (Suzuki *et al.*, 2004). Moreover, lovastatin treatment had a fast, but transient, negative effect on sterol

content in Arabidopsis seedlings, suggesting compensatory IPP and DMAPP flux from the plastids into the cytosol (Laule *et al.*, 2003).

The subsequent assembly of IPP and DMAPP into FPP can be impaired by inducible gene silencing of both Arabidopsis FPSs resulting in a reduction of sterol levels, but also defects in plastidial terpene metabolism (Manzano *et al.*, 2016). The complete loss of FPS function was embryonic lethal (Closa *et al.*, 2010). While human FPS can be potently inhibited by nitrogen-containing bisphosphonates (Drake et al., 2008), these inhibitors are currently not commonly used in plants.

The subsequent condensation of FPP into squalene depends on a single functional SQS in Arabidopsis (SQS1; (Busquets *et al.*, 2008). This SQS can complement a *S. cerevisiae erg9* null mutant and displayed the expected biochemical activity *in vitro* (Busquets *et al.*, 2008; Kribii *et al.*, 1997). No mutants have been characterized to date. Squalestatins (also called zaragozic acids), are highly potent and specific competitive inhibitors of rat SQS, with apparent subnanomolar *Ki* values (Baxter *et al.*, 1992; Bergstrom *et al.*, 1993). Also in plants, squalestatins are highly potent inhibitors that inhibit SQS in BY-2 cell suspensions with an IC_{50} value of 5.5 nM, possibly via an irreversible inhibition mechanism (Hartmann *et al.*, 2000; Wentzinger *et al.*, 2002). Interestingly, exogenous application of squalestatins to Arabidopsis plants impairs the plants' fertility and induces transcriptional responses that are also induced in lovastatin-treated plants (Suzuki *et al.*, 2004). The transcriptional changes seen after squalestatin or lovastatin treatment were proposed to reflect a response to defective isoprenoid or non-brassinosteroidal steroid accumulations, via an unknown mechanism rather than an effect of reduced cytokinin biosynthesis (Suzuki *et al.*, 2004). One possible explanation is that sterol deficiency differentially regulates the activities of homeodomain containing transcription factors that contain a lipid/sterol binding StAR-related lipid transfer (START) domain (Schrick *et al.*, 2014; Schrick *et al.*, 2004). An interesting consequence of inhibiting SQS by squalestatin is the increased availability of FPP for sesquiterpene biosynthesis (Kobayashi *et al.*, 2017).

Committing to sterol biosynthesis – Squalene Epoxidase

The subsequent, oxygen-dependent epoxidation of squalene into 2,3-oxidosqualene by SQUALENE EPOXIDASEs (SQEs), is a key step in eukaryotic sterol biosynthesis (Thimmappa *et al.*, 2014). Of three functional SQEs in Arabidopsis that can rescue SQE-deficient yeast (Laranjeira *et al.*, 2015; Rasbery *et al.*, 2007), only SQE1 seems to play the most predominant function, as a single *sqe1* mutant already displays pleiotropic phenotypes in the root and shoot (Posé *et al.*, 2009; Rasbery *et al.*, 2007). However, these phenotypes

were not due to the reduced sterol content of the mutant, but because of hyperaccumulation of its substrate, squalene (Doblas *et al.*, 2013).

Docking analyses on modelled SQE suggest that the allylamine fungicide, terbinafine, causes a conformational change that blocks one mode of substrate binding, while changing the geometry of another (Nowosielski *et al.*, 2011). Although conventional plant SQEs can complement yeast SQE deficient mutants (Rasbery *et al.*, 2007), they are not highly sensitive to these inhibitors (Wentzinger *et al.*, 2002). This is not surprising as single amino acid substitutions in SQE are sufficient to confer terbinafine resistance to yeast mutants (Leber *et al.*, 2003). Yet, the *sqe1-5* mutant is hypersensitive to terbinafine (Posé *et al.*, 2009). On the other hand, some organisms, such as the diatom *P. tricornutum*, are completely insensitive to terbinafine (Fabris *et al.*, 2014; Pollier *et al.*, 2019). This can be easily explained by the presence of an alternative SQE, that belongs to the fatty acid hydroxylase superfamily, instead of to the common flavoprotein monooxygenase-type SQEs (Pollier *et al.*, 2019), thus requiring a specific pharmacology.

Committing to sterol biosynthesis – Oxidosqualene Cyclases

The biosynthetic step that forms the branch point between primary sterol biosynthesis and specialized triterpene metabolism is the cyclization of oxidosqualene catalyzed by specialized oxidosqualene cyclases (OSCs) (Fig. 2). These OSCs evolved from bacterial squalene/hopane synthases, and the Arabidopsis genome contains 13 of them. In contrast to animals and yeast that use lanosterol as the first cyclic intermediate in cholesterol and ergosterol biosynthesis, respectively, plants use cycloartenol as the main cyclic intermediate in phytosterol and cholesterol biosynthesis (Fig. 2). This 2,3-oxidosqualene cyclization product is produced by the enzyme cycloartenol synthase 1 (CAS1) in Arabidopsis (Gas-Pascual *et al.*, 2014; Thimmappa *et al.*, 2014). In addition to CAS, Arabidopsis also encodes lanosterol synthase (LAS), lupeol synthase (LUP1), and β-amyrin synthase (bAS), OSCs that show a widespread occurrence throughout the plant kingdom (Miettinen *et al.*, 2018; Thimmappa *et al.*, 2014) with a suggested role in phytosterol biosynthesis (LAS) or plant defense (LUP1, bAS). In addition to these “universal” plant OSCs, Arabidopsis also encodes a set of OSCs that are specific to *Brassicaceae* or even to Arabidopsis as a species. These OSCs include thalianol synthase (THAS), marneral synthase (MRN1) and arabidiol synthase (PEN1), OSCs with a suggested role in plant defense and modulation of the root microbiome (Huang *et al.*, 2019).

While strong *cas1* mutant alleles are male gametophytic lethal, hypomorphic *cas1-1* mutants displayed organ-specific albinism (Babiychuk *et al.*, 2008). Strikingly, although these hypomorphic mutants accumulate 2,3-oxidosqualene, the total phytosterol levels, albeit with

a reduction in β-sitosterol, remained unchanged in *cas1-1* mutants, suggesting the existence of alternative pathways contributing to phytosterol biosynthesis (Babiychuk *et al.*, 2008). With exception of a significant reduction in β-sitosterol content, similar results were obtained by overexpression of CYTOKININ-INDUCED F-BOX (CFB) that stimulates CAS1 turnover (Brenner *et al.*, 2017). Interestingly, *cas1-1* also had reduced cholesterol levels (Babiychuk *et al.*, 2008), consistent with the recent identification of cycloartenol as a precursor in cholesterol biosynthesis in plants (Fig. 3) (Sonawane *et al.*, 2016).

A possible alternative route for phytosterol biosynthesis is LAS-dependent (Fig. 2). The Arabidopsis genome encodes for one functional LAS gene that can complement a lanosterol synthase deficient yeast strain (Kolesnikova *et al.*, 2006; Sawai *et al.*, 2006; Suzuki *et al.*, 2006). However, the *las1* knock-out, besides a reduction in sitostanol, did not display gross changes in total endogenous sterol content (Suzuki *et al.*, 2006). Tracer feeding confirmed that the LAS pathway accounts for +/-1.5% of β-sitosterol biosynthesis in Arabidopsis, which could be increased to 4.5% by overexpression of LAS1 (Ohyama *et al.*, 2009). How lanosterol is converted into phytosterols remains currently unknown.

In humans, the benchmark OSC inhibitor is the competitive inhibitor RO 48-8071 (Thoma *et al.*, 2004). Similar to *cas1-1* mutants, this inhibitor causes the accumulation of 2,3-oxidosqualene in Arabidopsis seedlings and in plant cell suspensions (Gas-Pascual *et al.*, 2015). Only after prolonged treatments (6 days), it also caused reductions of β-sitosterol and stigmasterol, and an increase of campesterol, that was probably due to a transcriptional downregulation of SMT2 (see further) (Gas-Pascual *et al.*, 2015). The stronger effect on phytosterol content by the inhibitor treatment than in the *cas1* mutant might be explained by additional inhibition of other OSCs that contribute to phytosterol biosynthesis, such as LAS1. Recently, LaurylDiMethylAmine Oxide (LDAO) was characterized as potent CAS1 inhibitor in Arabidopsis, but also the more downstream CPI activity (Darnet *et al.*, 2020).

Diversification of the sterol end-products - side chain metabolism

Cycloartenol is a precursor for the biosynthesis of phytosterols as well as of cholesterol in plants, respectively by methylation at the C24-position by the C-24 sterol methyltransferase SMT1, or by Δ24 reduction via STEROL SIDE CHAIN REDUCTASE 2 (SSR2) (Fig. 3) (Sawai *et al.*, 2014; Sonawane *et al.*, 2016).

In Arabidopsis, SMT1 catalyzes the formation of Δ^5 C-24 alkyl sterols that are precursors for campesterol, β-sitosterol and stigmasterol. Its enzymatic activity was demonstrated via biochemical assays and via an *ERG6*–deficient yeast complementation assay (Diener *et al.*,

2000). At the level of the sterol content, *smt1* mutants showed a reduction in β-sitosterol, but not in campesterol content (Diener *et al.*, 2000; Schrick *et al.*, 2002; Willemsen *et al.*, 2003). The normal campesterol levels are surprising as campesterol should derive from the same SMT1-dependent pathway as β-sitosterol. One explanation could be that the diverged SMT2 and SMT3 can partially substitute for the lack of SMT1 at the expense of their normal, more downstream function in producing ethylsterols, such as β-sitosterol, resulting in a higher flux of precursors into campesterol biosynthesis.

Later in the pathway, from 24-methylenelophenol onwards, the pathway bifurcates via two separate branches, to form the 24-ethylsterols, β-sitosterol and stigmasterol, or the 24-methylsterol, campesterol, as end-products, respectively (Fig. 3,4). The bifurcation is due to the formation of an ethyl side-chain at the C-24 position, via a second type of SMTs (in Arabidopsis, SMT2/COTYLEDON VASCULAR PATTERN 1 (SMT2/CVP1) and SMT3) (Carland *et al.*, 2010; Schaeffer *et al.*, 2001). Therefore, the activity of these SMT enzymes controls the balance of ethylsterol and methylsterols. Consistently with the proposed position of SMT2/3 in the pathway, the *cvp1* single and *cvp1smt3* double mutants accumulate 24-methylenelophenol, and campesterol biosynthesis at the expense of stigmasterol and β-sitosterol. Intriguingly, the sterol profile of the *cvp1smt3* double mutant showed marked resemblances to that of *smt1*, such as increased cholesterol and 24-methylenecholesterol levels, suggesting partially overlapping biochemical functions at the first methylation step (Carland *et al.*, 2010).

In contrast to mammals that synthesize cholesterol via a lanosterol intermediate, plants mainly initiate cholesterol biosynthesis using cycloartenol as a precursor (Sawai *et al.*, 2014; Sonawane *et al.*, 2016). The responsible gene was identified in a homology blast using the human DHCR24 amino acid sequence as a query (Sawai *et al.*, 2014). While two homologs with different specificities were identified in potato and tomato, SSR1 and SSR2, Arabidopsis contains only SSR1, previously identified as DWF1, an enzyme that was characterized in a more downstream step of phytosterol biosynthesis. Gene-silencing of SSR2 in potato caused a reduction in cholesterol, and cholesterol-derived steroidal glycoalkaloids, and an increase in campesterol and β-sitosterol levels (Sawai *et al.*, 2014). Conversely, cholesterol became the most abundant sterol in the Arabidopsis *smt1* alleles (Diener *et al.*, 2000; Schrick *et al.*, 2002; Willemsen *et al.*, 2003), a phenomenon that is also observed in a bermudagrass mutant with reduced *SMT1* expression (Chen *et al.*, 2018). The increase in cholesterol levels is consistent with SMT1 competing with SSR2 for the precursor cycloartenol. Notably, Arabidopsis contains no known SSR2 enzyme that could explain the cholesterol accumulation in *smt1* mutants, suggesting that its SSR1 displays some SSR2 activity.

Several side-chain azasterols, carbocationic transition state analogues of the substrates of SMT, were previously identified as SMT inhibitors in yeast (Renard *et al.*, 2009). Feeding experiments allowed for the identification of several azasterols that selectively inhibit either SMT1 or SMT2/SMT3 activities (Darnet *et al.*, 2020). The SMT1-selective azasterols caused sterol profiles typically observed in *smt1* mutants, including accumulation of cholesterol and cycloartenol. The homology of SSR to human DHCR24 suggests that DHCR24 inhibitors could be used as templates to develop selective SSR1 and SSR2 inhibitors, similarly as was done for SMT1 and SMT2/3.

A double C4-demethylation via distinct sets of Sterol-4α-Methyl Oxidases

While in mammals and yeast a double demethylation at the C4 position is executed consecutively by a single multi-enzyme complex, plants have up to four distinct C4-demethylation complexes, acting at different positions in the pathway (Fig. 3) (Darnet and Schaller, 2019). The highly conserved C4-demethylation complex (C4-DMC) consists of sterol-4α-methyl oxidase (SMO), 4α-carboxysterol-C3-dehydrogenase/C4-decarboxylase (CSD) and sterone ketoreductase (SR), tethered together via ERG28 (Mialoundama *et al.*, 2013; Sonawane *et al.*, 2016).

Plant SMOs can largely complement the 4,4-dimethyl sterol accumulation in the *erg25* mutant yeast strains (Darnet *et al.*, 2001). The first family, SMO1, is specifically involved removal of a single methyl group from the C-4 position of the 4,4-dimethyl sterol, 24-methylenecycloartenol, generating the 4-methyl sterol, cycloeucalenol. The second family (SMO2) is specifically active in removing the second methyl at the C4-position, at more downstream steps of phytosterol biosynthesis (Fig. 4). The tomato cholesterol biosynthetic pathway involves another pair of SMOs (SMO3 and SMO4 that evolved from SMO1 and SMO2 respectively) at analogous positions in the cholesterol pathway (Fig. 4) (Sonawane *et al.*, 2016).

Using tissue cultures of *smo1-1smo1-2*/+ roots, and virus-induced gene silencing (VIGS) it was shown that *smo1* activity is required for biosynthesis of β-sitosterol, stigmasterol, campesterol and cholesterol, which was accompanied with accumulation of the SMO1 substrate 24-methylenecycloartenol and its precursor cycloartenol (Darnet and Rahier, 2004; Song *et al.*, 2019). Moreover, VIGS of SMO2 genes led to accumulation of 4α-methylsterols in *N. benthamiana* leaves (Darnet and Rahier, 2004), which could be confirmed by analyzing *smo2-1smo2-2*/+ and *smo2-1*/+ *smo2-2* (Zhang *et al.*, 2016). Jointly, these knock-down strategies provide *in planta* support for their biochemical function.

Plant CSDs have been shown to complement ergosterol biosynthesis in *erg26* yeast mutants (Rahier *et al.*, 2006), putatively acting in conjunction with the distinct plant SMO-types in phytosterol and cholesterol biosynthesis (Rahier *et al.*, 2006; Sonawane *et al.*, 2016). Additionally, silencing of a putative *SR* gene in tomato further supported a role of distinct C4-demethylation complexes involved in phytosterol and cholesterol biosynthesis (Sonawane *et al.*, 2016).

Importantly, despite their ability to complement the *erg25* mutant, plant SMOs display marked differences in substrate specificity, and are 500-fold less sensitive to the antifungal SMO inhibitor APB (6-Amino-2-*n*-pentylthiobenzothiazole) (Darnet and Rahier, 2003). This suggests that new and subtype-specific SMO inhibitors need to be developed to target these steps in phytosterol and cholesterol biosynthesis. Additionally, inhibitors of CSD and SR activity that were recently identified, remain to be evaluated for interference with C4-demethylation in plants (Darnet and Schaller, 2019).

Processing of C4-methylsterols– cycloeucalenol cycloisomerase (CPI1)

The opening of the cyclopropane ring of cycloeucalenol by CYCLOEUCALENOL CYCLOISOMERASE1 (CPI1) subsequently leads to the production of obtusifoliol (Fig. 4). Consistent with its biochemical function, the *cpi1* mutant is characterized by increased levels of cycloeucalenol and a strong decrease in β-sitosterol and stigmasterol (Men *et al.*, 2008). Additionally the mutant accumulates some abnormal sterols, such as 24-methylpollinastanol. Silencing of the single copy of CPI in tomato revealed an analogous biochemical function in cholesterol biosynthesis, as indicated by the accumulation of the 31-norcycloartenol substrate (Sonawane *et al.*, 2016).

Currently, no specific CPI inhibitors are available. CPI1 catalyzes similar reactions as C-14 sterol reductase and C-8,7 sterol isomerase, making them shared targets for molecular inhibition by morpholines such as fenpropimorph (Taton *et al.*, 1987). Moreover CPI was found to be a secondary target of the CAS1 inhibitor LDAO (Darnet *et al.*, 2020).

Processing of C4-methylsterols – obtusifoliol 14α-demethylase (CYP51G1)

Obtusifoliol then undergoes demethylation and reduction at its C-14 position, via an obtusifoliol 14α-demethylase to form 4α-Methyl-5α-ergosta-8,24(28)-trien-3β-ol (Fig. 4). This enzymatic activity is exerted by CYP51, a cytochrome P450-dependent monooxygenase that is conserved across phyla (Lepesheva and Waterman, 2007). Arabidopsis contains a single, functional sterol C14-demethylase, CYP51 (renamed CYP51G1; (Bak *et al.*, 2011)), that can

complement the *erg11* yeast mutant, deficient in lanosterol C14-demethylase (Kushiro *et al.*, 2001).

Consistent with its metabolic function in phytosterol biosynthesis, a *cyp51* mutant accumulated its substrate obtusifoliol, at the expense of downstream phytosterols, campesterol, β-sitosterol and stigmasterol (Kim *et al.*, 2005). CYP51 also catalyzes cholesterol biosynthesis at an analogous step in Arabidopsis, tomato and *N. benthamiana* (Fig. 4)(Kim *et al.*, 2005; Sonawane *et al.*, 2016).

CYP51 belongs to the cytochrome P450s, a large group of enzymes that are often sensitive to two types of azoles; the imidazoles, such as clotrimazole, oxiconazole, and ketoconazole and the triazoles, such as triadimenol, voriconazole and fluconazole. These are nitrogen-containing heterocyclic compounds that non-competitively bind to the ferric ion of the heme group of the cytochrome P450 thus preventing it from binding its substrate (Warrilow *et al.*, 2013). Despite being primarily used as fungicides, several azoles also cause growth inhibition in plants, which may be due to interference with downstream BR biosynthesis (Rozhon *et al.*, 2013). The fungicidal activity of azoles derives from a combination of depleting ergosterol, and accumulating the toxic obtusifoliol-metabolite 14α-methyl ergosta 8,24(28)-dien-3β-6α-diol (Martel *et al.*, 2010a; Martel *et al.*, 2010b). One of the potential problems of working with azoles is their promiscuity towards diverse CYPs, and thus their potential for off-target effects, especially at higher concentrations, which are often needed to obtain a significant inhibitory effect on phytosterol biosynthesis in plants and diatoms (Fabris *et al.*, 2014). Indeed, uniconazole does not only inhibit CYP707A, that is involved in abscisic acid catabolism, it can also inhibit CYP90B1/DWARF4, that is involved in brassinosteroid biosynthesis (Fujiyama *et al.*, 2019).

Processing of C4-methylsterols – sterol C14-reductase (FACKEL /FK) and C-Δ8,Δ7 sterol isomerase (HYDRA1 /HYD1)

In the subsequent steps a sterol C14 reductase generates 4α-methylfecosterol that is converted into 4α-methylenelophenol by a C-Δ8,Δ7 sterol isomerase (Fig. 4). Both enzymatic steps are encoded by single genes in Arabidopsis, *FACKEL/HYDRA2* (*FK/HYD2*) and *HYDRA1* (*HYD1*) respectively, and in tomato (Sonawane *et al.*, 2016). Both types of enzymes can complement the ergosterol biosynthesis in corresponding mutant yeast strains, *erg24* (Schrick *et al.*, 2000) and *erg2* (Grebenok *et al.*, 1998; Sonawane *et al.*, 2016) respectively. Moreover, the sterol profiles are markedly similar, showing defects that are more prominent in actively dividing mutant calli than in the corresponding mutant seedlings

(Schrick *et al.*, 2000; Schrick *et al.*, 2002; Souter *et al.*, 2002). Calli of *fk* and *hyd1* are mainly affected in their campesterol levels, and to a lesser extend in their β-sitosterol levels, while stigmasterol levels remaini largely unaffected and cholesterol levels even increase (Schrick *et al.*, 2000; Schrick *et al.*, 2002). The largely specific effect on campesterol is difficult to explain as the other phytosterols and cholesterol all depend on precursors generated via these single gene-encoded enzymatic activities (Sonawane *et al.*, 2016).

Together with CPI, FK/HYD2 and HYD1 are targeted by a largely overlapping pharmacology (Fig. 4). An important class of inhibitors that target these enzymes are the morpholine fungicides, such as fenpropimorph. These compounds inhibit C-8,7 sterol isomerases (nM concentrations) and/or C14 sterol reductases (µM concentration) in fungi and yeast (Mercer, 1993). In plants, morpholines inhibit HYD1, FK and CPI (He *et al.*, 2003; Rahier *et al.*, 1986; Taton *et al.*, 1987). While fenpropimorph is the most commonly used morpholine in plants, it requires relatively high concentrations to function (30-100 µM), it is unstable and relatively expensive. Plants treated with morpholines have a disturbed sterol profile and growth impairments, similar to those observed in mutants defective in the targeted enzymes (He *et al.*, 2003).

A strong, more specific inhibitor of C-14 sterol reductases is the antifungal agent 15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol (15-azasterol) (Fig. 4), which causes similar changes in the sterol profiles as observed in the *fk* mutant (Schrick *et al.*, 2002).

Final processing steps in phytosterol and cholesterol biosynthesis

After the second C4-demethylation (cfr. C4-demethylation), the subsequent production of campesterol and stigmasterol is effected by three enzymes acting in both branches of the pathway at corresponding steps: Δ^7-sterol-C5-desaturase (C5-SD1) (in Arabidopsis DWARF7/STEROL1 (DWF7/STE1), sterol Δ^7-reductase (7-DR1) (in Arabidopsis, DWARF5 (DWF5)) and Δ^{24}-sterol-Δ^{24}-reductase/SIDE CHAIN REDUCTASE1 (SSR1) (in Arabidopsis DIMINUTO/DWARF1 (DIM/DWF1) (Fig. 5). Cholesterol biosynthesis in tomato involves the diverged SMO4, C5-SD-2 and 7-DR-2 (Sonawane *et al.*, 2016) (Fig. 5).

The genes *DWARF7/STEROL1* (*DWF7/STE1*), *DWARF5* (*DWF5*) and *DIMINUTO/DWARF1* (*DIM/DWF1*) encode the C5-SD1, 7-DR1 and the SSR1 enzymes in Arabidopsis, and are required for the production of campesterol and β-sitosterol. The *dwf7/ste1* mutants accumulate Δ7-sterols, and are strongly defective in campesterol, β-sitosterol and stigmasterol (Silvestro *et al.*, 2013). The *dwf5* mutant also accumulates Δ7, Δ5,7 and Δ8-sterols and has strongly reduced levels of campesterol, β-sitosterol and stigmasterol (Silvestro *et al.*, 2013). In *dim/dwf1* mutants, campesterol, β-sitosterol and

stigmasterol were reduced, while the β-sitosterol and campesterol precursors isofucosterol, and 24-methylene cholesterol, respectively, were increased (Klahre *et al.*, 1998). Interestingly, besides acting as a sterol C24-reductase, DWF1 was found to also display brassinosteroid C24-reductase activity (Youn *et al.*, 2018). Currently, no inhibitors are available for this part of the phytosterol biosynthesis pathway.

In the ethylsterol branch, β-sitosterol undergoes C-22 desaturation by the C-22 sterol desaturase CYP710A1, resulting in the end-product of this pathway: stigmasterol (Morikawa *et al.*, 2006). However, not many details are known about this desaturation reaction in higher plants. Interestingly, in Arabidopsis, a second CYP710 enzyme (CYP710A2) is also able to produce stigmasterol from β-sitosterol, and can also produce brassicasterol from 24-epi-campesterol (Benveniste, 2002; Morikawa *et al.*, 2006). Given that CYP710 belongs to the cytochrome P450 family, it is likely that this enzyme is sensitive to azoles, as indicated in the CYP51 section.

Sterol conjugation and oxidation

Besides through biosynthesis, sterol levels can be regulated via conjugation, to form fatty acid acyl sterol esters (SE), steryl glucosides (SG) and acyl steryl glucosides (ASG). The SEs are major constituents of cytosolic lipid droplets (Shimada *et al.*, 2019) and are proposed to serve as storage forms that can be rapidly mobilized. SGs and ASGs contribute to the membrane organization eg. as important constituents of plasma membrane microdomains. For details about the biosynthesis and physiological function of SEs, SGs and ASGs, we refer the reader to some excellent recent reviews (Ferrer *et al.*, 2017; Korber *et al.*, 2017; Mamode Cassim *et al.*, 2019; Zhang *et al.*, 2020)

While the hydrolases and glycosidases remain elusive, several other key biosynthetic enzymes have been identified and characterized. In brief, SEs are formed by activities by two distinct types of acyltransferases: acylCoA:sterol acyltransferases (ASAT) and phospholipid:sterol acyltransferases (PSAT), using respectively long-chain acyl-CoA and unsaturated fatty acyl groups from phospholipids as a donors (Chen *et al.*, 2007). Although belonging to a family of six related proteins, Arabidopsis is thought to have only one PSAT (PSAT1) (Ferrer *et al.*, 2017). Similarly, only one member of the membrane-bound O-acyltransferases (MBOAT) was found to display ASAT activity (Chen *et al.*, 2007). Mutants in *psat1* have early leaf senescence but *asat1* mutants lack an obvious morphological phenotype (Bouvier-Nave *et al.*, 2010). At the level of SE formation, analysis of these mutants showed major contributions of PSAT1 over ASAT1.

Several inhibitors of human ASAT enzymes have been identified (Ohshiro *et al.*, 2020a; Ohshiro *et al.*, 2020b; Ohtawa *et al.*, 2018), but their activity and specificity in plants remains to be demonstrated. The recently resolved structure of human Sterol O-acyltransferase 1 revealed that the small molecule mode CI-976 blocks the accessibility of several active site residues (Guan *et al.*, 2020), and may serve as a template for docking analyses to assess the activity of such inhibitors on plant enzymes.

Two steryl glycosyl transferases (UGT80A2/B1), both related to the yeast steryl glycosl transferase UGT51A1, are jointly required for SE formation in Arabidopsis (Stucky *et al.*, 2015), by adding a sugar moiety, usually glucose, to the C3-hydroxyl group of the sterol. However, while steryl glycosyl acyltransferase activity could be detected, its molecular constituents remain to be identified. Mutants defective in the SGT enzymes, display moderate phenotypes, ranging from some seed coat and embryo phenotypes to a reduced root elongation (Stucky *et al.*, 2015). Recently, reduced root hair production in *ugt80b1* mutants was connected to mislocalisation of the root hair cell fate regulator SCRAMBLED (Pook *et al.*, 2017). The residual SG and ASG accumulation in these mutants suggests additional, redundant SGT enzyme activities remain to be identified (Stucky *et al.*, 2015). Also for UDP-glycosyl transferases, several inhibitors can be found (Lv *et al.*, 2019; Oda *et al.*, 2015), that were not yet used or evaluated to inhibit steryl glycosyl transferase activities in plants.

Conclusions

Deriving from an ancestral pathway, each of the three eukaryotic kingdoms gave sterol biosynthesis its own flavor with an end-result that is tailored to the specific needs inherent to the adopted life-style. In plants, animals and fungi, the sterol composition is a key determinant of the physico-chemical properties and spatial organization of membranes. Additionally, sterols have a serious impact on physiology and signaling processes as precursors of potent hormones, such as brassinolide (Vriet *et al.*, 2013). However, it is becoming more and more obvious that sterols are key components of plant development, in addition to their role in brassinolide biosynthesis (Boutté and Jaillais, 2020). Currently, most work has been done using Arabidopsis as a model. It is, however, easy to envision that much remains to be learnt about the roles of the multitude of diverse sterols that exists within the green lineage. The most straightforward way to achieve this is through interference with the biosynthetic pathway using inhibitors. Several enzymatic steps are sufficiently conserved to justify the use of inhibitors that were developed in yeast and mammals, while others are not. In combination with a tremendous diversification of the enzymes, and even novel

enzymes being recruited as seen in diatoms, it is clear that a more targeted pharmacology is needed to meet specific needs. On the other hand, the existence of plant-specific, or even species-specific enzymatic activities represents attractive opportunities for the development of highly selective herbicides.

Most of our inferences about the physiological and cell biological roles of phytosterols derive from studies in mutants that accumulate C4-dimethylated sterol biosynthesis intermediates. These mutants, such as *smt1* (Diener *et al.*, 2000; Schrick *et al.*, 2002; Willemsen *et al.*, 2003), *smo1-1smo1-2* (Song *et al.*, 2019), *cyp51* (Kim et al., 2005), *fk/hyd2* (Schrick *et al.*, 2000), *hyd1* (Topping *et al.*, 1997), *cpi1* (Men *et al.*, 2008), *smt2smt3* (Carland *et al.*, 2010; Short *et al.*, 2018; Souter *et al.*, 2002), and *smo2-1smo2-2* (Zhang *et al.*, 2016), display severe developmental defects, and are often associated with misregulated auxin and cytokinin homeostasis, and with defects in endocytosis and cellular polarization mechanisms. However, mutants defective in later steps of sterol biosynthesis and not accumulating C4-(di)-methylated sterol biosynthesis intermediates, *dwf7/ste1*, *dwf5* and *dim/dwf1* mainly display typical brassinosteroid deficiency phenotypes, that can be partially reversed by exogenous application of brassinolide (Vriet *et al.*, 2013).This discrepancy in phenotypes indicates that the observed, strong, phenotypes reflect a specific toxicity, or signaling function related to the accumulation of the sterol biosynthesis intermediates, rather than to defects in phytosterols. This calls for caution when inferring physiological roles of sterols in plants, as most available inhibitors result in accumulation of C4-(di)methylated sterol biosynthesis intermediates that have severe impact on biological processes in plants, animals and yeast (Darnet and Schaller, 2019).

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FIGURE LEGENDS

Fig. 1. Key steps in squalene biosynthesis with indication of inhibitor targets

In the cytoplasm, IPP and DMAPP mainly derive from MVA, which depends on HMGR activity. Additional contributions to the cytoplasmic IPP and DMAPP pools, are phosphorylation of IP and DMAP, and IPP and DMAPP that was synthesized in the plastids via the MEP pathway. Inhibitors are indicated in red. DMAP, dimethylallyl phosphate; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; FPPS, farnesyl pyrophosphate synthase; GA-3P, glyceraldehyde 3-phosphate; GGPP, geranylgeranyl pyrophosphate; GPP, geranyl pyrophosphate; GPPS, geranyl pyrophosphate synthase; HMG, 3-Hydroxy-3-methylglutaryl; HMGR, HMG-CoA reductase; HMGS, HMG-CoA synthase; IP, isopentenyl phosphate; IPP, isopentenyl pyrophosphate; MEP, methylerythritol phosphate; MVA, mevalonate

Fig. 2: Conversion of squalene into cycloartenol and lanosterol.

SQE, squalene epoxidase; alt. SQE, alternative SQE (found in diatoms); LAS, lanosterol synthase; CAS, cycloartenol synthase, THAS, thalianol synthase. Terbinafine inhibits SQE, but not alt. SQE. RO 48-8071 inhibits the 2,3-oxidosqualene cyclases LAS and CAS.

Fig. 3. Scheme of C4 and C24 metabolism in cycloartenol-derived phytosterol and cholesterol biosynthesis.

SMO1/2/3/4, sterol-4α-methyl oxidase 1/2/3/4; C4-DMC, C4-sterol demethylation complex; SMT1/2/3, C-24 sterol methyltransferase 1/2/3; SSR1/2; Sterol Side chain reductase 1/ 2. Blue circles indicate the target sites of SMTs and SSRs at distinct positions in the sterol biosynthesis pathway. Green circles indicate the target sites of C4-DMCs. C4-(di)methylated sterol intermediates are indicated in the dashed-line box.

Fig. 4. Parallel processing of C4-methyl sterols in phytosterol and cholesterol biosynthesis.

CPI, cycloeucalenol cycloisomerase; CVP1, cotyledon vascular pattern 1; CYP51G1, cytochrome P450 51G1; CYP710A1, cytochrome P450 710A1; DIM, DIMINUTO; DWF1/5/7, DWARF1/5/7; FK, FACKEL; HYD, HYDRA; SMO, sterol-4α-methyl oxidase. C14-R, C14 reductase, C14-DM, C14 demethylase; 8,7SI C-8,7 sterol isomerase (green-yellow background correspond to Fig.3)

Fig. 5: Schemes of the final enzymatic conversions to generate stigmasterol, campesterol and cholesterol.

DIM, DIMINUTO; DWF1/5/7, DWARF1/5/7; HYD, HYDRA; STE1, STEROL1; C5-SD1/ 2; 7-DR1/ 2; SSR1; C22-SD; CYP710A1. Left: ethylesterol branch, middle, methyl-sterol branch; Right, cholesterol branch. (light and dark green-yellow backgrounds correspond to Fig. 3)

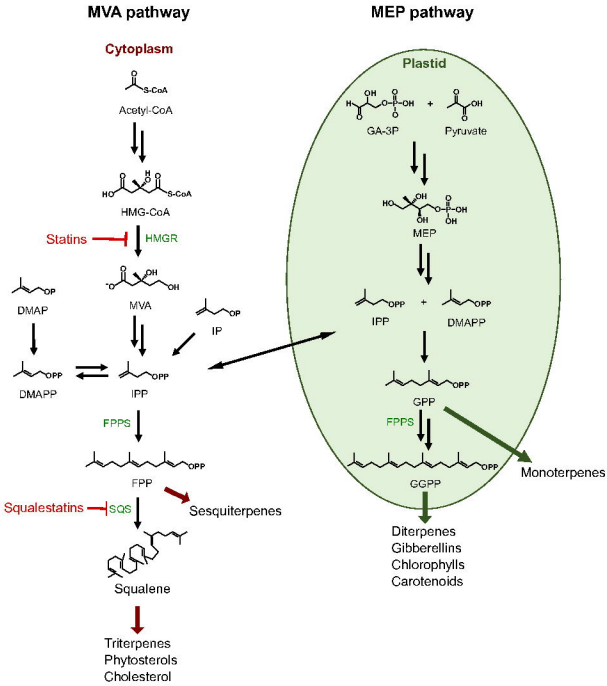


Fig. 1. Key steps in squalene biosynthesis with indication of inhibitor targets

Fig. 2: Conversion of squalene into cycloartenol and lanosterol

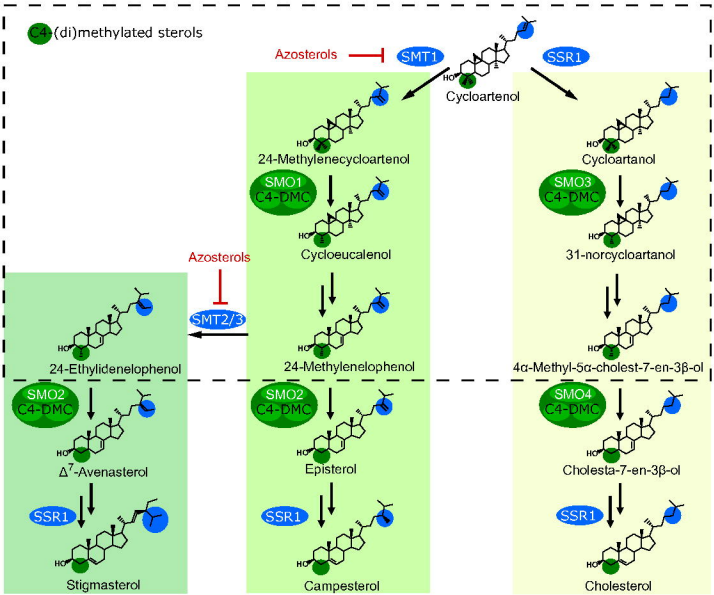
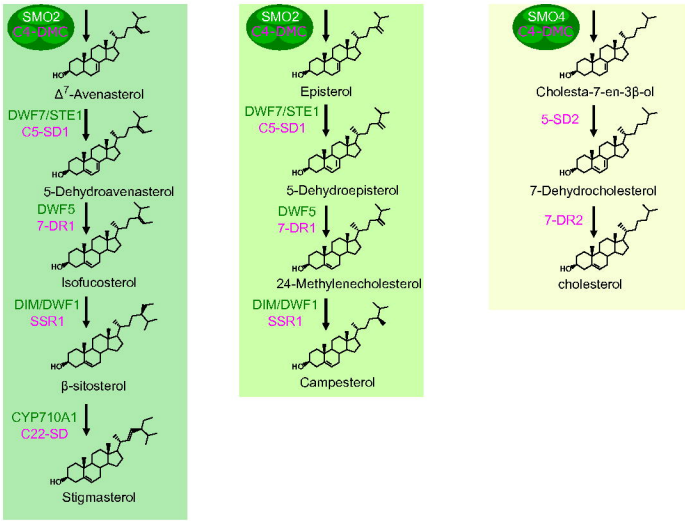


Fig. 3. Scheme of C4 and C24 metabolism in cycloartenol-derived phytosterol and cholesterol biosynthesis.

Fig. 4. Parallel processing of C4 -methyl sterols in phytosterol and cholesterol biosynthesis.



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