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1 Title page

2 Title: The use of mutants and inhibitors to study sterol
3 biosynthesis in plants

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5 Authors: Kjell De Vriese[1,2], Jacob Pollier[1,2,3], Alain Goossens[1,2], Tom Beeckman[1,2], Steffen
6 Vanneste[1,2,4,*]

7 Affiliations:

8 1: Department of Plant Biotechnology and Bioinformatics, Ghent University, Technologiepark 71, 9052 Ghent,
9 Belgium

10 2: VIB Center for Plant Systems Biology, VIB, Technologiepark 71, 9052 Ghent, Belgium

11 3: VIB Metabolomics Core, Technologiepark 71, 9052 Ghent, Belgium

12 4: Lab of Plant Growth Analysis, Ghent University Global Campus, Songdomunhwa-Ro, 119, Yeonsu-gu, Incheon
13 21985, Republic of Korea

14

15 e-mails:

16 K.D.V: kjvri@psb.vib-ugent.be

17 J.P: japol@psb.vib-ugent.be

18 A.G. algoo@psb.vib-ugent.be

19 T.B. tobee@psb.vib-ugent.be

20 S.V. stnes@psb.vib-ugent.be

21

22 *Corresponding author

23 Tel: +32 9 33 13844

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29

30 # The use of mutants and inhibitors to study sterol biosynthesis in
31 plants

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33 Kjell De Vriese, Jacob Pollier, Alain Goossens, Tom Beeckman, Steffen Vanneste

34

35 **ABSTRACT**

36 Sterols are very well known for their important roles in membranes and signaling in eukaryotes.
37 Plants stand out among eukaryotes by the large variety of sterols that they can produce, and
38 employing them across a wide spectrum of physiological processes. Therefore, it is critical to
39 understand the wiring of the biosynthetic pathways by which plants generate these distinct
40 sterols, to allow manipulating them and dissect their precise physiological roles. Many
41 enzymatic steps show a deep evolutionary conservation, while others are executed by
42 completely different enzymes. Here, we review the complexity and variation of the biosynthetic
43 routes of the most abundant phytosterols in the green lineage and how different enzymes in
44 these pathways are conserved and diverged from humans,yeast and even bacteria. Based on
45 their evolutionary conservation we discuss the use of human and yeast sterol biosynthesis
46 inhibitors in plants, as an argument for the development of plant-tailored inhibitors of sterol
47 biosynthesis.

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56 ## Introduction

57 Sterols are a class of triterpenoid lipids that consist of a hydrated phenanthrene group
58 and a cyclopentane ring that have been a topic of great interest for researchers for many
59 decades due to their essential physiological roles in eukaryotic organisms (Benveniste, 2004;
60 Hartmann, 1998).

61 For instance, the sterol composition in membranes has a crucial impact on membrane
62 fluidity and transmembrane export and import processes, and some sterols can even act as
63 second messengers or signaling molecules during developmental and cellular signaling
64 processes. The importance of sterols for eukaryotic organisms is even more apparent when
65 looking from an evolutionary point of view, since the occurrence of sterol biosynthesis is thought
66 to be a key evolutionary step in the advent of eukaryotic life (Galea and Brown, 2009).

67 Indeed, the ancient rise in atmospheric O_2 levels to the current 21% O_2 not only drove
68 the evolution of the earliest eukaryotic single-cell organisms, it also allowed for the occurrence
69 of sterol biosynthesis pathways, which require O_2 (Galea and Brown, 2009; Mouritsen, 2005).
70 This is contrasted by the occurrence of hopanoids in prokaryotes, which are ring-structured
71 molecules that look similar to sterols, but that do not require O_2 for their biosynthesis and lack a
72 3β-hydroxyl group, but exert analogous functions in the membranes as cholesterol (Berry *et al.*,
73 1993; Mangiarotti *et al.*, 2019; Saenz *et al.*, 2015). Intriguingly, the advent of sterol biosynthesis
74 may also have acted as an early defense mechanism protecting against oxidative damage in
75 these primitive eukaryotes, since sterols have been shown to function as a primitive cellular
76 defense against O_2 and reactive oxygen species (ROS) and are able to regulate cellular and
77 organellar O_2 entry (Galea and Brown, 2009). It is thus possible that primitive eukaryotes
78 evolved sterols as an adaptive response to the rising atmospheric O_2 levels, instead of just a
79 consequence of it like previously assumed. Notably, some bacteria also produce sterols,
80 presumably due to horizontal gene transfer (Bode *et al.*, 2003; Rivas-Marin *et al.*, 2019).

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81 While sterols occur in all eukaryotic organisms, the types and amounts of sterols varies
82 considerably between the different kingdoms. For instance, cholesterol is the major sterol
83 produced in animals, whereas fungi mainly produce ergosterol. Plants, on the other hand,
84 produce a wide variety of sterols (or phytosterols), with over 200 kinds known to date
85 (Benveniste, 2004; Guo *et al.*, 1995; Schaller, 2004). Within the phytosterols, campesterol,
86 stigmasterol and β-sitosterol make up the predominant molecules of the sterol profile in plants
87 (Benveniste, 2004; Hartmann, 1998): e.g. 64% campesterol, 6% stigmasterol and 11% β-
88 sitosterol in Arabidopsis (Benveniste, 2004; Schaeffer *et al.*, 2001). These three phytosterols
89 have either a methyl group (campesterol) or an ethyl group (β-sitosterol and stigmasterol) on
90 their C-24 position, and thus are also called 24-methylsterols and 24-ethylsterols, respectively
91 (Schaller *et al.*, 1998). The balance between 24-methylsterols and 24-ethylsterols differs
92 between plant species and is highly regulated, since their ratio has an important effect on
93 several cellular processes (Schaller, 2003). For instance, reproductive organs such as flowers
94 and seedpods are negatively affected by moderate changes in the campesterol/β-sitosterol
95 ratio, while more severe changes in the campesterol/β-sitosterol ratio have no significant effect
96 on stem elongation (Schaller, 2003).

97 The main function of phytosterols is the regulation of the fluidity and permeability of
98 membranes (Schaller, 2003). They achieve this by interacting with the saturated alkyl chains of
99 the phospho- and sphingolipids that make up the membrane bilayers, thus limiting their mobility
100 and permeability depending on the type and amount of sterols (Hartmann, 1998). While all of
101 the phytosterols are able to regulate membrane fluidity and permeability, their efficiency in doing
102 so varies (Hartmann, 1998; Schuler *et al.*, 1990; Schuler *et al.*, 1991). For instance, cholesterol
103 has the largest stabilizing effect on membranes, followed by campesterol, β-sitosterol, and
104 stigmasterol (Grunwald, 1971; Hodzic *et al.*, 2008). Therefore, changes in the membrane sterol
105 composition have an effect on the membrane permeability and function (Valitova *et al.*, 2010).

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106 While phytosterols are mainly present in the PM, small amounts of them have also been found
107 in membranes of the ER (Hartmann and Benveniste, 1987), mitochondria (Meance *et al.*, 1976),
108 vacuole (Yoshida and Uemura, 1986) and chloroplasts (Hartmann and Benveniste, 1987).
109 Another function in membranes to which phytosterols contribute is the formation of so-called
110 "lipid rafts". These lipid rafts are small, dynamic membrane domains rich in phytosterols and
111 sphingolipids, in which certain enzymes and signaling complexes are gathered (Laloi *et al.*,
112 2007; Malinsky *et al.*, 2013; Simon-Plas *et al.*, 2011; Simons and van Meer, 1988). Lipid rafts
113 have been successfully identified and isolated in several plant species and detailed analyses of
114 their composition confirmed the presence of the main phytosterols campesterol, β-sitosterol and
115 stigmasterol, as well as other sterols, sterol glycosides and sphingolipids (Cacas *et al.*, 2012;
116 Mongrand *et al.*, 2004; Simon-Plas *et al.*, 2011). Consequently, the phytosterol content of
117 membranes indirectly affects enzyme activity, signal transduction, ion transport, and protein-
118 protein and protein-lipid interactions that take place in and over these membranes
119 (Grandmougin-Ferjani *et al.*, 1997; Schaller, 2003).

120 This is evidenced by the wide range of severe phenotypes that were reported for
121 mutants defective in sterol biosynthesis. Phenotypes of such mutants include extreme dwarfism
122 and disturbances in embryogenesis, vascularization, fertility, cell differentiation and proliferation,
123 depending on the sterol biosynthesis step that is disturbed (Azpiroz *et al.*, 1998; Catterou *et al.*,
124 2001; Clouse, 2000; Guo *et al.*, 1995; He *et al.*, 2000; Piironen *et al.*, 2000; Schaller, 2003).
125 Currently, the origin of these sterol mutant phenotypes is poorly understood. Some can be
126 explained by defects in auxin transport (Men *et al.*, 2008; Pan *et al.*, 2009; Titapiwatanakun *et*
127 *al.*, 2009; Willemsen *et al.*, 2003; Yang *et al.*, 2013) or ethylene signaling (Souter *et al.*, 2002),
128 whereas others derive from defects in brassinosteroid signaling as campesterol serves as a
129 biosynthetic precursor of the brassinosteroid brassinolide. Furthermore, there are indications
130 that phytosterols can act as signaling/regulatory molecules during plant growth and

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131 development (Fujioka and Sakurai, 1997; Guo *et al.*, 1995; Lindsey *et al.*, 2003; Vriet *et al.*,
132 2013).

133 In conclusion, phytosterols not only are vital structural components of membranes, they
134 also play key roles during plant growth and development. Therefore, the large variety of plant
135 sterols allows plants to adapt to constantly changing environmental conditions.

136

137 Conservation and divergence in the early sterol biosynthesis pathway

138 The initial pathway from which all triterpenes (including phytosterols, lanosterol and
139 cholesterol) are derived is called the mevalonate (MVA) pathway, which is largely conserved
140 across eukaryotes and archaea (Buhaescu and Izzedine, 2007; Lombard and Moreira, 2011)
141 (Fig. 1). The end products of the MVA pathway are isopentenyl pyrophosphate (IPP) and
142 dimethylallyl pyrophosphate (DMAPP), which form the primary building blocks of all isoprenoids
143 (Goldstein and Brown, 1990).

144 The MVA pathway starts with the condensation of two acetyl-CoA molecules into
145 acetoacetyl-CoA by acetoacetyl-CoA thiolase. An additional condensation in the next step
146 catalyzed by HMG-CoA synthase (HMGS) results in the formation of 3-hydroxy-3-
147 methylglutaryl-CoA (HMG-CoA). Subsequent reduction of HMG-CoA by HMG-CoA reductase
148 (HMGR) leads to the production of mevalonate. In contrast to humans, plants often have
149 multiple HMGR isoforms in their genomes. For instance, the *Arabidopsis* genome contains two
150 HMGR genes that encode for three HMGR isoforms, of which HMG1 is most abundantly
151 expressed (Enjuto *et al.*, 1994; Enjuto *et al.*, 1995). Consistent with its biochemical role in the
152 mevalonate pathway, the pleiotropic *hmg1* phenotype can be rescued by exogenous application
153 of squalene (Suzuki *et al.*, 2004).

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154 In the last steps of the eukaryotic MVA pathway, MVA undergoes two phosphorylations
155 at its 5-OH position (catalyzed by mevalonate-5-kinase (MK) and phosphomevalonate kinase
156 (PMK)), followed by a decarboxylation (catalyzed by mevalonate 5-diphosphate decarboxylase
157 (MDD)), resulting in IPP. This IPP, together with its derivative DMAPP that is synthesized by
158 IPP isomerase (IDI), form the starting molecules of the pathways leading to the production of a
159 large variety of isoprenoids (Goldstein and Brown, 1990). Archaea use a modified MVA pathway
160 in comparison to eukaryotes, in which the last three enzymes have been replaced by other
161 enzymes (Boucher *et al.*, 2004).

162 Interestingly, unlike animals and fungi, plants also have the ability to produce IPP and DMAPP
163 via an alternative pathway: the methylerythritol phosphate (MEP) or non-mevalonate pathway
164 (Banerjee and Sharkey, 2014; Chappell, 2002), which takes place in the plastids and is mostly
165 used for the biosynthesis of various mono-, di- and tetraterpenoids (Laule *et al.*, 2003; Zhao *et*
166 *al.*, 2013) (Fig. 1). The MEP pathway is the main pathway for IPP and DMAPP biosynthesis in
167 bacteria, with some exceptions (Lombard and Moreira, 2011), and is obtained by plants during
168 the endosymbiosis event with cyanobacteria that originated the plastids (Lange et al., 2000).

169 On the other hand, the IPP and DMAPP produced by the cytosolic MVA pathway are mainly
170 used for the production of phytosterols, triterpenoids and sesquiterpenoids. Interestingly, many
171 green algae species do not possess the MVA pathway and are solely reliant on the MEP
172 pathway for isoprenoid biosynthesis (Lohr *et al.*, 2012). Notably, there are indications of
173 crosstalk between the cytosolic MVA and plastidial MEP pathways in plants (Mendoza-
174 Poudereux *et al.*, 2015; Tansey and Shechter, 2001). Furthermore, it was recently shown that
175 plants express a functional homolog of the isopentenyl phosphate kinase (IPK) that was
176 originally identified in archaebacteria as part of their modified MVA pathway (Dellas and Noel,
177 2010; Henry *et al.*, 2015). This enzyme catalyzes the phosphorylation of isopentenyl phosphate
178 (IP) and dimethylallyl phosphate (DMAP) into IPP and DMAPP, respectively, thus increasing

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179 their availability for terpenoid production (Henry *et al.*, 2015; Henry *et al.*, 2018). Interestingly,
180 IPP can be dephosphorylated back to IP by a subset of Nudix superfamily hydrolases (Henry et
181 al., 2018). Together, these findings illustrate the highly complex metabolic regulation of IPP and
182 DMAPP levels for terpenoid biosynthesis in plants.

183 Subsequently, in the cytosol, farnesyl pyrophosphate (FPP) is formed by two sequential
184 condensation reactions, in which two IPP molecules are added to DMAPP. These condensation
185 reactions are catalyzed by farnesyl pyrophosphate synthase (FPPS) (Kulkarni *et al.*, 2013). In
186 the plastidial MEP pathway, on the other hand, FPP is synthesized in two steps, in which IPP
187 and DMAPP are first converted to GPP by GPPS followed by the formation of FPP from GPP
188 and IPP by a plastidial FPPS (Manzano *et al.*, 2016). In the cytosol, FPP can either enter the
189 sesquiterpene biosynthesis pathway, or be further converted to squalene, a C-30 molecule
190 which is a condensation of two FPP units catalyzed by squalene synthase (SQS) (Tansey and
191 Shechter, 2001). Squalene is produced via this pathway in both pro- and eukaryotes, where it is
192 the universal precursor of hopanoids and steroids, respectively. In plants, animals and fungi,
193 squalene is further converted to 2,3-oxidosqualene by squalene epoxidase (SQE; see further)
194 (Thimmappa *et al.*, 2014).

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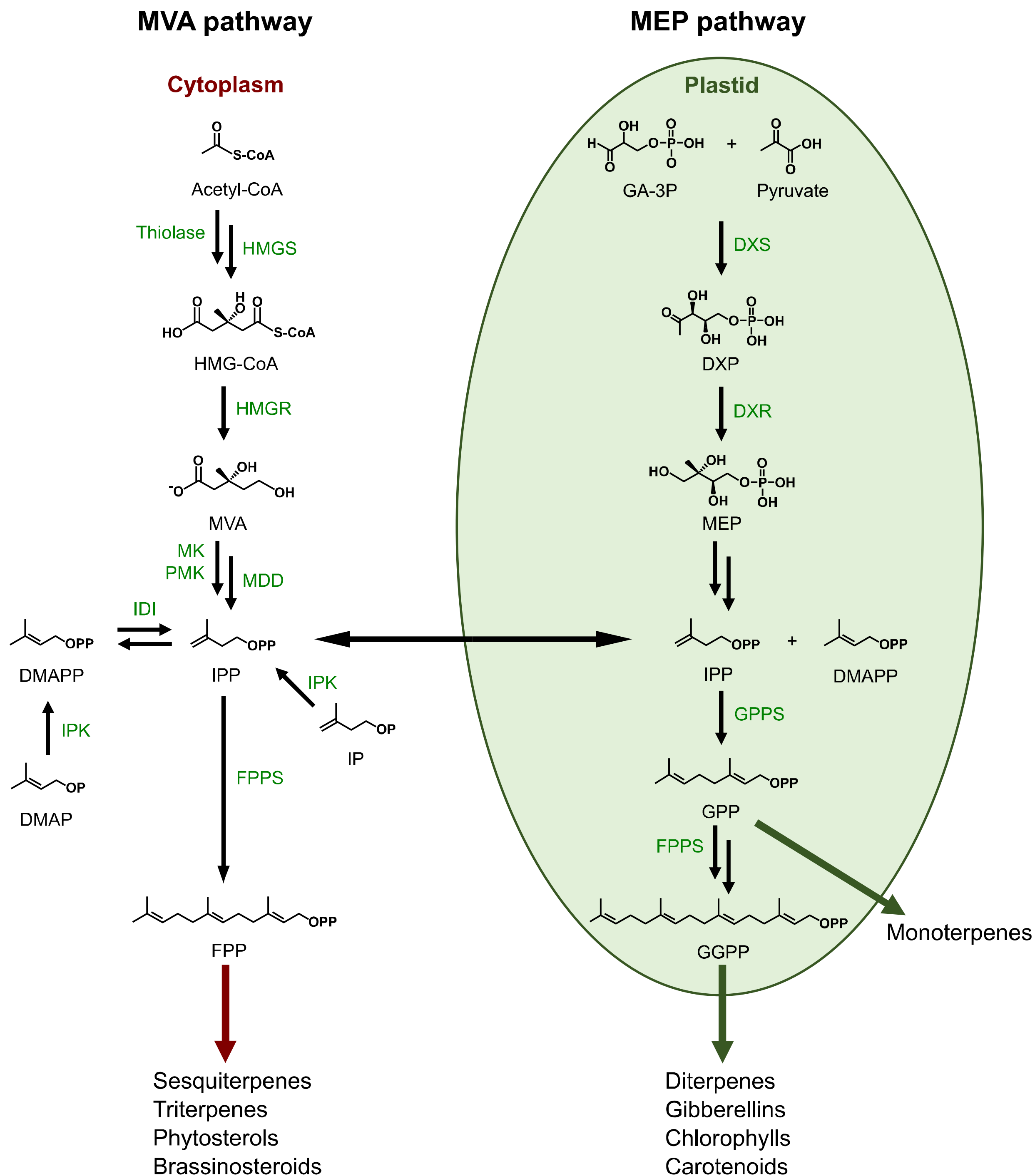

195
196 **Fig. 1. Schematic overview of the MVA and MEP pathways in *Arabidopsis thaliana*.** CoA, Coenzyme A; DMAP,
197 dimethylallyl phosphate; DMAPP, dimethylallyl pyrophosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, DXP
198 reductoisomerase; DXS, DXP synthase; FPP, farnesyl pyrophosphate; FPPS, farnesyl pyrophosphate synthase; GA-
199 3P, glyceraldehyde 3-phosphate; GGPP, geranylgeranyl pyrophosphate; GPP, geranyl pyrophosphate; GPPS,
200 geranyl pyrophosphate synthase; HMG, 3-Hydroxy-3-methylglutaryl; HMGR, HMG-CoA reductase; HMGS, HMG-
201 CoA synthase; IDI, IPP isomerase; IPK, isopentenyl phosphate kinase; IP, isopentenyl phosphate; IPP, isopentenyl
202 pyrophosphate; MDD, mevalonate 5-diphosphate decarboxylase; MEP, methylerythritol phosphate; MK, mevalonate-
203 5-kinase; MVA, mevalonate; PMK, phosphomevalonate kinase.

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204

205 Early Phytosterol biosynthesis – 2,3-oxidosqualene as a precursor for 206 phytosterols

207 As mentioned before, the biosynthesis of sterols in eukaryotes begins with the
208 epoxidation of squalene into 2,3-oxidosqualene by SQUALENE EPOXIDASEs (SQE)
209 (Thimmappa *et al.*, 2014). Of three functional SQEs in *Arabidopsis* that can rescue SQE
210 deficient yeast (Laranjeira *et al.*, 2015; Rasbery *et al.*, 2007), SQE1 seems to play the most
211 predominant function, as a single mutant displays pleiotropic phenotypes in the root and shoot
212 (Pose *et al.*, 2009; Rasbery *et al.*, 2007). However, these phenotypes were not due to the
213 reduced sterol content of the mutant, but rather due to its hyperaccumulation of squalene
214 (Doblas *et al.*, 2013). Moreover, the *sqe1* phenotypes could be explained by misregulation of
215 ROS production (Pose *et al.*, 2009), unlike later sterol biosynthetic mutants that display
216 misregulated ethylene production and auxin transport (See further) (Souter *et al.*, 2002; Souter
217 *et al.*, 2004). This observation gives further credibility to the hypothesis that sterol biosynthesis
218 may have evolved as an adaptation to oxidative stress (Galea and Brown, 2009). Furthermore,
219 these data provide evidence for a primordial role for a conserved oxidosqualene biosynthesis
220 pathway acting as the earliest section of the phytosterol biosynthesis pathway. However, the
221 absence of completely predictable reductions in the total phytosterol levels upon interference
222 with enzymes involved in oxidosqualene biosynthesis indicates an important gap in our
223 understanding of how early phytosterol biosynthesis is regulated. Indeed, recently, an
224 alternative SQE has been identified in the diatom *Phaeodactylum tricornutum*, that belongs to
225 the fatty acid hydroxylase superfamily instead of to the flavoprotein monooxygenases like the
226 conventional SQEs (Pollier *et al.*, 2019). This suggests that different enzymatic reactions in
227 plant phytosterol biosynthesis can be mediated by a wider palette of enzymes than would be
228 expected based on sequence homology to yeast and human sterol biosynthetic genes.

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229 Depending on the plant species, there are multiple cyclization pathways that convert 2,3-
230 oxidosqualene into different cyclic triterpene derivatives, based on the oxidosqualene cyclases
231 (OSCs) that are present. These OSCs evolved from bacterial squalene/hopane synthases, and
232 include cycloartenol synthase (CAS), lanosterol synthase (LAS), thalianol synthase (THAS) and
233 β-amyrin synthase (bAS) (Sawai *et al.*, 2006; Thimmappa *et al.*, 2014). The most prominent of
234 these pathways starts with the cyclization of 2,3-oxidosqualene into cycloartenol, which is
235 catalyzed by the enzyme cycloartenol synthase 1 (CAS1) in *Arabidopsis* (Gas-Pascual *et al.*,
236 2014; Rees *et al.*, 1969; Thimmappa *et al.*, 2014). This pathway mainly produces the three
237 major phytosterols as end-products, namely campesterol, β-sitosterol and stigmasterol, via a
238 complex series of enzyme-catalyzed conversions. Interestingly, the bacterium *Stigmatella*
239 *aurantiaca* also produces cycloartenol via a CAS enzyme that is similar to that of plants (Bode
240 *et al.*, 2003), and a squalene monooxygenase and an OSC were found to be essential for
241 lanosterol biosynthesis in the bacterium *Gemmata obscuriglobus* (Rivas-Marin et al., 2019).

242 Cycloartenol is first converted to 24-methylenecycloartenol by the addition of a methyl-
243 group at the C-24 position by C-24 sterol methyltransferase 1 (SMT1), which is a key regulatory
244 step of phytosterol biosynthesis (Neelakandan *et al.*, 2009; Shi *et al.*, 1996). In the next step,
245 removal of a methyl group from the C-4 position of 24-methylenecycloartenol leads to in
246 cycloeucalenol. In Arabidopsis, this step is catalyzed by three members of the sterol-4α-methyl
247 oxidase 1 (SMO1) enzyme family (Darnet and Rahier, 2004). The opening of the cyclopropane
248 ring of cycloeucalenol by cycloeucalenol cycloisomerase (CPI1) subsequently leads to the
249 production of obtusifoliol (Benveniste, 2002). Obtusifoliol then undergoes demethylation of its C-
250 14 position, which results in the formation of 4α-methyl-5α-ergosta-8,14,24(28)-trien-3β-ol
251 (Rahier and Taton, 1986). This reaction is catalyzed by obtusifoliol 14α-demethylase (CYP51G1
252 in Arabidopsis), a cytochrome P450 enzyme. Next, 4α-methyl-5α-ergosta-8,14,24(28)-trien-3β-
253 ol is converted to 4α-methylfecosterol by the sterol C-14 reductase FACKEL (FK). In the

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254 following step, the C-7 double bond of 4α-methylfecosterol undergoes a reduction catalyzed by
255 the C-8,7 sterol isomerase HYDRA1 (HYD1) (Souter *et al.*, 2002), which leads to the formation
256 of 24-methylenelophenol.

257 This part of the phytosterol pathway in Arabidopsis is encoded by single genes and the
258 corresponding mutants often display strong phenotypes, including *cpi, smt1*, *cyp51*, *hyd1* and
259 *fk*. The *cpi* mutant is characterized by increased levels of cycloeucalenol and its derivatives, and
260 has severe defects in its growth and development (Men *et al.*, 2008). The *smt1, hyd1* and *fk*
261 mutants have reduced phytosterol and BR levels, and are severely impaired in embryogenesis,
262 cell polarity, root growth, gravitropism and vascular development (Diener *et al.*, 2000; Schrick *et*
263 *al.*, 2000; Souter *et al.*, 2002; Topping *et al.*, 1997; Willemsen *et al.*, 2003). The abnormal
264 vascular development phenotype of the *hyd1* and *fk* mutants could be partially rescued by
265 crossing these mutants with auxin-resistant mutants, indicating that the *hyd1* and *fk* mutants
266 may have disturbed auxin signaling or transport (Souter *et al.*, 2002). Similarly, the abnormal
267 root phenotype in *hyd1* and *fk* could be rescued by crossing these mutants with a dominant
268 ethylene-resistant mutant, suggesting they also have disturbed ethylene signaling (Souter *et al.*,
269 2002). Recently, tissue-specific complementation of the *hyd1* mutant suggests that many of its
270 phenotypes can be explained by defective patterning of PIN auxin transporters, and associated
271 defects in auxin transport (Diener *et al.*, 2000). Interestingly, the *smt1* mutant is hypersensitive
272 to Ca^{2+} ions, since lowering the Ca^{2+} concentration in the growth medium of this mutant resulted
273 in improved root growth, probably due to alterations in membrane permeability (Diener *et al.*,
274 2000).

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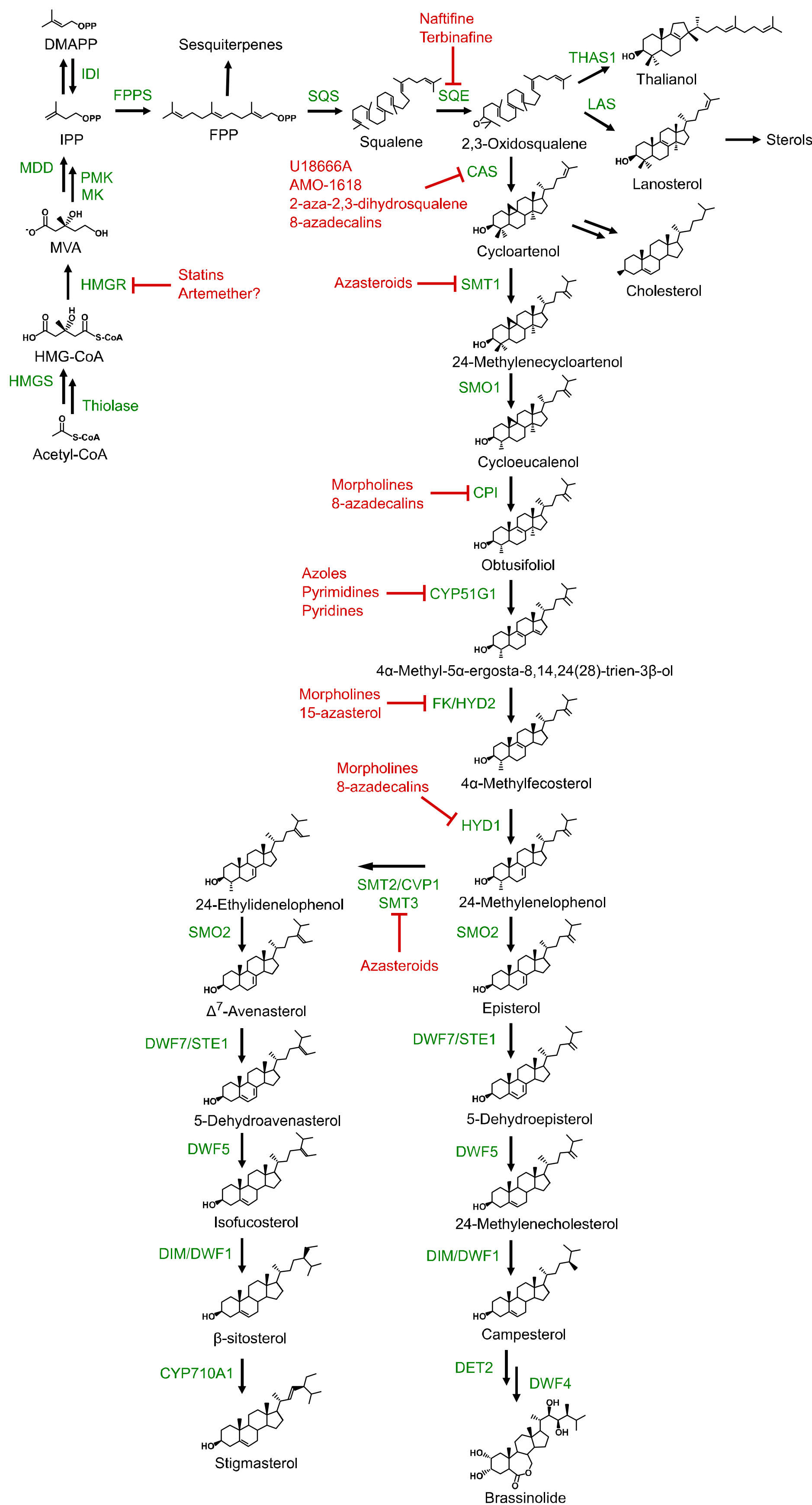

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278 **Fig. 2. Schematic overview of the main sterol biosynthesis pathway in *Arabidopsis thaliana* and putative**
279 **target sites of inhibitors.** CAS, cycloartenol synthase; CoA, Coenzyme A; CPI, cycloeucalenol cycloisomerase;
280 CVP1, cotyledon vascular pattern 1; CYP51G1, cytochrome P450 51G1; CYP710A1, cytochrome P450 710A1;
281 DET2, DEETIOLATED2; DIM, DIMINUTO; DMAPP, dimethylallyl pyrophosphate; DWF1/5/7, DWARF1/5/7; FK,
282 FACKEL; FPP, farnesyl pyrophosphate; FPPS, farnesyl pyrophosphate synthase; HMG, 3-Hydroxy-3-methylglutaryl;
283 HMGR, HMG-CoA reductase; HMGS, HMG-CoA synthase; HYD, HYDRA; IDI, IPP isomerase; IPP, isopentenyl
284 pyrophosphate; LAS, lanosterol synthase; MDD, mevalonate 5-diphosphate decarboxylase; MK, mevalonate-5-
285 kinase; MVA, mevalonate; PMK, phosphomevalonate kinase; SMO, sterol-4α-methyl oxidase; SMT1/2/3, C-24 sterol
286 methyltransferase 1/2/3; SQE, squalene epoxidase; SQS, squalene synthase; STE1, STEROL1; THAS1, thalianol
287 synthase.
288

289 Phytosterol biosynthesis – parallel branches for stigmasterol and campesterol

290 From 24-methylenelophenol onwards, the pathway bifurcates via two separate
291 branches, eventually resulting in either 24-ethylsterols (β-sitosterol and stigmasterol) or 24-
292 methylsterols (campesterol) as end-products, respectively. Campesterol can subsequently be
293 used as a precursor for brassinosteroid biosynthesis.

294 The 24-ethylsterol branch pathway begins with a second methylation of the C-24
295 position of 24-methylenelophenol by the enzymes C-24 sterol methyltransferase 2/cotyledon
296 vascular pattern 1 (SMT2/CVP1) and C-24 sterol methyltransferase 3 (SMT3), which results in
297 24-ethylidenelophenol (Bouvier-Nave *et al.*, 1998; Carland *et al.*, 2010). Like with SMT1, the
298 reaction catalyzed by SMT2/CVP1 is an important regulatory step in sterol biosynthesis, since it
299 determines the ratio of 24-methyl- and 24-ethylsterols, which affects several developmental
300 processes in plants (Bouvier-Nave *et al.*, 1997; Carland *et al.*, 2002; Schaeffer *et al.*, 2001).
301 Interestingly, it is thought that SMT2/CVP1 is also able to catalyze the primary C-24 methylation
302 catalyzed by SMT1, albeit to a lesser extend (Schaeffer *et al.*, 2001). A following double
303 demethylation of 24-ethylidenelophenol by sterol-4α-methyl oxidase 2 (SMO2) results in the
304 formation of Δ^7-avenasterol (Darnet and Rahier, 2004), which is subsequently converted to 5-
305 dehydroavenasterol by the Δ^7-sterol-C5-desaturase DWARF7/STEROL1 (DWF7/STE1) (Choe
306 *et al.*, 1999b; Gachotte *et al.*, 1996). Next, the sterol Δ^7-reductase DWARF5 (DWF5) converts

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307 this 5-dehydroavenasterol into isofucosterol (Choe *et al.*, 2000). Finally, a C-24 reduction of
308 isofucosterol by the Δ^{24}-sterol-Δ^{24}-reductase DIMINUTO/DWARF1 (DIM/DWF1) leads to the
309 generation of β-sitosterol (Choe *et al.*, 1999a), which can then undergo a further C-22
310 desaturation by the C-22 sterol desaturase CYP710A1, resulting in the end-product of this
311 pathway: stigmasterol (Morikawa *et al.*, 2006). However, not many details are known about this
312 desaturation reaction in higher plants. Interestingly, in Arabidopsis, a second CYP710 enzyme
313 (CYP710A2) is also able to produce stigmasterol from β-sitosterol, and can also produce
314 brassicasterol from 24-epi-campesterol (Benveniste, 2002; Morikawa *et al.*, 2006).

315 The 24-methylsterol branch pathway starting from 24-methylenelophenol that eventually
316 leads to the production of campesterol is similar to the first one and mostly uses the same
317 enzymes. However, instead of first being methylated at the C-24 position by SMT2/CVP1 and
318 SMT3 during the first step of this branched pathway, 24-methylenelophenol is directly
319 demethylated by SMO2. This causes 24-methylenelophenol to be converted to episterol (Darnet
320 and Rahier, 2004). The rest of the pathway consists of the same steps as the first branched
321 pathway. First STE1 causes a desaturation of the C-5 position of episterol, which results in 5-
322 dehydroepisterol. This is followed by a reduction of its C-7 position by DWF5, leading to 24-
323 methylenecholesterol (Choe *et al.*, 2000). Finally, a reduction of the C-24 double bond of 24-
324 methylenecholesterol by DIM/DWF1 yields the end-product of this pathway: campesterol.
325 Besides its function as a structural phytosterol in membranes, campesterol also acts as a
326 precursor for the brassinosteroid biosynthesis pathway (Choe *et al.*, 1999a; Clouse, 2011). For
327 details about brassinosteroid biosynthesis, we refer to dedicated reviews (Choe *et al.*, 1999a;
328 Clouse, 2011).

329 The *smt2*/*cvp1* mutant has increased campesterol levels and reduced β-sitosterol levels,
330 and is characterized by moderate developmental defects, such as disturbed venation patterns in
331 its cotyledons, serrated floral organs and a reduced stature (Carland *et al.*, 2010; Carland *et al.*,

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332 2002). Unlike the early sterol biosynthesis mutants *smt1*, *hyd1* and *fk*, more downstream sterol
333 biosynthesis mutants such as *smt2/cvp1*, *dim/dwf1*, *dwf5* and *dwf7/ste1* show no defects in
334 embryogenesis. The *smt2/cvp1* mutant is smaller than the wild type, but it doesn't demonstrate
335 extreme dwarfism (Carland *et al.*, 2002). Although *dim/dwf1*, *dwf5* and *dwf7/ste1* affect
336 successive steps in the conversion of episterol to campesterol, and Δ^7-avenasterol to β-
337 sitosterol (Choe *et al.*, 1999a; Choe *et al.*, 1999b; Clouse, 2002), the phenotypes of these
338 mutants resemble those of brassinosteroid-deficient mutants, reflecting the importance of
339 campesterol as a precursor of the most biologically active brassinosteroid, brassinolide.
340 However, while these mutants are significantly smaller than wild-type plants, they don't display
341 the extreme dwarfism that is typical of BR biosynthesis mutants. Furthermore, the sterol profile
342 of these mutants is vastly disturbed, with *dwf7/ste1* being almost completely devoid of
343 campesterol (Choe *et al.*, 1999b; Choe *et al.*, 2000). These macroscopic phenotypes can be
344 partially rescued by external application of BRs (Choe *et al.*, 1999a; Choe *et al.*, 1999b; Choe *et*
345 *al.*, 2000; Klahre *et al.*, 1998; Schaller, 2003), demonstrating that they are largely caused by an
346 impairment in downstream BR synthesis, rather than a direct effect of campesterol deficiency.
347 However, since DIM/DWF1, DWF5 and DWF7/STE1 also catalyze the conversion steps of Δ^7-
348 avenasterol to β-sitosterol (Fig. 2), their respective mutants are not only deficient in
349 campesterol, but also in β-sitosterol and stigmasterol, suggesting that the resulting defects in
350 membrane integrity are at least partially responsible for the observed phenotypes of these
351 mutants. This is presumably the case for the observed fertility defects, since BR application
352 does not restore fertility in these mutants, suggesting that phytosterols play an important role
353 during the plant reproduction that is independent from BRs (Schaller, 2004).

354 Furthermore, unlike *dim/dwf1*, *dwf5* and *dwf7/ste1*, the phenotypes of *smt2/cvp1* and the
355 early sterol biosynthesis mutants *smt1*, *cpi*, *fk* and *hyd1* cannot be rescued by BR treatment
356 (Carland *et al.*, 2002; Diener *et al.*, 2000; Schrick *et al.*, 2000). Since these phenotypes are

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357 independent from the downstream BR pathway, it is possible that early synthesized sterols
358 (sterol biosynthesis intermediates) can act as signaling molecules themselves, similar to what
359 has been shown for cholesterol in animals (Edwards and Ericsson, 1999; Farese and Herz,
360 1998; Vriet *et al.*, 2013). For example, accumulation of the sterol biosynthesis intermediate 4-
361 carboxy-4-methyl-24-methylenecycloartanol (CMMC), which accumulated in a mutant defective
362 in tethering the sterol C4-demethylation complex, interferes with auxin transport (Edwards and
363 Ericsson, 1999; Farese and Herz, 1998; Vriet *et al.*, 2013). Also of note is that the sterol
364 biosynthesis pathways are relatively conserved between Eukaryotes, with diatoms and yeast
365 using mostly similar or identical enzymes as the higher plants, albeit sometimes in a different
366 order, which explains the difference in end products obtained (e.g. ergosterol in yeast and
367 brassicasterol/campesterol in diatoms) (Fabris *et al.*, 2014). Overall, these mutants of early and
368 late steps of the sterol biosynthesis pathway have been excellent tools in aiding our
369 understanding of plant sterol biosynthesis and the role of sterols in plant growth and
370 development.

371

372 **Cholesterol biosynthesis in plants**

373 The major sterols in plants are β-sitosterol, campesterol and stigmasterol, but many
374 plants also produce cholesterol to some degree (Behrman and Gopalan, 2005). While the
375 cholesterol levels in plants are usually low (100 - 1000 times lower compared to animals),
376 cholesterol makes up a significant portion of the sterol content in some plant species (e.g. more
377 than 10% in *Solanaceae*) (Sonawane *et al.*, 2016). Furthermore, it has been shown to serve
378 several functions in various plant species, including as membrane component, leaf surface lipid,
379 and precursor for several plant metabolites such as steroidal glycoalkaloids (SGAs) and
380 phytoecdysteroids (Cardenas *et al.*, 2015; Dinan, 2001; Japelt and Jakobsen, 2013; Milner *et*
381 *al.*, 2011).

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382 Cholesterol is the major sterol in animals, in which the cholesterol biosynthesis pathway
383 has been extensively studied and characterized (Nes, 2011), while cholesterol biosynthesis in
384 plants is still not fully understood. Recently, several genes and enzymes involved in cholesterol
385 biosynthesis in tomato plants were identified by analyzing transcript and protein co-expression
386 data, as well as a combination of functional assays (Sonawane *et al.*, 2016). These data
387 demonstrated the involvement of 12 enzymes in the tomato cholesterol biosynthesis pathway, of
388 which several also function in the phytosterol biosynthesis pathway to catalyze highly related
389 enzymatic conversions. Furthermore, the other enzymes that are specific for the cholesterol
390 biosynthesis pathway seem to have evolved through gene duplication and divergence from
391 phytosterol biosynthetic enzymes (Sonawane *et al.*, 2016). Unlike animals, cholesterol
392 biosynthesis in plants does not seem to start from 2,3-oxidosqualene cyclization into lanosterol
393 by LAS (Sonawane *et al.*, 2016). Instead, the OSC involved is CAS, after which cycloartenol is
394 not only used for phytosterol biosynthesis, but also cholesterol biosynthesis (Fig. 2). Indeed, in
395 tomato and potato plants it was shown that sterol side chain reductase 2 (SSR2) is a key
396 enzyme in cholesterol biosynthesis that catalyzes the conversion of cycloartenol into
397 cycloartanol, the first committed step in cholesterol biosynthesis (Sonawane *et al.*, 2016).
398 However, while LAS probably doesn't contribute significantly to cholesterol biosynthesis, LAS
399 genes were identified in several plant species, including Arabidopsis (Kolesnikova *et al.*, 2006;
400 Sawai *et al.*, 2006; Suzuki *et al.*, 2006). Furthermore, it was shown that LAS1 overexpression in
401 Arabidopsis significantly increases the phytosterol levels while *las1* knockout mutants do not
402 have phytosterols derived from lanosterol, indicating that there exists an alternative phytosterol
403 biosynthesis pathway that is dependent on LAS (Ohyama *et al.*, 2009). The existence of
404 alternative pathways contributing to phytosterol biosynthesis could explain why phytosterol
405 levels in *cas1* mutants remain unchanged, despite a strong defect in cycloartenol synthase
406 activity as indicated by the accumulation of 2,3-oxidosqualene (Babiychuk *et al.*, 2008).

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407

408 Chemical inhibitors of key steps in the plant sterol biosynthesis pathway

409 Besides mutants, another way in which sterol biosynthesis can be disrupted is through
410 the action of chemical inhibitors that target specific steps of the sterol biosynthesis pathway
411 (Fig. 2, Fig. 3). Indeed, sterol biosynthesis inhibitors have proven to be effective tools to probe
412 and investigate sterol biosynthesis pathways across the different kingdoms. Many of the
413 currently used sterol biosynthesis inhibitors have seen commercial use as fungicides and
414 antimycotic drugs, and some can even be used to regulate plant growth (Lenton, 1987; Leroux
415 *et al.*, 2008). Since the sterol biosynthesis pathways of plants, animals and yeast share many
416 similar conversion steps that are catalyzed by semi-conserved enzymes, several of the most
417 used sterol biosynthesis inhibitors function across kingdoms (Ator *et al.*, 1992). Nevertheless,
418 there still exist clear differences in the sterol biosynthesis pathways between the kingdoms,
419 leading to different sensitivities and specificities of sterol biosynthesis inhibitors (Nes, 2011).
420 The following paragraphs will go into more detail about some of the most active and most used
421 sterol biosynthesis inhibitors in plants, and their presumed targets. The compounds discussed
422 and their presumed targets in Arabidopsis are indicated in Fig. 2. The numbers in brackets
423 behind the discussed compounds correlate to their numbers in Fig. 3.

424 Statins potently inhibit human HMGR activity by occupying the HMG-CoA binding site
425 (Istvan and Deisenhofer, 2001). Because HMGR is a rate-limiting enzyme in MVA biosynthesis,
426 statin-based medication is widely used to lower cholesterol levels (reviewed in (Davies *et al.*,
427 2016). In several plant species, statins, such as lovastatin **(1)** (or mevilonin) and mevastatin **(2)**
428 (or compactin), reduce root growth and sterol biosynthesis (Bach and Lichtenthaler, 1982, 1987;
429 Kim *et al.*, 2014; Soto *et al.*, 2011), demonstrating that statins can also be used as HMGR
430 inhibitors in plant sterol research.

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431 Two allylamine fungicides, namely naftifine **(3)** and terbinafine **(4)**, are potent non-
432 competitive SQE inhibitors in fungi (Birnbaum, 1990; Ryder, 1991; Nowosielski et al., 2011).
433 Docking analyses on modelled SQE suggest that terbinafine binding causes a conformational
434 change that blocks one mode of substrate binding, while changing the geometry of another.
435 (Nowosielski *et al.*, 2011). Although plant SQEs can complement yeast SQE deficient mutants
436 (Rasbery et al., 2007), they are not highly sensitive to these inhibitors (Yates et al., 1991, 1992;
437 Wentzinger et al., 2002). This is not surprising as single amino acid substitutions in yeast SQE
438 are sufficient to confer terbinafine resistance (Leber *et al.*, 2003). Yet, the *sqe1-5* mutant is
439 hypersensitive to terbinafine (Pose et al., 2009). On the other hand, some organisms such as
440 the diatom *P. tricornutum* are completely insensitive to terbinafine as they use alternative SQEs
441 (Fabris et al., 2014; Pollier et al., 2019).

442 Squalestatins (also called zaragozic acids), are highly potent and specific competitive
443 inhibitors of rat SQS, with apparent subnanomolar *Ki* values (Baxter et al., 1992; Bergstrom et
444 al., 1993). Also in plants, squalestatins are highly potent, as they inhibit SQS in BY-2 cell
445 suspensions with an IC_{50} value of 5.5 nM, possibly via an irreversible inhibition mechanism
446 (Hartmann et al., 2000; Wentzinger et al., 2002). Exogenous application of squalestatin
447 activates transcriptional responses also seen in lovastatin-treated plants and impairs the plants
448 fertility (Suzuki et al., 2004). The Arabidopsis genome encodes only a single functional SQS
449 (SQS1; Busquets et al., 2008), but has not yet been subjected to mutant analysis.

450 Over the years, several compounds have been identified that inhibit OSCs to varying
451 degrees by mimicking the carbocationic intermediates formed during the cyclization of 2,3-
452 oxidosqualene. Some examples of OSC inhibitors that have been successfully utilized in plants
453 are 2-aza-2,3-dihydrosqualene **(5)** (Duriatti et al., 1985; Cattel et al., 1986), U18666A **(6)**
454 (Duriatti et al., 1985; Cattel et al., 1986) and AMO-1618 **(7)** (Douglas and Paleg, 1978, 1978,
455 1981). Another class of OSC inhibitors are the 8-azadecalins, such as 4,4,10β-trimethyl-trans-

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456 decal-3β-ol (TMD) **(8)** and its derivatives (Ruhl et al., 1989; Raveendranath et al., 1990;
457 Hoshino et al., 1995). However, the 8-azadecalins also inhibit other enzymes besides OSCs
458 (such as cyclopropyl sterol isomerase, C-14 sterol reductase and C-8,7 sterol isomerase), thus
459 potentially leading to off-target effects.

460 In Arabidopsis, the C-24 sterol methyltransferase SMT1 catalyzes the transfer of a
461 methyl group from S-adenosyl-L-methionine to cycloartenol (Benveniste, 1986; Bouvier-Nave et
462 al., 1998; Diener et al., 2000), leading to the formation of Δ^5 C-24 alkyl sterols. Since SMT1 only
463 occurs in plants and fungi, and not in animals, it is an interesting target for studying phytosterol
464 biosynthesis. SMT2/CVP1 and SMT3 are mainly responsible for a second methyl addition, thus
465 resulting in an ethyl side-chain addition on the C-24 (Schaeffer et al., 2001; Carland et al.,
466 2010). Therefore, the regulation of the SMT enzymes determines the sterol composition in
467 plants. Many compounds have been designed over the years to act as SMT inhibitors (Nes,
468 2000). These inhibitors can be broadly classified in three groups: 1) substrate analogues that
469 act as inactivators of the enzyme, 2) substrate analogues that resemble high-energy
470 intermediates, and 3) product analogues. While these compounds are generally designed in
471 fungal systems, some of them have been shown to inhibit SMT1 and SMT2/CVP1 in plants as
472 well, including the azasteroid inhibitors 25-azacycloartenol **(9)** (Rahier et al., 1980; Schmitt et
473 al., 1981; Rahier et al., 1986; Mangla and Nes, 2000), 25-azalanosterol **(10)** (Rahier et al.,
474 1984) and 24-epiiminolanosterol **(11)** (Tal and Nes, 1987), which are carbocationic transition
475 state analogues of the substrates of these enzymes (Rahier et al., 1984).

476 The 14α-methylsterol demethylase enzyme in plants (obtusifoliol 14α-demethylase)
477 catalyzes the demethylation of obtusifoliol (Lepesheva and Waterman, 2007). This enzyme is a
478 cytochrome P450 dependent monooxygenase (CYP51G1 in Arabidopsis) (Benveniste, 1986;
479 Lepesheva and Waterman, 2007). In fungi and animals, the best studied and most widely used
480 inhibitors of P450s are the azoles, which are a popular type of antifungal compounds that are

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481 used for both agricultural and medical purposes (Becher and Wirsel, 2012). Two subclasses of
482 the azoles are the imidazoles, such as clotrimazole **(12)**, oxiconazole **(13)**, ketoconazole **(14)**,
483 imazalil (enilconazole) **(15)** and prochloraz **(16)**, and the triazoles, such as triadimenol **(17)**,
484 voriconazole **(18)** and fluconazole **(19)**. They are nitrogen-containing heterocyclic compounds
485 that form an effective class of fungicides by non-competitively binding to the ferric ion of the
486 heme group of fungal CYP51, thus preventing it from binding its substrate (Rogerson et al.,
487 1977; Warrilow et al., 2013). Despite being primarily used as fungicides, several azole
488 compounds also have an effect on plants to a varying degree, where they generally cause
489 growth inhibition, which may be due to interference with downstream BR biosynthesis
490 (Scheinpflug and Kuck, 1987; Vanden Bossche et al., 1987; Rozhon et al., 2013; Fabris et al.,
491 2014). However, while nanomolar concentrations of azoles are usually sufficient to inhibit
492 ergosterol biosynthesis in fungi, micromolar concentrations or higher are often needed to obtain
493 a similar inhibitory effect on phytosterol biosynthesis in plants and diatoms (Vanden Bossche et
494 al., 1987; Fabris et al., 2014). The cytochrome P450s are a superfamily of enzymes (Xu et al.,
495 2015), that are often sensitive to imidazoles (Murray, 1999). Of note is that azoles can be found
496 or even designed that display a certain degree of preference towards specific cytochrome P450
497 enzymes. Well-known examples in plants are uniconazole as an inhibitor of CYP707As that are
498 involved in abscisic acid catabolism (Saito *et al.*, 2006), and brassinazole as an inhibitor of
499 CYP90B1 that is involved in brassinosteroid biosynthesis (Asami *et al.*, 2000). Importantly, both
500 uniconazole and brassinazole can inhibit CYP90B1 activities, suggesting that one should be
501 wary of off-target side effects when using these inhibitors, especially when using them at high
502 concentrations. Recently, analysis of crystals of CYP90B1 in complex with uniconazole and
503 brassinazole demonstrated important differences in binding conformation (Fujiyama *et al.*, 2019)
504 which highlighted the importance of using crystal structures of plant CYPs to guide the design of
505 new, more-specific inhibitors.

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506 Besides the azoles, also pyrimidine-type fungicides, such as fenarimol **(20)**, nuarimol
507 **(21)** and triarimol **(22)**, and pyridine-type fungicides are thought to affect the CYP51 ortholog
508 and other cytochrome P450 enzymes, such as CYP710A1 in plants (Shive and Sisler, 1976;
509 Schmitt and Benveniste, 1979; Buchenauer and Rohner, 1981; Burden et al., 1987; Scheinpflug
510 and Kuck, 1987; Mercer et al., 1989; Leroux et al., 2008; Oh et al., 2015). Overall, these
511 compounds all cause strong reductions in root- and shoot growth with varying potency, and are
512 phytotoxic at high concentrations due to a severe reduction in phytosterols and an accumulation
513 of 14α-methylsterols (Burden et al., 1987; Lurssen, 1987).

514 CPI, C-14 sterol reductase and C-8,7 sterol isomerase are enzymes that catalyze similar
515 reactions at different stages of the sterol biosynthesis pathway (Benveniste, 1986), making them
516 shared targets for molecular inhibition. An important class of inhibitors that target these
517 enzymes are the morpholine fungicides, such as fenpropimorph **(23)**, dodemorph **(24)**,
518 aldimorph **(25)** and tridemorph **(26)** (Kerkenaar et al., 1981; Baloch et al., 1984; Baloch and
519 Mercer, 1987; Mercer et al., 1989; Marcireau et al., 1990). These compounds exert their
520 fungitoxicity by inhibiting C-8,7 sterol isomerase (nM concentrations) and/or C-14 sterol
521 reductase (µM concentration) in fungi and yeast, with different morpholines having different
522 specificities (Kerkenaar et al., 1981; Baloch et al., 1984; Baloch and Mercer, 1987; Kerkenaar,
523 1987; Marcireau et al., 1990). For instance, while fenpropimorph can effectively inhibit both the
524 C-8,7 sterol isomerase and C-14 sterol reductase in fungi, tridemorph primarily inhibits the C-8,7
525 sterol isomerase (Baloch et al., 1984; Kerkenaar, 1987). However, morpholines also function in
526 plants, albeit less potently and less specifically, where they have been shown to inhibit HYD1
527 (the plant C-8,7 sterol isomerase) (Rahier et al., 1986; Taton et al., 1987), FK (the plant C-14
528 sterol reductase) (Mercer et al., 1989; Taton et al., 1989; He et al., 2003) and CPI (Taton et al.,
529 1987) to a varying degree. Similarly, fenpropimorph treatment caused alterations in the sterol
530 content of the diatom *P. tricornutum* that could be explained by inhibition of multiple enzymes

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531 involved in its sterol biosynthesis pathway (Fabris et al., 2014). While fenpropimorph is the most
532 active and commonly used morpholine in plants, it requires relatively high concentrations to
533 function (30 - 100 μM), is unstable and relatively expensive. Plants treated with morpholines
534 have a disturbed sterol profile and growth impairments, similar to mutants defective in the
535 targeted enzymes (Bladocha and Benveniste, 1983; Burden et al., 1987; He et al., 2003).
536 However, while the morpholine compounds disturb the normal sterol profile of plants, they are
537 generally not phytotoxic (Bladocha and Benveniste, 1983; Taton et al., 1987, 1987).
538 Interestingly, in plants, 8-azadecalins such as N-benzyl-8-aza-4α,10-dimethyl-trans-decal-3β-ol
539 **(27)** and N-(1,5,9-trimethyldecyl)-4α,10-dimethyl-8-aza-trans-decal-3β-ol (TMDAD) **(28)** have
540 been shown to be more potent inhibitors of HYD1 and CPI than the morpholines (Rahier et al.,
541 1985; Taton et al., 1987). A strong, more specific inhibitor of C-14 sterol reductases is the
542 antifungal agent 15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol (15-azasterol) **(29)**,
543 which has been used to inhibit FK in several plant species, including Arabidopsis (Schrick et al.,
544 2002) and bramble cells (Schmitt et al., 1980).

545 While most of the abovementioned sterol biosynthesis inhibitors have been used to
546 inhibit plant growth and study plant and diatom sterol biosynthesis to some degree, it is clear
547 that many of these compounds originate as antifungal compounds for which the effect in plants
548 is often not completely understood. Indeed, much of the underlying mechanisms of these
549 inhibitors in plants and diatoms are still not completely clear and are often presumed based on
550 their function in fungi and/or animals. It should also be noted that only limited recent data is
551 available for most of these inhibitors in plants, as evidenced by the relatively old sources
552 referenced in the last paragraphs. This further supports the notion that the current knowledge
553 and toolset of sterol biosynthesis inhibitors in plants is lacking. The identification of more active
554 compounds that selectively target specific enzymes in the plant sterol biosynthesis pathway

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555 through a systematic approach, informed by crystal structures, would therefore be highly
556 welcomed to study sterol biosynthesis in the green lineage.

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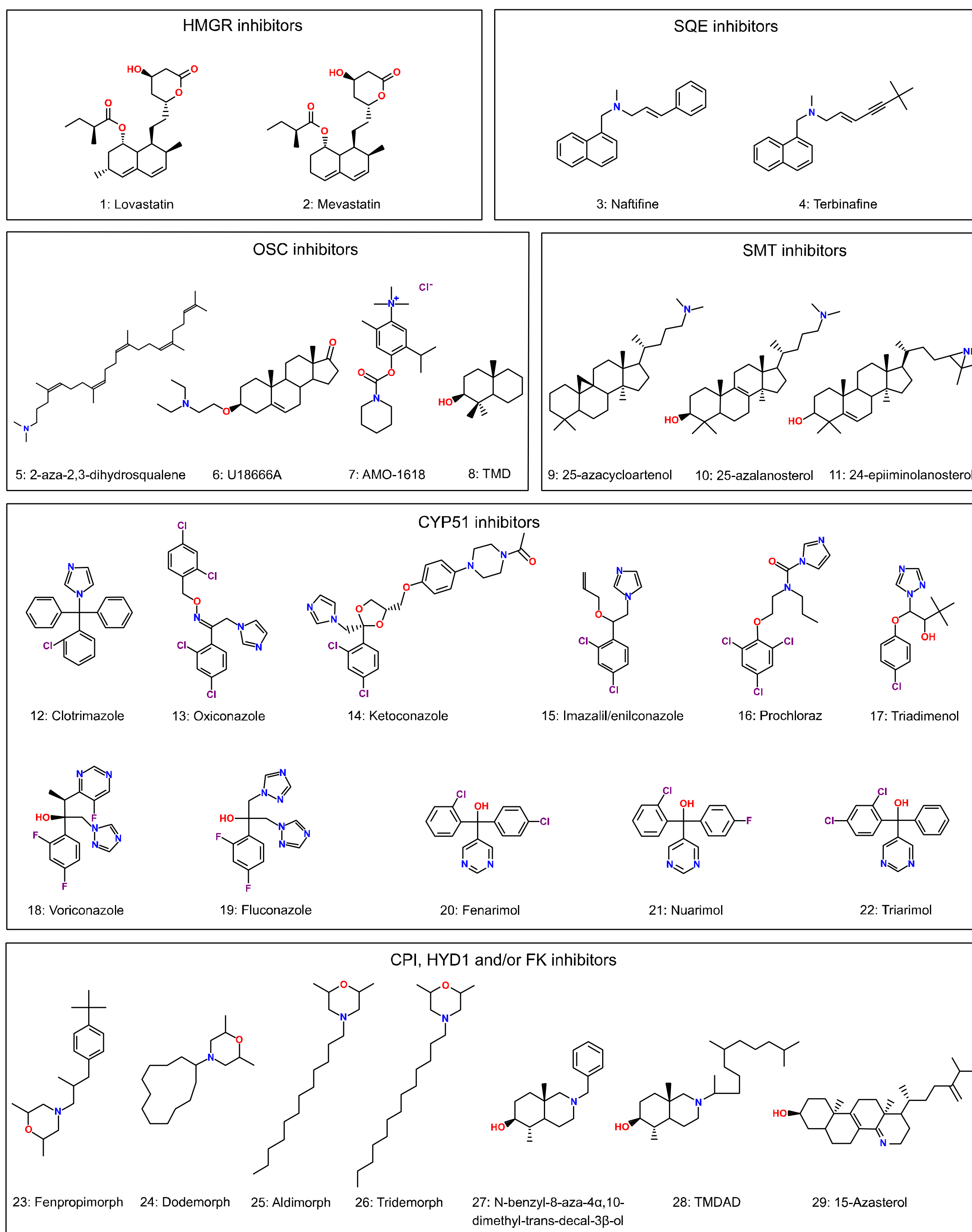

557

558 **Fig. 3. Structures of sterol biosynthesis inhibitors organized according to their putative targets.**

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