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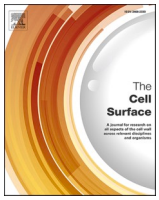

Sterols in plant biology – Advances in studying membrane dynamics

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ABSTRACT

Plants sense their environment at the cell surface, i.e. the plasma membrane, where extracellular signals are perceived and transduced. Together with the cortical cytoskeleton and the cell wall, membrane lipids can influence these processes by acting on protein dynamics at the plasma membrane. Among these lipids, sterols regulate membrane fluidity and thus, protein functions. However, plant sterols are diverse in structure and particularly difficult to study due to technical limitations. Nevertheless, advances in sterol imaging, sterol-protein interaction studies, and sterol perturbation methods have resulted in a better understanding of their functions in plant development and physiology. Here we summarize the current knowledge and the latest breakthroughs, and discuss future challenges, in the field of plant sterol biology and cell surface organization.

1. Introduction

Plant cells perceive and transduce an enormous number of external cues at their cell surface including hormonal signals, cell wall changes and mechanical stresses. In addition, vesicular trafficking events, as well as synthesis of cellulose and callose, occur at the plasma membrane (PM). The PM is a highly heterogeneous and dynamic environment, organized into subdomains which can act as landmarks (Jaillais and Ott, 2020). This organization might support perception and transduction of external stimuli by coordinating encounters between different signaling pathway components, as well as the precise targeting of membrane and cell wall components to the cell surface.

Sterols, which collectively make up about a third of the PM lipids, are key to the membrane organization due to their ability to modulate membrane fluidity (Bahammou et al., 2024; Grosjean et al., 2015). In contrast to animal membranes, which only contain cholesterol, plant PM comprises a complex sterol composition, predominantly featuring β-sitosterol, stigmasterol, and campesterol (Grison et al., 2015; Kierszniowska et al., 2009; Spector and Yorek, 1985). As an indicator of the importance of sterols in cellular functions, perturbations of sterol biosynthesis causes various developmental defects, including impaired shoot and root length, vascular patterning, seed and embryo development, cell division and often result in lethality (Carland et al., 2010; Cheon et al., 2010; Choe et al., 2000; He et al., 2003; Kim et al., 2005; Schaller, 2003). While sterols are regarded as fundamental to membrane organization and signaling, their small size and position inside the membrane leaflets have challenged precise mechanistic studies. This review synthesizes our current understanding of plant sterols at the cell surface, while highlighting emerging methodological advances that promise to shed new light on these essential membrane components.

2. The complex sterol system in plants

Sterols are amphiphilic molecules composed of three main structural elements: a hydrophilic hydroxyl group, a rigid tetracyclic steroid ring system, and a hydrophobic alkyl side chain (Fig. 1). Unlike animals and fungi, which contain predominantly cholesterol and ergosterol in their cell membranes respectively, plants feature a diverse variety of sterol species (Zhang et al., 2020). This variety is largely driven by the alkyl side chain, which varies in structure, presence of double bonds and length among sterol species, resulting in different steric conformations. In plants, β-sitosterol, stigmasterol and campesterol are the most abundant sterols at the PM (Bahammou et al., 2024). Sterol variants carrying a 3β-hydroxyl group are referred to as free sterols (FSs). Modifications of that group result in so-called sterol conjugates (Fig. 1). Plants typically contain three classes of sterol conjugates: Steryl esters (SEs), steryl glycosides (SGs), and acyl steryl glycosides (ASGs) (Fig. 1). As the name implies, SGs are sterols that have been glycosylated, most frequently

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glucosylated (Fig. 1). From such SGs, ASGs can be produced by a subsequent acylation of the sugar residue. The diversity of plant sterols has been hypothesized to reflect the need to withstand varying temperatures due to the sessile lifestyle of plants. Indeed, biological membranes containing phytosterols can more effectively buffer membrane fluidity across wide temperature ranges than cholesterol alone (Beck et al., 2007; Dufourc, 2008; James et al., 2014; Uemura et al., 1995).

2.1. Sterol composition and characteristics of the PM

Free sterols accumulate at the PM, making up around one third of the PM lipids. By contrast, internal membranes contain only just over 10 % sterols (Bahammou et al., 2024). However, the composition of sterols may differ among plant species and ecotypes. For example, stigmasterol is the second most abundant sterol species at the PM in *Arabidopsis thaliana* Landsberg erecta (Ler) suspension cells (Grison et al., 2015), but recent in-depth PM lipidomic analyses of *Arabidopsis* Columbia (Col-0) suspension cells indicated that β-sitosterol and campesterol are the most common sterol species, with the former being the most abundant (Bahammou et al., 2024). Similar variations in sterol composition were also noted across different organs and tissues, e.g. comparing pollen and leaves of different plant species (Furse et al., 2023), suggesting that sterol composition at the PM might be finely tuned during plant development.

SGs and ASGs represent the major sterol conjugates at the PM, constituting 4 % and > 1 % of total lipid mol% respectively, although this can vary drastically between different plant species and tissues (Bahammou et al., 2024). However, the relative proportions of different sterol species appear to be maintained across FSs and SG/ASG forms (Wewer et al., 2011).

In animals, cholesterol induces a thick and stiff membrane with reduced fluidity, even though the specific effects may depend on other interacting lipids (Beck et al., 2007; Marsh, 2006; Ovečka and Lichtscheidl, 2005). The interaction of the rigid and planar cholesterol with long fatty acid chains of phospholipids promotes membrane partitioning into more densely packed regions enriched in cholesterol known as "liquid-ordered phases" in contrast to "disordered-phases" that contain less cholesterol (Hjort Ipsen et al., 1987). In plants, the diverse array of sterols leads to a more complex impact on membrane properties and behavior. For example, campesterol mimics cholesterol's strong ordering effect (higher packing and slower dynamics) on membranes, whereas β-sitosterol and stigmasterol have much less pronounced effects on membrane ordering (Beck et al., 2007; Grosjean et al., 2015; Rujanavech et al., 1986). The conjugated sterol species SG and ASG, together with FS, synergistically increase membrane ordering, indicating an intricate interplay between different sterols species (Grosjean et al., 2015). Thus, the membrane ordering may be more nuanced in plant cells, perhaps fine-tuned by changes in FSs and conjugates. This complexity extends beyond the simple paradigm established in animal cells where increased cholesterol content directly correlates with increased membrane stiffness.

2.2. Biosynthesis of free sterols and conjugates

The biosynthesis pathway of plant sterols has been described in detail in recent reviews (Bajguz et al., 2020; De Vriese et al., 2021). We will therefore only briefly describe the synthesis routes of the main FSs (Fig. 2a). Sterol biosynthesis begins in the endoplasmic reticulum (ER) with Acetyl-CoA as the precursor, proceeding through the mevalonate and squalene pathways to yield cycloartenol. From here, the sterol biosynthetic pathway separates into two branches: the first branch leads to the production of cholesterol while the second branch divides again to give campesterol and β-sitosterol. Finally, stigmasterol is derived from β-sitosterol. Sterol biosynthesis is intertwined with the biosynthesis of other important isoprenoid-derived compounds, including the major plant hormones brassinosteroids (Bajguz et al., 2020). Indeed,

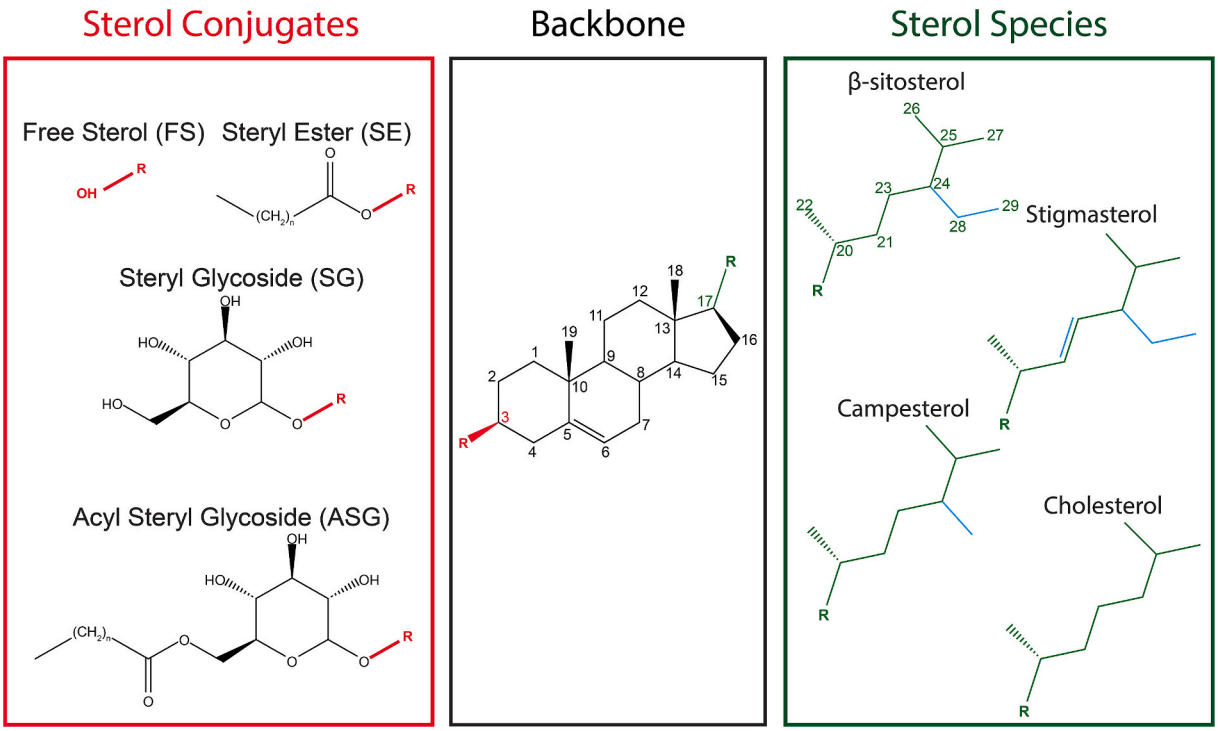

Fig. 1. General structure of the main sterol variants in plants. The tetracyclic steroid ring system forms a consistent backbone, with carbon atoms numbered according to the typical sterol numbering system (shown on backbone structure). The residue at the C3 position (red R groups) determines the type of sterol conjugate: Free Sterol (FS), Steryl Ester (SE), Steryl Glycoside (SG), or Acyl Steryl Glycoside (ASG). The alkyl side chain attached to C17 (green) defines the sterol species (β-sitosterol, stigmasterol, campesterol, or cholesterol). Key structural differences between sterol species are highlighted in blue. Adapted from (Ferrer et al., 2017). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

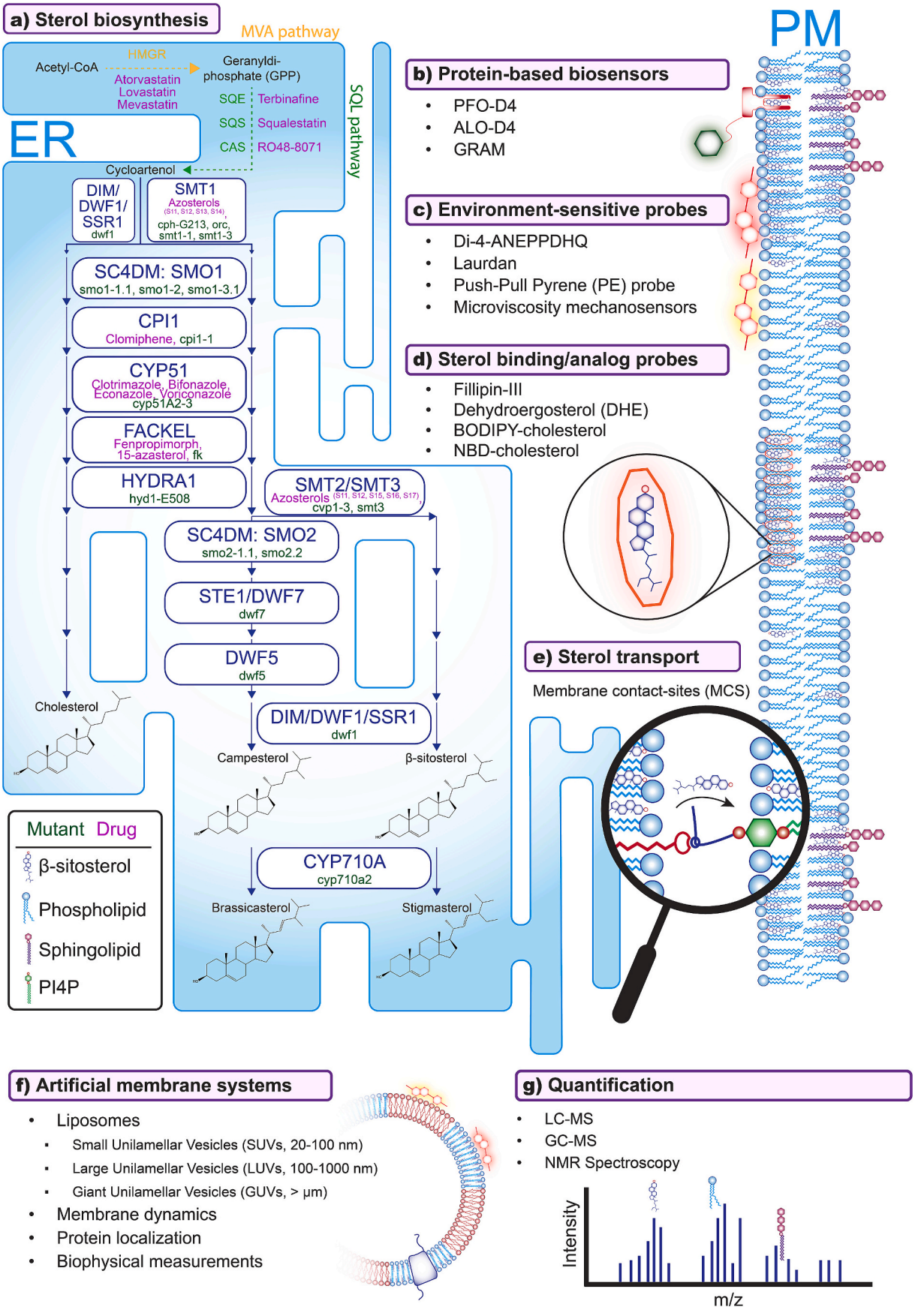

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Fig. 2. Comprehensive methods for studying plant sterols. a) Sterol biosynthesis pathway highlighting key enzymes, mutants (green), and inhibitory drugs (orange). 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), geranyldi-phosphate (GPP), squalene epoxidase (SQE), cycloartenol synthase (CAS), STEROL METHYLTRANSFERASE1 (SMT1), STEROL C-4 DEMETHYLASE (SC4DM), STEROL 4-ALPHA-METHYL OXIDASE1 (SMO1), CYCLOPROPYL ISOMERASE1 (CPI1), CYTOCHROME P450 51 (CYP51), STEROL C-14 REDUCTASE (FACKEL), C-8,7 STEROL ISOMERASE (HYDRA1), STEROL 4-ALPHA-METHYL OXIDASE2 (SMO2), STEROL C-24 REDUCTASE/DWARF7 (STE1/DWF7), DWARF5 (DWF5), DIMINUTO/DWARF1/STEROL SIDE CHAIN REDUCTASE1 (DIM/DWF1/SSR1), and CYTOCHROME P450 710 A (CYP710A) b) Protein-based biosensors include Perfringolysin O Domain 4 (PFO-D4), Anthrolysin O Domain 4 (ALO-D4), and the Glycosyltransferases, Rab-like GTPase activators and Myotubularins (GRAM) domain. c) Environmental-sensitive probes include di-4-aminonaphthylethenylpyridinium (Di-4-ANEPPDHQ), push-pull pyrene (PE). Sterol binding probes shown are nitrobenzoxadiazole (NBD) and boron-dipyrromethene (BODIPY). d) Sterol binding and analog probes including Filipin-III, dehydroergosterol (DHE), BODIPY-cholesterol, and NBD-cholesterol for sterol visualization and tracking. e) Membrane contact sites (MCS) as potential mediators of sterol transport between cellular membranes and as such possibly novel targets for sterol perturbation. Depicted is the potential transport via the counter-transport principle utilizing OSBP (red) and VAPA (dark blue) proteins and involving PI4P (green) as described for animals. f) Artificial membrane systems including different classes of liposomes (SUVs, LUVs, GUVs) for studying membrane dynamics and protein localization. g) Quantification methods include liquid-chromatography mass spectrometry (LC-MS), gas-chromatography mass spectrometry (GS-MS), and nuclear magnetic resonance (NMR) spectroscopy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

campesterol serves as a precursor to produce brassicasterol, which through multiple enzymatic steps is converted into brassinolide, the most bioactive brassinosteroid (Nolan et al., 2020).

While the biosynthesis of free sterols has been extensively studied, the formation of sterol conjugates remains less understood. Ferrer et al. provide a comprehensive review of our current knowledge regarding sterol conjugate biosynthesis (Ferrer et al., 2017). Sterol acyltransferases catalyze an esterification reaction to attach a fatty acid typically with a length of C12 to C22 (palmitic, stearic, oleic, linoleic or linolenic acids) to the hydroxyl group of a sterol (Dyas and Goad, 1993). The enzymes involved appear to have some specificity in their choice of substrate and localize mainly to the ER (Chen et al., 2007; Lara et al., 2018). Lipid bodies, also known as lipid droplets, emerge from specialized ER compartments. These droplets contain high amounts of sterol esters, possibly as a form of storage regulating sterol homeostasis, as well as an abundance of acyltransferases (Chapman et al., 2012; Lara et al., 2018; Shimada et al., 2019). SGs are formed by the attachment of sugar moiety to the hydroxyl group of sterols. These are most commonly glucose, but can also be xylose, galactose and mannose as well as di-, and triglucosides (Grille et al., 2010). These reactions are carried out by sterol glucosyltransferases, which despite the lack of a transmembrane domain can loosely associate to the PM, but are also found in the cytoplasm, at the ER and the tonoplast, and show some substrate specificity, indicating a dynamic regulation of SGs (Ramirez-Estrada et al., 2017; Stucky et al., 2015). Steryl glycoside acyltransferases modify SGs further into ASGs by esterification of various fatty acids to the sugar moiety (Schrick et al., 2011). So far, no genes encoding steryl glycoside acyltransferases have been identified, even though one study biochemically characterized such activity in carrots (Eichenberger and Siegrist, 1975; Ferrer et al., 2017).

The evolutive origin of sterol biosynthesis has been debated in recent years and is summarized in an excellent overview by Ferreira-Guerra et al. (Ferreira-Guerra et al., 2025). Steranes are organic compounds found in sedimentary rocks that present a tetracyclic ring system fully saturated with hydrogen. They are hypothesized to represent "molecular fossils" of sterols and, as such, can be used to map the occurrence of different sterol species to the emergence of eukaryotic life (Brunoir et al., 2023). Sterols as components of cell membranes are often considered as an eukaryotic feature (Desmond and Gribaldo, 2009). However, new evidence suggests that sterol biosynthesis genes have evolved in bacteria before being transferred to early eukaryotic lineages by bacterial symbiotic partners rather than being independent eukaryotic innovations (Hoshino and Gaucher, 2021; Santana-Molina et al., 2020). This transfer event occurred most likely before the divergence of eukaryotes, with the last eukaryotic common ancestor already displaying the majority of sterol biosynthetic genes. The different sterol systems across kingdoms were shown to be mostly a result of gene-loss and duplications. Thus, the group of diatoms generally displays most features of plant sterol biosynthesis, but the demethylation at C4 and reduction of the C24 double bond rely on fungal and animal enzymes (Gallo et al., 2020). Another example are the SMT genes that were recently suggested to have been inherited by the last eukaryotic common ancestor and later on lost in many animal phylae as a result of shifting feeding strategies, while being retained in plants (Brunoir et al., 2023).

The ability to flexibly modulate biophysical properties of cell membranes via sterols could have played a distinguishing role in the success of eukaryotes (Du et al., 2022; Ferreira-Guerra et al., 2025). In particular, plants have evolved varying sterol profiles as adaptations. In tomato and potato a duplication of the DWF1/SSR1 gene called SSR2 causes high levels of cholesterol, which is a precursor in glycoalkaloid synthesis that plays an important role in the defense against pathogens (Sawai et al., 2014).

2.3. *Transport of free sterols from the ER to the cell surface*

As indicated above, sterols are synthesized in the ER in plant cells. Indeed, protein extracts from ER fractionations produced sterol intermediates from precursors (Hartmann and Benveniste, 1987), and key enzymes in the sterol biosynthesis pathway appear to localize to the ER (Silvestro et al., 2013). This is in line with the biosynthesis of cholesterol in animals that also takes place in the ER (Krisans, 1996; Lusa et al., 2003). Interestingly, sterol biosynthesis enzymes have also been observed to localize outside the ER, e.g. at the PM, or the cell plate during cell division, but the cell biological relevance of such dual localization is not fully understood as there is so far no evidence that these enzymes produce sterols directly at the cell surface (Ohta et al., 2024; Silvestro et al., 2013). The lower abundance of sterols in the ER membrane system compared to the high abundance in the PM suggests efficient secretion of the sterols. Compartmental fractionation of leek seedlings revealed that Brefeldin A, an inhibitor of vesicular transport, perturbs sterol trafficking from the ER to the PM (Moreau et al., 1998). This led to the hypothesis of exocytosis via the Golgi apparatus being the main sterol trafficking route (Ovečka and Lichtscheidl, 2005). However, recent findings may also indicate vesicular trafficking-independent pathways for sterol transport in plants (Kumar et al., 2021).

An alternative pathway for rapid transfer of sterols between ER and PM could take place at ER-PM Membrane Contact Sites (MCS). MCS can allow for direct lipid transfer as membranes from two distinct cellular compartments come into close contact (10–30 nm) (Prinz, 2007). In animal cells, sterol transfer proteins exchange sterols at MCSs (Luo et al., 2019). Three distinct families of proteins can transfer lipids in animals and yeast (Luo et al., 2019):

1. Oxysterol-binding protein (OSBP)-related protein (**ORP**) family members.
2. Steroidogenic acute regulatory protein (StAR)-related lipid transfer (START)-related domain (**STARD**) protein family members.
3. Lipid transfer proteins anchored at a membrane contact site (**Lam**) family members.

Members of these three protein families transfer sterols between specific intracellular membranes. For instance, Oxysterol-binding

protein-Related Protein 1 L (ORP1L) tethers the late endosome/lysosome membrane to the ER and promotes cholesterol transport (Zhao and Ridgway, 2017). Lipid transfer can be driven by a counter-transport principle. This mechanism was elucidated in detail for the oxysterol binding protein (OSBP), the original namesake for the eponymous protein family. OSBP tethers Golgi membranes with the ER by binding an internal phospho-site to the ER-anchored vesicle-associated membrane protein (VAMP)-associated protein A (VAPA) while its pleckstrin homology (PH) domain binds to Golgi-residing phosphatidylinositol 4-phosphate (PI4P). The ORD domain of OSBP subsequently transfers a cholesterol from the ER to the Golgi membrane, after which a PI4P is transported in the opposite direction and is then dephosphorylated by Phosphoinositide-4-phosphatase Sac1 (Mesmin et al., 2013). Consequently, PI4P is continuously depleted from the ER, creating a PI4P gradient used to drive cholesterol transport.

In yeast, Lam1/3p protein from the Lam family tethers ER membranes with the PM and could be one of the carriers responsible for the rapid transport of newly synthesized sterols to the PM (Gatta et al., 2018). While evidence for homologous mechanisms in plants remain sparse, both the ORP and the STARD protein families are conserved in plants with 12 proteins of the ORP family found in *Arabidopsis* and six proteins with a START domain structurally similar to the mammalian one (Schrick et al., 2004b; Skirpan et al., 2006). In addition, the oxysterol-binding protein-related protein 2 A (ORP2A) localizes to MCSs, interacts with vesicle associated protein 27–1 (VAP27–1) and is proposed to act during autophagy (Ye et al., 2022). Furthermore, transport of diacylglycerol by Synaptotagmin 1 (SYT1) at ER-PM contact sites occurs in *Arabidopsis* (García-Hernández et al., 2024). These recent findings indicate that the lipid-transfer pathway at MCSs may also occur in plants.

2.4. Role of sterols at the plant PM

The interplay of sterols and sphingolipids, promoted through the interaction of the sterol hydroxyl group with the sphingolipid amide group, coined initially the principal of “lipid raft”, where patches of densely packed lipids can diffuse through less densely packed regions of the PM (Brockerhoff, 1974; Brown and London, 1998). The concept is today revised and nanodomains or “lipid rafts” are not defined anymore by the mere presence of sterols. Rather, nanodomain refers to any protein or lipid accumulating in specific domains within the local membrane environment (Jaillais et al., 2024). Nevertheless, sterols can play a significant role in signal transduction by forming interaction platforms in some nano- and microdomains for membrane proteins that require sterol-rich regions (Sulkarnayeva et al., 2014; Tang et al., 2021). For instance, the membrane-bound receptor kinase FLAGELLIN SENSING 2 (FLS2), involved in innate immunity responses, changes its PM dynamics upon perturbation of sterols, resulting in impaired signaling and immune response (Cui et al., 2024; Cui et al., 2018). The sterol content of the PM may also influence plant pathogen-interactions in other ways. Reduced sterol content at the PM increases permeability, which pathogens exploit to induce nutrient efflux into the apoplast where they thrive (Sulkarnayeva et al., 2014; Wang et al., 2012). Furthermore, *Solanum tuberosum* REMORIN group 1 isoform 3 (StREM1.3) restricts the movement of viruses between cells by reducing the permeability of plasmodesmata in a sterol-dependent manner, indicating a multifaceted role of sterols in the plant innate immunity (Gronnier et al., 2017).

As mentioned before, lipid droplets emerge from the ER and are important structures involved in lipid homeostasis including sterol conjugates homeostasis. While the exact mechanism behind lipid droplet formation is still not fully understood, altered sterol composition was shown to perturb the biogenesis of lipid droplets in seeds (Yu et al., 2021).

Sterol composition and cell wall synthesis have some intriguing connections. Cellulose synthesis was first suggested to be initiated by a sitosteryl glucoside primer. However, this hypothesis has since then been ruled out as cellulose synthesis can be achieved in vitro without a sitosteryl glucoside primer (Cho et al., 2017; DeBolt et al., 2009; Peng et al., 2002; Purushotham et al., 2016). Nevertheless, sterol biosynthesis mutants exhibit reduced cellulose content. While some of the phenotypes observed in those mutants were rescued by addition of brassinosteroids, cellulose defects were not restored, suggesting that cellulose synthesis depends on membrane sterol composition (Schrick et al., 2012; Schrick et al., 2004a).

Interestingly, sterol biosynthesis enzymes can localize to the cell plate, providing evidence for the long-hypothesized role of sterols in regulating cell division and apposition of newly synthesized cell walls (Frescatada-Rosa et al., 2014; Ohta et al., 2024; Schrick et al., 2000). These results point towards a complex relationship between different sterols and downstream biosynthetic pathways, which might complicate interpretations. Current evidence points to a model where sterol-rich membrane domains facilitate localization and function of the Cellulose Synthase Complex (CSC), though the precise molecular details remain to be elucidated (Bessueille et al., 2009; Ohta et al., 2024). One key factor could represent the over 100 S-acylation sites in a single CSC, a post-translational modification (PTM), that is required for correct membrane localization of the CSC and is associated with sterol-rich regions (Chamberlain and Shipston, 2015; Hemsley, 2009; Kumar et al., 2016). It is thought that sterols might also be involved in the perception of the plant cell wall integrity. A possible connection lies in proteins involved in cell wall integrity sensing, such as the receptor-like kinase FERONIA (FER), that induces responses to cell wall perturbation by Ca^{2+} signaling and direct phosphorylation of COMPAGNON OF CELLULOSE SYNTHASE 1 (CC1) (Liu et al., 2024; Shih et al., 2014; Wang et al., 2022). The two GPI-anchored proteins LORELEI and LORELEI-like Glycosylphosphatidylinositol-anchored protein 1 (LLG1) potentially function as co-receptors and help chaperone FER during transport to the PM (Li et al., 2015; Liu et al., 2016). Since GPI-anchored proteins are typically involved in delivering proteins to sphingolipid and sterol-rich regions (Saha et al., 2016; Stulnig et al., 1997), the FER microdomain might depend on sterol dynamics. This is further supported by promotion of sterol-rich regions via C2 domain ABA-related (CAR) proteins upon FER activation (Chen et al., 2023). However, while the precise molecular mechanisms that would underpin such hypothesis are lacking, the strong connection between GPI-anchored proteins and sterols appears to link phytosterols cell wall integrity sensing and FERONIA. Another connection between cell wall integrity and sterols was recently made in yeast, where the yeast cell wall integrity sensors WSC1, WSC2 and WSC3 interacted with ergosterol to promote signaling (Bernauer et al., 2023). Homologs for these proteins are not present in *Arabidopsis*, but similar processes might exist.

Sterol conjugates play crucial roles in sterol homeostasis and maintaining membrane integrity, particularly during stress responses (Ferrer et al., 2017; Rogowska and Szakiel, 2020; Singh et al., 2018). Both SGs and ASGs act synergistically with FSs to markedly increase lipid order in biological membranes (Grosjean et al., 2015). Consequently, they can be seen as crucial modulators of the plant PM involved in a variety of processes. As such they have been hypothesized to play a role in nano- and microdomains (Ferrer et al., 2017). Their role is well established in responses to abiotic and biotic stressors. Silencing of sterol glycosyltransferases in *Withania somnifera* results in reduced SG levels lead to higher susceptibility to heat stress, suggesting possibly a role of SGs in maintaining membrane integrity during temperature changes (Mishra et al., 2015; Saema et al., 2016; Singh et al., 2017). Furthermore, the overexpression of sterol glycosyltransferase enzymes conferred improved immunity against *Alternaria brassicicola* fungus as well as improved temperature and salt tolerance (Mishra et al., 2017; Mishra et al., 2013). An increase in in SGs and ASGs at the PM under hypergravity conditions of azuki beans, suggests a possible link between conjugated sterols and gravity response (Sakaki et al., 2023). A recent analysis in tomato revealed a possible connection between fruit development and SGs by means of silencing a sterol glycosyltransferase

(Chávez et al., 2023). However, single mutants in SG/ASG biosynthesis often show surprisingly mild phenotypes, suggesting functional redundancy or context-specific roles (DeBolt et al., 2009; Mishra et al., 2015; Stucky et al., 2015). SEs appear to serve primarily in sterol homeostasis rather than direct membrane function (Ferrer et al., 2017; Lara et al., 2018). Studies on *Arabidopsis* mutants defective in SE formation show accumulation of free sterols and formation of abnormal lipid bodies, suggesting that SE formation is important for cells to manage excess sterols, potentially storing them for later use (Lara et al., 2018; Shimada et al., 2020; Shimada et al., 2019). While less common, modified sterols such as sterol esters and sterol glucosides also exist in mammals. Recently, cholesterol glucosides have been identified in mammalian cells, particularly during stress responses like heat shock, suggesting some conservation of these modification strategies across kingdoms (Grille et al., 2010; Shimamura, 2020).

3. Methods to study sterols at the PM

3.1. Studying sterols using genetics and molecular biology

Sterol biosynthesis mutants are valuable tools to elucidate the role of sterols in cellular processes. The severe dwarf phenotypes of some of the mutants were published many decades ago, long before the biosynthesis steps of sterol were fully resolved but provided precious clues to the order of the enzymatic steps (Gachotte et al., 1995; Mirza and Maher, 1987). One major challenge when studying sterol biosynthesis mutants

Table 1
List of the key mutants in the sterol biosynthesis pathway of *Arabidopsis thaliana*.

Locus	Gene	Name	Type of mutant	Phenotype	Rescued by BL	Reference
At5g13710	SMT1	*cph-G213*	EMS	Embryonic patterning defects, seedling patterning defects, short hypocotyl and root, malformed cotyledons	No	(Schrick et al., 2000)
At5g13710	SMT1	*orc*	EMS	Reduced root and shoot growth, defects in cell polarity and auxin transport, altered embryo and root patterning, and abnormal cotyledon and vascular development. It also shows reduced fertility and altered gravitropic responses.	No	(Willemsen et al., 2003)
At5g13710	SMT1	*cph-T357*	EMS	Embryonic patterning defects, seedling patterning defects, short hypocotyl and root, malformed cotyledons	No	(Schrick et al., 2002)
At5g13710	SMT1	*smt1–1*	Ac transposon	Reduced fertility, stunted siliques, shorter petioles, smaller/rounder leaves, compact rosette, shorter stems, aberrant embryo development, conditional root growth defects on agar media	No	(Diener et al., 2000)
At5g13710	SMT1	*smt1–3*	Ac transposon	Similar to *smt1–1*, but with somewhat better overall growth in soil compared to other *smt1* alleles.	No	(Diener et al., 2000)
At4g12110	SMO1–1	*smo1–1.1*	T-DNA insertion: SALK_021399	Single mutant shows no phenotype. *smo1–1 smo1–2* is embryo lethal. *smo1–1.1 smo1–3.1* has a mild phenotype with shorter siliques and smaller plants.	–	(Song et al., 2019)
At4g22756	SMO1–2	*smo1–2*	T-DNA insertion: CSHL_GT13595	Single mutant shows no phenotype. *smo1–1 smo1–2* is embryo lethal.	–	(Song et al., 2019)
At4g22755	SMO1–3	*smo1–3.1*	T-DNA insertion: CSHL_ET12310	Single mutant shows no phenotype. *smo1–1.1 smo1–3.1* has a mild phenotype with shorter siliques and smaller plants.	–	(Song et al., 2019)
At5g50375	CPI	*cpi1–1*	Ds transposon insertion line: GT_5_12417	Clustered small cells and stomata.	No	(Men et al., 2008)
At1g11680	CYP51	*cyp51A2–3*	T-DNA insertion	Seedling lethal, pale yellow plants die at a variety of seedling stages.	No	(Kim et al., 2005)
At3g52940	FK/HYD	*fk-J3158*	EMS, weak allele	Clusters of small cells and stomata in leaf epidermis, defects in vascular patterning, root growth and embryo development	No	(Qian et al., 2013)
At1g20050	HYD1	*hyd1-E508*	EMS	Embryonic and seedling patterning defects, short hypocotyl and root	No	(Schrick et al., 2002)
At1g20050	HYD1	*hyd1-R216*	EMS	Embryonic and seedling patterning defects, short hypocotyl and root	No	(Schrick et al., 2002)
At2g29390	SMO2–1	*smo2–1.1*	T-DNA insertion: SALK_105017	Single mutant no obvious phenotype, slightly shorter hypocotyls and roots. *smo2–1 smo2–2* homozygous double mutant is embryo lethal, heterozygous shows severe dwarf phenotype.	–	(Zhang et al., 2016)
At1g07420	SMO2–2	*smo2–2*	T-DNA insertion: SALK_030719	Single mutant no obvious phenotype, slightly shorter hypocotyls and roots. *smo2–1 smo2–2* homozygous double mutant is embryo lethal, heterozygous shows severe dwarf phenotype.	–	(Zhang et al., 2016)
At1g20330	SMT2	*smt2*	T-DNA insertion: GABI_443_F03	Normal leaf morphology, slightly irregular leaf vascular development, normal plant stature.	No	(Nakamoto et al., 2015)
At1g20330	SMT2	*cvp1*–3	EMS	Round, cupped epinastic cotyledons, fragmented and less reticulated cotyledon vein pattern, misshapen vascular cells, serrated sepal and petal tips, slightly dwarf plants, reduced stature.	No	(Carland et al., 2010)
At1g76090	SMT3	*smt3*	T-DNA insertion: SALK_085292	Single mutant indistinguishable from wildtype. *cvp1–3/smt2 smt3* double mutant: Enhanced cotyledon, vein, and root defects compared to cvp1; dwarfism; loss of apical dominance; reduced fertility; abnormal floral development including organ spacing defects and homeotic transformations; and epidermal cell pattern defects similar to smt1 mutants.	No	(Carland et al., 2010; (Nakamoto et al., 2015)
At3g02580	DWF7	*dwf7–1*	T-DNA insertion	Severe dwarfism compared to wild-type plants, with significantly slower rates of cell division in callus tissue and impaired shoot regeneration.	Yes	(Choe et al., 1999)
At1g50430	DWF5	*dwf5–7*	T-DNA insertion: SALK_127066	Exhibits a semi-dwarf growth habit with reduced rosette width, plant height, silique length, and seed number compared to wild-type plants.	Yes	(Du et al., 2016)
At3g19820	DWF1	*dwf1*	T-DNA insertion: SALK_006932	Dwarf plants with dark-green colour, photomorphogenesis in the dark, delayed senescence, reduced apical dominance and fertility. Homozygous dwf1 mutants are infertile. The dwarf phenotype can be rescued by exogenous brassinolide application.	Yes	(Du, 2005)

lies in the pathway being closely intertwined with that of brassinosteroids (Bajguz et al., 2020). Indeed, as brassinosteroids are derived from the sterol biosynthetic pathway, observed phenotypes of sterol biosynthesis mutants may result from BR perturbation and not from perturbation of sterol levels. In Table 1, we listed some of the key mutants for sterol biosynthetic enzymes and the phenotype associated to the loss-of-function. As expected, many mutants present strong phenotypes, most notably the *dwarf* mutants (*dwf1*, *dwf5* and *dwf7*), which affect downstream parts of the pathway and that can be partially or fully rescued by the exogenous application of brassinolide (BL), the most abundant brassinosteroid derivative

Fig. 2, Table 1) (Carland et al., 2002). By contrast, mutations in a number of proteins further upstream in the pathway cannot be rescued with brassinolides (Table 1). While laborious, careful dissection of the pathway through the help of the mutants could therefore aid in deciphering which phenotypes can be attributed to perturbations in brassinosteroid signaling, and which ones are due to perturbation of sterol composition. However, it is of course also possible that sterol composition at the PM participates in the fine tuning of brassinosteroid signaling and vice versa.

3.2. Perturbation of sterols by pharmacological approaches

Apart of the genetic approach, several drugs are available to target different steps in the sterol biosynthesis pathway, representing powerful tools in dissecting the sterol biosynthesis pathway. Excellent reviews have recently described inhibitors of the sterol biosynthesis pathway and their targets in great detail (Bajguz et al., 2020; De Vriese et al., 2021). In this section, we summarize the available inhibitors by following their action along the biosynthetic pathway. The inhibitors mentioned in this review are listed in Table 2.

Statins are a group of compounds originally discovered as human cholesterol-lowering drugs, that act by inhibiting the 3-Hydroxy-3-Methyl-Glutaryl-coenzyme A Reductase (HMGR), a rate-limiting step in the mevalonate pathway upstream of sterol biosynthesis (Chester and El Guindy, 2021; Endo, 2010; Endo et al., 1976a, 1976b). Among the HMGR inhibitors used in plant research, lovastatin has been the most widely employed, followed by mevastatin and atorvastatin, with their application consistently resulting in severe growth defects and reduced phytosterol levels (Boutté et al., 2011; Frescatada-Rosa et al., 2014; Shimada et al., 2019; Suzuki et al., 2003). Recent work resolved the crystal structure of AtHMG1 in complex with a statin revealing the mode of action of statins in detail (Haywood et al., 2022). Although statins are effective at reducing sterol levels in plants, it has to be noted that because of its central role in the synthesis of various compounds such as for example chlorophyll and gibberellins, inhibition of the MVA pathway will inevitably have pleiotropic effects (Chatterjee and Kundu, 2015; Kasahara et al., 2002).

Terbinafine belongs to the allylamines, a class of fungicides inhibiting ergosterol synthesis, and blocks the activity of Squalene Epoxidase (SQE) in both fungi and plants (Laranjeira et al., 2015; Ryder, 1992). Squalestatins (also called zaragozic acids) are potent inhibitors of squalene synthase (SQS) in mammals, though their phenotypic effects in plants are less well characterized, although it is known that treatment causes infertility in *Arabidopsis* (Baxter et al., 1992; Hartmann et al., 2000; Wentzinger et al., 2002). Ro 48–8071 is another drug originally developed for lowering human cholesterol levels, but targeting the cyclization step yielding lanosterol and cycloartenol in animals and in plants, respectively (Morand et al., 1997). The crystal structure of a prokaryotic squalene cyclase has been resolved in complex with Ro 48–8071 (Lenhart et al., 2002). Interestingly, while Ro 48–8071 effectively inhibits Cycloartenol Synthase (CAS) in *Arabidopsis,* it also lowers the expression of *SMT2* resulting in a disproportional reduction of phytosterols, providing an interesting opportunity to selectively inhibit membrane sterols with smaller effects on brassinosteroid biosynthesis (Gas-Pascual et al., 2015).

Table 2
List of drugs used to study sterol and brassinosteroid biosynthesis. EA.: Enzymatic assay, PA.: Phenotypic analysis and comparison to mutants, MS.: Analysis of accumulation of precursor compounds using MS, CS.: Crystallographic analysis of mechanism.

Compound	Description	Target validation	References
Mevastatin	HMG CoA reductase (HMGR) inhibitor	EA, PA, MS, CS (AtHMGR with different statin)	(Kasahara et al., 2002)
Atorvastatin	HMG CoA reductase (HMGR) inhibitor	EA, PA, MS, CS (AtHMGR with Atorvastatin superimposed)	(Haywood et al., 2022)
Lovastatin	HMG CoA reductase (HMGR) inhibitor	EA, PA, MS, CS (AtHMGR with different statin)	(Suzuki et al., 2003)
Squalestatin	Squalene Synthase (SQS) inhibitor	EA, PA, MS	(Baxter et al., 1992; Hartmann et al., 2000; Wentzinger et al., 2002)
Terbinafine	Squalene epoxidase (SQE) inhibitor	EA, PA, MS	(Laranjeira et al., 2015; Ryder, 1992)
Ro 48–8071	Inhibits Cycloartenol synthase (CAS) inhibitor	EA, PA, MS, CS	(Gas-Pascual et al., 2015; Lenhart et al., 2002; Morand et al., 1997)
Clomiphene	CPI1	PA, MS	(Wang et al., 2024)
Clotrimazole	Cytochrome P450 inhibitor	EA, PA, MS	(Sohrabi et al., 2015; Wang et al., 2024)
Bifonazole	C-14 demethylase (CYP51) inhibitor	PA, MS	Wang et al., 2024)
Econazole	C-14 demethylase (CYP51) inhibitor	PA, MS	Wang et al., 2024)
Voriconazole	C-14 demethylase (CYP51) inhibitor	PA, MS	(Rozhon et al., 2013)
Fenpropimorph	C-14 reductase inhibitor (FACKEL)	EA, PA, MS	(He et al., 2003; Taton et al., 1989; Schaller et al., 1992)
15-azasterol	C-14 reductase inhibitor (FACKEL)	EA, PA, MS	(Schrick et al., 2002; Schmitt et al., 1980; Taton et al., 1989)
Azosterols	Sterol Methyltransferase (SMT) inhibitors	PA, MS	(Darnet et al., 2020)
S15, S16, S17	SMT2/SMT3	PA, MS	(Darnet et al., 2020)
S11, S12	SMT1 and SMT2/SMT3	PA, MS	(Darnet et al., 2020)
S13, S14	SMT1	PA, MS	(Darnet et al., 2020)
Spironolactone	Inhibits brassinosteroids, downstream of campesterol	–	(Asami et al., 2004)
Triadimefon	Inhibits DWF4 downstream of Campesterol	–	(Asami et al., 2003)
Brassinoazole	Inhibits Brassinolide synthesis	–	(Asami et al., 2000)
Brassinolide	To complement brassinosteroid related phenotypes	–	(Tang et al., 2020)

A recent study by Wang et al. (2024) systematically evaluated mammalian cholesterol biosynthesis inhibitors for their potential to target analogous steps in plant sterol synthesis (Wang et al., 2024). Bifonazole, clotrimazole, and econazole were identified as potent inhibitors of CYP51G1 (14α-demethylase) in *Arabidopsis*, causing growth

inhibition leading to accumulation of 14α-methyl sterol intermediates. Direct binding of clotrimazole to CYP51G1 was confirmed through drug affinity-responsive target stability assays, and this compound also inhibited CYP710A sterol C22-desaturase based on altered β-sitosterol/stigmasterol ratios. Notably, the selective estrogen receptor modulator clomiphene was found to have an unexpected target in plants, inhibiting CPI1 (cyclopropyl-cycloisomerase) as evidenced by accumulation of cycloeucalenol and other 9β,19-cyclopropylsterols.

The most widely used sterol inhibitor is Fenpropimorph that inhibits the C-14 reductase FACKEL (FK)(Grandmougin et al., 1989; He et al., 2003; Laloi et al., 2007; Qian et al., 2013; Schrick et al., 2004a; Wang et al., 2021). Plants treated with fenpropimorph exhibit, phenotypically similar to the *fk* mutants, reduced length of shoot, petiole and roots that cannot be rescued by application of brassinolide. Furthermore, compounds upstream of the C-14 reduction step accumulate, while phytosterols and brassinosteroids are severely decreased (He et al., 2003). An alternative C-14 reductase inhibitor represents 15-azasterol, that was proposed to be more specific and act at lower concentrations, although much less characterized (Schrick et al., 2002; Schmitt et al., 1980; Taton et al., 1989).

Independent of enzymatic activity, sterols can be depleted from the PM by short treatment with sterol-binding compounds such as methyl-β-cyclodextrin (MβCD). MβCD forms a cyclic glucose oligomer and can bind to endogenous sterols (Furt et al., 2010; Kierszniowska et al., 2009; Valitova et al., 2014). This approach avoids perturbing other biosynthetic pathways and reduces the risk of indirect effects due to the short treatment time.

3.3. Quantification of sterols

Plant sterol quantification requires complementary analytical approaches to capture both total sterol content and conjugated forms. Gas chromatography-mass spectrometry (GC–MS) following acid methanolysis and derivatization provides accurate quantification of total sterols using internal standards but hydrolyzes conjugates, thus losing information about their original forms. Therefore, parallel liquid chromatography-mass spectrometry (LC-MS) analysis of non-hydrolyzed samples is necessary to profile intact sterol esters, glycosides, and other conjugates (Bahammou et al., 2024; Child and Kuksis, 1983; Jouhet et al., 2017; Khoury et al., 2018; Wewer et al., 2011; Zhang et al., 2005).

While most studies quantify total sterols in whole-plant or specific tissues without fractionation (Aboobucker et al., 2021; Carland et al., 2010; Diener et al., 2000; Wewer et al., 2011), this approach does not reveal the actual stoichiometry of sterols at the PM, as demonstrated by a recent comprehensive analysis of (Bahammou et al., 2024). To draw accurate conclusions about membrane composition and function, it is essential to employ subcellular fractionation coupled with targeted lipid analysis, as changes in total lipid content may not reflect changes in specific cellular compartments.

3.4. Sterol-protein associations

The detergent-resistant membrane (DRM) approach has been used extensively to decipher which part of the membrane proteome associates with sterol-enriched membrane. DRM involves treating cell membranes with non-ionic detergents like Triton X-100 at 4 °C, where some membrane components resist solubilization and can be isolated by centrifugation (Brown and Rose, 1992). These DRMs are typically enriched in sterols, sphingolipids, and certain proteins, leading researchers to interpret DRM association as evidence for proteins residing in sterol/sphingolipid-rich "lipid rafts" (Borner et al., 2005; Brown and London, 1998; Kierszniowska et al., 2009). While this method is widely used to study sterol-protein interactions, concerns have been raised of how to interpret DRMs and how well the approach reflects pre-existing membrane organization (Brown, 2006; Lichtenberg et al., 2005). Consequently, DRMs should be interpreted cautiously and validated with complementary, detergent-free approaches that can study membrane organization under more physiological conditions. Styrene Maleic Acid Lipid Particle (SMALP) can solubilize proteins in a close to native lipid environment and has been proposed as an alternative to traditional DRM methods (Teo et al., 2019).

3.5. Visualization of sterol dynamics

Another approach to determine if a protein of interest is associated with membrane-based sterols consists in using sterol probes or biosensors to colocalize sterols with the fluorescent signal of the protein of interest. These probes may also give some insight on the sterol dynamics at the PM. Sterol probes are molecules that bind sterols or probes that change behavior depending on the physical properties of the membrane. In plants, the most widely used molecule is Filipin-III, a naturally fluorescent polyene macrolide antibiotic. Upon binding to the free hydroxyl group of sterols, Filipin-III exhibits a shift in its excitation spectrum towards lower wavelengths, which can be used to visualize filipin-sterol fluorescence as depicted in Fig. 3a (Boutté et al., 2011; Castanho et al., 1992). However, its photosensitivity resulting in rapid bleaching can be challenging during imaging experiments. Filipin-III is also highly cytotoxic and has been shown to disintegrate membranes (Robinson and Karnovsky, 1980). For these reasons, it is unclear how accurately sterol dynamics observed using filipin-III relate to native dynamics.

Phase-sensing probes like Di-4-ANEPPDHQ, which change their emission spectrum based on the lipid environment and membrane fluidity, have emerged as useful sterol markers. They can be used to assess sterol-rich regions and have been successfully employed in plants (Xu et al., 2020; Zhao et al., 2015). By imaging the sample in two channels, a ratio can be calculated through generalized polarization that indicates the degree of membrane order (Fig. 3c). When using these probes, one must consider that there is no direct interaction with sterols, which could lead to unwanted artifacts such as differences in membrane fluidity due to temperature changes rather than sterol content. Although Di-4-ANEPPDHQ is less toxic to cells than Filipin-III, it is not taken up as easily, which can cause problems when imaging cells that are buried deep in tissues (Zhao et al., 2015).

Laurdan functions similarly to Di-4-ANEPPDHQ but has less sensitivity to sterol content in the membrane and has been less used in plant research (Amaro et al., 2017). Recently, a novel probe called push-pull pyrene (PA) was developed. This probe acts like Laurdan, but is proposed to have several key advantages (Niko et al., 2016) including increased brightness, better photostability and better uptake due to the smaller size, and has been used in *Arabidopsis* (Tang et al., 2021). A related group of probes are mechanoprobes that can measure the viscosity of membranes and thus indirectly sterol dynamics, as they typically are associated with higher viscosity. A set of four probes that target vacuole, cytosol, PM, and cell wall has been used successfully in *Arabidopsis* (Michels et al., 2020).

In animal research, fluorescent cholesterol analogs are heavily used for sterol visualization. Popular compounds include dehydroergosterol (DHE), NBD-cholesterol, and BODIPY-cholesterol (Barrantes, 2022). However, how closely cholesterol analogs can resemble native cholesterol dynamics is not certain. In plants, this question mark is even more pronounced due to the high variety of sterol species and low abundance of cholesterol. However, some attempts in plants have been made and, if meticulously designed, such experiments may certainly yield valuable data (Kumar et al., 2021; Michels et al., 2020).

Protein-based biosensors that utilize Cholesterol binding Domain 4 of Perfringolysin O (PFO-D4), Anthrolysin O Domain 4 (ALO-D4) or the Glycosyltransferases, Rab-like GTPase activators and Myotubularins (GRAM) domain of GRAMD1s/Asters were developed in mammalian cells. Those domains bind accessible cholesterol, a small fraction of membrane sterols that are not tightly bound to other membrane lipids (Koh et al., 2023). In an attempt to adapt the system for phytosterols, the

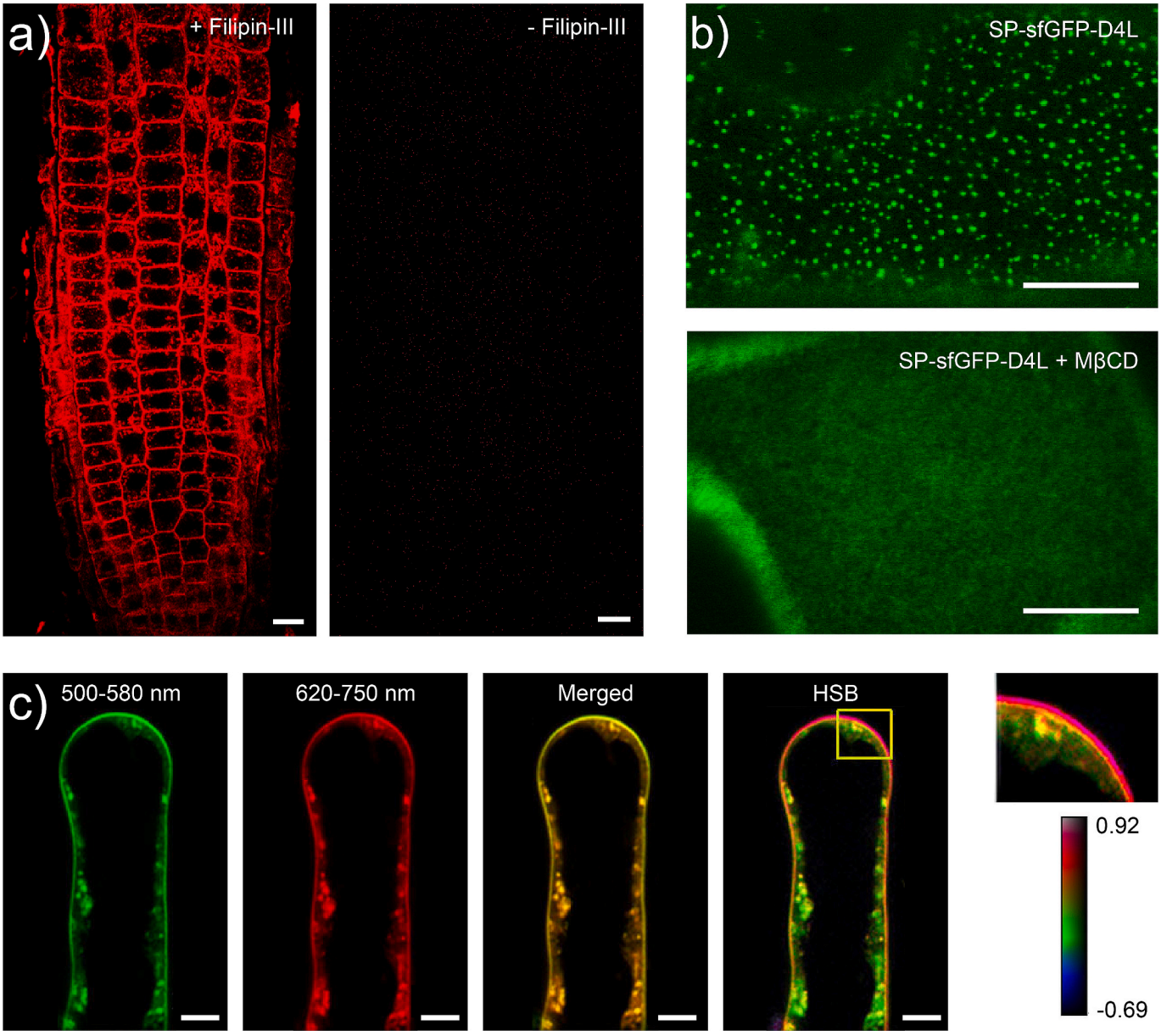

Fig. 3. Microscopic visualization of sterols in Arabidopsis. a) Filipin-III staining of the Arabidopsis root tip. Epidermal and cortical cells are labeled upon filipin-III staining when excited at 364 nm, visualizing 3-β-hydroxysterols in complex with filipin-III (left panel). Untreated cell show no fluorescence signal when imaged with the same settings (right panel). Adapted from (Boutté et al., 2011). b) The protein-based biosensor SP-sfGFP-D4L localizes to potential sterol-rich punctae in Arabidopsis cotyledons. The cholesterol-binding domain 4 (D4) of perfringolysin O (PFO) from Clostridium perfringens with a D44L mutation was fused to a secretion peptide (SP) and a superfolder green fluorescent protein (sfGFP) yielding a potential sterol biosensor: SP-sfGFP-D4L. Expressed in 8-day old Arabidopsis cotyledons results in localization of the biosensor to punctae that could resemble sterol-rich nanodomains (upper panel). Treatment with the sterol-depleting drug methyl-β-cyclodextrin (MβCD) causes punctae to diffuse. Adapted from (Ukawa et al., 2022). c) Quantitative visualization of membrane order using the environment-sensitive probe di-4-ANEPPDHQ in Arabidopsis root hair. Using excitation at 488 nm, emissions in two channels were recorded with windows of 500–580 nm and 620–750 nm (first and second panel from the left). The merged image of the two emission channels does not give immediate inside in the distribution of lipid order (third panel from the left). To spatially visualize the degree of membrane order, generalized polarization (GP) processing was performed to quantitatively infer the degree of membrane order from the ratio of the two emission channels. GP values are indicated by Hue-Saturation-Brightness (HSB) with values ranging from −0.69 (lower order, blue) to 0.92 (higher order, pink) showing higher lipid order at the root tip (last three images on the right). Adapted from (Zhao et al., 2015). Scale bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

D4 domain was mutated to facilitate binding to campesterol and sitosterol and successfully used to visualize sterol dynamics in *Arabidopsis* as depicted in Fig. 3b (Ukawa et al., 2022). These novel sterol probes may advance sterol research through their ability to be genetically encoded. Compared to traditional methods using Filipin-III or other probe-based approaches, they could also offer a less invasive alternative.

3.6. Sterol dynamics in model membranes

Model membranes have been long in use, and provide powerful tools to understand the biophysical properties of membrane lipids (Reeves and Dowben, 1969). Unlike the complex and dynamic environment of native cellular membranes, artificial model membranes allow precise control over lipid composition and environmental conditions (Rumiana and Carlos, 2022). These systems are classified primarily by size and

lamellarity. Each liposome class offers distinct advantages for sterol research: Small Unilamellar Vesicles (SUVs, 20–100 nm) are useful for studying sterol distribution in high curvature membranes, transmembrane movement and vesicle fusion (Backer and Dawidowicz, 1979; Chappa et al., 2021; Compassi et al., 1997; Salman et al., 1991; Schroeder et al., 1987; Wei et al., 2022); Large Unilamellar Vesicles (LUVs, 100–1000 nm) provide a more physiologically relevant system for studying sterol-induced membrane ordering and domain formation, being particularly useful for fluorescence spectroscopy (Coste et al., 2006; Grosjean et al., 2015; Kaiser et al., 2009; Ragaller et al., 2024); Giant Unilamellar Vesicles (GUVs, >1 μm) allow direct visualization of sterol-rich domains and phase separation through confocal microscopy and precise stochiometric ratios, often in the form of a ternary mix comprised of lipids, sterols and sphingolipids (Ariola et al., 2009; Bacia et al., 2005; Grosjean et al., 2015; Ragaller et al., 2024; Rumiana and Carlos, 2022). The distribution and behavior of sterols can here be monitored using a range of different probes like environment-sensitive fluorescent probes such as Di-4-ANEPPDHQ or Laurdan, or fluorescent lipid analog labeled with e.g. BODIPY (Ariola et al., 2009; Leonard et al., 2015). Recently published work on push-pull probes employed fluorescence lifetime imaging (FLIM) to conduct membrane fluidity measurements in GUVs (Ragaller et al., 2024). More advanced protocols allow for studying of protein-lipid interactions by fusing living cells and cell-derived membrane vesicles with artificial vesicles (Bahadori et al., 2018; Biner et al., 2016; Moreno-Pescador et al., 2023). This could potentially be used in plant biology to study membrane proteins in controlled lipid environments by fusing e.g. suspension cell culture cells or protoplast with GUVs. Bending rigidity and other mechanical properties of lipid membranes can be measured using GUV-based techniques such as fluctuation spectroscopy, micro-aspiration and optical manipulation (Dimova, 2019; Dimova, 2014; Garten et al., 2017; Prévost et al., 2017; Solmaz et al., 2012; Yamada et al., 2014).

Multilamellar vesicles (MLVs), as used by Beck et al., are particularly valuable for Solid-state deuterium NMR spectroscopy (^{2}H NMR) studies of sterol-membrane interactions because their multiple bilayers provide a high lipid concentration needed for good signal-to-noise ratio (Beck et al., 2007). ^{2}H NMR provides unique insights into sterol-membrane dynamics by measuring order parameters and molecular motions in model membrane systems (Léonard and Dufourc, 1991).

Supported lipid bilayers (SLBs), where a lipid bilayer is deposited onto a solid support, represent another valuable model membrane system for sterol research (Castellana and Cremer, 2006). Unlike freely suspended vesicles, SLBs enable the use of surface-sensitive analytical techniques such as atomic force microscopy, quartz crystal microbalance with dissipation monitoring, and various spectroscopic methods (Bar et al., 2023; Lee and Bain, 2005; Lv et al., 2018). Recent advances in SLB fabrication methods, particularly the solvent-assisted lipid bilayer (SALB) technique, have enabled the creation of sterol-rich bilayers containing up to ~60 mol% sterol content - far exceeding what is possible with traditional vesicle fusion approaches (Jackman and Cho, 2020). This allows SLBs to more accurately mimic the sterol-enriched domains found in biological membranes while maintaining the ability to precisely control composition. The planar geometry and stability of SLBs also make them particularly well-suited for studying how sterols influence membrane organization, protein-lipid interactions, and the formation of ordered membrane domains under carefully controlled conditions (Legrand et al., 2023).

4. Future perspectives in the field of membrane sterols

While sterols are major constituents of the cell surface, the extent of their influence on cellular processes remains largely unknown. Recent advances in imaging and lipid quantification could bring a fresh perspective. We think that some of the main future research directions in plant sterol biology could include:

1. The role of membrane sterol in plant development and physiology:

Clarifying the interconnection between the BR and sterols biosynthetic pathways would help better manipulate membrane sterol composition and thus understand its importance for plant development. Furthermore, a comprehensive analysis of sterol composition and dynamics in different plant tissues and developmental stages, using advanced mass spectrometry techniques coupled with subcellular fractionation would help elucidating the exact PM sterol composition during cell division, growth and differentiation. Moreover, the development of more specific and less disruptive probes for visualizing sterol dynamics in plant membranes would allow us to understand the role of sterol during cell differentiation events that require membrane partitioning (root hair growth, pollen tube growth, xylem formation etc.), or during responses to developmental signal or environmental cues.

2. The mechanisms of sterol-enriched nanodomain formation:

Investigating the influence of sterol composition on the biophysical properties of plant membrane will be primordial to further understand the mechanism behind sterol-enriched nanodomain formation. In addition, further investigation into the subcellular localization of sterol biosynthesis enzymes, particularly during cell division, is needed to elucidate if those enzymes directly participate to regulation of the spatial distribution of sterol at the plasma membrane.

3. The importance of sterol-enriched nanodomain for cell signaling:

Mechanistic studies to understand how sterol membrane organization is coordinated to cytoskeleton organization and cell wall synthesis will help elucidating the regulation of plasma membrane protein diffusion and subsequent signaling, including the potential role of membrane sterol on BR signaling.

In conclusion, while significant progress has been made in understanding plant sterols, many challenges and open questions remain. The continued development of new tools and techniques, combined with interdisciplinary approaches, will be crucial for advancing the knowledge of these essential membrane components and their diverse roles in plant biology.

CRediT authorship contribution statement

Paul Vogel: Writing – review & editing, Writing – original draft, Visualization. **Staffan Persson:** Writing – review & editing, Writing – original draft, Supervision. **Guillermo Moreno-Pescador:** Writing – review & editing. **Lise Noack:** Writing – review & editing, Writing – original draft, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tcsw.2025.100147.

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