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Review

# Emerging roles for conjugated sterols in plants

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ABSTRACT

In plants, sterols are found in free form (free sterols, FSs) and conjugated as steryl esters (SEs), steryl glycosides (SGs) and acyl steryl glycosides (ASGs). Conjugated sterols are ubiquitously found in plants but their relative contents highly differ among species and their profile may change in response to developmental and environmental cues. SEs play a central role in membrane sterol homeostasis and also represent a storage pool of sterols in particular plant tissues. SGs and ASGs are main components of the plant plasma membrane (PM) that specifically accumulate in lipid rafts, PM microdomains known to mediate many relevant cellular processes. There are increasing evidences supporting the involvement of conjugated sterols in plant stress responses. In spite of this, very little is known about their metabolism. At present, only a limited number of genes encoding enzymes participating in conjugated sterol metabolism have been cloned and characterized in plants. The aim of this review is to update the current knowledge about the tissue and cellular distribution of conjugated sterols in plants and the enzymes involved in their biosynthesis. We also discuss novel aspects on the role of conjugated sterols in plant development and stress responses recently unveiled using forward- and reverse-genetic approaches.

## 1. Introduction

Sterols are essential components of eukaryotic cell membranes that determine their physicochemical properties and, consequently, their biological function [1]. However, important new roles for sterols have been discovered in recent years. For instance, sterols interact with sphingolipids in the plasma membrane (PM) to form liquid-ordered microdomains (lipid rafts) that are involved in different biological processes [2,3]. Sterols also serve as precursors for the biosynthesis of brassinosteroids, a group of hormones that are essential for the regulation of plant development and morphogenesis [4]. Besides these functions, sterols are also involved in plant growth and development [5–13] as well as in plant responses to biotic and abiotic stresses [14–22].

The common structure of sterols is based on the cyclopentane-perhydrophenanthrene ring system (formed by four rigid rings) with a hydroxyl group at position C3 and a lateral chain of variable length (8–10 carbons) attached to carbon 17 (Fig. 1). In contrast to animals and fungi, plants produce a complex mixture of sterols that mainly differ in the nature of the side chain at position C17 and the number and position of double bonds in the rings or the lateral chain. β-Sitosterol, stigmasterol and campesterol (Fig. 1) are the most abundant sterols found in plants. Whereas β-sitosterol and stigmasterol have a major role in maintaining the structure and function of cell membranes, campesterol acts as the precursor of brassinosteroids [6,7]. Aspects related with the chemistry and biosynthesis of plant sterols have extensively been reviewed in recent years [23–26].

In plants, sterols are present not only as free sterols (FS) but also conjugated in form of steryl esters (SEs), steryl glycosides (SGs) and acyl steryl glycosides (ASGs) (Fig. 2). In SEs the hydroxyl group at C3 position is esterified with a fatty acid. SGs are characterized by having a sugar linked to the C3 hydroxyl group of the sterol moiety through a β-glycosidic bond. ASGs are derivatives of SGs in which the hydroxyl group of the C6 position of the sugar moiety is esterified with a fatty acid. The structural diversity of SEs, SGs and ASGs has extensively been reviewed [23,27–29]. In a restricted number of plants (mainly cereals) sterols are also found conjugated in form of steryl ferulates [30,31]. Ferulic acid is a hydroxylated derivative of cinnamic acid, a metabolic intermediate in the phenylpropanoid pathway. Because steryl ferulates seem to be present only in a limited number of plant species, studies

*Abbreviations:* ASG, acyl steryl glycoside; DIM, detergent-insoluble membrane; ER, endoplasmic reticulum; FS, free sterol; LD, lipid droplet; SE, steryl ester; SG, steryl glycoside; PM, plasma membrane

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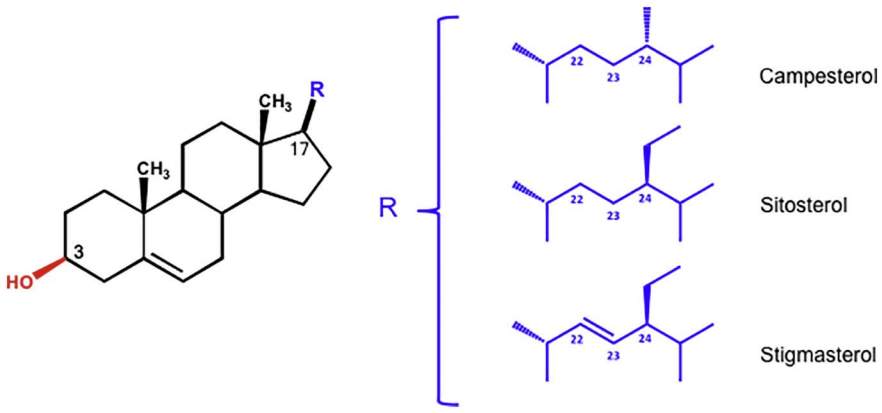


**Fig. 1.** Chemical structure of the major plant free sterols.

related with this type of conjugated sterols are very scarce.

In contrast to the increasing evidences supporting a relevant role of SEs, SGs and ASGs in a variety of cellular and biological processes in plants very little is known about their metabolism. This review provides an overview of the tissue and cellular distribution of these conjugated sterols and summarizes the knowledge about the genes and the corresponding enzyme activities currently identified in plants for the biosynthesis of SEs and SGs. New information about the function of conjugated sterols derived from functional genomic studies targeting sterol acyltransferases and sterol glycosyltransferases is also discussed.

## 2. Steryl esters

### 2.1. Tissue and cellular distribution of plant steryl esters

SEs are found in all plant tissues. However, their relative content usually differ among organs and tissues and may change in response to developmental and environmental factors. The sterol moiety of SEs usually corresponds to the same sterols present in the FS fraction, but in some tissues they may contain unusual sterols as well as sterol biosynthetic intermediates. The fatty acids found in SEs cover a wide range of lengths (from C12 to C22), being palmitic, stearic, oleic, linoleic and linolenic acids the most common species [28]. The high structural diversity of the SEs present in plants highlights the relevance of the sterol esterification process regarding their specialized function in different plant tissues.

SEs are remarkably abundant in particular plant tissues such as the tapetal cells of anthers, pollen grains, seeds, and senescent leaves [28,32,33]. High levels of SEs have also been reported in mutant and transgenic plants overproducing sterols [34,35]. It has recently been reported that SEs are also present at relatively high levels in the phloem sap, being cholesterol the most abundant phytosterol in this fraction [36].

It is widely accepted that SEs perform a primary role in cell membrane sterol homeostasis [6]. However, in some plant tissues SEs also represent a storage pool of sterols that can be used during growth and development. For instance, seeds store high amounts of SEs that, together with triacylglycerols, are mobilized during germination to support seedling growth during the early stages of development [37]. This is in agreement with the increase of SE content during seed development [38–40], which in developing tobacco and rape seeds occurs in parallel with the synthesis of triacylglycerols [40].

Early cell fractionation studies in different plant tissues indicated that SEs were mainly enriched in mitochondrial, nuclear and microsomal fractions [28]. However, more recent reports have shown that, like in other eukaryotic organisms, plants accumulate SEs in cytoplasmic particles known as lipid droplets (LDs) [41]. LDs (also known in plants as oleosomes, lipid bodies and oil bodies) are evolutionary conserved organelles present in almost all organisms and cell types [42]. Like in yeast and mammals, plant LDs consist of a neutral lipid core surrounded by a membrane monolayer derived from the endoplasmic reticulum (ER) and are stabilized by proteins bound to the LD surface [41]. Evidences available so far support the view that LDs are generated at specific subdomains of the ER in which triacylglycerols and possibly SEs are actively synthesized [41,43]. The induction of LD formation in mutant and transgenic plants overproducing sterols [6] supports the role of SEs in promoting LD biogenesis.

The proteomic analysis of LDs from mammals and yeast has led to the identification of proteins involved in a variety of cellular processes, indicating that these subcellular structures may have multiple functions in addition to their role as storage of lipids [44]. In contrast, only a few LD associated proteins have been identified in plants [45]. The best characterized ones are oleosins, caleosins and steroleosins, which correspond to the most abundant proteins present in LDs isolated from seeds [41]. Despite the widespread occurrence of LDs in plants their associated proteins in vegetative tissues remain largely unknown. Recently, a new class of LD associated proteins (LDAPs) required for the regulation of neutral lipid compartmentation has been identified in non-seed cell types [46,47].

The observation that many LD proteins are related with lipid and

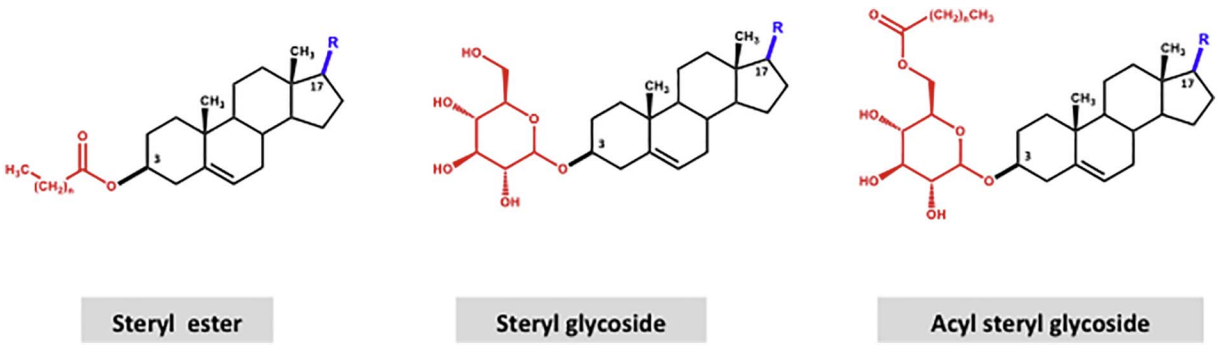


**Fig. 2.** Chemical structure of conjugated sterols.

sterol metabolism in mammals and yeast has reinforced the view that LDs may also have a relevant function in neutral lipid metabolism and membrane lipid homeostasis in plants [41,48]. In contrast, only two LD associated proteins related with sterol metabolism have been identified in plants: steroleosins, which have hydroxysteroid dehydrogenase activity and are likely involved in brassinosteroid metabolism [49,50] and the sterol biosynthetic enzyme Δ7-sterol-C5-desaturase, which is targeted to leaf lipid particles in addition to the ER [51].

SEs have been identified as a main structural component of the pollen coat of some *Brassica* species and are synthesized in the elaioplasts present in the tapetal cells of the anthers [32,33,52]. Nevertheless, a significant amount of SEs is also found in the intracellular domain of the pollen grain [32], suggesting that this SE pool could provide sterols for the synthesis of new membranes or to maintain sterol homeostasis during the active growth of the pollen tube. It has recently been reported that pollen from a variety of plant species show great differences in the composition of the SE fraction [53]. Moreover, the sterol composition of the SE fractions differs from that of the corresponding FS fraction. Thus, whereas Δ5-sterols (sterol end products) are predominant in the FS fraction, SEs are usually enriched in 9β,19-cyclopropyl sterol precursors [53]. The biological significance of the SE diversity found in pollen is currently unknown but has been related with specific plant-insect interactions or the optimization of the contact between the pollen grain and the stigma to promote germination [53,54].

In senescing tissues SEs have been proposed to participate in the cellular recycling and transport of sterols when FSs are released from the degenerating cell membranes [28,55]. In agreement with this tobacco leaves show a clear increase in SE levels which parallels a decrease in the FSs and ASGs levels during senescence [56]. The role of SEs in leaf senescence is also supported by the characterization of Arabidopsis mutant plants defective in SE biosynthesis [57].

### 2.2. Biosynthesis of steryl esters

Sterol acyltransferase activity (Fig. 3) has been detected in a variety of plant tissues and early studies suggested that the plant enzyme(s) were using acyl donors different from those previously described in mammals and yeast. In mammals, cellular cholesterol is esterified by the action of acyl CoA cholesterol acyltransferase (ACAT), that uses acyl-CoA as fatty acid donor [58], but blood cholesterol is esterified by a lecithin cholesterol acyltransferase (LCAT), which uses lecithin as acyl donor [59]. In *S. cerevisiae*, ergosterol is esterified by two isoforms of ACAT, ARE1 and ARE2 [60]. ACAT and LCAT are completely different proteins and their amino acid sequences have no significant similarity.

Early studies in plants showed that phosphatidylcholine and phosphatidylethanolamine were efficiently used as acyl donors in enzyme assays using microsomal preparations obtained from spinach leaves [61]. However, it was subsequently found that these phospholipids were rapidly hydrolyzed under the assay conditions and that 1,2-diacylglcerol was the true acyl donor in the reaction [62]. Sterol acyltransferase activity was also measured in tobacco leaf microsomal fractions using dipalmitoylglycerol as acyl donor [63]. Enzyme assays using cell-free extracts prepared from white mustard (*Sinapis alba*) roots showed that triacylglycerols were used as a source of fatty acids for sterol esterification [64,65]. Sterol acyltransferase activity using both triacylglycerols and diacylglycerols as acyl donors was also measured in *Zea mays* root extracts [66]. An alternative mechanism for the synthesis of SEs has been described in cell-free extracts of white mustard roots where a reversible trans-esterification process involving wax esters and FSs has been described in addition to the reported synthesis of SEs using triacylglycerols as acyl donors [67] (Fig. 4).

In contrast with all these biochemical data, the sterol acyltransferases currently characterized in plants after the cloning of the corresponding genes in Arabidopsis are a phospholipid:sterol acyltransferase (PSAT) and an acyl CoA:sterol acyltransferase (ASAT) (Fig. 4).

In Arabidopsis PSAT is encoded by the gene *PSAT1* (*At1g04010*) and was first characterized using microsomal fractions obtained from leaves of transgenic Arabidopsis plants constitutively overexpressing the *PSAT1* transcript [68]. The highest level of PSAT activity was found when phosphatidylethanolamine was used as acyl donor. The enzyme showed high preference for *sn-2* fatty acids (both saturated and unsaturated) and was able to acylate several sterols and sterol intermediates. The observation that PSAT1 did not use neutral lipids as acyl donor suggested that the polar head of phospholipids is a relevant factor in substrate recognition [68]. Although sterol intermediates were poor substrates for PSAT1, they were preferentially used when sterol end products (mainly sitosterol) were present in the enzyme reaction. This observation led to suggest the allosteric regulation of PSAT activity by sterol end products. Thus PSAT activity could modulate the level of FSs in the membrane through a mechanism involving the sequestration of biosynthetic intermediates in form of SEs. This is in agreement with the high enrichment of sterol precursors (mainly cycloartenol) in the SE fraction of mutant and transgenic plants overaccumulating sterols [34,35]. PSAT1 fused to GFP localizes in spherical structures of unknown identity that do not seem to correspond to LDs [69].

The Arabidopsis gene encoding ASAT (*At3g51970*) was cloned by Chen et al. [70] and the corresponding enzyme was characterized by expression of the *ASAT1* transcript in a *S. cerevisiae* mutant strain in which the two endogenous genes encoding ASAT (*ARE1* and *ARE2*) were disrupted. The characterization of the enzyme activity in cell extracts of the transformed yeast cells indicated that cycloartenol was the preferred acyl acceptor. Seeds from transgenic Arabidopsis overexpressing ASAT under the control of a seed-specific promoter showed an increase in total sterol content associated to an increase in the SE levels and a small reduction of FSs. Cycloartenol esters were highly increased at expenses of a significant decrease of campesterol and sitosterol esters. The SE fraction extracted from the transgenic seeds was enriched in saturated and long-chain fatty acids [70].

### 2.3. Genetic analysis of steryl ester function

The biological significance of the occurrence of two alternative enzymatic systems for the synthesis of SEs in plants has been investigated by Bouvier-Navé et al. [57] using Arabidopsis T-DNA insertion mutants defective in the expression of *PSAT1* and *ASAT1* genes. It was found that SE levels were strongly reduced (5- to 10-fold) in seeds of the *psat1-1* and *psat1-2* mutants but not in the *asat1-1* mutant, thus suggesting a major role of PSAT1 in the synthesis of SEs in seeds. However, *psat1-1*, *psat1-2* and *asat1-1* mutant showed a decrease in the level of SEs in leaves. In contrast, the SE levels in the flowers of all mutant lines were similar to those of wild type plants. These results point towards the existence of at least another sterol acyltransferase that remains to be identified in Arabidopsis. One possibility could be an enzyme phylogenetically related with PSAT or ASAT. The *PSAT1* gene is a member of the Arabidopsis PDAT/LCAT-like gene family composed by six genes (*At1g04010*, *At5g13640*, *At3g03310*, *At4g19860*, *At3g44830* and *At1g27480*) encoding proteins with similarity to human LCAT and lysosomal phospholipase A2 [71]. The genes *At5g13640*, *At3g03310* and *At4g19860* encode a phospholipid:diacylglycerol acyltransferase (PDAT) [71], a phospholipase A1 [72] and a phospholipase A2 [73], respectively. The protein encoded by *At3g44830* shows high similarity with PDAT and therefore it is unlikely to encode an enzyme with sterol acyltransferase activity. The remaining member of the PDAT/LCAT-like gene family corresponds to *At1g27480*, which encodes a protein showing high similarity to human LCAT. Surprisingly, no function for this protein could be determined after its expression in yeast [72].

Arabidopsis ASAT1 belongs to the superfamily of membrane-bound O-acyltransferases (MBOATs) [74]. When the fifteen members of the Arabidopsis MBOAT gene family were tested for complementation of a

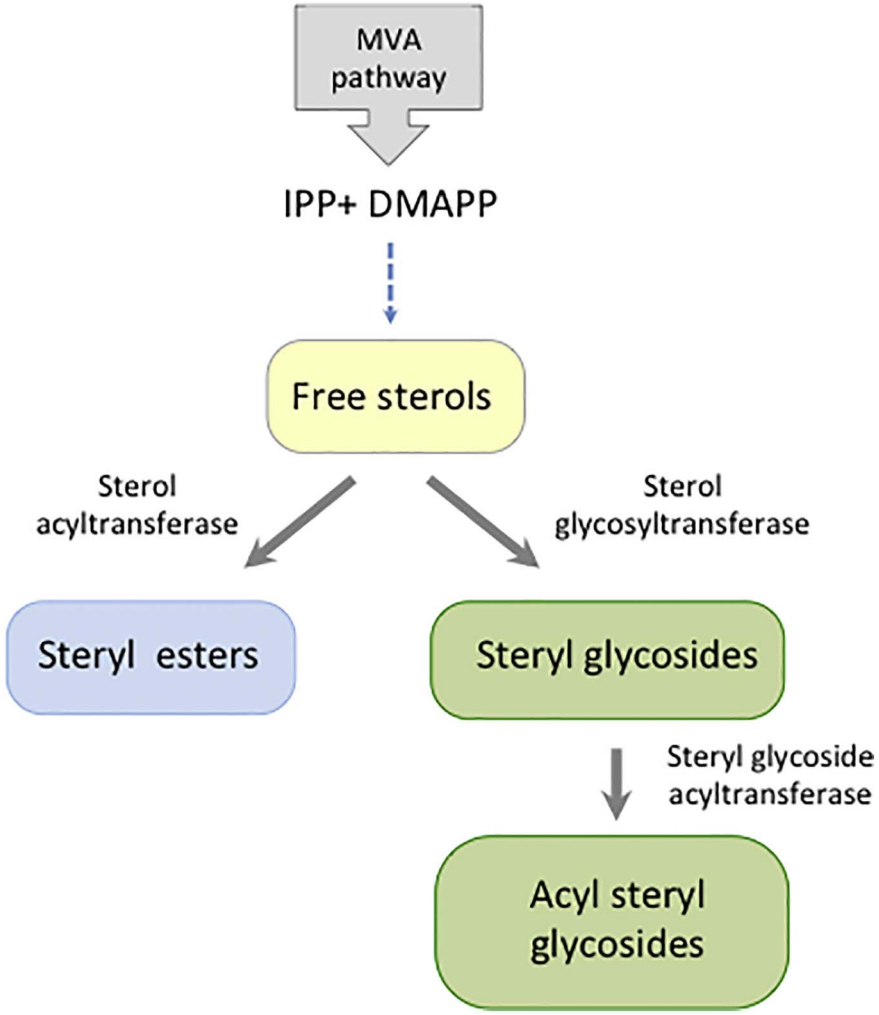


**Fig. 3.** Schematic representation of the biosynthesis of conjugated sterols showing the enzymatic activities involved. Free sterols derive from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) produced by the mevalonic acid (MVA) pathway [26]. The dashed arrow indicates multiple steps.

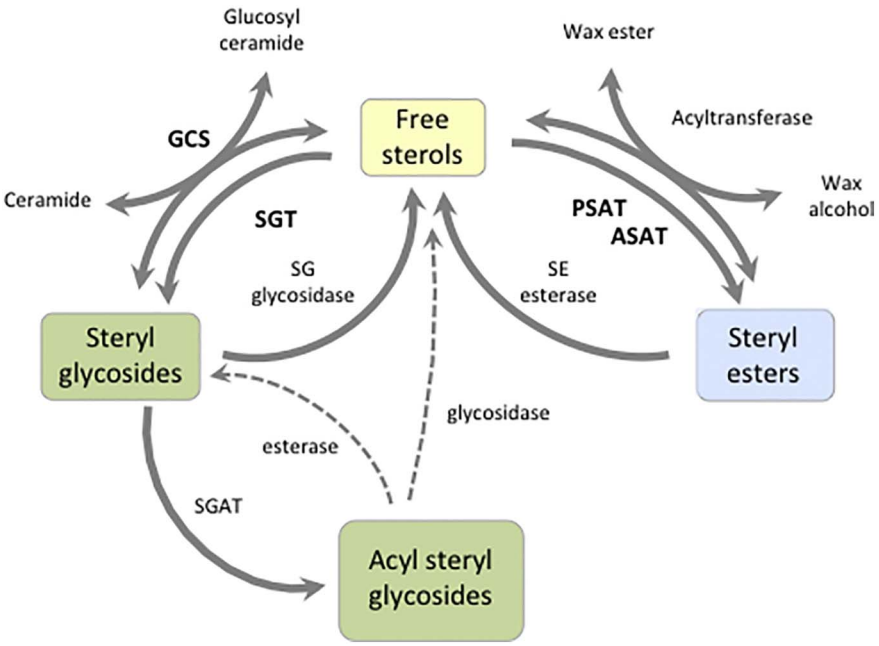


**Fig. 4.** Schematic overview of conjugated sterol metabolism in plants. Those enzymes for which the corresponding genes have been cloned are indicated in bold: ASAT, acyl-CoA:sterol acyltransferase; PSAT, phospholipid:sterol acyltransferase; SGT, UDP-glucose:sterol glycosyltransferase; SGAT, steryl glycoside acyltransferase; GCS, glucosylceramide synthase. Dashed arrows indicate enzymatic conversions not confirmed at experimental level.

yeast strain defective in SE biosynthesis only the expression of the protein encoded by *At3g51970* (ASAT1) was able to restore SE biosynthesis [70]. These results indicate that ASAT1 is the only member of the Arabidopsis MBOAT gene family having ASAT activity.

As mentioned above, several reports have shown sterol acyltransferases activity in plants using triacylglycerols and diacylglycerols as acyl donors. However, neither ASAT1 nor PSAT1 can efficiently use these compounds as fatty acid donors [68,70]. Therefore it is likely that plants could contain other genes encoding at least one sterol acyltransferase different from PSAT and ASAT.

The involvement of PSAT1 and ASAT1 in sterol homeostasis has been evaluated in the Arabidopsis *psat1-1*, *psat1-2* and *asat1-1* mutant lines. Feeding experiments using mevalonolactone and squalene (metabolic precursors known to enhance the metabolic flux towards sterols) revealed that *psat1-1* and *psat1-2* plants were highly sensitive to these compounds, whereas only minor effects were observed in the *asat1-1* plants [57]. This was explained by a limitation of the PSAT1 defective lines to transform the overproduced sterols into SEs. These results are in agreement with a major contribution of PSAT1 in maintaining FS homeostasis in plant cell membranes [57]. Furthermore, the observation that PSAT1 is activated by sterol end products [68] has led to suggest that this enzyme could also act as a cellular sensor for FS levels in the membrane [57].

The only visible phenotype associated with reduced levels of SEs was observed in the *PSAT1* defective mutants, which showed early leaf senescence [57]. Whereas SE levels increased about three-fold in detached leaves of wild type plants only minor changes were observed in the leaves of *psat1-1* and *psat1-2* plants. This is in accordance with the increase of PSAT1 transcript levels detected in the detached leaves of wild type plants. However, no changes were observed in the level of the *HMGR1* and *ASAT1* transcripts. The lack of induction of the *HMGR1* gene (which encodes HMG-CoA reductase, a key regulatory enzyme in sterol biosynthesis [75]) during the aging period suggests that the increase of SE levels in the senescent leaves derives from the endogenous recycling of membrane sterols.

Taken together, current data derived from the characterization of sterol acyltransferase defective mutants in Arabidopsis highlight a major involvement of PSAT1 in the synthesis of SEs and FS homeostasis and raise the question about the real contribution of ASAT1 in SE metabolism.

## 3. Glycosylated sterols

### 3.1. Tissue and cellular distribution of steryl glycosides and acyl steryl glycosides

As described above for SEs, glycosylated sterols (SGs and ASGs) are also widely distributed in plants and their relative levels differ among tissues and may change in response to developmental and environmental cues. SGs and ASGs usually have a sterol composition similar to that of the corresponding FS fraction [27]. Glucose is the predominant monosaccharide found in plant glycosylated sterols, but conjugation with other monosaccharides such as galactose, xylose and mannose have also been described [27,29,76]. Unusual SGs containing up to four sugar residues have been reported [77]. ASGs usually contain saturated and unsaturated C16 and C18 fatty acids, but some plants accumulate ASGs with atypical fatty acid chains [27].

SGs and ASGs are usually minor components of the total sterol fraction present in most plant species [27,78]. However, a remarkable exception is provided by plants of the genus *Solanum*, which show a very high content of glycosylated sterols [78,79]. Thus, whereas in most plant tissues the amount of SGs and ASGs is relatively low (usually less than 20% of total sterols), the SG + ASG fraction of tomato fruit may account for more than 85% of total sterols [80]. Although the evolutionary and biological significance of the high content of glycosylated sterols in a restricted number of plant species is not know with certainty, such a high content of glycosylated sterols in *Solanum* species has been suggested to play a role in protecting cell membrane integrity against the disruptive effect of the high levels of steroidal glycoalkaloids present in these plant species [81–83].

Along with phospholipids and sphingolipids, sterols represent a main component of the lipids present in the PM (20–50% of total lipids). In contrast to mammals and yeasts, the plant PM contains SGs and ASGs in addition to FSs. The analysis of the sterol composition of PM fractions from different plant tissues and growth conditions has revealed striking differences in the relative amount of free and glycosylated sterols [84]. Thus, while FSs represent the most abundant sterol in the PM of most plant species (with values higher than 90% in some cases), in oat and some *Solanum* species, the major sterols found in the PM correspond to ASGs (up to 78% in potato leaves) [84].

It is known that the presence of a sugar moiety attached to the sterol backbone alters its physicochemical properties. Because of this, the presence of SGs and ASGs in plant cell membranes raises the question about how are they organized in the lipid bilayer and the way in which they interact with other membrane lipids to modulate membrane function. SGs are likely oriented with the sterol moiety buried in the hydrophobic core of the lipid bilayer and the sugar located in the plane of the polar head groups of the membrane. In the case of ASGs, both the sterol moiety and the fatty acid chain are probably embedded in the hydrophobic phase of the membrane with the sugar oriented to the hydrophilic surface [84].

The classical model of PM organization suggests that sphingolipids associate among them through interactions between the polar heads and their long and saturated acyl chains, whereas sterols fill voids between the fatty acid chains of phospholipids and sphingolipids. Studies on the effect of plant sterols in modulating membrane properties have shown that β-sitosterol and stigmasterol, which only differ by a double bond at position C22 in stigmasterol (Fig. 1), behave in a very different way [85]. Thus it is likely that changes in the level and relative ratios of FSs, together with the presence of glycosylated sterols, may have a strong influence on the physicochemical properties and function of the cell membranes. Recent studies have shown that glycosylated sterols, alone and in synergy with FSs, have a major role in modulating plant PM organization [86]. Taken together, these observations suggest that specific changes in the relative level and profile of free and glycosylated sterols in the PM may be relevant in the adaptive responses of plants to environmental changes.

Over the last decades the classical view of cell membrane organization, in which the inserted proteins and lipids could diffuse freely (fluid mosaic model), has changed to a model based on a dynamic segregation of membrane components (lipids and proteins) to form microdomains which exhibit a composition, structure and biological function different from the surrounding membrane [2,87]. These membrane microdomains (also known as membrane or lipid rafts) have been involved in a variety of biological processes such as cell-to-cell interactions, membrane transport, protein trafficking, signal transduction, stress responses and polarized growth [2,3].

Lipid rafts are mainly composed by sphingolipids, sterols and proteins and their formation relies on the physicochemical properties of sphingolipids and sterols that allow the formation of liquid-ordered domains that promote the clustering of specific proteins at defined regions of the cell membrane [3]. PM microdomains have been characterized at biochemical level because of their insolubility in non-ionic detergents at cold temperatures. This feature allows the isolation of membrane fractions called detergent-insoluble membranes (DIMs) or detergent-resistant membranes (DRMs). Plant DIMS are enriched in sterols and sphingolipids. The sterol composition of PM and DIM samples isolated from different tissues of a variety of plant species has revealed a general enrichment of free and glycosylated sterols [88,89], thus highlighting the contribution of SGs and ASGs in the organization of plant PM microdomains.

In addition to lipids, DIMs also contain proteins. The proteomic

analysis of plant DIMs has shown that their protein profiles are different from that of the whole PM. The DIM associated proteins identified so far suggest their involvement in a variety of cellular processes [3,87,90,91]. It has also been reported that the DIM proteome of some plants dynamically respond to external stimuli [90]. Thus, it is likely that the enrichment of free and glycosylated sterols in DIMs represents an important factor in the recruitment of specific proteins into particular membrane microdomains during plant development and in response to environmental challenges.

Like in animal cells, the lipids of the plant PM also show an asymmetrical distribution between the cytoplasmic and the apoplastic leaflets [84]. The asymmetric distribution of lipids in the membrane bilayer affects the biophysical properties of cell membranes and influence numerous cell functions [84]. Free and glycosylated sterols are among the membrane lipids having an asymmetrical distribution in the plant PM [92,93]. Labeling studies using filipin (a fluorophore that specifically reacts with sterols) has revealed an enrichment of sterols (about 3-fold) in the apoplastic leaflet of the PM [92]. However, the relative distribution of FSs, SGs and ASGs between the cytoplasmic and the apoplastic leaflets has not yet been established. The mechanisms underlying the asymmetrical distribution of free and glycosylated sterols in the PM is not clear but could be associated to the interconnection of SG and glucosylceramide metabolism in the membrane [94] (Fig. 4).

In addition to their relevance in cell membranes, glycosylated sterols have been described in other plant tissues and cell compartments playing different roles. Glycosylated sterols are present in the pollen grain of Arabidopsis and have shown to be critical for pollen fitness by supporting pollen coat maturation [95].

Glycosylated sterols also represent the major sterol fraction found in the phloem sap, being cholesterol the dominant sterol form [36]. The biological significance of the sterols present in the phloem sap remains to be elucidated but may be relevant in physiological and ecological aspects related with phloem-feeding insects.

SGs have since long been proposed to act as primers for the synthesis of cellulose [96]. However the participation of SGs in cellulose biosynthesis is still a controversial matter [97].

### 3.2. Biosynthesis of glycosylated sterols

The synthesis of SGs is catalyzed by sterol glycosyltransferases (Fig. 3). In this reaction a sugar, mostly glucose, is transferred to the C3-hydroxyl group of the sterol backbone [76,98]. Sterol glycosyltransferase activity has been determined in different plant species and found to be associated to cell membranes [99–103]. However, cytosolic sterol glycosyltransferases have been identified in *Withania somnifera* [104] and cotton [105]. Biochemical studies using partially purified enzyme preparations revealed that sterol glycosyltransferase activity was enhanced by negatively charged phospholipids [100,103,106]. Although the mechanism underlying this activation was not elucidated this observation suggests that sterol glycosyltransferase activity could be modulated *in vivo* by changes in the lipid environment of the cell membrane. It has been proposed that plants can also synthesize SGs by the action of the enzyme UDP-glucose-dependent glucosylceramide synthase [107] (Fig. 4).

The first plant sterol glycosyltransferase cDNAs were cloned in oat and Arabidopsis by Warnecke et al. [108]. In recent years, other sterol glycosyltransferase cDNAs have been cloned in *Withania somnifera* [109,110], *Gymnema sylvestre* [111] and cotton [105]. Functional studies using the recombinant enzymes expressed in *E. coli* demonstrated that a general feature of the plant sterol glycosyltransferases characterized so far is the highest preference for UDP-glucose as a sugar donor and the capacity to use a wide spectrum of sterols as sugar acceptors [76,105,108–111].

Two genes encoding sterol glycosyltransferases (*At3g07020* and *At1g43620*) have been identified in Arabidopsis. They encode sterol 3-β-glycosyltransferases UGT80A2 and UGT80B1 respectively (http://www.p450.kvl.dk/At_ugts/family.shtml) and have been designated as *UGT80A2* and *UGT80B1* [112]. A recent report has shown that the related protein encoded by the gene *UGT713B* (*At5g24750*) does not have sterol glycosyltransferase activity [113]. *UGT80A2* and *UGT80B1* are differentially expressed in distinct plant organs and tissues [112] and their transcript levels are coordinately down-regulated short after seed imbibition [113].

Concerning ASG biosynthesis, steryl glycoside acyltransferase activity (Fig. 3) has been measured in cell free extracts from a variety of plant tissues [114–122]. In addition, partially purified enzyme preparations were obtained and characterized from carrot roots [123] and eggplant leaves [124]. These studies unveiled a complex scenario concerning the properties of the enzymes involved in ASG biosynthesis as well as the nature of the acyl-donors used by these enzymes. At present, no genes encoding steryl glycoside acyltransferases have yet been identified in any organism producing ASG, including animals, algae, fungi and bacteria. Therefore, the understanding of plant ASG biosynthesis has to wait until the identification and cloning of the genes encoding the corresponding enzymes.

### 3.3. Genetic analysis of steryl glycoside function

The characterization of Arabidopsis T-DNA insertion mutants defective in the *UGT80A2* and *UGT80B1* genes has provided interesting clues about the role of steryl glycosides in plants. Arabidopsis mutants *ugt80A2, ugt80B1* and the *ugt80A2,B1* double mutant are viable and fertile [112,113]. However, the *ugt80B1* mutant displays an array of phenotypes that are particularly pronounced in the embryo and seed [112]. The most remarkable feature of *UGT80B1* defective plants is the transparent testa phenotype and the reduction of seed size. Actually, the mutation *ugt80B1* was shown to be allelic to *transparent testa 15* (*tt15*) [112]. In the *ugt80A2,B1* double mutant background, the *UGT80B1* deficiency is also associated to other seed related defects such as small embryo size, defects in flavonoid deposition and loss of suberization [112]. In contrast, *UGT80A2* defective plants show a small reduction of seed size but not the transparent testa phenotype. At the seedling stage, *ugt80A2 and ugt80B1* mutants show a reduced elongation of the root, a phenotype that is clearly enhanced in the *ugt80A2,B1* double mutant [112]. The *ugt80A2,B1* double mutant is also affected in pollen coat maturation [95]. Neither the single *ugt80A2* and *ugt80B1* mutants nor the *ugt80A2,B1* double mutant show differences with respect to wild type plants in cold acclimation experiments [112].

The sterol analysis of *UGT80A2* and *UGT80B1* defective plants has provided valuable information about the biological significance of the functional redundancy of plant sterol glycosyltransferases. Leaf, stem and inflorescence/silique samples show a strong reduction of the SG + ASG contents in all the defective lines, although the largest effect is found in the *ugt80A2,B1* double mutant [112]. Unexpectedly, the FS + SE content was significantly increased in the inflorescence/silique samples but not in leaves and stems, thus suggesting the operation of a regulatory network in particular tissues affecting the homeostasis of free and conjugated sterols. In all cases, sitosterol and campesterol were the most abundant sterols found in the SG + ASG fraction [112]. Altogether, these results indicate that UGT80A2 and UGT80B1 have a similar contribution to the production of glycosylated sterols in vegetative and reproductive tissues. However, a more complex scenario was revealed when sterol profiling was focused on seeds [113]. It was found that the total SG content was strongly reduced in the seeds of *ugt80A2* and *ugt80A2,B1* plants but not in *ugt80B1* plants, thus suggesting a major role of UGT80A2 in the synthesis of SGs in seeds. Surprisingly, ASG levels were not significantly affected in the seeds of *ugt80A2* and *ugt80B1* plants but were decreased in the *ugt80A2,B1* double mutant. These results unveil an unexpected combined role of UGT80A2 and UGT80B1 in the biosynthesis of ASGs, at least in seeds.

A detailed analysis of the sterol composition of SG and ASG fractions of seeds from *ugt80A2* and *ugt80B1* mutants, together with the

biochemical characterization of the recombinant UGT80A2 and UGT80B1 proteins, uncovered differences in the substrate specificity of these enzymes towards the major plant sterols [113]. Thus, whereas UGT80A2 activity seems to account for most of the β-sitosteryl and stigmasteryl glycoside production in seeds, UGT80B1 preferentially forms brassicasteryl esters. These differences in substrate specificity have been related with the fact that UGT80A2 represents the more ancient UGT80 enzyme in plants, whereas UGT80B1 may have arisen during the evolution of vascular plants to play a more specialized role in the synthesis of SGs [113].

It is surprising that the phenotypes observed in the seeds of the *ugt80B1* mutant occur in the absence of significant changes in SG and ASG levels [113]. Thus it is likely that the seed phenotypes associated to UGT80B1 deficiency may derive from minor changes affecting the composition of SG and/or ASG rather than the overall ratios of SG and/or ASG in the total sterol fraction. Since SG and ASG are components of lipid rafts it is likely that alterations in the sterol profile of the PM could modify its function in specific cell types. Alternatively, UGT80B1 could be implicated in the synthesis of an as yet unidentified sterol glycoside relevant for seed development.

It is worth noting that the *ugt80A2,B1* double mutant has residual levels of SG and ASG [112,113,125]. This suggests the presence of additional enzymes involved in the synthesis of glycosylated sterols in Arabidopsis. Such enzyme activity could be provided, at least in part, by glucosylceramide synthase, which has been shown to glycosylate sterols *in vitro* [107]. The characterization of Arabidopsis mutants defective in *At2g19880*, the only gene encoding glucosylceramide synthase in Arabidopsis, has revealed an essential role for this enzyme in cell-type differentiation and organogenesis [126]. The fact that *At2g19880* null mutants show seedling lethality makes difficult to evaluate the hypothetical contribution of glucosylceramide synthase in the synthesis of steryl glycosides *in vivo*. The existence of other enzymes having sterol glycosyltransferase activity cannot be ruled out.

## 4. Conjugated sterols and plant stress responses

As indicated in Section 3.1, changes in the relative composition of sterols in cell membranes affect their biophysical properties and hence their biological functions. Because of this, it has been assumed that sterols play a prominent role in plant stress responses. Concerning conjugated sterols, there are many reports correlating changes in their profile with specific responses to different types of stress [69,105,127–141]. However, the biological basis underlying the role of conjugated sterols in plant stress is just starting to be uncovered.

The biochemical characterization of the Arabidopsis chilling-sensitive mutant *chs1* correlated this phenotype to an increase in SE levels [129]. However, it could not be established whether the increase in SE levels was a direct consequence of the *chs1* mutation or a secondary response associated to chilling injury. It has recently been reported that the *chs1* mutant is affected in the CHS1 gene (*At1g17610*), which encodes a TIR-NB type protein that induces different defense responses under chilling stress [142]. It has also recently been described that impaired SE synthesis in Arabidopsis is associated with the resistance to *Phytophtora infestans* [69]. However, the fact that the decrease in the SE levels, derived from a loss of function of PSAT1, is associated to unexpected overall changes in glycosylated sterol levels (increase of SGs and decrease of ASGs) does not allow to conclude whether the resistance phenotype is due to a decrease in SE levels or to changes in the glycosylated sterol levels.

With regard to glycosylated sterols, different reports have shown the induction of genes encoding sterol glycosyltransferases in *W. somnifera* and cotton in response to high and low temperatures [104,105,110]. The differential expression of sterol glycosyltransferase isoforms in *W. somnifera* suggested their specific functional recruitment in response to particular environmental challenges [110]. Furthermore, the rapid increase in the transcript level of some members of the sterol glycosyltransferase gene family in response to MeJA or SA treatments has led to propose their role in both biotic and abiotic stresses [110].

The role of SGs in stress responses has been further established using forward- and reverse-genetic approaches in different plant species. In Arabidopsis it has been reported that a T-DNA insertion mutant defective in the UGT80B1 gene shows increased sensitivity to cold and heat stress [143]. Furthermore, transgenic Arabidopsis plants overexpressing WsSGTL1, a sterol glycosyltransferase from *W. somnifera* [109], show increased salt, heat and cold tolerance [144]. Salt stress tolerance was also observed in transgenic tobacco plants overexpressing WsSGTL1 [145], while the overexpression of this gene in *W. somnifera* led to enhanced tolerance to cold [146]. In both cases, the plants overexpressing WsSGTL1 also presented enhanced resistance against the insect *Spodoptera litura* [146]. It has also been reported that down-regulation of sterol glycosyltransferase in *W. somnifera* results in increased susceptibility to the fungal pathogen *Alternaria alternate* [147] and heat stress [148]. However, it is possible that the changes in biotic stress tolerance reported in *W. somnifera* could be due not only to changes in SG and ASG levels but rather to changes in the level of whitanosides, a type of glycosylated sterol-related secondary metabolite present in this plant species [146,147].

## 5. Hydrolysis of conjugated sterols

Sterol homeostasis in eukaryotic cells relies on the availability of enzymatic systems catalyzing the interconversion of free and conjugated sterols. This is well documented in *S. cerevisiae* where sterol homeostasis is achieved through the operation of a complex regulatory network involving the sterol acyltransferases ARE1 and ARE2 acting in concert with the steryl ester hydrolases Yeh1p, Yeh2p and Tgl1p [149]. At present, little is known about SE hydrolases in plants. SE hydrolase (esterase) activity has been measured in microsomal fractions of tobacco plants [63] and the enzyme has been partially purified from roots of *S. alba* [150]. However, no genes encoding SE esterases have yet been identified in plants. Searches of plant genome databases using yeast SE hydrolases Yeh1p, Yeh2p and Tgl1p as a bait retrieve proteins with significant similarity to the yeast enzymes, thus suggesting the existence of genes encoding similar enzymes in plants (Fig. 4).

Glycosidase activities able to hydrolyze SGs have been reported in plants (Fig. 4). A membrane bound steryl β-glycoside hydrolase was purified from *S. alba* seedlings by Kalinowska and Wojciechowski [151]. This enzyme activity was later shown to be enhanced by zwitterionic phospholipids (such as phosphatidyl choline and phosphatidyl ethanolamine), suggesting that steryl β-D-glycoside hydrolase activity may be influenced by the membrane lipid environment [152]. Genes encoding steryl β-glucoside hydrolases have recently been identified in the yeasts *Cryptococcus neoformans* and *Saccharomyces cerevisiae* [153,154]. However, in contrast to SE hydrolases, no orthologs of the *S. cerevisiae* EGH1 gene (encoding ergosteryl-β-glucosidase Egh1) seem to be present in plant genomes.

To the best of our knowledge no hydrolase activities (either esterases or glycosidases) acting on ASGs have yet been reported in plants (Fig. 4). However, it has been shown that mammalian digestive enzymes such as cholesteryl ester hydrolase (also known as lysosomal acid lipase) and pancreatin (a mixture of pancreatic enzymes) are able to hydrolyze the fatty acyl moiety of ASGs [155]. No hydrolase activity acting on SGs was detected in these enzyme preparations. Interestingly, the gene encoding mammalian cholesteryl ester hydrolase (LIPA) is conserved in many eukaryotic organisms, including plants. Thus, it is likely that the plant orthologs of the mammalian LIPA gene could encode enzymes involved in ASGs deacylation. The SGs formed from the deacylation of ASGs could eventually be converted to FSs by the action of SG glycosidases (Fig. 4).

## 6. Conclusions and future directions

Conjugated sterols are ubiquitously present in plants and are known to be involved in a variety of biological processes. However, the biochemical, molecular and cellular bases underlying the role of conjugated sterols in plants have just started to emerge in recent years. The cloning of genes encoding sterol acyltransferases and sterol glycosyltransferases in Arabidopsis has been a major achievement to identify the nature of the enzymes involved in the biosynthesis of SEs and SGs and allow their biochemical characterization. Furthermore, forward- and reverse-genetic approaches targeting these genes in different plant species has also provided new insights into the role of conjugated sterols. However, current data support the existence of additional enzymes involved in the biosynthesis of SEs and SGs that remain to be identified. In addition, no genes encoding enzymes participating in ASG biosynthesis or implicated in the hydrolysis of conjugated sterols in plants have yet been described. Therefore, the identification of the full set of genes/enzymes participating in conjugated sterol metabolism in model plant species represents a major milestone to undertake functional genomics approaches to fully elucidate the role of conjugated sterols in plants. Another challenging aspect is to define the subcellular compartments involved in the metabolism of conjugated sterols and the mechanisms involved in their intracellular trafficking during plant growth and development and in response to environmental challenges.

## Conflict of interest statement

The authors declare that they have no conflicts of interest with the contents of this review.

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