Abstract

Isoprenoids represent the oldest class of known low molecular-mass natural products synthesized by plants. Their biogenesis in plastids, mitochondria and the endoplasmic reticulum–cytosol proceed invariably from the C5 building blocks, isopentenyl diphosphate and/or dimethylallyl diphosphate according to complex and reiterated mechanisms. Compounds derived from the pathway exhibit a diverse spectrum of biological functions. This review centers on advances obtained in the field based on combined use of biochemical, molecular biology and genetic approaches. The function and evolutionary implications of this metabolism are discussed in relation with seminal informations gathered from distantly but related organisms. © 2005 Elsevier Ltd. All rights reserved.

Keywords: Isoprenoids; Carotenoid; Tocopherol; Phylloquinone; Plastoquinone; Chlorophyll; Phytosterol; Prenyl derivatives; Enzymes; Genes; Cellular compartmentation

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1. Introduction

It has been estimated that 15–25% of plant genes are dedicated to plant secondary metabolism pathways [1]. The latter comprise three major class of products including isoprenoids, alkaloids and flavonoids. When considering the presence of these products among different taxa, it is apparent that the isoprenoid pathway is the oldest acquisition. In supporting this idea, one could note that plant dihydroflavonol reductase, the first specific enzyme of anthocyanin biosynthesis has extensive homology with isoprenoid dehydrogenases [2]. Isoprenoids are compounds built up of simple or multiple C5 units and are divided into different subgroups synthesized in different plant cell compartments including plastids, mitochondria and the endoplasmic reticulum–cytosol (Fig. 1). Their synthesis is often tightly linked to the function, differentiation state and organization of the compartment with which they are associated.

Here, we review our current understanding of plant isoprenoid biogenesis integrating biochemical, molecular and functional data. The metabolic engineering will not be covered, as this was comprehensively addressed [3]. For information concerning plant protein prenylation, readers are referred to previous reviews [4,5].

2. Biogenesis of IPP and DMAPP building blocks

2.1. Plastid and cytosolic pathways

It is now well established that in cyanobacteria and plants, isopentenyl diphosphate (IPP) is synthesized in the plastids through 1-deoxy-D-xylulose-5-phosphate (DXP) pathway and in the cytosol through the mevalonic acid (MVA) pathway according to the pathways outlined in Fig. 2 (for a review, see [6–10]). It is worth noting that alga belonging to the chlorophyta have apparently lost the mevalonic pathway and use exclusively the DXP pathway [11]. Similarly, the malaria parasite Plasmodium and other apicomplexa synthesize their isoprenoids from the DXP pathway in the non-green plastid-type apicoplast [12]. All plant genes encoding enzymes of the DXP and MVA pathways have been cloned and functionally analyzed. It has been observed that the plant genes of the DXP pathway are more phylogenetically close to other bacteria than to cyanobacteria [13]. The DXP pathway generate IPP as well as dimethylallyl diphosphate (DMAPP), whereas only IPP is released from the MVA pathways. One could note that the DXP pathway do not apparently involve phosphorylation of pathway intermediates as envisioned previously [14].

In Arabidopsis, the expression of 1-deoxy-D-xylulose-5-phosphate synthase (DXS), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), 2-C-methyl-D-erythritol-4-phosphate cytidyl transferase (MCT),
Nomenclature

AACT acetoacetyl-CoA thiolase
ABA abscisic acid
ADP adenosine 5'-diphosphate
ALAD 5-aminolevulinate dehydratase
AO aldehyde oxidase
aPS apophytoene synthase
ASC− ascorbate
ASCH ascorbic acid
ATP adenosine 5'-triphosphate
BS botryococcene synthase
CAO chlorophyll a oxygenase
CASE chlorophyllase
CBR chlorophyll b reductase
CCS capsanthin-capsorubin synthase
CDP-ME 4-(cytidine 5'-diphospho)-2-C-methyl-d-erythritol
CDP-MEP 2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-d-erythritol
CHB carotenoid β-hydroxylase
CHE carotenoid ε-hydroxylase
CLD chain-length determination domain
CMK 4-(cytidine 5'-diphospho)-2-C-methyl-d-erythritol kinase
CMP cytidine 5'-monophosphate
CPO coproporphyrinogen III oxidase
CPP chrysanthemyl diphosphate
CPPS chrysanthemyl diphosphate synthase
CPS ent-copalyl diphosphate synthase
CRTISO carotenoid isomerase
CS chlorophyll synthase
CTP cytidine 5'-triphosphate
cZ cis-zeatin
cZR cis-zeatin riboside
cZMP cis-zeatin riboside monophosphate
DBMIB 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone
DCMU 3-(3,4-dichlorophenyl)-1,1-dimethyl-urea
DCR divinyl protochlorophyllide reductase
DHA dehydroascorbate
DHAR dehydroascorbate reductase
DHNA 1,4-dihydroxy-2-naphtoate
DHNAPT DHNA phytol transferase
DihydroGG-Chl dihydrogeranylgeranyl-chlorophyll
DihydroGGPP dihydrogeranylgeranyl diphosphate
DMAPP dimethylallyl diphosphate
DMGGBQ 2,3-dimethyl-5-geranylgeranyl benzoquinol
DMPBQ 2,3-dimethyl-5-phytyl benzoquinol
DMPQ demethylphyloquinone
DMPQMT demethylphyloquinone methyltransferase
DMNT (E)-4,8-dimethyl-1,3,7-nonatriene
DPMDC 5-diphosphomevalonate decarboxylase
DXP 1-deoxy-d-xylulose-5-phosphate
DXR 1-deoxy-d-xylulose-5-phosphate reductoisomerase
DXS  1-deoxy-d-xylulose-5-phosphate synthase
FARM first aspartate-rich domain
FCC  fluorescent chlorophyll catabolite
FPP  farnesyl diphosphate
FPPS farnesyl diphosphate synthase
GA  gibberellin
GA12S GA12-aldehyde synthase
GA7OX GA 7-oxidase
GA13H GA 13-hydroxylase
GG-Chl geranylgeranyl-chlorophyll
GGPP geranylgeranyl diphosphate
GGPPS geranylgeranyl diphosphate synthase
GGR geranylgeranyl reductase
GPP  geranyl diphosphate
GPPS geranyl diphosphate synthase
GR  glutathione reductase
GSAT glutamate-1-semialdehyde aminotransferase
GSH  reduced glutathione
GSSG oxidized glutathione
GTR glutamyl-tRNA reductase
GTS glutamyl-tRNA synthetase
HBP450 heme-binding cytochrome P450
HCR  hydroxychlorophyll a reductase
HDR 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase
HDS 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase
HGA  homogentisic acid
HGGT homogentisate geranylgeranyl transferase
HMBPP 1-hydroxy-2-methyl-2-(E)-butenyl 4-phosphate
HMG-CoA 3-hydroxy-3-methylglutaryl-CoA
HMGR 3-hydroxy-3-methylglutaryl-CoA reductase
HMGS 3-hydroxy-3-methylglutaryl-CoA synthase
HPP  p-hydroxyphenylpyruvate
HPPD p-hydroxyphenylpyruvate dioxygenase
HPT homogentisate-phytyl transferase
HST 3, homogentisate solanesyl transferase
IP  isopentenyladenine
IPP  isopentenyl diphosphate
IPPI IPP-DMAPP isomerase
iPR isopentenyladenine riboside
iPRMP isopentenyladenine riboside monophosphate
IPT isopentenyl transferase
IS  isoprene synthase
KAO ent-kaurenoic acid oxidase
KET  β-carotene ketolase
KO  ent-kaurene oxidase
KS  ent-kaurene synthase
LCB lycopene β-cyclase
LCE lycopene e-cyclase
LHC light-harvesting complex
LSU large subunit
MBS methylbutenol synthase
MCH magnesium chelatase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
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<tbody>
<tr>
<td>MCS</td>
<td>2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase</td>
</tr>
<tr>
<td>MCT</td>
<td>2-C-methyl-D-erythritol-4-phosphate cytidyl transferase</td>
</tr>
<tr>
<td>MDCH</td>
<td>magnesium dechelatase</td>
</tr>
<tr>
<td>MECPP</td>
<td>2-C-methyl-D-erythritol-2,4-cyclodiphosphate</td>
</tr>
<tr>
<td>MEP</td>
<td>2-C-methyl-D-erythritol-4-phosphate</td>
</tr>
<tr>
<td>MGDG</td>
<td>monogalactosyldiacylglycerol</td>
</tr>
<tr>
<td>MGGBQ</td>
<td>2-methyl-6-geranylgeranyl benzoquinol</td>
</tr>
<tr>
<td>MGGBQMT</td>
<td>2-methyl-6-geranylgeranyl benzoquinol methyltransferase</td>
</tr>
<tr>
<td>MK</td>
<td>mevalonate kinase</td>
</tr>
<tr>
<td>MPBQ</td>
<td>2-methyl-6-phytyl benzoquinol</td>
</tr>
<tr>
<td>MPBQMT</td>
<td>2-methyl-6-phytyl-1,4-benzoquinol methyltransferase</td>
</tr>
<tr>
<td>MPK</td>
<td>mevalonate kinase</td>
</tr>
<tr>
<td>MSBQ</td>
<td>2-methyl-6-solaneyl-1,4-benzoquinol</td>
</tr>
<tr>
<td>MSBQMT</td>
<td>2-methyl-6-solaneyl-1,4-benzoquinol methyltransferase</td>
</tr>
<tr>
<td>MTC</td>
<td>magnesium protoporphyrin IX monomethylester cyclase</td>
</tr>
<tr>
<td>MTF</td>
<td>magnesium protoporphyrin IX methyltransferase</td>
</tr>
<tr>
<td>MVA</td>
<td>mevalonate</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>nicotinamide adenine dinucleotide phosphate, oxidized form</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate, reduced form</td>
</tr>
<tr>
<td>NCCs</td>
<td>non-fluorescent chlorophyll catabolites</td>
</tr>
<tr>
<td>NCED</td>
<td>nine-cis-epoxycarotenoid dioxygenase</td>
</tr>
<tr>
<td>NHDIM</td>
<td>non-heme-di-iron monooxygenase</td>
</tr>
<tr>
<td>NPQ</td>
<td>non-photochemical quenching</td>
</tr>
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<td>NSY</td>
<td>neoxanthin synthase</td>
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<td>OSC</td>
<td>(3S)-2,3-oxidosqualene cyclase</td>
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<tr>
<td>PAO</td>
<td>pheophorbide a oxygenase</td>
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<tr>
<td>PBD</td>
<td>porphobilinogen deaminase</td>
</tr>
<tr>
<td>PD</td>
<td>phytoene desaturase</td>
</tr>
<tr>
<td>PHB</td>
<td>p-hydroxybenzoate</td>
</tr>
<tr>
<td>PHBPT</td>
<td>p-hydroxybenzoate polypropenyl diphosphate transferase</td>
</tr>
<tr>
<td>Phytyl-Chl</td>
<td>phytyl-chlorophyll</td>
</tr>
<tr>
<td>PhytylPP</td>
<td>phytyl diphosphate</td>
</tr>
<tr>
<td>PMK</td>
<td>5-phosphomevalonate kinase</td>
</tr>
<tr>
<td>PPHB</td>
<td>polypropenyl-(p)-hydroxybenzoate</td>
</tr>
<tr>
<td>PolypropenylPP</td>
<td>polypropenyl diphosphate</td>
</tr>
<tr>
<td>POR</td>
<td>NADPH-protoporphyrilide oxidoreductase</td>
</tr>
<tr>
<td>PP</td>
<td>diphosphate</td>
</tr>
<tr>
<td>PPPP</td>
<td>prephytoene diphosphate</td>
</tr>
<tr>
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<td>protoporphyrinogen IX oxidase</td>
</tr>
<tr>
<td>PQ</td>
<td>plastoquinone</td>
</tr>
<tr>
<td>PS</td>
<td>phytoene synthase</td>
</tr>
<tr>
<td>PSPP</td>
<td>presqualene diphosphate</td>
</tr>
<tr>
<td>RCC</td>
<td>red chlorophyll catabolite</td>
</tr>
<tr>
<td>RCCR</td>
<td>RCC reductase</td>
</tr>
<tr>
<td>QTL</td>
<td>quantitative trait loci</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosyl-L-methionine</td>
</tr>
<tr>
<td>SAM γ-MT</td>
<td>S-adenosyl-L-methionine γ-tocopherol methyltransferase</td>
</tr>
<tr>
<td>SAM γ-MT3</td>
<td>S-adenosyl-L-methionine γ-tocotrienol methyltransferase</td>
</tr>
<tr>
<td>SARM</td>
<td>second aspartate-rich domain</td>
</tr>
<tr>
<td>SC</td>
<td>squalene cyclase</td>
</tr>
<tr>
<td>SDR</td>
<td>short-chain dehydrogenase/reductase</td>
</tr>
<tr>
<td>SMO</td>
<td>sterol-4(z)-methyl-oxidase</td>
</tr>
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</table>
4-(cytidine 5′-diphospho)-2-C-methyl-d-erythritol kinase (CMK), 2-C-methyl-d-erythritol-2,4-cyclodiphosphate synthase (MCS), 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase (HDS) is induced under light conditions [15–17], while the expression of 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (HDR) is constitutive [17]. Also, the expression of DXS, DXR, MCT and CMK is slightly induced in the dark in the presence of sucrose [17]. Similarly, DXS is up regulated during chloroplast to chromoplast differentiation in pepper fruits [18] and tomato fruits [19]. At the protein level, in Arabidopsis, MCT, DXS, HDS and DXR are highly expressed in young photosynthetic tissues and in flower organs [16,20,21]. Interestingly, overexpression of DXS in Arabidopsis [22], DXR in peppermint [23] or and HDR in Arabidopsis and tomato [24] led to an increased accumulation of diverse plastid isoprenoids. Indirect evidences suggest that DXR, HDS and HDR are potential targets of thioredoxin, and could be activated under light conditions [25,26]. In this context, it is interesting to note that Thermosynechococcus elongatus HDS has been characterized as a ferredoxin-dependent enzyme [27]. Thus the activity of HDS could be modulated via the light-dependent reduction of ferredoxin.

In vivo feeding experiments pointed to the fact that the DXP pathway leading to the synthesis of isoprene is feedback regulated at the level of DXS [28]. It has also been suggested that the accumulation of DXP pathway enzymes was regulated by post-transcriptional mechanisms that could operate at the level of DXS [29]. It is interesting to note that electrospray mass spectrometry combined with NMR showed that in vitro 2C-methyl-d-erythritol-2,4-cyclodiphosphate synthase (MCS) could bind potential downstream products such as IPP/DMAPP, geranyl diphosphate (GPP), and farnesyl diphosphate (FPP) in a ratio of 1:4:2 [30]. When transposed to the in vivo situation these data are consonant to a possible metabolic retrocontrol of the DXP pathway. Further testing this phenomenon could be achieved through the use of isolated plastoplasts which have been used to analyze different sequences of the pathway [31].

Concerning the MVA pathway, previous and recent data available from Arabidopsis suggest that phytochrome and crytochrome photoreceptor could be implicated in its regulation [32,33]. In addition it has been shown that in Arabidopsis cotyledons, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) is not predominantly localized in the endoplasmic reticulum (ER), but within undefined spherical vesicles located in the cyto-
plasm and within the central vacuole [34]. Finally, the physiological function of HMGR has been clarified through the analysis of two hmgr mutants. Arabidopsis hmg2 mutant showed no visible phenotype while hmg1 mutants exhibited dwarfism, early senescence and sterility [35].

2.2. Plastid–cytosol interaction

Cross-talk between the plastidial and the cytosolic pathways has been envisioned from two previous studies using feeding experiments with stable isotopes. In one case, the biosynthesis of the diterpene ginkgolide was analyzed using Ginkgo biloba seedlings [36]. These studies revealed that in the presence of 13C glucose, three isoprene units were incorporated according to the cytosolic (MVA pathway) and the fourth residue via the DXP pathway. In a similar vein, when Catharanthus roseus cell cultures were incubated with 1-deoxy-D-xylulose, it was found that part of the precursor was incorporated into sitosterol [37]. This behavior suggested a potential exchange of isoprenoid between the plastid and the cytosol. At the other extreme, in vivo feeding of glucose revealed that the contribution of mevalonate and the non-mevalonate pathways to the synthesis of sitosterol was equal in green callus culture of Croton sublyratus [38]. When C. roseus cell cultures were grown in the presence of glucose, it was shown that the plastid product phytol derives 60% from the DXP pathway and 40% from the MVA pathway. Additional studies revealed that biosynthesis of abietane diterpenoids in Torreya nucifera operates via the DXP and MVA [39]. Feeding experiments also indicated that in Arabidopsis ent-kaurene and campesterol derived from 1-deoxy-D-xylulose (68–87%) and (3–7%), respectively, and from MVA (5–8%) and (80%), respectively [40]. In Euglena the carotenoids diadinoxanthin and β-carotene were synthesized from DXP as well as MVA, while the C20 phytol side-chain derived exclusively from MVA [41]. In liverworts and in hornworts the FPP portion of the phytol chain derived from MVA, while the terminal IPP was from DXP [42–44]. Further evaluation of this cross-talk using 13C-labeled serine reveals that IPP formed in the chloroplast was not used for the biosynthesis of cytosolic isoprenoids in the cultured cells of the liverwort Heteroscyphus planus [44]. Also it has been shown that MVA was incorporated into the side-chain of plastoquinone-9 (PQ-9) when tobacco BY2-cells were cultivated in the presence of fosmidomycin. On the
other hand, in the presence of mevilonin, the DXP contributed to the synthesis of the cytosolic sterols [45]. Using the same strategy, it was shown that the plastid–cytosol cooperation was relatively modest [46]. This phenomenon also influences the biogenesis of lower terpenoids. In carrot leaves and roots, although monoterpenes were synthesized exclusively via the DXP pathway, the sesquiterpenes derived from the MVA as well as DXP pathways [47]. In grape leaves, the DXP pathway was prominently used for the biogenesis of monoterpenes and exceeded the MVA pathway by a factor around 100 [48]. In the epidermis of snapdragon flower, the DXP pathway provided IPP used both for monoterpenes and sesquiterpenes synthesis [49].

In Arabidopsis, the albino phenotype observed in several mutants, dxr, mct, hds and hdr [9,15,17,20,50] suggests that IPP derived from MVA could not efficiently compensate for the lack of the plastid derived IPP. This was also illustrated by the inefficient rescue of dxx mutants [15,51] and in Nicotiana benthamiana when the DXS-pathway specific genes were silenced [52]. Indeed, the silencing of DXR and HDR in N. benthamiana is paralleled by chlorotic phenotypes and drastic loss of photosynthetic pigments (Fig. 3, unpublished data). Data from isoprene-emitting plants revealed that the cross-talk between chloroplastic and extrachloroplastic pathways of isoprenoid formation is marginal. The evidence is explained when two facts are considered. First, isoprene synthesis consumes much more carbon precursors than other plastid isoprenoid synthesis [53]. Second, when the plastid pathway of isoprenoid was inhibited by fomidomycin, the synthesis of isoprene remained extremely low [54–56]. Thus available data indicate that when the plastid pathway is blocked, the amount IPP shuttled from the cytosol into the plastid could not compensate the deficiency.

The mechanism regulating this cross-talk is unknown. It has been observed that when the cytosolic and the plastid pathways of isoprenoid synthesis were alternatively blocked by lovastatin and fomidomycin, there was no correlation between the pattern of isoprenoid changes and gene expression. This led to suggest that post-transcriptional control may operate [46]. Also, because the labelling pattern varied according to the precursors used and the nature of the products analyzed, it has been suggested that the cross-talk between plastid and the cytosol required additional factors and could not be assimilated to a simple shuttle between the plastid and the cytosol [57].

Besides the above unresolved questions, the question remains, how prenyl diphosphate is transported between the plastid and the cytosol compartment. Uptake experiments using isolated plastids suggested that the plastid envelope possesses a putative IPP transporter [58]. However, the protein responsible for this transport has not been identified. In more recent studies, prenyl diphosphate transport activities have been measured employing liposomes containing chloroplast envelope proteins. This led to the suggestion that a unidirectional proton symport gated by Ca$^{2+}$ could be involved in the plastidial export of prenyl diphosphate to the cytosol [59]. The transport efficiency was shown to decrease in the following order: isopentenyl diphosphate, geranyl diphosphate, farnesyl diphosphate and dimethylallyl diphosphate. With this system, geranylgeranyl diphosphate (GGPP) was not transported [59]. The mechanism could involve a unidirectional proton symport system [59]. In another study, in vitro experiments have been performed using isolated intact chloroplasts, liposomes containing chloroplast envelope membrane proteins and several pathway intermediates including: DXP, 1-hydroxy-2-methyl-2-(E)-butenyl 4-phosphate (HMBPP) and IPP. These studies revealed that HMBPP was not exchanged between the cytosol and the chloroplasts while IPP was preferentially transported by the plastid.

![Fig. 2. Cytosolic MVA and plastidial DXP pathways of IPP and DMAPP synthesis in plants.](image-url)
The complexity of the putative plastid–cytosol cooperation is paralleled in some plant tissues by an intercellular cooperation. It has been shown using Northern blot and in situ hybridization experiments, that *C. roseus*, the transcripts of DXP-pathway genes, DXS, DXR, MCS and geraniol 10-hydroxylase that catalyzes the first committed step in the formation of iridoid monoterpenoids were localized in the internal phloem parenchyma. It has been hypothesized that under these conditions the intermediates are translocated sequentially from the internal phloem parenchyma to the epidermis and, finally, to laticifers and idioblasts during monoterpenoid indole alkaloids [61].

2.3. Non-dispensability of IPP isomerase

Because the DXP pathway generates IPP as well as DMAPP (Fig. 2), the role of plastid IPP isomerase has been questioned. Two types of isopentenyl diphosphate isomerase (IPPI) have been characterized in living organisms. Type 1 is the enzyme that reversibly catalyzes the conversion of IPP into DMAPP in most eucaryotes and some bacteria [62]. This enzyme contains an essential atom of zinc [63]. More recently, type 2 IPPIs have been characterized in archaea and some bacteria [64]. Type 2 is not homologous to type 1. Type 2 enzyme requires redox co-enzymes, i.e., flavin mononucleotide (FMN) and NAD(P)H, for activity. During the catalysis, FMN is reduced by NAD(P)H [65].

In plant type organisms it has been shown previously that *Synechocystis* strain PCC 6803 lacks isomerase I activity due to the absence of type-1 IPP isomerase gene [66]. In contrast ORFs homologous to type II IPP isomerase are present in several cyanobacterial genomes and shown to be inactive [67]. However, in further analysis the activity could be demonstrated [68]. These data revealed that IPPI is an essential gene. These trends were supported by *N. benthamiana* plants in which IPPIs have been silenced. These plants had mottled white-pale green leaves concomitantly to a drastic alteration of the plastid structure and photosynthetic pigments were decreased by 80%. Chloroplasts from silenced plants displayed a high IPP phosphatase activity [52]. Thus there is no redundancy between the function of IPPI and HDR (Fig. 2) which independently catalyze the synthesis of DMAPP.

In tobacco, two IPPIs genes, *IPPI1* and *IPPI2* have been characterized [69]. IPPI1 encodes the previously characterized plastid isoform, while IPPI2 encodes a cytosolic enzyme. The expression of *IPPI1* was induced...
by high-salt concentrations and high light stress. On the other hand, the expression of IPPI2 was induced under high-salt concentrations and cold stress conditions [69]. In a similar vein, the presence of two differentially regulated IPPIs has been reported from Cinchona robusta. Suspension-cultured cells of C. robusta, the source of pharmaceutically active alkaloids, produce large amount of anthraquinone alkaloids when challenged with phytopathogenic fungus Phytophtora cinnamomi [70]. Under these conditions, the activity of IPPI is transiently induced. Two isoforms of IPPI (IPPI1 and IPPI2) have been characterized in C. robusta and IPPI2 is specifically induced following the fungal elicitation. This phenomenon is paralleled by a decreased FPP synthase (FPPS) activity [71]. These data suggest that IPPI2 is dedicated to the induced biosynthesis of anthraquinone phytoalexins [72].

3. Prenyltransferases as main trunk enzymes

3.1. General aspects

The prenyltransferase reaction involves the condensation of an acceptor isoprenoid or non-isoprenoid molecule to an allylic diphosphate. Three types of reactions have been described [73]. The first and most studied reaction involves a head-to-tail or 1'-4 condensation of the 5-carbon IPP, to DMAPP or to an elongating allylic diphosphate chain. Depending on their specificities these prenyltransferases yield linear trans- or cis-prenyl diphosphates. The second class of prenyltransferases reaction involves head-to-head or head-to-middle condensations that lead to irregular terpenoids such as presqualene diphosphate, prephytoene diphosphate and chrysanthemyl diphosphate which serve as pathway-specific intermediates. The third type of prenyltransferases reaction involves the alkylation of a non-isoprenoid acceptor as observed in common prenyl lipids such as chlorophylls, tocopherols, ubiquinone and diverse secondary metabolites. Mechanistically, the reaction catalyzed by prenyltransferases and terpenoid cyclases proceed according to the same mechanism. In both cases, a new carbon–carbon bond is generated through the addition of a reactive electron-deficient allylic carbocation to an electron-rich carbon–carbon double bond [74].

The synthesis linear polyprenyl chain is catalyzed by trans- and cis-prenyltransferases [75,76]. Prenyltransferases belonging to the trans serie are usually subdivided into two subgroups [75]. One subgroup leads to GPP(C10), FPP(C15), GGPP(C20), and the other comprises octaprenyl diphosphate (C40), nonaprenyl diphosphate also known as solanesyl diphosphate (SPP) (C45) and higher molecular weight homologues. Although GPP, FPP and GGPP are the main precursors of monoterpenes, sesquiterpenes and diterpenes there are exceptions peculiar to plants of the apiaceae, asteraceae and lamiaceae (Fig. 4). Plants belonging to these families synthesize irregular monoterpene, sesqueripenes and diterpene that derive from head-to-head coupling of DMAPP in the case of chrysanthemane and lavandulane-type (Fig. 4) [77] between DMAPP and GPP in the case of allergenic sesqueripene lactone, anthecotuloide [78] and between two GPPs in the case of anisotomenes (Fig. 4) [79].

In plants, FPPS and GGPP synthase (GGPPS) generate the key branchpoint intermediates of cytosolic and plastid isoprenoids. Arabidopis FPPS [80] and pepper GGPPS [81,82] were characterized previously at the biochemical and molecular levels. Few inhibitors of these enzymes are available except phosphonate derivatives or aza-GGPP [83] and the fungal product gerfelin [84]. Seven genes encoding GGPPS have been characterized from Arabidopsis [85–87]. The in vitro activity of GGPPS isoenzymes (1,3–6) has been demonstrated [85–87]. Using green fluorescent protein (GFP) fusion protein, GGPPS1 and GGPPS3 have been localized in the chloroplasts. GGPPS2 and GGPPS4 were localized in the endoplasmic reticulum and GGPPS6 in the mitochondria [85]. GPP synthase (GPPS) has been isolated from Arabidopsis and shown to be potentially processed into a plastid and a cytosol isoform [88]. The cytosolic form is consonant with the fact, that in some plants monoterpene derivatives are synthesized from the MVA pathway [89]. Interestingly, when Perilla frutescens limonene synthase was routed either to the plastid or the cytoplasmic compartment of tobacco, limonene synthesis was detectable in the resulting transgenic plants. The amount of plastid derived limonene was 3.5-fold higher than that produced in the cytoplasm [90].

In peppermint, GPPS has been characterized as heterodimer composed of a large subunit (LSU) and a small subunit (SSU) and both subunits were required for activity [91,92]. The LSU is highly homologous and phylogenetically related to GGPPS (Fig. 5) and has no enzymatic activity per se. This scenario is
Fig. 4. Structure of some isoprenoid derivatives branching at different steps of the pathway. Enzyme abbreviations. GPPS, geranyl diphosphate synthase; FPPS, farnesyl diphosphate synthase; SQS, squalene synthetase; GGPPS, geranylgeranyl diphosphate synthase. Substrate abbreviations. DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GPP, geranyl diphosphate; IPP, isopentenyl diphosphate.

reminiscent of heteromeric bacterial prenyltransferase subgroups that lead to hexaprenyldiphosphate (C30) and heptaprenyldiphosphate (C35). These enzymes consist of two non-identical subunits that can be dissociated. None of the isolated subunit has the catalytic activity [75]. Thus further characterization of peppermint
GGPPS will be necessary to substantiate the model. In contrast, the GGPPS of *Abies grandis* (AgGGPS, AF513112) was characterized as a homodimer [93] and clusters with GGPPS (Fig. 5). In *Clarkia* and *Anthurium* flowers, a functional GGPPS has been characterized in parallel with two proteins sharing 53% identity with peppermint GPPS-SSU [94]. When *Clarkia* and *Anthurium* GGPPS and GPPS-SSU were incubated together, GPP was synthesized. Further informations concerning SPP synthases (SPPS), *cis*-polyprenyl synthases, irregular terpene synthases and prenyltransferases alkylating non-isoprenoid acceptors are reported later in appropriate sections.
3.2. Seminal informations gathered from FPP synthase and GGPP synthase

3.2.1. Enzyme structure and classification

Crystallographic studies using avian FPP synthase as a prototype reveal that trans-prenyl transferases are entirely composed of α-helices that form a large central cavity. The cavity consists of 10 antiparallel α-helices containing conserved residues [95]. Unexpectedly, squalene-hopane cyclase, pentalenene synthase and protein farnesyl transferase have the same fold, thus leading to the concept of “terpenoid synthase fold” [96]. Indeed these 3D data are reminiscent of previous studies demonstrating that under constrained conditions, avian liver FPP synthase can in addition to FPP produce cyclic sesquiterpenes [97].

Trans-Prenyltransferases display several conserved regions and in particular two aspartate-rich domains characterized as the first aspartate-rich motif (FARM) and the second aspartate-rich motif (SARM) which is strongly conserved (Fig. 6). The FARM and the SARM are placed on distinct α-helices and are positioned on opposite walls of the central cavity of FPPS. As might be inferred from previous studies using avian FPPS, in trans-prenyltransferases, the pyrophosphate group of the allylic substrate is bound with Mg$^{2+}$ chelated by the FARM motif and the pyrophosphate of IPP, the homoallylic substrate is bound with Mg$^{2+}$ coordinated by the SARM [95,98]. Based on the FARM and SARM motifs, trans-prenyltransferases could be grouped into several types.

Type I FPPSs also termed (eukaryotic) have a FARM motif (DDXXD) and a two aromatic amino acid residues at the fourth and fifth positions upstream from the FARM. On the other hand, type II FPPSs also termed (eubacterial FPPS) have a four amino acid insertion in the FARM DDXXXXD and a single aromatic amino acid motif upstream from the FARM. A third FPPS group has been functionally identified recently in Mycobacterium tuberculosis [99]. In the latter case, the FARM motif has a DDXXXD sequence but deviates from type I because only one aromatic amino acid is at the fifth position. The mycobacterial FPP is more closely related to archaeal type I GGPPS.

In the case of type I GGPPS also termed archaeal GGPPS, only two amino acids are inserted in the FARM and one aromatic amino acid residue at the fifth position upstream from the FARM. Plant GGPPS like eubacterial GGPPS belong to type II GGPPS. In these enzymes, four amino acids are inserted in the
FARM, like type II FPPS. Two of these inserted amino acids have been shown to be important for the catalytic activity [100]. Type II GGPPS is devoid of two aromatic amino acid residues at the fourth and fifth positions upstream from the FARM. Type III GGPPS (eukaryotic except plants) contains only two inserted amino acids like type I GGPPS. But unlike type I GGPPS, one of the inserted residues in type III GGPPS is glutamic acid. Type III GGPPS is also devoid of the characteristic aromatic upstream to the FARM. In addition, it has been shown that this class of prenyltransferases did not accept DMAPP as a substrate [101].

3.2.2. Mechanism of chain-length control

Data based on crystallographic studies [95,98,102–104] and site-directed mutagenesis of FPPS established that the product chain-length was mainly governed by amino acid residues located in the fourth and fifth positions before the FARM. Thus, this region and the FARM have been considered as the chain-length determination domain (CLD) of trans-prenyltransferases. Basically, when a mutation is introduced at these positions, the chain-length of the prenyl diphosphate resulting from the catalytic activity is inversely proportional to the steric hindrance of the introduced amino acid residue. For instance, the mutants F112A and F113S that introduce small residues in the FARM of avian FPPS led to FPPS mutants that produced, respectively, GGPP and or geranylgeranyl diphosphate [98]. Further shortening of the active cavity of avian FPPS by introducing the mutation A116W reduced the binding of GPP to the allylic site [105]. Also, a mutation S113F of Bacillus stearothermophilus, a position equivalent to F113 in avian FPPS, resulted in the following distribution of products: GPP/FPP: 27:1 [106]. Interestingly, the first animal GGPS involved in pheromones biosynthesis has been isolated from the bark beetle Ips pini [107]. This enzyme produced almost exclusively GPP. Although this enzyme is structurally related to avian FPPS [95], its FARM structure possesses F and Y residues at the fourth and fifth positions, consistent with the fact that relatively bulky residues play an important role in shortening the prenyl chain-length. In the same line of reasoning, when bulky residues, i.e.; M135Y/S136F were introduced to the FARM of GGPPS of the diterpene producing plant Scoparia dulcis, the mutant GGPPS lost the ability to use FPP as an allylic substrate. Under these conditions, DMAPP was accepted and led to 3% GGPP while FPP and GPP represented 55.3% and 9.3% of the final products [108]. This trend was also verified in the case of Arabidopsis thaliana SPPSs (AtSPPSs). The fourth and the fifth regions upstream from the FARM of AtSPPS1 and AtSPPS2 do not possess aromatic residues [109]. However additional amino acid residues play a key role in determining the chain-length. When the P143 and the C144 residues were deleted in the FARM domain of S. dulcis, the percentage of the products were: GGPP, 1%; FPP, 20.9%; and GPP, 5.5% [108]. The difficulty in predicting the chain-length was also illustrated by the fact that unlike the plants GGPPS, archebacterial GGPPS have an aromatic residue at the fifth position upstream from FARM and have only two amino acid residues inserted in the FARM, instead of four like Arabidopsis GGPPS. Also, it has been noted that the chain-length was changed when mutations were introduced in GGPPS, outside of the CLD domain and before the conserved G(Q/E) motif [110,111].

Alternatively, the ratio between the concentration of IPP and DMAPP could alter the product specificity of FPPS and GGPPS. At higher DMAPP concentration, FPPS produces increased amount of GPP [100]. In a similar vein, at high DMAPP concentrations, pepper chromoplast GGPPS produced significant amount of GPP [88]. Interestingly, it has been shown that the concentration of plastid DMAPP could reach millimolar value in isoprene- and methylbutenol-emitting and non-emitting plants [112]. Also, the DMAPP concentration was light-dependent [112] and could reach concentrations similar to that of other plastid metabolites such as 3-phosphoglycerate, dihydroxyacetone phosphate and fructose1,6-bisphosphates [113,114]. Under these conditions, the allylic substrate could compete with nascent polyprenyl chain and thus induces the abortion of the condensation reaction and the subsequent release of GPP. Alternatively, it has been shown that when the rate of the allylic carbocation prone to condensation was accelerated by Ca$^{2+}$ and Mn$^{2+}$, the formation of long prenyl diphosphate was favored [115]. Finally, it is worth noting that irregular terpene synthases that cluster close to FPPSs (Fig. 6) could catalyze the synthesis of prenyl diphosphate having different chain-lengths [116,117].
4. Polyprenyl polymer biogenesis

4.1. cis-Polyprenyltransferases

In eucaryotes, cis-prenyltransferases are involved in the synthesis of C55–C100 dolichols, which serve as sugar carriers in protein glycosylation [118, 119]. A dehydrodolichyl diphosphate synthase has been characterized from Arabidopsis [120]. The Arabidopsis gene was mainly expressed in roots [120]. In general, these prenyl transferases are activated by lipids and detergents. The peptide sequence of these enzymes do not have the most conservative FARM and the SARM that are characteristic of trans-type prenyltransferases, although they require Mg$^{2+}$ for activity. Data gathered from Escherichia coli undecaprenyl diphosphate synthase revealed that the active-site is organized differently from trans-prenyl transferase. In cis-prenyltransferases, the active-site is organized as a hydrophobic tunnel composed of $\alpha$-helices and $\beta$-strands [121]. Recent crystallographic data using undecaprenyl diphosphate synthase revealed that no metal was detected in the FPP active-site in complex with FPP. Only the binding of IPP and the condensation reaction require Mg$^{2+}$. FPP is bound to positively charged arginine residues and the hydrocarbon moiety interacts with hydrophobic amino acids [122, 123].

4.2. Rubber transferases

Despite the fact that more than 2000 plant species produce natural rubber, a cis-1,4-polyisoprenoid, *Hevea brasiliensis* is the only commercial source of rubber [124]. In this plant, rubber prenyl-transferase synthesizes a polymer containing thousands of IPP units. A small rubber particle protein (SRPP) gene has been cloned from *H. brasiliensis* [125]. SRPP is homologous to the rubber elongation factor previously characterized from *H. brasiliensis* rubber particles [126] and to a protein recently characterized from Guayule (*Parthenium argentatum*) [127]. Like the SRPP, the guayule homologue increased the incorporation of IPP into high molecular weight rubbers [127]. Search of the protein databases revealed that the deduced peptide sequences of the above depicted proteins have up to 47% identity to stress-related proteins from Arabidopsis and Phaseolus vulgaris, which apparently do not produce rubber [127]. Thus the specific role of such protein in rubber biosynthesis requires further investigations. Subsequent studies indicated that Arabidopsis possesses a cis-prenyltransferase that catalyzed the formation of polyprenyl chains ranging number from C100 to C130 [128]. Because rubber prenyl-transferase synthesizes a polymer containing thousands of IPP units, the Arabidopsis cis-prenyltransferase alone is not competent for rubber synthesis. Recently, a cDNA coding for a novel cis-prenyl chain elongating enzyme has been identified from *H. brasiliensis*. The corresponding gene is expressed predominantly in the latex compared with other *Hevea* tissues examined. The recombinant protein catalyzed the cis-1,4-polymerization of isoprene units and yielded a polyprenyl chain of $2 \times 10^3$–$1 \times 10^4$ Da. The highest polymerization activity required the addition of soluble factors present in the washed particles [129].

5. Lower terpenoid synthases

5.1. Isoprenoids branching from DMAPP and GPP

5.1.1. Isoprene and methylbutenol

Several plants belonging to mosses, ferns, gymnosperms and angiosperms emit isoprene and methylbutenol (for a review, see [130]; Fig. 4). The reactions are catalyzed by isoprene synthase (IS) and methylbutenol synthase (MBS), two plastid enzymes that convert DMAPP to isoprene and methylbutenol [130]. In contrast to several plastid biosynthetic enzymes, IS and MBS possess a relatively high $K_m$DMAPP. The gene encoding IS has been characterized from poplar species [131–133]. IS display significant identity and strong similarity to monoterpene synthases and particularly limonene synthases. This trend is reinforced by the fact that IS has a marginal limonene synthase activity [131]. The expression of IS is induced by heat stress and light, but not by jasmonic and salicylic acids [133]. Although Arabidopsis does not emit isoprene, IS has extended homology to Arabidopsis myrcene/ocimene synthase. One key difference between IS and monoterpene syn-
thase is the exclusive presence of two phenylalanine residues in IS active-site that could narrow and limit the interaction with longer allylic diphosphates [132].

5.1.2. Cytokinins

The two physiologically active plant cytokins, trans-zeatin and isopentenyladenine and the less active cis-zeatin are prenylated by DMAPP (Fig. 7) [134–136]. The reaction is catalyzed by specific prenyltransferases. In Arabidopsis, four plastid adenosine phosphate-isopentenyl transferases divert the DXP-derived DMAPP to the synthesis of trans-zeatin, whereas cis-zeatin is prenylated using MVA-derived DMAPP by a cytosolic prenyltransferase [137] (Fig. 7).

Overexpression of Arabidopsis FPPS1 isoform in Arabidopsis did not modify the sterol content but induced premature cell death and senescence-like phenotype in transgenic plants compared to the control. This phenotype has been attributed to reduced levels of endogenous zeatin-type cytokinins in leaves, due to reduced availability of IPP and DMAPP for cytokinin biosynthesis [138]. A quantitative trait loci (QTL) approach has been used to characterize the gene encoding cytokinin oxidase/dehydrogenase (OsCKX2) from rice [139]. OsCKX2 degraded cytokinins by cleavage of the unsaturated N6-isoprenoid side chains [136]. In rice, when the expression of OsCKX2 was downregulated, the number of reproductive organs and the production of grains were enhanced [139].

5.1.3. Phenylpropanoid derivatives

Several flavonoids are prenylated, for a review see [140]. Feeding experiments revealed that prenylated flavanones from Glycyrrhiza glabra [141] and Sophora flavescens [142], humulone in Humulus lupulus [143],

![Fig. 7. Cytokinin biosynthetic pathways. Enzyme abbreviations. IPT, isopentenyltransferase. Substrate abbreviations. ATP, adenosine 5'-triphosphate; cZ, cis-zeatin; cZR, cis-zeatin riboside; cZRMP, cis-zeatin riboside monophosphate; DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose-5-phosphate; IPP, isopentenyl diphosphate; iP, isopentenyladenine; iPR, isopentenyladenine riboside; iPRMP, isopentenyladenine riboside monophosphate; MVA, mevalonate; tRNA transfer RNA; tZ, trans-zeatin; tZR, trans-zeatin riboside; tZRMP, trans-zeatin riboside monophosphate.](image-url)
furanocoumarins in *Apium graveolens* [144] and the prenyl group of several isoprenyl phenyl ethers [145] derive from the plastidial DXP pathway. These data contrast with the fact that in shikonin, the geranyl group derive from the MVA pathway [89]. Because the flavanone ring is formed in the extraplastidial compartment, this suggests that a tight cooperation could exist between plastid and ER–cytosol. A very similar mechanism could operate for glycocollin and phaseollin, two prenylated isoflavonoid phytoalexins produced by elicited soybean and bean plants [146]. To facilitate the connexion between these spatially distinct compartments, it has been reported that an elicitor inducible flavonoid–DMAPP transferase is associated with the chloroplast membrane envelope [146].

5.2. Semio-terpene synthases and traumato-terpene synthases

5.2.1. General characteristics

The function lower terpene synthases is prominently dedicated to the synthesis of volatile and non-volatile chemicals used for communication and defense responses (Fig. 4). Thus they are referred as semio-terpene synthases and traumato-terpene synthases from the Greek root “semion” and “traumatos” meaning, respectively, mark or sign and wound or injury. Operationally these enzymes function as monoterpene, sesquiterpene and diterpene synthases. In earliest studies, monoterpen synthases were found in a soluble fraction and the role of plastids was challenged. On the other hand, sesquiterpene synthase and diterpene synthase were assigned, respectively, to the cytosolic and the plastid compartments (for a review, see [147]). Our appreciation of this compartmentation has changed through the use of genetic, molecular biology and more refined biochemical techniques. The plastid is now recognized as a main site of the initial steps monoterpene synthesis in lower and higher plants, whether or not they differentiate resin ducts, secretory pockets or trichome structures [88,148,149].

Mechanistically these enzymes proceed via several transient steps including: ionization of the diphosphate moiety with the aid of divalent cations, rearrangement of the resulting carbocation, deprotonation or nucleophile capture. The process is basically similar to that of prenyltransferases. Although endogenous phosphatase could yield terpenols [150], the synthesis of geraniol is catalyzed by specific monoterpen synthases designated geraniol synthase [151,152]. Thus the biogenesis of geraniol like that of linalool and methybutenol is based on hydration of transient allylic carbocation. Detailed aspects concerning the biosynthesis and the molecular biology of semio-terpene synthases and traumato-terpene synthases have been largely reviewed [153–155].

The number of semio-terpene synthases and traumato-terpene synthases in nature is presently unknown. However, studies from known examples indicate that multiple products could be generated by the same enzyme. For instance, (trans)-β-farnesene synthase, a sequiterpenoid cyclase, produced 5% of δ-cadinene and 8% of (cis)-β-farnesene in addition to (trans)-β-farnesene [156]. At the other extreme, the δ-selinene and γ-humulene synthases from grand fir generated at least 34 and 52 products, respectively [157]. In the genome of *Arabidopsis* 32 putative members could be identified [158]. Available data indicate that most of these genes were almost expressed in the flower organs [159]. Recent data indicate that only two sesquiterpene synthases catalyze the 20 sesquiterpene volatiles produced in *Arabidopsis* flowers [160].

Despite the fact that diverse sesquiterpene cyclases, FPPS and squalene synthetase (SQS) lack significant amino acid sequence identity, their X-ray structures reveal that they adopt the terpenoid synthase fold. The presently available crystal structure reveal that they have the same three-dimensional structures and differ by the specific arrangements of the amino acid side chains in the active-site [96,161–163]. It has been suggested that starting conformation of FPP in the active-site coupled to limited changes in the active site residues is a critical determinant of product diversity [164,165]. Thus mutant aristolochene synthase Y92A, a sesquiterpene synthase from *Penicillium roqueforti*, produced mostly the linear product (trans)-β-farnesene (72.6%), due to the increased freedom of FPP and its derived transient intermediates [166].

5.2.2. Pathway induction by biotic and abiotic factors and biological roles

In addition to being implicated in the discrimination between host and non-host plants for pollinisation, volatile isoprenoids are implicated in indirect defense. Most of the volatile emitted isoprenoids belong to the monoterpen and sesquiterpene series but homoterpenes such as (3E)-4,8-dimethyl-1,3,7-nonatriene
(DMNT) and 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) are also synthesized, respectively, from FPP and GGPP (Fig. 4) [167]. The latter were synthesized via a cleavage pathway including the action of cytochrome P450 oxygenases [168,169]. Interestingly, feeding experiments revealed that under these stressing conditions, DMNT and TMTT were flexibly synthesized according to the mevalonate and the non-mevalonate pathways [167]. Volatile isoprenoids emitted by herbivore infested plants serve as attractants for herbivore predators or parasites of plants and provide indirect defense against herbivores (for a review, see [170–174]). Their biosynthesis and emission are highly regulated. In brussels sprouts, the same volatile isoprenoid blend was emitted following wounding and herbivore feeding [175]. In maize, soybean, lima bean, cotton and cucumber, isoprenoid volatiles emitted during wounding were different from those due to herbivore feeding [173]. Among these monoterpenes, β-ocimene plays a key role because of its specific emission pattern and wide spread roles following herbivore feeding (indirect defense), resistance to pathogens [176] and tolerance to high temperature [177]. These molecules could potentiate defense response as shown by the fact that in uninfested lima bean leaves exposed to DMNT, TMTT and β-ocimene, the expression of several defence genes and FPPS were induced [178].

In the Arabidopsis genome, 40 semio-terpene synthases and traumato-synthase-like genes have been identified by genome in silico analysis [158,179]. Among these A. thaliana terpene synthases, (AtTS3) and (AtTS10) have been characterized at the molecular level. AtTS10 encodes a monoterpene synthase that catalyzes mainly the synthesis of myrcene (56%) and diverse monoterpenes including (trans)-β-ocimene (20%) [180], while AtTS3 is almost dedicated to the synthesis of (trans)-β-ocimene (94%) and minor terpene including (cis)-β-ocimene (4%) and myrcene (2%) [181]. Arabidopsis infested by Pieris rapae emitted several volatiles that serve to attract its parasitic predator Cotesia rubecula [182]. Under these conditions, the expression of AtTS3 and AtTS10 was induced but only myrcene was emitted in the atmosphere [182]. On the other hand, in response to jasmonic acid and mechanical wounding, the expression AtTS3 was induced in parallel to the emission of (trans)-β-ocimene [181]. This indicates that the expression of AtTS3 was finely tuned according to a complex and unknown mechanism. Furthermore, this phenomenon was regulated by a diurnal rhythm [183].

The accumulation of semio-isoprenoids and traumato-isoprenoids is often induced in stressed plants (for a review, see [184]). This phenomenon is often paralleled by pathway redirections as observed in diverse plants including tobacco [185,186], potato [187], pepper [188] and cell-suspension cultures of Tabernaemontana divaricata [189] and Ammi majus [190]. Finally, the secretion of oleoresin [a mixture of monoterpenic olefins (turpentine) and diterpene resin acids (rosin)] represents the primary response of conifers to wounding. Subsequent volatilization of turpentine results in crystallization and polymerization of the nonvolatile resin acid fraction and subsequent sealing of the wound site [191].

The molecular control of semio- and traumato-terpene synthases is poorly understood. The pathway is regulated by light through the action of phytochrome, at least in thyme seedlings [192] and Satureja douglasii [193]. Calcium influxes, in combination with phosphorylation dephosphorylation cascades have also been implicated [178]. The first transcription factor implicated in monoterpenoid metabolism was termed ORCA. ORCA is a member of the AP2/ERF-domain family of plant transcription factors. ORCA regulates the biosynthesis of terpenoid indole alkaloids [194]. Detailed work has also been performed using cotton. Sesquiterpenoid aldehydes including gossypol are common phytoalexins of cotton. Their formation through the (+)-δ-cadinene skeleton is developmentally and spatially regulated [195,196]. (+)-δ-Cadinene synthase (CAD) is encoded by a gene family divided into two subgroups that include CAD1-A represented by a single member and CAD1-C which comprises several members. Members of the CAD1-C are highly expressed in flower organs and in developing seeds concomitantly to the pigmentation of the glands and the accumulation of sesquiterpenoid aldehydes [196]. On the other hand, CAD1-A is weakly expressed in these tissues, but is highly expressed in elicited suspension-cultured cells of cotton that produce sesquiterpene aldehydes [196]. Recent data demonstrate that under these conditions, a specific cotton WRKY transcription upregulates the expression of CAD1-A [197].

Like other aspects of isoprenoid synthesis in plants, further work is required to master the control of the pathway. This will help explain why the overexpression of S-linalool synthase gene in Petunia hybrida not only resulted in trace amount of volatile S-linalool but induced the accumulation of S-linalyl-β-d-glucopyranoside,
its conjugated derivative [198]. Also, Arabidopsis overexpressing strawberry linalool/nerolidol synthase produced linalool, in addition to its glycosylated and hydroxylated derivatives [199]. Similarly, ripening tomato fruit expressing Clarkia breweri S-linalool synthase accumulated trace amounts of S-linalool and hydroxylinalool [200]. At the other extreme, transgenic tobacco expressing three monoterpene synthases γ-terpinene synthase, limonene synthase and β-pinene synthase produced 10–25-fold monoterpenoids [201]. Collectively, these findings are reminiscent of the fact that high level production of novel fatty acid in plants induced a futile cycle of fatty acid synthesis and degradation [202]. Thus one may anticipate that obtaining increased and unmodified final products will implicate genes which induce the differentiation of sequestery structures such as trichomes, resin ducts and secretory pockets.

6. Post-squalene sterol biogenesis

6.1. Background

Sterols are essential components of fungal, animal and plant membranes. They regulate membrane fluidity and permeability and interact with lipids and proteins within the membrane. These structural and membrane roles of sterols in different organisms or models, including the formation of “rafts”, have been reviewed elsewhere in a number of well documented papers [203–209], including some dedicated more specifically to plant sterols [210]. The sterols and the different biosynthetic routes operating in various organisms are now well established (Fig. 8) and have been the object of a number of reviews [211,212] including plants [213–217]. Sterols are precursors of a vast array of compounds involved in important cellular and developmental processes in animals (reviewed in [218]) and plant sterols are particularly linked to brassinosteroids synthesis for review: [219–221]. More recently, functional analysis of sterol function in plants has involved a range of approaches including genetic studies. Indeed, mutational and transgenesis studies have given new insights into the role of sterols in plant development and have been reviewed elsewhere [222–225]. In yeast, all relevant genes of the ergosterol pathway have been cloned allowing isolation of mutants by gene disruption 24, [226] for review. More recently in plants, functions of sterol biosynthetic enzymes have been confirmed by cloning and characterization of eleven of the putative seventeen genes in the portion of the pathway committed to the transformation of oxidosqualene to sitosterol (4) (Fig. 9) and reviewed elsewhere [179,217,227].

6.2. Pathway of phytosterol synthesis

The 21 enzymatic steps in the synthesis of phytosterols from oxidosqualene are all catalyzed by membrane-bound enzymes of the endoplasmic reticulum [214,228]. The transformation of cycloartenol (6) to phytosterols has been mapped by a wealth of isotopic labeling experiments mainly based on (+)-R-mevalonic acid incorporation [211,229] and, or, determination of sterol profile following treatment with inhibitors of sterol biosynthesis [230,231]. Another approach consisted in isolation, biochemical and enzymological characterization of microsome-bound enzymes that catalyze sterol transformation [214,228]. However, until very recently little had been known about the active-site of any of these enzymes. More recently, the genes coding for a number of enzymes in the oxidosqualene to phytosterol segment of the pathway has been expressed in the corresponding yeast defective mutants [216,227] allowing molecular and structure–function studies of the corresponding enzymes. Fig. 8 represents a comparative pathway of sterol biosynthesis in mammals, fungi and higher plants. As most pathways of terpenoids biosynthesis, enzymes involved are grouped into two major classes of reactions. A first major group comprises reactions involving a common carbocationic mechanism and catalyse electrophilic cyclizations, alkylations, reductions, and isomerizations, and ultimately quenches the initially produced positive charge by proton elimination, alkyl group or hydride addition (Fig. 10). Enzymes from the second group catalyze key oxidation reactions involving the insertion of molecular oxygen (Fig. 11). They are, in part, mediated by members of the extremely broad family of cytochrome P450 monooxygenases, or as shown later in this report, by several members of a small but increasing family of membrane-bound non-heme iron oxygenases involved in lipid biosynthesis. In these lines it should be emphasized the key role of the insertion of oxygen en route to the remodeling of bioactive natural terpenoids as shown in Fig. 12.
Fig. 8. Compared sterol biosynthetic pathway in different organisms.

R = CH₃  
R = C₂H₅  
R = C₂H₅, Δ22  

cholesterol (1)  
ergosterol (2)  
campesterol (3)  
sitosterol (4)  
stigmasterol (5)
6.3. Electrophilic processes in phytosterol synthesis

6.3.1. Oxidosqualene-cyclases

(3S)-2,3-Oxidosqualene cyclases (OSC) are versatile catalysts in plants which together are responsible for the formation of a vast array of tetra- and pentacyclic triterpenoids. Since the first hypothesis concerning the course of cyclization of squalene followed by rearrangements to lanosterol [232], and the proposed stereochemical rules for the process [233,234], the tools of synthetic chemistry, protein chemistry, molecular biology, and structural biology have been used to unravel the subtle interactions between oxidosqualene and its cyclases. Most of the work has been done with the mammalian and prokaryotic enzymes and has been reviewed comprehensively in several papers [235,236]. The last 10 years have witnessed the cloning, primary amino acid sequences and putative function elucidation of more than 15 plant cyclases. In addition to the formation of cycloartenol (6), higher plant OSCs catalyze the cyclization of oxidosqualene into a variety of pentacyclic triterpenoids. The first plant OSC was cloned from A. thaliana, and encoded a cycloartenol

Fig. 9. Chemical structure of the sterols and triterpenoids considered in the present work. Numbers are those used in the text.
More recently, a number of plant triterpene synthases genes have been cloned and functionally characterized in plants. This includes for example: α-β-amyris (10) synthase from Panax ginseng [238], lupeol (11) synthase, α- (12) and β-amyrins, Ψ-taraxasterol (13), taraxasterol (14), bauerenol (15), multiflorenol (16), butyrospertol (17), tirrucalla-7,21-dienol (18) synthases from A. thaliana [239–241], Medicago truncatula and Lotus japonicus [242,243], and cucurbitadienol synthase from Cucurbita pepo [243].

The polypeptide encoded by the A. thaliana lupeol synthase was shown to be a novel multifunctional triterpene synthase. Schuffling domain mutagenesis indicated that the second quarter from the N-terminus of the synthase was shown to have a crucial importance in determining the cyclization toward lupeol or amyrin

(6) synthase [237].
Similarly, a new α-amyris producing enzyme from *Pisum sativum* was shown to be a multifunctional triterpene synthase [245].

(3S)-2,3-Oxidosqualene is also channeled to the synthesis of triterpene saponins. Triterpene saponins are restricted to a large class of dicotyledonous plants, that exclude *Arabidopsis*. Although cereals are deficient in saponins, oats synthesize specific saponins termed avenacins [246]. Their synthesis from 2,3-oxidosqualene involves cyclization of (3S)-2,3-oxidosqualene to give primarily oleanane (β-amyris) or dammarane triterpenoid. The resulting terpenoid skeleton is subject to various modifications including oxidation, substitution and glycosylation, catalyzed by cytochrome P450-dependent monoxygenases, glycosyltransferases and other enzymes [247]. In oats these genes are clustered in the genome, thus giving a rare example of gene clustering in the biosynthesis of plant secondary metabolites [248].

In 1999 the first three-dimensional structure of a triterpene cyclase (squalene hopene cyclase from *Alicyclobacillus acidocaldarius*) was obtained [249]. However X-ray structural information on OSC is still lacking. Based on the structure of *A. acidocaldarius* squalene hopene cyclase and by exploiting homologies between the different OSC, homology modeling studies on human OSC lanosterol cyclase have been presented [250]. The model is in accordance with previously performed studies with mechanism-based suicide inhibitors and the aforementioned mutagenesis experiments. It offers another way to apply structure-based drug-design methods aiming to develop novel antifungal, hypocholesterolemic and phytotoxic compounds, an interest which is still unbroken. This combination of structural and biological studies has produced important mechanistic insights into both squalene cyclase (SC) and OSC and the data have led to a unified mechanism that is consistent with the chemical and structural findings.

The relaxed substrate and product specificities of a number of plant oxidosqualene cyclases, and also bacterial squalene-cyclases, point to the possibility of generating novel triterpenes by the directed evolution of these enzymes. Directed evolution approaches have been described to find mutations that allow *A. thaliana* or *Dictyostelium discoideum* cycloartenol synthase to biosynthesize lanosterol (19) [251,252], parkeol (20) [253] or other altered triterpene product profiles [254–256]. These precise analyses of mutations responsible for altered product profile may provide important insights into the role of residues close or distant from the active-site of the cyclases.

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[Fig. 12. Examples of highly oxidized bioactive terpenoids.]
6.3.2. Other enzymes

Other carbocationic steps in the conversion of cycloartenol to stigmasterol include: methylation of cycloartenol (6) to 24(28)-methylenecycloartanol (21); cleavage of the 9β,19-cyclopropyl ring of cycloeucalenol (22) to produce a Δ8(9)-sterol, obtusifoliol (23); reduction of Δ14(15)-bond of Δ8,14-sterols to produce a Δ8(9)-sterol; isomerization of the 8(9)-bond to the 7(8)-position; methylation of the side-chain Δ24(28)-bond to produce a 24(28)-ethylidene derivative; reduction of the Δ7-double bond; isomerization of the Δ24(28)-bond to the 24(25)-position and reduction of the Δ24(25)-double bond. In addition to early work concerning the enzymology of the two methylation steps in plants [257,258], more recent data concerning this step present only in plants and fungi, have been extensively reviewed [216,259,260]. Enzymology of other anaerobic steps in plant sterol biosynthesis has been reviewed previously [216,230,231], and a lot of gaps still remain in the knowledge of molecular events involved in the course of these enzymatic reactions in plants. Molecular biology and mutagenesis analysis should yield new insights in defining the course of these mechanistically linked enzymes.

6.3.3. Carbocationic transition state analogs

The first described nitrogen containing sterol biosynthesis inhibitor is triparanol that was shown to lower serum cholesterol in hypercholesterolemic rats and to lead to an accumulation of 24-dehydrocholesterol (desmosterol) (24) in the liver and serum of these rats, suggesting blockade of the sterol C24-reductase [261]. The first sterol biosynthesis inhibitor which was proposed to function as a carbocationic high energy intermediate analog (HEIA) (transition state analog; TSA), was 25-azacycloartanol (25) which was found to be a potent inhibitor of SAM-cycloartenol-C24-methyltransferase and SAM-24-methylenelophenol-C28-methyltransferase [262]. Indeed, further studies [257,258] confirmed that (25) and analogs did not behave as substrate for N-methylation catalyzed by the C24 methyltransferase, as it was proposed for the inhibition of C24-methyltransferase by triparanol [263]. Subsequently a vast array of rationally designed carbocationic transition state analogs possessing a heteroatom with a steady positive charge in the place of the carbenium-C were synthesized and could simulate HEIs and potently inhibit specific enzymes of terpenoids biosynthesis including: oxidosqualene cyclases, C24- and C28-methyl transferases, cycloeucalenol isomerase, Δ8,14-sterol Δ14 reductase, Δ8-Δ7-sterol isomerase and Δ5,7-sterol Δ7 reductase, for example, for review: [228,230,231,263], supporting the carbocationic mechanism of the reactions catalyzed by these enzymes. Interest to use such an approach to design novel antifungal compounds is still unbroken [264,265].

6.4. Membrane-bound non-heme iron oxygenases involved in phytosterol synthesis

A key step in terpenoid biosynthesis is the insertion of oxygen en route to the formation of endpathway products. Such a process is involved in the synthesis of many bioactive terpenoids possessing additional hydroxyl or carboxylic-acid groups in their structure (Fig. 12). In addition, it is also involved in the remodeling of sterol precursors to furnish the final physiologically active sterols, i.e., cholesterol, ergosterol or sitosterol. Indeed, removal of the three extra methyl groups at C4 and C14 and insertion of the Δ5 and Δ22 double-bonds in sterol precursors result from such an oxidative process. This process is often mediated by members of the extremely broad and thoroughly characterized protein family of cytochrome P450 monoxygenases, including obtusifoliol-14α-demethylase and sterol-Δ22-desaturase of plant sterol synthesis. However, the past decade has shed light on another smaller but increasing family of membrane-bound non-heme iron oxygenases catalysing highly stereoselective and regioselective O2 dependent hydroxylations, desaturations, epoxygenations and acetylenations of lipids. These oxygenases are mostly metabolizing fatty acids; however, recent studies indicate that oxygenases involved in the C4-demethylation and C5(6)-desaturation of sterol precursors are members of this family. While considerable experimental data are available on the biochemistry and the role of residues affecting the activity of P450 monoxygenases, less is known about the enzymology and the molecular determinants affecting the activity of these non-heme iron oxygenases.

6.4.1. Membrane-bound Δ7-sterol C5(6)-desaturase

Very few enzymatic systems able to carry out in vitro lipid desaturation reactions have been described so far. We have recently shown in higher plants [266] that Δ7-sterol-C5(6)-desaturase (EC 1.3.3.2) (5-DES)
processes the desaturation of Δ7-sterol precursors to produce Δ5,7-sterols, as in mammals [267] and yeast [268] (Fig. 8). We succeeded in isolating microsomal preparations from a higher plant (Zea mays), and from an erg3 yeast strain defective in endogenous 5-DES and expressing the A. thaliana 5-DES, which functioned in the C5(6)-desaturation reaction. Enzymatic activity requiring molecular oxygen, was strongly promoted by addition of NADH or NADPH, was inhibited by cyanide, hydrophobic metal chelators and cytochrome c, and was insensitive to carbon monoxide. In addition, the maize microsomal 5-DES was strongly and completely inhibited by IgG raised against plant cytochrome bs [269] indicating that membrane-bound cytochrome bs is an obligatory electron carrier from NAD(P)H through NAD(P)H cytochrome bs reductase to the plant 5-DES as in the mammalian 5-DES process [270]. These properties showed strong similarities to those of soluble and membrane-bound fatty acid desaturases such as the membrane-bound Δ12-desaturase from safflower for example. They were not consistent with the involvement of cytochrome P450 in the reaction. Taken together, these biochemical data suggested that the 5-DES is catalyzed by a non-heme iron oxygenase [266]. The substrate selectivity study for the maize 5-DES indicated that the presence of a C7–C8 double bond was necessary for desaturation [266]. The C7 double bond may be required for the activation of the allylic C6 hydrogen atom. Alternatively, the enzyme may be highly specific, in which case the absence of a double bond at C7–C8 would lead to alterations of the orientation of the substrate binding during catalysis and particularly of the hydrogen atoms at C5 and C6, thus affecting the course of the reaction. Taken together, the results demonstrated directly that during plant sterol synthesis the Δ5-bond is introduced via the sequence Δ7-sterol → Δ5,7-sterol → Δ5-sterol (Fig. 8).

A cDNA encoding 5-DES has been cloned in A. thaliana by functional complementation of a defective yeast mutant (erg3) [271]. The A. thaliana 5-DES protein shares 29%, 35% and 29% identity with the yeast [272], human [273] and Candida 5-DES, respectively, which differ slightly in size. The heterologous expression of A. thaliana 5-DES in the yeast erg3 mutant led to a functional microsomal preparation of recombinant 5-DES, and constituted, to our knowledge, the first in vitro functional recombinant plant membrane-bound lipid desaturation yeast system described so far. It allowed isolation of sufficient amounts of 5-DES for enzymological and mechanistic studies, and site-directed mutational analysis.

Investigation of the role of a variety of evolutionary conserved residues in the 5-DES reaction was carried out using site-directed mutagenesis and expression of the mutated enzymes in the erg3 yeast strain defective in 5-DES. The first group of mutants was affected in the eight conserved histidines from three histidines-rich motifs. Site-directed mutagenesis experiments in which each of the eight conserved histidines residues (His 147, 151, 161, 164, 165, 238, 241 and 242) was individually converted to leucine (which is hydrophobic and electrically neutral), totally eliminated the 5-DES activity both in vivo and in vitro, and established that all the histidines are essential for catalysis [274]. The role of the above mentioned histidine-rich motifs was probed by mutating them to glutamic acid residues, which could provide ligands for a possible Fe center and change the reactivity of the 5-DES, as has been proposed for soluble desaturases, the R2 subunit of ribonucleotide reductase [275,276] and the methane monooxygenase hydroxylase [277]. However, glutamic acid substitutions led to completely inactive mutants. In contrast, mutation of conserved histidines or other residues not part of the consensus motifs indicated that these residues were not essential for the catalysis but contributed to the activity through conformational or other effects [274]. Hydropathy plot of the plant 5-desaturase from different sources indicated that the three histidine-rich motifs were located in hydrophilic domains from the enzyme. One possible function for the tripartite consensus sequence, HX3H, HX3HH and HX2HH, of the 5-DES, consistent with the mutagenesis and biochemical data, would be to provide the ligands for a presumed catalytic Fe center, as previously proposed for a number of integral membrane enzymes catalysing desaturation and hydroxylations [278,279] and is discussed later.

A nuclear and recessive mutant of A. thaliana, ste1, defective in the sterol-C5-desaturase has been isolated and identified [280]. This mutant accumulates Δ7-sterols, (24R)-24-ethyl-5α-cholest-7-en-3β-ol (7), and (24ξ)-24-methyl-5α-cholest-7-en-3β-ol (8) at the expense of the normally occurring campesterol (3) and sitosterol (4). The 5-DES ORF from the A. thaliana leaky mutant ste1, contains a single mutation, T114I, which was proposed to be responsible for the 5-DES deficiency of this mutant. The T114I mutant was expressed in the erg3 yeast mutant and further enzymatically analyzed in the corresponding microsomal preparation. Threonine 114 was found not to be essential for the 5-DES activity. However, replacement of
Threonine 114 by the functionally non-conservative isoleucine led to a 10-fold reduced catalytic efficiency of the 5-DES and the corresponding transformed yeast accumulated substantial amounts of Δ5-sterols at the expense of Δ5,7-sterols in accordance with data obtained with the ste1 mutant [271,274]. An hydroxyl function is conserved at position 114 in the ERG3 family. Thr114 was replaced by the functionally conservative serine residue and the catalytical properties of mutants T114S were compared to that of the wild-type 5-DES. Mutant enzyme T114S displayed a 28-fold higher $V_{\text{max}}$ value than the wild-type enzyme. Moreover, $V/K$ values were 4-fold higher than that of the native 5-DES, indicating that a hydroxyl containing sidechain at position 114 substantially increased the catalytic rate of the 5-DES. This data supported the role of threonine 114 in the stabilization of the transition state of a rate-controlling step in the 5-DES catalytic pathway [274].

It has been previously proposed that enzymes maximize rates by binding transition states strongly and substrate weakly [281], and that they should have evolved in this way [282]. From this point of view, a serine residue at position 114, brings an apparent additional stabilization of the transition state and decreases the apparent binding energy of the substrate in the ground-state. In contrast, for mutant T114I, the free energy of the substrate complex both in the ground-state and in the rate determining transition state was approximatively uniformly increased. The result was a marked decrease in the activation energy to reach the transition state and a substantial improvement of the reaction rate of this mutant.

The ultra-selective oxidation chemistry and the detailed mechanism of the sterol-C5(6)-desaturase was probed using an approach based on intermolecular primary deuterium kinetic isotope effects. Deuterium-labeled 5α-cholest-7-en-3β-ol (9) bearing one or two deuteriums at the C-5α and (or) C-6α positions were synthesized in high isotopic and chiral purity. These compounds were used as substrates with the microsomal wild-type Z. mays and recombinant A. thaliana Δ7-sterol-C5(6)-desaturases to probe directly the stereochemistry and the mechanism of the enzymatic reaction. Clearly, in the conversion of (9) by both 5-DESs, the 6α-hydrogen was removed [283]. This data indicated a stereospecific syn desaturation during catalysis by both plant 5-DES. It is in accord with former labeling pattern data obtained in vivo in plants [284], fungi [285] and rat liver [286]. 6α-2H-5α-cholest-7-en-3β-ol showed a substantial intermolecular deuterium kinetic isotope effect (DKIE) on $V$ and $V/K$, $D^5V = 2.6 ± 0.3$, $D^6V/K = 2.4 ± 0.1$ and $D^6V = 2.3 ± 0.3$, $D^6V/K = 2.3 ± 0.2$ for the Z. mays and A. thaliana wild-type 5-DES, respectively. In contrast, negligible or minor isotope effects, $D^5V = 0.99 ± 0.04$, $D^5V/K = 0.91 ± 0.08$; and $D^5V = 0.93 ± 0.06$, $D^5V/K = 0.96 ± 0.04$, respectively, were observed with 5α-2H-cholest-7-en-3β-ol. The observed pattern of isotope effects indicated that: (i) the desaturation involves asynchronous scission of the two C–H bonds at C5 and C6. The data clearly indicated a stepwise mechanism, and ruled out a concerted mechanism, (ii) the 5-DES initiates oxidation by cleavage of the chemically activated C6α–H bond, (iii) this chemical step involving considerable C6α–H bond stretching, is partially rate-limiting the overall desaturation process. A possible mechanism for the 5-DES consistent with the data would involve an initial, energetically difficult C6α–H activation step, executed by a compound Q-type oxidant [287] to produce a C6 carbon-centered radical/FeOH pair (Fig. 13). This step would be similar to that proposed for a number of reactions catalyzed by cytochrome P450-dependent hydroxylations and desaturations involving an hypervalent iron-oxo species [288,289]. This radical could collapse to olefin via different pathways. Obtained experimental evidences favored a disproportionation reaction (pathway a) to give an olefinic product and iron-bound water [283] (Fig. 13). In the case of the plant 5-DES, obtained data did not favor pathway c including a C6α- or C6β-hydroxyl intermediates previously suggested [290], since such intermediates could not be detected during the reaction, and second, synthetic samples of these intermediates were not converted into 7-dehydrocholesterol by the enzymatic preparation [283]. The mechanism found for the present steroid desaturase is similar with that recently demonstrated for a number of membrane-bound fatty-acid desaturases and hydroxylases which also involve a large deuterium KIE for one of the C–H cleavage step while a negligible effect is found at the proximal carbon [291,292]. The switch controlling the ratio of desaturation versus hydroxylation remains an intriguing question. What governs the orientation of the reaction pathway to desaturation at C5(6) versus possible hydroxylation at C6 is not currently understood and presumably reflects the precise positioning of the substrate relative to the oxidant species within the Michaelis complex, to produce a preferential loss of the C5 hydrogen to form an olefin, instead of a C–O bond formation to form an alcohol at C6. That is, as suggested before, if access to the proximal carbon hydrogen is hampered, then hydroxylation of substrate would be favored (negative catalysis) [293].
The preceding methodology was combined with variation of enzyme structure to find information on the role of threonine 114 in catalysis by the 5-DES from *A. thaliana*. The data indicated the same stereochemical integrity for the T114 mutants and a primary DKIE pattern of desaturases that is qualitatively similar to that observed for the wild-type desaturase, suggesting a similar reaction pathway for these isoforms. In addition, the data indicated that a hydroxyl-containing side-chain at position 114 substantially increases the catalytical rate of the 5-DES and makes the deuterium sensitive step less rate limiting. There is no evidence that the intrinsic isotope effect is changed in the mutants. Rather it is likely that the chemistry is slower in the T114I mutant (STE1), decreasing the commitment and resulting in a more fully expressed KIE, closer to the intrinsic value. Conversely, in the case of mutant T114S, the chemistry is faster but subsequent steps, as product release, change less, so that the observed KIEs are smaller.

6.4.2. Membrane-bound sterol-4α-methyl-oxidases from the C4-demethylation complex

In all organisms, the sterol molecule becomes functional only after removal of the three methyl groups at C4 and C14. The inefficiency of sterol precursors possessing C4-methyl groups has been shown particularly in the yeast *Saccharomyces cerevisiae*, where ERG25 the gene coding the sterol-4α-methyl-oxidase (SMO) is essential. In animal and yeast, following removal of the C14 methyl group of lanosterol, both

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**Fig. 13.** Pathway a: proposed mechanism for Δ7-sterol-C5(6)-desaturase; experimental data are not consistent with pathways b or c.
C4-methyl groups are sequentially eliminated by the same C4-demethylation enzymes complex involving a single C4-oxidase gene, ERG25 [295,296]. The C4 demethylation process is central in sterol biosynthesis and involves 10 of the 20 enzymatic steps from oxidosqualene to phytosterols. It is energetically costly since it requires six upon the eleven oxygen molecules and eight upon the eighteen NAD(P)H molecules consumed in the post squalene segment of sterol biosynthesis. Moreover, since at least three crucial enzymes are involved, there are more opportunities for regulation and inhibition.

During the conversion of cycloartenol to phytosterols in photosynthetic eukaryotes, early in vivo labelling experiments [297,298] and more recent biochemical data [299] strongly suggested that two biosynthetically distant and distinct oxidative systems are involved in the C4-demethylation of sterol precursors and thus, is profoundly different with the animal and fungal systems. It has been enzymatically demonstrated in animals and in plants that the C4-demethylation complex involves a SMO which produces a 4α-carboxy-sterol derivative [299,300]. It is subsequently oxidatively decarboxylated by the 4α-carboxysterol-C3-dehydrogenase/C4-decarboxylase (4α-CD) [301–303] to produce a 3-oxosteroid which is stereospecifically reduced by a NADPH-dependent sterone reductase [304–306] (Fig. 14). In yeast, the genes encoding these components have been cloned and identified, that is, ERG25 [295], ERG26 [302] and ERG27 104 [306]. Recently the enzymatic system corresponding to the SMO component of this complex in yeast has been described [307]. It allowed to specifically investigate the enzymological properties of the yeast SMO and, particularly, to detect and identify the immediate oxidized metabolite by the yeast SMO, that is a C4-hydroxymethyl-sterol derivative which was identified by MS analysis of its diacetate derivative. Thus, in yeast, as well as in animal [300] and plants [299], the SMO is a multifunctional oxidase catalyzing three successive monooxygenations of the C4-methyl group to produce the C4-carboxysterol-derivative. Finally, similarly as for the 5-DES, biochemical properties of the plant and yeast SMOs were consistent with the involvement of a non-heme iron oxygenase [299,307].

Fig. 14. Putative multienzymatic complex involved in sterol C4-demethylation.
By combining homology and sequence motif searches with knowledge relating to sterol biosynthetic genes across species, five SMO cDNAs from *A. thaliana* (*AtSMO*) belonging to two families have been recently cloned [308, 309].

The two families of plant SMOs share only 37% sequence identity to each other and 26–34% identity to the yeast and mammalian SMOs. They cluster well together and with ERG25 from animals and yeast, as shown in the phylogenetic tree of Fig. 15. They also cluster with other identified membrane-bound non-heme iron hydroxylases such as cholesterol 25 hydroxylase (CH25H) [310] or C4 sphingolipid hydroxylase (SUR2) [311]. Heterologous expression of these cDNAs in a yeast *erg25* ergosterol auxotroph, lacking sterol-4α-methyl-oxidase activity, led to two groups of complementation: The SMO2 group of complementation

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**Fig. 15.** Phylogenetic tree for selected members of the membrane-bound non-heme iron oxygenases family. SMO, sterol methyl oxidase; *AtSMO21*, *At1g07420*; *AtSMO22*, *At2g29390*; *LeSMO21*, TC125471; *TaSMO2*, TC66074; *OsSMO1*, OSJNBA001014.3; *HsSMO*, NM006745; *ScSMO*, NP013157; C25H, cholesterol 25 hydroxylase; *HsC25H*, AF059214; *MmC25H*, AAC87480; SUR, C4-shingolipid hydroxylase; *ScSUR2*, AAA16608; *AtSUR21*, At1g14290; *AtSUR22*, At1g09640; SDES, Δ7-sterol-Δ5(6) desaturase; *AtSDES2*, AAD20458; *NtSDES*, AAO4034; *AtSDES1*, At3g02580; *AtSDES2*, At3g02590; *HsSDES*, NP008849; *MmSDES*, BAA33730; *CgSDES*, ABO2330; CYMAA, p-cymene monoxygenase, hydroxylase; *PcCYMAA*, AAB62299; ALK, alkane-1-monoxygenase; *PsALK*, AAC40951; D12FA, Δ12-fatty acid epoxygenase; *CpD12A*, Y16283; 12AC, Δ12-fatty acid acetylase; *Ca12AC*, CAA76158; FAD2, ω-3 fatty acid desaturase (endoplasmic reticulum); *AtFAD2*, P48623; FAD7, ω-3 fatty acid desaturase (chloroplast); *AtFAD7*, NM111953; *At3g11170*; FAD6, ω-6 fatty acid desaturase (chloroplast); *AtFAD6*, NP_194824; FAT, ω-3 fatty acid desaturase; *CeFAT1*, AAA67369; *hSDES*, Δ8-shingolipid desaturase; *AtbSDES*, At2g46210; Δ8SDES, Δ8 desaturase; *AtΔ8SDES*, At3g15870; Δ8DES, Δ8 sphingolipid desaturase; *HsΔ8DES*, S68358; Δ6DES, fatty acid Δ6-desaturase; *BoΔ6DES*, AF007561.1; FAH, fatty acid hydroxylase; *AtFAH1*, At4g20870; *AtFAH2*, At2g34770; *PfFA*, fatty acid desaturase, CAD77651; *Syn29SDES*, Δ9-desaturase; *ScOLE1*, fatty acyl-CoA Δ9-desaturase, NP011460.1; *PoCRTW*, β-carotene ketolase, BAA09591.1; *CAH*, carotenoid hydroxylase; *CaCAH1*, Y0225; *CaCAH2*, CAA70888; CER, aldehyde decarbonylase; *AtCER1*, At1g02205.2; *AtCER2* like, At2g37700; *AtCER2* like, At1g02190.
restored growth, but only low levels of ergosterol biosynthesis. The SMO1 group did not complement erg25-25c [308,309]. This data suggest that only one family of SMOs is functional for the oxidation of 4,4-dimethylzymosterol, the physiological substrate of the yeast SMO accumulating in erg25. These results represent the first functional identification of sterol-4α-methyl-oxidase genes from plants. All AtSMOs amino-acid sequences were characterized by the presence of three histidine-rich motifs, HX3HX8 or 9 HX2HHX73 or 75 HX2HH, which exhibit a topology and spacing of amino acids within the histidine motifs typical of the ERG3–ERG25 family, PFAM01598. Moreover, the spacing and topology of the histidine motifs are clearly distinct from those found in the extended family of membrane-bound fatty acid desaturases/hydroxylases. The identification of conserved motifs in different enzymes implies that they are evolutionarily related provided that the structure and relative spacing of the motifs is comparable. Indeed, elements of the motif are equally spaced in the ERG3–ERG25 family and are also placed in equivalent positions with respect to potential membrane-spanning domains [274,296,308]. In the case of fatty acid desaturase–cytochrome bs fusion proteins, the motif is predicted to occur on the same face of the membrane as the cytochrome bs [312,313]. Because the histidine-rich motifs are essential to 5-DES as well as to other enzymes of the family, it has been proposed that they are involved in coordinating an active-site metal center [278,279]. Moreover, spectroscopic studies of alkane α-hydroxylase provided evidence that a di-iron center is present in at least one member of this class of integral-membrane enzymes [314]. In the absence of crystallographic data for this membrane-bound desaturase, no information about specific details of the active-site including the proposed histidine coordinating ligands and the location of the hydrophobic binding pocket is available. The only data in this context have been obtained from the crystal structure of the related soluble castor seed Δ9 stearyl-ACP-desaturase. In that case, the iron atom is buried in the middle of the structure, resulting in an isolated local environment suitable for reactive oxygen chemistry [315].

To elucidate the precise functions of SMO1 and SMO2 genes families, their expression was reduced by using a virus-induced gene silencing (VIGS) approach in N. benthamiana. Two cDNA fragments of 387 and 498 bp corresponding, respectively, to NtSMO1 and NtSMO2 were cloned from N. tabacum and were inserted into the viral TTO1 vector, and N. benthamiana inoculated with the viral transcripts. The sterol profile of infected plants was analyzed: following silencing with SMO1, a substantial accumulation of 4,4-dimethyl-9β,19-cyclopropyl-sterols (i.e., 24-methylenecholestanol, (21)) was obtained, while qualitative and quantitative levels of 4α-methylsterols were not affected. In the case of silencing with SMO2, an important accumulation of 4α-methyl-Δ7-sterols (i.e., 24-ethyldienelophenol (27) and 24-ethyllophenol) was found, with no change in the levels of 4α-dimethylsterols.

These clear and distinct biochemical phenotypes demonstrate that, in contrast to animals and fungi, in photosynthetic eukaryotes, these two novel families of cDNAs are coding two distinct types of C4 methylsterol-oxidases controlling, respectively, the level of 4,4-dimethyl- and 4α-methylsterols precursors [309].

To our knowledge, plant mutants affected in SMO activity have not been reported yet. In this respect, the present study could give important clues for the elucidation of the physiological roles of 4,4-dimethyl- and 4α-methyl-sterols, and of the biological significance of the existence of two distinct C4-demethylation complexes in photosynthetic eukaryotes.

Finally, the phylogenetic tree of the membrane-bound non-heme iron oxygenases family, shown in Fig. 15 clearly reveals the diversity of their functions.

6.4.3. Other components of the C4-demethylation complex

The enzymes corresponding to the remaining two steps in the plant C4-demethylation process have been identified and their properties studied in maize. Evidence for the existence of a microsome-bound NAD+-dependent 4α-carboxysterol-C3-dehydrogenase/C4-decarboxylase (4α-CD) in plant was directly obtained from a maize microsomal preparation able to oxidatively decarboxylate several substrates, including 4α-carboxy-cholesterol-7-en-3β-ol (26). The BriJ W-1 solubilized enzyme was partially purified 290-fold and the apparent molecular mass of 4α-CD in sodium cholate was estimated to be 45 kDa [303]. The results supported the contention that cleavage of the C4–C32 bond of plant C4 methylsterols resulted of two separate processes: An oxygen and NAD(P)H-dependent oxidation of the 4α-methyl group to produce the corresponding 4α-carboxylic acid, followed by oxygen-independent cleavage that is consistent with the conclusion that this
oxosteroid reductase was associated with the C4-demethylation process. In plants, study of genes for decarboxylation and ketone reduction remain to be carried out. Together with the SMOs genes, these genes are interesting because no corresponding plant mutants have been detected in *A. thaliana*. This might mean that the ability to demethylate is essential for viability of the plant cell in accordance with the essentiality of ERG25, ERG26 and ERG27 in yeast [295,301,306]. In addition, a recent *S. cerevisiae* microarray expression study indicated that an ORF, YER044C, designed ERG28, was strongly coregulated with ergosterol synthesis [316]. Disruption of the ERG28 gene in yeast results in slow growth and accumulation of sterol intermediates of the C4 demethylation process. Two-hybrid analysis indicated that Erg25p, Erg26p, and Erg27p components required for the C4-demethylation, form a complex within the yeast cell [317]. Coimmunoprecipitation studies suggested that Erg28p works as a transmembrane scaffold to tether Erg27p and possibly other C4 demethylation proteins (Erg25p, Erg26p), forming a demethylation complex in the yeast endoplasmic reticulum [318] (Fig. 14).

Although, there are at least three enzymes involved in the C4 demethylation complex, no agriculturally or medically useful inhibitors such as the widely used triazoles or morpholines series [230,231] have emerged. However, the antifungal agent, 6-amino-2-n-pentylbenzothiazole was shown to directly inhibit, in vitro, the yeast SMO, but was inactive with the plant SMOs [307]. In addition, fenhexamid, a recently developed botryticide was shown to inhibit sterol biosynthesis in the fungus *Botryotinia fuckeliana* and led to the accumulation of 3-oxo compounds suggesting an inhibition of the 3-oxosteroid reductase [319].

6.5. Kinetically favored pathway in the conversion of cycloartenol to Δ5-sterols

Most of the reports on the enzymology of sterol transformation in plants have been obtained with a microsomal preparation isolated from *Z. mays* seedlings. The described data include apparent kinetical parameters, $K_m$ and $V_{max}$ for substrate, cofactors, and a variety of substrate analogs, allowing the substrate specificities of these distinct enzymes to be determined. Since the enzymes were all studied in the same microsomal preparation isolated from the same plant tissue, the different $K_m$ and $V_{max}$ values may reveal, together with the control by the substrate specificity, firstly, the kinetically favored pathway which leads directly to the end-product during active cell proliferation, and secondly, the rate limiting steps in the pathway. Examination of the enzymological literature includes studies of S-adenosylmethionine-sterol-C24- and C28-methyl-transferases [257–260,320], 4,4-dimethylsterol- and 4α-methyl-sterol-C4-methyl-oxidases [299], 4α-carboxysterol-C3-dehydrogenase/C4-decarboxylase [303], 3-oxosteroid-reductase [305], cycloeucalenol-isomerase [321], obtusifoliol-14α-methyl demethylase [322], Δ8,14-sterol-Δ14 reductase [323], Δ8-Δ7-sterol isomerase [324,325], Δ7-sterol-C5(6)-desaturase [266], and Δ5,7-sterol-Δ7-reductase [326] (Table 1, Fig. 16). Firstly, it turns (Table 1) that maize possesses a number of enzymes that exhibit a high degree of substrate specificity, including particularly monoxygenases involved in the removal of the C4 and C14 methyl groups. The specificity of these enzymes is particularly strict for structural motifs of the tetracyclic nucleus while variation of the side-chain structure are more often accepted. For example, SMO1 requires a 9β,19-cyclopropyl-ring [299], SMO2 a Δ7-unsaturation [299] and 14DM absence of a 4β-methyl group [322]. If minor pathways are poorly active in the case of these enzymes so that only certain intermediates may finally lead to Δ5-sterols, affecting their rate should be associated with a change of the amount of end pathway Δ5-sterols. Secondly, another control of the tetracyclic compounds pathway could be provided by enzymes with low turnover such as the C24-methyl transferase, the 4α-methyl oxidases and the 14α-demethylase.

This kinetic control of the pathway is exemplified by recent studies of plant mutants affected in postqualine enzymes. A first example is given by the biochemical characterization of a tobacco mutant (LAB 1–4) that overproduces 10-fold more sterols [327] due to an increase of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) activity [328]. In this mutant, high accumulation of cycloartenol, 24-methylenecloroartanol (21) and 24-ethylidenelophenol (27), and substantial accumulation of obtusifoliol (23) were observed in calli. More recently, co-expression of HMGR and C24-sterol methyltransferase type 1 in transgenic tobacco enhanced carbon flux towards end-product sterols and resulted in elevated accumulation of substrates of
the C4-demethylation reactions [329]. These data are consistent with the conclusion that the methylation of cycloartenol, the two C4-demethylation and the C14 demethylation reactions limit carbon flow to end-product sterols, at least in a physiological situation when the carbon flow is upregulated, in accordance with kinetic data obtained in vitro in the above mentioned maize enzymatic preparations.

Another example is given by the sterol profile of a number of *A. thaliana* mutants in the sterol synthetic pathway. This is the *SMT1*, *cephalopod* mutant which encodes a defective SAM-cycloartenol synthase

![Fig. 16. Kinetically favored pathway to 24-ethyl sterols in *Zea mays*. Numbers with the reaction refer to the catalytic constant $V_{\text{max}}$ (nmol mg\(^{-1}\) h\(^{-1}\)) of the corresponding enzymatic reaction in a *Zea mays* microsomal preparation, as mentioned in Table 1.](image-url)

Table 1

<table>
<thead>
<tr>
<th>Step</th>
<th>Enzyme</th>
<th>Favored substrate</th>
<th>$K_{\text{m}}$ ((\mu\text{M}))</th>
<th>$V_{\text{max}}$ ((\text{nmol mg}^{-1}\text{ h}^{-1}))</th>
<th>$V/K$ ((\text{h}^{-1}))</th>
<th>Substrate specificity</th>
<th>References</th>
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<td>Oxidosqualene</td>
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<td>++</td>
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<td>++</td>
<td>[257–260]</td>
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<td>0.06 +++++</td>
<td>++</td>
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<td>4α-Carboxy,4β-methylsterol</td>
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<td>126</td>
<td>nd +</td>
<td>+</td>
<td>[303]</td>
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<td>+</td>
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<td>++</td>
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<td>0.24 ++</td>
<td>++</td>
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<td>++</td>
<td>[266]</td>
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<td>36.6</td>
<td>0.32 ++</td>
<td>++</td>
<td>[326]</td>
</tr>
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</table>
C24-methyltransferase [330–332], the fackel (fk) mutant which encodes a defective sterol C14 reductase [333,334] and the hydra1 mutant which encodes a defective Δ8-Δ7-sterol isomerase [331,335,336]. The fackel callus accumulated up to 76% of Δ8,14-sterols including mainly Δ8,14-sitosterol (28) (59%) consistent with the pathway of Fig. 16 where the subsequent Δ8-Δ7-sterol isomerase is specific for Δ8-sterols. Accordingly, unmetabolized Δ8,14-sterol substrates, and no Δ7,14-sterols, were recovered in microsomal preparation or plant cell cultures containing both sterol C14 reductase and Δ8-specific inhibitor (15-azasterol) of the sterol C14 reductase [323,337]. The sterol profile of the fackel mutant totally confirmed the high level of Δ8,14-sterols, and reduction of end-products of sterol synthesis, obtained after treatment of plant cell cultures with an inhibitor of C14-reductase, 15-azasterol [337]. Although the detailed sterol profile of the hydra1 mutant was not determined, it showed a dramatic decrease in campesterol and sitosterol concentrations similar to that obtained in the fackel mutant [333,334]. Genetic interactions between cephalopod (CPH), fackel and hydra1, revealed that fackel and hydra1 act sequentially, thus confirming the previous biosynthetic data [323,337], while CPH acts independently of these genes to produce essential sterols [331]. The SMT1 null mutant was shown to still produce alkylated sterols [330], that is consistent with the previously observed overlap in the substrate specificity of sterol methyltransferases in phytosterol synthesis [258,338,339].

Taken together, the data obtained recently by powerful genetic analysis are globally consistent with the previous enzymological data of Table 1 and Fig. 16. They suggest a more linear pathway for transformation of the tetracyclic nucleus, and a more independent and parallel construction of the sterol side-chain in the case of a non-perturbated sterol biosynthesis. The modeling of the sterol structure is not only controlled by the substrate selectivity of the enzymes involved, but also by their high product selectivity as shown by mechanistic data on reaction course carried out in corn. Indeed, cleavage of the 9β,19-cyclopropane ring proceeds regioslectively to the Δ8(9)-double bond [340,341], demethylation at C14 produces a Δ4(15) double bond [322], and the Δ8 double bond is regioselectively isomerized to the Δ7 position without formation of a Δ8(14) double bond [325].

Beside this possible kinetic regulation of the plant sterol biosynthetic pathway, and in contrast to the situation in animals [342,343] and yeast [344,345], much less is known regarding how plant cells monitor their sterol content and regulate sterol synthesis.

A series of previous works have addressed the rate-limiting nature of HMGR for sterol biosynthesis in plants [328,346,347], and recently a feedback upregulatory effect occurring at the HMGR protein level in response to a depletion of end-products was reported in plants [348]. Squalene synthetase (SQS), a putative branch point diverting carbon from the central isoprenoid pathway to sterol has been investigated as a possible means by which sterol biosynthesis may be controlled. Data obtained suggested that the suppression of SQS in elicitor-treated tobacco cells was regulated at several different levels: probable decreased transcription of the SQS gene and no change in the absolute level of the SQS polypeptide, implicating a post-translational control for this enzyme activity [349]. It has been proposed that the C-24 methylation of cycloartenol (SMT1) is a major site of regulation in the sterol biosynthetic pathway [259,346,347,350]. While an Arabidopsis sterol methyltransferase 1 (SMT1) deletion mutant has been shown to still accumulate alkylated sterols, but at altered levels [330], overexpression of SMT1 in tobacco seed increased the amount of total sterols in seed tissue by up to 44% and decreased cycloartenol and cholesterol levels by up to 53% and 34%, respectively. A concomitant increase in endogenous HMGR activity, presumably upregulated by reduced level of cycloartenol, was also observed. Thereby SMT1 controls the flux of carbon into sterol biosynthesis in tobacco seed [329,351].

Several investigations have also sought to determine the contribution of specific biosynthetic steps by selection of genetic mutants or by the use of genetic engineering approaches previously mentioned in this report [330,331,333,334,336]. These studies have demonstrated the important role of sterols in plant growth and development but have not directly addressed how plant sterol biosynthesis is regulated. However, they suggested that sterols other than brassinolides may serve as novel signals controlling cell fate during plant embryogenesis [222,331,334].

It has been suggested that genes coding for enzymes making up a specific metabolon must have similar transcriptional networks to coordinate expression of the metabolic unit. Many enzymes involved in plant sterol biosynthesis are coded by multiple genes [216] and genes situated downstream of cycloartenol are involved
only in sterol biosynthesis. The biological significance of these paralogs remains to be elucidated. In these lines, study of the substrate specificities of the different SMO isoenzymes might reveal one possible role for this multiplicity.

Finally, further works on the role of the protein in the mechanism of the enzymes committed to the different reactions of the post-squalene sterol biosynthesis in plants, based on structural elucidation, mutants characterization and directed evolution, are required to enhance our understanding of these enzymes. These studies will be facilitated by the possibility to express cDNAs encoding these plant enzymes in heterologous systems allowing particularly the rigorous analysis of mutations responsible for altered enzymological properties. Precious clues could also be obtained from crystal structure of enzymes from bacterial sources such as the *A. acidocaldarius* hopene synthase [249] and the *M. tuberculosis* lanosterol 14-demethylase [352]. They could open new ways for the design of novel or more selective biocides affecting key enzymes of sterol biosynthesis, a field presenting a constant need for new compounds.

7. Biogenesis of mitochondrial isoprenoids

7.1. Ubiquinones

It can be inferred from previous studies that IPP is imported from the cytosol and used for ubiquinone biosynthesis [353] according to a pathway not yet definitively established in plants, but probably very similar to that operating in Gram-negative bacteria and yeasts [354] (Fig. 17). Feeding experiments show that the side-chain of ubiquinone is synthesized from mevalonate-derived IPP [355]. Although mitochondrial FPPS [356] and GGPPS [85] isoforms have been characterized, specific prenyltransferases catalyzing the synthesis of the side-chain of ubiquinone have not been characterized. It has been proposed that ubiquinone-9 (UQ-9) is synthesized in the ER–Golgi membranes of spinach leaves [357] and transported in the mitochondria [358]. Intracellular transport of UQs is supported by the fact that the inability of yeast coq mutants to grow on a non-fermentable carbon source was restored following the addition of UQ-6 in the medium [359]. Also, the uptake and transport of dietary UQ-8 to mitochondria prevented the growth arrest and sterility phenotypes of *Caenorhabditis elegans* clk-1 mutants [360]. In this context, it is interesting to note that *A. thaliana* possesses a plastidial and cytosolic solanesyl diphosphate synthases (SPPS) [109,361]. One could suggest that the cytosolic SPPS could be involved in the biogenesis of the prenyl side-chain of UQ-9. This hypothesis is strengthened by experiments in which *p*-hydroxybenzoate polyprenyl diphosphate transferase (PHBPT), encoded by *COQ2* was deliberately targeted to the ER of yeast and tobacco. Under these conditions, the UQ-6 content of transgenic yeasts and the UQ-10 content of transgenic yeasts and tobacco plants were 3- and 6-fold increased. The same trend was observed in transgenic yeasts and tobacco expressing *COQ2* properly targeted to the mitochondria [362]. The increased synthesis of UQ-10 in tobacco was paralleled by an increased tolerance to high-salt concentration and to oxidative stress induced by methylviologen [362].

Following the prenylation of *p*-hydroxybenzoate (PHB) three *C*-hydroxylations, *C*- and *O*-methylations and a decarboxylation steps are required (Fig. 17). How these individual steps are sequentially ordered in plants is not known. Genetic and biochemical information obtained from bacteria and eucaryotes [354,363] could now help clarify the sequence of the individual steps operating in plants. The gene encoding *Arabidopsis* PHBPT has been characterized [364]. PHBPT accepted geranyl diphosphate (GPP) as the allylic substrate and could direct in vivo the synthesis of UQ-6 and UQ-10, the main UQs that accumulate in *S. cerevisiae* and *S. pombe*, respectively [364]. This indicated that PHBPT has broad substrate specificity and suggests that the side-chain of ubiquinone is primarily determined by the specificity of the mitochondrial prenyltransferase generating the side-chain. The *PHBT* mRNA was predominantly expressed in the flower and disruption of the gene in *Arabidopsis* resulted in the arrest of embryo development at an early stage of zygotic embryogenesis. These results demonstrate that the *A. thaliana* *PHBT* gene involved in the biosynthesis of mitochondrial UQ plays an essential role in embryo development in *Arabidopsis* [364], in good agreement with previous data reported from animal studies [365,366]. The nature of the chain-length of plant ubiquinone varies according to species. For instance ferns accumulate mostly UQ-10, while most
other plants accumulate UQ-9 and UQ-10. In some plants, UQ-9 dominates while in others Q10 dominate. UQ-8 is also detected as a minor homologue in some plants. In Arabidopsis the main homologue is UQ-9, while in Capsicum organs, the main homologue is UQ-10, followed by minor UQ-11, UQ-9 and UQ-8 homologues [367]. Since the specificity polyprenyl diphosphate defines the UQ chain-length [368], one must consider the existence of diverse mitochondrial prenyltransferases dedicated to the synthesis of mitochondrial side-chain containing C8, C9, C10 and C11 isoprene units. Concerning the steps post-PHB, one could note that Arabidopsis AtCOQ3 homologous to yeast COQ3 has been cloned [369]. AtCOQ3 encodes 3,4-dihydroxy-5-hexaprenylbenzoate methyltransferase.

7.2. Isoprenoid oxidation

It has been reported that plant mitochondria is involved in the oxidative catabolism of isoprenoids in a pathway that requires in addition the interaction with plastids and microbodies [370–372]. This pathway is reminiscent of the modified β-oxidation pathway that operates in Pseudomonas citronellolis [373].
8. Biogenesis of plastid isoprenoids branching from GGPP

8.1. Diterpene biogenesis

The plastid is the main if not the unique site for the synthesis of GGPP that constitutes the backbone for the synthesis of diverse diterpenes. However, it must be mentioned that in the case of irregular diterpenes such as anisotomenes, two GPPs are used instead of one GGPP [79] (Fig. 4). The initial steps of GGPP transformation are catalyzed by diterpene cyclases and is usually followed by further modification in the ER–cytosol. The gibberellin pathway serves to illustrate this point (Fig. 18). Diterpene cyclases have been classified into cyclases A

![Diagram of Gibberellin biosynthetic pathway and compartmentation](image)

Fig. 18. Gibberellin biosynthetic pathway and compartmentation. Enzyme abbreviations: CPS, ent-copalyl diphosphate synthase; KS, ent-kaurene synthase; KO, ent-kaurene oxidase; KAO, ent-kaurenoic acid oxidase; GA\textsubscript{12}S, GA\textsubscript{12}-aldehyde synthase; GA7OX, GA 7-oxidase; GA13H, GA 13-hydroxylase. Substrate abbreviation. GGPP, geranylgeranyl diphosphate.
and B [374]. In type B, a N-terminal conserved aspartate motif E/DxD(D,N) is involved in the protonation-initiated cyclization of GGPP. In A type cyclase, a C-terminal conserved aspartate DDXXD motif is involved in the diphosphate ionization-initiated cyclization of the substrate [374,375]. Thus in the plant gibberellin pathway, the consecutive action copalyl diphosphate synthase (CPS) (B-type cyclase) and ent-kaurene synthase (KS) (A-type cyclase) are required to convert GGPP into kaurene (Fig. 18). On the other hand, in fungi that produce gibberellin, both activities reside on a single bifunctional protein (CPS/KS) that has CPS and KS [376,377]. Domains A and B are present in abietane synthase involved in the synthesis of resin acids. Interestingly, abietane synthase catalyzes two consecutive and mechanistically different cyclizations [375,378]. Taxadiene synthase [379] and levopimaradiene [380] that catalyze the first committed step of taxol and ginkgolide A have both domains.

With respect to the gibberellin pathway, ent-kaurene is further oxidized by a plastid envelope ent-kaurene oxidase (KO), a cytochrome P450 mono-oxygenase [381], that establishes a link between the plastid and the endoplasmic reticulum–cytosol for further modifications by membrane-bound cytochrome P450 mono-oxygenases and soluble or cytosolic 2-oxoglutarate-dependent dioxygenases (Fig. 18). The Arabidopsis genome contains only one gene encoding each of the diterpene biosynthetic enzyme CPS, KS and KO, whereas in the rice genome 3 CPS, 8 KS and 5 KO have been identified[382]. Based on expression and mutant analyses, it has been established that as in Arabidopsis, rice CPS, KS and KO specifically dedicated to gibberellin synthesis are encoded by single genes. This suggests that the remaining rice genes encode enzymes involved in the biosynthesis of rice phytoalexins [382]. Indeed, fifteen phytoalexins have been identified in rice and, with the exception of sakuranone, a flavonoid, all phytoalexins belong to the diterpene family [383]. Indeed, in suspension-cultured rice cells, diterpenoid phytoalexins were produced in response to UV and exogenously applied elicitors [384] according to a mechanism involving transcriptional expression of diterpene cyclases [385]. Thus, it has been shown that in rice, CPS\textsubscript{ent}1 and CPS\textsubscript{ent}2 were dedicated, respectively, to gibberellin and defensive phytoalexin synthesis [386,387]. In a similar vein, one could note that two kaurene synthase genes KS\textsubscript{1} and KS\textsubscript{2} have been characterized in Stevia rebaudiana leaves [388]. This phenomenon is reminiscent of the active biosynthesis of diterpene steviol glycosides, that accumulate in the leaves [388]. Similarly, it could be anticipated that the expression of specific diterpene cyclases accounts for the increased synthesis of diterpene antioxidants in plants belonging to the Labiate such as sage (Salvia officinalis) and rosemary (Rosmarinus officinalis) [389]. It has been shown that their synthesis is induced by light and drought stress [390,391]. The same mechanism could apply for 11E,13E-\textgamma-11,13-diene-8a,15-diol (WAF-1), an activator of MAPK cascade, involved in plant defenses [392].

Finally, either in the case of gibberellins or non-gibberellin-related diterpenoids, the later steps of diterpene synthesis often involve modifications of plastid products by cytosolic enzymes. How this export is mediated is not known. Potential plant diterpene ABC transporters have been identified in Nicotiana plumbaginifolia [393,394]. However presently identified proteins are located in the plasmalemma.

8.1.1. Reduction of geranylgeranyl chain to phytyl chain

In plastids, GGPP is in channeled toward the synthesis of the phytyl side-chain of several prenyllipids including chlorophylls, tocopherols and phylloquinone (Fig. 19). The reduction of the geranylgeranyl chain to phytyl chain is catalyzed by geranylgeranyl reductase (GGR). The bacterial and cyanobacterial and plant genes encoding GGR were identified previously [395–397]. Consistent with its role in the reduction of the geranylgeranyl chain [398], deletion of GGR induced the accumulation of GG-prenylated chlorophyll (GG-Chl) [396,397]. We have shown that GGR sequentially catalyzes the reduction of geranylgeranyl-prenylated chlorophyll (GG-Chl) \textit{a} into phytyl-Chla as well as the reduction of free GGPP into phytyl-PP [399]. In plastids, the same geranylgeranyl reductase is recruited into the chlorophyll, the tocopherol and the phylloquinone pathways (Fig. 19). The GGR gene is up-regulated during etioplast to chloroplast and chloroplast to chromoplast development [399]. In contrast to presently known GGRs, Rhodospirillum rubrum GGR only catalyzes the reduction of geranylgeranyl-prenylated phophythin into phytyl-prenylated phophythin [400].

The expression of an antisense GGR construct in tobacco resulted in a decreased content of tocopherol and chlorophyll (Chl). It was observed that up to 58% of the Chls were mainly in the form of GG-Chl [401]. The deficiency of tocopherol was compensated by a selective accumulation of xanthophyll cycle carotenoids [402]. The photosynthetic capacity of these transgenic plants was not altered under optimal growing conditions, thus
suggesting that the geranylgeranyl side-chain had no influence on the transfer of light energy [403]. A very similar situation has been described using the rice mutants M249 and M134 [404,405]. The rice mutants were devoid of tocopherol, and accumulated GG-Chls and phylloquinones prenylated with GG and partially reduced GG [404]. In a similar vein, the inactivation of \( \text{GGR} \) in \( \text{Synechocystis} \) \( \text{sp. PCC 6803} \) resulted in the accumulation of GG-Chl, and low amount of tocotrienol instead of tocopherol [406]. Also, the mutant could not grow photoautotrophically due to instability and rapid degradation of the photosystems in the absence of added glucose [406]. The influence of the side-chain of chlorophylls seems also marked for the organization of antenna complexes LHC1 and LHC2 in purple photosynthetic bacteria. For instance in the \( \text{GGR} \) mutant of \( \text{R. sphaeroides} \), that produces GG-bacteriochlorophyll (GG-Bchl) in place of phytyl-Bchl, the assembly of the antenna complex LHC2 was altered [396]. These changes are usually explained by the fact that phytyl side-chain is more flexible, or less rigid than the unsaturated geranylgeranyl chain. In addition to being involved in the reduction of GG-Chl, it has been demonstrated that \( \text{R. sphaeroides} \) \( \text{GGR} \) could also reduce the side GG-prenylated bacteriopheophytin (GG-Ph) into phytyl-Ph [400]. When transposed to higher plants, and more generally to organisms possessing two photosystems, these data strongly suggest the phytyl side-chain of phytyl-pheophytin is formed directly via \( \text{GGR} \) [400]. In other words, phytyl-pheophytin is not only formed via magnesium de-chelation of preformed chlorophylls.

8.2. Carotenoid biogenesis

8.2.1. Phytoene backbone and mechanistically related irregular isoprenoids

The conversion of geranylgeranyl pyrophosphate into phytoene is catalyzed in two distinct steps by phytoene synthase (PS). Previous studies have shown that phytoene synthase from tomato [407] and pepper [408] are integrated into a high molecular mass complex of 190–200 kDa. PS has been characterized as, a bifunctional enzyme [409]. The first catalytic step involves the abstraction of a diphosphate of a GGPP donor and is...
followed by a 1–1 or head to head condensation with a GGPP acceptor, thus giving a stable prephytoene PP (PPPP). During the second step, PPPP undergoes a complex concerted rearrangement involving removal of the diphosphate group and a carbocation neutralization (Fig. 20). Plant [409,410] and bacterial PS [411] are strictly dependent upon Mn$^{2+}$ and are stimulated by neutral detergents [411]. The reaction catalyzed by PS and squalene synthetase (SQS) are essentially identical, except that SQS requires Mg$^{2+}$ for activity and catalyzes in addition the NADPH-dependent reduction of the central bond of dehydrosqualene to give squalene. This mechanistic similarity is reflected by the fact that SQS produces dehydrosqualene, a C30 phytoene analogue, when incubated in the presence of Mn$^{2+}$ and in the absence of NADPH [412] or in the presence of dihydroNADPH, an unreactive analogue [413]. Also in the absence of NADPH and in the presence of Mg$^{2+}$, SQS produces 12-hydroxysqualene and 10-hydroxybotryococcene in addition to dehydrosqualene [414]. No obvious sequence similarity to any known NADPH binding site could be found in the peptide sequence of SQS. This led to the proposal that the NADPH-binding site is fulfilled by the most flexible part of SQS, i.e., the J–K loop (VKIRK) and part of the K helix which are not present in phytoene synthase [415]. These data point to convergent evolution between SQS and PS. Interestingly, *Staphylococcus aureus* and phototrophic bacteria (*Heliobacillus mobilis*, *Heliophilum fasciatum*, *Heliobacterium chlorum*, *Heliobacterium modesticaldum*, and *Heliobacterium gestii*) synthesize C30 carotenoids [416,417]. Available data from *S. aureus* indicate that these carotenoids derive from diapophytoene, a C30 homologue of phytoene. Diapophytoene results from the head-to-head condensation of two FPPs through a reaction catalyzed by diapophytoene synthase [418]. Indeed, data gained from bacterial diapophytoene synthase using mutagenic libraries and domain-swapping revealed

Fig. 20. Biosynthesis of phytoene and mechanistically related isoprenoids. *Enzyme abbreviations*. PS, phytoene synthase; CPPS, chrysanthemyl diphosphate synthase; SQS, squalene synthetase; aPS, apophytoene synthase; BS, botryococcene synthase. *Substrate abbreviations*. CPP, chrysanthemyl diphosphate; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; OPP, prephytoene diphosphate; PPPP, prephytoene diphosphate; PSPP, presqualene diphosphate.
that there is much more constraints to convert phytoene synthase into diapophytoene synthase than the
reverse. For instance a single change of Phe26 by non-aromatic amino acid residue in the peptide sequence
of diapophytoene allowed diapophytoene desaturase to produce the C40 phytoene [419]. It has been also shown
from E. coli complementation that diapophytoene synthase has low phytoene synthase activity, while phyto-
ene synthase has no diapophytoene synthase activity [420]. On the other hand, the desaturases seem more flex-
ible since phytoene desaturase and diapophytoene desaturase could use phytoene as well as diapophyto-
ene [420]. Because the active-site of SQS is organized into α-helices [415], like other trans-prenyltransferases
and terpene cyclases, one may suggest that PS also adopt the same folding [96].

Mechanistically the 1’–1 fusion between GGPP or FPP to give phytoene or squalene could be envisioned
for the biosynthesis of irregular or non-head-to-tail derived isoprenoids [77]. This class of compounds include
the monoterpeine chrysanthemic ester which is a typical component of natural pyrethrin insecticide extracted
from Chrysanthemum cinerariaefolium flower heads [421] (Fig. 20). Also, botryococcene, a component of the
hydrocracking oil produced by the green alga Botryococcus braunii [422] belongs to this class.

The initial step of chrysanthemic ester is catalyzed by chrysanthemyl diphosphate synthase (CPPS) which
condenses two DMAPPs into CPP. Surprisingly, CPPS has no homology to SQS and PSY, but displayed
extended homology to FPPS (up to 80%) [423]. However, there are two main differences between CPPS and
the orthodox FPPS family of prenyltransferases. CPPS possesses a plastid targeting sequence, thus suggest-
ing a new role of C. cinerariaefolium chromatoplasts in the synthesis of pyrethrins. In this connection, it is
worth noting that a maize sesquiterpene synthase displays a transit peptide-like motif [424] and that the
immunochemical localization of FPPS in rice, wheat and tobacco chloroplasts has been reported [425].
In addition, the second aspartate-rich domain DDXXD of FPPS is changed into NDXXD in CPPS
[423]. These above characteristics are shared with Artemisia tridentata CPPS. When incubated with
DMAPP, A. tridentata CPPS produced CPP and lavandulyl diphosphate. However, A. tridentata CPPS cat-
alyzed the synthesis of lavandulyl diphosphate and geranyl diphosphate in the ratio: 4:1:1 when incubated
with IPP and DMAPP [117]. Further studies using chimeric FPPS and CPPS substantiated the mechanistic
relationship between the cyclopropanation and the chain elongation activities of prenyltransferase [116] and
this led to suggest that phytoene and squalene synthetase emerged from an ancestry chain elongation
enzyme [116].

Apparently the lavandulyl chain could be formed according to another mechanism as shown in synthesis of
sophoraflavanone G, a branched monoterpenoid-conjugated flavanone characteristic to S. flavescens [426].
Here, two plastidial prenyltransferases were involved. The first condenses DMAPP at position 8 of leachian-
one G and the second prenyltransferase transfers a second DMAPP to the 2’ position of the dimethylallyl
group attached at position 8 of leachianone G to form sophoraflavanone G [426].

Several lines of evidence based on feeding [427] and in vitro studies [428] suggest that botryococcene is syn-
thesized from FPP by an enzyme similar to SQS or phytoene synthase. The reaction proceeds through the con-
densation of two FPPs and is dependent upon Mg2+ and NADPH or NADH. Botryococcene synthesis is
inhibited by squalestatin like SQS. In addition, botryococcene synthase activity is stimulated by Tween 80,
as shown in previous studies with SQS [429] and PS [430].

Phylogenetic tree analysis reveals that higher plant phytoene synthases are evolutionary related to algae and
cyanobacteria phytoene synthase (Fig. 21). On the other hand, plant SQSs cluster with diapophytoene
synthase.

Arabidopsis possesses only one PS gene, while tomato and tobacco have two genes. The duplication of PS
gene seems more widespread in plants belonging to the poaceae and both genes are functional [431]. Finally,
available evidence implicates PS as a primary determinant in the control of carotenoid accumulation in sink
tissues [432–437].

8.2.2. Phytoene desaturation

In oxygenic photosynthetic organisms, the desaturation of cis-phytoene to trans-lycopen involves four
steps that are catalyzed by phytoene desaturase (PD) and ζ-carotene desaturase (Fig. 22). This situation con-
strasts with that occurring in non-photosynthetic bacteria and fungi that use only one desaturase CrtI. How-
ever more recently a CrtI-type desaturase has been characterized from the cyanobacterium Gloeobacter
violaceus PCC 7421 [438]. Thus G. violaceus is the first oxygenic photosynthetic organism possessing a
CrtI-type desaturase and that does not need a carotenoid isomerase which is apparently present in the *G. violaceus* genome [438]. This may be related to the fact that *G. violaceus* belongs to an early group of the cyanobacterial phylum. This phylum is characterized by the fact that the photochemical reaction takes place in the

![Diagram of phylogenetic tree](image)

**Fig. 21.** Unrooted phylogenetic tree of phytoene synthases and related homologues. Alignments were performed with ClustalX and visualized with TreeView. Distances between sequences are expressed as 0.05 changes per amino acid residue. *Accession Numbers and abbreviations.* Dc (BAA84763); Os (AAK07735); Np (S54135); Te (AAM45379); Ha (CAC19567); Ca (CAA48155); Le (CAA42969); At (At5g17230); Zm (S68307); Db (T10702); No (NP_485873); Sn1 (NP_441168); Sn2 (CAA45530); Cha (ZP_00359166); SadPS (CAA52097.1); AtSQS (P53799); NtSQS (U60057); CaSQS (AF124842); Tt (YP_006040); Rc (P17056); Bl (AAF65581); Hb (NP_280449); Pa (BAA14128); Mt (NP_276914.1). At, *Arabidopsis thaliana*; Bl, *Brevibacterium linens*; Ca, *Capsicum annuum*; Cha, *Chloroflexus aurantiacus*; Db, *Dunaliella bardawil*; Dc, *Daucus carota*; Ha, *Helianthus annuus*; Hb, *Halobacterium* sp.; Le, *Lycopersicum esculentum*; Mt, *Methanothermobacter thermoautotrophicus*; No, *Nostoc* sp.; Np, *Narcissus pseudonarcissus*; Ni, *Nicotiana tabacum*; Os, *Oryza sativa*; Pa, *Pantoea ananatis*; Rc, *Rhodobacter capsulatus*; Sa, *Staphylococcus aureus*; Sn1, *Synechocystis* sp.; Sn2, *Synechococcus* sp.; Te, *Tagetes erecta*; Tt, *Thermus thermophilus*; Zm, *Zea mays.*

CrtI-type desaturase and that does not need a carotenoid isomerase which is apparently present in the *G. violaceus* genome [438]. This may be related to the fact that *G. violaceus* belongs to an early group of the cyanobacterial phylum. This phylum is characterized by the fact that the photochemical reaction takes place in the
periplasmic membrane due to the lack of thylakoid membranes [439]. One may envision that a very similar situation exists in the case of lycopene cyclase, because none of the known cyanobacterial lycopene cyclase genes [440,441] are observed in the entire genome of *G. violaceus* PCC 7421 [439].

The situation is different in non-oxygenic photosynthetic organisms such as *Rhodobacter capsulatus* and *R. sphaeroides* and in non-photosynthetic organisms such as fungi. In general these organisms possess a PD that could lead to lycopene. However in *R. capsulatus* and *R. sphaeroides*, the reaction yields mainly neurosporene en route to the synthesis of the acyclic xanthophyll spheroidenone. However exceptions could be observed in the case of the green sulfur bacterium *Chlorobium tepidum*, a strict anaerobe and an obligate photoautotroph. Here, the PD pathway is similar to that of cyanobacteria and plants [442]. On the other
hand, in Neurospora five desaturations lead to the formation of 3,4-dihydrolycopene, an intermediate of torulene synthesis [443]. The reaction sequence 15-cis-phytoene to trans-lycopene obviously proceeds via an isomerization step. The point at which in the biosynthetic sequence the cis to trans isomerization occurs and how this transition is accomplished is not entirely resolved. Earlier studies have shown that several intermediates accumulate during in vitro studies. In particular, cis- and trans-phytofluenes, cis- and trans-ζ-carotenes were formed under these conditions [444]. Also, it is well known that carotenoids possessing the trans-configuration are selected for light harvesting complexes while photoactive carotenoids having cis-configuration are usually incorporated into the reaction centers of photosynthetic organisms [445]. The stage at which this isomerization occur is also not known.

Etioplasts of the Arabidopsis crtiso mutant accumulates poly-cis-carotenoids in the dark and do not differentiate prolamellar bodies [446]. Similarly, tomato crtiso previously known as tangerine mutant accumulates prolycopene [447]. CRTISO encodes a carotenoid isomerase catalyzing the isomerization of prolycopene to lycopene (Fig. 22) [446,448]. CRTISO catalyzed in vitro the conversion of 7,9,9’-tri-cis-neurosporene to 9’-cis-neurosporene and 7’,9’,di-cis-lycopene into all-trans-lycopene [448]. When grown under light-activated heterotrophic conditions, the crtiso mutant of Synechocystis sp. PCC 6803 accumulated mainly cis-lycopene and traces of all trans-carotenoids including β-carotene and xanthophylls. Concomitantly, the D1 protein of the PSII was not detected and the PSII activity could not be measured [449]. Under continuous illumination the capacity to synthesize β-carotene and xanthophylls was restored as well as the PSII activity. This led to the conclusion that carotene isomerization was required for the assembly of PSII [449]. When R. sphaeroides, an organism devoid of CRTISO, was used for reconstitution experiments with trans-spheroidenone, the xanthophyll was converted to the 15,15′-cis configuration. This change occurred without any exogenous isomerase and in particular in the absence of a CRTISO [450]. Detailed analysis suggested that spontaneous thermal isomerization of trans-spheroidenone into 15,15′-cis-spheroidenone is required before the binding process [450].

In plants, the desaturation of phytoene is a target for photobleaching herbicides including pyridazinones, pyridinecarboxamides and phenoxybutanamides [451]. On the other hand, the phytoene desaturase from Pantoea that carries the four desaturation steps between phytoene and lycopene is not sensitive to the bleaching herbicide Norflurazon [452]. A mutated PD from Synechococcus PCC 7942 (mutant NFZ4) that conferred resistance to Norflurazon was introduced in tobacco. This conferred 58-fold and 3-fold resistance to the bleaching herbicide Norflurazon and fluridone [452]. This was paralleled by an increased stability of the PSII protein D1. On the other hand, the plants were not resistant to ζ-carotene desaturase inhibitors [453]. In an attempt to control the proliferation of invasive aquatic weed, a fluridone-resistant Hydrilla verticillata, has been naturally selected. The resistance has been ascribed to independent mutations of a conserved Arg residue: Arg304Ser, Arg304Cys, Arg304His [454,455]. The mechanism of phytoene desaturation and inhibition is largely unknown. Available data implicate plastoquinone-9 as a component of the carotene desaturase system [456,457]. Based on kinetic studies, it has been shown that the bleaching ketomorpholines do not act competitively with respect to phytoene [458]. On the other hand, it has been suggested that the bleaching herbicide Norflurazon acts competitively with respect to the plastoquinone cofactor on its PD binding site [459].

8.2.3. Lycopene cyclization

Lycopene cyclase (LC), catalyzes the cyclization of trans-lycopene at both ends [460,461] (Fig. 23). There is practically no similarity between the peptide sequences of LC belonging to different taxa. LC can be grouped into three main types. Type-1 LCs (or plant-type LCs) possess a pyridine nucleotide binding motif whose function is unknown [462]. It has been previously reported that NAD(P)H is required for the cyclization reaction in vitro [463]. However, these data showed that the hydrogen atom introduced at the position C(2) during the cyclization is derived from water and not from NADPH [463]. Thus no hydrogen was transferred from NADPH to the substrate. Type-1 LC can be further subdivided into lycopene β-bicyclases (LCB), lycopene ε-bicyclase (LCE), lycopene β-monocyclase and lycopene ε-monocyclase and bifunctional lycopene β,ε-bicyclase (Fig. 24). Type-2 LCs include the fungal enzymes. Here, lycopene cyclase is a bifunctional protein that has phytoene synthase activity at the carboxy terminus and lycopene cyclase activity at the amino terminus [464]. Type-3 LCs have been characterized from Gram-positive bacteria-like Brevibacterium linens. The lycop-
Fig. 23. Xanthophyll biosynthetic pathway. **Enzyme abbreviations.** LCB, lycopene β-cyclase; LCE, lycopene ε-cyclase; CHB<sub>HDM</sub> carotenoid β-hydroxylase non-heme-di-iron monooxygenase; CHB<sub>HBP450</sub> carotenoid β-hydroxylase heme-binding cytochrome P450; CHE<sub>HBP450</sub> carotenoid ε-hydroxylase heme-binding cytochrome P450; KET<sub>CrtW/CrtO</sub> β-carotene ketolase; ZEP, zeaxanthin epoxidase. VDE, violaxanthin de-epoxidase; NSY neoxanthin synthase; CCS, capsanthin-capsorubin synthase.
pene cyclase activity is the result of two distinct genes \textit{crtYc} and \textit{crtYd} encoding proteins of 125 and 107 amino acids \cite{465}. These two separable subunits are probably organized as a heterodimer. In archaea, the N-terminal and C-terminal halves of LC correspond to the subunits \textit{crtYc} and \textit{crtYd} of type-3 LCs, as shown in \textit{Sulfolobus solfataricus} \cite{466} and \textit{Halobacterium salinum} \cite{467}. The apparent flexibility of lycopene cyclase probably deserves further variation, since, sequence homologous to known plant or cyanobacterial LC could not be
identified in the sequenced genome of several cyanobacteria including *Synechocystis* sp. strain PCC 6803, *T. elongatus*, *Nostoc punctiforme* and *Anabaena* sp. Strain PCC7120, *G. violaceus* and *Trichodesmium erythraeum* [441,442]. Also, the genome of the green sulfur phototrophic bacterium *C. tepidum* did not encode any homolog of known types of LCs [442]. This led to the suggestion that the *CrtU* homolog that encodes a chlorobacterium synthase could fulfill this role [442]. This suggestion is reinforced by the fact, that cyanobacteria that possess an identifiable lycopene cyclase such as *Prochlorococcus marinus* and *Synechococcus* sp. (strain WH8102) lack homologs of *CrtU* [442]. All lycopene cyclases, irrespective of class, proceed via a carbocatician mechanism [468]. The mechanism is shared by capsanthin-capsorubin synthase [469]. Indeed, the primary structures of LC and CCS display 55% identity.

In higher plants, the formation of α-carotene requires the interaction of the lycopene β-bicyclase and the lycopene ε-monoacylase [470]. In *lactua sativa* a single lycopene bicyclase catalyzes the synthesis of ε-carotene, the precursor of luctuaxanthin [471]. Unlike higher plants, in *Prochlorococcus marinus* MED4 only a single lycopene bicyclase catalyzes the formation of the β-carotene, α-carotene and ε-carotene [441,472]. Functional studies revealed that the capacities to synthesize one or two ε-ring are specified by active amino acid residues located in the C-terminal domain of lycopene cyclase [471].

In *Synechocystis*, the lycopene β-monoacylase could be involved in the biosynthesis of the monocyclic myxol dimethyl-fucoside. This is reinforced by the fact that a marine bacterium, strain P99-3 produces myxol and possesses a lycopene β-monoacylase [473]. Genes encoding lycopene β-monoacylases that convert lycopene into γ-carotene have also been characterized from a marine bacterium, strain P99-3 [473], *Rhodococcus erythropolis* and *Deinococcus radiodurans* [474]. The most important diversity of LCs was encountered in cyanobacteria, belonging to the *Prochlorococcus* genus. Indeed, these cyanobacteria display a guenine isoprenoid metabolism due to the presence of chlorophyll *b* in addition to chlorophyll *a* and of α-carotene (63%), β-carotene (11%), ε-carotene epoxide (1%), δ-carotene (1%) β-cryptoxanthin (3%), 3-hydroxy-α-carotene (4%), zeaxanthin (16%) [441,472].

By virtue of its carbocatician mechanism, LC activity is strongly inhibited by amine derivatives positively charged at physiological pH [468,475–478].

### 8.2.4. Carotene to xanthophyll conversion

Two types of genes encoding oxygenases converting carotene into xanthophylls were first cloned in plants. These include β-carotene hydroxylases (*CHB*) and β-carotene ketolases (*KET*). Two genes encoding β-carotene hydroxylases (*CHB*1, *CHB*2) that convert β-carotene into C3-hydroxy-β-xanthophylls were first cloned from *Arabidopsis* [479] and pepper [395](Fig. 23). Genes encoding two structurally divergent ketolases that introduce a keto group in the C4 of carotenoid rings have also been cloned. These include the non-phylogenetically related CrtO and the CrtW genes (Fig. 25). The genome sequence of *Nostoc* sp. PCC7120 contains both CrtO and CrtW homologues. The CrtO type displays six conserved domains, including a putative FAD-binding domain [480]. These enzymes could function as monoketolase as shown for *Synechocystis* [481] or as mono and diketolase [480]. The CrtW ketolase could function as monoketolase or diketolase. These enzymes are found in *Agrobacterium aurantiacum* [482] and also in *H. pluvialis* [483] and *N. punctiforme* PCC 73102 [484]. Blast search revealed the presence of a gene homologous to *H. pluvialis* β-carotene ketolase in *Chlamydomonas reinhardtii*. Although this organism did not produce astaxanthin under trophic changes [485], the putative ketolase corresponded to an expressed gene, encoding a protein with a C terminal extension of 115 amino acid not observed in currently characterized ketolase [485]. However, *C. nivalis* under low temperatures and high level of UV irradiation produced astaxanthin as a natural screen and thus remain photosynthetically active [486,487]. Based on this evidence the putative *C. reinhardtii* could encode a true ketolase. Alternatively, due to the mechanistic similarity between CHB and KET emphasized below, the putative KET could catalyze an hydroxylation steps, possibly in the pathway leading to lorioxanthin. Blast search also revealed that a putative non-heme-di-iron CHB is present in the genome of the archaea *S. solfataricus* (Accession No. NP_344225).

It has been recognized that pepper CHB and algal KET of the CrtW type display similar histidine motifs: HX₃H, HX₂HH, HX₃H, HX₂HH for CHB and HX₃H, HX₂HHXH, HX₂HH for KET [395]. These histidine motifs are in relative positioning with respect to hydrophobic domains of the proteins in plant and bacterial CrtZ-type CHB [395]. Site-directed mutagenesis of residues within the conserved histidine motifs completely abolished the hydroxylase activity of pepper CHB [395]. This provided the experimental support that the
histidine motif of β-carotene hydroxylase was equivalent to those previously identified in fatty acid desaturases and alkane hydroxylases [278]. This suggested that CHB belong to the non-heme-di-iron monooxygenase (NHDIM) family (Fig. 23) where these histidine residues play a vital role such as coordinating the Fe ions in the active-site [279]. This assumption was strengthened by the fact that CHB was inhibited by iron chelating reagents such as o-phenanthroline and 8-hydroxyquinoline [395]. Based on this evidence, a mechanism was proposed [395] to explain the presence of iso-cryptoxanthin in place of echinenone in Dictyococcus cinnabari-
cell cultures treated with the carotenogenic inhibitor diphenylamine [488]. Mechanistically CHB and type-CrtW KET use activated molecular oxygen to break the C–H bond with the concomitant formation of unsaturated bonds, retention of hydroxyl or keto groups [489] as shown for fatty acids [490–492]. Also, it has been reported that the Δ5 desaturase [493] and the Δ22 desaturase [494] involved in yeast ergosterol biosynthesis could operate via hydroxy sterol intermediates. These considerations are supported by the recent identification of a *Brevundimonas* sp. hydroxylase that introduces a C2 hydroxy group into astaxanthin [495]. *Brevundimonas* sp. hydroxylase is phylogenetically more related closely related to sterol C5 desaturase than to other bacterial or plant carotenoid hydroxylases (Fig. 26). In a similar vein, it has been shown that a carotenoid ketolase isolated from *Adonis palaestina* operates via a desaturase-like mechanism [496]. Thus, through the subtle variation of one protein motif, nature has an invariant means to perform the large spectrum of reactions catalyzed by desaturases, epoxidases, acetylenases, conjugases, ketolases, decarbonylase and methyl oxidases (Fig. 27).

The above hydroxylation mechanism discussed above could not account for the hydroxylation of the ε-ring of β-carotene en route to lutein. Lutein biosynthesis involves hydroxylation of the two cyclohexene rings,

![Unrooted phylogenetic tree of non-heme-di-iron hydroxylases, ketolases and related homologues. Alignments were performed with ClustalX and visualized with TreeView. Distances between sequences are expressed as 0.1 changes per amino acid residue.](image_url)
β- and ε- of α-carotene. Although the β-ring of lutein is hydroxylated by a non-heme-di-iron monoxygenase, the ε-ring is not. This point was clarified following the isolation of the Arabidopsis mutant lut1 [497]. Lut1 mutant has decreased lutein content (up to 95%) and accumulated zeinoxanthin, a mono-hydroxy xanthophyll [497]. To identify the molecular basis of this biochemical modification, the LUT1 gene was cloned [498]. Molecular analysis revealed that the LUT1 gene encodes a cytochrome P450-type oxygenase, CYP97C1. The gene complemented the lut1 mutant, thus establishing that mixed function oxygenases such as P450s are involved in the hydroxylation of the ε-ring of lutein [498]. Further construction of an Arabidopsis mutant with all three known hydroxylase genes deficient, resulted in plant mutants still producing β-hydroxy xanthophylls [498]. This led to the hypothesis that Arabidopsis CYP97A3 which is 49% identical to LUT1, could encode an additional β-hydroxylase [498]. In this context, it is worth noting that CYP175A1 from Thermus thermophilus has been characterized as a CHB [499]. Thus the introduction of hydroxy group into the ring of carotenoid is catalyzed by structurally unrelated enzymes such as non-heme-di-iron oxygenases and cytochrome P450-type oxygenases (Fig. 23).

8.2.5. Xanthophyll interconversion

The gene encoding zeaxanthin epoxidase (ZEP) that catalyzes the conversion of zeaxanthin into antheraxanthin and violaxanthin has been first cloned from N. plumbaginifolia [500] (Fig. 23). ZEP catalyzes the epoxidation of 3-hydroxy-β-cyclohexenyl ring and requires the presence of FAD and ferredoxin for activity [501,502]. The resulting antheraxanthin and violaxanthin are subject to de-epoxidation by violaxanthin de-epoxidase (VDE). VDE was purified and its gene cloned from romaine lettuce [503]. VDE and ZEP are members of the lipocalin family [504]. Proteins belonging to this family are characterized by their ability to bind small lipophilic molecules [505]. Interestingly, VDE binds to monogalactosyldiacylglycerol (MGDG) at low pH [506]. VDE activity depends upon the availability of violaxanthin, the acidic pH of the lumen and protonated ascorbate [507] (Fig. 28). Thus pretreatment with 1-galactono-1,4-lactone, an ascorbic acid precursor, induces the accumulation of zeaxanthin in leaves [508]. Also VDE is inhibited by dithiothreitol, mercaptoethanol, cysteine and o-phenanthroline [509]. Available evidence shows that during the light period, VDE is bound to the thylakoid membranes [507,510] concomitantly to the acidification of the lumen. This agrees with the fact that in vitro VDE binds to the thylakoid membranes at pH 5.2 and is released at pH 7 [506]. VDE is specific for 3-hydroxy-5,6-epoxy-carotenoids which are in a 3S,5R,6S-configuration [506]. Thus epoxy-lutein could constitute an additional substrate for VDE in plants such as Cuscuta [511], oak [512], the tropical legume, Inga sp. [512] and plants belonging to the loranthaceae and viscaceae [513]. Recent data indicate that in woody plants, lutein-5,6-epoxide is compartmentalized mainly in the buds and leaf primordia [514] and is light-induced [514]. Except in vascular plants, sequences similar to VDE could not be identified in currently annotated cyanobacterial or algal sequences [485]. This could be due to the fact that algal VDE is structurally unrelated to vascular plant VDE. Indeed, in vitro studies demonstrated that VDE from the prim-
itive green alga *Mantoniella squamata* has reduced affinity for antheraxanthin and for mono epoxy-xanthophyll [515,516].

5,6-Epoxy cyclohexenyl xanthophylls are rearranged into \(\kappa\)-xanthophylls by the first characterized plant xanthophyll biosynthetic gene capsanthin-capsorubin synthase (CCS) [469]. The reaction yields capsanthin and capsorubin, the red pepper keto-carotenoids [469]. Mechanistically CCS operate like LC [468]. A CCS homolog displaying neoxanthin synthase (NSY) has been isolated from tomato [517] and potato tuber [518]. Although an *Arabidopsis* homologue has not been characterized, Blast search revealed that NSY is not chromoplast specific as anticipated [519], but is expressed in diverse tissues and organs. Mechanistically NSY operate like CCS, except we could not unambiguously demonstrate its cryptic lycopene cyclase.

8.2.6. The xanthophyll cycle

In photosynthetic organisms, the function of ZEP and VDE is integrated in the photoprotective xanthophyll cycle [520]. The xanthophyll cycle is present in vascular plants, green algae and some brown and red algae but not in cyanobacteria [520]. In marine diatoms, the xanthophyll cycle is termed the diadinoxanthin cycle and includes reversible reaction between diadinoxanthin and diatoxanthin [521] (Fig. 28).

Protection against excess light involves dissipation of light energy as heat and is known as the qE component of non-photochemical quenching (npq) of chlorophyll fluorescence. The process involves a buildup of a proton gradient across the thylakoid membrane [522], the presence of the PSII protein PsbS [523] and the deepoxidation of violaxanthin to zeaxanthin via antheraxanthin [520] (Fig. 28). *Arabidopsis* mutant devoid of VDE activity has been isolated using a screen for plants displaying lower npq [524]. Also, Tobacco plants expressing antisense VDE have reduced npq [525].

The isolation of *Arabidopsis* mutant deficient in vitamin C (*vtc1*) [526] suggests that a subtile regulation seems to operate between the xanthophyll cycle, ascorbate and ABA synthesis, at least in photosynthetic
tissues (Fig. 28). Indeed, the ABA content of vtc1 leaves is 60% greater than those measured in control plants [527]. These conditions favor the downregulation of VDE [528]. Thus reduced VDE activity could increase the flux of violaxanthin and neoxanthin used for ABA synthesis. This contention is supported by the fact that the expression of the 9-cis-epoxycarotenoid dioxygenase (NCED) catalyzing the synthesis of xanthoxin is increased in vtc1 [527].

8.2.7. Organization of carotenogenic enzymes

Earlier studies showed that in plastid the initial steps of carotenoid synthesis up to the level of phytoene could take place in the stroma while later steps require tight cooperation between the stromal phase and the membrane components [430,529]. However beyond this fact, we do not know how the carotenogenic enzymes are organized. This could have impact on the fate of the final products as shown by the accumulation of \( \gamma \)-carotene [473,530] or to \( \beta \)-cryptoxanthin [479].

Available data suggest that the xanthophyll cycle operate via binding and release from pigment protein complexes [531]. Also the lipid environment influences the activity of carotenogenic enzymes. For instance, VDE activity, is optimal when the concentration of MGDG relative to violaxanthin is 28:1 [532,533]. Also overexpression of PsbS, an intrinsic chlorophyll-binding protein of PSII [523] increased the VDE activity [534]. PsbS apparently binds free zeaxanthin [523] which otherwise could inhibit VDE [509,531].

8.2.8. Molecular regulation

Photosynthetic organisms control expression of photosynthesis genes in response to light intensity and redox conditions. In anoxygenic photosynthetic bacteria the biosynthesis of the photosynthetic apparatus and its isoprenoid components is upregulated by moderate light intensity and downregulated under aerobic conditions [535,536]. To this end, *Bradyrhizobium* and *Erythrobacter*, possess two spatially and genetically distinct carotenogenic pathways that operate concomitantly in the same cell during the photosynthetic growth. The photosynthetic pathway leads to spirilloxanthin which is incorporated in the photosynthetic membranes, while the other, a non-photosynthetic pathway leads to canthaxanthin or other ketocarotenoids [537] which are incorporated in non-photosynthetic membranes [538,539] to protect them by virtue of their efficient scavenging roles [540], 34 [541]. The regulation of the carotenoid biosynthesis under these conditions involves the homologous *ppsR/CrtJ*, and *tspO/CrtK* [542,543]. *TspO* and *CrtK* encode a sensory transducer that negatively regulate carotenogenic genes in response to light and oxygen [542]. *PpsR* and *CrtJ* possess a DNA and PAS binding domains. The DNA binding domain blocks the transcription of carotenoid genes by binding to the palindromic motif (TGTN\(_{12}\)ACA) of the promoters [544,545], while the PAS domain is involved in sensing light, redox potential and interacting with small ligands [546]. In the photosynthetic bacteria *Bradyrhizobium* strain ORS278 and *Rhodopseudomonas palustris*, the repressive effect of *PpsR* and *CrtJ* is antagonized by the red light absorbing form (Pr) of the bacteriophytochrome [538,547,548]. Interestingly, bacteriophytochrome also control the synthesis of the carotenoid deinoxanthin in the non-photosynthetic bacteria *Deinococcus radiodurans* [549].

The influence of light on carotenoid synthesis and carotenogenic gene expression has been reported in green tissues [550–555]. Overexpression of the *CHB* gene in *Arabidopsis* increased the xanthophyll cycle pool 2-fold. The resulting plants were more tolerant to high light and high temperatures as shown by reduced necrosis, reduced lipid peroxide accumulation and anthocyanin production [556]. Similarly, tobacco plants expressing a bacterial *CHB* produced more zeaxanthin and was more resistant when challenged by UV light [557]. On the other hand, expression of an antisense construct of *CHB* in *Arabidopsis* reduced the content of violaxanthin and neoxanthin, without changing the lutein content [558]. The level of lutein could be increased following the overexpression of lycopene \( \epsilon \)-cyclase [558].

Through the use of 3-(3,4-dichlorophenyl)-1,1-dimethyl-urea (DCMU) and 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB), two photosynthetic electron transport inhibitors that respectively inhibit the reduction of plastoquinone (PQ) and the oxidation of plastoquinol, it has been shown that the expression of *ZEP* and *CHB* are redox controlled. When the reduction of plastoquinone is inhibited by DCMU, the expression of *ZEP* and *CHB* is upregulated. Conversely, when the oxidation of plastoquinol is inhibited by DBMIB, the expression of *ZEP* and *CHB* is downregulated [555]. One could notice that in green tissue,
the reduction of PQ following DCMU treatment is paralleled by an increased accumulation of carotenoid [555]. These data based on studies with photosynthetic tissues and those previously reported for chromoplast containing tissue [489] enlarge the role of redox regulation of carotenogenic genes in plants. Demonstration of this type of regulation under natural condition is provided by the protective role of eschscholtzaxanthin, mono-anhydroescholtzaxanthin, anhydroescholtzaxanthin which accumulate in red leaves of Buxus sempervirens as a response to photoinhibitory conditions during winter [559].

In the case of chromoplast containing tissues, a transcriptional regulation of carotenogenesis seems to prevail [188,489,551,560]. Light also regulates the accumulation of carotenoids in non-photosynthetic tissues through the action of phytochrome [561]. Further experiments show that specific tomato fruit phytochrome controls the accumulation of lycopene during tomato ripening, independently of ethylene. This control is essentially due to phytochrome A since its mRNA accumulates 11.4-fold during the ripening period [562]. Furthermore, the availability of deposit structures characteristic of chromoplasts [563] influences the level of carotenoid that can be accumulated [564–566]. A mutation of the Or gene induces carotenoid accumulation in orange-curd-high carotene cauliflower inbreds [567]. The Or gene has been cloned [568] and shown to encode a plastid protein that display transmembrane domains. In addition, Or protein belongs to a protein complex in vivo [569].

QTL analysis reveals that several largely uncharacterized genes are involved in the regulation of carotenoid biosynthesis [570]. This is supported by recent data indicating a connection between carotenoid accumulation and photomorphogenesis in tomato. Analysis of tomato mutants high pigment (hp) 1 and 2 and dark green (dg) that induce increased photoresponsiveness and increased fruit pigmentation [571,572] shed new light. It has been shown that these mutations are not due to a lesion in structural carotenogenic genes, but to the tomato homolog of A. thaliana DE-ETIOLATED1 (DET1), a negative regulator of photomorphogenesis in the case of hp2 [573] and dg [574] and to the UV-DAMAGED DNA-BINDING PROTEIN1 (DDB1) [575] in the case of hp1 [576,577]. These data demonstrate that manipulation of the light signal cascade regulates the accumulation of carotenoid in tomato fruits. This is further strengthened by RNA interference repression of tomato DET1, DDB1 genes, as well as tomato COP1-like and HY5 [577–579]. Arabidopsis long hypocotyl (hy) HY5 is a basic leucine zipper transcription factor that promotes photomorphogenesis whereas COP1, is a negative regulator of photomorphogenesis that directly interacts with nuclear HY5 [580]. However, this regulation is not pathway specific, but participates in the pleiotropic regulation of antioxidant synthesis, because in hp2 mutant the concentration of carotenoid, tocopherol, ascorbic acid as well as phenolic derivatives possessing antioxidant effects were elevated [581,582]. Finally, the link between carotenogenesis and photomorphogenesis is also demonstrated by the fact that tomatoes overexpressing cryptochrome, a blue light receptor, overproduce chlorophylls in leaves and lycopene in fruits [583].

### 8.2.9. Integration of carotenoids in lipoprotein structures

In chloroplast approximatively, 80% of the xanthophyll are bound to pigment–protein complexes and thus are not involved in the lipid bilayer of the membranes [584]. Thus less than 20% of the xanthophyll help structure the membranes according to a rivet-like mechanism [585]. The nature and the amount of carotenoids that could be incorporated into plastid membranes is highly regulated in green tissues compared to non-green tissues that sequester excess carotenoids in specific structures [563]. Data demonstrating flexible regulation of this process could be inferred from previous studies. For instance when R. sphaeroides are grown semiaerobically, the reaction center contains spheroidenone [586], while spheroidene is incorporated the reaction center under anaerobic conditions [587]. Also non-native carotenoids can be functionally incorporated into photosynthetic membranes and complexes. In R. sphaeroides when the native phytoene desaturase leading to neurosporene is replaced by the 4-step phytoene desaturase from Pantoea phytoene desaturase, the resulting non-native lycopene is functionally integrated into the photosynthetic membranes [539,588]. Similarly, the chromoplast specific carotenoids capsanthin and capsorubin could be incorporated functionally in N. benthamiana [589]. Finally, evidence based on in vitro reconstitution experiments [590–593], algal mutants [594–596] and higher plant mutants [497,597–599] indicate that zeaxanthin could replace lutein in light harvesting complexes (LHC). In addition the relative proportions of the different chlorophyll antenna proteins are modified in these mutants [596,600]. These data collectively suggest that lutein, loroxanthin, violaxanthin and neoxanthin are not essential for the organization and the function of LHCII and LHCl in photosynthetic organisms.
The reassembly monomeric recombinant LHCIIb in the presence of diverse non-native xanthophylls, revealed a specific requirement for the presence of a hydroxy group at C-3 on a single β-end group [601]. However, the requirement for a 3-hydroxy-β-xanthophyll could be bypassed in cyanobacteria. For instance, in Synechocystis defective in CHB, the resulting absence of zeaxanthin and myxol dimethyl-fucoside, did not impair the growth and the photosynthetic capacities [602]. The isomeric configuration plays a decisive role as shown by the fact that cis-isomers of zeaxanthin, violaxanthin, and lutein could not be incorporated, in contrast to trans-neoxanthin [601]. However, one could note that some parasitic plants [603], such as Cuscuta reflexa, are devoid of neoxanthin [511]. In C. reflexa, neoxanthin is replaced in the LHCs by 9-cis-violaxanthin [604], in spite of the fact that neoxanthin binding site is highly selective for this xanthophyll [590]. It has also been reported that complexes containing 30–50% trans-neoxanthin are not stable and display low energy transfer at high temperatures [591]. On the other hand, lactuaxanthin and eschscholtzxanthin could be incorporated in the absence of lutein [601]. With respect to the binding mechanism, it has been demonstrated that intermolecular π–π stacking between carotenoids and the aromatic residues in LHC play key role in the binding of carotenoids to these proteins [605].

8.2.10. Carotenoid cleavage oxygenases

The rigid chromophore of carotenoids is attacked by several oxygenases to give apocarotenoids (Fig. 29) for a review, see [606,607]. In plants, these reactions lead to the formation of abscisic acid, an important plant hormone [608,609] and to several apocarotenoids derivatives that have diverse biological roles [607,610]. Carotenoid oxygenases have been characterized from Arabidopsis [611], tomato and peppers [612]. Similarly, carotenoid dioxygenases catalyzing the initial steps of crocin synthesis in Crocus sativus stigma and bixin synthesis in Bixa orellana seeds have been characterized. In C. sativus, a chromoplast zeaxanthin 7,8(7,8′)-cleavage dioxygenase initiates the reaction to yield crocetin dialdehyde [612] whereas a lycopene 5,6(5′,6′)-cleavage dioxygenase is involved in the initial step of bixin synthesis [613]. All carotenoid oxygenases proteins display four conserved histidines that could serve as ligands for non-heme iron. This is reinforced by the fact that the human β-carotene 15,15′-monooxygenase [614] which catalyzes the first step in the synthesis of vitamin A, is drastically inhibited by micromolar concentrations of the metal chelating agents α,α-bipyridyl and o-phenanthroline [615] and desferrioxamine [616]. X-ray analysis of the crystal structure of the apocarotenoid-15,15′-oxygenase (ACO) from Synechocystis sp. PCC 6803 reveals that ACO contains an Fe2+-4-His arrangement at high temperatures [591]. On the other hand, lactuaxanthin and eschscholtzxanthin could be incorporated in the absence of lutein [601]. With respect to the binding mechanism, it has been demonstrated that intermolecular π–π stacking between carotenoids and the aromatic residues in LHC play key role in the binding of carotenoids to these proteins [605].

With respect to the biological roles of apocarotenoids, recent studies have further substantiated their potential involvement in the control of Arabidopsis [619] pea [620] and Petunia [621] ramification. In particular, the putative apocarotenoid signal is modified via a cytochrome P450 enzyme [619].

Finally, like several membrane lipids with established functions, carotenoids are targeted to fulfill additional roles in other context (Fig. 30). In a more wider context, the process is triggered by lipid cleaving and/or oxidizing enzymes. Thus unsaturated plant fatty acids (linolenic acid), sterols (campesterol) and diverse carotenoids are, respectively, converted into jasmonic acid, brassinosteroids and apocarotenoids which regulate plant growth, development and response to biotic and abiotic factors (Fig. 30).

8.3. Chlorophyll biogenesis and degradation

The different steps of the pathway of chlorophyll biosynthesis and degradation have been elucidated recently through biochemical analysis and molecular cloning (Fig. 31), for reviews, see [622,623]. The last steps involve the prenylation of chlorophyllide by phytlyl diphosphate or geranylgeranyl diphosphate catalyzed by chlorophyll synthase (Fig. 31). In vitro studies revealed that chlorophyll a synthase accepts chlorophyllide a, but not bacteriochlorophyllide a, as a substrate, whereas bacteriochlorophyll a synthase accepts bacteriochlorophyllide a, but not chlorophyllide [624]. In addition, bacteriochlorophyll a synthase in Rhodobacter, Synechocystis and Arabidopsis have a preference for phytlyl diphosphate over geranylgeranyl diphosphate [624]. However in R. rubrum GGPP is used instead of phytlyl diphosphate [625]. In some lower photosynthetic organisms, farnesyl diphosphate is used as the prenyl donor. For instance, farnesyl diphosphate is used for the prenylation of bacteriochlorophyll g is Heliobacteria. This phenomenon is due to the fact that Heliobact-
teria are apparently unable to produce geranylgeraniol (C20) [416]. Beside farnesyl, several non-isoprenoid side chains could be used in photosynthetic bacteria. These include, dodecanyl-, hexadecenyl-, pentadecenyl-, tetradecenyl-side chains [626]. Mechanistic studies of the prenylation reactions in *R. rubrum* indicate that the process involves the nucleophilic attack by the carboxylate group of the chlorophyllide on the activated prenyl residue [627,628].

The first step of chlorophyll degradation involves the detachment of phytol by chlorophyllase (Fig. 31). This reaction is followed by the removal of the magnesium atom and partial oxygenolytic breakdown of...
the tetrapyrole macrocycle to give fluorescent chlorophyll catabolites (FCCs). These reactions are sequentially catalyzed by magnesium dechelatase (MDCH), pheophorbide oxygenase (PAO) and red chlorophyll catabolite (RCC) reductase (RCCR) [623,629]. The FCCs are finally exported to the vacuole to give the final products termed as non-fluorescent chlorophyll catabolites (NCCs) [630,631] (Fig. 31). The green alga *Chlorella protothecoides* is devoid of RCCR and RCC-like derivatives are excreted into the medium [632]. This catabolic pathway is enhanced during senescence and non-green plastid differentiation. The induction of PAO and the activity of PAO are restricted to senescing tissues [633]. In the case of senescing tissues, the degradation of chlorophyll is not associated to the general remobilization of nutrient occurring during this period as previously thought [634]. Chlorophyll catabolites are not exported from senescent leaves [635]. This catabolism most probably avoid the accumulation of photodynamic chlorophyll derivatives. The fate of the phytol chain is unknown. It has been proposed that the phytol chain is subject to a photooxidative degradation process [636,637]. Finally, one could note that chlorophyll-free chromoplasts, maintain the potential for chlorophyll biosynthesis as shown by in vitro incubation with exogenous chlorophyll precursors [638–640].

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**Fig. 30.** General role of membrane lipids in the formation of plant regulators. In addition to their structural roles, membrane lipids are targeted to generate bioactive derivatives. The process is initiated by lipid cleaving and/or oxidizing enzymes and culminates for instance with the production of jasmonic acid, brassinosteroids and apocarotenoids that serve diverse roles.
Fig. 31. Pathways of chlorophyll biosynthesis and catabolism. (A) Biosynthesis. Enzyme abbreviations. GTS, glutamyl-tRNA synthetase; GTR, glutamyl-tRNA reductase; GSAT, glutamate-1-semialdehyde aminotransferase; ALAD, 5-aminolevulinate dehydratase; PBD, porphobilinogen deaminase; UROS, uroporphyrinogen III synthase; UROD, uroporphyrinogen III decarboxylase; CPO, coproporphyrinogen III oxidase; PPX, protoporphyrinogen IX oxidase; MCH, magnesium chelatase; MTF, magnesium protoporphyrin IX methyltransferase; MTC, magnesium protoporphyrin IX monomethyl ester cyclase; DCR, divinyl protochlorophyllide reductase; POR, NADPH-protochlorophyllide oxidoreductase; GGR, geranylgeranyl reductase; CAO, chlorophyll a oxygenase; CBR, chlorophyll b reductase; HCR, hydroxychlorophyll a reductase. Substrate abbreviations. GGPP, geranylgeranyl dipiphosphate; PhytlIPP, phytol dipiphosphate. (B) Catabolism. Enzyme abbreviations. CASE, chlorophyllase; MDCH, magnesium dechelatase; PAO, pheophorbide a oxygenase; RCCR, red chlorophyll catabolite reductase. Substrate abbreviations. FCC, fluorescent chlorophyll catabolite; NCCs, non-fluorescent chlorophyll catabolites; RCC, red chlorophyll catabolite.
8.4. Tocopherol and tocotrienol biogenesis

The pathway of tocopherol and tocotrienol biosynthesis is shown in Fig. 32. It is apparent from structural considerations that the biosynthetic steps could be divided into the origin of the quinol ring, the methyl groups and the formation of the isoprenoid side-chain. Although the enzymatic steps and the compartmentation of component enzymes [641–643] of the tocopherol pathways and the purification of S-adenosyl methionine-γ-

![Fig. 32. Phylloquinone, vitamin E (tocopherols and tocotrienols) and plastoquinone biosynthetic pathways. Enzyme abbreviations. HPPD, p-hydroxyphenylpyruvate dioxygenase; HPT, 2, homogentisate phytol transferase; MPBQMT, 2-methyl-6-phytyl-1,4-benzoquinol methyltransferase; TC, tocopherol cyclase; SAM γ-MT, S-adenosyl-L-methionine γ-tocopherol methyltransferase; HGGT, homogentisate geranylgeranyl transferase; MGGBQMT, 2-methyl-6-geranylgeranyl benzoquinol methyltransferase; SAM γ-MT3, S-adenosyl-L-methionine γ-tocotrienol methyltransferase; HST, 3, homogentisate solanesyl transferase; MSBQMT, 2-methyl-6-solanesyl-1,4-benzoquinol methyltransferase. Substrate abbreviations. DHNA, 1,4-dihydroxy-2-naphtoate; DMGGBQ, 2,3-dimethyl-5-geranylgeranyl benzoquinol; DMBPQ, 2,3-dimethyl-5-phytyl benzoquinol; DMPQ, demethylphyloquinone; GPP, geranylgeranyl diphosphate; HGA, homogentisic acid; HPP, p-hydroxyphenylpyruvate; MGGBQ, 2-methyl-6-geranylgeranyl benzoquinol; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinol; MSBQ, 2-methyl-6-solanesyl-1,4-benzoquinol; PhytylPP, phytol diphosphate; SAM, S-adenosyl-L-methionine; SPP, solanesyl diphosphate.](image-url)
tocopherol (SAM-γ-T) [644] have been carried out previously, detailed progress in the elucidation of the pathways has been made through the use of *Arabidopsis* mutants (for a review, see [645]).

p-Hydroxyphenylpyruvate dioxygenase (HPPD) catalyzes the synthesis of homogentisic acid (HGA), a precursor of the aromatic ring of tocopherols, tocotrienols and plastoquinone (Fig. 32). However, it appears that HPPD is not required for plastoquinone biosynthesis in cyanobacteria and notably in *Synechocystis* [646]. Available data point to the fact that in contrast to downstream enzymes involved in the biosynthesis of plastid prenyllipids, HPPD is localized in the cytosol [647,648]. Thus we do not know how homogentisic acid (HGA) is recruited for tocopherol and tocotrienol biosynthesis.

HPPD is inhibited by several secondary metabolites produced by lichens and plants. These inhibitors include triketones, benzoquinones, naphthoquinones and anthraquinones. In vitro studies reveal that triketone natural products behave as competitive tight-binding inhibitors of HPPD [649]. HPPD is the target of several bleaching herbicides such as sulcotrione and isoxaflutole [650,651]. The herbicidal activities of these derivatives lead to the inhibition of tocopherol and plastoquinone biosynthesis and to concomitant build-up of phytoene [652]. These herbicides per se did not inhibit phytoene desaturase [653] but inhibit the synthesis of plastoquinone, a cofactor involved in the desaturation of phytoene [457].

The next step in the pathway is the prenylation of HGA acid by two distinct prenyltransferases. The prenylation of homogentisic acid by homogentisate-phytyl transferase (HPT) initiates the tocopherol branch. On the other hand, for the tocotrienol branch, the prenylation is carried out by homogentisate-geranylgeranyl transferase (HGGT) (Fig. 32). The tocotrienol pathway is particularly active in some plants belonging to monocot [654]. Either in the tocopherol or tocotrienol pathways, the resulting prenyl derivatives are subjected to methylation and cyclization as depicted in Fig. 32. Recent biochemical data indicate that *Capsicum* and *Arabidopsis* SAM-γ-T convert β- and γ- into β- and α-tocopherol, respectively. β-tocopherol is not accepted as a substrate. Kinetic analysis with the *Arabidopsis* suggests an iso-ordered βi-βi type reaction mechanism.

In *Arabidopsis* and in cyanobacteria the same methyltransferase catalyzes the methylation of 2-methyl-6-phytyl benzoquinol (MPBQ), the tocopherol precursor and 2-methyl-6-solanesyl-1,4-benzoquinol (MSBQ), the plastoquinone precursors [655,656]. Unexpectedly, the *Arabidopsis* and the cyanobacterial enzymes share only 18% identity [656]. Second, MPBQ and 2-methyl-6-geranylgeranyl benzoquinol (MGGBQ), which are, respectively, the tocopherol and the tocotrienol precursors are cyclized by the same enzyme [657].

Normal leaves are practically devoid of HGGT activity [641,658,659]. Recent data revealed that *Arabidopsis* HPT expressed in insect cells had approximately a 50- to 80-fold preference for Phytyl PP over GGPP [660]. These data may explain in part why leaves do not synthesize tocotrienols. However, it has been shown that tobacco leaves synthesized massive amount of tocotrienols under constrained conditions, i.e., when overexpressing *HPPD* and yeast prephenate dehydrogenase [661]. On the other hand, overexpression of tocopherol cyclase in *Arabidopsis* did not lead to the synthesis of tocotrienols [662]. The gene encoding HGGT has been cloned from cereals and is significantly different from HPT [663]. When HGGT was expressed in tobacco callus and in *Arabidopsis*, tocotrienol accumulated [663].

In the maize mutant Sucrose export defective *Sxd1* [664] which encodes tocopherol cyclase (TC) and in potato plants where the tocopherol cyclase is silenced using a RNAi-mediated silencing approach [665], the absence of tocopherol leads to pleiotropic effects. In these plants the plasmodesmata size exclusion limit or closure is altered by callose deposits. Under these conditions, the regulation of symplastic transport exerted by plasmodesmata is blocked in these plants. As a consequence, mutant and silenced plants accumulated starch and soluble sugars at high levels compared to wild-type plants [664]. In silenced potatoes, this phenomenon was paralleled by repression of photosynthesis genes (rbcS, *Cab* genes) and by an induced accumulation of defense-related genes such as the *proteinase inhibitor 1* and 2 [664]. These two features are characteristic of the molecular events induced by excess sucrose in plants [666]. However in the *vitamin E deficient1 Arabidopsis* mutant (*vte1*) that lacks tocopherol due to the deficiency TC activity, no such pleiotropic changes have been observed [657,667]. *vte1* is devoid of tocopherol but accumulates DMPBQ, the substrate of tocopherol cyclases [657,667]. It has been shown that DMPBQ could compensate for tocopherol deficiency in germinating seedlings [668]. It has been suggested that the absence of carbohydrate-deficient phenotype in *Arabidopsis* was due to the fact, that in this species, the phloem loading is predominantly apoplastic while in maize a symplastic pathway is preferentially used [669]. Callose deposition is usually associated with strong membrane lipid peroxidation [670]. Thus tocopherol may be required to counteract this deleterious effect [391,671–673].
Genes encoding enzymes of the initial steps of tocopherol and plastoquinone synthesis are subject to regulation under stress conditions. In Arabidopsis, the activity of tyrosine aminotransferase, which catalyzes the conversion of tyrosine to \( p \)-hydroxyphenylpyruvate, the precursor of HGA, is increased following jasmonic acid treatment. This trend is followed by an increased accumulation of tocopherol [674]. In a similar vein, the transcript level of barley 4-hydroxyphenylpyruvate dioxygenase HPPD is increased during senescence and this trend is induced by application of methyl jasmonate, ethylene as well as by treatment with the herbicides paraquat and DCMU or hydrogen peroxide [675]. It has been shown that in Arabidopsis overexpressing TC, the tocopherol content is increased 7-fold and is paralleled by a decrease in ascorbate and glutathione [662]. At the other extreme, the deficiency of TC is paralleled by an increase in ascorbate and glutathione [662]. Thus, these data suggest that a sensing mechanism operates that links the redox state of the cell to the ascorbate-glutathione cycle implicated in the regeneration of oxidized tocopherol [671].

Using hpt and tc mutants of Arabidopsis, it has been shown that tocopherols control during the mobilization of lipids during germination and early seedling development [668]. The seed longevity is reduced for both mutants compared to the wild-type. The hpt mutant totally devoid of tocopherols, accumulates high level of fatty acid hydroperoxides and their corresponding hydroxy derivatives. These changes were not observed in the tocopherol cyclase mutants that accumulate DMPBQ, probably due to the potential antioxidant effect of this compound [668]. These data are diagnostic features that the tocopherol content in germinating seeds is required to protect the unsaturated lipid reserves from oxidative degradation. This phenomenon could have a crucial role during the heterotrophic phase of oil seed germination. This contention is reinforced by the fact that the transcription of GGR is stimulated by light [399,676]. Interestingly, the protective role of tocopherol toward lipid oxidation is conserved in cyanobacteria [677] and algae. For instance, in Chlamydomonas mutant devoid of phytoene synthase, the lack of carotenoid is paralleled by partial deficiency in chlorophylls while the tocopherol content is increased 2-fold [678].

Knowledge gained from the molecular analysis of the tocopherol pathway has been exploited to increase the tocopherol content in model plants and agricultural crops (for a review, see [679]). In the absence of specific transcription factor, the upregulation of the expression of the SAM-\( \gamma \)-tocopherol gene has been achieved using a synthetic hybrid transcription factor [680]. The approach involved first a Dnase I hypersensitive mapping of the region of SAM-\( \gamma \)-tocopherol gene that could interact with the DNA binding domain of zinc finger proteins. Subsequently, a tripartite transcription factor construct was made that comprises a zinc finger protein designed to bind to the putative target sequence of SAM-\( \gamma \)-tocopherol methyltransferase promoter followed by a maize C1 activation domain and a nuclear targeting signal. When introduced in Arabidopsis, the hybrid transcription factor increased up to 20-fold the \( \alpha \)-tocopherol content of the seeds [680].

### 8.5. Phylloquinone and plastoquinone biogenesis

In photosynthetic membrane, phylloquinone also known as vitamin K1 functions as a bound one-electron cofactor in the A\(_1\) site of photosystem I (PSI). On the other hand, plastoquinone-9 (PQ-9) is implicated in the function of photosystem II (PSII), as a bound one-electron cofactor in the QA site and as an exchangeable two-electron two proton cofactor in the QB site. In its reduced form, PQH\(_2\)-9 diffuses in the membrane and is oxidized by the cytochrome b\(_{5}\)f complex. Their biosynthesis has not been fully investigated. Because bacterial menaquinones (vitamin K2) are structurally related to phylloquinone and because of the pathway leading to menaquinone is known (see http://metacyc.org), it has been hypothesized that a similar pathway operates in cyanobacteria and plants, according to 8 steps [681]. These informations led first to the cloning of 1, 4-dihydroxy-2-naphthoate phytlytransferase DHNA-phytlytransferase (DHNAPT) [681], 2-succinyl-6-hydroxyl-2,4-cyclohexadiene-1-carboxylate synthase and O-succinylbenzoic acid-CoA synthase from Synechocystis [682] (Fig. 32). An Arabidopsis mutant showing pale-green young leaves and white old leaves with a lesion in the gene designed abc4 has been isolated [683]. Cloning and subsequent functional characterization of the ABC4 gene indicated that it encoded the DHNAPT [683]. The mutation led to the absence of phylloquinone, and to a reduced PQ-9 content (about 3% of that of the wild-type). Similarly, in cyanobacteria when the phylloquinone biosynthetic gene encoding 2-succinyl-6-hydroxyl-2 4-cyclohexadiene-1-carboxylate synthase and O-succinylbenzoic acid-CoA synthase, DHNA synthase and DHNAPT were inactivated, no phylloquinone was produced. However, under these conditions, the PSII was not affected while the PSI activity was
50–60% preserved due to the recruitment of plastoquinone into the A1 site of PSI [681,684]. Obviously this compensation mechanism between phyloquinone and PQ-9 did not operate in higher plants. The binding flexibility of the A1 site of cyanobacteria is further strengthened by the fact that it could tolerate the biosynthetic intermediate demethylphyloquinone (DMPO) [685] nonnative quinones [686].

The apg1 mutant was isolated as a Ds-tagged Arabidopsis pale-green mutant. The mutant displays pale green leaves that turn albino in old leaves [687]. The apg1 mutant could not grow autotrophically but remain green when cultured in the presence of sucrose and under moderate light conditions (75 µmol quanta m–2 s–1). Under these conditions the mutant has qualitatively the same pigment composition than the wild-type. Under strong light conditions (400 µmol quanta m–2 s–1) the mutant was bleached [687]. In the apg1 mutant, the amount of D1, LHC and OE23 was drastically reduced. The APG1 encodes a plastid SAM-dependent methyltransferase highly identical to the 37 kDa chloroplast inner envelope membrane protein [688]. The apg1 mutant is deficient in PQ-9 and did not apparently accumulate 2-methyl-6-solanesyl-1,4-benzoquinol (MSBQ). Because the apg1 mutant was devoid of PQ-9, it has been inferred that APG1 protein catalyzed the last steps of plastoquinone synthesis, i.e., the methylation of MSBQ into PQ-9 [687]. Interestingly the PQ-9 deficiency was correlated with phytoene accumulation. This represents a diagnostic feature of the inhibition of phytoene desaturation as shown in the Arabidopsis mutant devoid of plastoquinone and tocopherol due to mutation in the HPPD gene [648]. Recent data based on transgenic expression of maize and Arabidopsis TC suggest that TC has a broader substrate specificity and catalyzed the formation of the chromanol ring of plastochromanol-8 from PQ-9 [689] (Fig. 32). These data could explain the presence of plastochromanol-8 as a minor component in seed oils [690].

PQ-9 are prenylated by solanesyl diphosphate (SPP), a C45 isoprenoid derivative. Previous studies have established that solanesyl diphosphate synthase (SPPS) and prenyltransferases catalyzing the synthesis of (all-trans)-long prenyldiphosphates are usually activated by exogenous activator that are thought to facilitate the desorption of the products from the active-site of the enzyme [691]. Two genes encoding SPPS have been characterized from A. thaliana and designed AtSPP1 and AtSPPS2 [109,361]. Recombinant AtSPPS accepted FPP and GGPP as allylic substrates but did not use DMAPP or GPP and the activity was stimulated by Triton X-100 [692]. When GGPP was used as the allylic substrate, only solanesyl diphosphate was synthesized. On the other hand, when FPP was used several C20–C40 intermediates were released [692]. The $k_{cat}/K_m$ value for GGPP was 5.1-fold higher than that of FPP [361]. AtSPPS2 is localized in the plastids, while AtSPS1 is localized in the endoplasmic reticulum [109,361]. Both genes are highly expressed in leaves compared in roots and the expression of AtSPPS1 is higher than that of AtSPPS2 [361]. The plastidial localization AtSPPS2 suggests that it is most likely involved in the synthesis of the side-chain of PQ-9. The existence of an extraplastidial SPPS is reminiscent of the fact that the endoplasmic reticulum–Golgi membranes of spinach leaves could synthesize plastoquinone [357] which is further transported to the plastids and mitochondria [358].

### 9. Conclusion

The regulation of the biosynthesis of isoprenoids in plant cells is poorly understood. In general, the amount of induced accumulation of isoprenoids in plant tissues is low. In fact we do not master several factors. We do not know how the metabolite fluxes between the primary and the secondary metabolisms are orchestrated. Practically, for all studied pathways, the concentration of the substrates and products of each step are presently unknown to evaluate which reactions are in equilibrium and which are displaced from the equilibrium. In addition, due to the complexity of the pathways, feedback control mechanisms should be envisioned. Also in several cases, the accumulation of secondary metabolites requires the differentiation of sequestery structures or conjugations and transport systems. All these specificities are difficult to delineate by insertion of limited number of genes.

Specific transcriptional regulators that could control one or multiple steps of the pathways have not been in depth characterized, as shown for the flavonoid pathway [693] and the jasmonate-responsive transcriptional regulators which induces the genes involved in alkaloid biosynthesis [694].

Finally, given the fact that agriculturally important traits are regulated by a number of genes known as quantitative trait loci (QTLs) derived from natural allelic variations [695], exploitation of QTLs and syntenic genomes would be a much powerful approach to control the pathway.
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References


