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Sterol Metabolism

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INTRODUCTION

Most eukaryotic cells contain and synthesize sterols. Whereas cholesterol is the unique sterol of vertebrates and ergosterol the major sterol of most fungi and some unicellular algae, most higher plants contain a complex mixture in which cholesterol is a minor component and 24-ethyl sterols (sitosterol and stigmasterol) account for more than 60% and 24-methyl sterols account for less than 40% (Table 1). Configuration at C-24 is 100% R in sitosterol. In contrast 24-methyl sterols are a mixture of 24-epimers where campesterol (24R-methyl cholesterol) and dihydrobrassicasterol (24S-methyl cholesterol) are in variable proportions (Benveniste, 1986; Nes, 1977). Important differences may occur in some plant families, for instance many plants belonging to the order of caryophillales contain large amounts of Δ7-sterols, spinach (*Spinacea oleracea*) and *Chenopodium rubrum* contain almost only Δ7-sterols such as spinasterol or 5α–stigmast-7-ene-3β–ol (Adler and Salt, 1987). Likewise, changes in the side chain structure may occur in some plant families. For instance sterols with a double bond at position C-25(27) occur often in cucurbitaceae and verbenaceae (Akihisa et al., 1987). The sterol composition of brassicaceae to which Arabidopsis belongs, presents a small difference to most plant species for an additional sterol, brassicasterol (ergosta-5,22E-diene-3β–ol), is found in the 24-methyl sterol fraction (Itoh et al., 1973; Schaeffer et al., 2001). It is noteworthy that insects, which constitute more than 80% of animal species, do not synthesize sterols. They use sterols from their diet. For instance, phytophageous insects are able to demethylate sitosterol into cholesterol, which is finally used to make ecdysteroids involved during embryonic and larval development (Svoboda and Weirich, 1995).

Sterols have multiple functions. First of all they are membrane constituents. They accumulate in the plasma membrane where they are present in concentrations similar to those of phospholipids (Hartmann-Bouillon and Benveniste, 1978). Several data obtained with model systems show that sterols in condensing the membrane bilayer regulate membrane fluidity and permeability (Bloch, 1983; Demel and De Kruyff, 1976). In addition to this role played by the bulk of sterols, a regulatory function has been assigned to sterols in various systems : i) covalent binding of cholesterol to HEDGEHOG is involved in embryonic development of vertebrates (Kip Guy, 2000) ; ii) interaction of cholesterol with caveolin induces the formation of membrane microdomains (caveolae), which may be signaling centers for multiple pathways (Karpen et al., 2001) ; iii) transport of plant sterols by elicitins from *Phytophtora* spp leads to a hypersensitive like response in tobacco (Mikes et al., 1998). These processes trigger signaling pathways involved in cell division, development or resistance to pathogens. Finally sterols are precursors of compounds with a high physiological activity such as brassinosteroids, an important class of hormones involved in higher plant growth and development (Li et al., 1996), ecdysteroids in invertebrates (Svoboda and Weirich, 1995), antheridiol and oogoniols, sexual pheromones of aquatic fungi belonging to the saprolegniale class (Brunt and Silver, 1991). Therefore, sterols, in addition to their own biological activity, are important interfaces of plant insect interactions.

In spite of this recent knowledge, several important questions remain open especially in plants : i) higher plants synthesize phospholipids containing polyunsaturated fatty acids and bulky 24-ethyl sterols, which are both incorporated into membranes in which they should interact (Schuler et al., 1991). Is the biosynthesis of both class of compounds co-regulated and, if so, how does such a coregulation work ? ii) Considering the regulatory processes enumerated above, what is the role of sterols in signal reception and transduction ? iii) It is well established that brassinosteroid biosynthesis constitutes one branch deriving from phytosterol biosynthesis. Whereas sterols are present usually in plants at concentrations in the range of several mg per g of dry weight, a few ng per g of dry

Table 1. Sterol profile of *Arabidopsis thaliana*[a]

Sterol	Wild-type plants $\mu g.g^{-1}$ dry weight
Cycloartenol	75
24-methylene cycloartanol	100
β-amyrin	30[b]
α-amyrin	25[b]
Lupeol	5[b]
Cycloeucalenol	tr
Obtusifoliol	25
4α-methyl-5α-ergosta-8,14,24(24^{1})-trien-3β-ol	nd
4α-methyl-5α-ergosta-8,24(24^{1})-dien-3β-ol	nd
24-methylene lophenol	25
24-ethylidene lophenol	25
Episterol	nd
Δ^{7}-sitostenol	25
Δ^{7}-avenasterol	25
24-methylene cholesterol	tr
Brassicasterol	**50**
Campesterol	275
Isofucosterol	75
Sitosterol	1600
Stigmasterol	150

[a] Data from Schaeffer et al. (2001). [b] Data from Husselstein et al. (2001)

Sterols (free and esters) were saponified with KOH in methanol to release the sterol moiety of steryl esters. Steryl glucosides have not been analyzed. nd : non detectable in wild-type plants ; tr : traces.

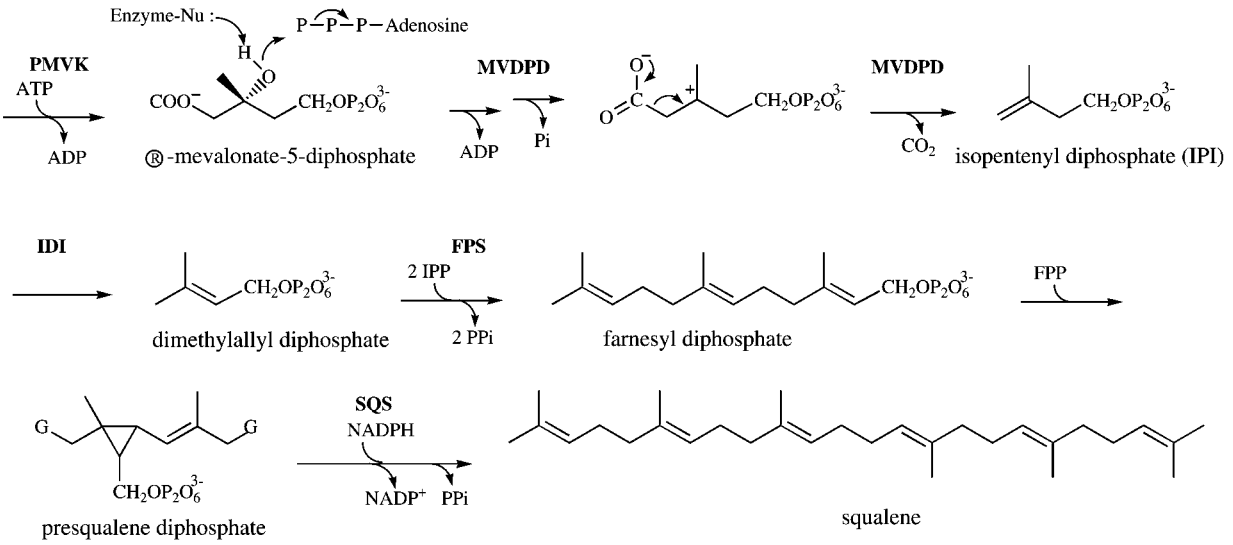

Fig. 1. Biosynthetic pathway leading to plant sterols. From mevalonate to squalene.

G = geranyl :

HMGR = (S)-3-hydroxy-3-methyl-glutaryl-CoA reductase ; MVK = mevalonate kinase ; PMVK : 5-phosphomevalonate kinase ; MVDPD = mevalonate diphosphate decarboxylase ; IDI = isopentenyl diphosphate-dimethylallyl diphosphate isomerase ; FPS = farnesyl diphosphate synthase ; SDS = squalene synthetase.

weight of brassinosteroids are generally found. Therefore a very small fraction of the phytosterols synthesized by the plant will be used for brassinosteroid biosynthesis. This leads to the existence of a tight control at the junction point between both pathways.

In order to answer these questions, a strategy of gain or loss of function has been applied recently to the sterol biosynthetic pathway. The use in the past of potent and specific inhibitors gave unvaluable informations (Benveniste and Rahier, 1992; Schmitt et al., 1981) which in some case were not reliable because of side effects of inhibitors. Recently the impressive development of molecular genetics, the availability of DNA tagged mutants, multiple possibilities offered by stable transformation in sense and antisense orientation and gene silencing, assorted with the sequencing of the Arabidopsis genome led to an important breakthrough in the field of sterol metabolism and functions.

Before entering into this field, a short recall of the state of the art of the sterol biosynthesis is needed. Sterol biosynthesis is complex and involves at least 25 steps from isopentenyl diphosphate, the commited precursor of all isoprenoids, to end pathway sterols. The biosynthetic scheme of Figure 1 is common to most higher plants and also applies to Arabidopsis. From isopentenyl diphosphate to 2,3-oxidosqualene (OS) the biosynthetic pathway is the same in eukaryotes, however profound differences exist downstream of OS (Figure 2). Whereas OS is cyclized to lanosterol in nonphotosynthetic eukaryotes, it is cyclized to cycloartenol, an isomer of lanosterol possessing a cyclopropane ring in place of the Δ^8 double bond, in photosynthetic eukaryotes. However some exceptions to this rule will be discussed below. As end pathway plant sterols do not contain a cyclopropane ring, an enzyme capable of opening it exist in photosynthetic eukaryotes. A third difference consists in the presence in higher plant sterols (sitosterol, stigmasterol…) of an extra 24-ethyl group, resulting from two methylation steps situated between cycloartenol and end pathway sterols. Sterols from vertebrates are not alkylated at C-24, whereas sterols from most fungi possess only one methyl at C-24. Finally the passage from cycloartenol to end pathway sterols involves three demethylation steps at positions C4 and C14 of the sterol skeleton. The order and the position of these steps strongly differ in non photosynthetic and photosynthetic eukaryotes.

In the present review we will present most significant progresses occurring between mevalonic acid and stigmasterol. As mevalonate is the precursor of sterols in most eukaryotes and especially in higher plants, we shall not

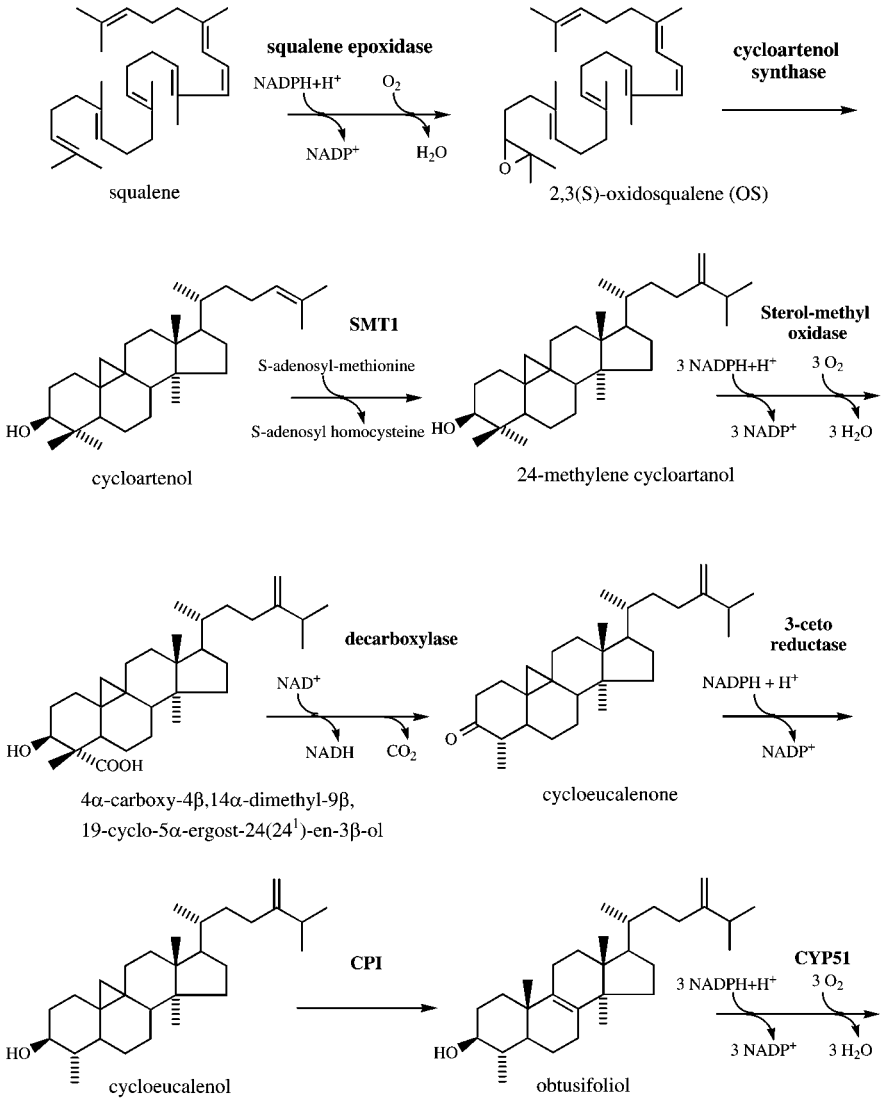

Fig. 2. Biosynthetic pathway leading to plant sterols. From squalene to end pathway sterols.
SMT1, SMT2 = sterol-methyltransferases ; FACKEL = $\Delta^{8,14}$-sterol- Δ^{14}-reductase ; STE1/DWARF7/BUL1 = Δ^{7}-sterol-C5(6)-desaturase ; DWARF5 = $\Delta^{5,7}$-sterol- Δ^{7}-reductase ; DWARF1/DIM = Δ^{5}-sterol- Δ^{24}-reductase (isomerase) ; CPI = cyclopropyl sterol isomerase ; CYP51 = obtusifoliol-14α-demethylase.

consider here the non mevalonate (2-C-methyl-D-erythritol 4-phosphate) pathway leading to isoprenoids (Rohmer et al., 1993) which will be treated separately. Because genetical data obtained in recent years repeatetly show that brassinosteroid and campesterol biosynthesis are intimately linked, this chapter on sterol metabolism will include a few considerations concerning brassinosteroid biosynthesis. A specific chapter is devoted to brassinosteroid biosynthesis.

FROM HMGCoA TO SQUALENE

3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, EC 1.1.1.34) catalyzes the two-step reduction of (S)-3-hydroxy-3-methylglutaryl coenzyme A (*S*-HMG-CoA) into *R*-mevalonate (Fig 1) and is generally considered to be a regulatory enzyme in sterol biosynthesis. All *HMGR*

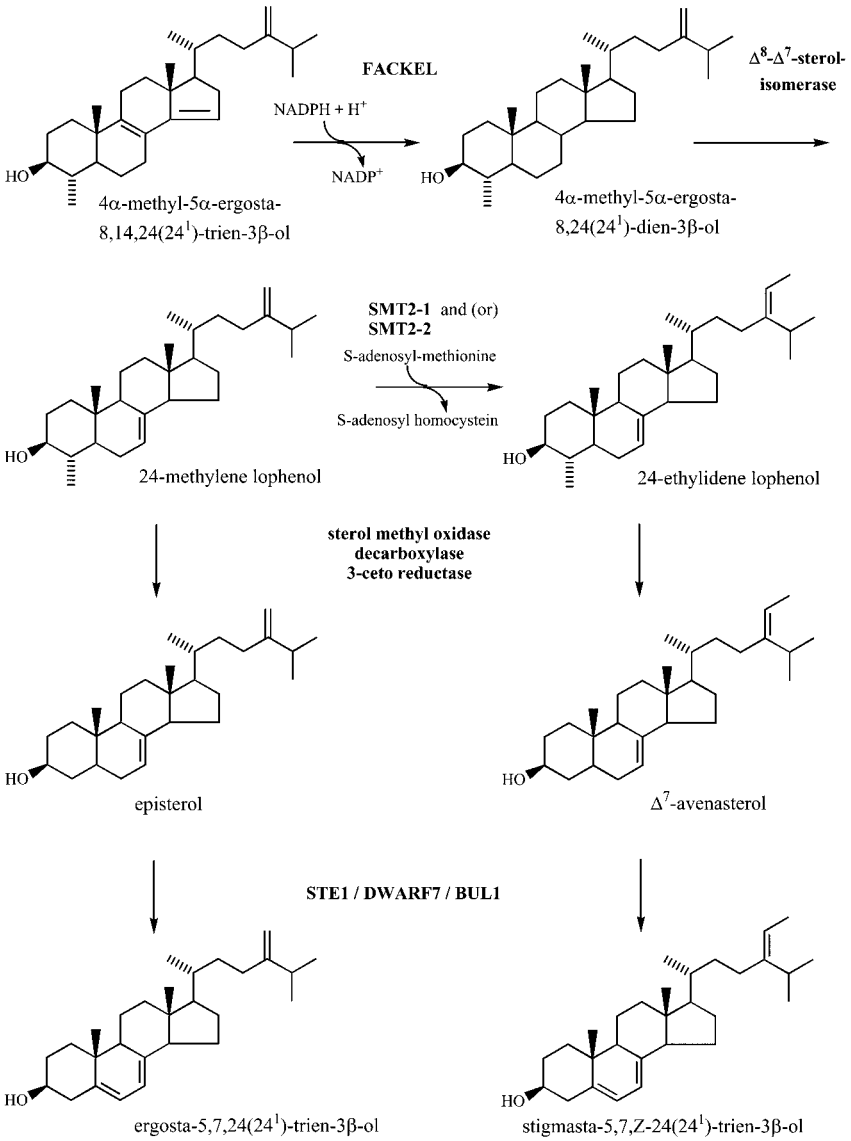

Fig. 2a.

genes from plants isolated so far code for proteins having the same general organization, a highly conserved membrane-spanning domain consisting of two hydrophobic amino-acid sequences that are separated from the globally hydrophilic and highly conserved catalytic domain by a poorly conserved linker region. It appears that plant HMGR isozymes are encoded by a small family of genes and a large body of evidence has been presented that these genes and their products are placed under developmental or environmental control (Bach, 1987; Choi et al., 1994; Enjuto et al., 1994). An Arabidopsis cDNA encoding HMGR has been cloned for the first time (Learned and Fink, 1989) by functional complementation of a yeast strain (JRY 2394) which was defective in mevalonic acid synthesis (Basson et al., 1988). Later on Arabidopsis was shown to contain two genes *HMG1* and *HMG2* (At1g76490 and At2g17370) that encode HMGR. *HMG1* mRNA is detected in all tissues, whereas the presence of *HMG2* mRNA is restricted to young seedlings, roots and inflorescences. *In vitro* translation products of these genes were shown to be efficiently inserted into endoplamic reticulum-derived microsomal membranes (Enjuto et al., 1994). Similar results were obtained with full-length cDNAs (*HMG1* and *HMG2*) from *Raphanus sativus*, another brassicaceae (Vollack et

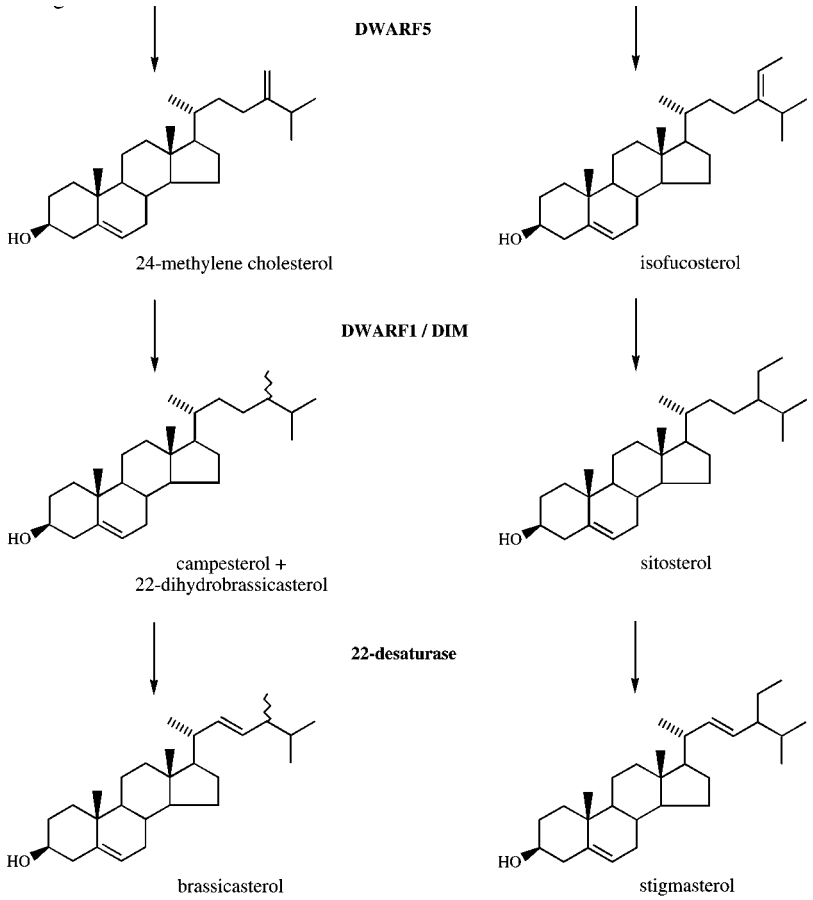

Fig. 2b.

al., 1994). Attemps to express a full-length cDNA (*HMG1*) encoding an isozyme of radish HMGR in *Escherichia coli* resulted in the production of catalytically inactive enzyme, while expression of a truncated form comprising only the soluble domain led to the formation of a highly active enzyme (Ferrer et al., 1990). Bacterial expression of the catalytical domain of HMGR (isoform HMG1) from Arabidopsis, and its inactivation by phosphorylation at S577 by *Brassica oleracea* 3-hydroxy-3-methylglutaryl-CoA reductase kinase were also reported (Dale et al., 1995). The use of an alternative promoter in the Arabidopsis *HMG1* gene was shown to generate an mRNA that encodes a novel HMGR isoform (HMG1L) with an extended N-terminal region (Lumbreras et al., 1995). Results of this study support the conclusion that the ER is the only cell compartment for the primary targeting of HMGR in Arabidopsis and that the three HMGR isoforms are cotranslationally inserted into the ER membrane, where they behave as integral membrane proteins (Lumbreras et al., 1995). Despite the house keeping role that has been ascribed to *HMG1*, both the quantitative and qualitative features of its expression are modulated in response to a variety of cellular and environmental signals and especially to light (Korth et al., 2000). More precisely, light-mediated regulation of *HMG1* expression has been shown to be an organ-autonomous response and indicated that the high levels of HMG CoA reductase expression detected in immature leaves may be primarily attributed to the dark-induced expression of *HMG1*, and that *HMG1* is expressed at low levels throughout the plant in response to light (Learned and Connolly, 1997). Finally HMGR is a rate-limiting enzyme for higher plant sterol biosynthesis (Chappell et al., 1995; Schaller et al., 1995; Schaller et al., 1993) ; its regulation is complex and occurs at transcriptional, translational and post-translational (Sugden et al., 1999) levels. Enzymological studies of the plant enzyme are still needed to better understand features of post-translational regulation. Recent crystal structures of the catalytic portion of human (Istvan et al., 2000) and *Pseudomonas mevalonii* (Bochar et al., 1999), HMGRs

have given precious clues to better understand some aspects of Arabidopsis HMGR regulation.

Mevalonate kinase (MVK), the enzyme that catalyzes the phosphorylation of *R*-mevalonate by ATP to produce *R*-mevalonate 5-phosphate and ADP, is considered as a potential regulatory enzyme of the isoprenoid biosynthetic pathway. Mevalonate kinase has recently been very much studied in animals because mutations in the gene encoding this enzyme have been shown to be responsible of several pathologies such as the periodic fever syndrome, mevalonic aciduria and hyperimmunoglobulinaemia (Houten et al., 2001). In contrast few studies have been reported in plants. Using an Arabidopsis cDNA library constructed in the yeast expression vector pFL61 (Minet et al., 1992), a cDNA of 1.64-kb encoding a protein of 378 amino acid residues with significant similarity to yeast and mammalian enzymes, was isolated and characterized by genetic complementation of the *erg12-1* mutation in yeast (Riou et al., 1994). Recently molecular cloning and expression analysis of the mevalonate kinase gene from Arabidopsis (AT5g27450) have been reported (Lluch et al., 2000). The expression pattern of the *MVK* gene suggests that the role of the encoded MVK is the production of a general pool of mevalonate 5-phosphate for the synthesis of different classes of isoprenoids involved in both basic and specialized plant cell functions. Overexpression of both yeast or Arabidopsis cDNAs in the yeast had no significant effect on ergosterol accumulation (Riou et al., 1994). DNA hybridization blots and Arabidopsis complete genome sequencing show that only one locus exists in the Arabidopsis genome. The single gene from Arabidopsis contains five exons and four introns (Lluch et al., 2000).

cDNAs or genes encoding **5-phosphomevalonate kinase** (PMVK, EC 2.7.4.2), the enzyme which catalyzes the phosphorylation of R-mevalonate 5-phosphate to R-mevalonate 5-diphosphate, has still not been isolated and characterized in higher plants. However complete sequencing of Arabidopsis genome has revealed the existence of a gene (At1g31910) whose encoded protein has 30% identity with the yeast PMVK (*ERG8*) (Tsay and Robinson, 1991). Therefore, with the availability of the *erg8-1* yeast mutant, cloning by complementation seems feasible. Interestingly, so far, two nonorthologous genes encoding PMVK have been described, the *Saccharomyces cerevisiae ERG8* gene and the human PMVK gene (Houten and Waterham, 2001). Molecular cloning of human phosphomevalonate kinase allowed the identification of a consensus peroxisomal targeting sequence suggesting that the conversion of mevalonate to farnesyl diphosphate occurs in parallel in the peroxisomes of mammalian cells (Biardi and Krisans, 1996).

Mevalonate diphosphate decarboxylase (MVDPD, EC 4.1.1.33) catalyzes the ATP-dependent conversion of mevalonate diphosphate into isopentenyl diphosphate (IPP). In *Saccharomyces cerevisiae* this enzyme is encoded by the *ERG*19 gene (Servouse et al., 1984). Sequence comparison with MVDPD amino acid sequence of *S.cerevisiae* identified an EST clone corresponding to a cDNA that may encode Arabidopsis MVDPD. The predicted amino acid sequence presents about 55% identity with the yeast, and human MVPDs. When expressed in yeast, the Arabidopsis cDNA complemented an *ERG19* deleted strain. However the wild type sterol content was not fully restored, suggesting that the Arabidopsis MVPD activity may not be optimal in yeast (Cordier et al., 1999). Complete sequencing of the Arabidopsis genome revealed the existence of two genes (At2g38700 and AT3g54250). Using a two-hybrid assay, it was shown that the *S.cerevisiae* MVDPD forms homodimers *in vivo* and a single substitution impairs dimerization. The Arabidopsis MVDPD forms also homodimers *in vivo*. Heterodimer formation between the plant and the wild-type yeast enzymes was detected by these assays (Cordier et al., 1999). Crystal structure would be an attractive goal in order to better understand the intricate mechanism of action of this enzyme.

Isopentenyl diphosphate (IPP) : dimethylallyl diphosphate (DMAPP) isomerase (IDI, EC 5.3.3.2) catalyzes the conversion of IPP to its electrophilic isomer DMAPP, which is then used to prime the prenyltransferase for chain elongation with IPP. Two Arabidopsis cDNAs (*IDI1* and *IDI2*) encoding isopentenyl diphosphate isomerase (IDI) were isolated by complementation of an IPP isomerase mutant strain of *S. cerevisiae* (Campbell et al., 1997). Both cDNAs encode enzymes with an amino terminus that may function as a transit peptide for localization in plastids. Total sequencing of the Arabidopsis genome and DNA blot analysis confirm that *IDI1* and *IDI*2 are derived from two genes (AT5g16440, AT3g02780) in Arabidopsis. This seems to be a general feature in most higher plants examined so far and suggests that a single gene would encode plastid and cytosolic IDI in plants (Cunningham and Gantt, 2000). The intracellular localization of isoprenoid synthesis enzymes has been reconsidered in mammalian cells and IDI has been shown to be localized predominantly in peroxisomes and not in the cytosol (Olivier and Krisans, 2000).

Farnesyl diphosphate synthase (FPS, EC 2.5.1.1) catalyzes the condensation of one molecule of dimethylallyldiphosphate (DMAPP) with two molecules of isopentenyl diphosphate (IPP) to give farnesyl diphosphate (FPP). Farnesyl diphosphate is a very important compound in the plant (especially Solanaceae) cell economy because it is

located at a branch point that leads to the formation of sterols and sesquiterpenoid phytoalexins. FPS is encoded by the gene *ERG* 20 in yeast. Disruption of the yeast *ERG* 20 gene is lethal (Blanchard and Karst, 1993). A cDNA encoding Arabidopsis FPS1 was subsequently isolated by complementation of the *erg* 20 deletion yeast mutant (Delourme et al., 1994) with the cDNA expression library cloned into pFL61 (Minet et al., 1992). This cDNA encodes a polypeptide of 343 amino acids residues presenting about 50% identity with yeast, rat and human FPSs. A second cDNA corresponding to the *FPS*2 was isolated by RT-PCR, it codes for a protein having 342 amino acid residues. *FPS*1 and *FPS*2 share an overall amino acid identity of 90.6% . The *FPS*1 gene (AT5g47770) consists of 12 exons and 11 introns whereas the *FPS*2 gene (AT4g17190) consists of 11 exons and 10 introns. In both genes, introns are located at equivalent positions relative to the coding sequences (Cunillera et al., 1996). *FPS*1 mRNA accumulates preferentially in roots and inflorescences whereas *FPS*2 mRNA is predominantly expressed in inflorescences. Later on it has been shown that the *FPS*1 gene generates a novel mRNA that encodes a mitochondrial farnesyl diphosphate synthase isoform (FPS1L) (Cunillera et al., 1997). The FPS1L mRNA accumulated preferentially in inflorescences whereas the previously reported FPS1 mRNA (FPS1S mRNA) is predominantly expressed in roots and inflorescences. Translation of the FPS1L mRNA from the upstream ATG start codon generates a novel FPS1 isoform (FPS1L) with an NH2-terminal extension of 41 amino-acid residues, which has all the characteristics of a mitochondrial transit peptide. The functionality of this extension as a mitochodrial transit peptide was demonstrated by *in vitro* import experiments using purified plant mitochondria (Cunillera et al., 1997). Spatial and temporal patterns of the β-glucuronidase (GUS) reporter gene expression directed by 5' regions of the Arabidopsis *FPS1* and *FPS2* genes have been studied. The highest levels of FPS2 : GUS activity were detected in flowers, especially in pollen grains. Overall, the pattern of expression of *FPS2 :GUS* suggests a role for *FPS2* in the synthesis of particular isoprenoids with specialized functions. In contrast, the *FPS1S :GUS* gene is widely expressed in all plant tissues throughout development, thus supporting a role for *FPS1*S in the synthesis of isoprenoids (for instance sterols) serving basic plant cell functions (Cunillera et al., 2000). Recently cDNA sequences encoding FPS have been cloned from several plant species such as *Lupinus albus* (Attucci et al., 1995), *Zea mays* (Li and Larkins, 1996), *Artemisia annua* (Matsushita et al., 1996), *Hevea brasiliensis* (Adiwilaga and Kush, 1996), and *Parthenium argentatum* (Pan et al., 1996). According to these studies, it seems that plant FPS would be encoded by multigene families like other key enzymes of the isoprenoid biosynthesis such as HMGR, IDI and several others (*vide infra*). The biological significance of the occurrence of similar isoforms in plants will be discussed in a conclusive paragraph. Nevertheless, FPSs are considered to play a key role in isoprenoid biosynthesis. It has been shown that in mammals FPS is a highly regulated enzyme involved in the control of the sterol biosynthetic pathway (Clarke et al., 1987). Relevant to this role are recent studies showing that : i) farnesol induces cell death and stimulation of HMGR in tobacco cv Bright Yellow-2 cells (Hemmerlin and Bach, 2000) ; ii) farnesol is utilized for isoprenoid biosynthesis in plant cells via FPP formed by successive monophosphorylation reactions (Thai et al., 1999).

Squalene synthase (SQS ; EC 2.5.1.21) catalyzes the reductive condensation of two molecules of FPP to squalene in the presence of NADPH and Mg^{2+}, via the intermediate presqualene diphosphate (Fig. 1). In contrast to preceding enzymes, SQS catalyzes the first committed step in sterol formation and for this reason may be highly regulated (Kennedy and Bard, 2001; Radisky and Poulter, 2000). A SQS probe from *Mus musculus* was successfully used to isolate two overlapping Arabidopsis cDNA clones coding for a protein of 410 amino acids with about 40% identity with mammalian and yeast squalene synthetases (Nakashima et al., 1995). The *Arabidopsis* SQS enzyme was expressed in *Escherichia coli*. A cell-free extract of *E.coli* transformed with a recombinant plasmid containing the *Arabidopsis* SQS cDNA efficiently converted [^{14}C]farnesyl diphosphate into squalene in the presence of NADPH and Mg^{2+}. This study revealed that both the structures and reaction mechanisms of squalene synthases were markedly conserved between plants and mammals. Later on it was shown that *Arabidopsis* SQS is encoded by a small gene family of two genes : SQS1 (AT4g34640) and SQS2 (AT4g34650), which are organized in a tandem array (Kribii et al., 1997). SQS1 and SQS2 have an identical organization regarding intron positions and exon sizes and encode SQS isoforms showing a high level of sequence conservation (79% identity). Remarkably the SQS1 isoform is unable to complement the SQS-defective (gene disrupted) *Saccharomyces cerevisiae* strain 5302, although SQS activity was detected in the microsomal fraction of the transformed yeast strain. However a chimeric SQS resulting from the replacement of the 66 C-terminal residues of the *Arabidopsis* enzyme by the 111 C-terminal residues of the *Schizosaccharomyces pombe* enzyme was able to confer ergosterol prototrophy to strain 5302. This and other expriments indicated that the C-terminal region of the enzyme is involved in the channeling of squalene through the yeast sterol pathway (Kribii et al., 1997). Squalene synthase cDNAs were isolated also in *Glycyrrhiza glabra*, *Nicotiana tabacum*, *Nicotiana benthamiana*, *Orizum sativa*, *Zea may, Solanum tuberosum*

and *Glycine max* (Devarenne et al., 1998; Hata et al., 1997; Hayashi et al., 1996; Yoshioka et al., 1999). Treatment of tobacco cell suspensions cultures with a fungal elicitor dramatically reduced SQS enzymatic activity. Analysis of SQS mRNA levels in elicitor-treated cells indicated that the suppression of SQS enzyme activity may result from a post-transcriptional modification of SQS (Devarenne et al., 1998). Squalestatin S1, a potent inhibitor of SQS in mammalian cells, has been shown to strongly inhibit sterol biosynthesis in tobacco BY-2 cells. Conversely enzymatic activity and mRNA levels of HMGR were several-fold increased (Hartmann et al., 2000). Finally a crystal structure of human squalene synthase has been recently reported and should be useful in the design of new inhibitors of potential therapeutic importance (Pandit et al., 2000).

FROM SQUALENE TO STEROLS

Squalene epoxidase (EC 1.14.99.7) catalyzes the conversion of squalene to 2,3(S)-oxidosqualene (OS) (Fig 2) which is the first oxygenation step in the sterol biosynthetic pathway. This microsomal-bound enzyme has been thoroughly studied in animal cells (Yamamoto and Bloch, 1970). Rat squalene epoxidase requires FAD, NADPH-cytochrome P450 reductase, NADPH and a supernatant protein factor (SPF). Rat squalene epoxidase cDNA was isolated by selecting *S.cerevisiae* transformants expressing rat cDNAs in the presence of terbinafin, a potent fungicide and inhibitor of fungal squalene epoxidase (Sakakibara et al., 1995). Rat squalene epoxidase, as deduced from the nucleotide sequence, contains 573 amino acids (Sakakibara et al., 1995). The amino acid sequence reveals one potential transmembrane domain and a FAD binding domain, suggesting that the squalene epoxidase is a flavoprotein. This deduced sequence is 30% identical to *ERG1*, the gene encoding squalene epoxidase in *S.cerevisiae* (Jandrositz et al., 1991). Rat squalene epoxidase expressed in *E.coli* was shown to catalyze the *in vitro* conversion of squalene to 2 ,3(S)-oxidosqualene, thus confirming the polypeptide as a functional squalene epoxidase (Sakakibara et al., 1995). The gene encoding the supernatant protein factor (SPF) that promotes the squalene epoxidation catalyzed by rat liver microsomes has been recently cloned (Shibata et al., 2001). The encoded protein of 403 amino acids belongs to a family of cytosolic lipid-binding/transfer proteins. Only a few studies concerning squalene epoxidase have been reported in plants. The sequencing of the Arabidopsis genome has revealed 6 genomic clones (AT1g58440, AT2g22830, AT4g37760, AT5g24140, AT5g24150 and AT5g24160) whose deduced polypeptidic sequences possess significant identities with the rat liver enzyme. The amino acid sequences reveal one potential transmembrane region and a FAD binding domain suggesting the existence in Arabidopsis of a family of genes encoding squalene epoxidase. However no data dealing with the function of theses genes has been reported so far. Sequences of 3 Arabidopsis and 2 *Brassica napus* cDNAs encoding squalene epoxidase homologues have been reported (Schäfer et al., 1999). Comparison of cDNA and genomic sequences indicate that the 3' splice site of an intron in these genes has undergone junctional sliding (Schäfer et al., 1999), a phenomenon having interesting evolutionary significance.

2,3(S)-oxidosqualene-cycloartenol cyclases (cycloartenol synthase). The 2,3(S)-oxidosqualene (OS) cyclases compose a family of biocatalysts that convert (S)-2 ,3-oxidosqualene (OS) to polycyclic triterpenes. The mammalian (Abe and Prestwich, 1995) and fungal (Shi et al., 1994) OS cyclase (EC 5.4.99.7) produces lanosterol, precursor of cholesterol in vertebrates and of ergosterol in most fungi. The higher plant OS cyclase (EC 5.4.99.8) catalyzes the formation of cycloartenol, precursor of phytosterols (Benveniste, 1986; Nes, 1977). This latter enzyme must break 11 bonds and form 11 new ones to form cycloartenol, which is a pentacyclic cyclopropyl triterpene containing 9 chiral centers. In addition to cycloartenol synthase, higher plants contain other OS-cyclases converting OS into a vast array of pentacyclic triterpenoids (oleanane, ursane, lupane, baccharane, friedelane derivatives). The stereoelectronic factors that understate the catalytic mechanism of these reactions have been discussed elsewhere (Abe et al., 1993). The formation of lanosterol and cycloartenol is initiated in the chair-boat-chair-like conformation of OS whereas the formation of amyrins and related pentacyclic triterpenes is initiated in the chair-chair-chair-like conformation of OS. In all cases the proton-initiated cyclization is postulated to proceed through the following events : i) a series of antiperiplanar 1,2 additions ; 1,2 rearrangements and 1,2 eliminations ; ii) the formation of conformationally rigid and partially cyclized carbocationic intermediates ; iii) stabilization of the developing carbocationic centers on the substrate by negative point charges at the active site of the enzyme (Johnson et al., 1987). As suggested by several authors (Feil et al., 1996; Shi et al., 1994) the electron density required for carbocationic stabilization could arise from anionic and (or) aromatic residues. These cyclization intermediates could be stabilized by cation-π interactions (Dougherty, 1996). The fact that OS mimics possessing positively charged nitrogen atoms in place of potential carbocation behave as potent inhibitors is consistent with the above views (Taton et al., 1992). Most prokaryotic organisms do not synthesize sterols, but many of them synthesize hopanoids that are derived from pentacyclic triterpenic hydrocarbons

which originate from squalene cyclisation owing to squalene-hopene cyclases (Wendt et al., 1997). A 2,3-OS-lanosterol cyclase (lanosterol synthase) gene (ERG7) from *S. cerevisiae* was isolated and encoded a 83-kDa protein (Corey et al., 1996; Shi et al., 1994). Later on a splendid approach led to the isolation of an Arabidopsis cDNA (ATCYC) encoding a cycloartenol synthase (Corey et al., 1993). This was achieved by functional expression in a yeast mutant lacking lanosterol synthase (GL7) by the use of a chromatographic screen. This cDNA contained a 2277-bp open reading frame encoding a 86-kD protein which was about 35% identical with *S. cerevisiae* lanosterol synthase and remarkably 46% identity with a *Rattus norvegicus* lanosterol synthase (Abe and Prestwich, 1995) showing that the plant cycloartenol synthase has more identity with a mammalian than a fungal lanosterol synthase. A single mutation (adenine1362guanine) occurring on the Arabidopsis cycloartenol synthase gene has been shown to confer to cycloartenol synthase the ability to form 25% lanosterol and 21% parkeol in addition to cycloartenol. This mutation leads to a I454V change in the peptidic sequence (Hart et al., 1999). A yeast mutant (*erg7*) defective in lanosterol synthase was transformed with a plasmid harbouring the cycloartenol synthase cDNA. The transformed yeast was still auxotrophic to ergosterol because cycloartenol is not usable by yeast to make sterols. *Erg7* did not need any more ergosterol if transformed with a cycloartenol synthase cDNA possessing the A1362G mutation (Hart et al., 1999). Likewise, a tyrosine-to-threonine mutation was shown to convert the Arabidopsis cycloartenol synthase synthase to an OS cyclase that forms lanosterol as its major product (Herrera et al., 2000). Such experiments have far-reaching evolutionary implications. The corresponding single gene (At2g07050) consists of 18 exons and 17 introns. The Arabidopsis genome sequencing program reveals the presence of a gene (AT3g45130) whose deduced polypeptide sequence possessed 62% identity with the cycloartenol synthase protein and appeared closest to cycloartenol synthase in a phylogenetic tree containing many of the triterpene synthases already known (Fig. 4). The function of the protein encoded by this gene is still unknown.

In recent years a breakthrough has been achieved in the field of triterpene synthases. A cDNA encoding β-amyrin synthase (*PGAM*) has been characterized in *Panax ginseng*. Expression of the cDNA in yeast led to formation of a polypeptide capable of converting OS into β-amyrin (Kushiro et al., 1998). A cDNA encoding a lupeol synthase (*ATLUP1*) has been identified in Arabidopsis after expression in *S.cerevisiae* (Herrera et al., 1998; Husselstein-Muller et al., 2001). The protein (ATLUP1) encoded by this cDNA was shown to convert OS into lupeol, minor triterpenoid compounds were also identified (Herrera et al., 1998). Another cDNA (*ATLUP2*) was subsequently cloned from Arabidopsis and expressed in *S. cerevisiae* (Husselstein-Muller et al., 2001; Kushiro et al., 2000). The transformed *S. cerevisiae* was shown to cyclize OS into α-, β-amyrins and lupeol (Husselstein-Muller et al., 2001). During parallell experiments performed in another laboratory, taraxasterol, Ψ-taraxasterol, bauerenol, multiflorenol, butyrospermol and tirrucalla-7,21-dienol were identified in addition to α- and β-amyrins and lupeol (Kushiro et al., 2000). Therefore the polypeptide (ATLUP2) encoded by this gene is a novel multifunctional triterpene synthase. Shuffling domain mutagenesis has been done on ATLUP1 and PGAM in order to investigate the region important for protein specificity. The second quarter from the N-terminus of the protein was shown to have a crucial importance in determining the enzymatic process toward lupeol or amyrin (Kushiro et al., 1999). The genome sequencing project has revealed a rather complex situation. *ATLUP1* and *ATLUP2* are representatives of a small family of five genes (*ATLUP1-5*), in addition seven genes (*ATPEN1-7*) whose deduced polypeptide sequences have about 55% identity with the *ATLUP* family and which could encode triterpene synthases have been also identified (Husselstein-Muller et al., 2001). All genes encoding demonstrated or postulated triterpene synthases have been gathered in the Table 2. Each genomic sequence is labeled with a code and is also characterized by the number of exons and introns. In order to obtain information on evolutionary relationships, a phylogenetic tree was constructed using a heuristic search (Doyle and Gaut, 2000) (Fig. 4). In this tree the prokaryotic hopane synthases from *Alicyclobacillus* (*AAHOP*) was considered as reference taxa. According to this tree, the seven pentacyclic triterpene synthases from Arabidopsis (*ATPEN1*to *7*) form a group of genes clearly distinct from the five lupeol synthases (*ATLUP1* to *5*). Interestingly, the *ATLUP* subfamily also encompasses the two β-amyrin synthases of *Panax ginseng* (*PGAM*) and of *Pisum sativum* (PSAM) and is clearly distinct from the lupeol synthases from *Olea europaea* (*OELUP*) and *Taraxacum officinalis* (*TOLUP*) (Kushiro et al., 1999).

S-Adenosylmethionine-sterol-C-methyltransferases. Sterols from fungi and higher plants differ from vertebrate sterols by the presence of an extra alkylgroup at C-24 (Nes, 2000). Whereas most fungi sterols possess a methyl group at C-24, higher plants contain both 24-methyl- and 24-ethyl sterols. This alkylation of the side chain is catalyzed by S-adenosylmethionine (AdoMet)-sterol-C-methyltransferases (SMTs). In *S.cerevisiae*, the SMT converts zymosterol into fecosterol (Moore and Gaylor, 1969). In higher plants (and therefore in Arabidopsis), the presence of 24-ethylsterols results from two distinct methyltransfers from AdoMet (Nes, 2000; Rahier et al., 1984). According to the chemical structures of intermediates of

Table 2. Summary of genomic sequences found in databases and corresponding to enzymes of sterol biosynthesis and metabolism

Function	Accession number	Chromosome	Exon number
3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG1)	At 1g76490	1	4
3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG2)	At 2g17370	2	4
Mevalonate kinase	At 5g27450	5	5
5-phosphomevalonate kinase	At 5g27450	1	nd
Mevalonate diphosphate decarboxylase 1	At 2g38700	2	9[b]
Mevalonate diphosphate decarboxylase 2	At 3g54250	3	9[b]
Isopentenyl diphosphate: dimethylallyl diphosphate isomerase 1 (IDI1)	At 5g16440	5	6[b]
Isopentenyl diphosphate: dimethylallyl diphosphate isomerase 2 (IDI2)	At 3g02780	3	6[b]
Farnesyl diphosphate synthase 1	At 5g47770	5	12
Farnesyl diphosphate synthase 2	At 4g17190	4	11
Squalene synthase 2	At 4g34640	4	13
Squalene synthase 1	At 4g34650	4	13
Squalene epoxidase	At 1g58440	1	8[b]
Squalene epoxidase	At 2g22830	2	6[b]
Squalene epoxidase	At 4g37760	4	6[b]
Squalene epoxidase	At 5g24140	5	8[b]
Squalene epoxidase	At 5g24150	5	8[b]
Squalene epoxidase	At 5g24160	5	8[b]
2,3(S)-oxidosqualene-cycloartenol cyclase	At 2g07050	2	18
Lupeol synthase (ATLUP1)	At 1g78970	1	17
Multifunctional triterpene synthase (ATLUP2)	At 1g78960	1	17
Putative lupeol synthase (ATLUP3)	At 1g78950[c]	1	17
Putative lupeol synthase (ATLUP4)	At 1g78950[c]	1	17
Putative lupeol synthase (ATLUP5)	At 1g66960	1	17
Putative pentacyclic triterpene synthase (ATPEN1)	At 4g15340	4	14
Putative pentacyclic triterpene synthase (ATPEN2)	At 4g15370	4	14
Putative pentacyclic triterpene synthase (ATPEN3)	At 5g48010	5	14
Putative pentacyclic triterpene synthase (ATPEN4)	At 5g36150	5	13[b]
Putative pentacyclic triterpene synthase (ATPEN5)	At 5g42600	5	14
Putative pentacyclic triterpene synthase (ATPEN6)	At 1g78500	1	14[b]
Putative pentacyclic triterpene synthase (ATPEN7)	Not registred	3	13[b]
S-adenosyl methionine-cycloartenol-C24-methyltransferase (ATSMT1)	At 5g13170	5	14
S-Adenosyl methionine-24-methylene lophenol-C-24(24[1])-methyltransferase (ATSMT2-1)	At 1g20330	1	1
S-Adenosyl methionine-24-methylene lophenol-C-24(24[1])-methyltransferase (ATSMT2-2)	At 1g76090	1	1
Obtusifoliol-14α-demethylase (CYP51) 1	At 1g11680	1	2
Obtusifoliol-14α-demethylase (CYP51) 2	At 2g17330	2	2
$\Delta^{8,14}$-sterol-Δ^{14}-reductase	At 3g52940	3	13
Δ^{8}-Δ^{7}-sterol isomerase	At 1g20050	1	4
Δ^{7}-sterol-C5-desaturase 1	At 3g02580	3	3
Δ^{7}-sterol-C5-desaturase 2	At 3g02590	3	3
$\Delta^{5,7}$-sterol-Δ^{7}-reductase	At 1g50430	1	13
Δ^{5}-sterol-Δ^{24}-reductase (isomerase)	At 3g19820	3	2
UDP-glucose : sterol glucosyltransferase	At 1g43620	1	14
UDP-glucose : sterol glucosyltransferase	At 3g07020	3	14

[a] This gene presents significant homology with mammalian 5-phosphomevalonate kinase
[b] Hypothetical values given in data bases (genome sequencing program)
[c] These genes have not been separated in data bases (genome sequencing program)

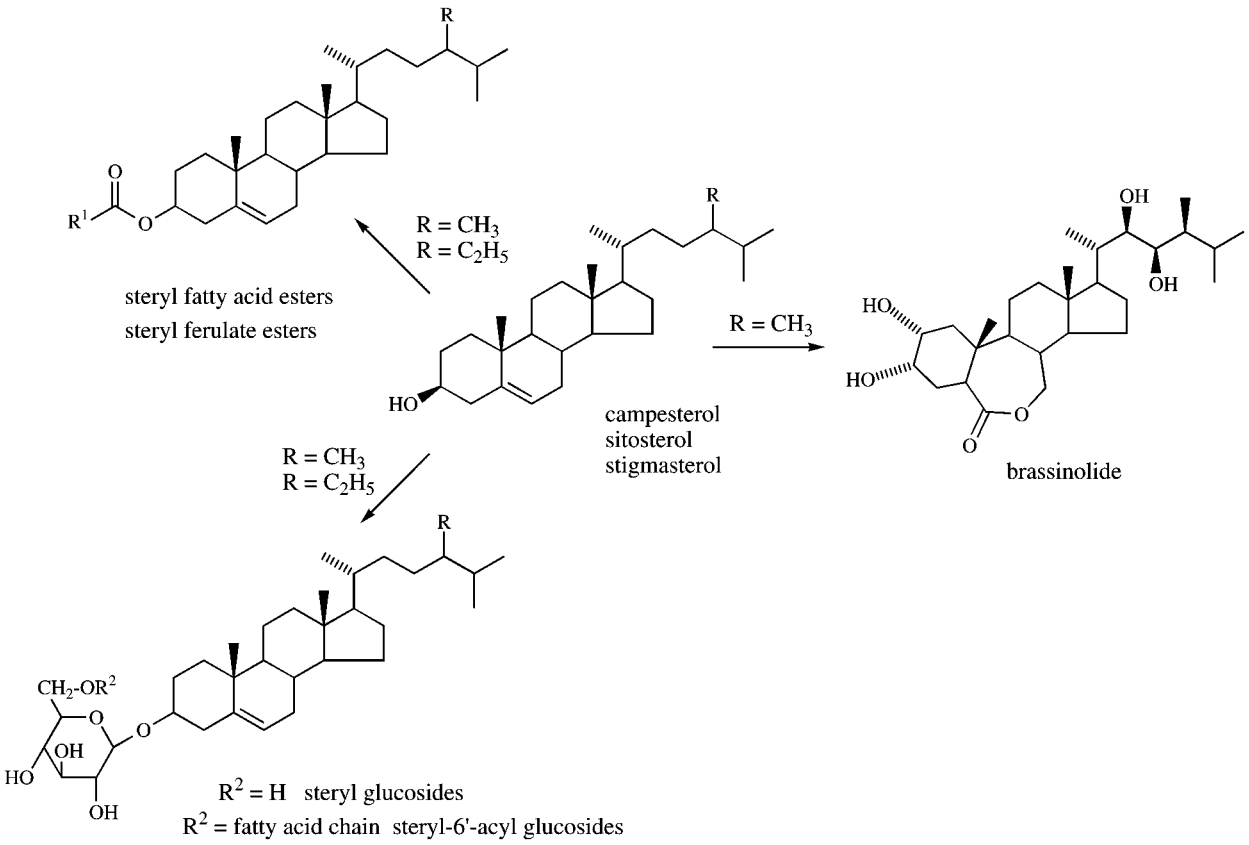

Fig. 3. Plant sterol metabolism.

plant sterol biosynthesis and substrate specificity studies, it is generally assumed that cycloartenol (Fig2) is the substrate of the first methylation reaction, resulting in 24-methylene cycloartanol (Wojciechowski et al., 1973), whereas 24-methylene lophenol is the preferred substrate for the second methylation, yielding 24-ethylidene lophenol (Fonteneau et al., 1977). Because the chemical structures of 24-methylene cyclartanol and 24-methylene lophenol are very different, it has been suggested that the two methylation ractions would be catalyzed by two different enzymes (Rahier et al., 1986). However, since no plant SMT has been purified so far, the hypothesis of an unique plant SMT catalyzing both alkylations (Nes et al., 1991) should also be considered. The first SMT gene (*ERG6*) cloned was from *S.cerevisiae* (Gaber et al., 1989; Hardwick and Pelham, 1994). Later on complete identification was performed by enzymatic assays on the protein derived from *ERG6* after expression in *E.coli.* According to these studies the best substrate of the recombinant enzyme was zymosterol which was methylated to give fecosterol in agreement with previous enzymatic assays performed on microsomes from WT *S.cerevisiae* (Venkatramesh et al., 1996). A *SMT* cDNA of 1411 bp has been cloned from an Arabidopsis cDNA library, the deduced amino acid sequence of this cDNA showed three conserved regions found in AdoMet-dependent methyltransferases (Kagan and Clarke, 1994) and 38% identity with *ERG6*. This cDNA was used to transform a wild-type *S.cerevisiae* as well as the yeast null mutant *erg6*, which is deficient in the SMT-zymosterol-C-24 methyltransferase. In both cases, several 24-ethyl and 24-ethylidene sterols were synthetized, indicating that the Arabidopsis cDNA encodes a plant SMT capable of performing two sequencial methylations at C24 and C24-1 of the yeast sterols (Husselstein et al., 1996). Microsomes from *erg6* expressing the Arabidopsis SMT were shown to possess AdoMet-dependent sterol-C-methyltransferase activity. Delipidated preparations of these microsomes converted cycloartenol into 24-methylene cycloartanol and 24-methylene lophenol into 24-ethylidene lophenol. The catalytic efficiency of the expressed SMT was 17-times higher with 24-methylene lophenol than with cycloartenol. This result provides evidence that the

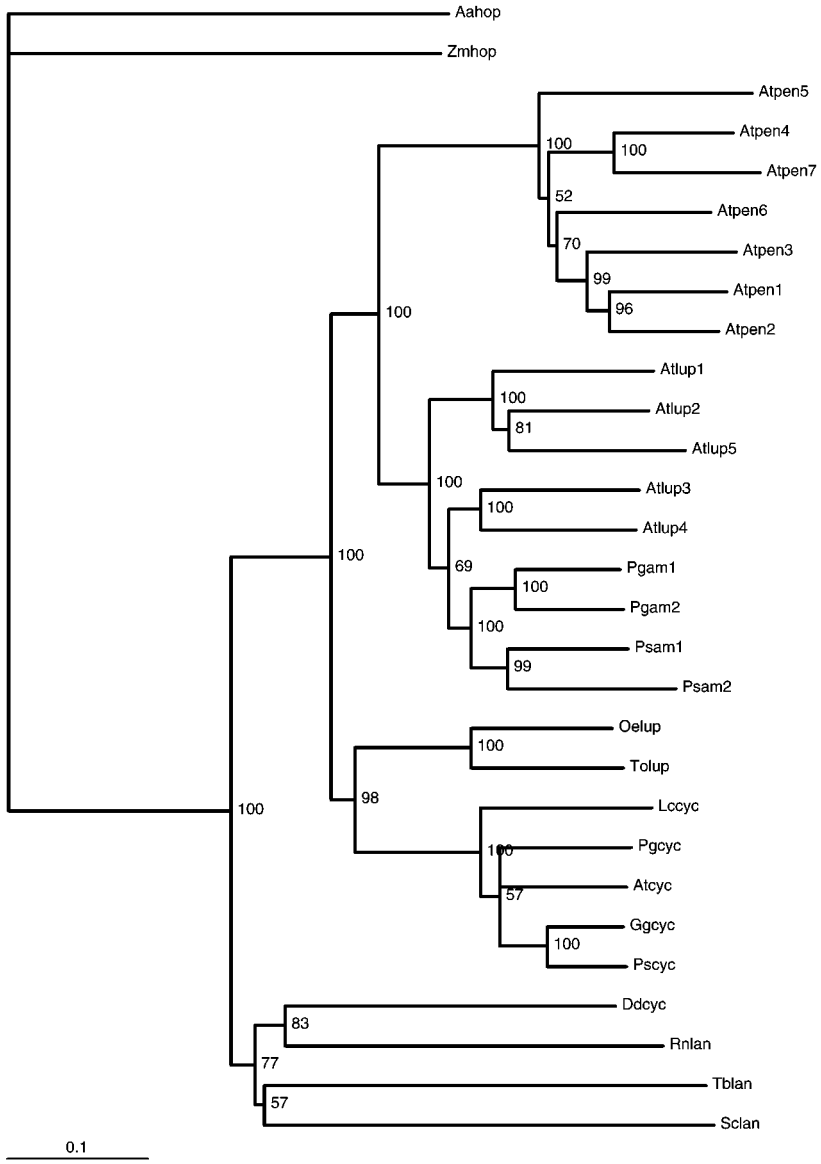

Fig. 4. Phylogenetic tree constructed for several plant, animal, fungal and bacterial triterpene synthases. AAHOP and ZMHOP : *Alicyclobacillus acidocaldarius* and *Zymomonas mobilis* hopene synthases (Wendt et al., 1997), RNLAN, SCLAN and TBLAN : *Rattus norvegicus* (Abe and Prestwich, 1995), *Saccharomyces cerevisiae* (Shi et al., 1994) and *Trypanosoma cruzi* (Joubert et al., 2001) lanosterol synthases. ATCYC, DDCYC, PSCYC, GGCYC, PGCYC, LCCYC : *Arabidopsis thaliana* (Corey et al., 1993), *Dictyostelium discoidum* (Godzina et al., 2000), *Pisum sativum, Glycyrrhiza glabra, Panax ginseng* (Kushiro et al., 1998), *Luffa cylindrica* cycloartenol synthases (Kushiro et al., 1999). ATLUP1 to 5 : *Arabidopsis thaliana* triterpene synthases which cyclize 2,3-oxidosqualene into various pentacyclic triterpenes (Herrera et al., 1998 ; Husselstein-Muller et al., 2001 ; Kushiro et al., 2000). PGAM1 and PGAM2 : *Panax Ginseng* β-amyrin synthase (Kushiro et al., 1998). PSAM1 and PSAM2 : *Pisum sativum* β-amyrin synthase and multifunctional triterpene synthase respectively. OELUP and TOLUP : *Olea europeae* and *Taraxacum officinalis* lupeol synthases (Kushiro et al., 1999) ; ATPEN1 to 7 ; *Arabidopsis thaliana* putative triterpene synthases. Accession Numbers for ATLUP1-5, ATPEN1-7, and ATCYC are given in Table 1. The phylogenetic tree has been rooted with AAHOP as outgroup. The numbers at the nodes of the dendogram are bootstrap which indicate the frequencies of occurrence of partitions found in the tree. The phylogenetic tree was developed with the aligned sequences from GCG files (version 10.1 of UNIX) and the PAUP program according to Doyle and Gaut (2000). The phylogenetic tree was drawn with TreeView.

Arabidopsis cDNA encodes a 24-methylene-lophenol-C-24[1]-methyltransferase catalyzing the second methylation step of plant sterol biosynthesis (Bouvier-Navé et al., 1997). This cDNA was named *ATSMT2-1*. A second cDNA (*ATSMT2-2*) of 1249 bp was cloned and was shown to encode a protein of 359 amino acids, 82% identical with the protein deduced from *ATSMT2-1*. Meanwhile two cDNAs from *Nicotiana tabacum* (*NTSMT2-1* and *NTSMT2-2*) and one from *Oriza sativa* (*OSSMT2*) were characterized and were shown to be about 80% identical to the Arabidopsis cDNAs. The cDNA *NTSMT2-1* was expressed in *erg6* and the corresponding protein was shown to convert 24-methylene lophenol into 24-ethylidene lophenol as efficiently as ATSMT2-1 (Bouvier-Navé et al., 1997). Meanwhile cDNAs from *Glycine max* (Shi et al., 1995; Shi et al., 1996), *Ricinus communis* (Bouvier-Navé et al., 1997), *Zea mays* (Grebenok et al., 1997), *N.tabacum* and *O.sativa* (Bouvier-Navé et al., 1998) and Arabidopsis (Diener et al., 2000; Schaeffer et al., 2001) were isolated and characterized. The proteins encoded by these cDNAs are about 80% identical in all possible combinations, but have only 40% identity with SMT2 and 48% identity with ERG6. They constitute therefore a group of cDNAs (*SMT1*) distinct of the group formed by *SMT*2. *SMT1* from *Z.mays* (Grebenok et al., 1997), from Arabidopsis (Diener et al., 2000) and from *N.tabacum* (Bouvier-Navé et al., 1998) were shown to partially restore the abillity of *erg6* to make ergosterol. The *G.max* SMT1 was expressed in *E.coli* and the recombinant protein was shown to alkylate lanosterol (an unnatural substrate in plants) to give eburicol (Shi et al., 1996). In the presence of AdoMet, delipidated microsomes from *erg6* transformed with tobacco *SMT1* efficiently converted cycloartenol into 24-methylene cycloartanol, but did not produce any 24-ethylidene lophenol upon incubation with 24-methylene lophenol. This demonstrated that cDNA *NTSMT1* (and most probably the other plant *SMT* of the same group such as *ATSMT1*) encoded a cycloartenol-C24 methyltransferase (Bouvier-Navé et al., 1998). Sequence alignment of SMT1 and SMT2 reveal three highly conserved domains. One of them (LDXGCGXGGPXRXI) corresponds to the G-rich consensus motif described for all AdoMet-dependent methyltransferases (Kagan and Clarke, 1994). A second (IEATCHAP) and a third (YEW/F/YGWGXSFHF) motives are believed to be typical of methyltransferases acting on a sterol substrate (Bouvier-Navé et al., 1997; Nes, 2000). Whereas SMT2 possess a hydrophobic domain of approximately 25 amino acids at the N-terminal position, SMT1 are devoid of such a hydrophobic domain (Bouvier-Navé et al., 1997). As confirmed by the *Arabidopsis* sequencing project and the above results, the Arabidopsis genome contains three distinct genes (At5g13710, At1g20330, At1g76090) encoding sterol-C24 methyltransferases(ATSMT1, ATSMT21, ATSMT22 respectively). *smt1* mutations, which lack SMT1, were isolated from a transposon Ac transgenic line (Diener et al., 2000). The *smt1* plants have pleiotropic defects: poor growth and fertility, root sensitivity to Ca^{2+} ions, and a loss of proper embryo morphogenesis. *smt1* was shown to have an altered sterol content. It accumulates cholesterol , has much less 24-ethyl sterols, but its campesterol content is similar to this in control plants, reflecting that methylation of endogenous sterols is still active even in the absence of any functional SMT1 present. Actually, if SMT1 were the only enzyme responsible *in planta* for the first methylation reaction of cycloartenol to produce 24-methylene cycloartanol, *smt1* should be completely deficient for C-24 alkylation. Because the majority of sterols in *smt1* plants are alkylated, SMT1 cannot be the only enzyme *in* Arabidopsis capable of the first C-24 methylation step (Diener et al., 2000). One explanation for the presence of 24-methylated sterols in *smt1* is that in the absence of *SMT1*, these methylations would be performed by SMT21 or SMT22 (that do not ordinarily perform this reaction in the wild type because of their preference for 24-methylene lophenol) even though at a much slower rate. The expression of *SMT2* and *SMT1* was also studied in plants (Schaeffer et al., 2000; Schaeffer et al., 2001; Schaller et al., 1998). The expression of *ATSMT21* was modulated in *35S : :SMT21* Arabidopsis in order to study its physiological function. Plants overexpressing the transgene accumulated sitosterol at the expense of campesterol. These plants displayed a reduced stature and growth that could be restored by brassinosteroid treatment. Plants showing co-suppression of *SMT21* were characterized by a high campesterol content and a depletion in sitosterol. Pleiotropic effects on development such as reduced growth, increased branching, and low fertility of high campesterol plants were not modified by exogenous brassinosteroids, indicative of specific sterol requirements to promote normal development. Thus *ATSMT21* has a crucial role in balancing the ratio of campesterol to sitosterol in order to satisfy both growth requirements and membrane integrity (Schaeffer et al., 2001).

4,4-dimethyl sterol and 4α-methyl sterol 4-demethylation. The passage of 24-methylene cyclartanol to end-pathway sterols involves removal of two methyls at position 4 and one methyl at position 14. The steps involved in these operations are depicted in Fig.2. The enzymatic system operating in the removal of the two methyls at C-4 in plants presents profound differences with the fungal or mammalian systems (Pascal et al., 1990; Pascal et al., 1993). In contrast to animals and fungi where the two C-4-methyl group are sequentially removed, a series of results have unequivocally established that two distinct oxidative systems are involved in the removal of the first and the second C4-methyl of phytosterol precursors. The first will act on 24-methylene cycloartanol whereas the second will operate on 24-methylene and 24-ethylidene lophenol (Fig

2). In the case of the first enzymatic system it has been shown that the oxidative conversion of 24-methylene cycloartanol to cycloeucalenol exhibits similar cofactor requirements, and inhibitor sensitivity as do corresponding animal systems. The demethylation is specific for the C-4α methyl group and is initiated by the C-4 methyl oxidase, which converts the methyl group to the alcohol, the aldehyde and finally to the carboxylic acid. This sequential oxidation was shown to require NADH, oxygen and cytochrome b5 as an electron carrier between the terminal oxidase and NADH. It is not inhibited by CO but is susceptible to cyanide, indicating that a cytochrome P-450 is not involved. In the next reaction, the carboxyl group is removed by a second enzyme, the C4 decarboxylase acting on 4α-carboxy-4β,14α-dimethyl-9β,19-cyclo-5α–ergost-24(24^1)-en-3β–ol and resulting in formation of cycloeucalenone possessing a keto group at C-3 (Rondet et al., 1999). A third enzyme, 3-keto reductase, then reduces the keto group of cycloeucalenone to give cycloeucalenol (Pascal et al., 1994). This process is repeated with the second methyl group but the substrate is then 24-methylene or 24-ethylidene lophenol and the product is then episterol or Δ^7-avenasterol, respectively. Cloning and characterization of *ERG25*, the *S.cerevisiae* gene encoding C4-methyl oxidase has been reported (Bard et al., 1996). This gene encodes a 309-amino acid polypeptide showing a C-terminal endoplasmic reticulum (ER) retrieval signal KKXX and three histidine-rich clusters found in eukaryotic membrane bound fatty acid desaturases (Shanklin et al., 1994). A human homologue of *ERG25* was cloned and sequenced. It encodes a polypeptide of 293 amino acids with an identity of 38% to *ERG25* and contains histidine clusters (Li and Kaplan, 1996). Very recently the functional identification of sterol-4α-methyl oxidase cDNAs from *A. thaliana* was achieved by complementation of a yeast *erg25* mutant lacking sterol-4α-methyl oxidation (Darnet el al., 2001).

Cycloeucalenol obtusifoliol isomerase. One of the most striking event occurring during sterol biosynthesis is the opening of the cyclopropane ring of cycleucalenol to give obtusifoliol, a step that is restricted to the plant kingdom and catalyzed by an enzyme, the cycloeucalenol-obtusifoliol isomerase or cyclopropyl sterol isomerase (CPI), the catalytic mechanism of which has been thoroughly studied (Heintz and Benveniste, 1974; Rahier et al., 1989) (Fig. 2). Photosynthetic eukaryotes and certain non photosynthetic protists such as *Acantamoeba polyphaga* (Raederstorff and Rohmer, 1987) or *Dictyostelium discoidum* (Godzina et al., 2000; Nes et al., 1990) use CPI (EC 5.5.1.9) to convert pentacyclic cyclopropyl sterols to tetracyclic end-pathway sterols. Arabidopsis CPI was cloned by functional complementation of a *S.cerevisiae* mutant (Lovato et al., 2000). Expression of an Arabidopsis cycloartenol synthase cDNA in a *S.cerevisiae* lanosterol mutant (*erg7*) provided a sterol auxotroph because cycloartenol is not usable by yeast to make ergosterol. This yeast strain was transformed with an Arabidopsis expression library constructed in a yeast vector (pFL61) and sterol prototrophs were selected. A strain accumulating biosynthetic ergosterol was obtained. The novel phenotype was conferred by an Arabidopsis cDNA (CPI) that encoded a 36-kDa protein of 280 amino acids. It was assumed that the *S.cerevisiae* sterol C4 demethylase complex (Erg25p, Erg26p, and Erg27p) that normally metabolize lanosterol would have sufficiently broad substrate specificity to accept its isomer cycloartenol. Thus cycloartenol would be demethylated at C4 (at least partially) to give small amounts of 31-nor-cycloartenol which was shown to be readily transformed into 31-nor lanosterol (Heintz and Benveniste, 1974), which could then enter the normal yeast sterol pathway (Lovato et al., 2000). CPI is encoded by a unique gene (At 5g50375) possessing 8 exons.

Obtusifoliol-14α-demethylase. In animals and fungi, the 14α–methyl group is the first of the C14 and C4 methyls to be removed; however in higher plants, the 14α–methyl is removed after one C-4 methyl has disappeared (Fig.2). The mechanism of the 14α–methyl removal involves two oxidation steps leading to an alcohol , then an aldehyde at C-29 and a further oxidative step involving a deformylation leading to formic acid and the sterol product with a typical 8,14-diene (Aoyama et al., 1987). The three steps are catalyzed by a single enzyme, which has been shown to be a cytochrome P-450 (Trzaskos et al., 1986). Lanosterol is the substrate of this enzyme in *S.cerevisiae* and in mammals (Aoyama et al., 1987; Shyadehi et al., 1996), eburicol would be the preferred substrate in filamentous fungi (Delye et al., 1998), while obtusifoliol is the substrate in higher plants (Taton and Rahier, 1991). Much attention has been payed to the 14α–demethylase since the enzyme is the target of a vast array of compounds having antimycotic effect and used in agriculture (Benveniste and Rahier, 1992) and medicine (Vanden Bossche and Janssen, 1992). These compounds, which are derivatives of pyridine, pyrimidine, imidazole, triazole…interact by their nitrogen sp2 doublet with the protohemic iron of cytochrome P-450 and hamper the access to oxygen. As shown above, the plant pathway differs from fungal and animal pathways downstream of 2(3)-oxidosqualene. No lanosterol is present in plants to serve as substrate for the 14-demethylation step. It is obtusifoliol that is the substrate for 14-demethylation. A thorough enzymological study has defined the stuctural requirements of this enzyme, which was shown to be remarkably specific for the structure of obtusifoliol and which does not use lanosterol (Taton and Rahier, 1991). A panoply of azole derivatives were shown to inhibit this enzyme ; some of them, developed as herbicides, were shown to be specific

to obtusifoliol-14-demethylase (OBT14DM) (Benveniste and Rahier, 1992; Salmon et al., 1992). Tobacco calli resistant to phytotoxic azole derivatives have been isolated in mutagenesis experiments. The mechanism of resistance consists of an overproduction of sterols, which is not due to OBT14DM overproduction or modification but to an increase of HMGR enzymatic activity (Gondet et al., 1992; Schaller et al., 1994). cDNAs encoding lanosterol, eburicol and obtusifoliol 14-demethylases have been isolated from mammals (Aoyama et al., 1994), fungi (Kalb et al., 1986) and plants (Bak et al., 1997; Cabello-Hurtado et al., 1999) respectively . They shared a remarkable amino acid identity ranging from 38% to 65% though they belong to widely diverging species. Therefore they were classified in the same family, which is CYP51 (Nelson et al., 1996). A functional *Sorghum bicolor* OBT14DM was cloned and expressed at high levels in *E.coli*. The recombinant enzyme was shown to catalyze efficiently the 14α–demethylation of obtusifoliol (Bak et al., 1997). Likewise a *Triticum vulgare* OBT14DM was expressed in a *S.cerevisiae* mutant (*erg11*) defective in lanosterol 14-demethylase. Appropriate engineering of the *TvOBT14DM* cDNA and of the recipient *erg11* mutant allowed to optimize expression (Cabello-Hurtado et al., 1999). Experimental structural informations on the active sites of the fungal, plant, and mammalian CYP51 would greatly facilitate developing more efficient antifungal drugs. However all forms of CYP51 were membrane-bound microsomal enzymes which complicates structural studies of this protein by X-ray crystallography. A soluble CYP51 ortholog has been discovered recently in *Mycobacterium tuberculosis* (Bellamine et al., 1999). It has been shown to demethylate lanosterol and obtusifoliol and to be inhibited by azole antifungals. *E.coli*-expressed MTCYP51 has been recently crystallized in the presence of fluconazole and a crystal structure at 2.2 A has been reported (Podust et al., 2001). This new structure provides a basis for rational design of more efficacious antifungal agents or new herbicides (Salmon et al., 1992; Grausem et al., 1995) as well as insight into the molecular mechanism of P450 catalysis. Arabidopsis genome sequencing has revealed the existence of two genes (At2g17330, At1g11680) encoding proteins having 65-75 % identity with an already characterized OBT14DM from *Sorghum bicolor* (Bak et al., 1997). Both genes possess two exons and an intron. The cDNA (ATCYP51) corresponding to At1g11680 has been cloned and has been shown to be able to sustain growth of the *erg11* mutant suggesting ATCYP51 to be a functional sterol 14α–demethylase. Arabidopsis plants were transformed with AtCYP51 in antisense orientation. The resulting transgenic plants showed a semi-dwarf phenotype in the early growth stage. Their obtusifoliol content increased while campesterol and campestanol levels were unchanged (Kushiro et al., 2001). As their 24-ethyl sterols content was not reported, it is still not possible to draw conclusions as to the link between growth reduction and sterol biosynthesis inhibition in these transgenic plants.

$\Delta^{8,14}$-sterol-Δ^{14}-reductase. 4α,14α-dimethyl-5α-ergosta-8,14,24(24^1)-trien-3β–ol is the product of the C14 demethylation of obtusifoliol in plants. The following step is the hydrogenation by NADPH of the Δ^{14} double bond to give fecosterol (4α–methyl-5α–ergosta-8,24(24^1)-dien-3β–ol) (Fig. 2). The product of the C14 demethylase reaction in *S.cerevisiae*, 4,4-dimethyl-cholesta-8,14,24-trienol, is reduced at the C-14 position to form 4,4-dimethylcholesta-8,24-dienol by the action of the product of the *ERG24* gene (Lorenz and Parks, 1992; Marcireau et al., 1992). *ERG24* encoded a protein of 438 amino acids. The C14 reductase is inhibited by Fenpropidine and Fenpropimorph, two fungicides used in agriculture (Baloch and Mercer, 1987). By chromosomal gene disruption a *S.cerevisiae* defective in 14-reductase has been constructed. This strain was shown to grow in aerobiosis if it beared an additional mutation allowing sterol uptake. In this last growth condition, the cells required a sparking ergosterol supplementation and accumulated 5α-ergosta-8,14-dienol as the end-product of the sterol pathway (Marcireau et al., 1992). Most interestingly it has been shown that the human lamin B receptor exhibited sterol C-14-reductase activity in *S.cerevisiae* (Silve et al., 1998). In fact, the sterol C14 reduction step and ergosterol prototrophy were restored in lamin B receptor-producing *erg24* transformants which lack endogenous C14-reductase. C14 reductase was studied in plants. *Rubus fruticosus* suspension cell cultures were highly susceptible to A25822B, an azasterol antimycotic agent and accumulated 5α–stigmasta-8,14-dien-3β–ol and 5α–stigmasta-8,14,Z-24(24^1)-trien-3–ol at the expense of Δ5-end pathway sterols (Schmitt et al., 1980). An enzymatic assay for 8,14-sterol 14-reductase was devised allowing to characterize this enzyme and to study its inhibition by analogues of a presumptive carbocationic intermediate of the reduction reaction (Taton et al., 1989). Cloning of the gene (*FACKEL*) encoding this enzyme was performed recently through the isolation of dwarf mutants (EMS and TDNA insertion mutants). *FACKEL* (At 3g52940) was isolated and was shown to encode a predicted integral membrane protein of 369 amino acids with eight to nine transmembrane segments related to the vertebrate lamin receptor and several sterol C-14 reductases including *S.cerevisiae* sterol C-14 reductase (Jang et al., 2000; Schrick et al., 2000). The Arabidopsis protein possessed a signature motif (LLXSGYWGXXRH) of sterol reductases. Functional evidence that *FACKEL* encoded a sterol C-14 reductase was provided by complementation of *erg24* (Schrick et al., 2000). GC/MS analysis confirmed that *fk* mutations lead to accumulation of Δ8,14-sterol intermediates in the biosynthetic pathway preceding the C-14 reductase step. The sterol profile of *fk* calli was sim-

ilar to that of plant cells treated with A25822B (Schmitt et al., 1980; Schaller et al., 1994). The *fk* mutation resulted also in reduction of brassinosteroid (BRs) content (Jang et al., 2000). However, unlike other BR-deficient mutants, the defect of hypotyl elongation could not be overcome by exogenous BRs (Jang et al., 2000; Schrick et al., 2000). Thorough microscopical and cytological observations indicated that mutations in the *FACKEL* gene affect body organization of the Arabidopsis seedlings and that *FACKEL* was required for cell division and expansion and was involved in proper organization of the embryo. These results indicated a novel role for sterols in the embryogenesis of plants (Clouse, 2000).

Δ^8-Δ^7-sterol isomerase. When the 14α–methyl group is removed and the 14 double bond is reduced, the resulting Δ^8-sterols are isomerized to Δ^7-sterols in mammals, fungi, and higher plants. This process is catalyzed by a Δ^8-Δ^7-isomerase. The reaction involves two steps : first a protonation of the 8 double bond leading to an intermediate bearing a carbocation at C8 then the elimination of a proton at C7 leads to the Δ^7-sterol (Akhtar et al., 1970; Goad et al., 1969). From enzymatic assays and biogenetic considerations it appears that zymosterol, fecosterol and 4α–methyl-5α–stigmasta-8,Z-24(24^1)-dien-3β–ol are the substrates of this enzyme in vertebrates (Yamaga and Gaylor, 1978), *S.cerevisiae* (Yabuzaki et al., 1979) and higher plants (Taton et al., 1987) respectively. A mutant (*erg2*) defective in Δ^8-Δ^7-sterol isomerase was described for *S.cerevisiae* (Barton et al., 1974). The *erg2* mutation leads to the accumulation of sterols containing the Δ^8-double bond. *ERG2*, the gene encoding the Δ^8-Δ^7-sterol isomerase (SI) was cloned by functional complementation of *erg2* (Arthington et al., 1991). Later, SI-encoding genes were isolated from the rice blast fungus *Magnaporthe grisea* and the maize pathogen *Ustilago maydis* (Keon et al., 1994). Genetic and biochemical evidence showed that *ERG2* would encode an enzyme catalyzing conversion of Δ^8-sterols to Δ^7-sterols. Recently a *S.cerevisiae* mutant defective in the internalization step of endocytosis was shown to contain defects in *ERG2* leading to a lack of Δ^8-Δ^7-isomerase activity (Munn et al., 1999). A murine SI encoding cDNA has been cloned by functional complementation of the corresponding deficiency (*erg2*) in *S.cerevisiae*. The amino acid sequence deduced from the cDNA open reading frame was shown to be highly similar to human emopamil-binding protein (EBP), a protein which is the target for neuroprotective drugs (Silve et al., 1996). A yeast strain in which the SI coding sequence has been replaced by that of human EBP or its murine homologue, recovered the ability to convert Δ^8-sterols to Δ^7-sterols both *in vivo* and *in vitro*. Interestingly, the amino acid sequence deduced from the murine (SI) or human (EBP) failed to reveal any striking similarity with *S.cerevisiae* Erg2p (13% identity). Mutations in the gene encoding *Homo sapiens* SI were shown to cause X-linked dominant Conradi-Hunermann syndrome (Braverman et al., 1999). Alanine scanning mutagenesis was used to identify residues in the four putative transmembrane α-helices of human EBP that are required for Δ^8-Δ^7-sterol isomerase catalytical activity and binding of inhibitors. Out of 64 alanine mutants of EBP mutants H77A, E81A, T126A, N194A, W197A, contained less than 10% end-pathway 5,7-sterols. Therefore amino acids H77, E81, T126, N194 and W197 should play an important role in sterol Δ^8- Δ^7 isomerization (Moebius et al., 1999). An Arabidopsis Δ^8-Δ^7-sterol isomerase cDNA has been isolated by functional complementation of the corresponding *S.cerevisiae* sterol mutant (*erg2*). The full length Arabidopsis cDNA that complement the *erg2* mutation contained an open reading frame encoding a protein of 223 amino acids sharing 35% amino acid identity to the *Mus musculus* SI and the *H.sapiens* EBP and very low identity (less than 15%) with Erg2p. The sigma ligands, haloperidol, ifenprodil and verapamil inhibited the production of ergosterol in wild-type *S.cerevisiae* and in the *erg2* mutant complemented with the Arabidopsis SI (Grebenok et al., 1998). The Arabidopsis protein contained three transmembrane segments and presented several structural and biochemical similarities with the mammalian EBP. It is encoded by an unique gene (At1g20050) possessing 4 exons and 3 introns.

Δ^7-sterol-C5(6)-desaturase. Δ^7-Sterol products of the Δ^8-Δ^7-sterol isomerase are transformed into Δ^5-sterols through two reactions : the first one involves a desaturation step at C5 leading to a $\Delta^{5,7}$-sterol, then the Δ^7-double bond of $\Delta^{5,7}$-sterols is reduced to give Δ^5-sterols (Fig. 2). Insertion of the 5,6-double bond has been shown to involve stereospecific removal of the 5 (4-proR in MVA) and 6 (5-proS in MVA) hydrogen atoms during ergosterol biosynthesis in *S.cerevisiae* (Bimpson et al., 1969). The same stereospecificity is seen during the insertion of the 5,6-double bond during sterol biosynthesis in rat liver, higher plants and *Ochromonas malhamensi*s (Goodwin, 1979). The close similarity of fatty acid desaturation in rat liver microsomes to the introduction of the sterol 5 ,6-double bond has been pointed out (Reddy et al., 1977). Both systems require NADH and molecular oxygen, and involve a cyanide-sensitive factor ; they both abstract cis-oriented hydrogens, occur in the microsomes and are inhibited by cyanide and thiol-blocking agents, but not by carbon monoxide. Further evidence of this came witth the demonstration of the participation of cytochrome b5 in the conversion of cholest-7-en-3-ol into cholesta-5,7-dien-3-ol (Osumi et al., 1979). The characterization of a Δ7-sterol C5(6)-desaturase from *Zea mays* has been reported (Taton and Rahier, 1996). This desaturase presents features similar to those reported for rat liver and yeast. It is strongly inhibited by cyanide, is sensitive to 1,10-phenanthroline and to salicylhydroxamic acid, but is insensitive to carbon

monoxide, thereby suggesting the involvment of a metal ion, presumably iron, in an enzyme-bound form in the desaturing system (Taton and Rahier, 1996). More recently the *ERG3* gene from *S.cerevisiae* has been cloned by complementation of an *erg3* mutant defective in C5-sterol desaturase. The functional gene contained an open reading frame of 365 amino acids. Gene disruption demonstrated that *ERG3* is not essential for cell viability (Arthington et al., 1991). A nuclear and recessive mutant of Arabidopsis affected in sterol biosynthesis (*ste1*) has been isolated and identified. This mutant accumulated Δ^7-sterols (24R)-24-ethyl-5α-cholest-7-en-3-ol, and (24ξ)-24-methyl-5-cholest-7-en-3β-ol at the expense of the normally occurring campesterol and sitosterol. It has been suggested that *ste1* is defective in the sterol-C5-desaturase (Gachotte et al., 1995). To check this hypothesis *ste1* was transformed with *ERG3* encoding a Δ^7-sterol-C5-desaturase from yeast, which resulted in partial recovery of wild-type sterol composition. To definitely identify *ste1,* homologous complementation was necessary. To this end an Arabidopsis cDNA encoding a Δ^7-sterol-C5-desaturase was isolated and characterized by functional complementation of *erg3*. This was achieved by transformation of *erg3* with an Arabidopsis cDNA library inserted in a yeast vector (pFL61). Transformants were screened for cycloheximide resistance and resistant clones were analyzed to determine their sterol profile. The presence of ergosterol was detected in one clone in which a plasmid containing a cDNA of 1141bp was found. The 1141 cDNA allowed the deduction of an open reading frame of 843 bp encoding a 281 amino acid polypeptide (Gachotte et al., 1996). Three histidine-rich motifs (HX_3H, HX_2HH and HX_2HH) and three transmembrane segment were found in the Arabidopsis polypeptide and are also present in Erg3p as well as in *Nicotiana tabacum* (Husselstein et al., 1999) and *Homo sapiens* (Husselstein et al., 1999; Matsushima et al., 1996; Nishi et al., 2000) orthologues. Histidine-rich motifs are also characteristic of many membrane-bound fatty acid desaturases from higher plants (Shanklin et al., 1994). Overexpression of the Arabidopsis desaturase cDNA driven by a 35S promoter in transgenic *ste1* plants led to full complementation of the mutant. This result demonstrated that *STE1* was the impaired component in the desaturation system. The *ste1*-derived open reading frame has been cloned by RT-PCR. Alignment of the wild-type with the *ste1* derived protein sequences revealed a single amino acid substitution : T114 in the wild-type is changed to I114 in Ste1p. The presence of this mutation in the mutant *STE1* genomic sequence demonstrated that the change of the T114 to I was necessary and sufficient to create the leaky allele *ste1* (Husselstein et al., 1999). The role of 15 residues in the reaction catalyzed by Arabidopsis Δ^7-sterol-C5(6)-desaturase (5-DES) was investigated using site-directed mutagenesis and expression of the mutated enzymes in an *erg3 S.cerevisiae* strain defective in 5-DES. One group of mutants was affected in the eight invariant histidine residues from three histidine-rich motifs. Replacement of these residues by leucine completely eliminated the desaturase enzymatic activity both *in vivo* and *in vitro* (Taton et al., 2000) in agreement with the hypothesis that the function of histidine-rich motifs would be to provide the ligands for a presumed catalytic Fe center, as previously proposed (Shanklin et al., 1994). Another group of mutants was affected in residue 114 based on previous observations indicating that mutant T114I (*ste1*) was deficient in 5-DES. It was shown that the enzyme T114I had an 8-fold higher Km and 10-fold reduced catalytic efficiency. Conversely, the functionally conservative substituted mutant enzyme T114S displayed a 28-fold higher Vmax value and an 8-fold higher Km value than the wild-type enzyme. The data suggested that T114 strongly affected the reactivity of the desaturase and gave clues to get engineered 5-DES with much higher performances (Taton et al., 2000). Deuterated 7-cholestenol analogues were used as mechanistic probes for wild-type and mutated Δ^7-sterol-C5(6)-desaturase. Deuterium kinetic isotopic effects for the removal of 6α- and 5α-hydrogens were measured. The observed pattern of isotope effects allowed Rahier (2000) to propose a mechanism for the desaturation of cholest-7-en-3β-ol. The Arabidopsis 5-DES considered above is encoded by one gene (At3g02580). Sequencing of Arabidopsis genome has revealed the existence of a second gene (At3g02590), the deduced protein sequence of it having 80% identity with Ste1p. Both genes possess three exons and two introns. Screening of about 50,000 M2 seeds of an EMS mutant population allowed the isolation of 43 dwarf mutants. Two of them (dwf7-1 and dwf7-2) were biochemically complemented by brassinolide (BR) and early BR biosynthetic precursors. Furthermore, results from feeding studies with ^{13}C-labeled MVA and mevastatin and from sterol analysis suggested that the defective step was specifically the Δ^7-sterol C5(6) desaturation. Sequencing revealed a premature stop codon in exon1 (*dwf7-2*) and in exon 3 (*dwf7-1*) of the the *5-DES* gene confirming that *dwf7-1* and *dwf7-2* were null alleles of *STE1*. This work showed for the first time that the reduction of BR biosynthesis in *dwf7* was due to a shortage of substrate sterols (campesterol) and was the direct cause of the dwarf phenotype (Choe et al., 1999b). An extremely dwarf mutant (*bul1-1*) of Arabidopsis was recently isolated and shown to contain as much as 90% of Δ^7-sterols and less than 2% of Δ^5-sterols. It was therefore defective in the Δ^7-sterol-C5-desaturation step leading to brassinosteroid biosynthesis. Cellular characterization and rescue experiments with brassinosteroids demonstrated the involvment of the 5-DES in brassinosteroid-dependent plant growth processes (Catterou et al., 2001a). Indirect immunofluorescence of α-tubulin in *bul1-1* revealed a total lack of the

parallel microtubule organization that is typical of elongating cells in the wild-type. Rescue experiments with brassinosteroids and subsequent molecular analyses suggested that a brassinosteroid-responsive pathway exists, which allows microtubule nucleation/organization and cell elongation without activation of tubulin gene expression (Catterou et al., 2001b).

$\Delta^{5,7}$-sterol Δ^{7}-reductase (Δ7-SR) catalyzes the reduction of the Δ^{7}-double bond of the $\Delta^{5,7}$-sterols into Δ^{5}-sterols. This step occurs only in vertebrates and higher plants. A microsomal preparation from seedlings of *Zea mays* catalyzed the NADPH dependent reduction of the Δ^{7}-bond of $\Delta^{5,7}$-cholestadienol (Taton and Rahier, 1991). Novel 6-aza-B-homosteroids were shown to strongly inhibit (Δ^{7}-SR) and to behave as analogues of a high energy carbocationic intermediate occurring during the reaction (Rahier and Taton, 1996). The gene encoding the Δ^{7}-SR was cloned by transformation of wild-type *S.cerevisiae* with a cDNA library of Arabidopsis constructed in the yeast vector pFL61 and the subsequent selection of cells producing Δ^{5}-sterols at the expense of ergosterol (a $\Delta^{5,7}$-sterol) by nystatin selection. These cells were selected due to their resistance to nystatin, a polyene fungicide that is highly toxic to ergosterol producing cells. This clever strategy allowed recovery of a plasmid bearing a 1290-bp cDNA open reading frame, which encoded a protein of 430 amino acids that had significant sequence identity (32%) with Arabidopsis sterol Δ^{14}-reductase and the previously reported LLXSGWWGXXRH signature of sterol reductases. Cholesta-5,7-dien-3β-ol was shown to be reduced in vitro to cholesterol by an enzymic preparation from a yeast strain expressing the Arabidopsis (Δ^{7}-SR) (Lecain et al., 1996). Screening of a population of dwarf mutants led to a series of mutants (*dwf5*) presenting a defect in the gene encoding the Δ^{7}-SR. *dwf5* plants display the characteristic dwarf phenotype typical of BR mutants. This phenotype included small, round, dark-green leaves, and short stems, pedicels, and petioles. *dwf5* mutants were biochemically complemented with exogenous BRs. Metabolite tracing with ^{13}C-labeled precursors in *dwf5* and sterol analysis suggested a deficiency in a Δ^{7}-SR activity. In particular sterol analysis showed that $\Delta^{5,7}$-campestenol (a possible substrate of Δ^{7}-SR) accumulated. Surprisingly, the major compound analyzed was shown to be 5α-ergost-7-en 3β-ol. To explain this result authors suggested that a Δ^{5}-reductase (*det2*) acting downstream of campesterol could reduce the Δ^{5} double bond of $\Delta^{5,7}$-sterols which accumulated in *dwf5.* Deficiency in Δ7-SR activity was verified by DNA sequencing showing that all six independent alleles contained loss-of-function mutations in the Δ7-SR gene. The *dwf5* plant could be restored to wild type by ectopic overexpression of the wild-type copy of the gene (Choe et al., 2000). This gene (At1g50430) was shown to contain 13 exons and 12 introns. The Smith-Lemli-Opitz syndrome (SLOS) is an inborn disorder of sterol metabolism. All patients suffer from mental retardation. The SLOS gene has been shown to be a Δ^{7}-SR (EC 1.3.1.21) required for the *de novo* cholesterol biosynthesis.The protein encoded by the *Homo sapiens* Δ^{7}-SR has 35% identity with the Arabidopsis Δ^{7}-SR (Moebius et al., 1998). Results showed also that defects in the Δ^{7}-SR gene (several missense, nonsense, and splice site mutations) caused the SLOS (Fitzky et al., 1998).

Δ^{5}-sterol Δ^{24}-reductase(isomerase) catalyzes the reduction of the Δ^{24} double bond of the side chain of sterols. This reaction uses a substrate possessing one double bond in animals and in higher plants. In animals this substrate is desmosterol, a $\Delta^{24(25)}$ sterol, whereas in higher plants, substrates are 24-methylene cholesterol and isofucosterol (Fig. 2) and Δ24(24^{1})-sterols are probably isomerized in $\Delta^{24(25)}$-sterols prior to be reduced. However, reduction of the Δ^{24} double bond proceeds on sterols possessing two conjugated double bonds such as ergosta-5,7,22,24-tetraen-3β-ol in *S.cerevisiae* and other organisms producing ergosterol. The *ERG4* gene provides the enzyme that reduces the double bond at C24(24^{1}), the final step in ergosterol biosynthesis. Disruption of *ERG4* led to a mutant (*erg4*) which was able to grow similarly to the wild-type showing that the C24 reductase was not essential for viability (Lai et al., 1994). As discussed earlier (Lees et al., 1995), the sterols accumulated in *erg4* mutants (e.g. ergosta-5,7,22,24-tetraen-3β-ol) are similar in structure to ergosterol and could fulfil functions normally attributed to ergosterol. The *diminuto* mutant (*dim*) was initially isolated as a slowly growing dwarf Arabidopsis mutant with greatly reduced fertility (Klahre et al., 1998). The *DIM* gene was cloned, and genomic analysis and gene expression studies strongly suggested that *dim* did not produce any DIM protein. The *DIM* gene (AT3g19820), also referred to as *DWF1* (Choe et al., 1999b) or *CBB1* (Kauschmann et al., 1996), is composed of two exons and one intron. Both the mutant phenotype and gene expression could be rescued by the addition of exogenous BRs. Analysis of endogenous sterols demonstrated that *dim* accumulated 24-methylene cholesterol but was deficient in campesterol, an earlier precursor of BR and in BRs as well. Feeding experiments using deuterium-labeled 24-methylenecholesterol and 24-methyl desmosterol confirmed that *DIM*/*DWF1* is involved in both the isomerization and reduction of the 24(241) bond and encoded a sterol C24(241) reductase isomerase (S24REDISO). Transient expression of a green fluorescent protein-DIM/DWF1 fusion protein and biochemical experiments showed that DIM/DWF1 is an integral membrane protein that most probably is associated with the ER (Klahre et al., 1998). Several *dwf1* alleles were also characterized. 7 of 10 *dwf1* mutations were shown to directly affect a flavin adenin dinucleotide binding domain conserved in various oxidoreductases (Choe et

al., 1999b). The proteic sequence of S24REDISO from Arabidopsis was shown to have 41% identity with an *Homo sapiens* ortholog (seladin-1) but no significant identity with ERG4. No ortholog of S24REDI has been reported in the *S.cerevisiae* genome. Thus the C24 reduction step seems to be performed by completely different enzymatic systems in higher plants and animals on one hand and in *S.cerevisiae* (and probably most fungi) on the other hand. The human *DIM/DWARF1* homolog seladin-1 has been shown to confer resistance to Alzheimer's disease-associated neurodegeneration and oxidative stress (Greeve et al., 2000). This exciting result would attribute a novel role of cholesterol in neurodegenerative diseases. However the involvment of seladin-1 in cholesterol biosynthesis has not been demonstrated so far. Finally mutations in the 3β-hydroxysterol Δ^{24}-reductase gene were shown to cause desmosterolosis, an autosomal recessive disorder of cholesterol biosynthesis (Waterham et al., 2001).

Sterol-Δ^{22}-desaturase. Ergosterol from *S.cerevisiae*, most fungi and several algae, and stigmasterol : (24S)-24-ethyl-cholesta-5,E-22-dien-3β-ol from most higher plants possess a double bond at C-22. Δ^{22}-sterols are not present in vertebrates. This double bond results from a desaturation step. This step is the antepenultimate during ergosterol biosynthesis, it is probably the last step of plant sterol biosynthesis. The substrate of Δ^{22}-desaturase is ergosta-5,7,24(24^1)-trien-3β-ol in yeast, which is transformed into ergosta-5,7,22,24(24^1)-tetraen-3-ol by yeast microsomes. This reaction has been shown to involve molecular oxygen, NADPH, and is inhibited by CO and metyrapone. Thus it involves a cytchrome P-450 species (Hata et al., 1983). The gene (*ERG5*) encoding this reaction has been recently cloned by complementation of an *erg5* mutant (Skaggs et al., 1996). The deduced amino acid sequence (538 amino acid residues) presented general features common to most cytochrome P-450 open reading frames and exhibits the highest homology with some animal cytochrome P-450 proteins of the family 2. Surprisingly, the highest homology was not with CYP51A1 (lanosterol-14-demethylase) (Skaggs et al., 1996). Thus *ERG5* is the first member of the *CYP61* family. Enzymatic properties and inhibition by azole antifungal agents of CYP61 have been studied. Results revealed CYP61 to have a similar affinity to azole drugs (fluconazole, ketoconazole) when compared with data available for CYP51 (Kelly et al., 1997). The results of gene disruption demonstrated that *ERG5* is not essential for cell viability suggesting that the Δ22 would not be essential. Most biosynthetic studies show that sitosterol would be the substrate for a C-22 desaturase leading to stigmasterol (Benveniste, 1986; Nes, 1977). Very little is known about this step in higher plants, since the *in vitro* conversion of sitosterol to stigmasterol has never been shown enzymatically. Arabidopsis is remarkable among higher plants since it was shown to contain brassicasterol (ergosta-5,E-22-dien-3-ol) in addition to stigmasterol, indicating that both 24-methyl and 24-ethyl sterols could be substrates of the C-22 desaturase. No cDNA encoding this proteins from Arabidopsis (or any other higher plant) has been reported so far.

STEROL METABOLISM

Previous studies performed with a tobacco mutant *sterov* overproducing sterols have shown that the free sterol content of this mutant remained close to this of wild-type plants and that the excess of sterols was converted into steryl esters (mostly steryl palmitate, oleate, linoleate and linolenate) which accumulated in lipid droplets (Gondet et al., 1994; Schaller et al., 1994). Such a result stressed the importance of sterol metabolization in order to maintain a level of free sterols in membranes compatible with their vital functions. At least three pathways are universally involved in sterol metabolization in plants and especially in Arabidopsis : i) sterol acylation ; ii) sterol glycosylation ; iii) oxydative conversion of sterols into brassinosteroids. Other pathways also exist such as formation of ecdysteroids, steroidal alkaloids, cardiotonic steroidal glucosides etc…but they belong to the secondary metabolism in specific plant families and therefore would not be relevant of the general plant metabolism.

Sterol esterification. Steryl esters are present in all plants (Dyas and Goad, 1993) and are most often localized in the cytoplam of plant cells. They accumulate also as lipid bodies in the elaioplasts of the tapetum in developing *Brassica napus* anthers (Hernandez-Pinzon et al., 1999; Wu et al., 1999). In most cases sterols are esterified by fatty acids but in some plants (*Oriza sativa*) sterols are esterified by phenylpropanoids and especially by ferulic acid (Akihisa et al., 2000). A large number of studies have established that phytosterol and phytostanol fatty acid esters have serum cholesterol-lowering properties and are inhibitors of cholesterol absorption in human small intestine (Gylling et al., 1999; Jones, 1999; Normén et al., 2000). Triterpene and phytosteryl ferulates from rice bran have been shown to have anti-inflammatory effects against 12-*O*-tetradecanoylphorbol-13-acetate-induced inflammation in mice (Akihisa et al., 2000). Two enzymatic systems are involved in sterol esterification : In mammals cellular cholesterol is esterified by a fatty acid CoA derivative and this step is catalyzed by an acylCoA cholesterol acyltransferase (ACAT) (Farese Jr, 1998; Lin et al., 1999; Yang et al., 1997), whereas blood cholesterol is esterified by a fatty acid from lecithin using a lecithin cholesterol acyltrans-

ferase (LCAT) (Jonas, 2000; Peelman et al., 1999). In *S.cerevisiae* ergosterol is esterified by two polypeptides encoded by two ACAT related genes (Yang et al., 1996). Deletion of both ACAT genes resulted in a viable cell with undetectable esterified sterol (Yang et al., 1996). ACAT and LCAT are completely different proteins and their amino-acid sequences have no significant identity. The enzymatic system operating in higher plants is still not clearly defined. The reaction seems to be associated with the microsomal fraction that is able to convert phytosterols into steryl esters (Bouvier-Navé and Benveniste, 1995). However, several acyl donors : triacylglycerol (Zimowski and Wojciechowski, 1981), diacylglycerol (Garcia and Mudd, 1978), and lecithin (Bouvier-Navé and Benveniste, 1995; Garcia and Mudd, 1978) have been reported so far. Still, no cDNA encoding a sterol acyltransferase has been characterized. A polypeptide from Arabidopsis possessing 25% identity with the human ACAT1 was shown to be in fact a diacylglycerol acylCoA acyltransferase (DGAT) (Bouvier-Navé et al., 2000). The Arabidopsis genome sequencing program has revealed the existence of several candidate genes encoding proteins having significant identity (higher than 25%) with human LCAT.

Sterol glycosylation. Extensive reviews on sterol glycosylation have been published (Ullmann et al., 1993; Wojciechowski, 1991). This reaction consists of the reaction of free sterols with UDP-glucose to give sterylglycosides and UDP in the presence of a UDP-glucose :sterol glucosyltransferase. This reaction has been shown to be associated with membranes in higher plants and more precisely with the plasma membrane in *Zea mays* (Hartmann-Bouillon and Benveniste, 1978). Such a reaction plays an important role in eukaryotic organisms. It is obvious that the attachment of a glycosyl moiety to the sterol backbone alters the physical properties of this lipid. Such changes have been studied with artificial membranes (Grunwald, 1975; Webb et al., 1995). To evaluate the impact of these changes in the living cell a genetic approach would be needed. For this purpose isolation and characterization of sterol glycosyltransferases from eukaryotes have been undertaken. A purified enzyme (Warnecke and Heinz, 1994) has been used for the cloning of a corresponding cDNA from oat. Amino acid sequences derived from the amino terminus of the purified protein and from peptides of a trypsin digestion were used to construct oligonucleotide primers for PCR experiments. Screening of oat and Arabidopsis cDNA libraries with amplified labeled DNA fragments resulted in the isolation of sterol glucosyltransferase-specific cDNAs with inserts lenghts of ca. 2.3 kb for both plants. These cDNAs encode polypeptides of 608 (oat) and 637 (Arabidopsis) amino acid residues with molecular masses of 66kDa and 69kDa, respectively (Warnecke et al., 1997). Genes from *S.cerevisiae*, *Pichia pastoris*, *Candida albicans, Dyctiostelium discoidum* (Warnecke et al., 1999) and cDNAs from oat (*Avena sativa*) and Arabidopsis (Warnecke et al., 1997) have been cloned and were expressed in *E.coli*. *In vitro* enzyme assays with cell-free extracts of transgenic *E.coli* strains showed that the genes encoded UDP-glucose :sterol glucosyltransferases which can use different sterols such as cholesterol, sitosterol, and ergosterol as sterol acceptors. The genes from Arabidopsis (At3g07020 and At1g43620) are composed of 14 exons and 13 introns.

Conversion of campesterol into brassinosteroids will be developed in another chapter of this book.

DISCUSSION

Since the publication of a review dealing with cloning of cDNAs or genes encoding enzymes of sterol biosynthesis from plants (Bach and Benveniste, 1997), impressive progress has been in that field, due to important advances achieved in the following three directions : i) cloning of most cDNAs and genes encoding enzymes of sterol biosynthesis and metabolism ; ii) isolation and characterization of mutants defective in several key steps of these pathways ; iii) total sequencing of the Arabidopsis genome. The main informations already obtained or expected concern : j) gene organization and expression ; jj) mechanism of action of enzymes ; jjj) functions of sterols.

Organization and expression of genes

A general picture concerning regulation of plant sterol biosynthesis is still far from being available. Whereas important work has already been done on the first steps of isoprenoid synthesis : HMGCoA synthase and reductase (Alex et al., 2000; Lumbreras et al., 1995), farnesyl diphosphate synthase (Cunillera et al., 2000) and squalene synthase (Devarenne et al., 1998), such a study is still in its infancy in the post-squalene block of steps. Some interesting informations arise from Table 2 which gathers data concerning the organization of most of genes encoding sterol biosynthesis and metabolism in Arabidopsis. According to Table 2, genes are scattered among the five chromosomes and no block of genes corresponding to segments of the biosynthetic pathway are detectable. The number of genes present for a given enzymatic step is variable. As far as Arabidopsis is concerned, most

enzymes listed on Table 2 are encoded by one gene (MVK, PMVK, OS-cycloartenol cyclase, SMT1, $\Delta^{8,14}$-sterol-Δ^{14}-reductase, Δ^{8}-Δ^{7}-sterol isomerase, Δ^{7}-SR, Δ^{5}-sterol-Δ^{24}-reductase (isomerase) and UDP-glucose : sterol glucosyltransferase) or by two genes (HMGR, MVDPD, IDI, FPS, SQS, SMT2, CYP51, 5-DES). Exceptions are squalene epoxidase (6 genes) and the triterpene synthases (12 genes). Gene multiplicity seems to be frequent in the plant kingdom. According to a recent study performed on HMGR, FPS and SQS, one gene would be expressed in all parts of the plant and would have a « house keeping function » while a paralogue would be expressed selectively in some organs or tissues (Cunillera et al., 2000). As such genes are situated at the beginning of the sterol pathway, it could be suggested that paralogous genes may be involved in the synthesis of different isoprenoids other than sterols. This explanation does not apply to genes encoding enzymes situated downstream of cycloartenol that are involved only in sterol synthesis. However, it is reasonable to assume that one gene should be involved in the main pathway, whereas its paralogue could be linked to a parallel pathway. This could occur if the substrate specificity of the protein encoded by the second gene was different from that of the enzyme encoded by the first one. Such a situation is not unlikely in sterol synthesis, where several minor parallel pathways may exist beside the main pathway. The number of exons (one to eighteen) is extremely variable and does not seem to be typical of a defined class of enzyme. For instance, group of genes encoding enzymes having similar mechanism of action such as IDI, FPS and SQS on one hand or methyltransferases on the other hand contain different number of exons. As only a few genes encoding sterol biosynthesis enzymes have been sequenced in other plants than Arabidopsis, it is still difficult to hypothesize on whether the exon-intron pattern is conserved in orthologous genes or not. Likewise, no phylogenetic rules arise from comparison of exon number. Important points remain to be solved : i) how is the genetic machinery involved in sterol synthesis informed when the steady-state level of sterols is reached in cellular membranes ? ; ii) how is this information transmitted from membranes to the genetic machinery ? ; iii) what is the type of regulation involved : transcriptional, translational or post-translational ? ; iv) if transcription constitutes a major site of regulation, what are the *cis* and *trans* regulatory elements involved ? To answer this last question, regulation studies should include isolation and careful analysis of gene promoter sequences in order to disclose regulatory elements and their corresponding binding proteins.

Mechanisms of enzymatic catalysis

The possibility to express cDNAs encoding enzymes of sterol biosynthesis and metabolism in heterologous systems (*S. cerevisiae* mutants, *E. coli*, and *Sodoptera frugiperda* cultured cells) has opened new avenues to perform structural studies on enzymes as well as studies of their catalytical mechanism and inhibition by tailor-made inhibitors (transition-state analogues or suicide inhibitors). However only few enzymological data have been reported on plant enzymes so far. Recently site-directed mutagenesis of recombinant Arabidopsis 5-DES has given precious clues to do deeper studies on the catalytical mechanism of this enzyme (Rahier, 2001; Taton et al., 2000). As shown above, crystal structures have been successfully got in the case of human (Istvan et al., 2000) and *Pseudomonas mevalonii* (Bochar et al., 1999) HMGRs, human squalene synthetase (Pandit et al., 2000), *Alicyclobacillus acidocaldarius* hopene synthase (Wendt et al., 1999), and *Mycobacterium tuberculosis* lanosterol 14-demethylase (Podust et al., 2001). It is expected that crystal structures of Arabidopsis enzymes of sterol biosynthesis will be obtained in the next future.

Functions of sterols

A breakthrough on sterol biosynthesis and function was possible due to the isolation and biochemical and molecular characterization of Arabidopsis sterol mutants. Here we consider only mutations affecting enzymes of sterol biosynthesis leading to the synthesis of the so-called end-pathway sterols (campesterol, sitosterol and stigmasterol). We are not considering the steps involved during the conversion of e.g. campesterol into brassinolide. A major insight obtained from these mutants was that most « sterol » mutants had a dwarf phenotype and that a wild-type size could be at least partially recovered following treatment of the mutant with exogenous brassinosteroids (BRs). The extent of recovery was highly variable and dependent of the gene affected by the mutation. For instance, *dwarf1, dwarf5* and *dwarf7* mutants defective in the conversion of 24-methylene cholesterol to campesterol (Choe et al., 1999b), in the Δ^{7}-reductase (Choe et al., 2000) and the Δ^{7}-sterol-C5(6)-desaturase (Choe et al., 1999a) respectively, were shown to be totally rescued by BRs. In contrast, the phenotype of *smt1* plants which are defective in the sterol methyltransferase involved in the

first methylation occuring during sterol biosynthesis is poorly rescued by exogenous brassinosteroids (Diener et al., 2000). Likewise the phenotype of *fackel* which is defective in the $\Delta^{8,14}$-sterol C-14 reductase was not rescued by feeding with BRs (Jang et al., 2000; Schrick et al., 2000). Although a complete sterol profile (including 24-ethyl sterols in addition of 24-methyl sterols) was not reported in the articles dealing with *dwarf1, dwarf5*, and *dwarf7*, it is possible to suggest that sterols which accumulate in these mutants could fulfil most of the functions attributed to sterols in the cell economy and in development. The fact that some plants belonging to the order of caryophyllales contain 100% of Δ^{7}-sterols (Adler and Salt, 1987) and have a sterol profile similar to *dwarf7* is in agreement with this suggestion. In contrast sterols which are present in *fackel* ($\Delta^{8,14}$-sterols) (Schrick et al., 2000), in *smt1* (Diener et al., 2000), and in transgenic plants where *SMT2* is silenced (Schaeffer et al., 2001) should not satisfy membrane integrity. As Arabidopsis mutants *fackel* and *smt1* have defects associated with body organization of the seedling, it was suggested that plant sterols, in addition of their structural role in membranes, may be **key signaling molecules** influencing position-dependent cell-fate during embryonic development (Clouse, 2000; Jang et al., 2000; Schrick et al., 2000). A role in embryonic development was already proposed for cholesterol in mammalian cells (Kip Guy, 2000). In this context *S. cerevisiae* sterol mutants (*erg2*) (Munn et al., 1999) and *erg6* (Hardwick and Pelham, 1994) were shown to present defects in membrane dynamics. In addition, membrane receptors such as the lamin B receptor (Silve et al., 1998) or emopamil binding proteins (Moebius et al., 1999; Silve et al., 1996) were shown to catalyze $\Delta^{8,14}$-sterol-14-reduction and Δ^{8}-Δ^{7}-sterol isomerisation respectively. These observations stress the importance of accurately determining the cellular localization of enzymes of sterol biosynthesis. This goal could be reached using enzymes of sterol biosynthesis fused to the green fluorescent protein and suitable fluorescent antibodies against markers for components of the endomembrane system. Such techniques could also apply to detect the formation of multienzyme complexes (metabolons) which could be independently regulated (Cunillera et al., 1996).

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