The genetics and molecular genetics of terpene and sterol origami

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Terpenes and sterols are complex molecules synthesized by equally complex biosynthetic pathways. Recent progress in using the tools of genetics, molecular genetics and genetic engineering to dissect triterpene metabolism in the cytosol, and terpene metabolism in the plastids, has opened up new strategies and avenues of investigation. Most importantly, these studies have enhanced our appreciation of the biological significance of these compounds for plants, and have revealed new secrets of this intriguing biochemistry for practical applications in agriculture and medicine.

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Abbreviations

cpd	*constitutive photomorphogenesis and dwarfism*
det2	*de-etiolated2*
fk	*fackel*
GPP	geranyl pyrophosphate
MEP	methyl-erythritol-phosphate
sad	*saponin-deficient*

Introduction

Mention terpenes, or more properly isoprenoids, to many biologists and they are likely to conjure up mental images of chemical structures with a striking similarity to hexamethyl chicken wire. Mention the same to chemists, and they are likely to imagine swirling electrons and protons within the complexities of the chicken wire. Yet, we all are familiar with terpenes. These are the compounds that give our foods their wonderful aromas and tastes, and many of our cleaning reagents their fresh scents. Probably somewhat less appreciated is the importance of plant terpenes to the nutritional quality of foods and as leads in drug-discovery programs. Carotenoids from plants are a primary source of vitamin A [1], and phytosterols are important cholesterol-lowering agents in our diets [2]. Taxol and artemisiniin are two good examples of terpenes that have stimulated new drug-development efforts [3]. These are just some of the obvious ways we have been able to take advantage of these compounds. Biologically, the terpenes are immensely important compounds to plants. They provide unique means for these organisms to sense and interact with their environment [4,5•,6], and serve as hormones that orchestrate developmental programs [7,8]. Impressive progress in understanding the complex biochemistries that underlie these roles [9–11,12••] has opened up unique opportunities to use genetic engineering to alter terpene metabolism for biological studies [13,14••] and possible industrial applications [15••,16••]. These are just some of the recent advances in the arena of terpene biosynthesis and metabolism, and the ones highlighted in this review.

The isoprenoid pathway

One of the more exciting recent advances in the field of plant biochemistry has been the discovery of the mevalonate-independent pathway for isoprenoid biosynthesis (Figure 1). This new pathway, referred to as the methyl-erythritol-phosphate (MEP) pathway for the first intermediate committed solely to the biosynthesis of isoprenoids, was first discovered in prokaryotes capable of

Figure 1

A schematic outline of the two terpene (isoprenoid) biosynthetic pathways known to operate in plants and their intracellular locations. End products and biochemical steps (arrows) highlighted in blue are the subjects of this review.

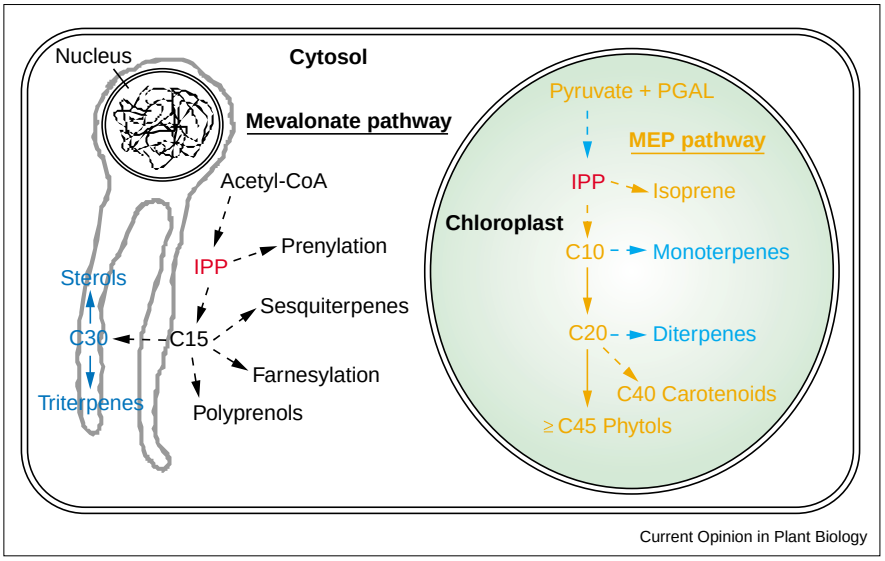

Figure 2

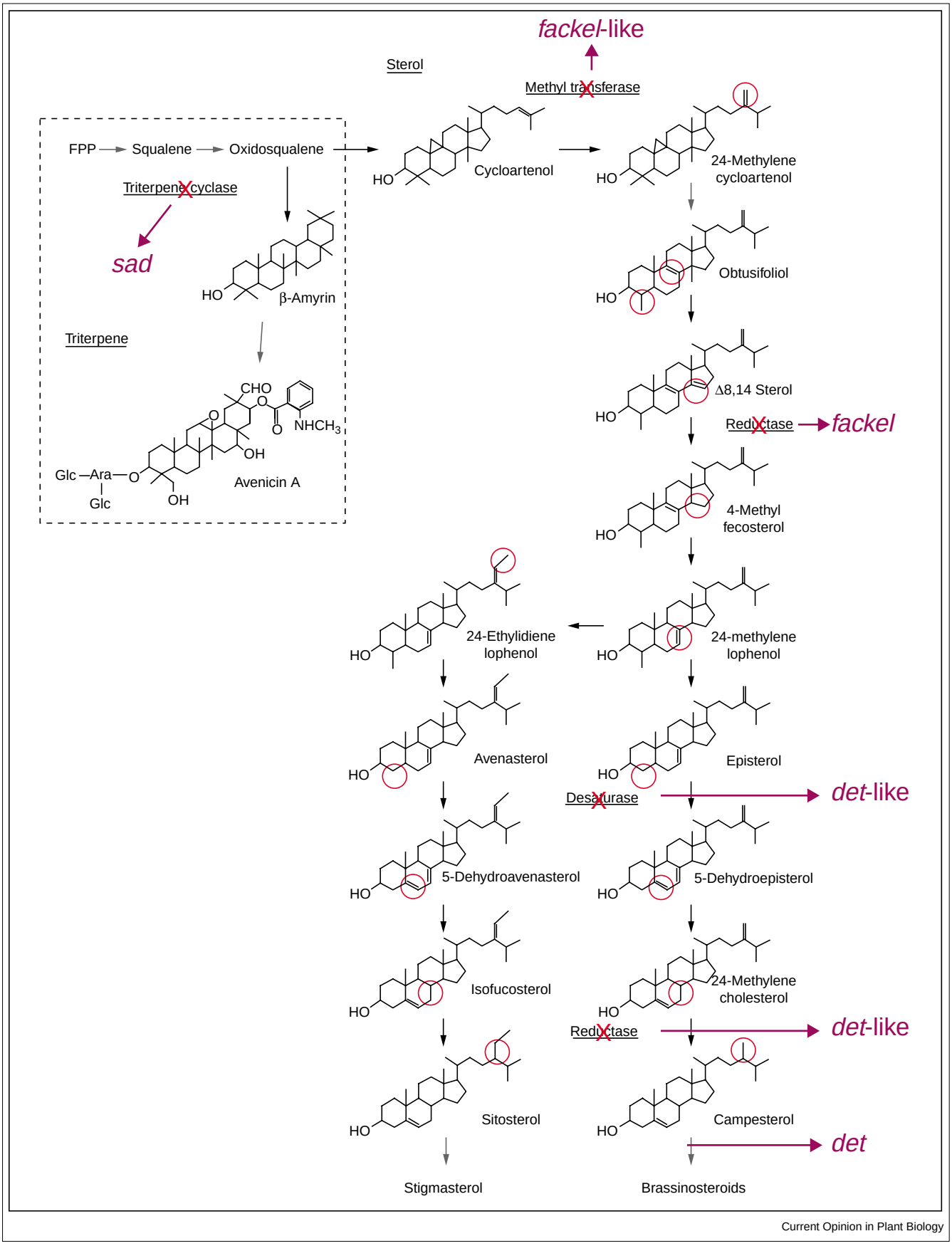
fackel-like
Sterol
Methyl transferase
FPP
Squalene
Oxidosqualene
Triterpene cyclase
sad
Cycloartenol
24-Methylene cycloartenol
Obtusifoliol
β-Amyrin
Triterpene
Δ8,14 Sterol
Reductase
fackel
Avenicin A
4-Methyl fecosterol
24-Ethylidiene lophenol
24-methylene lophenol
Avenasterol
Episterol
Desaturase
det-like
5-Dehydroavenasterol
5-Dehydroepisterol
Isofucosterol
24-Methylene cholesterol
Reductase
det-like
Sitosterol
Campesterol
det
Stigmasterol
Brassinosteroids
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Figure 2 legend

A schematic outline of the sterol and triterpene biosynthetic pathways. Genetic mutations correlated with lesions/deficiencies in particular enzymes are marked with red 'X's and are presented in a hierarchical fashion to emphasize the polarity and lack of polarity between mutants. For example, exogenous application of brassinolides completely restores the wildtype growth phenotype to *det* mutants and partially restores wildtype growth to the *det*-like mutants, but does not significantly affect the growth of *fackel* or *fackel*-like mutants. The *sad* mutations affect a branch pathway of triterpene metabolism that has little or no impact on the sterol biosynthetic pathway.

accumulating hopenes [17,18], which are the equivalent of eukaryotic sterols. The MEP pathway has since been confirmed to exist in plants and, not surprisingly, is localized to the chloroplasts [19]. Operation of the MEP pathway is intimately related to the reactions of carbon dioxide fixation and photosynthesis, as evidenced by the two immediate precursors for this pathway, pyruvate and phosphoglyceraldehyde. Two important features of this pathway are that mevalonate is not an intermediate in the plastidic synthesis of isopentenyl diphosphate (IPP) (and dimethylallyl diphosphate [DMAPP]), and that the MEP pathway appears to be largely responsible for the biosynthesis of monoterpenes, diterpenes, tetraterpenes (carotenoids), and polyprenols. Sesquiterpenes, sterols and triterpenes are synthesized in the cytosol via the mevalonate pathway [20], which is responsible for the synthesis of the key intermediate farnesyl pyrophosphate (FPP). Hence, specific classes of isoprenoids are derived from the independent operation of two very different pathways (Figure 1).

New biological roles for sterols?

Plants are known to accumulate a very diverse range of sterols and triterpenes, and there has been considerable speculation about why so many variants might be needed [21]. Sitosterol, stigmasterol and campesterol make up 60–80% of all sterols in almost all plants and plant tissues, a mixture of 6–12 other chemical variants make up the remainder. Although the biosynthesis of these compounds is still considered to be complex and incomplete, the basic outlines of their syntheses are fairly well understood (Figure 2). Until 1995, the biological importance of sterols was most often associated with their roles as structural components of membranes and their importance for altering the fluid dynamics of phospholipid bilayers. In 1996, this view of sterol metabolism was changed dramatically when two of the de-etiolated mutants of *Arabidopsis*, *de-etiolated2* (*det2*) and *constitutive photomorphogenesis and dwarfism* (*cpd*), were found to be caused by defects in genes that had a surprising degree of sequence similarity to genes for latter steps of sterol biosynthesis in man [7,8]. In turn, this led to experimentation which demonstrated that the *det* and *cpd* mutants were defective in their ability to synthesize brassinolides, which are derivatives of campesterol shown much earlier to have biological activity in rice seedlings [22]. Moreover, Li *et al.* [7] and Szekeres *et al.* [8] showed that the *det* and *cpd* mutants were deficient in brassinolides and that exogenous application of very low concentrations of brassinolides restored normal plant development in these mutants. Furthermore, the wildtype *DET2* and *CPD* genes were shown to encode enzymes that are responsible for two distinct steps in the conversion of campesterol to brassinolides.

The elucidation of the *det/cpd* mutants not only provided crucial evidence that validated the importance of brassinolides as growth regulators, but also created a conceptual shift in our thinking about the possibility of finding other 'hidden' growth regulatory molecules. The recent investigations of the *fackel* (*fk*) mutants are important examples of this shift. The *fk* mutant was originally identified as an embryonic-patterning mutant by Mayer *et al.* in 1991 [23]; and allelic variants were discovered by Schrick *et al.* [24••] and Jang *et al.* [25••]. Schrick *et al.* [24••] re-discovered the *fk* mutants while searching for novel mutations effecting the body organization of young seedlings, whereas Jang *et al.* were attempting to characterize a unique *Arabidopsis* mutant, *extra-long-lifespan*, that had abnormal cell division and expansion in embryonic and post-embryonic development. Both of these investigations subsequently demonstrated that the various mutants identified were allelic to the *fk* locus, and that the *fk* mutants mapped to a gene coding for a sterol C14 reductase (Figure 2). Exogenous application of brassinolides could not restore wildtype growth to the *fk* mutants as it did to the *det* mutants. Sterol analysis demonstrated considerable reduction in the content of sterols downstream of the mutated reductase in the *fk* mutants, but these reductions were not always consistent between the various *fk* mutants. More consistent, however, was the appearance in all *fk* mutants of four new sterol constituents, which appear to have typical sterol structures yet retain a double bond between carbons 14 and 15. These sterols have been referred to as *fk* sterols, and both groups of investigators interpreted their results as consistent with the existence of new or novel sterol signals that are important for embryogenic development. Nevertheless, the two reports do differ somewhat in their interpretation of the data. Jang *et al.* [25••] suggest that the *fk* mutation results in the production of *fk* sterols that might be biologically active in promoting developmental events during embryogenesis and post-embryogenesis. Alternatively, Schrick *et al.* [24••] suggest that the defects manifested in the *fk* embryos might arise because of the absence of an essential sterol signal molecule. Regardless of which model one considers, both investigations suggest the existence of new sterol signal molecules that are capable of controlling developmental programs.

As exciting as this prospect may appear, reservations about the possibility of uncovering new signal sterol molecules

based on the *fk* mutant have been raised [26]. The crux of this debate centers on whether the *fk* mutants reveal a need for sterol signal molecules or whether these mutants simply reflect general sterol deficiencies. Schrick *et al.* [24••] argued that the *fk* mutants do not simply result from a lack of structural sterols because cells in the mutants divide up to the 60-cell stage of embryogenesis. These authors do recognize, however, that cell expansion is limited in the *fk* mutants, inferring that membrane biogenesis might be limited by the availability of structurally suitable sterols. Perhaps more difficult to reconcile is the observation of a *fk*-like phenotype for another sterol biosynthetic mutant. In investigating sterol methyl transferases, Diener *et al.* [27•] developed anti-sense suppressed lines for one particular sterol methyltransferase and observed a phenotype that is essentially the same as that of the *fk* mutants. In contrast, *fk*-like phenotypes were not reported for mutations in genes downstream of *FK* in the sterol biosynthetic pathway (i.e. *STEROL1* [*STE1*], *DWARF7* [*DWF7*], *DWF5* and *DIMINUTO1* [*DIM1*]; [28,29]); and wildtype growth was completely restored when exogenous brassinolides were supplied to these mutants. Therefore, regardless of whether or not the *fk* mutants reveal the existence of new signal sterols or not, the mutants are telling us that viability is compromised by the structural variants of sterols that accumulate in the *fk* mutants.

Triterpenes – biochemical progress and biological activities

In addition to sterols, which are ubiquitous in eukaryotic cells, plants also accumulate triterpenes. These are 30-carbon structures that are derived from oxidosqualene, the last common intermediate for both sterols and triterpenes (Figure 2). Although the difference between sterols and triterpenes may appear trivial at first, the triterpenes are distinct from sterols in several important ways. Physiologically, triterpenes tend to accumulate in plants as conjugates with carbohydrates, alkaloids and other macromolecules, and generally they have a five-ring structure rather than the four-ring structure of sterols. In contrast to sterols, triterpenes are not essential, and not all plants accumulate triterpenes or their derivatives. Furthermore, it is unusual for triterpenes to serve as structural components of membranes. Biochemically, the cyclization reaction that converts oxidosqualene to cycloartenol, the committed precursor of sterol biosynthesis, is catalyzed by cycloartenol synthase (also referred to as oxidosqualene cyclase) ([30]; Figure 2). This enzyme is distinct from those catalyzing the cyclization of oxidosqualene to amyrin or lupeol, which are the common structural backbones of a large proportion of the triterpenes found in nature [9–11]. Although much of the early work on characterizing the triterpene synthases was hindered by their integral membrane association, rapid progress has been made with the development of yeast expression systems [30,31•].

Interest in saponins has also been heightened recently because of several observations concerning their biological activities. Saponins such as digitalis have long been known for their medicinal value, but recent pharmacological screens of plant natural products have identified the triterpenoid saponin fraction from *Acacia victoriae* as an effective inhibitor of tumor cell growth [32]. Further fractionation studies identified avicin D and G as the active components from these initial extracts [33•]. The medicinal properties of these saponins as specific agents that are capable of inducing apoptotic death in tumor cells — via a direct effect on mitochondrial function and as suppressors of several other indicators of cell multiplication [34] — is intriguing but the chemical complexities of these molecules are even more startling. Avicin D and G appear to be derived from a typical β-amyrin triterpene that is decorated by a trisaccharide at carbon 3, a tetrasaccharide at carbon 28, and a very unusual side chain at carbon 21 consisting of two acyclic monoterpene constituents connected by a quinovose sugar. The organic synthesis of such a complex structure would surely pose a serious challenge to even the most adept chemist. However, it is exciting to think that biochemical pathways responsible for the synthesis of such compounds are waiting to be discovered.

Assessing the biological significance of any natural product to a plant's fitness has been an important challenge that has often focussed on the so-called inducible defense compounds. Because one of the best-characterized plant responses to microbial challenge is the production of phytoalexins, a tremendous amount of experimental work has been directed at evaluating inducible defense responses and calculating the contribution of a particular inducible compound to the plant's defense capabilities [35,36]. However, plants are also known to accumulate a variety of secondary metabolites that possess antimicrobial activities during the normal course of growth and development. Although there has been some progress in assessing the contribution of these phytoanticipins to plant defenses, the contribution of these preformed defense compounds to a plant's susceptibility or resistance to microbial pathogens has been under studied [37]. This deficiency has recently been addressed in a series of decisive reports.

In 1995, Osbourn and colleagues demonstrated that a fungal pathogen that infects *Avena* species was considerably more pathogenic if it expressed an enzyme capable of detoxifying the saponins that are associated with oats [38]. However, a more direct assay of how important the oat-derived avenacins are for resistance was lacking. Using the diploid species *Avena strigosa*, Osbourn's group [13] developed a clever fluorescence screen to isolate chemically induced mutants that did not accumulate avenacins (i.e. *saponin-deficient* [*sad*] mutants) (Figure 2). These mutants were then examined for disease severity after challenge by a fungal pathogen for a specified length of time. Papadopoulou *et al.* [13] observed that, in general, *sad* mutants with reduced avenacin concentrations were more susceptible. Furthermore, the reduced avenacin content and disease susceptibility traits co-segregated in

F_2 progeny of crosses between wildtype and mutant plants. Several of the *sad* mutants have subsequently been mapped to mutations within a novel triterpene cyclase that is involved in avenacin biosynthesis [12••].

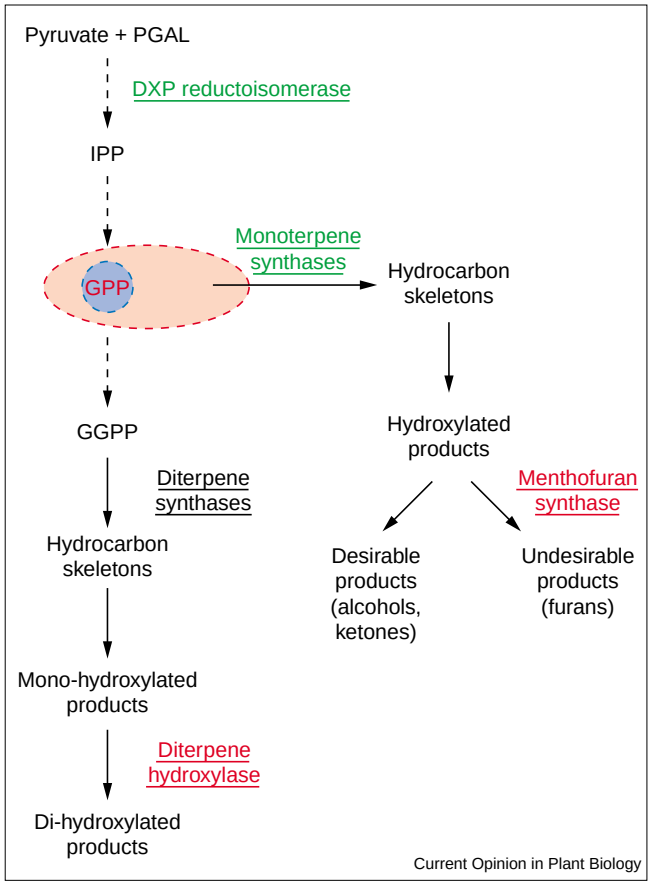
Yin and yang of engineering terpene metabolism

A true test of our understanding of any biochemical process is reflected in our ability to fiddle with that pathway in predictable ways using the tools of genetic engineering. Although there has been some success in manipulating the cytosolic mevalonate pathway [4], recent progress by Lewinsohn *et al.* [15••], Mahmoud and Croteau [16••] and Wang *et al.* [14••] represents strides forward in manipulating the MEP pathway (Figure 3). In a larger context, the work of these groups addresses a broader conceptual issue of how the flux of carbon down the MEP pathway is controlled or regulated.

Although increases in carotenoid levels in rice [1], tomato [39] and rape [40] have been achieved, the manipulation of aroma characteristics remains an attractive challenge. In one sense, the challenge in manipulating aroma and fragrance is not to divert too much of the carbon entering the MEP pathway, just enough for the production sufficient fragrant molecules to allow their detection by the olfactory senses. Aromas and fragrances are detectable at concentrations of parts per million or parts per billion. Hence, Lewinsohn *et al.* [15••] rationalized that even when only a small amount of geranyl pyrophosphate (GPP) is available in developing tomato fruits, a properly engineered monoterpene synthase might be able to siphon away enough carbon from the MEP pathway for the production of volatile monoterpenes. These investigators [15••] fused a linalool synthase gene from *Clarkia* that had been well characterized previously [41] to the E8 promoter element known to direct gene expression during fruit development [42]. They then examined the level of linalool production by the transgenic fruits. Interestingly, not only did the fruits accumulate linalool during ripening but they also accumulated a hydroxylated derivative of linalool at approximately 25–50% of the concentration of linalool. This work has obvious practical implications and demonstrates that endogenous GPP is available to be accessed by an appropriately targeted monoterpene synthase.

The results of Lewinsohn *et al.* [15••] are somewhat unexpected. Until now, many investigators have tacitly assumed that the flux of intermediates down the MEP pathway is directed or channeled. That is, that the rate of precursors entering the pathway is limiting and, therefore, that neither intermediates nor pools of GPP would accumulate. Mahmoud and Croteau [16••] examined this issue from a slightly different perspective. These investigators introduced the gene encoding 1-deoxy-D-xylulose 5-phosphate (DXP) reductoisomerase into peppermint under the control of a strong constitutive promoter, and examined the complete terpene profile of the resulting transgenic lines.

Figure 3



The flow of carbon and intermediates through the MEP pathway can be altered by selective genetic engineering of discrete steps in this pathway. In separate studies [14••–16••], the expression levels of the genes encoding the enzymes depicted in green (upregulated) and red (downregulated) have been manipulated and the consequences for the flow of carbon to end products assessed. The blue and red ovals represent hypothetical pools of GPP available to several branch pathways, one for monoterpene and the other for diterpene metabolism. DXP, 1-deoxy-D-xylulose 5-phosphate; GGPP, geranylgeranyl diphosphate; IPP, isopentenyl diphosphate; PGAL, phosphoglyceraldehyde.

This gene catalyzes a putative rate-limiting step early in the MEP pathway [19], and its introduction resulted in an increase in the total monoterpene content of about 30% (an average from ten independent transgenic lines) without any consistent change in the overall profile of monoterpenes. Combined with the observations by Lewinsohn *et al.* [15••], Mahmoud and Croteau's [16••] results indicate that the MEP pathway is more malleable than once thought.

Mahmoud and Croteau [16••] also demonstrated the feasibility of an antisense approach to enhancing the quality of peppermint oil. Monoterpene hydrocarbons resulting from the action of the monoterpene synthases are often modified in very specific ways: most often these include the insertion of hydroxyl groups and the oxidation of these hydroxyl groups to ketones. Additional modifications

may also introduce a second hydroxyl function in close enough proximity to a ketone that furan-type structures can arise. In peppermint, menthofuran is derived from such a series of modifications to a monoterpene backbone, and is considered undesirable because it alters the flavor and color characteristics of the peppermint oil. Upon expressing an antisense form of the menthofuran synthase gene in transgenic peppermint, Mahmoud and Croteau [16••] observed a reduction of about 50% in the menthofuran content of the peppermint oil. This occurred without a decrease in the overall amount of oil produced or other alterations in the monoterpene profile of the oil.

A final example of elegant tinkering with the MEP pathway was recently reported by Wang *et al.* [14••]. These investigators have had a long-standing interest in leaf-surface chemistry; in particular, in how the diterpene constituents are synthesized by leaf hairs or trichomes and accumulate on the surface of tobacco leaves [43]. Earlier studies had hinted at the possibility that several leaf-surface compounds, especially the cembratriene mono-alcohols (diterpenes) (Figure 3), might serve as a deterrent to insect pests such as aphids [44]. Using a P450 gene that was isolated on the basis of its exclusive expression in trichomes and a suspicion of its responsibility for encoding a terpene hydroxylase, these investigators engineered both sense and antisense constructs of this gene under the direction of a strong constitutive promoter into transgenic plants. Wang *et al.* [14••] observed that at least one of the antisense transgenic lines and two of the sense (co-suppressed) transgenic lines had levels of cembratriene-diols that were reduced by approximately 40%, which correlated with a reduction in the level of the corresponding P450 mRNA. Importantly, these transgenic lines also had greatly enhanced levels of the cembratriene mono-alcohols, the direct precursors of the cembratriene diols. The suppression of diol levels in the transgenic lines did not effect any morphological traits, except that the transgenic lines were not colonized by aphids to the same extent as the control plants were. The transgenic lines were just as attractive to aphids as wildtype plants, but the toxicity of the transgenic lines to colonizing aphids was enhanced, probably due to the increased levels of mono-hydroxylated cembratrienes.

Conclusions

The isoprenoid or terpene biosynthetic pathway in plants is probably one of the most complex and elaborate pathways known. It actually consists of two pathways that operate independently of each other by virtue of being localized to separate intracellular compartments. Moreover, each of these pathways gives rise to a unique spectrum of products that include structural components, such as sterols, and other compounds whose functions are not immediately apparent, such as saponins, monoterpenes and diterpenes. Sorting out the biological significance of this complexity is clearly a major challenge. The effort to meet this challenge is benefiting from the use of conventional genetic mutants as well as those designed and constructed via genetic engineering.

Acknowledgements

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- •• of outstanding interest

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See annotation [24••].

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The authors created a lesion in a gene that controls an early step in the sterol biosynthetic pathway to study how sterol biosynthesis is controlled. They noted that the transgenic lines exhibited abnormal embryo development, much like the *fk* mutant. To some extent, the observations reported in this paper rebut the conclusions reached by Jang *et al.* [25••] and Schrick *et al.* [24••], who examined the genetic basis of the *fk* mutants.

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