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Terpenoid Metabolism

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INTRODUCTION

The terpenoids constitute the largest family of natural products; over 22,000 individual compounds of this class have been described (Connolly and Hill, 1991), and the number of defined structures has doubled every decade since the 1970s (Devon and Scott, 1972; Glasby, 1982). The terpenoids play diverse functional roles in plants as hormones (gibberellins, abscisic acid), photosynthetic pigments (phytol, carotenoids), electron carriers (ubiquinone, plastoquinone), mediators of polysaccharide assembly (polyprenyl phosphates), and structural components of membranes (phytosterols). In addition to these universal physiological, metabolic, and structural functions, many specific terpenoid compounds (commonly in the C_{10}, C_{15}, and C_{20} families) serve in communication and defense, for example, as attractants for pollinators and seed dispersers, competitive phytoxins, antibiotics, and herbivore repellents and toxins (Harborne, 1991). Terpenoids available in relatively large amounts as essential oils, resins, and waxes are important renewable resources and provide a range of commercially useful products, including solvents, flavorings and fragrances, adhesives, coatings, and synthetic intermediates (Zinkel and Russell, 1989; Dawson, 1994). Members of the terpenoid group also include industrially useful polymers (rubber, chicle) and a number of pharmaceuticals (artemisinin, taxol) and agrochemicals (pyrethrins, azadirachtin).

In spite of the economic significance of the terpenoids and their many essential functions, relatively little is known about terpenoid metabolism and its regulation in plants. There are several reasons for this. An overwhelming problem with the terpenoids is their sheer number. A given plant may synthesize (and catabolize) many different terpenoid types (from C_5 to C_{40} and higher) at different times and locations for many different purposes throughout the course of development. Because all terpenoids are produced by a common biosynthetic pathway, sophisticated control mechanisms must exist to ensure the production of appropriate levels of these often structurally complex compounds in the proper metabolic, developmental, and environmental context. Pathway elucidation for highly functionalized terpenoid metabolites is not trivial, and determining the enzymology of terpenoid metabolism has proven very challenging, both because relatively little enzymatic machinery is dedicated to terpenoid metabolism and because many of the reaction types involved (carbonium ion mechanisms) are unlike those of primary metabolism of carbohydrates, proteins, and lipids. Moreover, the genetics (let alone the molecular genetics) of terpenoid metabolism are insufficiently developed to provide generally useful tools for examining control of metabolism at the cell, enzyme, transcript, or DNA levels. Of comparable significance is the paucity of suitable model systems for examining either the induced regulation of multiple terpenoid pathways or the developmental regulation of even one isoprenoid pathway in a uniform, defined cell type and the lack of adequate bioanalytical methodologies for determining pathway flux or for examining intracellular metabolite concentrations. In this brief review, we provide an update of some recent advances in terpenoid metabolism with a focus on illustrative examples from this broad field of research that may serve to indicate trends and, perhaps, suggest future directions.

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BIOSYNTHETIC PATHWAYS

Early structural investigations led Wallach (1914) to formulate the "isoprene rule." This principle—that most terpenoids could be hypothetically constructed by a repetitive joining of isoprene (2-methyl-1,3-butadiene; see Figure 2) units—was a major advance in terpenoid chemistry because it provided the first unified concept for a common structural relationship among terpenoid natural products. The concept was subsequently refined by Ruzicka (1953) when he formulated the "biogenetic isoprene rule." This hypothesis, which ignores the precise character of the biological precursors and assumes only that they are "isoprenoid" in structure, is based largely on mechanistic considerations for the construction of these compounds involving electrophilic elongations, cyclizations, and rearrangements.

The biogenetic isoprene rule has proven to be amazingly prophetic. The biosynthesis of terpenoids, shown in Figure 1, can be conveniently divided into four major processes, the first of which involves the conversion of acetyl-coenzyme A (CoA) to the "active isoprene unit," isopentenyl pyrophosphate (IPP). The action of various prenyltransferases then generates from this precursor the higher order terpenoid building blocks, geranyl pyrophosphate (GPP; C_{10}), farnesyl pyrophosphate (FPP; C_{15}), and geranylgeranyl pyrophosphate (GGPP; C_{20}). These

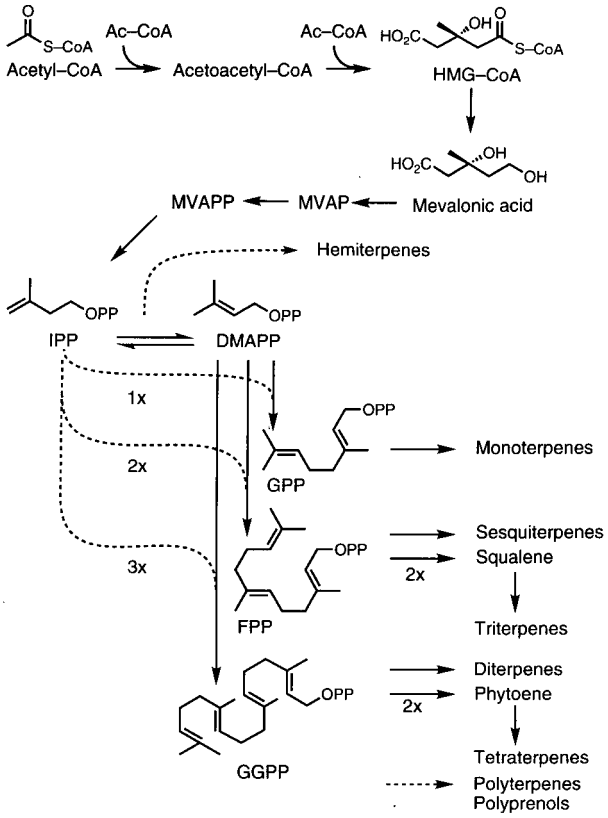

Figure 1. Outline of the Mevalonic Acid Pathway and Terpenoid Skeleton Biosynthesis.

1×, 2×, and 3× indicate the number of IPP units added.

branch point intermediates may then self-condense (to the C_{30} and C_{40} precursors of sterols and carotenoids, respectively), be utilized in alkylation reactions to provide prenyl side chains of a range of nonterpenoids (including proteins), or undergo internal addition (that is, cyclization) to create the basic parent skeletons of the various terpenoid families. Finally, oxidation, reduction, isomerization, conjugation, or other secondary transformations elaborate the unique and manifold character of the terpenoids. The biosynthetic pathway of terpenoid production, outlined in Figure 1, has been reviewed recently (Gershenzon and Croteau, 1993) and is considered only briefly here.

The initial steps of the pathway involve the fusion of three molecules of acetyl-CoA to produce the C_6 compound 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). The first two reactions, which in both animals and yeast are catalyzed by two separate enzymes, acetyl-CoA acetyltransferase and HMG-CoA synthase, have not been studied extensively in plants. However, both reactions are thought to operate through a similar carbanionic mechanism. In plants, these two reactions are apparently catalyzed by a single enzyme, utilizing Fe^{2+} and quinone cofactors (Bach et al., 1991; Weber and Bach, 1994).

The next step, which is catalyzed by HMG-CoA reductase, is of paramount importance in animals as the rate-limiting reaction in cholesterol biosynthesis (for review, see Goldstein and Brown, 1990). The regulation of this enzyme is complex, as discussed later. This enzyme catalyzes two reduction steps, each requiring NADPH. The mevalonate resulting from the reduction of HMG-CoA is sequentially phosphorylated by two separate soluble kinases, mevalonate kinase and phosphomevalonate kinase, to form 5-pyrophosphomevalonate. Formation of IPP is then catalyzed by pyrophosphomevalonate decarboxylase, which performs a concerted decarboxylative elimination. This enzyme requires ATP and a divalent metal ion. The tertiary hydroxyl group of pyrophosphomevalonate is phosphorylated before the concerted elimination, thus making it a better leaving group.

IPP is the basic C_5 building block that is added to prenyl pyrophosphate cosubstrates to form longer chains. IPP itself is insufficiently reactive to undergo ionization to initiate the condensation to higher terpenoids. Therefore, it is first isomerized to the allylic ester dimethylallyl pyrophosphate (DMAPP) by IPP isomerase. This enzyme, which has been described in plants (Ogura et al., 1968; Spurgeon et al., 1984; Dogbo and Camara, 1987; Lützow and Beyer, 1988), requires a divalent metal ion. Based on characterization of the animal and yeast enzymes, IPP isomerase operates through an unusual carbocationic mechanism.

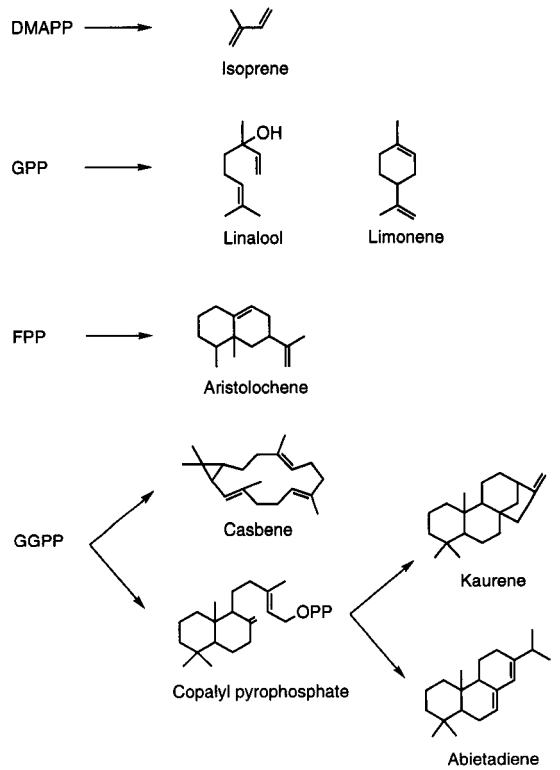

Figure 2. Representative Terpenoids.

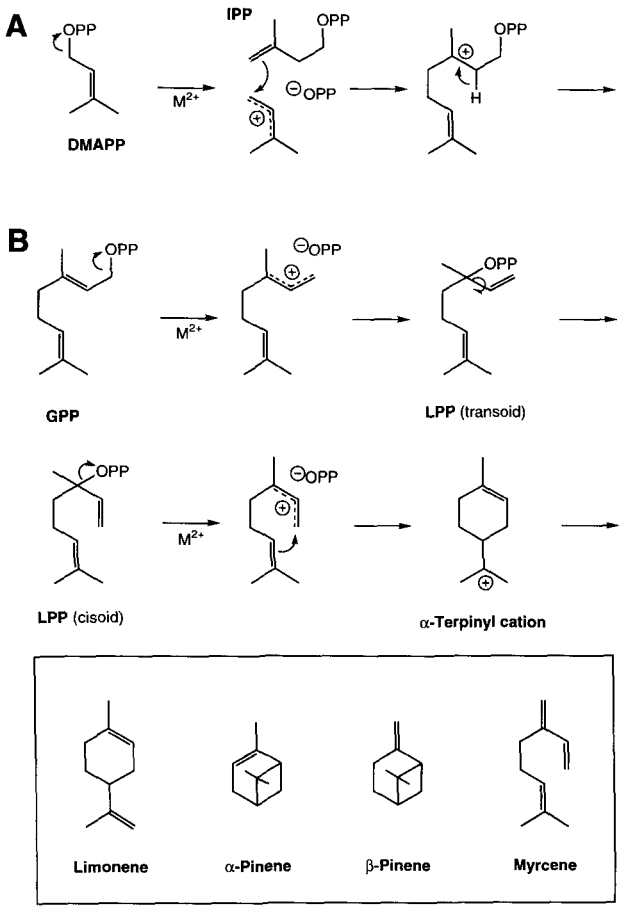

Figure 3. Enzyme Mechanisms of Two Representative Synthases.

(A) GPP synthase.

(B) Limonene synthase.

The final products from limonene synthase are limonene (94%), α-pinene (2%), β-pinene (2%), and myrcene (2%). M^{2+} indicates a required divalent metal cation, usually Mg^{2+} or Mn^{2+}. LPP and the α-terpinyl cation are enzyme-bound intermediates.

Isoprene, the simplest of the terpenoids (Figure 2), is synthesized directly from DMAPP by diphosphate elimination. The reaction is catalyzed by the enzyme isoprene synthase, which has been studied in aspen and velvet beans (Silver and Fall, 1991; Kuzma and Fall, 1993). The enzyme requires Mg^{2+} or Mn^{2+} for activity (Silver and Fall, 1991; Kuzma and Fall, 1993) and is probably localized in the chloroplasts (Mgaloblishvili et al., 1978). Isoprene is produced and emitted by the leaves of many plants and accounts for a significant proportion of atmospheric hydrocarbons (Zimmerman et al., 1978).

Higher terpenoids (Figure 2) are generated via the action of prenyl transferases, which perform multistep reactions beginning with DMAPP (or a longer allylic pyrophosphate) and IPP to form higher isoprenologs, generally with all *trans* (*E*) geometry. GPP synthase forms the C_{10} intermediate (GPP) from DMAPP and IPP (Figure 3A). This synthase has been characterized in a number of plant species and is clearly separable from FPP synthase (Croteau and Purkett, 1989; Heide and Berger, 1989; Feron et al., 1990; Suga and Endo, 1991; Endo and Suga, 1992; Soler et al., 1992). FPP synthase forms the C_{15} intermediate (FPP) in two discrete steps: first DMAPP and IPP form GPP, which remains bound to the enzyme; then another IPP is added to yield FPP. GGPP synthase operates in a similar manner, via three condensation steps, to form the C_{20} intermediate. These prenyltransferases are all homodimers of native size 70 to 80 kD that require either Mg^{2+} or Mn^{2+} for activity, with a stoichiometry of two metal ions per catalytic site. The divalent metal ion binds to the pyrophosphate moiety of the allylic cosubstrate so as to make it a better leaving group in the ionization step. Ionization of the allylic pyrophosphate leads to formation of a charge-stabilized allylic carbocation (see Figure 3). Electrophilic addition to the double bond of IPP generates a new carbocation intermediate that, after *trans* elimination of a proton, yields a new prenyl pyrophosphate five carbons larger. Such electrophilic additions are biochemically rare but occur in several reactions of terpenoid biosynthesis (see later discussion).

Other prenyltransferases form terpenoid chains with *cis* double bonds, the most notable being that involved in rubber biosynthesis. Rubber (*cis*-1,4-polyisoprene) is a linear polyprenoid consisting of 400 to more than 100,000 isoprene units. Rubber biosynthesis is primed by a *trans*-allylic diphosphate initiator that is then extended by a *cis*-prenyltransferase, the rubber transferase (Cornish, 1993). Rubber transferase has been studied in a number of species; it requires Mg^{2+} or Mn^{2+} for activity and is firmly attached to intracellular rubber particles (Cornish and Backhaus, 1990; Cornish, 1993; Cornish et al., 1994).

In contrast to the linear polymer rubber, most terpenoids are cyclic, and the various classes are formed from the branch point C_{10}, C_{15}, C_{20}, and C_{30} intermediates by enzymes called cyclases or synthases. Most enzymes of this type catalyze reactions that are the intramolecular equivalent of the prenyltransferase reaction (Croteau, 1987). Typical are the reactions of the monoterpene cyclases. In these reactions, GPP is first ionized and isomerized to enzyme-bound linalyl pyrophosphate (LPP), the tertiary allylic isomer (Figure 3B). This preliminary isomerization step is necessary because the *trans*-2,3-double bond of the geranyl precursor prevents direct cyclization. Ionization of LPP promotes cyclization to the terminal double bond to yield the enzyme-bound α-terpinyl cation, a universal intermediate of these cyclization reactions (Figure 3B). The different basic monoterpene skeletons are then delineated from this highly reactive intermediate by processes that may involve internal electrophilic addition to the remaining double bond, hydride shifts, and other rearrangements before termination of the reaction sequence by proton loss or capture of the carbocation by a nucleophile (water or the original pyrophosphate anion). An unusual feature of the monoterpene cyclases is that many of these enzymes produce multiple products (Wagschal et al., 1991; Colby et al., 1993). For example, limonene synthase, in addition to the principal cyclic product limonene, also

generates smaller amounts of myrcene and α- and β-pinene (Figure 3B).

The cyclization to many sesquiterpenes and some diterpenes is performed in a similar manner, although in most instances a preliminary isomerization step is not required because of the greater flexibility of the longer C_{15} or C_{20} chain. In the diterpene series, cyclization is often promoted by protonation of a double bond rather than by ionization of the allylic diphosphate ester. In the triterpene series, electrophilic cyclization is most commonly initiated by protonation of an epoxide, for example, in the cyclization of 2,3-oxidosqualene to cycloartenol in photosynthetic organisms (Parish, 1992). The exploitation of electrophilic additions for carbon–carbon bond formation is a biochemical rarity. Conservation of this unusual carbocationic mechanism suggests a common evolutionary origin of the prenyltransferases and the terpenoid cyclases.

Subsequent transformations of the basic parent skeletons involve oxidations, reductions, and isomerizations to form the myriad different terpenoids. Many of the hydroxylations or epoxidations involved are performed by cytochrome P-450 mixed function oxidases (for review, see Mihaliak et al., 1993). These secondary reactions are not unique to terpenoid biosynthesis and are not covered here.

REGULATION

The regulation of terpenoid biosynthesis is complex, in large part because of the manifold functions of this family of metabolites and the vast differences in control in both time and location throughout the course of plant development. In general terms, regulation of terpenoid biosynthesis falls under two broad categories, spatial and temporal control, by which we have organized the illustrative examples in this section.

Spatial Regulation—Specialized Cells and Tissues

Terpenoid biosynthesis may be delimited by physical structures at histological, cellular, and subcellular levels. Where large amounts of hydrophobic terpenoids are produced and accumulated, specialized secretory structures are usually required. Common among the conifers are systems of resin ducts and blisters (Penhallow, 1907; Fahn, 1979), and the relative complexity of these structures closely parallels their potential for monoterpene production. In conifer species such as *Thuja plicata* (Western red cedar), which contain only scattered resin cells, levels of endogenous monoterpenes are low, as are monoterpene cyclase activities (Lewinsohn et al., 1991b). In species with correspondingly more complex secretory structures, from resin blisters of *Abies* (fir) species to resin passages in *Picea* (spruce) and resin ducts of *Pinus* (pine), the corresponding levels of endogenous monoterpenes and monoterpene cyclase activities increase correlatively with the degree of specialization (Lewinsohn et al., 1991b).

In angiosperms that produce high levels of monoterpenes, sesquiterpenes, or diterpenes, the biosynthetic machinery is often sequestered in specialized glandular structures. In *Mentha* (mint) species, for example, monoterpenes are produced primarily in the glandular trichomes of the leaf (Fahn, 1979; Gershenzon et al., 1989). These trichomes are modified epidermal hairs consisting of a cluster of secretory cells, with an underlying stalk and basal cell, surmounted by a droplet of oil enclosed by the cuticle layer (Fahn, 1979). Latex vessels represent another highly specialized cell type involved in the production of terpenoids such as rubber.

Biosynthesis of other terpenoids is often restricted to specific tissues at their sites of utilization. For example, the monoterpene linalool, which is involved in attracting pollinators for *Clarkia breweri*, is produced primarily in the petals of the flower (Pichersky et al., 1994; and see later discussion). Carotenoids are produced in fruit chromoplasts of red peppers (*Capsicum anuum*) during ripening (Camara and Brangeon, 1981; see also Bartley and Scolnik, 1995, this issue). Biosynthesis of wound-induced monoterpenes and diterpenoid resin acids in conifers is localized to the proximity of the wound (Lewinsohn et al., 1991a); in some species, wounding leads to the formation of "traumatic resin ducts" near the site of injury (Blanchette, 1992).

Spatial Regulation—Subcellular Compartments

Debate has raged over the subcellular localization of the early steps of terpenoid biosynthesis leading to IPP. Whereas one model holds that IPP is synthesized in each subcellular compartment in which it is utilized, a competing model maintains that IPP is synthesized exclusively in the cytosol and is partitioned to other locations (Gray, 1987; Kleinig, 1989). HMG-CoA reductase, which is thought to be an important regulatory step in IPP biosynthesis, has been localized to both plastids and mitochondria of radish (Bach, 1986, 1987), although the Arabidopsis enzyme is thought to be localized only in the endoplasmic reticulum (ER), based on the insertion of the in vitro–expressed protein into mammalian microsomes (Enjuto et al., 1994). The issue is further complicated by the fact that the origin of IPP may differ with tissue type and developmental stage. A study with developing barley chloroplasts demonstrated that, whereas chloroplasts from young tissue are capable of synthesizing IPP, those from mature leaf tissue rely on import of cytosolic IPP (Heintze et al., 1990). On the other hand, in isolated glandular trichomes of peppermint, IPP formation in the cytosol is blocked at HMG-CoA reductase at the time when oil accumulation is most rapid; thus, the biosynthesis of both monoterpenes and sesquiterpenes relies exclusively on partitioning of plastid-derived IPP (McCaskill and Croteau, 1995). It seems unlikely that a general organizational model for the origin of IPP can apply to all tissues and stages of development. A full assessment of the interactions between and contributions of various compartments to the

supply of precursors for terpenoid metabolism is not possible until accurate flux measurements can be made for each locale.

In contrast with early steps in terpenoid biosynthesis, the compartmentation sites of subsequent steps in the biosynthesis of terpenoids seem clear. Synthesis of FPP (Belingheri et al., 1988; Feron et al., 1990; Hugueney and Camara, 1990) and its derived sesquiterpenes (Gleizes et al., 1980; Belingheri et al., 1988) takes place in the cytosol and the cytosol/ER boundary, as does the synthesis of triterpenes, including the phytosterols (Goodwin, 1979).

For monoterpenes, however, plastids are clearly implicated as the exclusive site of synthesis (for review, see Kleinig, 1989). Biosynthesis of GPP (Figure 3A) has been localized in plastids of *Vitis vinifera* (Feron et al., 1990; Soler et al., 1992). Isolated leucoplasts from *Citrofortunella mitis* and *Citrus unshiu* fruit are capable of monoterpene (but not sesquiterpene) biosynthesis from IPP (Gleizes et al., 1983; Pauly et al., 1986). Isolated chromoplasts of daffodil flowers (Mettal et al., 1988) and of *Citrus sinensis* fruits (Pérez et al., 1990) have a similar capacity for monoterpene formation. A study of 45 species of higher plants revealed a correlation between the levels of monoterpene (but not sesquiterpene) biosynthesis and the presence of leucoplasts (Cheniclet and Carde, 1985). Immunogold labeling has indicated that the monoterpene cyclase limonene synthase is located specifically in the leucoplasts of mint gland secretory cells (J. Gershenzon and R. Croteau, unpublished data).

Diterpenes are also biosynthesized in plastids (for review, see Kleinig, 1989). GGPP synthase activity has been demonstrated in proplastids of castor bean (Dudley et al., 1986) and in the stroma of daffodil chromoplasts (Laferrière and Beyer, 1991). The enzyme has been partially purified from fruit chromoplasts of tomato (Spurgeon et al., 1984) and purified to homogeneity from *C. annuum* chromoplasts (Dogbo and Camara, 1987) and the stroma of mustard (*Sinapis alba*) etioplasts (Laferrière and Beyer, 1991). GGPP synthase has been directly localized to the plastid stroma by immunocytochemical methods (Cheniclet et al., 1992; Kuntz et al., 1992).

The cyclase activity responsible for converting GGPP to casbene is associated with proplastids of castor bean (Dudley et al., 1986). The *ent*-kaurene synthase activity, which catalyzes an early step of gibberellin (GA) biosynthesis, is associated with chloroplasts (Simcox et al., 1975; Moore and Coolbaugh, 1976; Railton et al., 1984). Recently, the Arabidopsis gene for the A activity of *ent*-kaurene synthase has been cloned, and the in vitro–translated protein was shown to be efficiently processed and imported into isolated chloroplasts (Sun and Kamiya, 1994).

Carotenoids and chlorophyll are synthesized in the chloroplasts (for reviews, see Bartley et al., 1994; Bartley and Scolnik, 1995, this issue; von Wettstein et al., 1995, this issue). Prenylquinones and tocopherols are also biosynthesized in the chloroplasts (for reviews, see Schultz et al., 1985; Kleinig, 1989). The mitochondria appear to have an independent system for the biosynthesis of ubiquinones from IPP (Lütke-Brinkhaus et al., 1984). Ubiquinone and plastoquinone formation appears to take place in microsomes as well, possibly in the Golgi apparatus (Swiezewska et al., 1993).

In summary, there are at least three distinct semiautonomous subcellular compartments that segregate terpenoid biosynthesis: the cytosol/ER for sesquiterpenoid and triterpenoid biosynthesis; plastids for monoterpenoid, diterpenoid, and tetraterpenoid biosynthesis (as well as for the prenyl moieties of chlorophyll, plastoquinones, and tocopherols); and mitochondria (and/or Golgi apparatus) for ubiquinone biosynthesis. Control over biosynthesis of these classes of terpenoids can then be exercised by the combination of physical isolation of dedicated enzymes of a particular pathway branch and the rate of production of the basic precursor (IPP) within each compartment or the partitioning of IPP between subcellular compartments. For example, in glandular trichomes of peppermint, the cytosolic mevalonate pathway is blocked at HMG-CoA reductase, so IPP is produced exclusively in the leucoplasts. Although the cytosol has the potential to form high levels of sesquiterpenes, to judge from in situ assays of prenyltransferase and cyclase activity, sesquiterpene production is limited by partitioning of IPP from the plastids (McCaskill and Croteau, 1995). Similar interactions between organelles must occur in the transfer of metabolites from plastids to sites of secondary transformations, such as ER-bound cytochrome P-450 oxygenases and various cytosolic redox enzymes. In many cases, this intracellular exchange leads ultimately to the secretion of the terpenoid end products to various duct or glandular storage cavities.

Temporal Regulation

The developmental regulation of isoprene emission from velvet bean leaves has been examined by R. Fall and associates. Both isoprene emission and isoprene synthase activity increase over 100-fold as leaves develop, peaking at ~14 days after leaf emergence and declining thereafter (Grinspoon et al., 1991; Kuzma and Fall, 1993). These correlative results strongly suggest that the level of isoprene synthase in the leaf is a principal determinant of isoprene production during development.

The production of monoterpenoids in the floral scent of *C. breweri* has been studied in some detail by E. Pichersky (Raguso and Pichersky, 1995). Emission of the acyclic monoterpene linalool (Figure 2) and of several linalool oxides begins upon opening of the flowers and peaks on the second day (Pichersky et al., 1994). Most of the monoterpene emitted comes from the petals, with some coming from the style. Linalool synthase activity was measured and shown to parallel the emission patterns of linalool and the linalool oxides (Pichersky et al., 1994). These results suggest that the production of linalool and its derivatives is directly related to the level of the responsible synthase in the developing flower.

In the case of mint species, the level of monoterpenoid oil accumulated in oil glands over the course of leaf development appears to reflect directly the relative balance between levels of key biosynthetic enzymes (GPP synthase and limonene

cyclase; Figure 3) and catabolic processes (Gershenzon and Croteau, 1993). When considered together, these results strongly suggest that the developmental regulation of terpenoid metabolism resides, at least in part, at the level of the first dedicated steps of biosynthesis of the various structural classes. This is not to say, however, that precursor supply is not a factor in determining the overall rates of terpenoid production (see later discussion).

Aside from developmental regulation, many terpenoids are specifically induced in response to elicitors. C.A. West and associates have investigated the induction of biosynthesis of casbene (Figure 2), an antifungal and antibiotic diterpene of castor bean, in response to elicitor treatment. Fungal infection of castor bean seedlings results in no measurable change in the rate of conversion of mevalonate to IPP. However, the activities of FPP synthase, GGPP synthase, and the diterpene cyclase casbene synthase all increase markedly (Dudley et al., 1986). The increase in casbene synthase activity is preceded by an increase in the level of mRNA encoding the enzyme (Moesta and West, 1985). Nuclear run-on experiments have demonstrated that the increase in mRNA levels results from transcriptional activation of the casbene synthase gene (Lois and West, 1990). Elicitation of casbene biosynthesis in castor bean thus proceeds through at least two levels of control: general induction of the enzymes for biosynthesis of the immediate precursor of diterpenes (GGPP), and specific induction of the enzyme directly responsible for synthesis of casbene. The latter enzyme is regulated at the level of transcription, and the earlier steps may be as well.

Elicitor treatment also induces sequiterpene phytoalexin biosynthesis in tobacco cell suspension cultures, and J. Chappell has been studying induction in this model system. Upon treatment with elicitors, FPP, which is normally employed in sterol biosynthesis, is diverted from the sterol pathway as large amounts of antibiotic sesquiterpenes are produced (Chappell and Nable, 1987; Threlfall and Whitehead, 1988; Vögeli and Chappell, 1988). This diversion is accomplished by the induction of sesquiterpene cyclases and coeval suppression of squalene synthesis, the initial committed step of phytosterol production (Threlfall and Whitehead, 1988; Vögeli and Chappell, 1988). The induction of sesquiterpene cyclase activities, which parallels the increase in sesquiterpenoid phytoalexins, is preceded by a sharp increase in HMG-CoA reductase activity (Chappell and Nable, 1987; Vögeli and Chappell, 1988; Vögeli et al., 1990). Although the increase in HMG-CoA reductase activity is necessary for the induction of sesquiterpene biosynthesis (Chappell et al., 1991b), it does not appear to be the rate-limiting step (Vögeli and Chappell, 1988). Overexpression of a mammalian HMG-CoA reductase in transgenic tobacco plants also confirmed that reductase activity is not limiting for elicitor-induced sesquiterpene biosynthesis (Chappell et al., 1991a), in contrast to what might have been expected based on the mammalian model for the regulatory role of this enzyme (Goldstein and Brown, 1990).

One of the sesquiterpene cyclases, *epi*-aristolochene synthase (Figure 2), has been purified from tobacco cells and subsequently cloned (Vögeli et al., 1990; Facchini and Chappell, 1992). This cyclase produces 5-*epi*-aristolochene, a precursor to capsidiol and debneyol, two prevalent sesquiterpenoid phytoalexins in elicitor-induced tobacco (Whitehead et al., 1989). Induction of this cyclase is regulated at the level of mRNA accumulation, probably at the point of transcription (Vögeli and Chappell, 1990; Facchini and Chappell, 1992). Cloning of the gene, however, revealed the presence of a gene family, suggesting that regulation of different family members may be diverse (Facchini and Chappell, 1992).

Yet another aspect of induced terpenoid biosynthesis is provided by the wound response of some conifers. Research on this aspect of monoterpene and diterpene biosynthesis has been performed in our laboratory. Conifers produce oleoresin, a mixture of monoterpene olefins and diterpenoid resin acids, as a defense secretion that both is toxic to invaders and acts to seal wounds. In certain species, such as pines, oleoresin is produced constitutively at high levels and stored under pressure in ducts, whereas in other species, oleoresin formation is wound inducible at the site of injury (Lewinsohn et al., 1991a; LaFever et al., 1994). In species of *Abies* and *Picea*, wounding causes within 7 days a large increase in monoterpene cyclase activities, representing both the enhancement of constitutive cyclases and the induction of new activities for the production of different products (Gijzen et al., 1991; Lewinsohn et al., 1991a; Funk et al., 1994). Wounding also induces a 400-fold increase in the activities of diterpene cyclases, mainly abietadiene synthase (Figure 2) (LaFever et al., 1994), and a significant increase in the cytochrome P-450 oxygenases involved in the conversion of abietadiene to the principal resin acid, abietic acid (Funk et al., 1994). The coordinate induction of enzymes for the biosynthesis of monoterpenoid and diterpenoid components of oleoresin suggests a common regulatory mechanism, almost certainly at the level of transcription. Another layer of control is imposed by environmental conditions; light and water stresses reduce constitutive monoterpene cyclase protein and activity levels and abolish the wound response (Lewinsohn et al., 1993).

GENETICS

Limited application has been made of classical genetics to the field of terpenoid biosynthesis. Perhaps the most extensive studies of this type have been performed on monoterpene biosynthesis in *Mentha* species by M.J. Murray and co-workers. Using extensive crossing experiments, they identified a number of genes that are involved in the production of essential oil monoterpene components (Hefendehl and Murray, 1976; Lawrence, 1981). Phenotypes were scored only by compositional analysis of major oil constituents; however, some important intermediates are present at trace levels, and the genetic data were unable to order biosynthetic sequences unambiguously. Murray's extensive crossing experiments, for example, did not positively identify any genes regulating

formation of limonene, a key precursor of the major monoterpenes of *Mentha*. Failure to demonstrate, in any but trace quantities, supposed key intermediates of the biosynthetic pathway caused researchers to propose yet more biogenetic schemes (Hefendehl and Murray, 1976; Lawrence, 1981), further confusing the issue.

Biochemical analysis of a Scotch spearmint mutant helped to correct a number of these earlier biogenetic misconceptions. Examination of both oil composition and enzyme activities showed that hydroxylation of limonene at the C3 or C6 position of limonene is the crucial determinant of the respective *p*-menthane components of peppermint and spearmint species (Croteau et al., 1991). The enzymatic machinery preceding and subsequent to the regiospecific hydroxylation step is essentially identical for both types of mint. Experiments such as this demonstrate the necessity of coupling genetic evaluation to detailed biochemical analysis. Once crucial synthetic steps were elucidated through a direct biochemical approach, it was possible to interpret some of the earlier genetic data in terms of known enzymatic activities. A recent review correlates the genetic data with a biochemical evaluation of the monoterpene biosynthetic pathways in mint and proposes genetic associations for monoterpene biosynthetic enzymes (Croteau and Gershenzon, 1994).

Lack of fundamental biochemical studies has also hampered genetic investigations of monoterpene production in conifers. As with early studies in *Mentha*, investigations of genetic control in conifers have distinguished phenotypes largely through compositional analysis of resin. The inherent limitations of this approach are compounded by the fact that many conifer monoterpene cyclases produce more than one product. Interpretation of compositional data has also come under critical attack because of the use of inappropriate statistical methods and lack of consistency in reporting compositional data (for review and critique, see Birks and Kanowski, 1988).

Classical genetics and biochemical analyses have been successfully integrated in the study of GA biosynthesis (for review, see Graebe, 1987). Because properly controlled GA biosynthesis is necessary for normal development of plants, mutants are often readily identifiable, and a wide range of dwarf mutants in a number of species are available for study, including the *ga1* mutant of Arabidopsis and the *an1* mutant of maize. Recently, the *GA1* gene of Arabidopsis was cloned and characterized as encoding the A activity of *ent*-kaurene synthase, which catalyzes the conversion of GGPP to copalyl pyrophosphate (Figure 2), the first committed step in GA biosynthesis (Sun and Kamiya, 1994). The *ga1* mutants had been shown to be blocked in the conversion of GGPP to *ent*-kaurene (Figure 2), but until the gene was cloned and functionally expressed in *Escherichia coli*, it was unknown whether the A activity or the B activity (which catalyzes the conversion of copalyl pyrophosphate to *ent*-kaurene) was affected (Zeevaart and Talon, 1992). The *An1* gene of maize also encodes an *ent*-kaurene synthase. The gene was recently cloned by transposon tagging and is proposed to encode *ent*-kaurene synthase A (Bensen et al., 1995).

MOLECULAR BIOLOGY

In recent years, several genes involved in terpenoid biosynthesis have been cloned. A large number of plant genes for HMG-CoA reductase have been isolated and sequenced. In animals, this reductase catalyzes the regulatory step in the biosynthesis of sterols. Several feedback regulatory mechanisms control transcription of the reductase gene, and its catalytic activity is modulated by both covalent modification and noncovalent interactions (Goldstein and Brown, 1990). In contrast, plants, which produce much more diverse products from the mevalonate pathway in various tissues and organelles, contain multiple, differentially regulated genes for HMG-CoA reductase (Narita and Gruissem, 1989; Yang et al., 1991; Choi et al., 1992; Chye et al., 1992; Genschik et al., 1992; Park et al., 1992; Burnett et al., 1993; Enjuto et al., 1994). Different reductase genes are expressed in different parts of the plant (Chye et al., 1992; Genschik et al., 1992; Enjuto et al., 1994), at different stages of development (Narita and Gruissem, 1989), and in response to various stresses (Yang et al., 1991; Choi et al., 1992; Genschik et al., 1992). In potato tubers, for example, different reductase genes are induced in response to pathogen attack and wounding (Yang et al., 1991; Choi et al., 1992). With the availability of molecular probes, it is now possible to dissect the complex regulation of HMG-CoA reductase in plants.

Little is known about the genes encoding other enzymes of the mevalonate pathway leading to IPP. It is likely that differential expression of these genes, as with HMG-CoA reductase, in specialized tissues and subcellular compartments is an important feature in the global regulation of plant terpenoid metabolism. However, without accurate measurements of pathway flux and intermediate concentrations, it is difficult to assess fully which steps of the mevalonate pathway in plants are of greatest regulatory significance in the supply of IPP.

Few genes for prenyltransferases have been isolated from plant sources. Two GGPP synthase genes have been cloned, from *C. annuum* (Kuntz et al., 1992) and Arabidopsis (Scolnik and Bartley, 1994), but much of what we know about prenyltransferases is derived from work with animal and microbial systems. The prenyltransferases show significant conservation among plants, animals, fungi, and bacteria, with five distinct regions of high similarity at the amino acid level (Chen et al., 1994). Most of the sequences group by kingdom, suggesting a common ancestor for both prokaryotic and eukaryotic prenyltransferases (Chen et al., 1994). However, the gene for GGPP synthase from chromoplasts of *C. annuum* (Kuntz et al., 1992) segregates with the eubacterial prenyltransferases, suggesting that this gene may have been transferred from a bacterial symbiont (Chen et al., 1994).

Photoaffinity labeling identified an arginine residue at the putative active site of the prenyltransferase FPP synthase from avian liver (Brems and Rilling, 1979; Brems et al., 1981). A Lys → Glu mutation in this region of the yeast FPP synthase results in considerably decreased activity (Blanchard and Karst,

1993); however, directed mutagenesis of the putative active site arginine in the rat liver enzyme has little effect on activity (Joly and Edwards, 1993). Two highly conserved sequence domains of the prenyltransferases contain the consensus sequence DDXX(XX)D (Ashby and Edwards, 1990; Ashby et al., 1992; Chen et al., 1994), proposed as binding sites for the metal ion–complexed pyrophosphate moieties of the two diphosphate ester substrates (Ashby and Edwards, 1990). Site-directed mutagenesis of aspartate and arginine residues in these conserved regions of FPP synthases from yeast (Song and Poulter, 1994) and rat liver (Marrero et al., 1992; Joly and Edwards, 1993) results in greatly impaired catalysis. The K_m value for IPP in these mutants is elevated, but the K_m value for GPP, the allylic cosubstrate, remains relatively unchanged (Marrero et al., 1992; Joly and Edwards, 1993; Song and Poulter, 1994). Modification of the C terminus of FPP synthase from yeast (Song and Poulter, 1994) and *Bacillus stearothermophilus* (Koyama et al., 1993) also elevates the K_m value for IPP. These experiments suggest that at least one of the conserved DDXX(XX)D regions and a C-terminal domain play a role in IPP binding, the alteration of which would be expected to compromise the condensation and product release steps of the reaction cycle.

FPP synthases from chicken liver (Reed and Rilling, 1975; Tarshis et al., 1994) and *B. stearothermophilus* (Koyama et al., 1993) have been crystallized, and the x-ray crystal structure of the avian enzyme has been solved at high resolution (Tarshis et al., 1994). The crystal structure reveals a central cleft in the FPP synthase homodimer, with the conserved DDXXD regions located on opposite sides of this cleft (Tarshis et al., 1994). Furthermore, by substituting samarium as an analog of the normal Mg^{2+} cofactor, the crystal structure showed that these two regions are metal ion binding sites (Tarshis et al., 1994). These results are consistent with the mutagenesis data and have important implications for identifying critical regions of the plant prenyltransferases as well as of the terpenoid cyclases, most of which contain similar DDXXD sequences.

Several plant terpene synthase genes have been cloned, including the one for limonene synthase (Figure 3), a monoterpene synthase from spearmint (Colby et al., 1993). The *E. coli*–expressed protein produces the same distribution of multiple monoterpene products as the native enzyme, confirming that these result from the activity of a single enzyme (Colby et al., 1993). One other monoterpene synthase, a linalool synthase (Figure 2), has been cloned from *C. breweri* (E. Pichersky, personal communication). Three diterpene cyclases have also been cloned: casbene synthase from castor bean (Mau and West, 1994); abietadiene synthase (Figure 2) from grand fir (B. Stofer Vogel and R. Croteau, unpublished results); and two genes for the A activity of *ent*-kaurene synthase (Figure 2), the Arabidopsis *GA1* gene (Sun and Kamiya, 1994) and the maize *An1* gene (Bensen et al., 1995). A sesquiterpene cyclase from tobacco, *epi*-aristolochene synthase (Figure 2), has also been cloned (Facchini and Chappell, 1992). It was shown to be part of a tobacco gene family, and two of the genes are arranged in tandem (Facchini and Chappell, 1992). The genomic structures of the exons of tobacco *epi*-aristolochene synthase and castor bean casbene synthase are remarkably similar (Mau and West, 1994).

Comparing the deduced amino acid sequences of the terpene synthase genes reveals a fair degree of homology among them (Colby et al., 1993; Mau and West, 1994; Chappell, 1995). These synthases, however, bear little or no homology with fungal or bacterial terpenoid cyclases or with any of the prenyltransferases. Even the tobacco *epi*-aristolochene synthase bears little homology with the fungal aristolochene synthase. All of the plant protein sequences include DDXXD sequences, except the two *ent*-kaurene synthase genes (Sun and Kamiya, 1994; Bensen et al., 1995). The two *ent*-kaurene synthases and the abietadiene synthase, however, segregate together by sequence similarity (Figure 4). The *ent*-kaurene synthase A activity catalyzes the formation of (−)-copalyl pyrophosphate (Figure 2) from GGPP by a proton-initiated cyclization at the olefinic terminus of the substrate, without ionization and loss of the pyrophosphate moiety. On the other hand, abietadiene synthase from grand fir catalyzes the enantiomeric reaction to (+)-copalyl pyrophosphate followed by a cyclization that proceeds by ionization and loss of the pyrophosphate moiety (to yield pimeradiene and, by rearrangement, abietadiene) (Figure 2). The latter cyclization corresponds to the B-type activity of *ent*-kaurene synthase and is a more typical terpenoid cyclization reaction than the proton-initiated cyclization catalyzed by *ent*-kaurene synthase A. The abietadiene synthase contains a DDXXD region (B. Stofer Vogel and R. Croteau, unpublished data), as do the aforementioned monoterpene, sesquiterpene, and diterpene cyclases (limonene synthase, *epi*-aristolochene synthase, and casbene synthase) that involve the more typical cyclization initiated by ionization of the substrate pyrophosphate ester bond. This observation suggests that DDXXD regions are involved in binding the metal ion–complexed pyrophosphate group and assisting in substrate ionization.

Linalool synthase (Figure 2) is the most distantly related of the terpenoid synthase genes (Figure 4). This distant relationship is perhaps not surprising, however, because linalool synthase catalyzes a simple ionization with water trapping of the carbocation, rather than the inter- or intramolecular carbon–carbon bond formation catalyzed by the prenyltransferases and terpenoid cyclases. Nonetheless, the sequence similarity places linalool synthase closer to the other terpenoid synthases than to the prenyltransferases. The mechanisms of action of limonene synthase, *epi*-aristolochene synthase, and casbene synthase with their respective C_{10}, C_{15}, and C_{20} substrates are quite similar, and these cyclase genes, accordingly, segregate together.

FUTURE PROSPECTS

The field of terpenoid biochemistry has come a very long way since Ruzicka (1953) first proposed the active isoprene unit, and there has been a renaissance of activity over the last

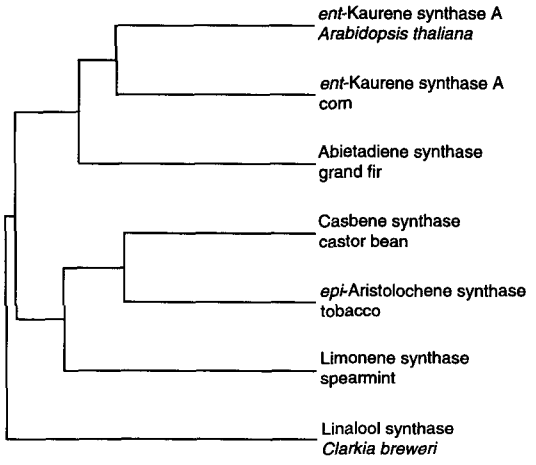

Figure 4. Sequence Relationships among Terpene Synthases.

Amino acid sequences were analyzed by the PILEUP program from the Genetics Computer Group Packet (Genetics Computer Group, 1994).

decade with the application of molecular biology to this area of research. The availability of the genes and overexpressed proteins for terpenoid cyclases allows mechanistic details and structure–function relationships to be deduced at a level heretofore impossible. Manipulation of these enzymes through directed mutagenesis, their use at substrate levels to study reaction parameters, and their x-ray crystallographic analysis can be expected to reveal a wealth of mechanistic richness in the construction of the various terpenoid types that goes well beyond the view now apparent from studies with animal and microbial enzymes, particularly prenyltransferases, with their more limited catalytic repertoire. Comparison of sequence and mechanism should also provide clues to the origin and evolutionary wellspring that gave rise to the enormous diversity of this family of plant products.

The cloning of plant HMG-CoA reductase genes based on homologies with their animal and microbial counterparts suggests that the remaining plant genes directing the synthesis of IPP can be similarly obtained. With nucleic acid probes and antibodies, it will be possible to explore the regulation of the IPP supply in greater detail and to define further the organization of terpenoid metabolism, especially those features of compartmentation and targeting that are clearly important for subsequent transgenic exploitation.

Classical biochemistry will continue to play an important role in determining cellular regulatory features (for example, possible feedback loops involving allosterism or covalent modification) and in measuring flux and intermediate concentrations to provide essential metabolic context. With this level of understanding, directed transgenic manipulation of terpenoid biosynthesis should be possible to enhance the flavor and color of foodstuffs, increase the yields of individual compounds of commercial significance, and engineer defenses against pests and pathogens. The possibilities, like the forms and functions of terpenoid biosynthesis, seem nearly limitless.

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