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REVIEW

Metabolic Engineering of Isoprenoids

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The metabolic engineering of natural products has begun to prosper in the past few years due to genomic research and the discovery of biosynthetic genes. While the biosynthetic pathways and genes for some isoprenoids have been known for many years, new pathways have been found and known pathways have been further investigated. In this article, we review the recent advances in metabolic engineering of isoprenoids, focusing on the molecular genetics that affects pathway engineering the most. Examples in mono- sequi-, and diterpenoid synthesis as well as carotenoid production are discussed.

Isoprenoids (also known as terpenoids) are a class of secondary metabolites found in all species. They derive their name from isoprene, a branched five-carbon molecule that is the basic building block of this class of compounds. Wallach noted in 1914 that many compounds, particularly the essential oils of plants, could be dissected into five-carbon isoprene units. These compounds were called terpenes, the name derived from the terebinth tree, *Pistacia terebinthus*, which contains a terpenoid resin. The "isoprene rule" was formulated by Wallach and modified by Ruzicka (Ruzicka, 1953) and states that terpenes are formed by the repetitive joining of isoprene units linked head to tail. Based on this rule, terpenes were classified based on the number of isoprene units present. Monoterpenes have 10 carbons (C_{10}), sesquiterpenes have 15 carbons (C_{15}), diterpenes have 20 (C_{20}), sesterterpenes have 25 (C_{25}), triterpenes have 30 (C_{30}), and tetraterpenes have 40 carbons (C_{40}). It became obvious that certain groups of compounds like steroids (C_{27}) and related compounds violated the rule. This was rationalized by the fact that the head to tail principle could be modified and the original skeleton could be cleaved or rearranged. This modifications lead to the vast number of compounds encompassed by the isoprenoids.

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There are many different types of isoprenoids that serve a number of different functions. Steroids are present in most eukaryotes and fulfill roles such as maintaining membrane fluidity and acting as hormones and bile acids. Carotenoids are necessary for photosynthetic organisms and can also act as antioxidants. Ubiquinone, menaquinone, and plastoquinone are involved in electron transport and dolichol is involved in protein glycosylation. The other terpenes (mono-, di-, tri-, etc.) play less essential roles, but can be found throughout nature, particularly in plants and marine invertebrates, and many have been found to have significant pharmaceutical importance. Isoprenoids attached to regulatory proteins have been shown to regulate cellular development. As is the case with many alkaloids and the quinones involved in electron transport, isoprenoids form parts of a number of different classes of metabolites constructed by mixed biosynthesis.

Since the development of new genomic techniques, metabolic engineers have been empowered with the ability to move ahead at much faster speeds. A great deal of work has already been accomplished in the metabolic engineering of polyketides. This work has shown great pharmaceutical promise and has been aided by the sequencing of genomes of the microorganisms that produce these powerful antibiotics. Terpenoids have been much more difficult to work with since they are more common in higher organisms whose genomes are not as well investigated. Most of the work in the metabolic engineering of isoprenoids has been focused on carotenoids as the genes involved in their biosynthesis have become more widely available and product identification is readily achievable by color screening. Since the terpenoids encompass such a vast number of bioactive compounds, there is a great deal of ongoing basic research into the genetics of these secondary metabolites and as the information becomes more available, the metabolic engineering of isoprenoids will flourish. This review will focus on the engineering that has been done in the major classes of isoprenoids and will touch on the developments in basic research that will drive metabolic engineering in the future.

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1. DIFFERENT PATHWAYS FOR ISOPRENOID BIOSYNTHESIS

For many years, scientists believed that the pathway of isoprenoid compounds was well understood. Labeling experiments with ^{14}C-labeled substrates indicated that the pathway was as shown in Fig. 1B, beginning with acetyl-CoA and proceeding through mevalonic acid to isopentenyl pyrophosphate (IPP). This path to isoprenoids was known as the mevalonic acid (MVA) pathway.

Since the 1950s, many discrepancies appeared that jeopardized the role of MVA as the sole isoprenoid precursor. For example, isotopically labeled MVA and acetate were usually not well incorporated into carotenoids and monoterpenes in plant systems while they were incorporated quite well into other isoprenoids such as steroids. In 1993, Rohmer *et al.* discovered a separate pathway by doing ^{13}C NMR labeling experiments with eubacterial hopanoids using glyceraldehyde 3-phosphate (G3P) and pyruvate as precursors (Rohmer *et al.*, 1993). This pathway (see Fig.

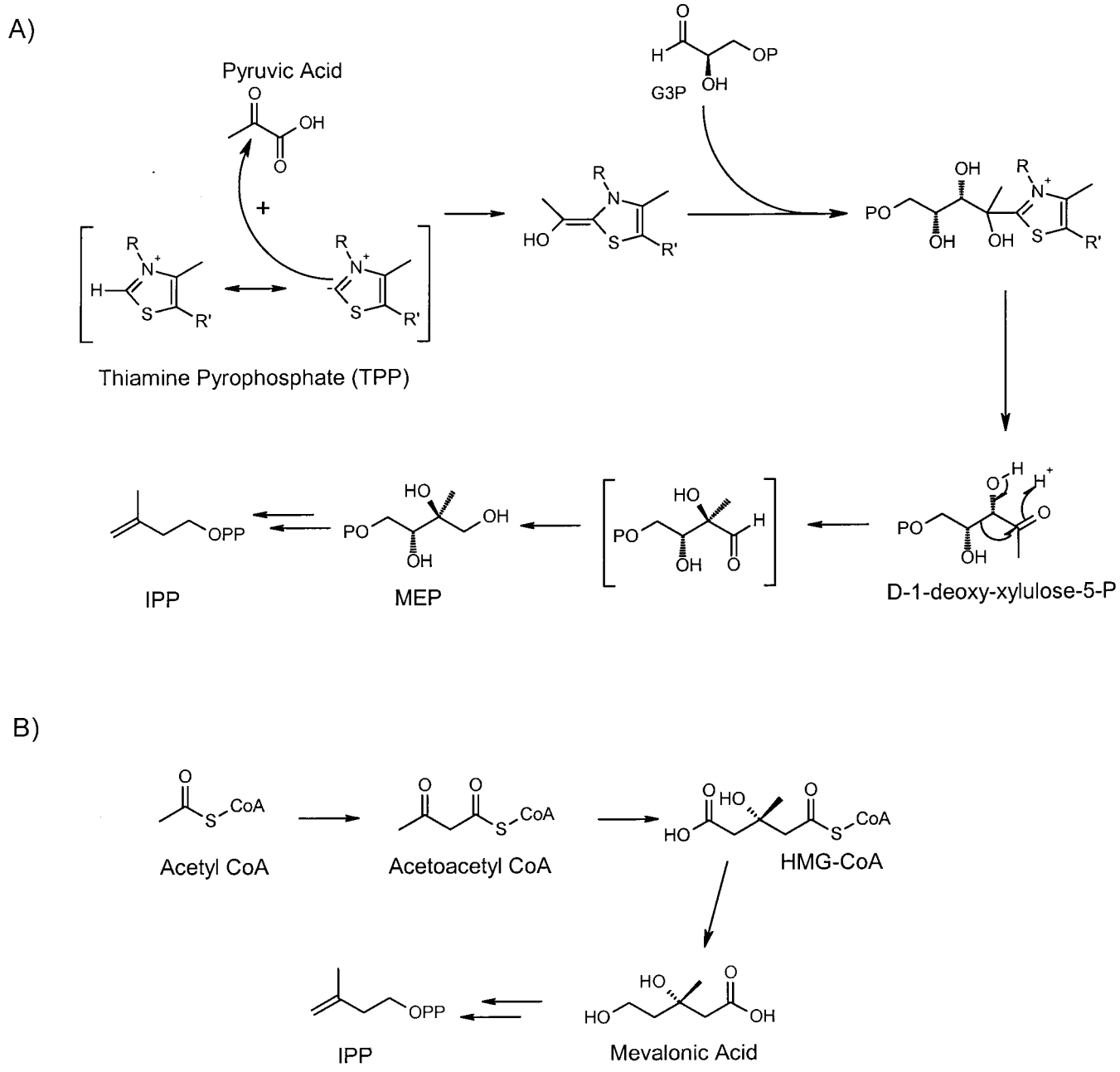

FIG. 1. Pathways to isopentenyl pyrophosphate (IPP). (A) G3P/pyruvate pathway. (B) Mevalonic acid (MVA) pathway.

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1A) proceeds through 1-deoxy-d-xylulose-5-phosphate (DXP) and 2-methyl-d-erythritol-4-phosphate (MEP) via the enzymes 1-d-deoxyxylulose 5-phosphate synthase (*dxs*) (Sprenger *et al*., 1997; Lois *et al*., 1998; Lange *et al*., 1998; Bouvier *et al*., 1998) and 1-d-deoxyxylulose 5-phosphate reductoisomerase (*dxr*) (Takahashi *et al*., 1998; Schwender *et al*., 1999), respectively. The history and mechanism of this pathway has been nicely presented in a recent review (Rohmer, 1999). During the review process of this article, additional genes in this pathway have been identified, which include *ygbP* (Rohdich *et al*., 1999; Kuzuyama *et al*., 2000a), *ychB* (Luttgen *et al*., 2000; Kuzuyama *et al*., 2000b), and *ygbB* (Takagi *et al*., 2000; Herz *et al*., 2000). These genes have been renamed *ispD*, *ispE*, and *ispF*, respectively (Rohdich *et al*., 2000).

For the purposes of metabolic engineering, it is useful to know which isoprene units are derived from the MVA pathway and which are derived from the G3P/pyruvate pathway. Many species of bacteria have been shown to utilize the G3P/pyruvate pathway for isoprenoid biosynthesis, but the MVA pathway is also used in some bacteria. Labeling experiments have proved the MVA pathway exists in *Staphylcoccus carnosus* (Horbach *et al*., 1993), *Myxococcus fulvus* (Lois *et al*., 1998), *Chloroflexus aurantiacus* (Rieder *et al*., 1998) and several species of archaebacteria (Moldoveanu and Kates, 1988; Kakinuma *et al*., 1988; Eguchi *et al*., 1998). In addition to labeling studies, two metabolic inhibitors are available to help establish the identity of the operative pathway, mevinolin for the MVA pathway and fosmidomycin for the pyruvate/G3P pathway. Gene searching also provides a strong evidence for the existence of a pathway. A nice example is the finding of a cluster of MVA pathway genes in *Streptomyces* (Takagi *et al*., 2000). While there is not enough evidence to make any conclusions about the general use of either pathway, suffice it to say the G3P/pyruvate pathway is widespread amongst eubacteria.

There has been a great deal of research done on eukaryotic phototrophic organisms that shows widespread use of the G3P/pyruvate pathway. Experiments performed with the green alga *Scenedesmus obliquus* (Schwender *et al*., 1996) showed incorporation of ^{13}C-labeled acetate or glucose identical to that of bacteria. The same labeling experiments were performed with higher plants (Lichtenthaler *et al*., 1997) with surprising results. Some of the isoprenoids such as sterols were found to be formed via the MVA pathway, while phytol, carotenoids, and plastoquinone were formed via the G3P/pyruvate pathway. This led to the conclusion that isoprenoids formed in the cytoplasm, such as sterols and triterpenes, utilized the MVA pathway, while isoprenoids formed in the chloroplasts (phytol, carotenoids, plastoquinone, and many smaller terpenes) utilized the G3P/pyruvate pathway. It has been shown that isoprenoid precursors, IPP, GPP, FPP, and GGPP, from one pathway can be incorporated into the other pathway (Schwarz, 1994). Even though bacteria and plants show evidence of the G3P/pyruvate pathway, it is interesting to note that fungi and yeast do not seem to use this pathway at all (Disch and Rohmer, 1998).

After either the G3P/pyruvate pathway or the MVA pathway has synthesized IPP, the next step is chain elongation. Due to its lack of reactivity, IPP must first be isomerized to dimethylallyl pyrophosphate (DMAPP), a transformation catalyzed by the enzyme isopentenyl pyrophosphate isomerase (*idi*). These two species then condense to form longer chain isoprenoids. The enzyme used to catalyze this reaction varies depending upon the chain length needed.

The first condensation reaction forms geranyl pyrophosphate and is catalyzed by the enzyme geranyl pyrophosphate synthase. DMAPP and other allylic phosphates are potent alkylating agents based on the strength of the phosphate as a leaving group and the carbonium ion stabilization provided by charge delocalization. The allylic cation intermediate is attacked by IPP to form the product, geranyl pyrophosphate. To make longer chain lengths, further iterations of this process are required.

There are many types of diphosphate (pyrophosphate) synthases which are classified based upon the length of the chain (Ogura and Koyama, 1998). The short-chain prenyl diphosphate synthases consist of GPP-, FPP-, and GGPP-synthases. These species are the starting materials for the monoterpenes, sesquiterpenes, and diterpenes, respectively. FPP and GGPP are also used to prenylate a number of proteins utilized in cellular processes (Grunler *et al*., 1994). In addition, FPP is the precursor to squalene and the entire steroid family while GGPP is the precursor to the carotenoids. All of these species can be attached together to form longer prenyl chains. The medium-chain prenyl diphosphate synthases, including hexaprenyl, heptaprenyl, and octaprenyl pyrophosphate synthases, are used as side chains (tails) for biological quinones such as ubiquinone, menaquinone and plastoquinone. The chain length of these molecules is species-specific (Grunler *et al*., 1994). Long-chain prenyl diphosphate synthases include nonaprenyl (solanesyl/C_{45}) pyrophosphate synthase, decaprenyl (C_{50}) pyrophosphate synthase, and undecaprenyl (C_{55}) pyrophosphate synthase. These are used as tails for biological quinones, depending on the species involved. The last group includes *cis*-polyprenyl pyrophosphate synthases that are used to create dolichols and various rubbers. The protein sequences for a number of prenyl pyrophosphate synthases have been studied and their secondary structures were predicted (Chen *et al*., 1994). Many prenyl pyrophosphate

synthases have been purified and characterized in recent years and have been reviewed (Ogura and Koyama, 1998).

FPP synthase is an extremely important enzyme due to its role in sesquiterpene and steroid biosynthesis as well as protein farnesylation. FPP synthase from *Bacillus stearothermophilus* has been overexpressed in *Escherichia coli* (Koyama *et al*., 1993). These strains are able to produce thermostable FPP synthase in amounts as large as 20% of the total soluble proteins. In addition, it is easily purified due to its thermostability. Yeast FPP synthase has been overproduced and purified by epitope tagging (Song and Poulter, 1994), and synthases from rat, human, and avian livers have been overexpressed and purified (Tarshis *et al*., 1994; Marrero *et al*., 1992; Ding *et al*., 1991).

The use of random mutagenesis is a useful method for making a desired mutation if an effective screening method can be found. GGPP synthase of *Sulfolobus acidocaldarius* was cloned using the carotenoid biosynthetic genes of *Erwinia uredovora* to produce a red clone with its color dependent on the expression of GGPP synthase (Ohnuma *et al*., 1994). This same group mutagenized *B. stearothermophilus* using $NaNO_2$ to make a library of FPP synthase mutants. By utilizing the carotenoid screening method, they found 11 positive clones out of over 24,000 mutants (Ohnuma *et al*., 1996). Analysis of these mutants showed three amino acids were extremely important in producing the final chain length. Further studies of these mutants showed that amino acid changes at position 81 could produce chain lengths longer than GGPP (Ohnuma *et al*., 1996, 1998). An archaeal GGPP synthase from *Sulfolobus acidocaldarius* has been overexpressed in *E. coli* with higher specific activity and thermostability than the native enzyme (Ohto *et al*., 1998). A GGPP synthase from *Thermus thermophilus* has been cloned in *E. coli* with similar results (Ohto *et al*., 1999). Another archaeal GGPP synthase from *Archaeoglobus fulgidus* has been cloned in *E. coli* as an efficient way to synthesize GGPP from IPP for carotenoid production (Wang *et al*., 1999). GGPP synthases from rat and human have been cloned and expressed in *E. coli* even though the bacterial homologs have considerably fewer sequence similarities than those of lower eukaryotes (Kainou *et al*., 1999). Expression of the mouse and human genes was detected by the carotenoid method mentioned above.

In the study of ubiquinone biosynthesis, several cases of metabolic engineering of polyprenyls have been investigated. The *ispB* gene and *COQ1* genes encode octaprenyl pyrophosphate synthase in *E. coli* and hexaprenyl pyrophosphate synthase in *Saccharomyces cerevisiae*, respectively. The *ispB* gene complemented a *coq1* delete mutant of *S. cerevisiae* on nonfermentable media and produced ubiquinone-8 instead of the ubiquinone-6 that is normally found in this yeast, showing that the length of the chain was dependent on the polyprenyl pyrophosphate synthase involved (Okada *et al*., 1996). Similarly, homologs of *ispB* from *Haemophilus influenzae* and *Synechocystis* sp. strain PCC6803 produced their standard ubiquinone-7 and ubiquinone-9 when substituted for *ispB* in *E. coli* (Okada *et al*., 1997a,b). In a separate study, avian FPP synthase was converted to a GGPP synthase via site-directed mutagenesis (Tarshis *et al*., 1996). Solanesyl pyrophosphate synthase has been isolated from *Rhodobacter capsulatus* and expressed in both *S. cerevisiae* and *E. coli* (Okada *et al*., 1997a,b). Decaprenyl pyrophosphate synthase was cloned out of *Gluconobacter suboxydans* (Okada *et al*., 1998) and overexpressed in *E. coli*. It produced ubiquinone-10, which is the species found in humans.

2. MONO-, SESQUI-, AND DITERPENOIDS

Monoterpenoids are found most commonly in plants and insects as essential oils and hormones. They are formed from geranyl pyrophosphate (GPP) and often undergo cyclization reactions to form one of the many cyclic monoterpene skeletons. Each set of skeletons then undergoes further secondary transformations leading to the vast amount of monoterpenes we encounter in nature. Monoterpenes are biosynthesized in the plastids of plants from the G3P/pyruvate pathway and in other higher organisms and yeasts from the MVA pathway. Monoterpenes have a widespread economic value with uses ranging from flavors (e.g., limonene) and fragrances (e.g., geraniol, citranellol) to anticancer activity (Burke *et al*., 1997; Crowell, 1999). They also have numerous functions in the realm of chemical ecology as chemical defenses and as hormones (Hick *et al*., 1999). Many new monoterpene structures and synthases have been discovered in recent years. While it is not our purpose to review the many new chemical and genetic discoveries in monoterpenes, sesquiterpenes, and diterpenes, there are excellent reviews available (Grayson, 1998; Fraga, 1999; Hanson, 1999).

Most monoterpenes are formed by the conversion of GPP to (3*S*)- or (3*R*)-linalyl pyrophosphate followed by cyclization (see Fig. 2). The isomerization and cyclization reactions are often catalyzed by the same enzyme. Monoterpenes need not be cyclic as the biosynthesis of the monoterpene (*S*)-linalool proceeds only as far as linalyl pyrophosphate (LPP) before it is quenched by water. The coding sequence for (*S*)-linalool synthase from *Clarkia breweri* has recently been isolated and characterized (Dudareva *et al*., 1996).

The cyclase limonene synthase catalyzes the isomerization-cyclization of GPP to limonene. Limonene has considerable commercial value as a flavor and as a possible

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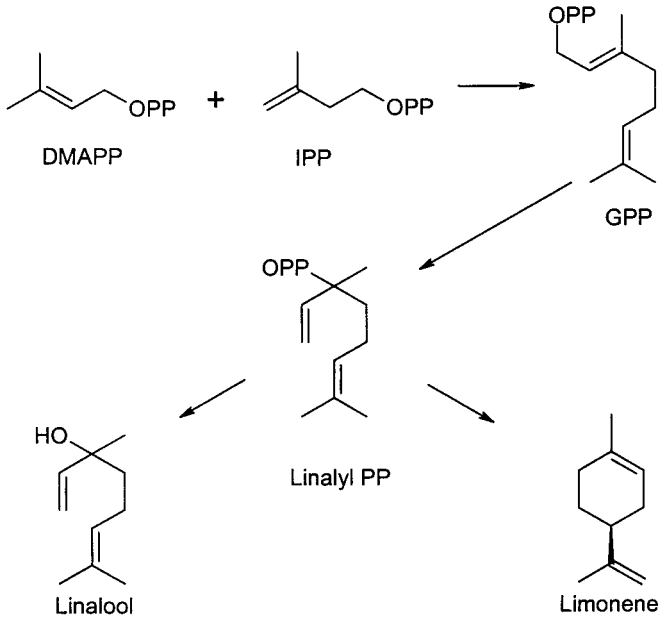

FIG. 2. Formation of monoterpenes from DMAPP and IPP. Linalool and limonene are shown as examples of cyclic and noncyclic monoterpenes.

anti-carcinogen. In the liverwort *Ricciocarpos natans*, in which limonene is the major monoterpene metabolite, a soluble limonene synthase has been purified and characterized (Adam *et al*., 1996). Ten cDNAs with homology to spearmint limonene synthase DNA have been isolated from *Perilla frutescens* and two have been cloned into *E. coli* (Yuba *et al*., 1996).

As the availability of new terpenoid genes increases, the rate of metabolic engineering studies will increase proportionately. In recent years, all of the rudimentary isoprenoid genes such as deoxyxylulose phosphate synthase and hydroxymethylglutaryl CoA reductase have been identified and characterized in *E. coli*, and much work has been done to characterize genes that code enzymes that are further downstream such as monoterpene cyclases and hydroxylases. As a result efforts to engineer these molecules in transgenic organisms has begun.

Monoterpenes are generally synthesized in different glandular structures of plants, but work has been done to change the site of synthesis of these compounds. One such study was performed in which phytoene synthase from daffodils was transformed into rice endosperm in order to overproduce vitamin A (Burkhardt *et al*., 1997). Specific promoters were used to target biosynthesis of the metabolites to a particular type of tissue. Changing the endogenous site of biosynthesis of a metabolite can also create difficulties. In one study, it was shown that the consequence of the overexpression of HMG CoA in tobacco was the overproduction of sterols by the cytosolic MVA pathway which led to accumulation of lipids in the cytoplasm (Schaller *et al*., 1995).

Another example of tissue-specific expression of monoterpenes is in the mint family. Calli (Spencer *et al*., 1993) and teratomas (Berry *et al*., 1996) have been transformed using *Agrobacterium tumefaciens* and expression of transient reporter genes has also been accomplished (Caissard *et al*., 1996). In 1998, transgenic peppermint was obtained by transformation with *A. tumefaciens* by two separate groups (Niu *et al*., 1998; Diemer *et al*., 1998). In addition, several promoters have been discovered that direct expression to specific areas of the plant including the epidermis (Canevascini *et al*., 1996; Bilang and Bogorad, 1996; Mandaci and Dobres, 1997; Hung *et al*., 1998), and nonglandular trichromes (Larkin *et al*., 1993; Pyee and Kolattukudy, 1995).

Sesquiterpenes are derived from farnesyl pyrophosphate (FPP) in the same way monoterpenes are derived from GPP. FPP can then be cyclized to form different sesquiterpenes as shown in Fig. 3. Some examples of useful sesquiterpenes are periplanone B, a cockroach hormone used for traps, the antimalarial drug artemisinin and the platelet-activating factor antagonist ginkgolide B.

(+)-Aristolochene has been found in a number of fungi and its antipode has been found in leaf oils from a number of plants (Cane, 1990). (+)-Aristolochene synthase has been isolated and purified from *Penicillum roqueforti* (Hohn and Plattner, 1989) and its cDNA overexpresse d in *E. coli* (Cane *et al*., 1993). *epi*-Arisotolochene synthase has been purified from tobacco (Vogeli and Chappell, 1990) and overexpressed in *E. coli* (Facchini and Chappell, 1992). Vetispiradiene synthase has been cloned from *Hyoscyamus muticus* and expressed in *E. coli* (Back and Chappell, 1995). Pentalenene is the precursor for the pentalenolactone family of antibiotics. Pentalenene synthase has also been purified and overexpressed in *E. coli* (Cane *et al*., 1994). δ-Cadinene is a metabolite produced in juniper leaf oil and is also produced as a precursor of defensive phytoalexins in cotton. The cDNA for cotton (+)-δ-cadinene synthase has been cloned and overexpressed in *E. coli* (Chen *et al*., 1995).

Trichodienes are a widely studied family of sesquiterpenes as they are a family of toxins biosynthesized by a number of fungi (Cane and Ha, 1988; Cane *et al*., 1992; Cane and Yang, 1994). Trichodiene synthase has been purified from *Fusarium sprotrichioides* and overexpressed in *E. coli* (Cane *et al*., 1993).

Geranyl geranyl pyrophosphate (GGPP) is the precursor of the diterpenes and is usually produced from GGPP synthase (Fig. 4). Casbene is a diterpene with antibacterial and antifungal activity that can be found in castor bean seedlings (*Ricinus communis*). Casbene is formed by the cyclization of GGPP to casbene by casbene synthase. This

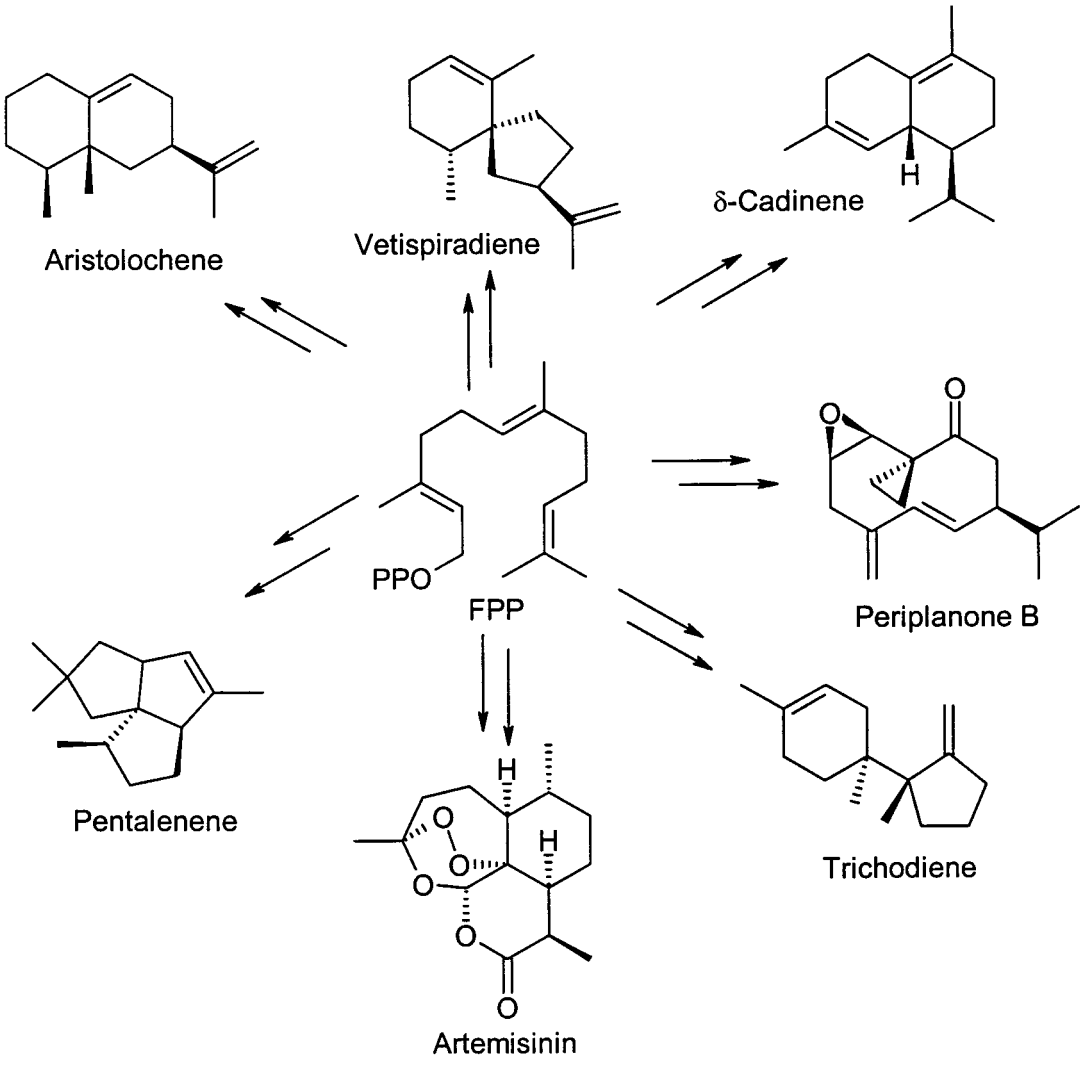

FIG. 3. Examples of sesquiterpenes. FPP is converted in several steps to the various sesquiterpenes shown above.

enzyme has been recently overexpressed and characterized in *E. coli* (Hill *et al.*, 1996). Abietadiene cyclase, which is involved in the synthesis of the tree resin abietadiene, has been partially purified from evergreen extracts (Lafever *et al.*, 1994) and its cDNA has been overexpressed in *E. coli* (Vogel *et al.*, 1996). Another diterpene of note is paclitaxel (Taxol). Many biosynthetic studies of paclitaxel have been performed in recent years and have been nicely reviewed (Hezari and Croteau, 1997). This work should lead to the metabolic engineering of Taxol if it continues on its present course.

3. CAROTENOIDS

Most of the work to date in the metabolic engineering of isoprenoids has been done using carotenoids primarily because of the easy color screening. Carotenoids are a class of tetraterpenoid pigments that are often associated with flowers and fruits and are known as potent antioxidants. All chlorophyll-containing photosynthetic organisms produce carotenoids, as do red yeasts and many types of fungi (Britton, 1983; Goodwin, 1980). They are also widely found in all photosynthetic and in many nonphotosynthetic eubacteria. They serve a number of industrial uses ranging from coloring agents for agriculture, food, and cosmetics (Goodwin, 1980; Bauernfeind, 1981), precursors of vitamin A in humans and animals (Livrea and Vidali, 1994), feed supplements for poultry and fish (Bauernfeind, 1981), and potential anticancer agents (Mayne, 1996).

The genetic pathway for the production of carotenoids has been studied extensively in a number of species, thus opening the door to the bioengineering of these compounds. Armstrong defined the pathway for the biosynthesis of

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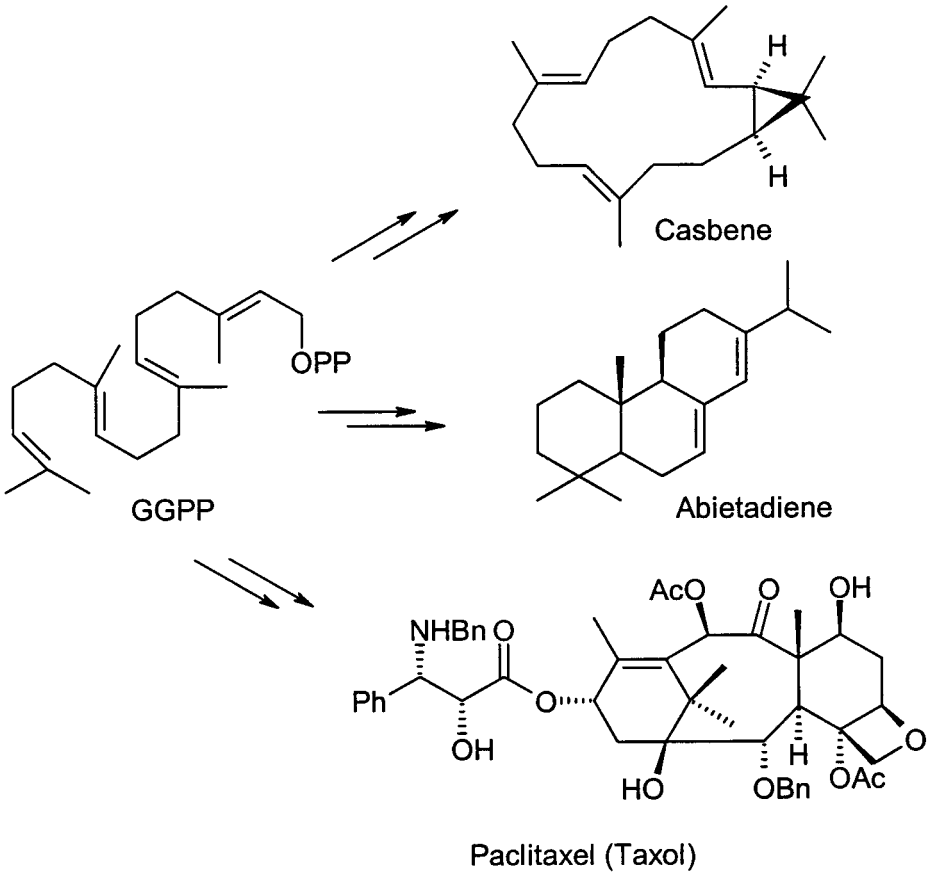

FIG. 4. All of these diterpenes are derived from GGPP, but they vary in the number of steps to the product. Casbene and abietadiene are formed from GGPP in several steps by the single enzymes casbene synthase and abietadiene synthase while paclitaxel is formed from a large number of enzymes.

carotenoids in *R. capsulatus* (Armstrong *et al*., 1989, 1990) and Misawa described the carotenoid pathway in *E. uredovora* (Fig. 5) (Misawa *et al*., 1990). Shortly afterward, the pathway for *Erwinia herbicola* was documented (Hundle *et al*., 1994; Schnurr *et al*., 1991).

Carotenoids are synthesized within the plastids of plants using G3P/pyruvate as the source of IPP (Lichtenthaler *et al*., 1997). IPP is isomerized to DMAPP by the *idi* gene product and then proceeds on to geranylgeranyl pyrophosphate (GGPP) via the enzyme GGPP synthase. The first committed step in carotenoid biosynthesis is the head-to-head condensation of two GGPP units to form phytoene. This step is catalyzed by the enzyme phytoene synthase (PSY in plants; CRTB in bacteria). Phytoene is converted to lycopene in two steps by the single enzyme phytoene desaturase (CRTI) in bacteria and by two separate enzymes, phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) in plants. The nomenclature used by cyanobacteria is somewhat different from that used for the plant and bacterial genes (Armstrong, 1997).

While there are relatively few genes available to produce C_{30} carotenoids (Wieland *et al*., 1994), there are a large selection available for the synthesis of C_{40} carotenoids. The majority of the work in carotenoid engineering in tetraterpene carotenoids has been done with the bacterial *crt* genes from *Erwinia* species. The first product in the carotenoid pathway is phytoene, formed via the *crtB* genes. There are a number of phytoene desaturase genes available to attain various levels of unsaturations in the chain. Genes for lycopene cyclase that can lead to β- or ε-carotenes have also been cloned and used in engineering studies. Of all the carotenoid genes, only zeaxanthin epoxidase from *Nicotiana* has been unable to be expressed in *E. coli* due to the need for ferredoxin which is absent in the bacteria (Marin *et al*., 1996; Bouvier *et al*., 1996). The *Erwinia* species *crt* genes have been used to produce lycopene, β-carotene, and zeaxanthin in *E. coli* and other bacteria (Misawa *et al*., 1990, 1991; Ausich *et al*., 1991).

One problem with metabolic engineering in general is the problem of competition of engineered pathways with

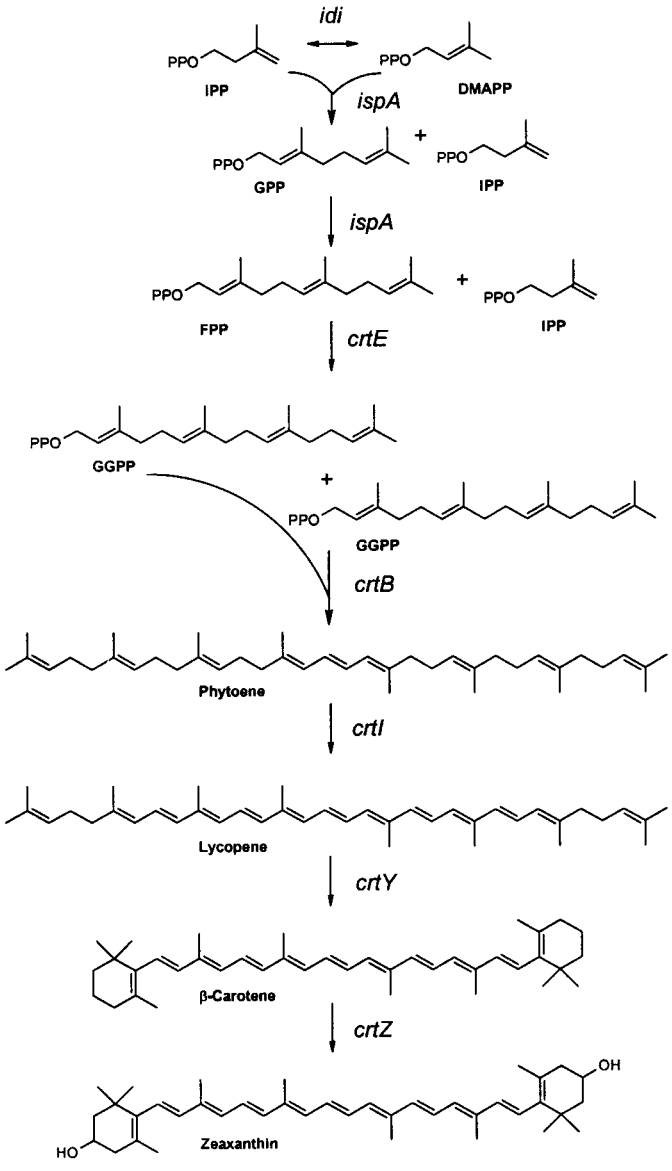

FIG. 5. Pathway to carotenoids.

endogenous pathways for precursors. Engineering the supply of prenyl pyrophosphate precursors for increased production of exogenous carotenoids is therefore necessary. It has been shown that a rate limiting step in terpene biosynthesis is the isomerization of IPP to DMAPP and that the IPP isomerase gene increases production of carotenoids (Kajiwara *et al.*, 1997). To characterize the pathway kinetics in detail, Wang *et al.* have systematically studied the limiting steps in GGPP synthesis in *E. coli* (Wang *et al.*, 1999). It was found that the conversion from FPP to GGPP is the first functional limiting step for the production of caratenoids in *E. coli*. After an *Erwinia* GGPP synthase (coded for by *crtE*), which converts FPP to GGPP, was overexpressed in *E. coli*, the limiting step shifted to the IDI reaction. After simultaneous overexpression of *E. coli idi* and *Erwinia crtE*, the limiting step then shifted to the formation of FPP.

There is also competition for the use of GGPP, the substrate for phytoene synthase and the tetraterpene carotenoids. It has been shown that overexpression of the *crtB* gene for phytoene synthase results in a slower growing *E. coli* strain due to the rerouting of GGPP from endogenous processes (Neudert *et al.*, 1998). The overexpression of GGPP synthase, however, increased the production of carotenoids by about 20% (Ruther *et al.*, 1997). When both GGPP synthase and IPP isomerase were increased, there was no problem with bacterial growth (Kajiwara *et al.*, 1997). Transformation of *E. coli* with the genes for overexpression of 1-deoxy-d-xylulose 5-phosphate synthase (*dxs*), 1-deoxy-d-xylulose 5-phosphate reductoisomerase (*dxr*) and isopentenyl pyrophosphate isomerase (*idi*) was found to increase production of carotenoids by a factor of 3.5 (Albrecht *et al.*, 1999). To avoid competition from other pathways and to relieve the limiting steps, a multifunctional GGPP synthase from *Archaeoglobus fulgidus* was cloned and overexpressed in *E. coli*, along with the *E. coli idi* gene (Wang *et al.*, 1999). This *E. coli* strain increased the astaxanthin production by a factor of 50 compared with other strains published in the literature. Since *E. coli* uses the pyruvate/G3P pathway for IPP synthesis, the balance between the two substrates becomes important. It has been shown that the overexpression of the phosphoenolpyruvate synthase (coded by *pps*) directs the excess pyruvate to G3P and thus increases the flux to the IPP pathway (Farmer and Liao, 2001). In addition, an artificial control loop was engineered to control the expression of the IPP pathway in E. coli (Farmer and Liao, 2000). The resulting strain produces much higher lycopene than the corresponding control. This example shows that proper regulation of metabolically engineered pathway is important.

Several hydroxylated carotenoid derivatives were recently produced by the combination of up to three plasmids containing several carotenogenic genes from *E. uredovora* and two different *Rhodobacter* species (Albrecht *et al.*, 1997). The industrially important carotenoid astaxanthin is not produced by *Erwinia* species, but rather is produced by marine bacteria such as *Agrobacterium aurantiacum* (Yokoyama *et al.*, 1994). A new gene named *crtW* was isolated from *A. aurantiacum* (Misawa *et al.*, 1995a,b) and was found to produce a *β*-carotene ketolase which was responsible for the catalysis of *β*-carotene to canthaxanthin.

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When the *crtZ* gene, which encodes a β-carotene hydroxylase, was added, canthaxanthin was converted to astaxanthin.

The budding yeast *S. cerevisiae* does not synthesize carotenoids, but does synthesize the steroid ergosterol in relatively large amounts. To redirect the metabolic flux of isoprenoid precursors from the steroid pathway to the carotenoid pathway, the *E. uredovora crtE*, *crtB*, and *crtI* genes were transformed into *S. cerevisiae*, thereby producing lycopene. The ergosterol content decreased by 10%, which was in proportion to the amount of carotenoids produced in the transformant (Yamano *et al.*, 1994).

The food yeast *Candida utilis* has been has also been found to produce a large amount of ergosterol (Miura *et al.*, 1998). The *E. uredovora crtE*, *crtB* and *crtI* genes, through the use of yeast promoters and terminators, were used to produce lycopene in *C. utilis*, with a 35% reduction in the amount of ergosterol (Miura *et al.*, 1998). β-Carotene and astaxanthin have also been produced in *C. utilis* by the use of *Erwinia crt* genes (Miura *et al.*, 1998). In an attempt to increase the metabolic flux of isoprenoids in *C. utilis*, the level of HMG-CoA reductase was increased. When this occurred, along with the disruption of the *ERG9* gene encoding squalene synthase, a seven-fold increase in lycopene synthesis was achieved (Shimada *et al.*, 1998).

Carotenoid engineering has been accomplished in the plant kingdom as well. The original efforts were not extremely successful. The attempt to increase carotenoid levels by overproducing PSY has led to dwarfism as a result of redirecting GGPP away from gibberellin synthesis (Fray *et al.*, 1995). Lycopene was produced earlier in development, but in the ripe fruits, the level was lower than the control (Bramley, 1997). Tobacco plants also showed an inhibition of carotenoid synthesis when PDSwas overexpressed after viral infection (Kumagai *et al.*, 1995). There have been some successes as well, as phytoene has been produced in transgenic rice transformed with daffodil PSY cDNA (Burkhardt *et al.*, 1997).

Expression of the Erwinia *crtI* gene encoding phytoene desaturase has led to tolerance of the herbicide norflurazon, which inhibits PDS but is ineffective against CRTI. Xanthophyll metabolism was also altered in the transgenic plants leaving more lutein and less violaxanthin (Misawa *et al.*, 1994).

The alga *Haematococcus pluvialis* contains a gene named *crtO*, which encodes a β-C-4-oxygenase needed for the biosynthesis of ketocarotenoids. This gene was cloned (Lotan and Hirschberg, 1995) and expressed in *Synechococcus* PCC7924, which endogenously produces β-carotene and zeaxanthin, allowing the *Synechococcus* strain to biosynthesize astaxanthin and other ketocarotenoids (Harker and Hirschberg, 1997).

4. RECENT ADVANCES IN ISOPRENOID METABOLIC ENGINEERING

The past few years have witnessed accelerated progress on isoprenoid metabolic engineering. The genes and enzymatic steps involved in the pyruvate/G3P (non-MVA) pathway have been largely identified (see above text). Various novel strategies to increase production of isoprenoids or synthesis of novel products have been reported. Using metabolic engineering, the β-carotene biosynthetic pathway was introduced into rice endosperm (Ye *et al.*, 2000) and tomato (Romer *et al.*, 200) and successfully elevated the provitamin A content. Transferring the β-carotene ketolase gene from the alga *H. pluvialis* to tobacco led to the production of astaxanthin in its flowers and changed the nectary color from yellow to red (Mann *et al.*, 2000). This technique enables future genetic manipulations of pigmentation in fruits and flowers. *In vitro* evolution has been applied to phytoene desaturase (*crtI*) and lycopene cyclase (*crtY*) in the context of a carotenoid pathway to generate novel carotenoids (Schmidt-Dannert *et al.*, 2000). *In vitro* evolution applied to a GGPP synthase, which has been shown to be one of the rate-controlling enzymes, improved the production efficiency of carotenoids (Wang *et al.*, 2000). Combinatorial biosynthesis by coexpressing different carotenoid desaturases resulted in novel hydroxycarotenoids with improved antioxidative properties (Albrecht *et al.*, 2000). In addition, engineering an artificial control loop was shown to be beneficial to the redirection of metabolic flux to the desired product, such as carotenoids (Farmer and Liao, 2000). These and other novel technologies are likely to further accelerate the progress of isoprenoid metabolic engineering.

Even though good progress has been made in the metabolic engineering of isoprenoids, it is still a largely undiscovered field. There are a large number of molecules with pharmaceutical promise that are either terpenoids or meroterpenoids (compounds in which terpenes constitute part of the entire molecule). Some prominent examples are the sesterterpenoid manoalide (Soriente *et al.*, 1999), which has both anti-inflammatory and analgesic properties, and the antimitotic diterpene paclitaxel (Hezari and Croteau, 1997).

Isoprenoid biosynthesis is also important for plant growth and biomass properties. Overexpression of gibberellin 20-oxidase in hybrid aspen has recently been shown to increase both the growth rate and xylem fiber length (Eriksson *et al.*, 2000). As more genes are characterized and the novel technologies become available, the field of isoprenoid metabolic engineering holds great promise.

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