Applications of Plant Metabolic Engineering

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INTRODUCTION

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In the past years the interest in plant secondary has increased rapidly. Three major reasons can be mentioned for this. First because plants are a major source for the production of medicines and the development of novel medicines; second because plants contain health promoting secondary metabolites, third because of the interest in the resistance of plants against pests and diseases in which the secondary metabolism plays a crucial role. Seven years ago we edited a book (Verpoorte and Alfermann 2000) on the engineering of plant secondary metabolism. A general overview was given of plant secondary metabolism, and the strategies one could envisage for engineering plant secondary metabolite pathways. Furthermore, a number of examples were presented describing the state-of-the-art of engineering plant secondary metabolism. Now we have again compiled a series of papers on the engineering of plant metabolism.

Obviously in the past period quite a few applications have been reported. Some of them were successful, others were less successful and the unsuccessful ones we will probably never hear of. Reasons for failure are often basic biological problems: the regeneration of transgenic plants from transformed cells, and the stability of transformed cell lines or transformed plants. The toolkit for transformation and overexpressing genes has improved and consequently the number of successful transformations increased. However, the major difficulties concern the fact that the biosynthetic pathways involved proved to be much more complicated than originally thought. Engineering a single step may result in an increase of the immediate product but not necessarily in an increase of the final product of the pathway. As we discussed in the previous book, problems of pathway architecture, interaction between various pathways in the total metabolic network, enzyme complexes, compartmentation, feedback inhibition, and regulation all play an important role. It means that unraveling pathways on all levels should have the highest priority. Eventually this might enable us to design efficient approaches to pathway engineering.

PATHWAY ELUCIDATION

The key to genetic engineering is the detailed knowledge of the pathways of interest. The step-by-step approach for elucidation of pathways remains an important, though elaborate, tool in biosynthetic studies. Retrobiosynthetic studies and labeling experiments have shown to be excellent tools to confirm pathways on the level of intermediates (e.g. Eisenreich et al., 2004). Once the intermediates are known, one has to identify the enzymes involved. However, the isolation of enzymes catalyzing the individual steps of a pathway is hampered by, among others, low levels of the enzyme, instability of the enzyme, and problems in obtaining the substrate for measuring activity. Consequently many of the secondary metabolite pathways still have quite a few black boxes, for which paper chemistry has proposed intermediates, but for which no actual experimental evidence exists.

To elucidate pathways various molecular biological approaches have been advocated. Many are based on making "mutants" by knocking out genes (transposon tagging, RNAi, etc.). However, the problem is the identification of the steps which have been blocked in a mutant. Plants in which an essential biosynthetic gene for the flower pigments is affected are immediately observed by eye. In a split second one can screen hundreds of plants for the flower color. However, in case of a colorless metabolite in roots or leaves elaborate analytical methods are needed to identify a mutant. This explains why the flavonoids/anthocyanin biosynthesis is one of best known biosynthetic pathways (Springob et al., 2003).

FUNCTIONAL GENOMICS

Because of the problems in pathway mapping, functional genomics was thought to be a way to elucidate secondary metabolite pathways on all levels from genes to products. Functional genomics aims at determining the function of genes. Transcriptomic data, proteomic data, metabolomic data and physiological functions are all matched through biostatistical methods and bioinformatics. In case of organisms with a known genome sequence such an approach may be successful. But lack of sequence data is a major constraint in studying secondary metabolism in non-model plants.

Proteomics is not the panaceae to solve these problems, as only a small percentage of all proteins will be observed. Particularly low abundance proteins will not be observed (Jacobs et al., 2000, 2005; Chen and Harmon 2006). Secondary metabolism often only represents a small part of the total metabolism, e.g. the energy needed for the biosynthesis of alkaloids was found to be less than 1% of the total metabolism in the development of *Cinchona* seedlings (Aerts et al., 1990, 1991). The enzymes involved may be below the level of detection. For example in proteomics of *Catharanthus roseus* cell cultures some 100 proteins were found to be induced when alkaloid biosynthesis was turned on. Only two of these are known indole alkaloid biosynthesis enzymes (Jacobs et al., 2000, 2005). About 60 had homology with peptide sequences from primary metabolite genes from other plants, whereas

the peptides of about 40 proteins did not match with any known sequence. To identify the genes encoding these proteins and determine their function would be quite difficult and elaborate.

Goossens and co-workers (Goossens et al., 2003; Oksman-Caldentey et al., 2004; Rischer et al. 2006) developed a cDNA-amplified fragment-length polymorphism method that in combination with targeted metabolomics can be used to identify genes involved in certain pathways. Indeed it was shown that in this way a number of genes involved with the induction of alkaloid biosynthesis in *Catharanthus roseus* can be identified, though many of them are primary metabolism related genes, and not directly involved in the pathway. Genes with sequences not matching any known genes are candidates for structural genes of species specific pathways, but it requires extensive further studies to identify the precise role.

Metabolomics, the latest of the – omics family, aims at the qualitative and quantitative analysis of all metabolites in an organism (Fiehn, 2001; Rochfort, 2005; Ryan and Robards, 2006). Metabolomics can be considered as the chemical characterization of a phenotype, and is thus an important tool in functional genomics. It can be used to measure the levels of compounds under different conditions. By correlating these data with proteomic and transcriptomic data one may get information about genes involved in the regulation of pathways and the structural genes involved.

The integration of all the – omics data and physiological data, i.e. taking a holistic view at the organism at all levels without a starting hypothesis, is a novel approach to biological research now known as systems biology. Also for plants this approach is now recognized as a very promising way to study for example plant interaction with insects or microorganisms (Oksman-Caldentey et al., 2004; Sweetlove and Fernie, 2005; Verpoorte et al., 2005).

Even though the various tools of functional genomics can be helpful in identifying genes involved in secondary metabolite pathways, none of them is capable of identifying all intermediates, proteins or genes involved in a pathway. Besides problems of low concentrations, the major problem is that in a living system, the changes in levels of transcripts, activity of enzymes and level of metabolites have different dynamics. The final result of an induction at gene level is only observed many hours or days later, if one even at all can speak about a final result in a dynamic system.

COMPARTMENTATION

The compartmentation of secondary metabolite biosynthetic pathways has received much attention in the past years. Several reviews on this topic have been published (e.g. Kutchan, 2005; Yazaki, 2005). If we take *Catharanthus roseus* as an example it has been shown that both intra- and intercellular compartmentation do play an important role. The early terpenoid precursors from the MEP-terpenoid pathway and geraniol-10-hydroxylase are made in different cells (internal phloem parenchyma) than the other important precursor tryptamine (epidermis). The last step of the

biosynthesis of the terpenoid precursor secologanin occurs also in these epidermis cells. Strictosidine synthase present in the vacuoles of these cells catalyzes the condensation of tryptamine and secologanin to yield the intermediate strictosidine, which is the starting point for several different pathways leading to different types of terpenoid indole alkaloid skeletons (for a review see van der Heijden et al. 2004). The branch leading to vindoline is present in other specialized cells (ideoblasts and laticifers), thus requiring intercellular transport of strictosidine or a later product from the vindoline branch.

Concerning the intracellular compartmentation, it is known that plastids are the source of the terpenoid precursors and tryptophan. Decarboxylation of tryptophan occurs in the cytosol, whereas strictosidine is produced in the vacuole from the precursors secologanin and tryptamine in the vacuole. Further steps are again outside the vacuole. The required glucosidase, for example, is localized in the ER (Geerlings et al, 2000), whereas a crucial step in the vindoline biosynthesis occurs in green chloroplasts (for a review see van der Heijden et al., 2004; Kutchan, 2005). This has implications for engineering alkaloid production in the native host of the pathway. One needs to express the gene in the correct compartment and the correct type of cell, otherwise no or little effect is achieved. But even more important, it means that the flux through a pathway is not only controlled by structural genes catalyzing a chemical reaction, but also by transport from the site of production of a precursor to the site of the next enzyme.

TRANSPORT

Because of the different compartments involved in biosynthetic pathways, the intermediates need to be reallocated to the proper compartment. Reallocation is a complex phenomenon in plants and plant cells. Diffusion is always involved in the reallocation of compounds. Affinity for lipid membranes (lipophylic properties of a compound) and intra- and extracellular fluids (hydrophilic properties of a compound) are important factors for diffusion driven transport through membranes (Blom et al. 1991). On top of that active transport through membranes may occur through e.g. a proton antiport mechanism or ABC-type of transporters (such as proteins belonging to the PDR, MRP and MDR families). For example from measuring transport of alkaloids and iridoids into isolated *C. roseus* vacuoles, we concluded that bidirectional transport occurs through different type of transporters (MRD out and ABC and MRP proteins in) with quite different rates for the different *C. roseus* alkaloids and secologanin (Roytrakul, 2004; Roytrakul and Verpoorte, 2007). In other cell organelles and the cell membranes similar processes might occur. Furthermore, conjugation of compounds with e.g. glutathione under the influence of glutathione transferases and peroxidases may play a role in the vacuolar transport of certain compounds (Dean and Devarenne, 1997; Grotewold 2004; Yazaki 2005). Transport is thus extremely complex as besides diffusion driven transport, different types of active transport are involved, with different directions

and for each single compound a different selectivity. Biosynthetic rates might thus very well be controlled on the level of transport.

Besides transport also storage is an important aspect of secondary metabolite production. Vacuoles are storage organelles, but import of the products is required. For example overexpression of the terpenoid indole alkaloid pathway genes encoding tryptophan decarboxylase and strictosidine synthase in tobacco cells in combination with feeding of the precursor secologanin did not result in any storage of the products. Instead the products were excreted into the medium, which is opposite to the situation in *C. roseus* cells where the alkaloids are stored in the vacuole (Hallard et al., 1997).

In this introduction I will not try to give a complete overview of all aspects of compartmentation, transport and storage. I only want to conclude that the green factory in many aspects is very similar to an industrial factory, (e.g. a factory assembling cars). Both require energy for the production process, transport from the sites of the production of building blocks to the site where these are assembled to yield the final product and a storage site for the stock of the final product. It might thus be possible to apply technical engineering strategies to plan plant metabolic engineering.

TARGETS FOR METABOLIC ENGINEERING

Metabolic engineering is possible, but what are the targets? Why should one like to alter the metabolism of plants?

The following goals can be mentioned:

- Improved quality for producer (farmer)
  - Improved yield
  - Improved resistance against pests and diseases
  - Improved traits for cultivation and harvesting
- Improved quality for processing (industry)
  - Storage of food
  - Suppress level of unwanted products (e.g. toxic compounds) or improve quality of product (e.g. starch, lignins)
  - Higher level of specialty chemicals, e.g. for medicines
  - Fiber quality
  - Biofuel viscosity, stability
- Novel compounds for drug development (industry)
- Improved quality for consumer
  - Taste of food
  - Color of food or flowers
  - Increased level of health improving compounds
  - Lower level of undesired compounds

Looking at this list of possibilities it is clear that the applications concern changes in primary metabolism or in secondary metabolism. It also implies that choices have to be made, e.g. does one go for yields or quality (Morris and Sands 2006; Singh et al. 2006). Secondary metabolism is per definition species specific, it serves the producing organism to survive in its ecosystem. In plants it is, among others, involved in defense against pests and diseases, and in attracting pollinators. Furthermore taste, flavor and color of our food are related to secondary metabolism. Also various health effects of food are connected with secondary metabolites. The defense compounds are of different character, some are constitutively expressed (phytoanticipins), others are only biosynthesized after wounding or in infection (phytoalexins) (Zhao et al. 2005). That means that the regulation of secondary metabolism in part is developmentally regulated, in part is dependent of external (stress) signals.

Starting from ubiquitous primary metabolites as precursors the number of steps in secondary metabolite pathways differs considerably. The biosynthesis of the phytoalexin resveratrol from ubiquitous primary metabolites consists of only a single step, catalyzed by one single enzyme, encoded by one single gene (Hain and Grimmig, 2000). Whereas the biosynthesis of an indole alkaloid like vinblastine, includes at least 30 different steps, at least three different cells types and four different cellular compartments, and consequently also is regulated by transport systems (van der Heijden et al. 2004; Pasquali et al., 2006). Because secondary metabolism is species-specific, the knowledge about most pathways is limited, and very few pathways in plants have been fully elucidated to all levels of intermediates, enzymes and genes.

STRATEGIES

For developing a strategy for metabolic engineering of plant secondary metabolism, one has to keep all the above mentioned aspects in mind. There is a clear difference in approach for increasing or decreasing the flux through a pathway.

Decreasing a flux could for example be of interest in case of undesired (toxic) compounds, or to cut off certain pathways that compete with the pathway of interest. Also catabolic pathways might be of interest to cut, in order to increase the level of a desired compound. To decrease a flux, the level of the protein of interest can be decreased by an antisense or RNAi approach or by overexpresssing an antibody of the selected enzyme of the target pathway. As long as not any vital pathway is knocked out, this approach should be easy with a good chance of success.

To increase the level of a compound, one needs to know the pathway into much detail to be able to select targets for engineering. This should result in the identification of possible sites for modification, e.g. overcoming limiting steps. As mentioned above, only a few genes of plant secondary metabolite pathways are known. Engineering long pathways thus requires extensive studies to elucidate the pathway. One may also consider the use of microbial genes to achieve certain reactions in plants for which the encoding plant genes are not known yet. The production of salicylate in plants by overexpression of microbial genes is such an example (Verberne et al. 2000).

One of the problems with pathway engineering is that many genes need to be transformed into the plant. Instead one may also consider the overexpression of regulatory genes. This may result in the induction of a series of genes of a secondary metabolite pathway (Grotewold et al. 1998). An example is the engineering of the signal transduction pathway leading to the induction of a pathway of interest, e.g. overexpression of a transcription factor (Gantet and Memelink, 2002; Memelink et al. 2001; Memelink 2005a, 2005b). This approach does not necessarily result in an increased level of the desired end products as it was found for alkaloids in *C. roseus*, other flux limiting steps may remain (Memelink et al. 2001).

Another possibility is to modify a constitutive pathway into an inducible one by introducing inducible promoters. This has the advantage that one can separate growth and secondary metabolite production, and avoiding a competition between the two processes for the limited energy and precursor pools in the plant cells. Highest production of secondary metabolites in a bioreactor can be achieved in a fed-batch-type of process in which growth and product formation are in different phases of the process (Verpoorte and ten Hoopen, 2006; Zhao and Verpoorte 2007).

NEW COMPOUNDS

To produce new compounds for a plant, one can add extra steps to an already existing route, or introduce a new enzyme for the plant that catalyzes an early step in a route. Examples are the production of alkaloids in the hairy roots of an iridoid producing plant *Weigela* "Stryriaca" by overexpression of tryptophan decarboxylase and strictosidine synthase (Hallard, 2000). Another example is the overexpression of a terpene synthase (see Luecker et al., Chapter 9; Aharoni et al., 2005; Dudareva et al., 2006), resulting in the formation of a novel molecular skeleton for the host plant. This molecule may serve as a scaffold for further reactions catalyzed by enzymes in the plant to yield a novel product for that plant, and maybe even a totally novel compound. Such a recombinatorial biochemistry approach aiming at production of novel compounds is of interest for developing novel leads for drug development (Julsing et al. 2006). It might also be of interest for increasing the resistance of the plant against pest and diseases. However, from the point of view of safety, it might need quite some work to proof that the new compound(s) is(are) not toxic for the consumer.

Producing a known compound in another plant might be of interest for several reasons. The most obvious is the introduction of health promoting compounds in food plants (Yonekur-Sakakibara and Saito, 2006). Golden rice is an excellent example of extra nutrional value that can be created by metabolic engineering to increase the vitamine A level (Al-Babili and Beyer, 2005). Flavonoid production is a target for metabolic engineering in plants, with the aim to increase antioxidant levels in food (Forkmann and Martens, 2001; Schijlen et al. 2004).

An other reason could be that the target plant has better properties for producing the compound than others. The *Atropa belladonna* plant producing scopolamine is such an example (Hashimoto et al. 1993).

In studies on metabolic engineering one often sees only small increases in fluxes if compared to wild type plants or plant cells. Apparently the complex metabolic networks do not allow major reshuffling of carbon fluxes. Therefore rebuilding pathways in the simplest possible cells is seen as a possibility to reach high productivity. Synthetic biology aims at the construction of such production cells in which all unnecessary genetic material has been cut to leave a cell rigged for growth. In such cells the desired pathways could be introduced. Obviously such cells will be of microbial origin. The possibility of using microorganisms for the production of plant products has been shown by several studies in the past years, e.g. terpenoids (Chemler et al., 2006; Kutchan, 1989; Martin et al., 2001; Ro et al., 2006; Szczebara et al., 2003; Withers and Keasling, 2007) and alkaloids (Kutchan, 1989; Geerlings et al., 2001). The total biosynthesis of hydrocortisone in yeast is an excellent example of the potential of this approach for production of medicines (Szczebara et al. 2003).

CONSTRAINTS

In principle there are infinite possibilities for applying metabolic engineering, but they are limited by:
– public acceptance
– safety issues
– lack of knowledge of biosynthetic pathways
– regeneration of genetically transformed plants
– viability of plants with altered metabolism
The major area for application on the short term is the production of medicinal compounds, either known or new, in plants or plant cell or tissue cultures. At present the commercial application of genetically modified secondary metabolism is in altered flower colors.

PERSPECTIVES

To end this introduction I want to mention the great perspectives of plant metabolic engineering. In comparing the present book with that of 7 years ago we can see that some dreams already have become true, but much more can be expected to become true in the coming years. Above I dealt with some of the results and quite extensively with the difficulties, as we need to identify the bottlenecks to further open the way for applications. Predicting the future is difficult, there will always be unexpected breakthroughs that will change the complete picture. But at least for certain technologies it is easy to predict that the methods, the tools, will become easier and faster. For example for sequencing genomes it is easy to predict that in 10 years it will be a minor project to sequence the genome of a plant, which means that for the functional genomics at least the problem of non-model plants will be solved. In the mean time the number of plant genes with a known function will also grow steadily, and consequently it will become easier to identify the function

of genes in other plants, and thus to elucidate pathways. Also metabolomics will further develop into fluxomics, instead of taking a "picture" at one single time point, one will make a "film" of the metabolic fluxes, in microorganisms this sort of studies are already in progress, for plants it will come in due time. Fluxomics will be of great use to identify the bottlenecks in a pathway that needs to be addressed to be able to increase the flux.

With such improved tools, the great chemical potential of plants for making complex chemical structures will be further developed. The advances made with recombinatorial biochemistry in microorganisms and metagenomics (Chemler et al., 2006; Ordovas and Moose, 2006) show us that we may expect a lot from exploring the metagenome of all plants for useful genes to make novel complex compounds with interesting biological activities.

Considering the various goals, one may expect that transgenic plant cell cultures producing medicines might be easily accepted by all people now opposing transgenic plants. In fact plant cell cultures might be an excellent platform for metabolic engineering using different modules containing sets of genes that could be used for recombinatorial biochemistry. Transgenic plants producing desired fine chemicals or with improved traits for non-food applications are other important areas where we may expect interesting applications. However, for food use it may take a longer time as in that case safety studies will be required, but on the longer term the perspectives seems bright for plants with increased levels of health promoting constituents.

Engineering the green cell factory is in its infancy, but it looks like a healthy baby with a bright future ahead!

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CHAPTER 1

BIOSYNTHESIS OF PLANT NATURAL PRODUCTS AND CHARACTERIZATION OF PLANT BIOSYNTHETIC PATHWAYS IN RECOMBINANT MICROORGANISMS

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Abstract: Plant natural products are important medicinal and flavor and fragrance compounds. Many of these metabolites have complex structures that cannot be produced economically through total chemical synthesis. The production of natural products for consumer use or as scaffolds for more complex molecules has to rely upon extraction from plant materials or the development of engineered production hosts. Limitations in the development of engineered biosynthetic pathways stem from incomplete knowledge of plant biosynthetic pathways. There is a strong dependence on recombinant microorganisms to elucidate plant biosynthetic pathways and characterize individual biosynthetic enzymes. As more pathways are characterized, the emphasis on microorganisms will shift from single enzyme studies to the construction of short biosynthetic pathways for the targeted overproduction of compounds

Keywords: recombinant; microbial pathway engineering; plant natural products; microbial gene expression

1. INTRODUCTION

Humans have been exploiting plant natural products for centuries as medicines, dyes and to improve food quality. The two largest industries that rely on plant extracts are the pharmaceutical and consumers products industry; jointly they possess a combined market value in 2002 of US $31.5 billion in the USA alone (Raskin et al., 2002; Papnikolaw 1998). Almost one quarter of all prescribed pharmaceuticals

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contain compounds that are directly or indirectly (through semi-synthesis) derived from plants (Cragg et al., 1997; Oksman-Caldentey et al., 2004). Industries such as food, beverage, pharmaceuticals, nutraceuticals, soaps and detergents, cosmetics and toiletries all rely on aroma additives derived from plants.

Many promising plant phytochemicals with prospects in the development of new pharmaceuticals have such complex chemical structures that total chemical synthesis is economically prohibitive. Thus, production of these compounds or precursor scaffolds (which can be further modified synthetically) has to rely on extraction from plant materials or the development of engineered production hosts for their targeted overproduction. Aroma and flavor compounds, on the other hand, typically have less complex chemical structures allowing their production via synthetic routes. However, consumers' concerns about health and environmental impact of synthetic chemicals and the production thereof has created a market for "natural" flavors and aromas (Vanderhaegen et al., 2003). Such "natural" ingredients can either be directly derived from plant materials or generated by enzymatic activities or fermentation, which includes the use of engineered hosts, according to the US Food and Drug Administration (Food and Drug Administration, 2001).

To develop consumer goods based on plant derived natural products it is important to understand metabolite biosynthesis on a molecular level. Knowledge of biosynthetic enzymes and their corresponding genes, as well as the complex underlying regulation of metabolic pathways in plants, would enable overproduction of plant phytochemicals of value in engineered plant or microbial cells. Moreover, designed biosynthetic pathways comprised of biosynthetic enzymes expressed at modified levels and/or enzymes with altered activities can create new structural diversity, encompassing structures not found in nature. However, genes of secondary metabolic plant pathways are not clustered as in microorganisms and the tremendous duplication of genes encoding certain biosynthetic enzyme classes creates significant challenges in the characterization of biosynthetic pathways despite the improved availability of plant genomic information. Genome sequences and EST data from plant model organisms such as *Arabidopsis thaliana*, *Medicago trunculata* and *Clarkia breweri* reveal that the most prolific enzyme families (cytochrome P450s, glycosyltransferases, and methyltransferases), known to be involved in the biosynthesis of natural products, have undergone extensive gene duplication (Kliebenstein et al., 2001; Pichersky et al., 2006; D'auria and Gershenzon 2005), which severely limits homology-based predictions of gene functions as well as comparative genomics approaches to decipher biosynthetic pathways (Frick and Kutchan, 1999). Hence, often tedious and time consuming genetic/molecular biology or metabolic/biochemistry approaches have been used to study plant pathways (Rohloff and Bones, 2005). More recent approaches utilize genomic, proteomic and metabolomic data (Sumner et al., 2003; Trethewey, 2004; Oksman-Caldentey and Saito, 2005; Fridman et al., 2005; Jacobs et al., 2005; Verpoorte and Memelink, 2002; Ounaroon et al., 2003) to study metabolic networks of plants. As part of these efforts and with the availability of EST and genome data, an increasing number

of plant genes have been cloned and expressed in microbial hosts for biochemical annotation of function (Aharoni et al., 2000; Achnine et al., 2005).

In this chapter we will review how recombinant microorganisms have been used for the elucidation of plant biosynthetic pathways as well as production of plant natural products. Although recombinant microorganisms have until now mostly been used for the characterization of catalytic activities of newly discovered biosynthetic enzymes, a number of recent examples demonstrate the feasibility of installing multi-step plant pathways in engineered microbial cells for the production of plant natural products. We will use these examples to illustrate the potential of engineered microbial hosts for the synthesis of diverse phytochemicals. Futhermore, we show how this approach forms a platform to further diversify the spectrum of plant natural products through techniques such as manipulation of enzyme functions and precursor supply, and combinatorial biosynthesis. We have organized this chapter according to natural product classes and their main industrial application – flavor and aroma or medicinal compounds. It should be noted though, that some natural product classes (e.g. terpenes, apocarotenoids, flavonoids) have compounds with applications in both consumer markets and will be discussed in this review under the market of which the majority of its members fall.

2. FLAVOR AND AROMA COMPOUNDS

Plant derived volatile aroma compounds are one of the best studied groups of secondary metabolites with over 1000 volatiles identified from plants (Pichersky et al., 2006). Volatiles serve important functions in plants acting as signaling molecules, phytoalexins, and attractants for pollinators (for reviews of plant derived volatile aroma compounds see (Pichersky and Gershenzon, 2002; Dudareva et al., 2004; Dudareva and Pichersky, 2000). Aroma synthesizing plants are used as flavorings, preservatives and herbal remedies and the production of flavor and fragrance compounds has many important industrial applications (Goff and Klee, 2006). Applications include: addition or replenishment of flavors and fragrances to processed foods with extended shelf lives, metabolic engineering of more fragrant or colorful flowers and fruits and increasing vitamin or pigment content of foods.

Biotechnological production of aroma compounds can be carried out through plant cell and tissue culture, biocatalysis and biotransformations by microorganisms or *de novo* synthesis (reviewed in (Krings and Berger, 1998; Vandamme, 2002)). Currently more is known about microbial production of aroma and flavor compounds than plant biosynthesis (Marasco and Schmidt-Dannert, 2003). Poor characterization of secondary metabolite pathways in plants is due in part to the minute quantities of aroma compounds found in plants that are often difficult to isolate. Conventional breeding is impeded by the complexity of the biochemical pathways involved in the biosynthesis of aroma and flavor formation, and the sensitivity to environmental conditions. Furthermore, there is a fundamental lack of established methodology to characterize flavor chemistry and organoleptic properties (Lewinsohn et al., 2001). An understanding of aroma secondary metabolite pathways

in plants on a molecular level similar to the level of understanding researchers have achieved for microbial systems will provide access to the genes and enzymes involved allowing for improvements and manipulations of the pathways.

Aroma compounds are divided into three major classes of compounds: phenylpropanoid (benzenoid) derivatives, fatty acid derivatives and isoprenoid derivatives. Plant medicinal natural products also derive from phenylpropanoid and isoprenoid pathways. Unique to aroma biosynthesis are fatty acid derivatives. In contrast, many medicinal phytochemicals are alkaloid derivatives, and few aroma compounds are generated from alkaloid precursors.

2.1. Fatty acid derived flavor and aroma compounds

Important fruit flavors with low odor thresholds are derived from unsaturated fatty acids during ripening and described as fruity, coconut-like, buttery, sweet or nutty aromas. β-oxidative cleavage and decarboxylations of fatty acids form compounds such as lactones and volatile esters. Flavor formation is initiated when free fatty acids are released from lipids by lipases and then specific double bonds are peroxidized by lipoxygenases to produce hydroperoxides. For example, in tomatoes, the cleavage by 13-hydroperoxide from linoleic or linolenic acids produces "grassy" or "green" notes from hexanal and *cis*-3-hexenal respectively. These aldehydes can be further reduced by an alcohol dehydrogenase to form hexanol and *cis*-3-hexanol (Vick and Zimmerman, 1986) (Figure 1). The availability of appropriate unsaturated fatty acids is an important factor affecting fruit flavor and aroma development because they provide the aliphatic esters, alcohols, acids and carbonyls that contribute to fruit flavors. Modifications of the unsaturated fatty acid pools can direct the formation of specific products of value.

Esters are the main component of apple (*Malus domestica)*, pear *(Pyrus communis)*, and banana (*Musa sapientum)* flavors. Esterification is the final step in volatile ester formation and results from trans-acylation by an acyl-CoA moiety to the alcohol and is carried out by alcohol acyltransferase (AAT) enzymes. (Other volatile esters that derive from methylsalicylate, most likely from the phenylpropanoid pathway, will be discussed in a later section). Several AAT genes (i.e. from melons, apples, bananas and strawberries) have been cloned and expressed in *E. coli* (Aharoni et al., 2000; Chau et al., 2004a; Yahyaoui et al., 2002; Souleyre et al., 2005; Beekwilder et al., 2004; D'auria et al., 2002; Shalit et al., 2003). Recombinant systems allow for different combinations of alcohols and acyl-CoAs to be studied to identify the specificity and flexibility of AAT activity. DNA microarrays were used to identify a strawberry AAT (SAAT) involved in the formation of over 100 fruity esters in strawberries. The SAAT enzyme is a member of the BAHD family of acetyltransferases whose members have diverse roles in secondary metabolism. Aharoni et al. (2000) expressed the recombinant protein in *E. coli* and found it had wide substrate specificity with a preference for medium chain length alcohols and that volatile ester formation depended on the availability of acyl-CoA molecules and alcohol substrates (Aharoni et al., 2000).

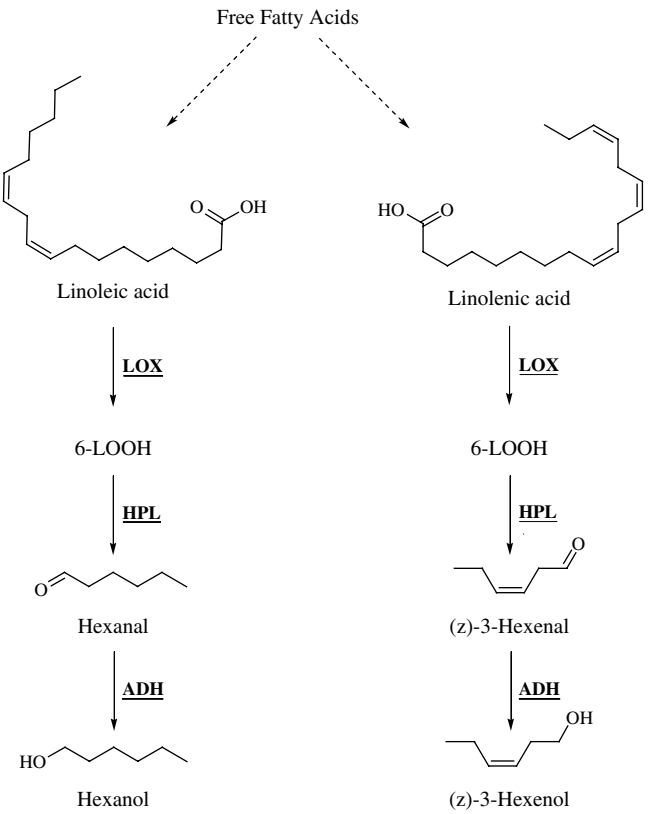

Figure 1. Example biosynthetic pathway for the formation of fatty acid derived volatile compounds (C5 and C6) starting from linoleic and linolenic acids. Enzyme abbreviations are as follows: LOX, lipoxygenase; HPL, hydroperoxide lyase; ADH, alcohol dehydrogenase

Beekwilder et al. (2004) compared wild strawberry (*Fragaria vesca*) and banana (*Musa sapientum*) AATs to the cultivated strawberry *Fragaria x ananassa*. They found substrate preference could not be determined by sequence similarity and that recombinant substrate preferences were not reflected in the fruit volatile profiles. This suggests that the volatile ester profile of a fruit species may be governed by the supply of precursors. A similar study created transgenic *P. hybrida* with the rose AAT and observed higher levels of benzyl acetate and phenylacetate demonstrating the possibility of directing the production of pathway volatiles (Guterman et al., 2006). Metabolic engineering of precursors within plants can create new ester profiles or conversely the AAT enzymes can be introduced into new precursor backgrounds to create novel esters. Directed biotransformation within engineered microbial cells is also a mechanism for creating new aroma products. Advantages to microbial production would be fewer volatiles that mask the scents, stricter control

over protein expression levels and less modification of compounds to non-volatile forms through glycosylation reactions (Dudareva and Negre, 2005).

2.2. Volatile benzenoids

Most phenylpropanoid and benzenoid compounds derive from the shikimate pathway via phenylalanine. The first committed step in phenylpropanoid and benzenoid formation is catalyzed by L-phenylalanine ammonium lyase (PAL), which is the deamination of phenylalanine to *trans*-cinnamic acid. *Trans*-cinnamic acid is the branchpoint between two pathways, one forming the volatile benzenoid compounds and the other medicinal compounds such as stilbenes and flavonoids discussed later.

Benzoic and salicylic (2-hydroxyl benzoic) acids are synthesized from *trans*-cinnamic acids (Boatright et al., 2004a) and are precursors for the synthesis of important floral scents such as methyl benzoate and methyl salicylate. *Trans*-cinnamic acid loses 2 carbon units in route to becoming volatile. Metabolic flux models suggest several mechanisms for the loss of the two carbon units (Boatright et al., 2004b). One mechanism is a CoA-dependent β-oxidation similar to the β-oxidation of fatty acids. The *trans*-cinnamic acid is activated to the cinnamoyl-CoA ester and then hydrated to form 3-hydroxy-3-phenylpropionyl-CoA. The hydroxyl group of the intermediate is oxidized to a ketone and the β-keto thioester is subsequently cleaved to form benzoyl-CoA. The alternative CoA-independent, non-β-oxidative pathway involves the hydration of the free cinnamic acid to 3-hydroxy-3-phenylpropionic acid. A reverse aldol reaction forms the benzaldehyde which is then oxidized to benzoic acid. Finally, a combination pathway has been described that is CoA-dependent and non-β-oxidative (Figure 2). Enzymes and genes involved in the early biosynthesis of benzenoids have not been decribed on a molecular level, analyses has been restricted to analyses of pathway intermediates and flux analysis (Boatright et al., 2004b). However, several methyltransferases that convert benzenoid compounds into the corresponding carboxyl-methyl esters have been identified. Also a benzoyl-CoA:benzyoyl:benzyl alcohol benzoyl transferase which forms benzylbenzoate has been identified (D'auria et al., 2002).

Methyl esters of benzenoids are responsible for the scents of many flowering plants and find applications as flavor and aroma compounds. For example, methyl jasmonate is used in the perfume industry; methyl salicylate found in leaves and flowers of wintergreen is a flavor ingredient in many types of candy, food and medicine; methyl cinnamate is found in basil; methyl benzoate is a major component of ylang-ylang oil and in the aroma and flavor of tropical fruits. Six benzenoid *O*-methyltransferase enzymes have been identified and heterologously expressed (reviewed in (Effmert et al., 2005)). All six enzymes belong to the same SAM dependent class of carboxyl O-methyltransferases, but recognize different benzenoid substrates which has been established by functional characterization of enzymes expressed in *E. coli*. The first methyltranferase identified was from *Clarkia breweri* and shown to involved in floral scent production (Ross et al., 1999).

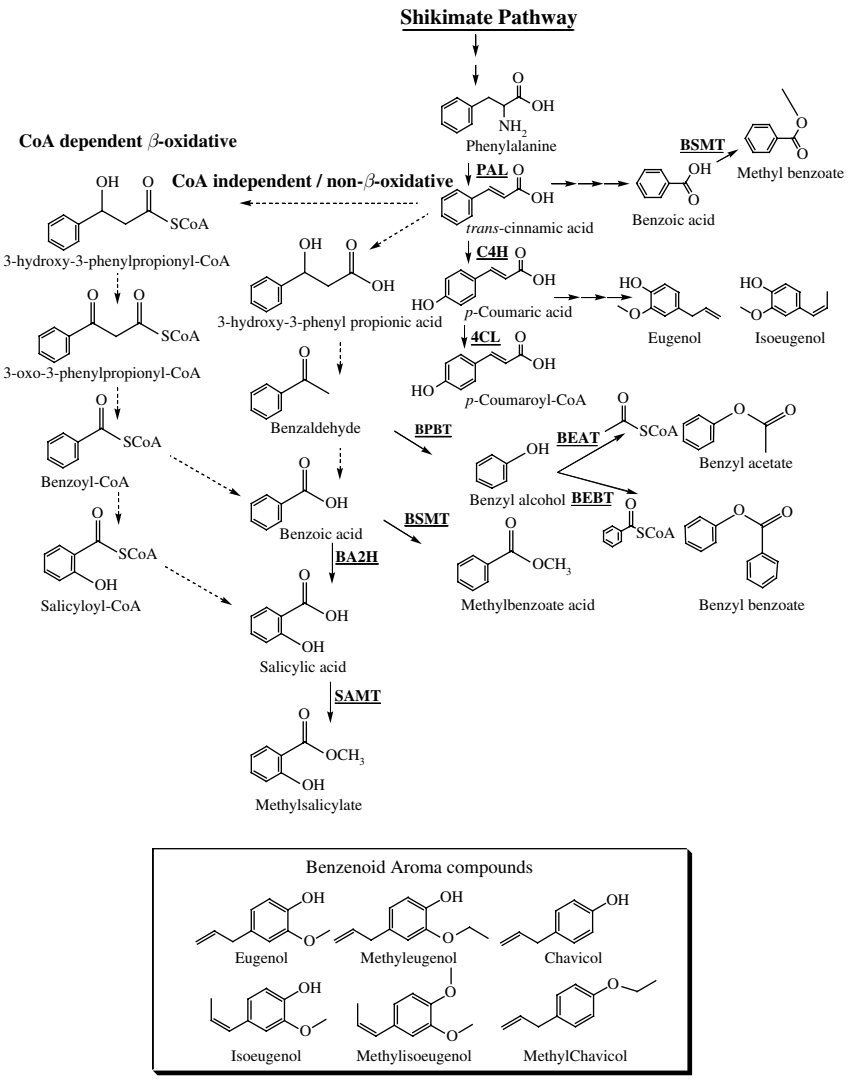

Figure 2. Proposed biosynthetic pathway of benzenoid compounds starting from the amino acid phenylalanine. Adapted from Boatwright et al. 2004. Cloned genes are bolded and underlined. Enzyme abbreviations are as follows: PAL, phenylalanine ammonium lyase; C4H, P450 cinnamate-4-hydroxylase; 4CL, 4-coumaroyl-CoA-ligase; BSMT, *S*-adenosyl-l-Met:benzoic acid carboxyl methyltransferase; BPBT, benzoyl-CoA:benzyl alcohol/phenylethanol benzoyltransferase; BEAT, acetyl-CoA:benzyl alcohol acetyltransferase; BEBT, benzoyl-CoA:benzyl alcohol benzoyl transferase; BA2H, benzoic acid 2-hydroxylase; SAMT, salicylic acid carboxyl methyltransferase

This enzyme was highly specific for the conversion of salycilate to its corresponding methylester, which was also observed for an enzyme identified from snapdragons (*Antirrhinum majus)* (Negre et al., 2002). In contrast, another methyltransferase

isolated from *Stephanotis floribunda* forms methyl benzoate but accepts several different benzoic and cinnamic acid derivatives (Pott et al., 2002). Other identified enzymes methylate benzoate (Murfitt et al., 2000); jasmonate (Seo et al., 2001); benzoate and salicylate (Chen et al., 2003),(Negre et al., 2003) or several benzoic and cinnamic acid derivatives (Pott et al., 2004).

2.3. Monoterpenes

Monoterpenes are the main component in the essential oils frequently used in flavorings, fragrances, and pharmaceuticals (Aggarwal et al., 2002; Croteau, 1998; Loza-Tavera, 1999; Mahmoud and Croteau, 2002; Pattnaik et al., 1997; Hohl, 1996) and are synthesized in the plastids of plants and are responsible for many of the odors associated with them (Colby et al., 1993). These compounds along with sesqui-, diterpenoids and carotenoids discussed later in the section on medicinal natural products, belong to the diverse class of terpenoid (or isoprenoid) natural products synthesized by plants and microorganisms.

2.3.1. Biosynthesis of terpenoid compounds

Over 30,000 terpenoid structures have been described from different terpenoid classes (Broun and Somerville, 2001), making terpenoids the largest group of natural products. These compounds are synthesized by successive condensations of C_5 isoprene units, isopentyl diphosphate (IDP) and dimethylallyl disphosphate (DMADP). Condensations are catalyzed by various chain length specific prenyltransferases in mainly head-to-tail reactions (Yan et al., 2001). A different class of enzymes is responsible for catalyzing the head-to-head condensation seen with carotenoids and sterols. Terpenoid classes are defined by the number of condensed isoprene units resulting in chains with 10, 15, 20, 30 or 40 carbons: In the case of volatile monoterpenoids, two isoprene units make up a ten carbon isoprenoid backbone (C_{10}) (Figure 3).

Additional modifications to the isoprenoid backbones including cyclizations, desaturations and oxidations produces the structural diversity in the terpenoid class (Dewick, 2002; Kuzuyama and Seto, 2003). Terpene synthases (also referred to as cylases, TPSs) catalyze cyclization of the linear isoprene diphosphate precursors geranyldiphosphate (GDP, C_{10}), farnesyldiphosphate (FDP, C_{15}), and geranylgeranyldiphosphate (GGDP, C_{20}) into mono-, sesqui-, and diterpenes respectively (Davis and Croteau, 2000; Bohlmann et al., 1998a). TPSs catalyze cyclization by first catalyzing removal of the diphosphate to form a highly reactive carbocation and then controlling carbocation migration as it steps through the backbone (involving hydride shifts, cyclizations, rearrangements and reprotonations) until final carbocation quenching and product release occurs. Terpene synthases all have a similar active site scaffold (Lesburg et al., 1997; Starks et al., 1997a), but different types of cyclases are categorized based on the isoprene chain length specificity, the way that they generate the carbocation, folding patterns and the quenching mechanism. The number of structures increases dramatically with

increasing backbone length and conjugation. For example, 1,000 monoterpenoid structures have been reported from C10 GDP, whereas there are 7,000 known sesquiterpene structures from C15 FDP (Bohlmann et al., 1998a). In the case of monoterpenes, several different structural types can be distuingished: acyclic (e.g. myrcene, linalool), monocyclic (*d*-limonene) or bicyclic(e.g. α- and β-pinene, 3-carene) monoterpenoids. Additional oxidation and reduction, and isomerization reactions result in more complex monoterpenes such as geraniol, menthol, and camphor.

Sequence information is not predictive of the terpenoid products from TPSs and as a result many plant terpene synthases have been cloned, expressed in heterologous hosts and functionally characterized (Pichersky and Gershenzon, 2002; Dudareva et al., 2004; Starks et al., 1997b; Faldt et al., 2003; Bohlmann et al., 2000; Phillips et al., 2003; Chen et al., 2004; Martin et al., 2004). Monoterpene synthase from species of *Pinaceae*, *Lamiaceae*, *Rutaceae*, *Myrtaceae*, *Asteraceae*, and other plants have been cloned and functionally expressed in *E. coli* (Bohlmann et al., 1999; Yuba et al., 1996; Bohlmann et al., 1997; Williams et al., 1998; Wise et al., 1998; Jia et al., 1999). *Arabidopsis thaliana* alone has 32 putative cyclase genes identified in its genome, including 6 proven monoterpene synthases and 2 sesquiterpene synthases (Aubourg et al., 2002; Tholl et al., 2005) which are responsible for some of the 102 volatiles identified in *A. thaliana* (Rohloff and Bones, 2005). Ongoing functional genomics projects including functional analysis of recombinant enzymes aim at understanding the AtTPS families (Chen et al., 2003; Faldt et al., 2003; Bohlmann et al., 2000; Chen et al., 2004; Aharoni et al., 2003).

2.3.2. Engineered terpenoid biosynthesis

With a cyclase gene identified it is possible to engineer recombinant microbial cells for terpenoid synthesis (Carter et al., 2003; Huang et al., 1998; Huang et al., 2001; Martin et al., 2003) from endogenous IDP and DMADP precursors. These precursors are produced through two different pathways: the mevalonate (MVA) or mevalonate independent pathway (DXP) (Rodriguez-Concepcion and Boronat, 2002). The MVA pathway is predominantly in eukaryotes, archaea, and some eubacteria. The DXP pathway is used by the majority of eubacteria, including *E. coli* and green algae (Boucher and Doolittle, 2000). Plants, however, use both pathways to produce sesqui- and triterpenes via the MVA pathway in the cytosol and mono-, diterpenes and carotenoids via the DXP pathway in the chloroplasts.

In *E. coli*, there have been many studies aimed at increasing IDP and DMAPP pools by diverting the precursors away from competing endogenous pathways and directing flux towards the engineered pathway (Estevez et al., 2001; Farmer and Liao, 2000; Kajiwara et al., 1997; Kim and Keasling, 2001; Lee and Schmidt-Dannert, 2002; Matthews and Wurtzel, 2000). Significant challenges to this engineering approach include the complex regulation of the DXP pathway, achieving significant expression of plant enzymes in the host and toxicity of some DXP pathway intermediates when present in high quantities. The engineering of the

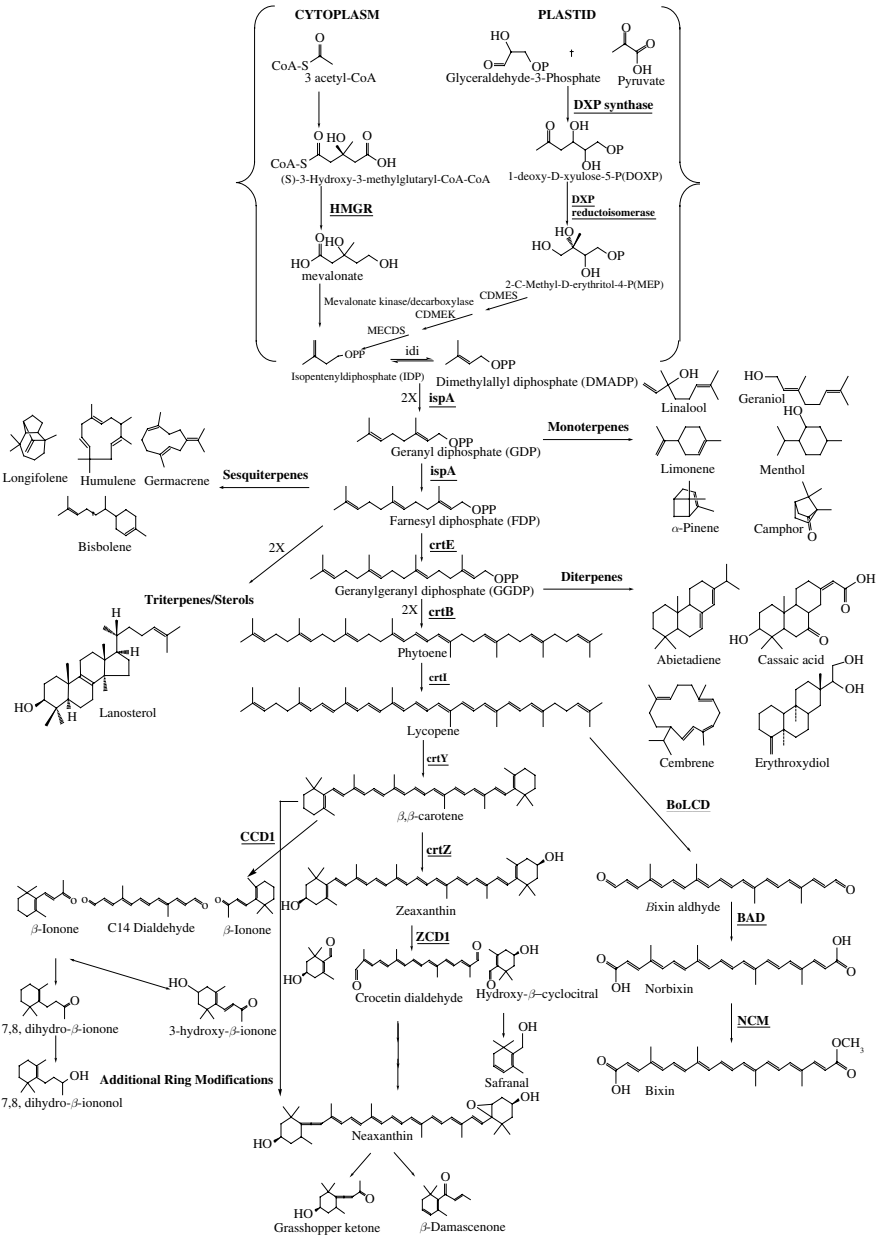

Figure 3. Biosynthetic pathways of isoprenoid derived compounds. Cloned genes are in bold and underlined. Enzyme abbreviations are as follows: HMGR, HMG-CoA reductase; Idi, IDP isomerase; Dxs, 1-deoxy-d-xylulose 5-phosphate synthase; IspA, FDP synthase; CrtE, GGDP synthase; CrtB, phytoene synthase; CrtI, phytoene desaturase; CrtY, lycopene cyclase; CrtZ, carotene hydroxylase; CCD, carotenoid cleavage dioxygenase; ZCD1, zeaxanthin cleavage enzyme; BoLCD, lycopene cleavage dioxygenase; BAD, bixin aldehyde dehydrogenase; NCM, norbixin carboxyl methyltransferase

eukaryotic MVA pathway into *E. coli* as an alternative pathway for the production of terpenoids was one successful attempt at alleviating the regulation and toxicity issues (Martin et al., 2003). Along with precursor engineering, stoichiometric flux balance analysis has been used, further increasing terpenoid production levels in recombinant cells (Alper et al., 2005a; Alper et al., 2005b). In addition to ensuring sufficient pools of IDP and DMADP in *E. coli*, production of terpenoids other than sesquiterpenoids (from endogenous FDP in *E. coli*) requires expression of heterologous prenyltransferases for synthesis of respective prenyldiphosphate precursors such as GDP (C_{10}) or GGDP (C_{20}) for mono-, diterpenoid and carotenoids production.

Synthesis of 10 out of 17 monoterpenoid compounds of lemon oil, widely used in the beverage and cosmetic industry, has been achieved in *E. coli* by cloning and expressing four *Citrus* monoterpene synthases (Lucker et al., 2002). These cyclases mainly synthesized (+)-limonene, (–)-β-pinene, and γ-terpinene, the major monoterpenpoids of lemon oil. cDNAs for various limonene synthases have also been cloned from *Mentha* species (Colby et al., 1993); *Abies grandis* (Bohlmann et al., 1997)*; Perilla frutescens* (Yuba et al., 1996); and *Schizonepeta tenuifolia* (Maruyama et al., 2001).

The formation of monoterpenes from several species of mint plants has been fully elucidated (Croteau et al., 2005). Menthol is a key monoterpene in peppermint (*Mentha piperita)* and provides its characteristic organoleptic cooling sensation. Biosynthesis of menthol involves cyclization of GDP by limonene synthase to limonene followed by hydroxylation by a P450 limonene-3-hydroxylase to *trans*-isopiperitenol (Lange and Croteau, 1999; Lupien et al., 1999; Alonso et al., 1992). *Trans*-isopipertitenol is then converted through the activity of a dehydrogenase, an isomerase and three reductases to (–)-menthol (Ringer et al., 2003; Ringer et al., 2005; Davis et al., 2005). In spearmint *(Mentha spicata)*, a homologous monoterpenoid hydroxylation reaction occurs but with differerent regiospecificity (C6) resulting in the formation of *trans*-carveol (Lupien et al., 1999; Haudenschild et al., 2000), which is converted to (–)-carvone by a dehydrogenase that is homologous to the peppermint enzyme (Ringer et al., 2005) (Figure 4). A single amino acid substitution has been found to convert the limonene-6-hydroxylase from spearmint into a limonene-3-hydroxylase (Schalk and Croteau, 2000).

The simpler spearmint pathway was installed in *E. coli* in a stepwise manner to develop a monoterpene production system (Carter et al., 2003). Overexpression of GDP synthase, limonene synthase, P450 hydroxylase and dehydrogenase in *E. coli* led to the secretion of 5 mg/L limonene into the culture medium. However, none of the targeted product carvone was detected, although feeding studies of recombinant *E. coli* with limonene demonstrated that the two last steps of the pathway were functional. Limited availability of isoprenoid precursors and the excretion of pathway intermediate limonene into the medium were identified as the main bottlenecks for efficient carvone production in *E. coli*. These problems could be overcome by increasing a) the isoprenoid precursor pool using the above described engineering strategies and b) activity of the two downstream pathway enzymes. It is well-known that high P450 activity is very difficult to reconstitute