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HARNESSING PLANT BIOMASS FOR BIOFUELS AND BIOMATERIALS

Biosynthesis of plant-derived flavor compounds

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Summary

Plants have the capacity to synthesize, accumulate and emit volatiles that may act as aroma and flavor molecules due to interactions with human receptors. These low-molecular-weight substances derived from the fatty acid, amino acid and carbohydrate pools constitute a heterogenous group of molecules with saturated and unsaturated, straight-chain, branched-chain and cyclic structures bearing various functional groups (e.g. alcohols, aldehydes, ketones, esters and ethers) and also nitrogen and sulfur. They are commercially important for the food, pharmaceutical, agricultural and chemical industries as flavorants, drugs, pesticides and industrial feedstocks. Due to the low abundance of the volatiles in their plant sources, many of the natural products had been replaced by their synthetic analogues by the end of the last century. However, the foreseeable shortage of the crude oil that is the source for many of the artifical flavors and fragrances has prompted recent interest in understanding the formation of these compounds and engineering their biosynthesis. Although many of the volatile constituents of flavors and aromas have been identified, many of the enzymes and genes involved in their biosynthesis are still not known. However, modification of flavor by genetic engineering is dependent on the knowledge and availability of genes that encode enzymes of key reactions that influence or divert the biosynthetic pathways of plant-derived volatiles. Major progress has resulted from the use of molecular and biochemical techniques, and a large number of genes encoding enzymes of volatile biosynthesis have recently been reported.

Keywords: aroma, flavor, terpenes, phenylpropanoids, fatty acid derivatives, furanones.

Introduction

Flavor is defined as the combination of taste and odor. It is, however, influenced by other sensations such as pain, heat, cold and tactile sensations, often referred to as the 'texture' of foods (Thomson, 1987). Due to the multitude of molecules that convey flavor, this review will only focus on flavor compounds that primarily impart smell, although several of them might also interact with taste receptors. Odorants are volatile chemical compounds that are carried by inhaled air to the olfactory epithelium located in the nasal cavities of the human nose (Buck and Axel, 1991). The odorant must possess certain molecular properties in order to produce a sensory impression. It must have a certain degree of lipophilicity and sufficiently high vapor pressure so it can be transported to the olfactory system, some water solubility to permeate the thin layer of mucus, and must occur at a sufficiently high concentration to be able to interact with one or more of the olfactory receptors.

The knowledge and use of plants as flavoring and seasoning to enhance the quality of foods, beverages and drugs is as old as the history of mankind. Plants used as spices and condiments are usually aromatic and pungent owing to the presence of varying types of essential oils. In addition, people have also used perfume oils and unguents made from plants on their bodies for thousands of years in lesser or greater amounts dependent on fashion whims. The first perfumes were all natural. In the 19th century, the commercialization of flavors and fragrances on an industrial scale started with the isolation of single chemicals responsible for the characteristic aroma of natural products (e.g. cinnamaldehyde isolated from cinnamon oil and benzaldehyde from bitter almond oil)

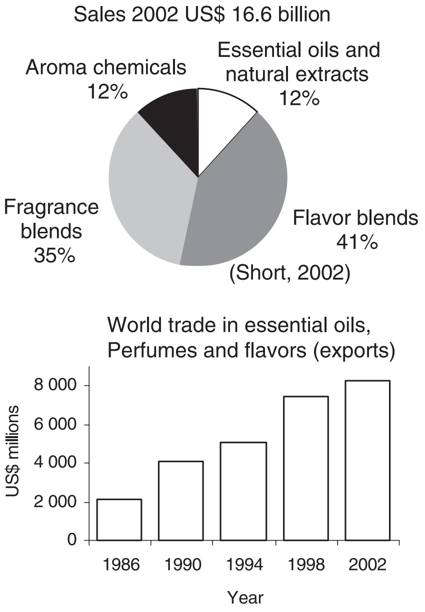

Figure 1. Overview of the flavor and fragrance industry.

at a time that is characterized by significant technological breakthroughs, largely in chemistry.

The first flavor and fragrance companies were founded by entrepreneurial scientists or business people, and many still exist. The synthesis of aroma chemicals that impart the characteristic odor and taste of natural products became commonplace and gave rise to a new branch of chemical specialities. Today, the total market for flavors and fragrances is estimated at US$18 billion, with market shares between the flavor and fragrance businesses being almost equal (Guentert, 2007). The largest markets are in Europe (36%) and North America (32%), followed by the Asian Pacific region (26%). Eight major global companies share 60% of the world market.

The flavor and fragrance industry is a composite of four closely inter-related and overlapping business sectors (Figure 1). Essential oils and other natural extracts are usually defined as aromatic materials obtained from botanical or animal sources by distillation, cold pressing, solvent extraction or maceration. Essential oils represent complex aroma mixtures of potentially hundreds of chemical constituents. Aroma chemicals are organic compounds with a defined chemical structure. They are produced by organic or biocatalytic synthesis or isolated from microbial fermentations, plants or animal sources, and are used to compound flavors and fragrances. Flavor blends and fragrance blends are complex formulations of aromatic materials such as essential oils and their natural derivatives as well as aroma chemicals containing up to 100 constituents. Formulated flavors are used by the food and beverage, tobacco and pharmaceutical industries, while formulated fragrances are used to give pleasant scents to fine fragrances, personal care and household products.

Limited information is available publicly on the size of the market for essential oils and plant extracts. Because flavor and fragrance trading is a multi-billion dollar business, the companies keep the compositions of their products confidential, thus the consumption values for aroma chemicals are only rough estimates and vary considerably from source to source. Information on the levels of the world's top 20 essential oils in volume terms was compiled some years ago (Table 1) (Lawrence, 1993). Orange (*Citrus sinensis*) oil production was 26 000 tonnes, followed by oils from cornmint (*Mentha arvensis*) and eucalyptus (*Eucalyptus globulus*) (cineole-type). Recent values for cornmint oil show that the market has grown substantially since then (Table 1). Experts forecast growth in global demand for flavor and fragrances of 5.4% per year, and predict that growth in demand for essential oils and natural extracts will outpace that for synthetic aroma chemicals over the next several years (Short, 2002). Worldwide transactions in the markets for essential oils show an annual average increase of 10% for exports (Figure 1). Flavor and fragrance blends that are composed of essential oils and synthetic aroma chemicals are still the largest product segments (in terms of sales) for the flavor houses (Figure 1). Although 2800 individual aroma chemicals are used in flavor and fragrance compositions, only a few hundred are offered on the merchant market and used in quantities larger than 50 tonnes per year.

Table 1 World's top essential oils in volume terms

Essential oil	Species	Volume (tonnes)[a]	Value[a] (US$ × 10^6)
Orange	*Citrus sinensis* (L.) Osbeck	26 000	58.5
Cornmint	*Mentha arvensis* L. f. *piperascens* Malinv. ex Holmes	4300 (20 000[b])	34.4
Eucalyptus cineole-type	*Eucalyptus globulus* Labill., *E. polybractea* R.T. Baker and other Eucalyptus species	3728	29.8
Citronella	*Cymbopogon winterianus* Jowitt and *C. nardue* (L.) Rendle	2830	10.8
Peppermint	*Mentha* × *piperita* L.	2367	28.4

[a] Lawrence (1993), except [b] Srivastava *et al.* (2002).

Table 2 Estimated world consumption of selected aroma chemicals in flavor and fragrance compositions

	Volume (tonnes)		
	1996[a]	2006[b]	Remarks
2-Phenylethyl alcohol and esters	7000	14 000	1 tonne natural; cost of natural: US$ 1000 per kg
Linalool and esters	6000	8000	
Esters of lower fatty acids	5800		
Vanillin	5500	10 000[c]	1800 tonnes natural; cost of natural: US$ 1000 per kg; cost of synthetic: US$ 11 per kg
Benzyl acetate	4000	12 000	
Menthol	4000	6300[d]	
Geraniol and esters	3500	12 000	
Ionones	3500	12 000	
Anethole	3200		
Cinnamaldehyde	2000	3000	
Aliphatic aldehydes	1450		
Benzyl salicylate	950	8000	
Benzyl benzoate	750	4000	
Jasmine derivatives	700	8000	
C_6 compounds and esters	400		10 tonnes natural; cost of natural: US$ 3000 per kg
Maltol	300		

[a]Somogyi (1996); [b]http://salesmanagement-info.blogspot.com/2007/07/top-30-aroma-chemicals-used-in-making.html, except [c]Schrader *et al.* (2004) and [d]http://de.wikipedia.org/wiki/Menthol.

Selected volatile substances even reach annual consumption rates of more than 5000 tonnes (Table 2). About 40% of the fragrance chemicals are also used in making flavors, but 80% of the global consumption of vanillin, menthol, eugenol, limonene, and esters of lower alcohols and lower fatty acids is used in making flavors.

Today, due to the high cost or lack of availability of natural flavor extracts, most commercial flavorants are 'nature-identical', which means that they are the chemical equivalent of natural flavors but are chemically synthesized, mostly from petroleum-derived precursors, rather than being extracted from the source materials. Because chemical synthesis often uses environmentally unfriendly production processes such as heavy metal catalysts, and crude oil represents a limited source, it is desirable to switch to bioproduction, including the extraction from natural sources, *de novo* microbial processes (fermentation), and bioconversion of natural precursors using micro-organisms or isolated enzymes (Guentert, 2007).

Biological functions of plant volatiles

Essentially all plant parts such as leaves, flowers, fruits and roots emit volatiles, which have multiple functions that are not always solely related to their volatility (Pichersky and Gershenzon, 2002). Because plant volatiles are involved in species-specific ecological interactions and are often restricted to specific lineages, they have been considered to be associated with defensive and attractive roles (Pichersky *et al.*, 2006). It is believed that they are not essential for plant survival but provide adaptive characteristics under strong environmental selection.

Compounds emitted by flowers most probably serve to attract and guide pollinators, but only a few studies have demonstrated the ability of individual substances to attract specific pollinators (Dudareva *et al.*, 2004). However, volatiles might also protect the carbohydrate-rich nectar by inhibiting microbial growth. Similar to humans, it is probably the qualitative and quantitative composition of the flavor molecules that imparts the specific sensory impression for the pollinators rather than the presence of a certain individual compound. Volatiles may be a better signal at night than floral color or shape to draw insect pollinators.

Because volatiles show anti-microbial and anti-herbivore activity, it is believed that they serve to protect valuable reproductive parts of plants from enemies. For example, one monoterpenol (*S*-linalool) and its derivatives significantly repelled an agricultural pest – the aphid *Myzus persicae* – in dual-choice assays (Aharoni *et al.*, 2003). Isoprene, a ubiquitous volatile hydrocarbon, acts to increase the tolerance of photosynthesis to high temperature by stabilizing the thylakoid membranes or quenching reactive oxygen species (Dudareva *et al.*, 2004).

A general property of vegetative plant tissue is the release of volatiles following herbivore damage (De Bruxelles and Roberts, 2001; Pichersky and Gershenzon, 2002). Some of these substances have been demonstrated to serve as indirect plant defenses through multi-trophic interactions because they attract arthropods that prey upon or parasitize the herbivores, thus minimizing further damage to plant tissue. However, volatiles also act as direct repellents or toxicants for herbivores and pathogens, and some have the potential to eliminate reactive oxygen species. This also includes root-emitted volatiles, which may function as anti-microbial or anti-herbivore substances or exhibit allelopathic activities that increase the ecological competitiveness of the plant (Steeghs *et al.*, 2004). Accordingly, plant volatiles can minimize the growth suppression of epiphytic bacteria by the phytopathogenic fungus *Botrytis cinerea* and thus affect population dynamics on leaf surfaces (Abanda-Nkpwatt *et al.*, 2006a), while simple alcohols emitted by leaves may provide a carbon and energy source for epiphytic methylotrophs (Abanda-Nkpwatt *et al.*, 2006b). Volatiles also attract female insects to lay eggs on flower buds and berries (Tasin *et al.*, 2007).

In fruits, volatile emission and accumulation have probably evolved to facilitate seed dispersal by animals and insects. For humans, volatiles in fruits have a considerable economic impact, as parameters of food quality and consumer preference. The function of fruit volatiles as a signal of ripeness and as an attractant for seed-dispersing organisms is supported by the fact that some substances are specifically formed by ripe fruits but are absent in vegetative tissues and non-ripe fruit. Unlike ripe fruits and flowers, vegetative tissues often produce and release many of the volatiles sensed as flavors only after their cells are disrupted. These volatile flavor compounds may exhibit anti-microbial activity and have anti-cancer activities but can be toxic at high doses (Goff and Klee, 2006).

From the chemical perspective, flavor molecules constitute a heterogeneous group of compounds, with straight-chain, branched-chain, aromatic and heteroaromatic backbones bearing diverse chemical groups such as hydroxyl, carbonyl, carboxyl, ester, lactone, amine, and thiol functions. More than 700 flavor chemicals have been identified and catalogued (Surburg and Panten, 2005; http://www.flavornet.org/index.html). Most are from various plant sources of diverse plant families and are major constituents of essential oils. The biosynthetic pathways of important plant volatiles have been traced back up to intermediates of primary metabolism (Croteau and Karp, 1991). It has been shown that carbohydrates, fatty acids and amino acids represent the natural carbon pools for flavor compounds, which can also be liberated from their polymers (Figure 2).

Biosynthetic pathways

As many plant flavor compounds are accumulated and biosynthesized in specialized anatomical structures (Figure 3) (Bagchi, 2000; Croteau *et al.*, 2000), the development of techniques for isolation of the secretory cells in such structures (Gershenzon *et al.*, 1992) has proven to be of crucial importance in our understanding of the key biosynthetic pathways and their regulation. Moreover, as these tissues contain many of the enzymes and significantly express many of the genes involved in the production of such metabolites, the isolation of secretory cells has greatly contributed to characterization of many of the enzymes and genes involved in the formation of many plant natural products in peppermint, sweet and lemon basil, as well as tomato and other crops (Fridman *et al.*, 2005; Gang *et al.*, 2002; Iijima *et al.*, 2004a,b; McConkey *et al.*, 2000). The following sections describe the biosynthesis of plant-derived flavor molecules grouped by their biogenetic origin.

Carbohydrate-derived flavor compounds

Furanones and pyrones. Furanones and pyrones (Figure 4) are important fruit constituents or have been isolated from the bark and leaves of several tree species (Schwab and Roscher, 1997). Although hexoses and pentoses are the primary photosynthetic products and serve as excellent flavor precursors in the Maillard reaction, only a limited number of natural volatiles originate directly from carbohydrates without prior degradation of the carbon skeleton. Such compounds include the furanones and pyrones (Bood and Zabetakis, 2002).

Substituted 4-hydroxy-3(2*H*)-furanones and the pyrone maltol constitute an uncommon group of flavor molecules with exceptional low odor thresholds. Furanones have been detected in a few plant species in which they are emitted only by the fruits. Maltol has been isolated from the bark and leaves of *Larix deciduas*, *Evodiopanax innovans*, *Cercidiphyllum japonicum* and four kinds of Pinaceae plants (Tiefel and Berger, 1993). Incorporation experiments using labeled precursors revealed that D-fructose-1,6-diphosphate is an efficient biogenetic precursor of furaneol. In strawberry (*Fragaria* × *ananassa*) and tomato (*Solanum lycopersicum*), the hexose diphosphate is converted by an as yet unknown enzyme to 4-hydroxy-5-methyl-2-methylene-3(2*H*)-furanone, which serves as the substrate for an enone oxidoreductase recently isolated from ripe fruit (Klein *et al.*, 2007; Raab *et al.*, 2006). A highly similar sequence was identified in an EST collection for pineapple (*Ananas comosus*), another species which produces furaneol in its fruits. In

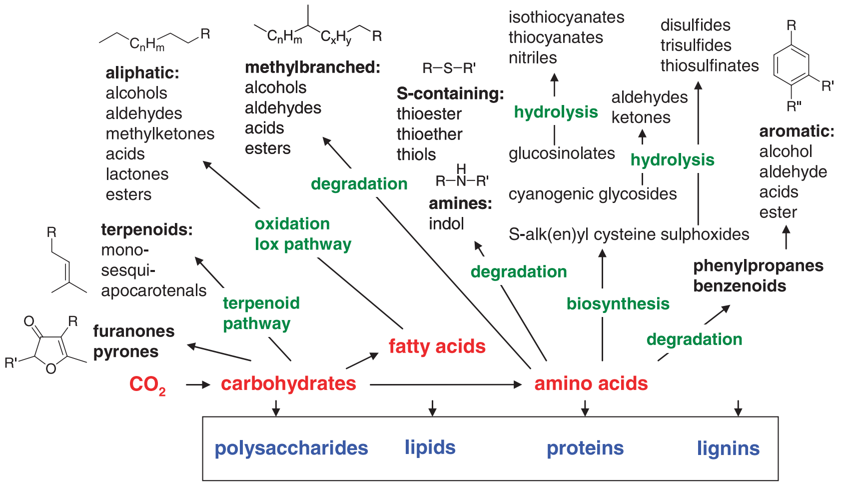

Figure 2. Natural carbon pools for the production of flavor compounds, and the pathways.

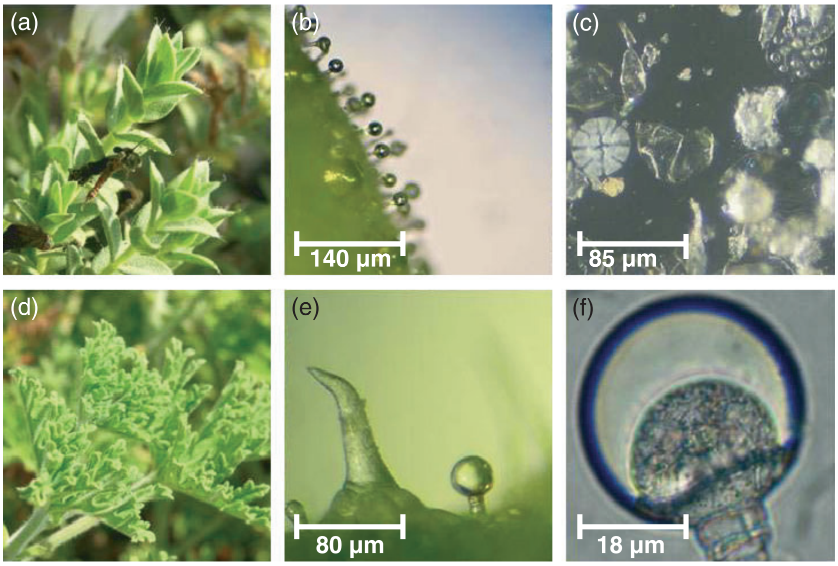

Figure 3. Glandular and non-glandular trichomes in *Origanum dayi* Post (Lamiaceae) (a–c) and *Pelargonium graveolens* (Geraniaceae) (d–f). Leaves (a, d) and light microscopy of the leaf surface (b,e) and isolated glandular trichomes (c, f) isolated according to the procedure first developed by Gershenzon *et al.* (1992).

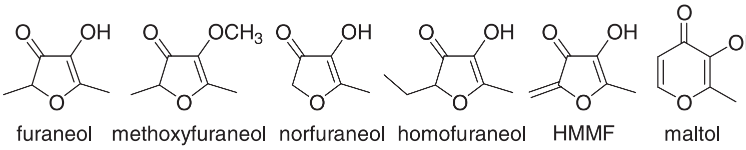

Figure 4. Carbohydrate-derived flavor molecules, including 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (furaneol), 2,5-dimethyl-4-methoxy-3(2*H*)-furanone (methoxyfuraneol), 4-hydroxy-5-methyl-3(2*H*)-furanone (norfuraneol), 2-ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone (homofuraneol), 4-hydroxy-2-methylene-5-methyl-3(2*H*)-furanone (HMMF) and 3-hydroxy-2-methyl-4*H*-pyran-4-on (maltol).

strawberry, furaneol is further metabolized by an *O*-methyltransferase (FaOMT) to methoxyfuraneol (Wein *et al.*, 2002). An *ortho*-diphenolic structure was identified as a common structural feature of the accepted substrates, and is also present in the dienolic tautomer of furaneol. Genetic transformation of strawberry with the *FaOMT* sequence in the antisense orientation, under the control of a constitutive promoter, resulted in a near total loss of methoxyfuraneol, demonstrating the *in vivo* methylation of furaneol by FaOMT (Lunkenbein *et al.*, 2006). However, the reduced level of methoxyfuraneol was only perceived by one third of the volunteer panelists, consistent with results obtained by aroma extract dilution assays. Norfuraneol and homofuraneol have been identified in tomato and melon fruits, respectively, but their biogenetic pathways and that of maltol remain unknown (Schwab and Roscher, 1997). However, studies in tomato and yeast have identified phosphorylated carbohydrates as potential precursors of the furanones (Hauck *et al.*, 2003; Sasaki *et al.*, 1991).

The furanones are mutagenic to bacteria and cause DNA damage in laboratory tests. However, they are also very effective anti-carcinogenic agents in the diets of animals, and their antioxidant activity is comparable to that of ascorbic acid (Slaughter, 1999). Norfuraneol has been identified as a male pheromone in the cockroach *Eurycolis florionda* (Walker), and furaneol deters fungal growth. Furaneol is also one of the key flavor compounds in the attractive aroma of fruits (Farine *et al.*, 1994). It has been proposed that the evolved biological function of the furanones is to act as inter-organism signal molecules in various systems. The 4-hydroxy-3(*2H*)-furanones associated with fruit aromas act to attract animals to the fruit, which ensures seed dispersal. In the case of humans, the coincidental chemical synthesis of these compounds in foods during preparation results in these foods appearing particularly attractive through transferred operation of the original signaling mechanisms (Slaughter, 1999).

Terpenoid pathway. Terpenoids are enzymatically synthesized *de novo* from acetyl CoA and pyruvate provided by the carbohydrate pools in plastids and the cytoplasm. Although fatty acid oxidation is one of the major pathways producing acetyl CoA, this process probably does not contribute to the formation of terpenoids as it takes place in peroxisomes. Terpenoids constitute one of the most diverse families of natural products, with over 40 000 different structures of terpenoids discovered so far. Many of the terpenoids produced are non-volatile and are involved in important plant processes such as membrane structure (sterols), photosynthesis (chlorophyll side chains, carotenoids), redox chemistry (quinones) and growth regulation (gibberellins, abscisic acid, brassinosteroids) (Croteau *et al.*, 2000). The volatile terpenoids – hemiterpenoids (C_5), monoterpenoids (C_{10}), sesquiterpenoids (C_{15}) and some diterpenoids (C_{20}) – are involved in interactions between plants and insect herbivores or pollinators and are also implicated in general defense or stress responses (Dudareva *et al.*, 2004; Pichersky and Gershenzon, 2002; Pichersky *et al.*, 2006). Terpenoids, mainly the C_{10} and C_{15} members of this family, were found to affect the flavor profiles of most fruits and the scent of flowers at varying levels (Figure 5). Citrus fruit aroma consists mostly of mono- and sesquiterpenes, which accumulate in specialized oil glands in the flavedo (external part of the peel) and oil bodies in the juice sacs. The monoterpene *R*-limonene normally accounts for over 90% of the essential oils of the citrus fruit (Weiss, 1997). The sesquiterpenes valencene and α- and β-sinensal, although present in minor quantities in oranges, play an important role in the overall flavor and aroma of orange fruit (Maccarone *et al.*, 1998; Vora *et al.*, 1983; Weiss, 1997). Nootkatone, a putative derivative of valencene, is a small fraction of the essential oils, but has a dominant role in the flavor and aroma of grapefruit (MacLeod and Buigues, 1964; Shaw and Wilson, 1981), while the monoterpene *S*-linalool was found to be an important general strawberry aroma compound (Aharoni *et al.*, 2004; Larsen and Poll, 1992) and is found in many other fruits including peaches, guavas, nectarines, papayas, mangoes, passion fruits, tomatoes, litchi, oranges, prickly pears and koubos (Baldwin *et al.*, 2000; Bernreuther and Schreier, 1991; Flath and Takahashi, 1978; Idstein *et al.*, 1985; Ninio *et al.*, 2003; Ong and Acree, 1998; Visai and Vanoli, 1997). The combination of the monoterpenes geraniol, citronellol and rose oxide is a key component of the characteristic aroma of aromatic muscat grapes as well as the special scent of roses (Bayrak, 1994; Dunphy and Allcock, 1972; Luan *et al.*, 2005).

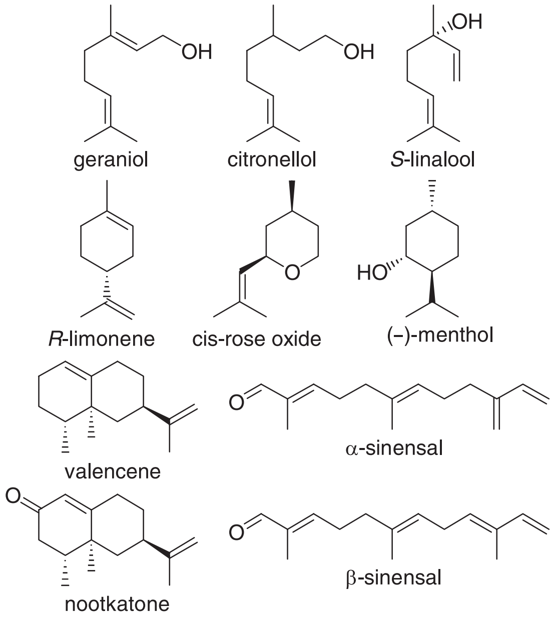

Figure 5. Important plant-derived volatile terpenoids.

Terpenoids are also the primary constituents of the essential oils of many types of herbs. The peltate glandular trichomes of peppermint produce copious amounts of a commercially valuable, menthol-rich essential oil, composed primarily of *p*-menthane monoterpenes (Turner and Croteau, 2004). The glandular trichomes of sweet basil (*Ocimum basilicum*) are rich in phenylpropenes as well as monoterpenes and sesquiterpenes (Iijima *et al.*, 2004a). Lemon-scented herbs of various plant families, such as lemon basil (*Ocimum* × *citratus*, Lamiaceae), lemongrass (*Cymbopogon citratus*, Poaceae) and lemon verbena (*Aloysia citriodora*, Verbenaceae), accumulate citral, a mixture of the *cis–trans* isomeric monoterpene aldehydes neral and geranial (Lewinsohn *et al.*, 1998; Iijima *et al.*, 2004a,b; Gil *et al.*, 2007). Therefore, many terpenoids are commercially important and are widely used as flavoring agents, perfumes, insecticides, anti-microbial agents and important raw material for the manufacture of vitamins and other key chemicals. Many terpenoids have medicinal properties; consequently they are of interest to the pharmaceutical industry as anti-retroviral agents or anti-malarial compounds (Modzelewska *et al.*, 2005). As a result, modulation of terpenoid biosynthesis in medicinal and aromatic plants has received much interest (Gómez-Galera *et al.*, 2007; Mahmoud and Croteau, 2001, 2002; Mahmoud *et al.*, 2004; Muñoz-Bertomeu *et al.*, 2006, 2007; Tadmor and Lewinsohn, 2007). Synthetic variations and derivatives of natural terpenes and terpenoids also greatly expand the variety of aromas used in perfumery and flavors used in food additives.

Despite their diversity, all terpenoids derive from the common building units isopentenyl diphosphate (IDP) and its isomer dimethylallyl diphosphate (DMADP) (Croteau and Karp, 1991; Croteau *et al.*, 2000; McGarvey and Croteau, 1995). In plants, both IDP and DMADP are synthesized via two parallel pathways, the mevalonate (MVA) pathway, which is active in the cytosol, and the methylerythritol 4-phosphate (MEP) pathway, which is active in the plastids (Lichtenthaler, 1999; Rodriguez-Concepción and Boronat, 2002; Rohdich *et al.*, 2002; Rohmer, 2003). It is generally recognized that the cytosolic pathway is responsible for the synthesis of sesquiterpenes, phytosterols and ubiquinone, whereas monoterpenes, gibberellins, abscisic acid, carotenoids and the prenyl moiety of chlorophylls, plastoquinone and tocopherol are produced in plastids (Lichtenthaler, 1999; Rodriguez-Concepción and Boronat, 2002; Rohdich *et al.*, 2002; Rohmer, 2003), but indications of cross-talk between the plastidic and cytosolic pathways have been found in tobacco, Arabidopsis and snapdragon petals (Aharoni *et al.*, 2004; Dudareva *et al.*, 2005; Ohara *et al.*, 2003). The direct precursors of terpenoids, linear geranyl diphosphate (GDP, C_{10}), farnesyl diphosphate (FDP, C_{15}) and geranylgeranyl diphosphate (GGDP, C_{20}), are produced by the activities of three prenyl transferases. Terpene synthases are the primary enzymes responsible for catalyzing the formation of hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}) or diterpenes (C_{20}) from the substrates DMADP, GDP, FDP or GGDP, respectively.

Prenyl transferases catalyze the addition of IDP units to prenyl diphosphates with allylic double bonds to the diphosphate moiety. Most of the prenyl transferases accept DMADP as the initial substrate, but they also bind GDP or FDP depending on the particular prenyltransferase (Greenhagen and Chappell, 2001; Tarshis *et al.*, 1994, 1996; Withers and Keasling, 2007). The availability of GDP and FDP are often the key factor in the production of monoterpenes and sesquiterpenes in plants. This problem was elegantly overcome in metabolic engineering experiments by the co-expression of GDP and FDP synthases with appropriate monoterpene and sesquiterpene synthases over-expressed in tobacco (Wu *et al.*, 2006). This strategy, together with targeting of the over-expression to the plastid compartment, resulted in increased synthesis of the sesquiterpenes amorpha-4,11-diene and patchoulol and the monoterpene *S*-limonene (Wu *et al.*, 2006).

The third phase of terpene volatile biosynthesis involves conversion of the various prenyl diphosphates DMADP, GDP, FDP and GGDP to hemiterpenes, monoterpenes, sesquiterpenes and diterpenes, respectively, by the large family of the terpene synthases. Triterpenes (and sterols) and tetraterpenes (such as carotenoids) are derived from the condensation of two molecules of FDP or GGDP, respectively. Plant hemiterpene, monoterpene, sesquiterpene and diterpene synthases are evolutionarily related to each other and are structurally distinct from triterpene or tetraterpene synthases. Much of the progress achieved in recent years in terpenoid metabolism is described elsewhere in this issue (Bohlmann and Keeling, 2008). Many terpene synthases have been isolated and characterized from various plant species (Bohlmann *et al.*, 1998; Tholl, 2006).

While many terpene volatiles are direct products of terpene synthases, many others are formed through transformation of the initial products by oxidation, dehydrogenation, acylation and other reactions (Croteau and Karp, 1991; Croteau *et al.*, 2000; Dudareva *et al.*, 2004; Pichersky *et al.*, 2006). For example, (–)-(*1R,2S,5R*)-menthol, the principal monoterpene of commercial peppermint essential oil and the component responsible for the familiar cooling sensation of peppermint and its products, is formed by eight enzymatic steps involving monoterpene synthases, isomerases and reductases (Ringer *et al.*, 2005; Turner and Croteau, 2004). The biosynthesis starts with the formation of 4*S*-limonene from GPP and ends with the reduction of (–)-menthone to (–)-menthol. *Mentha arvensis* is the primary species of mint used to make natural menthol crystals and natural menthol flakes. As with many widely used aroma chemicals, the annual demand for menthol of 6300 tonnes greatly exceeds the supply from natural sources.

Metabolic engineering of the terpenoid pathway is a constantly improving tool, used for the fundamental study of terpenoid biosynthesis (Lücker *et al.*, 2001, 2004; Ohara *et al.*, 2003). In addition, this tool is being used more and more for the understanding of chemical diversity in crops (Köllner *et al.*, 2004; Portnoy *et al.*, 2008), as well as improvement of traits in crops such as disease and pest resistance (Kappers *et al.*, 2005; Schnee *et al.*, 2006; Wu *et al.*, 2006), enhanced and altered aroma formation (Lavy *et al.*, 2002; Lewinsohn *et al.*, 2001; Mahmoud and Croteau, 2001) and production of medicinal compounds (Wu *et al.*, 2006). Most of the recent progress in this field has been summarized by Lücker *et al.* (2007). A recent example in which flavor engineering was detected by non-trained test panelists involved ectopic expression of the lemon basil geraniol synthase gene under the control of the fruit ripening-specific tomato polygalacturonase promoter (Davidovich-Rikanati *et al.*, 2007). This caused diversion of the plastidial terpenoid pathway for production of lycopene to the accumulation of high levels of geraniol and about ten novel geraniol derivatives, and had a profound impact on tomato flavor and aroma, as evaluated organoleptically.

Apocarotenoid formation. Carotenoids are tetraterpenoid pigments that accumulate in the plastids of leaves, flowers and fruits, where they contribute to the red, orange and yellow coloration. In addition to their roles in plants as photosynthetic accessory pigments and colorants, carotenoids are also precursors of apocarotenoids (also called norisoprenes) such as the phytohormone abscisic acid, the visual and signaling molecules retinal and retinoic acid, and aromatic volatiles such as β-ionone. Evidence, based on comparative genetics, has indicated that carotenoid pigmentation patterns have profound effects on the apocarotenoid and monoterpene aroma volatile compositions of tomato and watermelon fruits (Lewinsohn *et al.*, 2005a,b). This work indicated that the various flavors and aromas of otherwise similar fruit of different colors have a real chemical basis and are not solely due to psychological preconception. Indeed, enzymes capable of cleaving carotenoids at specific sites were found to be involved in the synthesis of a number of apocarotenoids. Carotenoid cleavage dioxygenases (CCDs) catalyze the oxidative cleavage of carotenoids, resulting in production of apocarotenoids (Schmidt *et al.*, 2006). CCDs often exhibit substrate promiscuity, which probably contributes to the diversity of apocarotenoids found in nature. Apocarotenoids are commonly found in the flowers, fruits, and leaves of many plants (Winterhalter and Rouseff, 2002), and possess flavor aroma properties together with low aroma thresholds. They are found among the potent flavor compounds in wines and contribute to floral and fruity attributes (Winterhalter and Schreier, 1994). Therefore, they have been subject to extensive research in recent years with regard to their structure and flavor potential (Winterhalter and Rouseff, 2002). The synthesis of β-ionone, geranyl acetone and 6-methyl-5-hepten-2-one in tomato fruits increases 10–20-fold during fruit ripening, and these compounds were produced by the activity of the genes Le*CCD1A* and Le*CCD1B* that were isolated from tomato fruits (Simkin *et al.*, 2004). In tomato fruit, β-ionone is present at very low concentrations (4 nl l^{-1}), but due to its low odor threshold (0.007 nl l^{-1}) is the second most important volatile contributing to fruit flavor (Baldwin *et al.*, 2000). Silencing of Le*CCD1A* and Le*CCD1B* resulted in a significant decrease in the β-ionone content of ripe fruits, implying a role for these genes in C13 norisoprenoid synthesis *in vivo* (Simkin *et al.*, 2004). Reduction of *Petunia hybrida CCD1* transcript levels in transgenic plants led to a 58–76% decrease in β-ionone synthesis in the corollas of selected petunia lines, indicating a significant role for this enzyme in volatile synthesis (Simkin *et al.*, 2004). Also, a potential CCD gene was identified among a *Vitis vinifera* L. EST collection, and recombinant expression of Vv*CCD1* confirmed that the gene encodes a functional CCD that cleaves zeaxanthin symmetrically yielding 3-hydroxy-β-ionone and a C_{14} dialdehyde (Mathieu *et al.*, 2005). CCDs were also found to be involved in the formation of important aroma compounds in melon (*Cucumis melo*) (Figure 6). The product of the *Cm*CCD1 gene, whose expression is up-regulated upon fruit development, was shown to cleave carotenoids, generating geranylacetone from phytoene, pseudoionone from lycopene, β-ionone from β-carotene, and α-ionone and pseudoionone from δ-carotene (Ibdah *et al.*, 2006).

Fatty acid-derived and other lipophylic flavor compounds

The majority of plant volatiles on a quantitative and qualitative basis originate from saturated and unsaturated fatty acids. Fatty acid-derived straight-chain alcohols, aldehydes, ketones, acids, esters and lactones are found ubiquitously in the plant kingdom at high concentrations, and are basically formed by three processes, α-oxidation, β-oxidation and the lipoxygenase pathway (Schwab and Schreier, 2002). In plants, fatty acids are stored as triacylglycerides and therefore enzymatic oxidative degradation of lipids is preceded by the action of acyl hydrolase, liberating the free fatty acids from acylglycerols. However, identification of a number of oxylipin-containing phosphatidylglycerols, monogalactosyldiacylglycerols and digalactosyldiacylglycerols demonstrated that direct oxidation of the fatty acid side chain in acylglycerides is possible (Buseman *et al.*, 2006).

Lipoxygenase pathway (in-chain oxidation). Saturated and unsaturated volatile C_6 and C_9 aldehydes and alcohols are important contributors to the characteristic flavors of fruits, vegetables and green leaves. They are widely used as food additives because of their 'fresh green' odor. The short-chain aldehydes and alcohols are produced by plants in response to wounding and play an important role in the plants defense strategies and pest resistance (Matsui, 2006; Stumpe and Feussner, 2006). At least four enzymes are involved in the biosynthetic pathway leading to their formation: lipoxygenase (LOX), hydroperoxide lyase (HPL), 3*Z*,2*E*-enal isomerase and alcohol dehydrogenase (ADH) (Figure 7).

LOX is a non-heme, iron-containing dioxygenase that catalyzes the regio- and enantio-selective dioxygenation of unsaturated fatty acids (e.g. linoleic and α-linolenic acid) containing one or more 1*Z*,4*Z*-pentadienoic moieties (Liavonchanka and Feussner, 2006). Numerous plant LOX have been characterized because they are essential components of the oxylipin pathway, converting fatty acids into hydroperoxides and finally flavors such as 3*Z*-hexenol, 2*E*-hexenal and 2*E*,6*Z*-nonadienal. Products of the LOX pathway

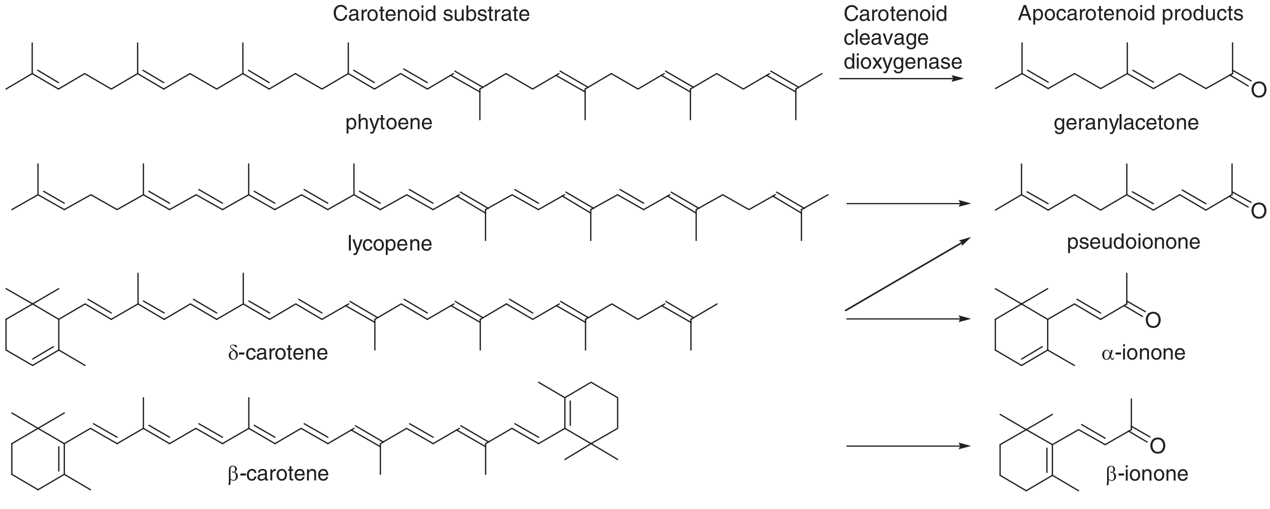

Figure 6. Carotenoids and their degradation products.
Carotenoid substrates (left) are oxidatively cleaved to yield the apocarotenoid derivatives (right).

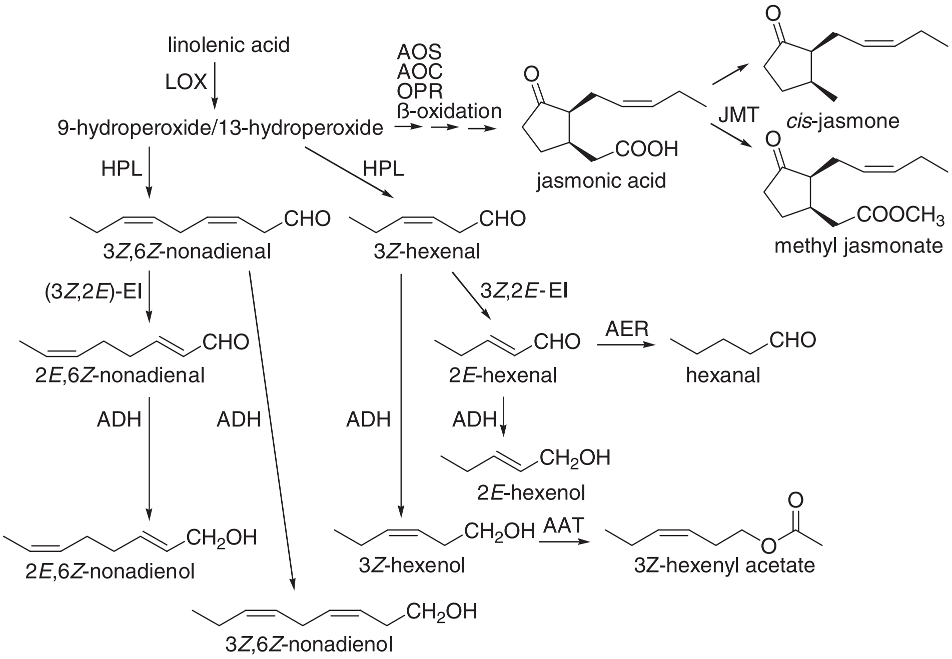

Figure 7. Linolenic acid-derived flavor molecules.
AAT, alcohol acyl CoA transferase; ADH, alcohol dehydrogenase; AER, alkenal oxidoreductase; AOC, allene oxide cyclase; AOS, allene oxide synthase; HPL, hydroperoxide lyase; JMT, jasmonate methyltransferase; LOX, lipoxygenase; OPR, 12-oxo-phytodienoic acid reductase; 3*Z*,2*E*-EI, 3*Z*,2*E*-enal isomerase.

are involved in wound healing, pest resistance and signaling, or have anti-microbial and anti-fungal activity. LOX enzymes have been classified with respect to their positional specificity with regard to fatty acid oxygenation. Oxygenation at C9 (9-LOX) or at C13 (13-LOX) of the hydrocarbon backbone leads to the (9*S*)- and (13*S*)-hydroperoxy derivatives, respectively. Plant LOX can also be grouped into two gene sub-families according to their overall sequence similarity. Enzymes carrying no plastidic transit peptide show a high sequence similarity (>75%) to one another and are designated type 1 LOX. Type 2 LOX harbor an N-terminal extension and have only a moderate overall sequence similarity (approximately 35%). The three-dimensional protein structures of soybean LOX-1 and -3 have been elucidated and essential amino acids identified (Liavonchanka and Feussner, 2006).

In vegetative tissues, LOX provides *Z,E*-configured hydroperoxides that can be metabolized to compounds that are crucial elements of plant defense. It is less clear why seeds and tubers have large amounts of LOX. Genetic removal of specific LOX isoforms appears not to compromise plant health (Baysal and Demirdöven, 2007). In tomato, five LOX genes (TomLoxA, B, C, D and E) are expressed during ripening. Antisense suppression of TomLoxA and B in tomato fruit resulted in no significant changes in the fruit flavor, but co-suppression of TomLoxC strongly affected the production of fatty acid-derived volatiles (Chen *et al.*, 2004).

HPL cleaves the LOX products, resulting in the formation of ω-oxo acids and volatile C_6 and C_9 aldehydes. Similar to LOX, HPL can be classified into two groups according to substrate specificity (Noordermeer *et al.*, 2001). HPL is a member of the cytochrome P450 family CYP74B/C, and acts on a hydroperoxy functionality in a lipid peroxide without any co-factor. Recently, a hemi-acetal has been identified as primary product of HPL (Matsui, 2006). Down-regulation of HPL has been performed in potato plants (Salas *et al.*, 2005). Such silencing of HPL induced an increase in LOX activity but a decrease of most of the C_6 volatiles.

The β,γ-unsaturated carbonyl functionality in the HPL products is prone to isomerization, either enzymatically catalyzed by a 3*Z*,2*E*-enal isomerase or non-enzymatically. Although 3*Z*,2*E*-enal isomerase activity has been described in soybeans (*Glycine max* L.) and alfalfa (*Medicago sativa* L.), neither a protein nor a corresponding gene has been cloned yet (Noordermeer *et al.*, 1999; Takamura and Gardner, 1996).

C_6 and C_9 aldehydes can be further metabolized by ADH to form the corresponding alcohols. *ADH* genes that are suspected to participate in the production of aromas are expressed in a developmentally regulated manner, particularly during fruit ripening (Manriquez *et al.*, 2006). Overexpression of the tomato *ADH2* gene has led to improved flavor of the fruit by increasing the levels of alcohols, particularly 3*Z*-hexenol (Speirs *et al.*, 1998). An acyltransferase catalyzes the formation of 3*Z*-hexenyl acetate from 3*Z*-hexenol and acetyl CoA, and 2-alkenal reductase can reduce 2*E*-hexenal to hexanal (D'Auria *et al.*, 2003; Mano *et al.*, 2002).

The reaction sequence leading from α-linolenic acid to the signaling molecule jasmonic acid involves the enzymes 13-LOX, allene oxide synthase, allene oxide cyclase and 12-oxo-phytodienoic acid reductase, followed by three successive β-oxidation steps (Figure 7) (Howe and Schilmiller, 2002; Li *et al.*, 2005). Jasmonic acid and its volatile methyl ester act as phytohormones and are involved in plant responses to stress and developmental processes. In addition, methyl jasmonate is the main component of the scent of jasmine flowers and contributes to the precious flavors of *Rosmarinus*, *Gardenia*, *Artemisia* and lemon oil. *cis*-jasmone, which acts as either an attractant or a repellent for various insects, is a decarboxylated derivative of jasmonic acid generated by oxidative degradation of jasmonate (Schaller *et al.*, 2005).

α- and β-oxidation. Although the degradation of straight-chain fatty acids by α- and β-oxidation is a major process for the formation of flavor molecules in all organisms, the specific pathways in plants are not well understood. The fatty acid α-oxidation mechanism in plants involves free fatty acids (C_{12}–C_{18}) that are enzymatically degraded via one or two intermediates to $C_{(n-1)}$ long-chain fatty aldehydes and CO_2. A dual-function α-dioxygenase/peroxidase and NAD^+ oxidoreductase catalyze the α-oxidation of fatty acids in plants (Saffert *et al.*, 2000). β-Oxidation results in successive removal of C_2 units (acetyl CoA) from the parent fatty acid. The detailed mechanisms of conventional β-oxidation are well established (Goepfert and Poirier, 2007). Forward and reverse genetic screens have revealed the importance of β-oxidation during plant development and in response to stress (Baker *et al.*, 2006). Combinations of mutations show much stronger phenotypes, but it is unclear whether the necessity for β-oxidation is to provide an energy source or a lipid-derived signal molecule.

Short- and medium-chain linear carboxylic acids that are formed by repeated β-oxidative cycles followed by the action of an acyl CoA hydrolase have been found in many essential oils isolated from different plant sources (Figure 8a). As a second pathway, *de novo* synthesis and hydrolysis of acyl acyl carrier protein (acyl ACP) can also provide volatile acids. Aliphatic acids up to C_{10} play a significant role in flavors due to their sharp, buttery and cheese-like odors, not only on their own, but particularly as substrates in the form of their acyl CoAs for biosynthesis of other flavors. Aliphatic short- and medium-chain aldehydes and alcohols are emitted by various plant parts and are probably formed by enzymatic reduction of the parent acyl CoAs (Flamini *et al.*, 2007). Alternatively, alcohols can also be formed by ADH-mediated hydrogenation of aldehydes, and medium-chain aldehydes are intermediates of the α-oxidation cycle starting with common fatty acids (Hamberg *et al.*, 1999). However, alcohols are less important as flavor molecules due to their high odor thresholds in comparison with their aldehyde homologues. Most plant ADHs are Zn-dependent medium-chain dehydrogenases that are thought to be involved in the response to a wide range of stresses, including anaerobiosis and elicitors (Chase, 2000). An ADH with specific substrate preference has been isolated from melons (Manriquez *et al.*, 2006). Specifically, flavor ester production relies upon the supply of acyl CoAs formed during β-oxidation and alcohols. Alcohol acyl transferases (AAT) are capable of combining various alcohols and acyl CoAs, resulting in the synthesis of a wide range of esters, thus accounting for the diversity of esters. Numerous *AAT* genes have been isolated and characterized in fruit and vegetables (Aharoni *et al.*, 2000; Beekwilder *et al.*, 2004; El-Sharkawy *et al.*, 2005). Aliphatic esters contribute to the aroma of nearly all fruits and are emitted by vegetative tissues. Some are responsible for a particular fruit aroma or for the smell of a flower. However, many of these esters possess a non-specific fruity odor. Another major group of fatty acid-derived flavor molecules are alkanolides, which have γ-(4-) or δ-(5-)-lactone structures (Figure 8a). Sensory important lactones usually possess 8–12 carbon atoms and some are very potent flavor components for a variety of fruits (Basear and Demirci, 2007). The fact that both the optical purity and the absolute configuration vary for identical lactones isolated from different sources supports the idea of different biosynthetic pathways. However, all lactones originate from their corresponding 4- or 5-hydroxy carboxylic acids, which are formed by either (i) reduction of oxo acids by NAD-linked reductase, (ii) hydration of unsaturated fatty acids, (iii) epoxidation and hydrolysis of unsaturated fatty acids, or (iv) reduction of hydroperoxides (Schöttler and Boland, 1996). Enzymes and genes specifically involved in the formation have not yet been reported. In contrast to 4- and 5-hydroxy fatty acids, 3-hydroxy acids, the

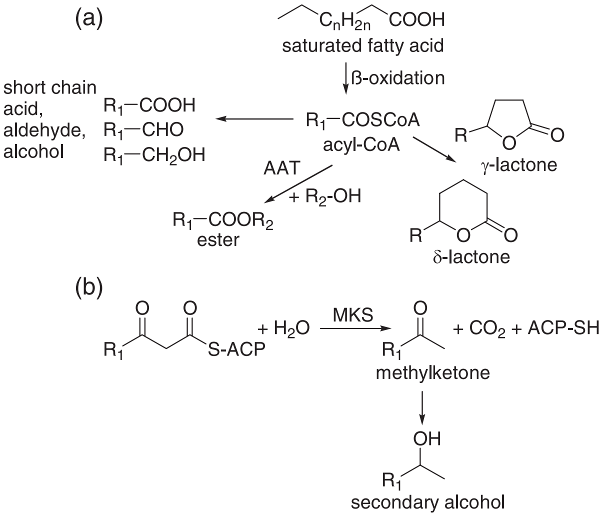

Figure 8. Biosynthesis of (a) short-chain acids, aldehydes, alcohols, esters and lactones, and (b) methylketones.
AAT, alcohol acyl CoA transferase; MKS, methylketone synthase; ACP, acyl carrier protein.

normal intermediates of the β-oxidation, do not form lactones. However, they are converted to methyl- or ethyl-3-hydroxyesters in plants and contribute to the aroma of fruits.

Short-length methylketones (C_5–C_{11}) are highly potent flavor molecules that have been found in numerous plants, while medium-length methylketones (C_7–C_{15}) are highly effective in protecting plants from numerous pests. Recently, the first methylketone synthase was isolated from tomato, which catalyzes the hydrolysis and subsequent decarboxylation of C_{12}, C_{14} and C_{16} β-ketoacyl ACPs to give C_{11}, C_{13} and C_{15} methylketones, respectively (Fridman *et al.*, 2005). In contrast, in fungi, methylketones are derived from β-oxidative degradation of fatty acids through β-ketoacyl CoA intermediates (Schwab and Schreier, 2002). Methylketones are assumed to be precursors of aroma-active secondary alcohols such as 2-pentanol and 2-heptanol, which are important flavor molecules produced by passion fruits (Figure 8b) (Strohalm *et al.*, 2007).

Amino acid-derived flavor compounds

Although aldehydes and alcohols derived from the degradation of branched-chain and aromatic amino acids or methionine constitute a class of highly abundant plant volatiles, their pathways have been barely analyzed in plants. Especially important are branched-chain volatiles derived from branched-chain amino acids. Isoamyl acetate, an ester with a strong fruity odor described as similar to banana or pear, is one of the key constituents of banana flavor (Surburg and Panten, 2005). 2-Methyl-butyl acetate has a strong apple scent and is associated with apple varieties that are rich in aroma such as 'Fuji', 'Gala' and 'Golden Delicious' (Dixon and Hewett, 2000; Holland *et al.*, 2005). Methyl 2-methyl butanoate determines the characteristic aroma of prickly pear (Weckerle *et al.*, 2001), while a combination of several volatile esters imparts the unique aroma of melons, with isoamylacetate and 2-methyl-butyl acetate being prominent in many varieties (Beaulieu and Grimm, 2001; Jordan *et al.*, 2001; Shalit *et al.*, 2001).

Acids, alcohols, aldehydes, esters, lactones and N- and S-containing flavor molecules. In micro-organisms, the catabolism of amino acids has been analyzed in detail and is initiated by aminotransferases forming 2-ketoacids that serve as substrates for three biochemical reactions: (i) oxidative decarboxylation to carboxylic acids, (ii) decarboxylation to aldehydes, and (iii) reduction to 2-hydroxyacids (Figure 9a) (Marilley and Casey, 2004). Compounds derived from leucine such as 3-methylbutanal, 3-methylbutanol and 3-methylbutanoic acid, as well as phenylacetaldehyde and 2-phenylethanol formed from phenylalanine, are abundant in various fruits such as strawberry, tomato and grape varieties (Aubert *et al.*, 2005). In addition, alcohols and acids derived from amino acids can be esterified to compounds with a large impact on fruit odor, such as 3-methylbutyl acetate and 3-methylbutyl butanoate in banana (Nogueira *et al.*, 2003).

Genes encoding enzymes responsible for the direct decarboxylation of phenylalanine have been isolated from tomato, petunia and rose, showing that alternative catabolic pathways exist in plants (Figure 9a) (Kaminaga *et al.*, 2006; Tieman *et al.*, 2006). Although the enzymes display subtle differences in sequences and enzymatic properties, their down-regulation led to reduced emission of 2-phenylacetaldehyde and 2-phenylethanol. Over-expression of the amino

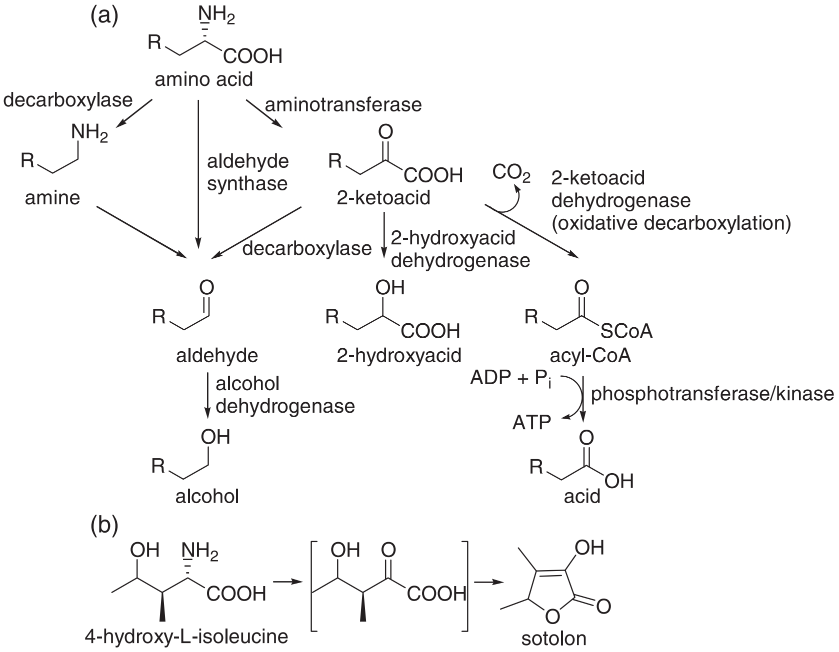

Figure 9. Biosynthesis of amino acid-derived flavor compounds.
(a) Catabolism of branched-chain amino acids leading to methyl branched flavor compounds, and (b) postulated biosynthesis of sotolon. Formation of aldehyde (a) from amino acids requires the removal of both carboxyl and amino groups. The sequence of these removals is not fully known and could be the opposite to that shown or aldehyde could be formed in one step by aldehyde synthase (Kaminaga *et al.*, 2006; Tieman *et al.*, 2006).

acid decarboxylase in tomato resulted in fruits with up to 10-fold increased levels of 2-phenylacetaldehyde, 2-phenylethanol and 1-nitro-2-phenylethane (Tieman *et al.*, 2006). The modulation of the emission of 2-phenylethanol and 2-phenylacetaldehyde is important because these substances exert a dual effect. At low concentrations, both compounds are associated with pleasant sweet flowery notes, while at high levels, the pungent aroma of 2-phenylacetaldehyde is nauseating and unpleasant (Tadmor *et al.*, 2002).

4,5-Dimethyl-3-hydroxy-2(5*H*)-furanone (sotolon) is the major flavor-impact compound of dried fenugreek seeds *Trigonella foenum-graecum* L., and is probably formed from 4-hydroxy-L-isoleucine (Slaughter, 1999). The supposed biogenetic origin is supported by stereochemical considerations (Figure 9b).

Sulfur-containing flavor compounds originating from methionine and cysteine are responsible for the odor of garlic (methanethiol, dimethyl disulfide, *S*-methyl thioacetate), onions (propanthial *S*-oxide), boiled potato (methional) and cooked cabbage (methanethiol) (Jones *et al.*, 2004). In Arabidopsis, the cleavage of methionine is catalyzed by methionine γ-lyase, resulting in the production of methanethiol, 2-ketobutyrate and ammonia (Rébeillé *et al.*, 2006). In onion (*Allium cepa*) and garlic (*A. sativum*), a series of volatile sulfur compounds is generated by cleavage of odorless *S*-alk(en)yl cysteine sulfoxide flavor precursors catalyzed by the enzymes allinase and lachrymatory-factor synthase (Jones *et al.*, 2004; Lanzotti, 2006). The biosynthetic pathway involves alk(en)ylation of the cysteine in glutathione, followed by cleavage and oxidation to form the sulfoxides or (thio)alk(en)ylation of cysteine or *O*-acetyl serine. Once the plant tissue is damaged, the flavor precursors are enzymatically cleaved by allinase to give a series of volatile sulfur compounds that undergo further vapor-phase chemical transformations (Figure 10a). Because the levels of the flavor precursors amount to 1–5% dry weight in certain *Allium* species, it is supposed that they play a major role for the plant. Two roles that have been ascribed are defense against pests and predation, particularly in the over-wintering bulb, and carbon, nitrogen and sulfur storage and transport (Jones *et al.*, 2004).

Volatile biogentic amines are another group of flavor molecules that are synthesized from amino acids or their precursors. The volatile indol is formed in maize by the cleavage of indole-3-glycerol phosphate, an intermediate in tryptophan biosynthesis (Figure 10b) (Frey *et al.*, 2000). Cruciferous vegetables such as mustard, broccoli, cauliflower, kale, turnips, collards, Brussels sprouts, cabbage, radish and watercress contain glucosinolates, which are natural precursors of flavor molecules. Glucosinolates,

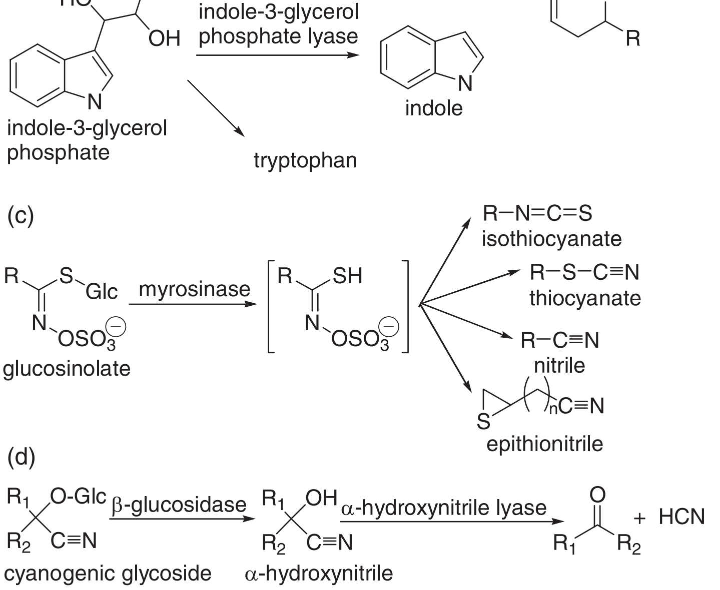

Figure 10. Biosynthetic pathways for (a) thiosulfinates and their degradation products, (b) formation of indole in maize, (c) volatiles produced from glucosinolates, and (d) cyanogenic glucosides.

which are synthesized from certain amino acids, are sulfur-rich, nitrogen-containing thioglycosides that, upon hydrolysis by endogenous thioglucosidases, produce volatile products such as isothiocyanates, thiocyanates and nitriles (Figure 10c) (Bones and Rossiter, 2006; Chen and Andreasson, 2001). These are the active substances that serve as defense compounds or attractants for the plant. For humans, they function as cancer-preventing agents, biopesticides and flavor compounds (Halkier and Gershenzon, 2006).

Cyanogenic glycosides, which are β-glycosides of α-hydroxynitriles, are another group of amino acid-derived flavor precursors (Figure 10d) (Bak *et al.*, 2006). Cyanogenesis is the process by which hydrogen cyanide and volatile ketones or aldehydes are released from cyanogenic glycosides and is dependent on glycosidase activities (Vetter, 2000). Enzymatic hydrolysis yields an unstable hydroxynitrile intermediate that spontaneously decomposes under certain conditions to hydrogen cyanide and a carbonyl compound. Alternatively, the intermediate can be broken down enzymatically by α-hydroxynitrile lyase. Ecological studies have shown that cyanogenic glycosides can act as either feeding deterrents or phagostimulants, depending on the insect species (Vetter, 2000).

Phenylpropenes and other aromatic derivatives. Benzenoid and phenylpropanoid volatile compounds, primarily derived from phenylalanine, contribute to the aromas and scents of many plant species and play important roles in plant communication with the environment (Dudareva and Pichersky, 2006; Knudsen and Gershenzon, 2006; Pichersky *et al.*, 2006). Several enzymes that catalyze the final steps in the biosynthesis of these compounds have been isolated and characterized. However, the early steps leading to the formation of the benzenoid backbone remain unclear (Beuerle and Pichersky, 2002; Schnepp and Dudareva, 2006; Wildermuth, 2006).

In general, biosynthesis of benzenoids from phenylalanine requires shortening of the carbon skeleton side chain by a C_2 unit, which can potentially occur via either the β-oxidative pathway or non-oxidatively (Boatright *et al.*, 2004). Experiments with stable isotope-labeled precursors in tobacco (*Nicotiana tabacum*) leaves (Ribnicky *et al.*, 1998) suggested that benzoic acid is produced from phenylalanine-derived cinnamic acid via the β-oxidative pathway, first yielding benzoyl CoA, which can then be hydrolyzed by a thioesterase to free benzoic acid. In contrast, labeling experiments, together with initial enzyme characterization, in *Hypericum androsaemum* cell cultures (Ahmed *et al.*, 2002) supported the existence of a pathway for non-oxidative conversion of cinnamic acid to benzaldehyde with subsequent formation of benzoic acid, which can be further converted to benzoyl CoA (Beuerle and Pichersky, 2002). *In vivo* isotope labeling and metabolic flux analysis of the benzenoid network in petunia (*Petunia hybrida*) flowers revealed that both pathways yield benzenoid compounds, and that benzyl benzoate is an intermediate between L-phenylalanine and benzoic acid (Boatright *et al.*, 2004). Transgenic petunia plants were generated in which expression of benzoyl-CoA: phenylethanol/benzyl alcohol benzoyltransferase (BPBT), the gene encoding the enzyme that uses benzoyl CoA and benzyl alcohol to make benzyl benzoate, was reduced or eliminated. Elimination of benzyl benzoate formation decreased the endogenous pool of benzyl acid and methyl benzoate emission but increased emission of benzyl alcohol and benzylaldehyde, confirming the contribution of benzyl benzoate to benzoic acid formation (Orlova *et al.*, 2006). Labeling experiments with $^2H_5$-phenylalanine revealed a dilution of isotopic abundance in most measured compounds in the dark, suggesting an alternative pathway from a precursor other than phenylalanine, possibly phenylpyruvate.

Phenylpropenes such as *t*-anethole, eugenol and isoeugenol are produced by plants as defense compounds against animals and micro-organisms and as floral attractants of pollinators. Moreover, humans have used phenylpropenes since antiquity for food preservation and flavoring and as medicinal agents (Gross *et al.*, 2002). Glandular trichomes of sweet basil (*Ocimum basilicum*), which synthesize and accumulate phenylpropenes, contain an enzyme that can use coniferyl acetate and NADPH to form eugenol (Koeduka *et al.*, 2006). Petunia (*Petunia hybrida* cv. Mitchell) flowers, which emit large amounts of isoeugenol, possess an enzyme homologous to the basil eugenol-forming enzyme that also uses coniferyl acetate and NADPH as substrates but catalyzes the formation of isoeugenol. The basil and petunia phenylpropene-forming enzymes belong to a structural family of NADPH-dependent reductases that also includes pinoresinol/lariciresinol reductase, isoflavone reductase and phenylcoumaran benzylic ether reductase (Koeduka *et al.*, 2006).

Phenylpropenes and benzoids are further subjected to methylation catalyzed by plant *O*-methyltransferases (OMTs) (Ibrahim *et al.*, 1998). Various OMTs involved in the biosynthetic pathways of floral scent components have been identified and characterized. For example, *S*-adenosyl-L-methionine (iso)eugenol OMT, which catalyzes the methylation of eugenol and isoeugenol to form the volatiles methyleugenol and isomethyleugenol, has been isolated from *Clarkia breweri* (Wang *et al.*, 1997). Eugenol OMT and chavicol OMT, which convert eugenol and chavicol to methyleugenol and methylchavicol, respectively, have been identified in *Ocimun basilicum* (Gang *et al.*, 2002; Lewinsohn *et al.*, 2000). Similarly, enzymatic activities able to convert *t*-anole to *t*-anethole and chavicol to estagole have been demonstrated in *Foeniculum vulgare* tissues (Gross *et al.*, 2002, 2006).

Another benzyl alcohol derivative, 1,3,5-trimethoxybenzene, has been identified as a key component of the odor of Chinese rose (Yomogida, 1992). This volatile is an effective sedative and has been used as a cosmetic additive (Shoji *et al.*, 2000). The biosynthesis pathway is thought to begin with phloroglucinol and includes three methylation steps. Many modern rose varieties synthesize a related compound, 3,5-dimethoxytoluene (Flament *et al.*, 1993), from orcinol (3,5-dihydroxytoluene) by two successive methylations. The orcinol *O*-methyltransferases responsible for these reactions have been characterized (Lavid *et al.*, 2002; Scalliet *et al.*, 2002). Orcinol *O*-methyltransferases can also carry out the last two methylation steps of 1,3,5-trimethoxybenzene biosynthesis but are incapable of efficiently methylating phloroglucinol, explaining the absence of 1,3,5-trimethoxybenzene from the floral scent of modern rose varieties. A novel phloroglucinol *O*-methyltransferase that methylates the first step to produce the intermediate 3,5-dihydroxyanisole has been isolated from rose petals, and the two previously described orcinol *O*-methyltransferases catalyze the subsequent steps (Wu *et al.*, 2004).

Methyl salicylate and methyl benzoate are common components of floral scent and are believed to be important attractants of insect pollinators (Dobson, 1994; Dudareva and Pichersky, 2000; Dudareva *et al.*, 1998, 2000). Enzymes that catalyze the formation of methyl salicylate and methyl benzoate from salicylic acid (SA) and benzoic acid (BA), respectively, have been characterized from flowers of *Clarkia breweri*, snapdragon (*Antirrhinum majus*), petunia (*Petunia hybrida*), *Arabidopsis thaliana* and *Stephanotis floribunda* (Chen *et al.*, 2003; Murfitt *et al.*, 2000; Negre *et al.*, 2002; Pott *et al.*, 2002; Ross *et al.*, 1999). While these enzymes use *S*-adenosyl-L-methionine as the methyl donor as do many previously characterized methyltransferases that act on a variety of substrates (e.g. DNA, protein, phenylpropanoids), these SA and BA carboxyl methyltransferases have primary amino acid sequences that show no significant sequence identity to other methyltransferases. Interestingly, a group of *N*-methyltransferases involved in biosynthesis of the alkaloid caffeine, including theobromine synthase, share sequence similarity with the SA and BA carboxyl methyltransferases (D'Auria *et al.*, 2003). These enzymes were therefore grouped into a new class of methyltransferases designated the SABATH methyltransferases, and this family now also includes jasmonic acid methyltransferase (Seo *et al.*, 2001), indole-acetic acid methyltransferase (Zubieta *et al.*, 2003) and cinnamic/*p*-coumaric acid methyltransferase (Kapteyn *et al.*, 2007). The recently obtained three-dimensional structure of *C. breweri* SA carboxyl methyltransferase (Zubieta *et al.*, 2003), combined with *in silico* modeling of the active site pocket in the *Nicotiana suaveolens* and *S. floribunda* enzymes (Pott *et al.*, 2004), also indicates that these enzymes have a unique structure that is distinct from those of unrelated methyltransferases found in plants (Noel *et al.*, 2003).

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the most widely used flavor compound in the world. It is the principal flavor component of the vanilla extract obtained from cured pods (beans) of the orchid *Vanilla planifolia* Andrews. Vanillin accumulates in the secretion around the seeds in the mature fruits. A unique secretory tissue composed of closely packed unicellular hairs is located in three gaps between the placentas along the central fruit cavity. These cells seem to be responsible for vanillin secretion (Joel *et al.*, 2003). Vanilla extract is valued as a natural flavor, but, because of its cost and limited availability, less than 1% of the annual world demand for vanillin is isolated from its natural source (Walton *et al.*, 2003). Most of the vanillin used by the flavor industry originates from chemical methods that use guaiacol, eugenol or lignin as starting materials (Rao and Ravishankar, 2000). Vanillin is believed to be synthesized from phenylpropanoid precursors, and various biosynthetic pathways have been proposed. A three-step pathway for vanillin biosynthesis from 4-coumaric acid has been proposed based on precursor accumulation and on feeding cell cultures of *V. planifolia* with the proposed precursors (Havkin-Frenkel *et al.*, 1999). In this pathway, 4-coumaric acid is first converted to 4-hydroxybenzaldehyde by 4-hydroxybenzaldehyde synthase through a chain-shortening step (Podstolski *et al.*, 2002). Then, hydroxylation at position 3 on the ring is performed by 4-hydroxybenzaldehyde synthase, converting *p*-hydroxybenzyl alcohol to 3,4-dihydroxybenzyl alcohol or aldehyde. The final enzymatic step was shown to be catalyzed by a multifunctional *O*-methyltransferase from *V. planifolia* that has a broad substrate range, including 3,4-dihydroxybenzaldehyde (Pak *et al.*, 2004).

Biotechnological processes

Natural aroma chemicals have been used for millennia and are still used today. However, over the past 50 years, due in part to the expense of natural ingredients, the flavor and fragrance industry has developed and uses synthetic products comprising nature-identical odorants and artifical compounds that are not found in nature. Today, 90% of the newly introduced fragrance ingredients are synthetic aroma chemicals (De Preville, 2006). However, the demand for natural aroma chemicals is growing fast, in response to both consumers, who are asking for a return to nature, as well as perfumers and flavorists, who are looking for novel creative ingredients. Therefore, the percentage of natural flavors with respect to all added flavors has increased to 90% (EU) and 80% (USA) in beverages, to 80% (EU and USA) in savory foods, and to 50% (EU) and 75% (USA) in dairy foods (Schrader, 2007). Increasing oil prices have put additional pressure on this trend. However, the quality and supply of traditional natural flavor and fragrance chemicals are limited. In addition to extraction from natural sources, viable alternative and innovative ways to provide flavor and fragrance chemicals include biotechnological routes, i.e.

microbial fermentation, biotransformation using whole cells, biocatalysis using isolated enzymes, plant tissue culture and transgenic plants.

By volume, short-chain acids and alcohols are the most prominent aroma-active compounds produced by biotechnology. The strong oxidative capabilities of *Acetobacter* and *Gluconobacter* strains are harnessed for the production of acetic acid, propionic acid, butanoic acid, 2-methylpropanoic acid, 2-methylbutanoic acid and 3-methylbutanoic acid, and the derived short-chain alcohols constitute cheap by-products during the distillation of 'bioethanol' (Schrader, 2007). These natural acids and alcohols are of great importance to the flavor industry (US$ 100 per kg), either because of their intense smell and sour taste, in the case of the acids, or as substrates for enzymatic synthesis of flavor esters (Schrader *et al.*, 2004). The derived aldehydes are efficiently produced by oxidation of the corresponding alcohols using alcohol oxidase- or alcohol dehydrogenase-expressing micro-organisms, e.g. *Pichia pastoris*.

Microbial biotransformation and biosynthesis of flavor and fragrance chemicals offer the additional potential benefits of producing optically active isomers that often have marked differences in flavor and fragrance quality and sensory intensity. 4*R*-decanolide (*R*-γ-decalactone), which imparts a fruity, peach-like aroma, has an annual market volume of several hundred tonnes. Natural 4*R*-decanolide was an extremely expensive, natural aroma chemical (US$ 10 000 per kg) in the early 1980s. Today, it is primarily produced by *Yarrowia lipolytica*, resulting in a drop of the price to US$ 300 per kg. The yeast degrades ricinoleic acid (12*R*-hydroxy-9*Z*-octadecenoic acid), the dominant fatty acid of castor oil, to the enantiomerically pure lactone (Schrader, 2007; Schrader *et al.*, 2004). Alternatively, optically pure aroma chemicals, e.g. (−)-menthol may be commercially synthesized by enantio-selective hydrolysis of racemic substrates, e.g. menthyl benzoate, by recombinant *Candida rugosa* lipase (Menzel and Schreier, 2007).

Natural 3*Z*-hexenol (US$ 3000 per kg) is also produced competively compared to its isolation from peppermint oil by a series of biocatalytic steps. The industrial process mimics the plant biochemical pathway starting from linolenic acid and exploiting crude plant enzyme preparations of lipoxygenase (soy flour) and hydroperoxide lyase (guava fruit homogenate) to produce the desired aldehyde. Additionally, baker's yeast is used as reducing catalyst to convert the aldehyde to the corresponding alcohol (Figure 7) (Schrader, 2007).

Biosynthetic vanillin has been marketed since 2000, and is prepared by the action of micro-organisms on ferulic acid extracted from rice bran (Barghini *et al.*, 2007). At US$ 1000 per kg, this product is not cost-competitive compared with petrochemical vanillin, which sells for around US$ 11 per kg, but it can be labeled as natural flavoring. Natural vanillin derived from *Vanilla* is only available in very low amounts and is therefore limited to a few selected food applications. Although *V. planifolia* cell cultures have been studied extensively, no economical feasible vanillin production has resulted from this (Korthou and Verpoorte, 2007).

The examples show that biotechnological production of flavor compounds is a mature discipline in the chemical industry. About 100 aroma chemicals in the market are already produced by enzymatic or microbial processes, including genetically engineered micro-organisms (Schrader, 2007). These production lines rely on plants as the source of the flavor precursors and most of the involved genes and enzymes originate from microbial sources, except for biotechnological production of the C_6 compounds. Successful metabolic engineering of the pathways of a number of plant flavor molecules has already been achieved in plants, but these transgencis are not yet economically competitive (Davidovich-Rikanati *et al.*, 2007; Lavy *et al.*, 2002; Lewinsohn *et al.*, 2001; Mahmoud and Croteau, 2001; Muñoz-Bertomeu *et al.*, 2006). In some cases, genetic manipulation led to changed phenotypes, indicating the depletion of essential intermediates through redirection of the carbon flux (Aharoni *et al.*, 2003). Thus, more research is necessary to elucidate the regulation of flavor formation in plants.

Plant volatile oils are synthesized, stored and released by a variety of specialized epidermal or mesophyll structures, probably because most of the flavor molecules are phytotoxic. The regulation and formation of these structures is not understood, but their manipulation, whether achieved through conventional means or modern molecular approaches, may be an additional potential approach for changing oil composition and quality.

Possible uses of flavor molecules

Aroma-active volatiles are important secondary plant metabolites that are traditionally used for their flavor and fragrance in the food, pharmaceutical and perfumery industries to improve the attractiveness of the products (Guentert, 2007). However, they also serve as precursor sources for the production of aroma chemicals, drugs and plant protection agents, particularly enantiomers, and are useful chiral building blocks in chemical and biotechnological syntheses (De Carvalho and da Fonseca, 2006). Identification of the biological and pharmacological activities of flavor compounds has been gathering momentum in recent years. The commercialization of plant volatiles is also focused on their bioactivities, including anti-microbial, anti-viral, anti-oxidative, analgesic, digestive, anti-carcinogenic, semiochemical and other activities (Koroch *et al.*, 2007). Aromatherapy is a form of complementary or alternative medicine that uses essential oils and other scented plant compounds for therapeutic purposes, affecting a person's mood or health (Kiecolt-Glaser *et al.*, 2008). Thus, essential oils as a rich source of flavor molecules are used widely to prevent and treat human diseases (Edris, 2007). The sesquiterpene artemisinin, derived from *Artemisia annua*, has attracted special attention due to its anti-malarial activity (Kindermans *et al.*, 2007). Alternative sources for the supply of the anti-malarial compound have been proposed due to shortage of the raw material (Chang *et al.*, 2007). In addition, volatiles may also be used to effectively manipulate rumen microbial fermentation to reduce the formation of ammonia and methane (Calsamiglia *et al.*, 2007). Thus, flavor compounds have numerous functional properties and exert their action in mammals as well as in other organisms. They will continue to be indispensable natural ingredients.

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