Recent Advances in Understanding the Regulation of Plant Flavour

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ABSTRACT

Plants emit a number of volatile organic compounds some of which are very attractive for perfume and cosmetic industry which reached about 11.6 billion US dollars in the global market in 2003. To date, about 17,000 plant volatile compounds have been identified. In the last two decades efforts have been made to understand the biosynthesis, functions and regulation of emission of plant volatiles and to target certain volatiles for exploitation at the commercial level. Work is still undergoing in several laboratories in this field by using the most advanced molecular techniques. Plant volatiles are mostly derived from three main classes of compounds: terpenoids, phenyl propanoids/benzenoids and fatty acid derivatives. This review deals with the function and regulation of plant volatiles highlighting the application of new biotechnological approaches to improve the aroma and scent of flowers and fruits with high commercial value.

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INTRODUCTION

Plants synthesize and emit three main classes of volatile compounds: terpenoids, benzenoids and fatty-acid derivatives. Plant volatiles (PVs) are typically lipophilic liquids with high vapor pressures and non-conjugated PVs can cross membranes freely and be released into the atmosphere or soil in the absence of a diffusion barrier (Pichersky *et al.* 2006). Whereas some volatiles are probably common to almost all plants, others are specific to only one or a few related taxa. These PVs have important functions in the form of reproduction, defense, plant–plant communication and tritrophic interaction.

FUNCTIONS OF PLANT VOLATILES

Reproduction

Many plants emit floral scents to attract and guide a variety of insect pollinators to improve reproductive success (Pi-

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chersky and Gershenzon 2002; Dotterl and Jürgens 2005). Floral scents serve as attractants for species-specific or species-non–specific pollinators (Knudsen *et al*. 1999). The scent production or emission in many flowers is reduced after pollination. In snapdragon (*Antirrhinum majus*) and petunia, a 70 to 75% pollination-induced decrease in methylbenzoate emission begins only after pollen tubes reach the ovary (Negre *et al*. 2003). Post pollination process initiates with the decrease in emission of the flower scents and in its attractiveness to pollinators, and increases an overall reproductive success of the plant by directing pollinators to unpollinated flowers (Negre *et al*. 2003; Verdonk *et al*. 2003).

Defense

It is now recognized that herbivore-induced PVs appear to mediate both direct and indirect defences and even signal to nearby plants. Volatiles emitted by vegetative tissue after hervivory can directly repel or intoxicate microbes and animals, or attract insects and mites that prey upon or parasitize herbivores, and thereby reduce further damage to the plant (Pare and Tumlinson 1999; Dicke and van Loon 2000). The composition of the volatiles emitted by damaged plants is specific for the plant species and the herbivore that damages the plant (Takabayashi and Dicke 1996; Turlings *et al*. 1998). Many floral volatiles also function as defense agent since they have anti-microbial or anti-herbivore activity (de Moraes *et al*. 2001; Hammer *et al*. 2003) and thus can protect valuable reproductive parts of plants from enemies.

PVs are induced by herbivory on aerial or below-ground plant tissues. These PVs include C_6 green-leaf volatiles (GLV, e.g. (*Z*)-3-hexenal and (*Z*)-3-hexenyl acetate), methyl salicylate, methyl jasmonate, indole, terpenes and others. These volatiles can act as direct defense compounds (de Moraes *et al*. 2001) or play a role in indirect defense (Dicke *et al*. 2003; Rasmann *et al*. 2005). GLV can account for >50% of the emissions from damaged plant parts and are typically released mostly from damaged plant cells within 1–2 seconds after the mechanical damage but some GLV are released from younger undamaged leaves of herbivore-damaged plant (Fall *et al*. 1999).

Terpene volatiles like monoterpenes and sesquiterpenes have frequently been shown to be emitted from flowers and other aerial parts of the plant, and their release is often developmentally regulated or induced by damage (Dudareva *et al*. 2003; Arimura *et al*. 2004). Conifer terpenoids have a variety of influences on forest insects by mobilizing a terpenoid called oleoresin (comprised of a diverse array of terpenoid compounds) to the site of wounding (Huber *et al*. 2004; Martin and Bohlmann 2005). Like isoprene, some herbivore-induced monoterpenes and sesquiterpenes have the potential to scavenge with various reactive oxygen species (Hoffmann *et al*. 1997; Bonn and Moortgat 2003), and so could protect against internal oxidative damage (Delfine *et al*. 2000; Loreto *et al*. 2004). Volatile monoterpenes and sesquiterpenes have also been reported to be synthesized and accumulated in roots and rhizomes of various plant species (Kovacevic *et al*. 2002) and because of their antimicrobial and antiherbivore activity, these substances may serve as direct defences of below-ground tissue when they accumulate.

Tritrophic interaction

The tritrophic plant-herbivore-carnivore interactions have been reported in more than 23 plant species in combination with a diverse range of herbivore and carnivores (Dicke 1999). One of the well-studied examples for this tritrophic interaction includes interactions between lima bean plants (*Phaseolus lunatus*), herbivorous spider mites (*Tetranychus urticae*), and carnivorous mites (*Phytoseiulus persimilis*). The attraction of the predatory mite *P. persimilis* to the sesquiterpene alcohol (3*S*)-(*E*)-nerolidol was recently demons-

trated with transgenic *Arabidopsis thaliana* overexpressing strawberry nerolidol synthase, a terpene synthase (TPS) (Kappers *et al*. 2005). The result suggested that (3*S*)-(*E*)-nerolidol is a component of the volatile signal that attracts the predatory mites to spider mite-infested plants. Overexpression in *A. thaliana* of another terpene synthase gene, the corn TPS10 gene which forms (*E*)-*β*-farnesene, (*E*)-*α*-bergamotene, and other herbivore-induced sesquiterpene hydrocarbons released from maize upon herbivory by lepidopteran larvae, increased attractiveness of the transgenic plant to the parasitic wasps, *Cotesia marginiventris* (Schnee *et al*. 2006).

Communication

PVs released from herbivore-infested plants mediate inter-organ and interplant interactions; These PVs may induce the expression of defense genes and the emission of volatiles in healthy leaves on the same plant or of neighboring non-attacked plants, thus increasing their attractiveness to carnivores and decreasing their susceptibility to the damaging herbivores (Arimura *et al*. 2004; Ruther and Kleier 2005). PVs also prime the neighboring plants to respond faster to future herbivore attack (Engelberth *et al*. 2004; Kessler *et al*. 2006). A recent investigation has shown convincingly that *Nicotiana attenuata* plants growing adjacent to artificially wounded *Artemisia tridentata* (sagebrush), with air but no soil contact between the plant species, suffered reduced levels of herbivore damage and exhibited increased levels of the defensive enzyme polyphenol oxidase, compared to *N. attenuata* growing adjacent to undamaged sagebrush (Karban *et al*. 2000). A small amount of volatile hormones such as ethylene and derivatives of salicylic acid or jasmonic acid serves roles in long-distance communication (Pichersky and Gershenzon 2002; Truman *et al*. 2007). The enormous variety of metabolites emitted by plants suggests that volatile compounds may provide a detailed language for communication. Very recently, it has been shown that the volatile, methyl benzoate emitted by snapgdragon flowers inhibits the root growth of neighboring *Arabidopsis* (Horiuchi *et al*. 2007). The importance of PVs in mediating interactions between plant species is much debated. It has been demonstrated that the parasitic plant *Cuscuta pentagona* (dodder) uses volatile cues for host location. For example, *C. pentagona* seedlings exhibit directed growth toward nearby tomato plants (*Lycopersicon esculentum*) and toward extracted tomato volatiles in the absence of other cues. Impatiens (*Impatiens wallerana*) and wheat plants (*Triticum aestivum*) also elicit directed growth. Moreover, the seedlings can distinguish tomato and wheat volatiles and preferentially grow toward the former. Several individual compounds from tomato and wheat elicit directed growth by *C. pentagona*, whereas one compound from wheat is repellent. These findings provide compelling evidence that volatiles mediate important ecological interactions among plant species (Runyon *et al*. 2006).

BIOCHEMISTRY OF PLANT VOLATILES

Types of volatile compounds

PVs are lipophilic molecules with high vapor pressure and serve various ecological roles. The headspace sampling and relatively inexpensive chromatography-mass spectrometry make it possible to identify thousands of floral scents. The solid-phase microextraction (SPME) process and proton-transfer-reaction mass spectrometry (PTR-MS) have emerged as useful tools for monitoring PVs (Steeghs *et al*. 2004; Pellati *et al*. 2005). Furthermore, this technique is sensitive enough to identify plant odors from specific tissues of floral organs (Barták *et al*. 2003). The substances reported are largely lipophilic products with molecular masses of 30-300 atomic mass units. Most can be assigned to the following classes (in order of decreasing size): terpenoids, fatty acid derivatives including lipoxygenase pathway pro-
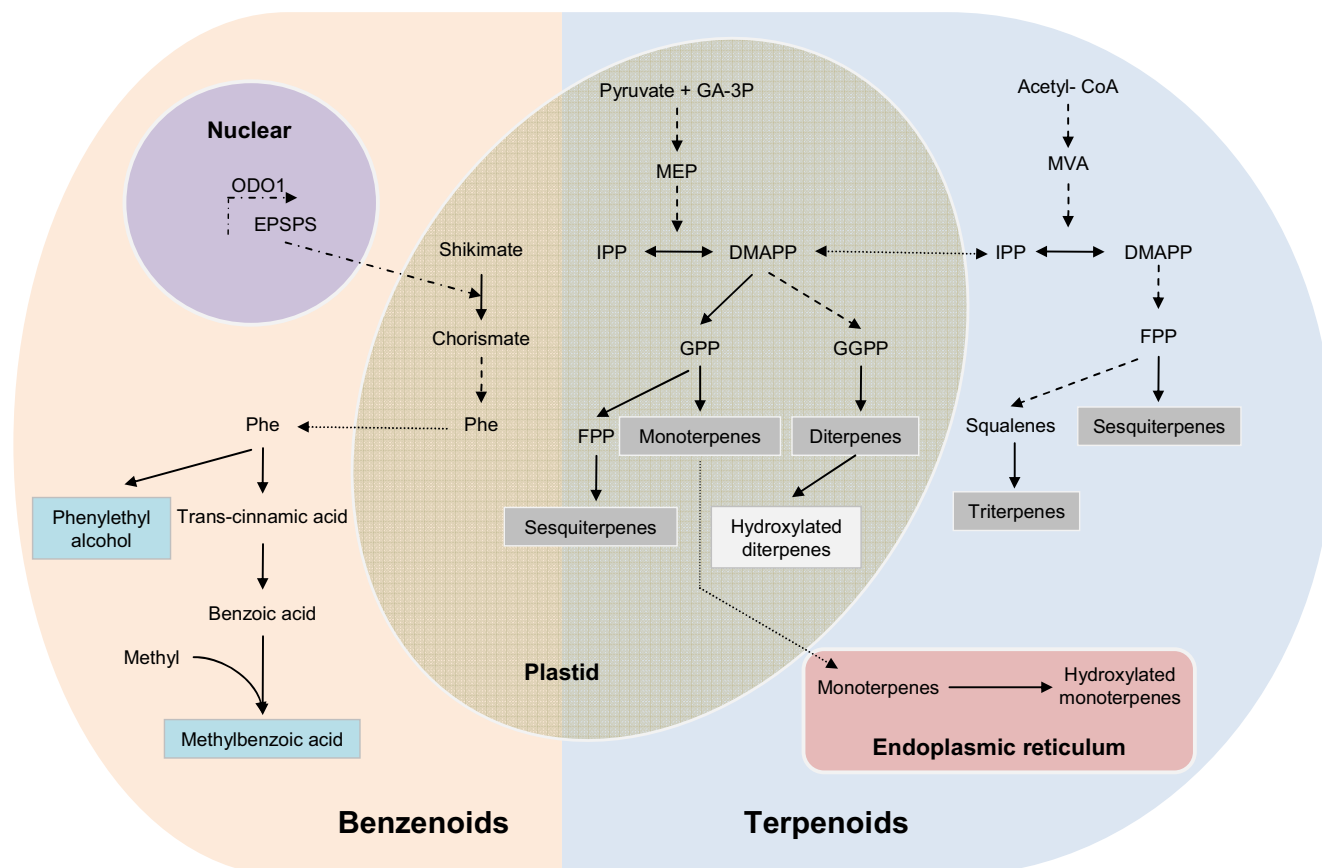
Fig. 1 Benzenoids and terpenoids biosynthesis in plants. Solid arrows, dashed arrows, dotted arrows, and dotted-dashed arrows indicate single enzymatic steps, multiple enzymatic steps, transportation, and activation, respectively. Final products are boxed. Abbreviations: Acetyl-CoA, Acetyl coenzyme-A; DMAPP, dimethylallyl diphosphate; EPSPS, 5-enol-pyruvylshikimate-3-phosphate synthase; FPP, farnesyl diphosphate; GA-3P, D-glyceraldehyde-3-phosphate; GGPP, geranylgeranyl diphosphate; GPP, geranyl diphosphate; IPP, isopentenyl diphosphate; MEP, methylerythritol 4-phosphate; MVA, mevalonic acid; ODO1, ODORANT1; Phe, phenylalanine.

ducts, benzenoids and phenylpropanoids, C_5-branched compounds, and various nitrogen and sulfur containing compounds which constitute about 1% of plant secondary metabolites (Dudareva *et al*. 2004). Nearly, all of these classes are emitted from vegetative parts as well as flowers (Knudsen *et al*. 1993), and some are even emitted from roots (Steeghs *et al*. 2004). So far, more than 1,700 compounds have been identified in the floral head space of 990 taxa belonging to 90 families and 38 orders (Knudsen and Gershenzon 2006).

Biosynthesis pathways

The number of PVs is very large (Knudsen *et al*. 1993) but surprisingly, these compounds are biosynthesized by a relatively small number of often overlapping metabolic pathways (Croteau *et al*. 2000). Some PV biosynthetic enzymes produce multiple products from a single substrate or act on multiple substrates. In general, most PVs are derived from three main classes of compounds-terpenoids, phenylpropanoids/benzenoids, and fatty acid derivatives-which are often greatly modified. Generally, the synthesis of PVs involves the removal of hydrophilic moieties and modifications such as oxidation/hydroxylation, reduction, methylation, and acylation reactions.

Terpenoids

Mono- and sesquiterpenes belong to the terpenoids, the largest group (more than 50,000) of natural products known (Croteau *et al*. 2000). These terpenes are synthesized from isopentenyl diphosphate (IPP) by different mono- and sesquiterpene synthases (Trapp and Croteau 2001). In plants, both the cytosolic mevalonate and the plastidic methylerythritol phosphate (MEP) pathways generate the five-carbon com-

pound, IPP and its isomer dimethylallyl diphophate (DMAPP) (**Fig. 1**). A plastidic prenyltransferase synthesizes the monoterpene starting material, geranyl diphophate (GPP) from the condensation of one IPP molecule and one DMAPP molecule. A second type of plastidic prenyltransferase condenses DMAPP with three IPP molecules to produce geranylgeranyl diphophate (GGPP) deriving diterpene. In the cytosol, the condensation of one DMAPP molecule with two IPP molecules results in farnesyl diphophate (FPP) deriving sesquiterpenes. These diphophate-containing compounds also serve as precursors of many primary metabolites such as carotenoids and quinones (Croteau *et al*. 2000).

Phenylpropanoids-benzenoids

Phenylpropanoids and benzenoids containing an aromatic ring consist of the second largest class of PV compounds. Most of these compounds are derived from intermediates in the pathway that leads from shikimate to phenylalanine and then to an array of primary (e.g. lignin) and secondary nonvolatile compounds (e.g. phenylpropanoid compounds) (Dudareva *et al*. 2004). The first step in the biosynthesis of some phenylpropanoids and benzenoids is catalyzed by the enzyme phenylalanine ammonia lyase (PAL). Eugenol (clove essence) is a reduced version of coniferyl alcohol, a lignin precursor (Gang *et al*. 2001). Phenylacetaldehyde, a compound of tomato fruit (Tadmor *et al*. 2002), is derived from phenylalanine by decarboxylation and oxidative removal of an amino group (Hayashi *et al*. 2004). The production of the C6-C1 benzenoids from C6-C3 phenylpropanoids is resulted from the shortening of two carbons of the three-carbon chain attached to the phenyl ring of phenylpropanoids. The mechanism by which this is achieved is not fully understood. Shortening of the three-carbon side chain of phenylalanine-derived hydroxycinnamates to one carbon

leads to aromatic building blocks such as benzoic acid and benzaldehyde (Boatright *et al*. 2004).

Fatty acid derivatives

Fatty acid derivatives are derived by oxidative cleavage and decarboxylation of various fatty acids, resulting in the production of shorter-chain volatiles with aldehyde and ketone moieties (Howe and Schilmiller 2002; Fridman *et al*. 2005), which often serve as precursors for the biosynthesis of other PVs (D'Auria *et al*. 2002). Short-chain alcohols and aldehydes are formed by metabolic conversion or degradation of phospholipids and fatty acids through the concerted action of lipoxygenases, hydroperoxide lyases, isomerases, and dehydrogenases (Croteau and Karp 1991). Similarly, some volatile terpenes are derived from the cleavage of carotenoids by carotenoid cleavage dioxygenases (Simkin *et al*. 2004).

Types of modifications

To enhance the volatility, PVs undergo various modification reactions through enzymes such as P450 cytochrome oxidases, oxidoreductases, methyltransferases and acyltransferases.

P450 cytochrome oxidases involved in volatile biosynthesis have been well characterized from a multitude of plant and animal species (Schuler 1996). The basic skeleton of the monoterpenes and sesquiterpenes is often modified by hydroxylation by this enzyme. This enzyme catalyzes the biosynethsis of volatile phenylpropenes such as eugenol and the benzenoid vanillin (Schoch *et al*. 2001; Gang *et al*. 2002).

NADP/NAD-dependent oxidoreductases have been implicated in the interconversion of volatile alcohols and aldehydes. For example, apparently, non-specific alcohol dehydrogenases can convert the short-chain aldehydes such as hexanal and 3-*cis*-hexenal to hexenol and 3-*cis*-hexenol, alcohols that are found in damaged leaves (Bate *et al*. 2002).

A methyltransferase (MT) catalyzes the methylation of hydroxyl groups, which has been observed in a large portion of PVs. *S*-adenosyl-l-methionnine (SAM) serves as the methyl donor. Type I methyltransferase family has been shown to catalyze the 4-hydroxyl methylation of eugenol to form methyleugenol in flowers of *C. breweri* (Noel *et al*. 2003). 3,5-Dimethoxytoluene, a major scent compound in many hybrid roses, is produced from orcinol in two successive methylation reactions catalyzed by two very similar MTs, orcinol O-methyltransferases (OMTs) (Lavid *et al*. 2002; Scalliet *et al*. 2002).

Methylation of carboxyl groups is also widespread in the plant kingdom. An enzyme, salicylic acid carboxyl methyltransferase (SAMT) capable of methylating salicylic acid was reported from *C. breweri* flowers (Ross 1999) and other plant species (Negre *et al*. 2003). Benzoic acid carboxyl methyltransferase (BAMT) catalyzes the methylation reaction to form the snapdragon floral volatile methylbenzoate (Dudareva *et al*. 2000).

Acylation, most often with an acetyl moiety but also with larger acyls such as butanoyl or benzoyl acyls, to make volatile compounds is also common. In all known examples, such plant volatile esters are synthesized by plant acyltransferases called BAHD, after the first letter of the first four enzymes identified and characterized: *C. breweri* benzyl alcohol O-acetyltransferase (BEAT), *Gentiana triflora* anthocyanin O-hydroxycinnamoyltransferase (AHCT), *Dianthus caryophyllus* anthranilate N-hydroxycinnamoyl/benzoyltransferase (HCBT), and *Catharanthus roseus* deacetylvindoline 4-*O*-acetyl-transferase (DAT) (St-Pierre and de Luca 2000). BAHD enzymes are involved in the synthesis of volatiles such as eugenol and (*Z*)-3-hexen-1-yl acetate (Gang *et al*. 2002; D'Auria *et al*. 2007). Benzyl alcohol acetyl-CoA transferase from *C. breweri* flowers produces benzyl acetate (Dudareva *et al*. 1998) and benzyl alcohol benzoyl-CoA transferase produces benzylbenzoate in flowers

of *Clarkia* (D'Auria *et al*. 2002) and petunia (Boatright *et al*. 2004).

REGULATION OF EMISSION

Floral scent bouquets may contain from one to a hundred volatiles, but most species emit between 20 and 60 different compounds (Knudsen and Gershenzon 2006). Last decade has made significant progress to understand the biosynthesis of floral scents and a few floral scent genes have been identified so far and cloned. It has been shown that PVs are synthesized de novo in damaged and undamaged tissues (Dudareva *et al*. 1996; Pare and Tumlinson 1997). Although there is no universal regulation of plant volatile emission, existing literatures showed that the volatile emission (synthesis/production) is regulated by developmental factors (temporal and spatial regulation) as well as environmental factor (e.g. light).

Spatial regulation

The gender and cultivar affected both the qualitative profile and the relative abundances of the volatiles of whole flowers and isolated floral organs (Custódio *et al*. 2006). Many recent investigations have demonstrated that all flower organs are not equally employed in scent emission. Spatial differences within a flower (perianth, gynoecium, and androecium) are quite common. Examination of spatial emission patterns of *C. breweri* revealed that petals are mostly responsible for S-linalool, methyl eugenol, and methyl iso-eugenol emission whereas linalool oxide is released from the pistil. In the case of benzenoid esters, the petal tissue was responsible for the benzylacetate and methylsalicylate emission, whereas the pistil was the primary source of benzyl benzoate release (Wang *et al*. 1997; Dudareva *et al*. 1999). Methylbenzoate is one of the most abundant scent compounds detected in the majority of snapdragon varieties and is produced in upper and lower lobes of petals in the reaction catalyzed by BAMT (Kolosova *et al*. 2001b). Comparative analysis of volatiles emitted from *Ranunculus acris* (buttercup) showed that petals, stamens, sepals, and gynoecium emit identical volatiles, although they contribute different amounts; petals and stamen contribute the most.

It has been suggested that the developmental biosynthesis of volatiles is regulated largely at the level of gene expression (Dudareva *et al*. 1996; McConkey *et al*. 2000; Guterman *et al*. 2002; Dudareva *et al*. 2003).

The expression of the genes encoding the enzymes for flower scent production is both temporally and spatially regulated. The highest level of expression for the majority of floral scent gene was found in the petal tissue (Dudareva *et al*. 2000; Lavid *et al*. 2002; Boatright *et al*. 2004; Pott *et al*. 2004) with the exception of *LIS* and benzoyl-coenzyme A:benzyl alcohol benzoyl transferase (*BEBT*) genes of *C. brewari* for which the highest level of the transcript was detected in the stigma (Dudareva *et al*. 1996; D'Auria *et al*. 2002). Expression of some floral scent genes occur exclusively in petal tissue like petunia benzoic acid/salicylic acid carboxyl methyltransferase (*BSMT*), benzoyl-CoA:benzyl alcohol/phenylethanol benzoyltransferase (*BPBT*), snapdragon *BAMT*, *S. floribunda SAMT*, ocimene synthase and mycrene synthase (Dudareva *et al*. 2000; Pott *et al*. 2002; Dudareva *et al*. 2003; Negre *et al*. 2003; Boatright *et al*. 2004. The biosynthesis of volatile compounds is present almost exclusively in cells of epidermal layer of petals from which they can easily escape into the atmosphere. For example, two genes *IEMT* and *LIS* have specific expression in the epidermal cells of petals in *C. breweri* and snapdragon flowers (Dudareva and Pichersky 2000; Kolosova *et al*. 2001a). Till to date, little is known about the subcellular localization of the biosynthesis of scent compounds, although it has been shown that methyl benzoate is made in the cytosol since its corresponding enzyme, BAMT is a cytosolic protein (Kolosova *et al*. 2001a).

Temporal regulation

The release of PVs is often temporally regulated. Plants tend to emit scents at maximum level when the flowers are ready for pollination and concomitantly when their potential pollinators are active. Newly opened and young flowers, which are not ready to function as pollen donors usually produce less scent (Jones *et al*. 1998; Dudareva *et al*. 2000). Analysis of the activities of the enzymes for the PV formation showed two different developmental regulation patterns. The enzyme activities of the first group, represented by *C. breweri* LIS and SAMT, and snapdragon BAMT increase in young flowers and decline in old flowers whereas *C. brewari* IEMT, BEAT, and BEBT, and petunia BPBT belonging to the second group show no decline through the life span of the flower, although emission of corresponding volatiles declines substantially (Dudareva and Pichersky 2000; D'Auria *et al*. 2002; Boatright *et al*. 2004).

In addition to the regulation of gene expression, the temporal level of substrate plays a key role for the emission of volatile compounds (Dudareva *et al*. 2000). The role of substrate in the regulation of the biosynthesis of volatile compounds was also recently confirmed by metabolic engineering. When the *LIS* gene was introduced under the control of the cauliflower mosaic virus (CaMV) 35S constitutive promoter into *P. hybrida* WI15, the differences between organs in the amount of the synthesized linalool or its glycosides depended more on the availability of the substrate GPP in the tissue than on expression of the *LIS* gene (Lucker *et al*. 2001).

Rhythmic regulation

Rhythmic emission of floral volatile is regulated by either light/dark cycle or endogenous circadian clock. The circadian rhythmic emission of volatile compounds shows a period of 24 hours and is even maintained under continuous light or dark condition (Altenburger and Matile 1990; Loughrin *et al*. 1991). Plants emit one set of compounds during the day and another at night (Matile and Altenburger 1988; Loughrin *et al*. 1992). Moreover, it has been found that some floral volatile compounds are emitted in a rhythmic manner whereas others are not (Loughrin *et al*. 1991; Nielsen *et al*. 1995). The diurnal or nocturnal rhythmic release of flower scent generally coincides with the visiting period of potential pollinators. Emission of methyl benzoate from bee-pollinated snapdragon flowers occurs in a rhythmic manner, with maximum emission during the day, and coincides with the foraging activity of bumblebees

(Kolosova *et al*. 2001b). A detailed time-course analysis showed that the oscillation of methyl benzoate is resulted from the rhythmic regulation of the substrate, benzoic acid, but not that of BAMT activity.

Monoterpenes can also be released rhythmically as was shown for *Artemisia annua* (Lu *et al*. 2002). A β-pinene synthase mRNA from *A. annua* has been shown to be regulated in a circadian rhythm, and its expression profile is correlated with the content and emission of the monoterpene produced by the corresponding enzyme (Lu *et al*. 2002). Thus, at least one layer of regulation is provided by the highly specific rhythmic expression of terpenoid synthases. However, *C. breweri* linalool synthase protein levels and enzyme activities remain high while linalool emission decreases (Dudareva and Pichersky 2000). This indicates that an additional layer of regulation exists in *C. breweri*, most likely in the pathways providing terpenoid precursors.

METABOLIC ENGINEERING

The importance of PVs is increasing day by day in perfume and food industry for their flavour and aroma. Traditional way to make good plants with strong flavored scent is time consuming and, therefore, to improve the overall process we need metabolic engineering approach. To date, the pioneering attempts on metabolic engineering of PVs were performed on *Arabidopsis*, petunia, carnation, tobacco, tomato, strawberry, and apple. Among these species, petunia is especially the favored model plant for volatile research. Since petunia contains very little monoterpenes, all monoterpenes detected may be considered as result of transgene expression (Gerats and Vandenbussche 2005). There are several target pathways for metabolic engineering of the PVs. The well-understood mevalonate and MEP pathways are important for engineering terpenoids, while shikimate pathway plays a central role in biosynthesis of phenylpropanoid/benzenoid (**Fig. 1**). Most of the works (**Table 1**) focused on these two compounds because they are major volatile classes in plant.

Metabolic engineering of terpenoid volatile compounds

Terpenoids are the most important class of PVs and therefore they take a central position among metabolic engineering works. It was suggested that geranyl diphosphate (GPP) synthase and monoterpene synthase are the most critical positions for regulation of terpene volatile synthesis. *C. breweri* linalool synthase (LIS) gene encodes the enzyme that converts geranyl diphosphate (GPP) to a cyclic mono-

Table 1 Summary of metabolic engineering approaches for plant volatile biosynthesis.

Species	Gene	Result	Human olfactory detection	Reference
Arabidopsis	FaNES1	Linalool and derivatives	ND	Aharoni *et al*. 2003
	FaNES1	Nerolidol and (*E*)-DMNT	ND	Kappers *et al*. 2005
Petunia	CbLIS	Linalool glucoside	No	Lucker *et al*. 2001
	RhAAT	Benzyl acetate and phenylethyl acetate	ND	Guterman *et al*. 2006
	Anti[a]-PhBSMT	Methylbenzoate decreased	Yes	Underwood *et al*. 2005
	Anti-PhPAAS	Phenylacetaldehyde and phenylethanol eliminated	ND	Kaminaga *et al*. 2006
	Anti-PhCFAT	Isoeugenol decreased	ND	Dexter *et al*. 2007
	PhODO1	Benzenoids volatile decreased	ND	Verdonk *et al*. 2005
Tobacco	AnBGL1	2-ethylhexanol, *trans*-caryophyllene, and cembrene	ND	Wei *et al*. 2004
	ClTER, ClLIM, ClPIN	γ-terpinene, limonene, β-pinene and side products	Yes	El Tamer *et al*. 2003; Lucker *et al*. 2004a
Tobacco TERLIMPIN[b]	MsLIM3H	Isopiperitenol and derivatives	ND	Lucker *et al*. 2004b
Carnation	CbLIS	Linalyl oxides	No	Lavy 2002
	Anti-DcF3'H	Methylbenzoate	Yes	Zuker *et al*. 2002
Tomato	CbLIS	Linalool & derivatives	Yes	Lewinsohn *et al*. 2001

Abbreviations: AnBGL1, *Aspergillus niger* β-glucosidase; ClLIM, *Citrus limon* limonene synthase; ClPIN, *Citrus limon* β -pinene synthase; ClTER, *Citrus limon* γ -terpinene synthase; CbLIS, *Clarkia breweri* linalool synthase; DcF3'H, *Dianthus caryophyllus* flavanoid 3'-hydroxylase; MsLIM3H, *Mentha spicata* limonene-3-hydroxylase; ND, not determined; PhBSMT, *Petunia hybrida* benzoic acid/salicylic acid carboxyl methyltransferase; PhCFAT, *Petunia hybrida* coniferyl alcohol acyltransferase; PhODO1, *Petunia hybrida* ODORANT1 transcription factor; PhPAAS, *Petunia hybrida* phenylacetaldehyde synthase; RhAAT, *Rosa hybrida* alcohol acetyltransferase.
(a) RNAi sequence
(b) Transgenic tobacco expressing ClTERM, ClLIM and ClPIN.

terpene volatile (3S)-linalool with sweet scent (Dudareva *et al.* 1996). This gene was introduced into petunia, tomato, and carnation. Petunia and carnation normally do not produce any linalool. In spite of high expression levels of LIS, transgenic petunia plant could not emit this flavor; linalool was rapidly converted to the non-volatile form of S-linalyl β-D-glucoside by endogenous glucosyltransferases (Lucker *et al.* 2001). The similar problem appeared in carnation, where S-linalool was mostly oxidized to linalool oxides. Nevertheless, linalool and its derivatives *cis*- and *trans*-linalool oxides were detected in leaves and flowers of transgenic carnation at such a low level that was not strong enough for human olfactory (Lavy 2002). In tomato, LIS was expressed in fruit using fruit specific E8 promoter which mainly works in the ripening stage. The expression of linalool and 8-hydroxy linalool were strong enough to be detected by human olfactory (Lewinsohn *et al.* 2001). These results suggest that the products of transgenic monoterpene synthase have usually been modified into less phytotoxic or less reactive forms in plants that do not accumulate linalool (Lucker *et al.* 2006).

Different expression levels of terpenoids are not only present in different tissues, but also present when the transgene was subcellularly localized into plastids, endoplasmic reticulum (ER), and cytosols. Geranyl diphosphate (GPP), the precursor of limonene, is produced by mevalonate pathway in the cytosol and by non-mevalonate pathway in plastids. The *Perilla frutescens* limonene synthase (LS) gene, encoding the enzyme catalyzes stereo-specific cyclization of GPP to form limonene, was transformed into tobacco. High, low and zero amount of limonene was produced when the enzyme was targeted to plastids, cytosol and ER, respectively. Among ER targeted plant lines, only one accumulates LS polypeptide, but the peptide did not show any enzymatic activity. These results suggest that GPP in both cytosol and plastid can be trapped by LS; and ER is not a suitable compartment because of the incorrect folding or instability of protein (Ohara *et al.* 2003). Attempts to engineer sesquiterpenes confirmed the advantage of subcellular organelles targeting strategy. By switching FaNES1, a strawberry linalool/nerolidol synthase, from cytosol to mitochondria or plastid, the expected sesquiterpene product (3S)-(*E*)-nerolidol was produced and emitted at 20-30 times higher (Aharoni *et al.* 2003; Kappers *et al.* 2005). So far, this emission of (3S)-(*E*)-nerolidol is highest in comparison with other metabolic engineering researches, implicating that the precursor fanesyl diphosphate (FPP) is readily available in mitochondria. In practical aspect, the carnivorous predatory mites (*Phytoseiulus persimilis*) were significantly attracted by the transgenic plants overexpressing (3S)-(*E*)-nerolidol, thus improving the plant defense (Kappers *et al.* 2005). It has been proposed that the herbivore induced PVs can be exploited in agricultural pest control to repel herbivores by attracting their enemies (Turlings and Ton 2006).

Introduction of multiple genes in a plant can combine multiple volatile compounds to ameliorate plant flavors. Three genes encoding three monoterpene synthases from lemon named *TER*, *LIM*, and *PIN* (Lucker *et al.* 2002) were introduced into tobacco. From leaves and flowers, transformed *TER* lines express γ-terpinene, small amount of α-terpinene and limonene; transformed *LIM* lines express only limonene; and transformed *PIN* lines express β-pinene and small amount of γ-terpinene. These monoterpene products were not produced by wild-type tobacco. By crossing, all three transgenes were combined into one plant. These plant lines successfully emitted three expected volatiles and a number of side products. The considerable emission of these compounds suggests that there is a sufficient amount of substrate pool for monoterpene production (Lucker *et al.* 2004a). Sensory analysis showed that the differential emission from leaves of transgenic and wild-type plants is strong enough for olfactory detection by human, but not from flowers (El Tamer *et al.* 2003).

One metabolic pathway includes several steps which may occur in different subcellular compartments. The most challenging target is engineering more than one step involved in one pathway, or regulating the metabolic network. P450, the gene encoding limonene hydroxylase from *Mentha spicata* L. 'Crispa', was introduced into the ER of tobacco which expressed three lemon monoterpene synthases as mentioned above. The transgenic plant highly emitted the new volatile compound (+)-*trans*-isopiperitenol, a product resulted from P450 hydroxylase activity on (+)-limonene, and some side products which include isopiperitenone and *p*-cymenne. In a previous research, the three lemon enzymes were targeted to plastids. However, P450 was most likely functions in endoplasmic reticulum. This result suggests that (+)-limonene was efficiently transported through a panel between plastid and endoplasmic reticulum. In this case, it is clear that two newly introduced steps, which happened in different locations, can work together (Lucker *et al.* 2004b).

Metabolic engineering of phenylpropanoid/benzenoid volatile compounds

Benzenoid compounds contribute the second most abundant volatile compounds in plants (van Schie *et al.* 2006). Unfortunately, because of the limited knowledge on biosynthetic pathway, candidate genes and regulation factors, there were very few attempts to engineer these volatile compounds. However, encouraging results have been obtained recently, regarding the biosynthetic pathway for the production of these volatile compounds.

In an effort to modify the color of carnation flower by using antisense method to suppress the flavanone 3-hydroxylase mediating a key step in the anthoxanthin pathway, the transgenic plants not only changed their color but also emitted five- to seven-fold more phenylpropanoids, methyl benzoate and 2-hydroxy methyl benzoate. However, the emission of other volatile compounds was not changed (Zuker *et al.* 2002). It is reasonable that the suppression of anthoxanthin synthesis redirected the pathway to produce more benzoic acid because both of them are derived from phenylpropanoid pathway.

In petunia, ethylene elicits a series of physiological and biochemical events in floral organs, ultimately leading to senescence of petals and successful fertilization. Treatment of petunia with ethylene showed a remarkable decrease in the expression level of two genes, benzoic acid/salicylic acid carboxyl methyltransferase (*PhBSMT1* and *PhBSMT 2*) with concomitant reduction in methyl benzoate and other volatile emission. The use of RNA-mediated interference technology (RNAi) as a powerful tool for modification of metabolic pathways has gained success. RNAi suppression of the above two genes also resulted in 75% to 99% decrease of methyl benzoate, even without ethylene. This change was detectable by human olfactory. The authors conclude that in petunia, *PhBSMT1* and *PhBSMT2* genes are involved in biosynthesis of methyl benzoate and down-regulated by ethylene (Underwood *et al.* 2005).

In general, more than one biochemical pathway is responsible for a blend of volatile compounds released from different plant tissues. A comparative analysis of the regulation of benzenoid and monoterpene emission in snapdragon flowers revealed that the orchestrated emission of phenylpropanoid and isoprenoid compounds is regulated upstream of individual metabolic pathways and includes the coordinated expression of genes that encode enzymes involved in the final steps of scent biosynthesis (Dudareva *et al.* 2000, 2003). Thus, transcription factors that regulate multiple biosynthetic pathways are good targets to be engineered for manipulation of the odor bouquet. Using targeted transcriptome analysis, the gene *ODORANT1* (*ODO1*) that encodes a novel R2R3-type MYB transcription factor was identified in petunia. Transcript level of *ODO1* correlates with level of volatile emission. Downregulation of this factor led to significant reduction of volatile benzenoids because the synthesis of their precursors in

the shikimate pathway was decreased. Moreover, RNAi suppression of *ODO1* also reduced transcript level of several genes in this pathway. From these data, the authors conclude that ODO1 is the key regulator of floral flavor biosynthesis (Verdonk *et al.* 2005).

Recently, a typical metabolic engineering work targeted to benzenoids was performed using rose alcohol acetyltransferase gene (*RhAAT*) in petunia. The gene was well expressed under the control of 35S promoter to produce larger amount of benzyl acetate and phenylethyl acetate. Interestingly, the enzyme used endogenous phenylethyl alcohol and benzyl alcohol as substrate (Guterman *et al.* 2006), while *in vitro* assay showed its preferred substrate is geraniol (Shalit *et al.* 2003), which is unavailable in the petunia cell.

Engineering plant cells culture to produce flavors

Similar to propagation of plants, cell and tissue cultures technique can be used for commercial production of natural products, including flavors and fragrances. The production of compounds by plant cell cultures has advantages over production by conventional agricultural practices. Unlike the production of plants that is seasonally limited, cell cultures provide a system that can be used year-round and that is independent of the geographic location, and political situations. Cells can be induced in a coordinated manner to produce metabolites of interest, and these can be isolated from the nutrient medium or from the cells (Murashige and Skoog 1962). However the method is expensive since the production of flavor compound or its precursors are relatively in low amount. The best results toward the production of flavor compounds by cell cultures have been achieved in cases which the characteristic aroma of the fruit or plant is caused mainly or entirely by the synthesis and accumulation of a single compound or only a few compounds with similar structures and properties e.g. vanilla for which the major flavor component is vanillin, and onions and garlic, for which the characteristic flavor derives from alliin derivatives (Hrazdina 2006). Although the production of the majority of aroma compounds by plant tissue culture methods is presently not feasible for either economic or physiological reasons, future developments may hold promise. Flavor and aroma compounds from plant tissue cultures may become available as we learn the details of their biochemical pathways and their genetic control.

CONCLUSION

Although plants emit volatile compounds for different purposes as searched by many scientists, it is not conclusive why plants lose a good percentage of carbon through emission. Nonetheless, whatever may be its function, these secondary metabolites are attractive for commercial exploitation in food and perfume industry and metabolic engineering seems to be a promising tool to achieve the enhancement and introduction of flavour and aroma in food stuffs.

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