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Chapter 12. Evolution of scent genes

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Abstract:

Plants produce a vast array of volatile organic compounds (VOCs), which play major roles in the interactions with their biotic and abiotic environments. In recent years, considerable research effort has aimed at the characterization of genes and enzymes involved in the biosynthesis of VOCs in plants. The elucidation of many VOC biosynthesis pathways in various plant species brought up the central question of the evolutionary origin of this remarkable chemical diversity. This chapter describes some of the molecular mechanisms, by which plants may acquire or lose the capacity to synthesize specific VOCs. On the one hand, many plant species have acquired the capacity to produce highly diversified blends of volatiles through different mechanisms leading to divergent evolution and pathway plasticity, such as the expansion of large gene families following gene duplication, catalytic promiscuity or easy change in substrate specificity within enzyme families. On the other hand, some compounds may be produced by unrelated enzymes, or even totally different pathways, which evolved independently in different plant species. As illustrations, examples have been selected from different categories of VOCs in various plant families, with an emphasis on scent and aroma compounds from plants of particular agronomical or horticultural interests.

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12.1 Introduction

Elucidation of biosynthesis pathways of volatile organic compounds (VOCs) in plants is a very active field of research. Since the initial isolation of the limonene synthase and the linalool synthase genes from *Mentha spicata* and *Clarkia breweri* (Colby et al., 1993; Dudareva et al., 1996; Pichersky et al., 1995), dozens of genes involved in the biosynthesis of terpenes, phenylpropanoids/benzenoids and fatty acid derivatives have been characterized in plants belonging to various families. Although most work has been done on angiosperms and conifers, the genetic basis of VOC diversity found in more basal plant lineages is also starting to be investigated (Jia et al., 2018; Kumar et al., 2016). Until recently, most studies were focused on identifying the genes and enzymes involved in the biosynthesis of aroma compounds. Pioneering works used model plants such as *C. breweri*, *Antirrhinum majus*, *Arabidopsis thaliana* or *Petunia x hybrida*. Some plants of agronomical or horticultural interests such as *Solanum lycopersicum* and *Rosa x hybrida* were also investigated in early works. During the past ten years, as more and more pathways were elucidated, the central question of the evolutionary origin of this chemical diversity arose. In a growing number of cases, impact of gene and/or allele evolution on specialized metabolism has been analyzed in detail. In this chapter, we will describe some of these molecular mechanisms by which a plant can acquire or lose the capacity to synthesize specific VOCs. As illustrations, examples will be selected from different categories of VOCs in various plant families, with an emphasis on scent and aroma compounds from plants of particular agronomical or horticultural interests.

12.2 The motors of natural selection

12.2.1 Friends and foes interacting with plants

Plants are sessile organisms, which have, even more than other organisms, exploited chemical diversity to cope with their changing environments. The metabolites that a plant is able to synthesize have crucial roles for its survival in challenging conditions, due to both biotic and abiotic factors. VOCs are particularly important for interactions with pollinators, mostly insects, and for defense against other organisms, including insects. It is generally admitted that plant defense was the initial function of VOCs and that scent emission for the attraction of pollinators evolved later (Raguso 2009).

VOCs emitted by the vegetative parts of plants are commonly assumed to play a role in the defense against various enemies, including herbivores and microbial pathogens. These VOCs may be emitted constantly or in response to an herbivore attack. Plant-derived metabolites may have direct effects on the herbivore through toxic properties or act as anti-digestive and anti-nutritive compounds. They may also attract enemies of the herbivores in the now well-known indirect defense phenomena (Turlings et al., 1995). Accordingly, any mutation of a gene leading to the production of an advantageous volatile compound, or eliminating a disadvantageous compound, increases the plant fitness and thus can be quickly selected. Some studies have evaluated the benefits and costs of vegetative VOCs on plant fitness in a natural environment (reviewed in Cipollini et al., 2014). Multiple herbivores attacking the same host exert conflicting selection pressures on the plant. More generally, this phenomenon is part of scenarios of diffuse coevolution (Wise and Rausher 2013), in which a community of plants may influence the evolution of a community of insects, and reciprocally.

Flower scent is a strong signal directing pollinators to flowers that can deliver a reward like nectar or pollen. Sometimes the reward for insects is a place used as a nest that favors mating and protection. Scent can also be deterrent or toxic to undesirable visitors like ants, caterpillars, or even bacteria (Muhlemann et al., 2014). Scent can direct pollinators based on short- and long-distance attractions, especially flowers that are visited at night by moths or

bats. In *Hesperis matronalis*, the fitness (measured by the number of seeds) is dependent on the level of scent emission and on the type of pollinators (day or night-pollinators) but is not dependent on the anthocyanin color (Majetic et al., 2009). In *P. axillaris*, scent is a major trait for attraction of pollinators such as the hawkmoth *Manduca sexta* (Hoballah et al., 2005). Scent is thus considered as a major fitness component, however, due to the complexity of the volatile profiles of many flowers, detailed analysis of the role of individual compounds is difficult.

The emission of VOCs undergoes temporal variations and emission rhythms. Some of them are emitted during blooming or after blooming, some of them are emitted during the night, the morning, the day or the evening, in relationship to the behavior of pollinators. For example, emissions are finely tuned in *Cirsium arvense* and *Nicotiana attenuata* flowers, according to the visit of pollinators or enemies (Kessler et al., 2010; Theis et al., 2007; Yon et al., 2017). Community-wide patterns of flower scent signals have rarely been explored. Nevertheless, scent advertisement in a plant community has been shown to be inversely proportional to the population size of pollinators: more scent is emitted by species blooming early in the season, when pollinators are scarce and the emission tends to decrease when more pollinators are present (Filella et al., 2013). Furthermore, flowering plants that are not abundant in the community have evolved specific strategies, for example the emission of generalist attractive compounds such as β -ocimene. The dynamics of seasonal emission of scent leading to the structuration of the plant-pollinator network is thus quite complex. Although it is generally assumed that floral volatiles provide fitness benefits for plants, VOCs may impact both mutualists that pollinate flowers and herbivores that damage them (Boachon et al., 2015; Kessler et al., 2013; Kessler et al., 2015). By silencing both benzylacetone biosynthesis and nectar production in *N. attenuata* by RNAi approaches, the costs and benefits of these traits for the plant could be evaluated, in relationships with two pollinators and one herbivore

(Kessler et al., 2015). This study concluded that both herbivores and pollinators shaped the evolution of floral traits and that it made little sense to study the effects of the ones without considering the effects of the others.

Besides the well described plant-insect interactions, more recent works highlight the influence of plant VOCs on the microbial communities present at the surface of plant tissues (Reviewed in Junker and Tholl 2013). For example, in *A. thaliana*, flowers of plants lacking (*E*)- β -caryophyllene emission showed greater bacterial growth on their stigmas than did wild-type flowers, which suggests that this compound serves as a defense against floral pathogens (Huang et al., 2012). If plant VOC emissions may impact the growth of bacterial communities, the reverse is also true, as microbial VOCs have been shown to influence plant growth or reproduction (Piechulla et al., 2017). The diversity of both beneficial and pathogenic organisms interacting with plants makes it complicated to understand the forces that drive the evolution of VOC biosynthesis and diversity. Furthermore, evolution may occur on genes that directly synthesize VOCs, but also on transcription factors and regulators that are linked to development cycle, to rhythmicity, or to response to environmental, biotic or abiotic factors.

12.2.2 Molecular mechanisms leading to biochemical diversity

The term of plant specialized metabolism was chosen to reflect that each species (or genus or family...) is capable of synthesizing its own bouquet of metabolites, which is supposed to be well adapted to its biotic and abiotic environment (Pichersky and Lewinsohn 2011). This implies that each plant has acquired the necessary set of genes and enzymes to produce the said compounds. And indeed, genes involved in the biosynthesis of specialized metabolites, including VOCs, are often restricted and specific to the lineages that produce these compounds. There are various mechanisms by which such genes evolve.

It is generally held that a great deal of plant gene diversity evolved through duplication and specialization of paralogs (Flagel and Wendel 2009). These duplications include whole-genome duplications, linked to polyploidization events, that are common in many plant lineages, as well as local duplications. It is beyond the scope of this chapter to describe the various mechanisms of gene duplication, so only a brief summary will be given here (for a review see Innan and Kondrashov 2010, Andersson et al., 2015 and Panchy et al., 2016). Briefly, new genes with novel functions can evolve from extra copies of duplicated genes, which may arise after replication errors or during meiosis after unequal crossing overs for example. These mechanisms may produce chromosome regions with tandem repeats, which are very frequent in plant genomes. Larger duplications, involving chromosome parts or whole chromosomes may also occur. Whole genome duplication and increase in ploidy levels are also major sources of gene duplication, especially in plants. Since Ohno's famous book "evolution by gene duplication" (Ohno 1970), more than ten theoretical models have been proposed to explain what happens after the duplication event (Fig. 12.1) (Andersson et al., 2015; Innan and Kondrashov 2010; Ober 2010). The differences between these models are based on the modes of selection (neutral, relaxed, purifying or positive), on the types of mutations (gain-of-function Fig. 12.1A, loss-of-function Fig. 12.1B, or no mutation), on the fact that duplicate genes immediately increase the fitness (e.g. concerted evolution Fig. 12.1C, or positive dosage) or increase fitness after a series of mutations, and on the ways of functional evolution (neofunctionalization Fig. 12.1D, subfunctionalization Fig. 12.1E, subneofunctionalization, or pseudogenization Fig. 12.1F). In neofunctionalization (Fig. 12.1D), one of the duplicated copy accumulates mutations, which eventually lead to a new function, while the other copy retains the original function. However, in this case it is highly probable that the randomly-accumulated mutations are deleterious, leading to non-functionalization of the gene, or pseudogenization (Fig. 12.1F). To circumvent this weakness

of the theory, another model was proposed, in which each duplicated copy was able to perform a part of the initial function(s) of the ancestral gene. This model is broadly referred to as subfunctionalization (Force et al., 1999) (Fig. 12.1E). When applied to metabolism, this view is supported by the fact that many enzymes are promiscuous and have the ability to catalyze more than one reaction. Mutations in regulatory elements of the duplicated copies may also lead to divergent patterns of expression. Other types of models, in particular hybrid ones that include both subfunctionalization and neofunctionalization, have been proposed. In the subneofunctionalization model, a short period of subfunctionalization precedes a longer neofunctionalization period (He and Zhang 2005).

Place Figure 12.1 here.

It is noticeable that concerning specialized metabolism, authors only described neofunctionalization or subfunctionalization in terms of an extended definition of these concepts, probably because the link between the theoretical models and the experimental observations is not obvious. Unfortunately, there are still too few descriptions of neo-, sub-, or subneofunctionalization scenarios for genes involved in VOC biosynthesis.

Conversely, such scenarios are well-documented for some non-volatile specialized compounds (Moghe and Last 2015). For example, capsaicin biosynthesis in pepper involves capsaicin synthase, which evolved in the genome of *Capsicum* species through multiple gene duplications in the acyltransferase gene family (Kim et al., 2014). Furthermore, evolution of this new function was accompanied by the evolution of a fruit-specific expression pattern, that did not occur in other Solanaceae. This allowed the authors to propose a true neofunctionalization scenario giving rise to capsaicin biosynthesis.

Evolutionary analysis of the *Ruby1* and *Ruby2* Myb transcription factors regulating anthocyanin biosynthesis in *Citrus sp.* has led to the hypothesis that both genes derive from the duplication of an ancestral *Ruby* gene (Huang, Wang, et al., 2018). *Ruby1* and *Ruby2* genes exhibit different expression patterns, in fruits and in leaves, respectively, thus illustrating a case of subfunctionalization. Repeated gain or loss of function of *Ruby1* and *Ruby2* genes during domestication of *Citrus* gave rise to complicated patterns of anthocyanin accumulation, which may occur in fruit peel, fruit flesh or leaves, depending on *Citrus* species.

Duplicated genes often revert back to a single copy and neofunctionalization models, subfunctionalization models and sub- and neofunctionalization models correspond to rare events (Moghe and Last 2015). Most often, duplicated copies accumulate mutation over time and evolve to pseudogenes without functions (Fig. 12.1F). Nevertheless, microRNAs, long noncoding RNA, transcription binding sites and Pfam domains (Punta et al., 2012) could originate from pseudogenes. For example, significant differences were found in the number of Pfam domains in the pseudogenes of terpene synthases (TPS) and P450 oxidases in the genomes of several plant species (Xie et al., 2019). Such creation of new regulation elements, like noncoding RNA or *cis*-acting sequences, could give rise to a “rewiring” of the pre-existing regulatory mechanism and/or de novo evolution of some genes. For example, a duplicated TPS pseudogene may act as a new *cis*-acting element in front of the older copy or give rise to a new noncoding RNA (Fig. 12.1G).

Apart from duplication and divergence, the evolution of an entirely new gene, without a functional ancestor, was first seen as highly improbable, but has been shown to occur in many organisms, including plants (Andersson et al., 2015; Donoghue et al., 2011). Finally, transposable elements (TE) are a source of diversification and creation of genes by providing regulatory elements and/or exons (Krasileva 2019). For example, TE may have participated to

the evolution of nicotine biosynthesis in the genus *Nicotiana*. Indeed, many transcription factor binding sites present in upstream regions of nicotine biosynthesis genes are likely derived from TE insertions (Xu et al., 2017). One example of the plausible involvement of retrogenes in VOC biosynthesis was recently described in *Prunus*. Analysis of the benzyl alcohol acetyltransferase gene (*BEAT*) family and revealed 44 *PmBEAT* genes in *Prunus mume*, but only two *PpBEAT* genes in *P. persica* (Bao et al., 2019). Some *PmBEAT* genes are expressed in flowers and associated to benzyl acetate biosynthesis. In contrast, *P. persica* flowers do not emit benzyl acetate and *PpBEAT* genes are not expressed floral tissues. The authors proposed that flower-expressed *PmBEAT* genes evolved through tandem duplication and retroduplication in the genome of *P. mume*. Surprisingly, they found that *PmBEAT* retrogenes were expressed, which is generally not the case because of the absence of regulatory elements. The cluster of *PmBEAT* genes therefore illustrates the contribution of retrogenes to VOC diversity.

Taken together, these examples illustrate the fact that novel functions may arise from duplications or de novo creations by different mechanisms. Whatever the evolution mechanisms involved, they can either directly affect the activity of the enzymes in biosynthetic pathways or modify the expression patterns of genes encoding these enzymes. These processes may create chemodiversity following divergent evolution, or lead to the production of the same volatiles by convergent evolution. Finally, single loss-of-function or gain-of-function mutations may result in substantial modifications of VOC profiles.

12.3 Divergent evolution: the creation of chemodiversity

As described above, various mechanisms, among which duplication and divergence are predominant, lead to new enzymes with new functions in plants. Lineage-specific new enzymes have the potential to synthesize novel compounds, ultimately leading to the great

diversity of plant-derived VOCs present in nature. Moreover, modification of expression patterns may lead to new products, due to the access to novel substrates. To illustrate these processes, we chose to describe in details a few examples of compounds from different chemical families.

12.3.1 Evolution of tea scent in rose

The rose is considered the queen of flowers and is widely used as a garden plant and for the cut flower market. Roses are also used for the production of essential oil for the cosmetic and perfume industries. The extraordinary diversity of modern rose varieties has been obtained, by intensive breeding, from a relatively small number of progenitors including both Chinese species, such as *R. chinensis* and *R. gigantea*, and European species or varieties such as *R. gallica* and *R. moschata*, the former contributing recurrent flowering and the latter cold and disease resistance characters (Krüssmann 1981). In addition, the European and Chinese progenitors each contributed distinctive scent characteristics. The major scent components provided by European roses include 2-phenylethanol (2PE) and a number of monoterpenes (see § 12.4.2). Chinese roses, on the other hand, produce phenolic methyl ethers such as 3,5-dimethoxytoluene (DMT) or 1,3,5-trimethoxybenzene (TMB), and hence contributed these scent components to modern rose varieties (Joichi et al., 2005). Various genomic approaches were first used to identify genes involved in rose scent biosynthesis, starting with the establishment of petal transcriptome databases (Channelière et al., 2002; Dubois et al., 2012; Guterman et al., 2002). Analysis of these databases allowed the identification of several genes potentially involved in phenolic methyl ethers production. Functional characterization of some of these genes led to the identification of two orcinol *O*-methyltransferases (OOMTs), that catalyze the successive methylations of the orcinol substrate to produce DMT (Lavid et al., 2002; Scalliet et al., 2002; Scalliet et al., 2006). TMB is synthesized by three successive

methyations of phloroglucinol, the first step being catalyzed by a phloroglucinol-*O*-methyl transferase (POMT) (Wu et al., 2004). The next steps of TMB biosynthesis are probably catalyzed by OOMT1 and OOMT2.

OOMT1 and OOMT2 are very similar enzymes (96.5% identity at the amino-acid level). They exhibit different substrate specificities, that are consistent with their operating sequentially in DMT biosynthesis. These substrate specificities were shown to depend mostly on a single amino acid polymorphism in the phenolic substrate binding site of OOMTs (Scalliet et al., 2008). An analysis of the *OOMT* gene family in 18 species representing the diversity of the genus *Rosa* indicated that the genomes of all rose species contained *OOMT2* genes. However, only Chinese roses, grouped under the Indicae section of the genus *Rosa*, possessed *OOMT1* genes in addition to *OOMT2* genes. Hence, the Chinese-rose-specific *OOMT1* genes most probably evolved, by duplication and divergence, from an *OOMT2*-like gene that has homologues in the genomes of all extant roses. As OOMT2 is capable of performing the first step of the pathway, although with less efficiency than OOMT1, the evolutionary mechanism involved is probably a case of subfunctionalization. The emergence of the *OOMT1* gene may have been a critical step in the evolution of scent production in Chinese roses, maybe in relationship with specific pollinators in their wild habitat. This gene and the “tea scent”, originally limited to two Chinese wild rose species, were thus transmitted during the breeding of modern roses and are found today in thousands of cultivars present in gardens all over the world.

Besides roses, the importance of *O*-methyltransferase enzymes (OMTs) as a source of VOC diversity has been documented since long in many plant species, including, for instance, *C. breweri*, *Ocimum basilicum*, *Fragaria x ananassa*, *Vitis vinifera* and *Coffea arabica* (Dunlevy et al., 2013; Frato 2019; Gang et al., 2002; Guillaumie et al., 2013; Wang and Pichersky 1999; Wein et al., 2002). In particular, pioneering molecular modeling studies identified a single

amino acid as responsible for the difference in substrate preference between the closely related chavicol-OMT and eugenol-OMT from sweet basil (Gang et al., 2002).

12.3.2 From primary to specialized metabolism: the example of green leaf volatiles

Oxylipins are fatty acid-derived metabolites present in plants and animals. Oxylipins encompass jasmonates, which are essential for plant development and thus belong to primary metabolism present in all plant species. On the other hand, the same oxylipin pathway also leads to the biosynthesis of green leaf volatiles (GLVs), which belong to specialized metabolism and differ among plant species. GLVs play important roles in plant defense and may be induced upon herbivore attack or in response to abiotic stresses for example (Scala et al., 2013; Wasternack and Feussner 2018). In addition, GLVs participate in the aromas of some fruits and vegetables (Du et al., 2011; Selli et al., 2014; Spyropoulou et al., 2017). GLVs are also present in flower scent, where they mediate interactions with pollinators (Brodmann et al., 2012) or repel florivores. Allene oxide synthase (AOS) and hydroperoxide lyase (HPL) catalyze the first committed steps to jasmonate and GLVs, respectively. Both enzymes belong to the cytochrome P450 family and, more precisely, to the CYP74 subfamily. Resolution of the crystal structure of *A. thaliana* AOS and analysis of the catalytic mechanism leading to the oxidation of its substrate, 13*S*-hydroperoxyoctadecatrienoic acid, pointed out the essential role of the Phe 137 amino acid (Lee et al., 2008). Replacing this single amino acid markedly reduced the activity of the protein and was sufficient to convert it into an HPL enzyme, capable of producing GLVs. HPLs are widely distributed in angiosperms. Characterization of two closely related CYP74 from the liverwort *Marchantia polymorpha* showed that they both exhibited AOS activity, but not HPL activity (Koeduka et al., 2015). Moreover, phylogenetic analyses suggested that CYP74 enzymes with AOS and HPL activities may have arisen multiple times independently, in the algae/moss/liverwort lineage,

and in the angiosperm lineage (Koeduka et al., 2015). More studies are needed to better understand the evolutionary origins of this essential pathway in plants.

12.3.3 Enzyme promiscuity and gene clusters: the example of terpene synthases

TPSs are key enzymes in plants, which catalyze the formation of the terpenoid backbone. Even though the primary products of TPS activity may be further metabolized, the diversity of terpenoids found in plants can largely be attributed to the activity of TPS. TPSs have been the subject of multiple studies, which led to a better understanding of their biochemical properties and environmental regulations (reviewed in Chen, Tholl, et al., 2011; Degenhardt et al., 2009). One of the characteristic features of TPSs is their promiscuity, which means that they are broad-specificity proteins (for a review on promiscuity, see Kreis and Munkert 2019). Indeed, a single TPS may synthesize multiple products from the same substrate, as for example the patchoulol synthase from *Pogostemon cablin* (Deguerry et al., 2006) or the TPS35 from *M. truncatula* (Garms et al., 2010), which catalyze the formation of 14 and 27 different sesquiterpenes, respectively. Conversely, other enzymes only produce one compound, such as the geraniol synthase of sweet basil (Iijima et al., 2004). Few amino acid changes may change drastically the products of a TPS, leading to a large diversity of terpene profiles in one species or different accessions. For example, amino acid changes at five positively selected sites in the *Oryza* TPS1 protein from rice are responsible for the diverse sesquiterpene blends produced by seven different *Oryza* species in response to insect damages (Chen et al., 2014). Similarly, maize plants respond to herbivory by lepidopteran larvae with the emission of a mixture of volatiles consisting mostly of the sesquiterpenes (*E*)- α -bergamotene and (*E*)- β -farnesene, which attract natural enemies of the larvae to the damaged plants. The damage-dependent emission of TPS10-related volatiles is an indirect defense trait conserved among maize and its wild relative teosinte. Sequence comparison in maize and

teosinte allowed the identification of a single amino acid in the active site of TPS10, which determines the ratio (*E*)- α -bergamotene to (*E*)- β -farnesene, and which has changed during the evolution of maize and teosinte species (Köllner et al., 2009). Moreover, recent advances in functional characterization of TPSs, with the use of prenyl diphosphates with different chain length or different (*E/Z*) configurations, led to the conclusion that many TPSs can accept more than one substrate (Pazouki and Niinemets 2016). Typical examples are the linalool/nerolidol synthases, which can accept both geranyl diphosphate (GPP) and farnesyl diphosphate (FPP) substrates, leading to formation of the monoterpene linalool or the sesquiterpene nerolidol (Aharoni et al., 2004).

As more and more genome sequences have become available, the organization of *TPS* genes has been investigated in many different plant species and families. These genes are frequently organized in clusters of tandemly duplicated genes, as observed in tomato (Falara et al., 2011) and grapevine (Martin et al., 2010), for instance. A survey of 13 angiosperm genomes showed that *TPS* genes were significantly more enriched in tandem duplicates compared to genome-wide average, across most of the analyzed species (Hofberger et al., 2015). This suggests that this gene family has experienced rapid evolution through gene duplication, which has led to divergence in enzyme functions resulting in a wide diversity of terpenes. The evolution of TPSs in cultivated tomato, *S. lycopersicum*, and in related wild species, *S. habrochaites*, and *S. pimpinelli*, has been studied in details. These species synthesize a range of terpenes in their glandular trichomes, which are implicated in defense against insects. The terpene chemical diversity in *S. habrochaites* could be attributed to TPSs related to TPS20, previously characterized in *S. lycopersicum* (Gonzales-Vigil et al., 2012). These TPS20-related enzymes, unlike most TPSs in other plants, use (*Z*)-prenyl diphosphates, like neryl diphosphate and (2*Z*, 6*Z*)-farnesyl diphosphate, as substrates and belong to TPS-e/f subfamily. They are clustered on chromosome 8 of the *S. lycopersicum* genome, together with other genes involved in

terpene biosynthesis. Combined biochemical, genomic and phylogenetic analyses on the tomato species led the authors to propose a complex evolutionary scenario (Matsuba et al., 2013). Briefly, in all *Solanum* species analyzed, the monoterpene synthase genes in the cluster evolved from a diterpene synthase by duplication and divergence. In *S. habrochaites*, a new sesquiterpene synthase gene was created following another duplication event involving a gene encoding a monoterpene synthase. This enzyme evolved to use (2Z, 6Z)-farnesyl diphosphate. This has led to an organization in which monoterpene and sesquiterpene synthases are clustered on the same chromosome locus and the prenyltransferases that provide their substrates are also part of the same gene cluster. This clustering of *TPS* genes with other genes in the terpenoid pathway seems quite common in angiosperms (Boutanaev et al., 2015). There are many hypotheses that could explain the clustering of genes in a metabolic pathway (Nützmann et al., 2016; Nützmann et al., 2018). One of these is the fact that it may facilitate coexpression of the genes in the cluster. The genomes of some species harbor much more compact *TPS* gene families. For instance, the genome of cultivated apple (*Malus domestica*) contains only 10 potentially functional *TPS* genes and a larger number of *TPS* pseudogenes were found. The genome duplication events that have shaped the organization of the apple genome appears to have significantly impacted the diversity of the *TPS* genes in this species (Nieuwenhuizen et al., 2013). In addition, the reduced number of *TPS*s in a modern cultivar, compared to heritage varieties, suggests that breeding strategies may have reduced the range of terpenes in the apple fruit.

12.4 Convergent evolution

Convergent evolution is an independent evolution resulting in similar traits in different organisms. For example, the biosynthesis of the same compound in different plant species may be catalyzed by phylogenetically unrelated enzymes. There are many examples of

convergent evolution in the plant specialized metabolism. For instance, a key reduction step in the biosynthesis of tropane alkaloids involves enzymes from the short-chain dehydrogenase/reductase family in the Solanaceae, whereas it involves unrelated enzymes from the aldo-keto reductase family in the Erythroxylaceae, suggesting that tropane alkaloid biosynthesis evolved independently in these plant families (Jirschitzka et al., 2012). Phylogenetic analysis of caffeine N-methyltransferases (NMTs) in *Coffea canephora* showed that these genes expanded through tandem duplications, independently of NMTs from *Theobroma cacao* and *Camellia sinensis*, indicating a convergent evolution of caffeine biosynthesis in different plant lineages (Denoëud et al., 2014). Similarly, for decades it was believed that nearly all VOCs were produced through a limited number of pathways shared by all plants. However, there is more and more evidences that different pathways leading to similar or identical compounds have evolved independently in plants. These alternative pathways often occur through convergent evolution (Sun et al., 2016).

12.4.1 Functional convergence: distinct enzyme lineages producing similar compounds

The monoterpene alcohol linalool is present in the floral scent of many species. Its diverse ecological functions have recently been reviewed (Raguso 2016a). This compound may be both an attractant for pollinators (in orchids for example) and a repellent for various herbivores. In a tritrophic interaction context, emission of linalool by *N. attenuata* leaves has been proposed to attract predators of the generalist herbivore *Manduca sexta* (He et al., 2019). When present in nectar, it could contribute to avoid microbe proliferation. Two enantiomeric forms of this molecule exist: (*R*)-(-)-linalool and (*S*)-(+)-linalool. Both forms have distinct biochemical properties and impacts on insects. For instance, female moths oviposit more on *Datura wrightii* plants emitting (*S*)-(+)-linalool over control plants, while plants emitting (*R*)-(-)-linalool are less preferred than control plants (Reisenman et al., 2010). These two

compounds may also be present differentially throughout flower development (Pragadheesh et al., 2017). Since the first characterization of *C. breweri* linalool synthase (Dudareva et al., 1996), TPSs able to catalyze the formation of linalool from GPP have been functionally characterized in many plant species, including *A. thaliana* (Chen et al., 2003; Ginglinger et al., 2013), *F. x ananassa* (Aharoni et al., 2004), *S. lycopersicum* (van Schie et al., 2007), *A. majus* (Nagegowda et al., 2008), *V. vinifera* (Martin et al., 2010), *Gossypium hirsutum* (Huang, Xiao, et al., 2018), *Rosa* genus (Magnard et al., 2018) and *N. attenuata* (He et al., 2019). Phylogenetic analysis indicates that these genes belong to four TPS subfamilies (Fig. 12.2). For example, in *R. chinensis*, three genes encoding potentially functional TPSs are expressed in petals, *RcLINS*, *RcLIN-NERS1*, and *RcLIN-NERS2*. The *LIN-NERS1/2* genes are clustered on chromosome 5 of the rose genome (Raymond et al., 2018) whereas *RcLINS* gene is on chromosome 2. There are also truncated version of linalool/nerolidol synthases in the rose genome, probably representing pseudogenes. *RcLINS* is responsible for the small amounts of (*R*)-(-)-linalool present in rose scent (Magnard et al., 2018). *RcLIN-NERS1*, and *RcLIN-NERS2* genes, although weakly expressed, are probably not active in planta as neither nerolidol, nor (*S*)-(+)-linalool are present in rose petals. A comparison of roses with strawberries, show that these two members of the Rosaceae family have evolved different enzymes to produce linalool. *F. x ananassa* uses the (*S*)-(+)-linalool /nerolidol synthase FaNES1 (Aharoni et al., 2004), whereas the orthologous *RcLIN-NERS2* is ineffective in rose flowers. Conversely, the small quantity of linalool produced in rose flowers derives from the activity of the *RcLINS* (*R*)-(-)-linalool synthase, which is unable to produce nerolidol. The gene encoding an enzyme showing extensive identity (78%) to *RcLINS* is present in the *F. vesca* genome (XP004289147.2), however, to our knowledge, its implication in linalool production has never been reported. More generally, in plant species that were studied so far, linalool or linalool/nerolidol synthase activities are generally well correlated with their

belonging to one of the TPS subfamilies (Fig. 12.2). Mono-functional linalool synthases cluster into the TPS-b subfamily. Like RcLINS, most of these b class enzymes with linalool synthase activity generally produce (*R*)-(-)-linalool. The bi-functional linalool/nerolidol synthases, including RcLIN-NERS1 and RcLIN-NERS2 belong to the TPS-g subgroup together with some linalool/nerolidol/geranylinalool synthases. The linalool isomer produced by synthases belonging to this group is usually (*S*)-(+)-linalool, like RcLIN-NERS1 and 2. Although not all studies report the linalool enantiomer that is produced by the different species, the general trend is that (*S*)-(+)- and (*R*)-(-)-linalool synthases have evolved independently, probably under the selection pressures exerted by both herbivores and pollinators.

Place Figure 12.2 here.

Another example of convergent evolution is the biosynthesis of phenylpropenes. These compounds are phenylpropanoids derived from phenylalanine, which participate in the unique aroma of many fruits, herbs and spices. In flowers, they attract pollinators (Tan et al., 2006) and may be used as defense compounds against fungi and bacteria (Atkinson 2018). Many plants synthesize volatile phenylpropenes in their floral and vegetative organs as a defense against insect pests and herbivores. For example, roses stamens emit eugenol (Yan et al., 2018). The last step of the biosynthesis pathway to eugenol and isoeugenol is performed by the NADPH-dependent reductases eugenol synthase (EGS) and isoeugenol synthase (IGS), respectively (Koeduka et al., 2006). These enzymes catalyze the elimination of an acyl moiety from coniferyl acetate. The enzymes belong to the PIP family of reductases, together with phenylcoumaran benzylic ether reductase (PCBER) and pterocarpan reductase (PTR). Phenylpropene synthases have been shown to be distributed in two distinct protein lineages in

C. breweri and *P. x hybrida* (Koeduka et al., 2008), suggesting independent evolutionary origins. Indeed, phylogenetic analysis has revealed that *C. breweri* CbIGS1 and CbEGS1 proteins are members of Class I in the EGS/IGS subfamily of the PIP family, together with basil EGS and petunia IGS. On the other hand, CbEGS2 and PhEGS1 cluster together with members of the Class II subfamily, with similarity to PCBER and PTR proteins. The two independent origins of phenylpropene synthase activities have since been supported by studies in a number of other plants such as *F. x ananassa* and *Daucus carota* (Aragüez et al., 2013; Koeduka 2014; Yahyaa et al., 2019).

12.4.2 Pathway convergence: different pathways producing the same compounds

Monoterpenes are well known as components of floral scent and also as constituents of essential oils of aromatics plants that are widely used in the cosmetic, perfume, food, and pharmaceutical industries. Because of their high economic relevance, monoterpene biosynthesis has been extensively studied. In rose, acyclic monoterpene alcohols, such as geraniol, nerol and β -citronellol and their aldehyde and acetate derivatives are especially abundant. In most plants studied so far, geraniol is synthesized in plastids by geraniol synthase (GES), belonging to the TPS family. GES genes have been cloned and functionally characterized in many plant species, including *O. basilicum* (Iijima et al., 2004), *Cinnamomum tenuipilum* (Yang et al., 2005), *Perilla sp.* (Ito and Honda 2007), *Valeriana officinalis* and *Lippia dulcis* (Dong et al., 2013), and *Catharanthus roseus* (Simkin et al., 2013). In roses, no such enzyme has been isolated and it has been recently shown that rose flowers use an alternative route to produce geraniol.

By comparing the volatile profiles and differential gene expression of scented and unscented rose cultivars, an unexpected enzyme – a Nudix hydrolase, RhNUDX1 – was found to be responsible for the formation of geraniol (Magnard et al., 2015). The function of RhNUDX1

was elucidated by testing the recombinant protein with several potential substrates, by co-localizing the gene with a major QTL for geraniol production, and by analyzing RNAi RhNUDX1 transgenic plants. The results indicated that RhNUDX1 used GPP as substrate, as classical TPSs, but hydrolyzed one phosphate resulting in geranyl monophosphate (Fig. 12.3). Thus, a further dephosphorylation reaction catalyzed by an unidentified phosphatase present in rose petals is required to form geraniol. This process differs from terpene synthase-catalyzed reactions, where a diphosphate group is removed in one reaction. In addition, the RhNUDX1 enzyme has been shown to be located in the cytosol, raising the question of the origin of its GPP substrate, which may be either transported from plastids or generated in the cytosol.

Place Figure 12.3 here.

Nudix hydrolases belong to a superfamily of pyrophosphatases found in animals, plants, bacteria and archaea. They generally catalyze the hydrolysis of nucleoside diphosphates linked to other moieties (X) and contain the Nudix domain defined by Bessman et al. (1996) as G(X₅)E(X₇)REUXEEXX. For example, the *E. coli* mutator protein MutT eliminates harmful oxidized nucleotides, such as 8-oxo-7,8-dihydrodeoxyguanosine triphosphate (8-oxo-dGTP), which may be misincorporated in DNA during replication (Maki and Sekiguchi 1992). Similarly, in humans, MTH1 is involved in cell sanitation as it also prevents the incorporation of oxidized nucleotides (Gad et al., 2014). In plants, Nudix hydrolases are poorly characterized, although up to 29 genes have been identified in the *A. thaliana* genome (Kraszewska 2008). Based on sequence similarities, several functional groups have been defined, including diphosphoinositol polyphosphate phosphohydrolases, coenzyme A pyrophosphatases, ADP-ribose pyrophosphatases and ApnA hydrolases. In *A. thaliana*, some

Nudix hydrolases have been linked to specific functions *in vivo*. For example, some act as regulators of plant defense responses and have also been implicated in plant abiotic stress responses (Fonseca and Dong 2014; Ishikawa et al., 2009). RhNUDX1 shows the closest similarity to AtNUDX1 from *A. thaliana*.

Although AtNUDX1 actual function is still controversial, this protein has been proposed to have a similar function to that of the MutT protein from *E. coli* (Jemth et al., 2019; Yoshimura et al., 2007). However, recent studies indicate that AtNUDX1 may be involved in the regulation of terpene synthesis. Indeed, AtNUDX1, in combination with another Nudix hydrolase, catalyzed the dephosphorylation of isopentenyl diphosphate (IPP), which is the common precursor of both mono- and sesquiterpenes, to isopentenyl phosphate (IP). IP is transformed to IPP by isopentenyl phosphate kinase (IPK) (Henry et al., 2015). The ratio of IPP to IP, modulated by activities of Nudix hydrolases and IPK, is supposed to regulate terpenoid biosynthesis (Henry et al., 2018). These results suggest that NUDX1 proteins may have been recruited to perform various functions in different plants species. In rose, one striking feature of RhNUDX1 is its very high level of expression in petals, whereas it is barely expressed in other plant organs. Upregulation of the expression in petals might thus have been an important step for the evolution of *RhNUDX1* function in scent production. It is also noteworthy that this gene is present in tandem repeated copies in the genome of *R. chinensis* cv. 'Old Blush' (Hibrand Saint-Oyant et al., 2018; Raymond et al., 2018). The repeated duplication of the gene may therefore have been selected possibly leading to higher transcription levels. Monoterpenes are present in the scent of many flowers. The discovery that very different pathways involving TPSs or Nudix hydrolases evolved independently to produce monoterpenes suggests that they may be important for plant fitness, as pollination or defense-related compounds. The Nudix pathway to monoterpene biosynthesis has so far only

been characterized in the *Rosa* genus. The questions of its evolutionary origin and of its presence in other members of the Rosaceae family still remain.

There are other examples of compounds formed by different ways in different species.

For example, geranial can be produced by two unrelated pathways. In *O. basilicum*, *Zingiber officinale* and *Perilla sp.*, geranial is formed from geraniol by alcohol dehydrogenases (Iijima et al., 2014; Iijima et al., 2006; Sato-Masumoto and Ito 2014). However, this monoterpenoid can also be produced from carotenoids. Indeed, carotenoid cleavage dioxygenase 1 (CCD1) from *O. sativa* may use the carotenoid lycopene as a substrate to generate geranial and some C8/C13 ketones (Ilg et al., 2009). CCDs may also catalyze the formation of a large number of tomato fruit volatiles, including neral and geranial (Ilg et al., 2014).

Some volatiles derived from Phenylalanine (Phe), for example phenylacetaldehyde (PAld) and 2PE, are important scent compounds in numerous flowers such as roses and *P. x hybrida*. 2PE is also a component of plant defenses (Günther et al., 2019). The genes and enzymes responsible for the biosynthesis of these phenylalanine-derived compounds have been identified and functionally characterized in a number of plant species. Interestingly, plants have evolved different pathways to convert Phe to PAld, (Fig. 12.4). In *P. x hybrida*, a bifunctional phenylacetaldehyde synthase (PAAS) can catalyze both decarboxylation and oxidative deamination reactions and convert Phe to PAld (Kaminaga et al., 2006) (Fig. 12.4, route 1). Aromatic aldehyde synthases (AASs) with the same catalytic properties as PAAS are also active in flowers or leaves of *A. thaliana*, depending on the ecotype (Gutensohn et al., 2011).

Place Figure 12.4 here.

In *R. x hybrida*, RhPAAS is functionally similar to PAAS from *P. x hybrida* (Farhi et al., 2010). Genetic analysis of 2PE production in a segregating rose population has recently shown that this trait depends on specific *RhPAAS* alleles (Roccia et al., 2019). In *S. lycopersicum* fruits, a similar pathway proceeds in two steps, Phe being first converted to 2-phenylethylamine by an aromatic amino acid decarboxylase (AADC) and then subjected to oxidation by an as yet to be identified monoamine oxidase (Tieman et al., 2006) (Fig. 12.4, route 2). AADCs and AASs are members of the same aromatic amino acid decarboxylase (AAAD) family. One particular amino acid, present in a catalytic loop of these enzymes determine whether they act as monofunctional AADCs (decarboxylation) or bifunctional AASs (decarboxylation-deamination). A mutation of this amino acid is sufficient to switch enzyme activities (Torrens-Spence et al., 2013). In addition, another pathway leading to PAld biosynthesis from Phe also exists in rose (Fig. 12.4, route 3). In this pathway, Phe is first converted into phenylpyruvic acid by an aromatic amino acid aminotransferase (AAAT3), and then undergoes decarboxylation to form PAld by a phenylpyruvic acid decarboxylase (PPDC) (Hirata et al., 2012; Hirata et al., 2016) (Fig. 12.4). Similarly, in the fruits of *Cucumis melo*, Phe is also converted by an amino acid transaminase (ArAT1) to phenylpyruvic acid (Gonda et al., 2010). Experiments with transgenic petunia plants producing high levels of phenylpyruvate suggest that such an aminotransferase pathway could also be active in *P. x hybrida* petals (Oliva et al., 2017).

Recently, the metabolism of Phe was studied in *Populus trichocarpa* (Günther et al., 2019). In this species, 2PE and its glycosylated derivative (2-phenylethyl- β -D-glucopyranoside) are released upon herbivory. Separate pathways have been shown contribute to the formation of these compounds in poplar leaves. PtAAS1 controls the emission of 2PE (Fig. 12.4, route 1), whereas PtAADC1 is involved in the formation of 2-phenylethylamine which can be further metabolized into 2-phenylethyl- β -D-glucopyranoside (Fig. 12.4, route 2). *PtAAS1* and

PtAADCI sequences are very similar and are clustered on the same chromosome, which suggests that they may have evolved from a common ancestor by gene duplication and neofunctionalization.

Lastly, in *P. trichocarpa* and *Plumeria rubra*, 2PE biosynthesis has been shown to proceed via a fourth route (Fig. 12.4, route 4), which involves the conversion of Phe into (*E/Z*)-phenylacetaldoxime by cytochrome P450s from the CYP79 family (Dhandapani et al., 2019; Irmisch et al., 2014; Irmisch et al., 2013). In all species, the conversion from PAld to 2PE is supposed to be catalyzed by a phenylacetaldehyde reductase (PAR), characterized in rose, poplar and tomato (Chen, Kobayashi, et al., 2011; Günther et al., 2019; Tieman et al., 2007).

2PE may be further converted into ester derivatives, as shown in rose (Shalit et al., 2003).

Biosynthesis of 2PE is thus another example of multiple routes used to produce the same compound, which is probably of high interest for pollination and/or defense in plants.

Interestingly in rose, the pathway which uses AAAT3 and PPDC becomes active only in summer under high-temperature conditions, in contrast to the pathway using RhPAAS, which is active throughout the year, regardless of temperature, and produces a nearly constant amount of 2PE. Several hypotheses have been proposed as to why roses use an alternative 2PE biosynthesis pathway in summer: the seasonally induced pathway may have originated in wild rose species as a heat adaptation when roses spread to low latitudes and/or low altitudes. Alternatively, this pathway may have been selected during cultivation to produce blossoms in summer. (Hirata et al., 2016).

12.5 Loss- or gain-of-function mutations as a source of VOC diversity

Several examples in wild and cultivated plant species show that local mutations in a single gene may result in loss- or gain-of-function alleles that may greatly impact VOC profiles.

12.5.1 Loss of floral scent production during pollinator-mediated speciation

As described in other chapters of this book, scent is one of the major components that mediate the interaction of flowers with their pollinators (Gervasi and Schiestl 2017). There are still too few examples to draw general conclusions but we present here three examples, in which modifications in flower scent profiles led to changes in pollinator communities.

The *Petunia* genus was used as a model to investigate the genetic basis of gain and loss of scent during the transition from bee to moth and from moth to hummingbird pollination (Amrad et al., 2016; Klahre et al., 2011). *P. inflata*, which is supposed to be ancestral to other species, has purple flowers, is pollinated by bees and emits the benzenoid compound benzaldehyde, in small amounts. Conversely, *P. axillaris* has white flowers and is nocturnally pollinated by hawkmoths. It emits large amounts of benzenoids such as methylbenzoate, benzylalcohol and benzaldehyde. Finally, the hummingbird-pollinated flowers of *P. exserta* are red and scentless. Here we will focus on the differences found between *P. axillaris* and *P. exserta*. At first, two QTLs linked to the capacity to produce benzenoid volatiles were detected in an F1 population derived from an interspecific cross between *P. axillaris* and *P. exserta* (Klahre et al., 2011). Using genetic approaches, one of these QTLs, present on chromosome VII, could be associated to the MYB transcription factor ODO1, which regulates several enzymes of the phenylpropanoids/benzenoid pathway (Verdonk et al., 2005). *ODO1* gene is highly expressed in *P. axillaris* that emit high amounts of volatiles, compared to *P. exserta* and *P. integrifolia* which emit much lower levels of VOCs. A combination of genetic and molecular approaches identified the gene underlying the QTL on chromosome II as cinnamate-Co-A ligase (*CNLI*) (Amrad et al., 2016). This enzyme conjugates cinnamic acid to coenzyme A, the first committed step in the benzenoid pathway. The decreased activity found in *P. exserta*, compared to *P. axillaris*, was attributed to multiple defects in the gene, including a nonsense mutation in the fourth exon, which is predicted to shorten the protein by

171 amino acids. These works concluded that functional inactivation in CNL1, together with the decrease expression of the MYB transcription factor ODO1, accounted for the loss of scent during the shift from hawkmoth to hummingbird pollination.

Another study reported that the loss of floral scent during the transition from outcrossing to selfing in the genus *Capsella* was also caused by loss of CNL function (Sas et al., 2016).

Indeed, the insect-pollinated *C. grandiflora* emits benzaldehyde as a major constituent of its floral scent, whereas it has been lost in the selfing *C. rubella*. By comparing CNL sequences in 207 accessions of the two species, they found that it was inactivated twice by independent mutations in *C. rubella*.

These studies on *Petunia* and *Capsella* suggest that loss-of-function of *CNL* genes has repeatedly occurred during evolutionary transition to scentless flowers, in two phylogenetically unrelated genera.

The genus *Mimulus* offers another example of loss of floral scent during pollinator mediated speciation. *Mimulus lewisii* is a bumblebee-pollinated alpine species, while its sister species, *M. cardinalis*, is a lower-elevation hummingbird-pollinated species. Three major floral volatiles are produced by *M. lewisii*, (*R*)-limonene, β -myrcene, and (*E*)- β -ocimene. *M. cardinalis* produces only (*R*)-limonene, but in much lower amounts than *M. lewisii* (Byers, Bradshaw, et al., 2014). Differences in *TPS* sequences were proposed to be responsible for the changes in floral scent composition. *M. lewisii* limonene myrcene synthase (*MILMS*) and ocimene synthase (*MIOS*) genes are strongly expressed in floral tissues. Conversely, *M. cardinalis* *LMS* (*McLMS*) gene is weakly expressed and has a nonsense mutation in exon 3. Like *MIOS*, *M. cardinalis* *OS* (*McOS*) gene is expressed in flowers, but the corresponding enzyme does not produce (*E*)- β -ocimene (Byers, Vela, et al., 2014). When (*E*)- β -ocimene emission was reduced in transgenic southern *M. lewisii* plants by targeting *OS* gene with RNAi, visitations by bumblebees were significantly decreased (Byers, Vela, et al., 2014),

suggesting that (*E*)- β -ocimene is a key component in the scent of *M. lewisii*, and that OS has influenced pollination syndrome evolution. Further experiments in related *Mimulus* species have shown that emission of (*E*)- β -ocimene was restricted to the flowers of *M. lewisii*, and that deletion, missense, or frameshift mutations were present in the *OS* gene of other species. It is thus likely that parallel loss-of-function in *OS* genes occurred in the *Mimulus* genus, probably as the result of natural selection (Peng et al., 2017). The selective forces driving scent losses during evolution of the pollination syndromes are not known. One of the possible explanations is that the plants must both attract pollinators and avoid attracting the florivores. In some ecological contexts, it may be advantageous for plants to reduce scent emissions, in order to minimize the cost of florivory (Raguso 2016b).

12.5.2 Loss- and gain-of-function mutations impacting scents and aromas in cultivated plant species

Gain or loss of scent and aroma attributes has frequently, and sometimes unintentionally, been selected during plant domestication. One of the first molecular analysis of such phenomenon characterized the fruit aroma of cultivated *F. x ananassa* in comparison to those of its wild relative *F. vesca*. The poor aroma of cultivated strawberries could be attributed to a two base-pair insertion causing a frameshift in a *TPS* gene, which was responsible for the biosynthesis of bouquet of monoterpenes containing α -pinene and β -myrcene in wild strawberries (Aharoni et al., 2004).

Rice (*O. sativa*) is a crucial food source for the majority of the world's population. Fragrance in the grain is highly valued by the consumers and has been the subject of intensive researches. Although rice fragrance may involve several compounds (Daygon et al., 2017), the major contributor is 2-acetyl-1-pyrroline (2AP), which possesses a popcorn-like aroma. Early analysis of the genetic basis of rice flavor led to the identification of a single locus on

chromosome 8 (*FGR*) associated with fragrance. The *FGR* locus encodes an amino aldehyde dehydrogenase (AADH) or betaine aldehyde dehydrogenase (BADH2), which catalyzes the oxidation of γ -aminobutaraldehyde, which is itself derived from putrescine, to γ -aminobutyric acid. However, the recessive allele *badh2.1* encodes an inactive AADH protein, due to an 8 bp deletion in exon 7 of the gene, that results in a premature stop codon and putatively truncated BADH2 protein. γ -aminobutaraldehyde thus accumulates in the grain and is transformed to 2AP, through yet unknown steps, leading to the typical fragrant phenotype (Chen et al., 2008). *O. sativa* consists of two major varietal groups, *Indica* and *Japonica*, that have been recognized in China since ancient times.

In-depth survey of the genetic of the *BADH2* gene in a large panel of genetically and geographically diverse rice germplasm showed that one particular *badh2.1* allele is predominant in all fragrant rice varieties today, such as Jasmine and Basmati varieties (Kovach et al., 2009). Sequencing of the *BADH2* gene in a panel of 242 *O. sativa* accessions, revealed a single origin of the *badh2.1* allele within the *Japonica* varietal group and showed that it was later introgressed in the *Indica* varietal group, presumably by breeders. This work also suggested that the *badh2.1* allele, associated with fragrance within a small set of varieties from China, was selected as a de novo mutation in *O. sativa* after domestication from its wild progenitor, presumably after the divergence of the *Japonica* populations. Other *BADH2* alleles conducting to fragrant varieties were later isolated (Bindusree et al., 2017; He et al., 2015). All these studies show that the *BADH2* gene was selected during rice domestication for its effect on the plant sensorial quality. Interestingly, in other plants producing 2AP, the genes and mechanisms involved were found to be similar. For example, in *Glycine max*, genetic mapping of a QTL for fragrance linked a *GmBADH2* allele to 2AP biosynthesis (Juwattanasomran et al., 2011). Similarly, sequencing the *BADH* gene in a fragrant cucumber (*Cucumis sativus*) cultivar revealed that a defective allele of *CsBADH* was responsible for

2AP accumulation (Yundaeng et al., 2015). These examples illustrate the fact that a loss-of-function mutation may lead to the synthesis of a new volatile compound, and it is striking to see that selection of fragrant cultivars in different plant species resulted in the selection of mutations in orthologous genes.

In a few cases, the fascinating aromas that characterize specific cultivars involve gain-of-function or enhanced-function mutations. For instance, the typical floral flavor of Muscat and Gewurztraminer grape varieties is greatly appreciated both in wines and in table grapes. Muscat flavor is due to high levels of monoterpenols (geraniol, linalool and nerol) accumulated in grapes. Genetic analyses of monoterpenol contents in Muscat and Gewurztraminer varieties revealed the co-localization of a gene encoding 1-deoxy-D-xylulose 5-phosphate synthase (*VvDXS*) with a major QTL positioned on chromosome 5 (Battilana et al., 2009; Duchêne et al., 2009). Furthermore, an association study showed that a single nucleotide polymorphism (SNP) responsible for the substitution of a lysine (K284) with an asparagine at position 284 (N284) of the *VvDXS* protein was significantly associated with Muscat-flavored varieties (Emanuelli et al., 2010). Expression of the Muscat-specific *VvDXS* allele in tobacco confirmed that the N284 mutation was sufficient to drive a massive accumulation of monoterpenes in the corresponding transgenic lines, compared to lines expressing the regular K284 allele. Kinetic analyses showed that the N284 mutation impacted the catalytic efficiency of the *VvDXS* enzyme, *VvDXS* N284 being twice as efficient as *VvDXS* K284 (Battilana et al., 2011). This represents a remarkable example of a natural enzyme variant with enhanced catalytic properties, causing terpene over-accumulation in the much-appreciated Muscat-flavored grapevine varieties.

12.6 Conclusion

The capacity to produce scent is a key component of plant fitness, together with other vegetative and floral traits. Although the picture is far from complete, in the past decade, many pathways that lead to the biosynthesis of terpenoids, phenylpropanoids/benzenoid compounds and fatty acid derivatives were deciphered. The availability of genomic sequences has allowed the analysis of the spatial and temporal regulation of these pathways. The knowledge of enzymes and regulator genes of VOC biosynthesis has permitted to address the crucial question of the evolutionary origin of these pathways. On the one hand, many plant species have acquired the capacity to produce highly diversified blends of volatiles through different mechanisms leading to divergent evolution and pathway plasticity, such as the expansion of large gene families following gene duplication, catalytic promiscuity or easy change in substrate specificity within enzyme families. On the other hand, some ubiquitous volatiles such as geraniol, linalool or 2PE are present in flower scent or vegetative VOCs of many plant species. Recent studies have shown that such compounds may be produced by unrelated enzymes, or even totally different pathways, which evolved independently in different plant species. Evolutionary convergence towards the biosynthesis of a limited number of ubiquitous volatiles suggests that these compounds may be especially important for plant fitness, as attractant for pollinators or for defense against herbivores or pathogens. Very recent studies analyzing the roles of VOCs in different ecological situations suggest that the response of a specialist herbivore to a single compound like linalool may be very complex, depending on enantiomer, plant genotype, and environmental conditions (He et al., 2019). Many more studies will undoubtedly aim at deciphering the ecological functions of plants volatiles in the future.

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Figure legends

Figure 12.1: Mechanisms of gene functionalization

A, Gain-of-function mutation (GOF) changes the ancestral function #1 giving a modified function #1', or a novel function #2; B, Loss-of-function mutation (LOF) suppresses the ancestral function, but is beneficial and selected. Thus, the absence of function #1 can be considered as a function #2, even if the gene becomes a pseudogene; C, Mutations on duplicated copies are eliminated during evolution because two copies of the gene, giving rise to the enhanced function #1', are beneficial. This can be considered as concerted evolution; D, GOF on one duplicated copy gives a new beneficial function #2: this is a neofunctionalization process. The other copy keeps the function #1; E, LOF on both copies splits the ancestral function #1 in two subfunctions #1' and #1'', but the "sum" of the functions is the function #1: this is a subfunctionalization process. Therefore, it is sometimes considered that the ancestral gene had two functions. The subfunctionalization event can be followed by a neofunctionalization event of one copy in a subneofunctionalization process; F, LOF on one copy gives rise to a pseudogene, but the ancestral function #1 is kept by the other copy; G, The pseudogenization process is not complete and some regulatory modules remain intact (transcription factor binding sites in the promoter, expression of a noncoding RNA...). This can lead to regulation of the other copy, giving rise to a function #1', or to regulation of other genes.

Figure 12.2: Diversity of linalool synthases characterized from different plant species

Unrooted Neighbor Joining tree depicting the classification of linalool synthases and linalool/nerolidol synthases from plants into terpene synthase (TPS) subfamilies.

Linalool/nerolidol/geranylinalool synthases were also included. Protein sequences were aligned with ClustalW and tree was constructed using Geneious software. Linalool isomers

are indicated by colors of the GenBank accession letters. Black letters, unidentified linalool isomer; blue letters, (*R*)-(-)-linalool synthase; orange letters, (*R/S*)-linalool synthase (racemic); pink letters, (*S*)-(+)-linalool synthase.

Figure 12.3: Biosynthesis of monoterpenes and sesquiterpenes in plants

Roles of NUDX proteins in terpene biosynthesis have been shown in *A. thaliana* (Henry et al., 2018) and *Rosa sp.* (Magnard et al., 2015). ADRO, acetyltransferases, dehydrogenases, reductases, and oxidases; FPP, farnesyl diphosphate; FPPS, farnesyl diphosphate synthase; GP, geranyl phosphate; GPP, geranyl diphosphate; DMAP, dimethylallyl diphosphate; DMAPP, dimethylallyl diphosphate; IP, isopentenyl phosphate; IPK, isopentenyl phosphate kinase; IPP, isopentenyl diphosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; MTPS, monoterpene synthases; MVA, mevalonic acid; NUDX, nudix hydrolase; Pase, phosphatase; STPS, sesquiterpene synthases; ?, unknown transporters; One arrow, one enzymatic step; Two arrows, several enzymatic steps; Solid circle, functionally characterized enzymes; Dashed circle, one or several hypothetical enzymes; Dashed box with opposite arrows, hypothetical transporters.

Figure 12.4: Biosynthesis of 2-phenylethanol and derivatives in plants

AAAT, aromatic amino acid aminotransferase; AADC, L-aromatic amino acid decarboxylase; AAT, alcohol acetyltransferase; β -glu, β -glucosidase; CYP79, cytochrome P450 family 79 enzyme; MAO, monoamine oxidase; PAAS, phenylacetaldehyde synthase; PAR, phenylacetaldehyde reductase; PPDC, phenylpyruvic acid decarboxylase; UGT, UDP-glycosyltransferase; TOX, transoximase. *At*, *Arabidopsis thaliana* (Gutensohn et al., 2011); *Cm*, *Cucumis melo* (Gonda et al., 2010); *Sl*, *Solanum lycopersicum* (Tieman et al., 2006; Tieman et al., 2007); *Pc*, *Populus x canescens* (Günther et al., 2019); *Ph*, *Petunia x hybrida*

(Kaminaga et al., 2006), *Pr*, *Plumeria rubra* (Dhandapani et al., 2019); *Pt*, *Populus trichocarpa* (Günther et al., 2019; Irmisch et al., 2014; Irmisch et al., 2013); *Ro*, *Rosa sp.* (different rose species and cultivars were used) (Chen, Kobayashi, et al., 2011; Farhi et al., 2010; Hirata et al., 2012; Hirata et al., 2016; Kaminaga et al., 2006; Roccia et al., 2019; Shalit et al., 2003). Solid lines, well established reactions with characterized enzymes; Dashed lines, Hypothetical reactions.

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