Floral Fragrance. New Inroads into an Old Commodity[1]

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The ability of flowering plants to prosper throughout their long evolution has been strongly dependent on the constant development of strategies to lure pollinators. This has led to the creation of elaborate perianth forms, splendid color patterns, and a broad spectrum of fragrances. Flower morphogenesis and pigmentation have been intensively studied in the last several decades, and today, the results of our deeper understanding of the underlying pathways have already been harnessed for crop improvement (Zuker et al., 1998). In contrast, knowledge of the biochemistry of fragrance production and the mechanism regulating its emission remains sketchy.

Flower scent is a composite character that is determined by a complex mixture of low-molecular-weight volatile molecules. Due to the invisibility of this character, to the shortcomings of humans' sense of smell, and to the highly variable nature of the trait (in part because of strong environmental influences), no simple, efficient, and reliable methods to screen for genetic variation have been developed. Moreover, to date, no convenient plant model systems that would enable biochemical and forward and reverse genetic studies of flower scent are available. For many years, the research into floral fragrance focused on its chemical elucidation, coupled with chemical synthesis to produce the large quantities demanded by the perfume and food industries. Indeed, hundreds of structures are known (Knudsen et al., 1993) and many are synthetically produced. Most fragrance compounds belong to three major groups: phenylpropanoids (including benzenoids), fatty acid derivatives, and terpenoids (Croteau et al., 2000). The elucidation of their pathways with respect to the enzymes and genes involved and the underlying molecular mechanisms controlling them has just begun, with an *Update* on the topic having recently appeared in this journal (Dudareva and Pichersky, 2000).

Here, we review some of the methodological issues involved in the study of floral scent and examine how the uniqueness and complexity of the trait necessitate the integration of modern techniques with non-conventional model systems. For example, several plant systems, including flowers of *Clarkia breweri*, snapdragon (*Antirrhinum majus*), and rose (*Rosa* spp.), have been chosen, not for their amenability to molecular studies, but mainly for their fragrance characteristics and their amenability to chemical and biochemical analyses (Fig. 1; Pichersky et al., 1994; Dudareva et al., 2000). The use of diverse plant systems in combination with modern metabolomic, genomic, and proteomic approaches is expected to lead to a detailed understanding of the underlying processes. Utilization of this knowledge to produce fragrance compounds in transgenic plants and to improve the often-lacking aroma characteristics of fruits, vegetables, and flowers clearly has great biotechnological potential, and we review some promising recent experimental attempts to engineer floral scent.

FLORAL SCENT AND POLLINATION

Fragrance compounds play numerous important roles in the interactions between plants and their surroundings, a major one being to attract pollinators, which are mostly, although by no means exclusively, insects (Dudareva and Pichersky, 2000). The unique combination of volatile molecules making the small and not-so-small differences in fragrance spectra among flowers of different species can be distinguished by the olfactory receptors of insect antennae enabling them to find and visit their flower(s) of choice (Pham-Delegue et al., 1990; Raguso et al., 1996). However, little is currently known about the innate ability of insects to detect specific volatiles or their innate and learned responses (attraction, repulsion, or indifference) to such compounds (Henning et al., 1992). This field of study should see much more activity in coming years.

Although all floral organs can emit fragrance compounds, petals are the main source of scent in most plants (Pichersky et al., 1994). Some plants have developed highly specialized anatomical structures, termed "scent glands," for fragrance production; in

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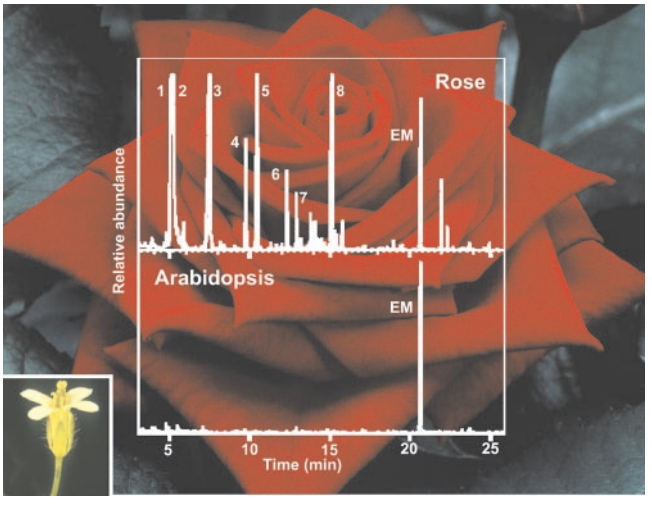

Figure 1. Headspace analysis of Arabidopsis (ecotype Colombia) and rose (cv Fragrance Cloud) flowers. Headspace was collected for 24 h (from a single rose flower and from approximately 40 Arabidopsis flowers) into a Porapak Q cartridge (Dudareva et al., 1998), and eluted with 1 mL of hexane containing 10 μg of ethyl myristate (EM) that served as an internal standard. Samples were analyzed as described in Lewinsohn et al. (2001), except that a Restek Rtx-5Sil MS (30 m $\times$ 0.25 mm) fused-silica capillary column was used. The main components were identified by comparing retention times and mass spectra with those of authentic standards and complemented with computerized libraries. The major volatiles identified in roses are **1:** 3-hexen-1-ol acetate, **2:** *n*-hexyl acetate, **3:** 2-phenylethyl alcohol, **4:** citronellol, **5:** 2-phenylethyl acetate, **6:** citronellyl acetate, **7:** neryl acetate, and **8:** germacrene D. In Arabidopsis, only traces of fatty acid degradation products and hydrocarbons were identified.

other plants, the non-specialized floral epidermal cells are recruited for fragrance production and emission (for review, see Dudareva and Pichersky, 2000). In some cases, floral scent emission shows diurnal rhythms; flowers that are pollinated at night often tend to have peak emissions at night, whereas for day-pollinated flowers, the situation is reversed. Whereas nocturnal emission of volatiles is controlled by an endogenous circadian clock, daytime emission in most cases is controlled directly by light (Jakobsen and Olsen, 1994). Nevertheless, circadian control of fragrance production in day-emitting plants has been shown for rose (Helsper et al., 1998) and snapdragon (Kolosova et al., 2001a).

Pollination by scent-guided insects is a critical step in the successful production of food on the farm, and lack of efficient pollination can lead to low crop yields. For example, fruit orchards in the United States are critically dependent on bee pollination, and the major reduction in the number of bees that has occurred over the last decade (due to disease) has caused a corresponding decrease in fruit yield (Kraus and Page, 1995). Some plants that are introduced into a new environment lacking suitable pollinators have very low pollination rates from the start; for example, alfalfa plants grown for seed in the southwestern United States have seed-set rates as low as 2% (Henning et al., 1992).

Several commercial products have been developed based on bee pheromones that are used to spray orchards to increase bee visitation rates, but recent analysis has failed to demonstrate any significant increase in fruit yield following their application (Ambrose et al., 1995). On the other hand, it has been proposed, based on differences in floral volatiles among alfalfa varieties and experiments with artificial flowers, that selection for alfalfa plants emitting more linalool, a monoterpene alcohol, will result in higher seed yield (Henning et al., 1992).

HEDONIC ATTRIBUTES OF FLORAL SCENT

Although flower fragrance has evolved for the evolutionary success of plants, mankind has long recognized its sensual pleasure. Man's admiration of flower fragrance rapidly turned volatile substances into a high-impact commercial commodity. Mainly synthetically produced, but also natural, volatiles are heavily used in the perfume, cosmetics, air freshener, laundry detergent, and the food and drink industries (Burdock, 1995).

Humans, like insects, strongly associate scent with specific flowers, e.g. rose, gardenia, and jasmine. Although the scent of certain flowers can generally be described as pleasant or revolting—the smell of roses being a simile for pleasantness versus the smell of *Hydrosme rivieri* flowers, which has been compared to rotten meat (Stransky and Valterova, 1999)—the pleasantness of other flowers to humans is specific to the individual. All the same, the very same fragrance compounds can be present in flowers that are perceived by humans as having quite different scents. For example, the monoterpene geraniol, a major volatile in rose flowers, is also emitted by the uniquely scented jasmine flowers (Croteau and Karp, 1991); furthermore, this compound contributes to the scents of more than 250 different plant species (Knudsen et al., 1993). Not only the composition of the fragrance pallette but also the overall level of the volatiles determine a scent's appeal to humans (Burdock, 1995). When grown outdoors, *Narcissus tazetta* is recognized by its pleasant scent, whereas the smell emitted by a bouquet of narcissuses, especially in a confined space where volatiles accumulate to high levels, becomes highly unpleasant. The concentration of a particular compound in a mixture of volatiles has dramatic effects; for example, a high level of indole has a very unpleasant odor, reminiscent of fecal matter, but at a high dilutions, it is perceived as floral and pleasant.

Since flower scent has almost never been a target trait in commercial breeding programs, which have traditionally concentrated on color, longevity, form, etc., it is lacking in most modern varieties (Zuker et al., 1998). For example, carnation (*Dianthus caryophyl-*

lus) flowers traditionally possessed a spicy/clove odor, which is determined by eugenol. In some old varieties, eugenol contributes up to 85% of total headspace volatiles. Most modern varieties, however, produce low levels of eugenol and lack the characteristic fragrance (Clery et al., 1999). Furthermore, not only may fragrance not have been selected for, it may actually have been unintentionally selected against, for example, due to the negative correlation between longevity and fragrance. One mechanistic explanation for such a correlation may have to do with the observation that the common floral scent compounds jasmonic acid and methyl jasmonate are known to promote flower senescence (Porat et al., 1993).

FRAGRANCE EVALUATION: OLFACTORY SENSING VERSUS ANALYTICAL TOOLS

For a molecule to impart an odor sensation in humans, it must have at least a certain degree of volatility to reach the olfactory epithelium located in the upper nasal cavity (Thomson, 1987). The human olfactory system can recognize and discriminate between a vast variety, on the order of thousands, of odorous molecules, due to the expression of an extremely large gene family of odorant receptors. A single olfactory neuron is believed to express a single odor receptor gene. These receptors, localized to the olfactory neuron cell surface, activate G-proteins and initiate a cAMP-mediated signal transduction cascade, leading ultimately to odor sensing (Zhao and Firestein, 1999). The organoleptic perception threshold depends on the volatile molecule, and for some it is extremely low. Several organic molecules can even be detected at a concentration of less than 10^{-8} M (Thomson, 1987). Hence, a subject's assessment of the influence of a specific fragrance compound in an aroma mixture on the overall sensation imparted by the scent does not necessarily reflect its absolute concentration in the scent, but depends on its perception threshold.

For some volatiles, the human nose, like the olfactory organs in insects, can be more sensitive than analytical tools in the laboratory (Hinterholzer and Schieberle, 1998). Nevertheless, attempts to characterize floral scent using humans as the "sensory equipment" face numerous difficulties, including the lack of specific words to characterize specific scents. In contrast to the general acceptance of, for example, words describing colors, scents are often described in the literature as "woody," "fruity," "musty," etc. (Burdock, 1995); there is no clear agreement among scientists as to what physical attributes such terms actually correspond to. Thus, for an objective evaluation of floral scent, some type of instrumentation is clearly needed.

Mass spectroscopy (MS) detectors coupled to gas-chromatography techniques have enabled chemical analyses of flower scent components with high levels of sensitivity (Van Beek, 1999). Sampling methods, however, have been somewhat more problematic. Since fragrance compounds are often emitted to the atmosphere, several methodologies to trap floral volatiles have been designed. They include headspace analysis and solid-phase extraction (Van Beek, 1999). These methods, used to collect and sample the volatiles emitted from flowers, are often qualitative in nature. This is because the methods used to trap the volatiles are often inefficient; thus, an indeterminate amount of material is not captured and escapes into the atmosphere, and due to the different physical properties of each compound in the scent, the proportion that escapes is different for each (Van Beek, 1999). To analyze volatiles accumulating in tissue, extraction using organic solvents or supercritical fluid CO_2, as well as steam and hydrodistillation procedures, are often performed (Van Beek, 1999). It is easy to quantify the different volatiles utilizing these latter methodologies, but they can also be problematic, since heat and adverse pH conditions may modify the original volatile composition present in the flowers. For example, phenethyl alcohol is largely lost during the distillation of rose essential oil (Weiss, 1997). Lavender flowers accumulate linalyl acetate, which is partially hydrolyzed during distillation processes (Morin and Richard, 1985). Moreover, these techniques are designed to analyze the volatiles that accumulate in the flower tissues, and not necessarily those emitted by the flowers, which constitute their scent.

It is thus apparent that the evaluation of fragrance is a highly complex matter and the linkage between olfactory sensing and chemical analyses is one of the main topics to be addressed. The first step in this direction was made by the development of an electronic nose that has three elements: an odor-sensor array, a data preprocessor, and a pattern-recognition engine (Craven et al., 1996). The signals that form the output of a sensor array do not provide a spectrum of scent constituents but rather information relating to the quality of the compounds, which are characterized by a particular sensor response signature. Thus, whereas gas chromatography-MS can detect individual volatiles quantitatively and qualitatively, the electronic nose can only make a cumulative analysis. Recently, a promising simple colorimetric sensor array for odor visualization was described (Rakow and Suslick, 2000). This novel "smell-seeing" device utilizes the color change induced in an array of metalloporphyrin dyes upon binding of volatiles.

IMPACT OF GENOMICS ON FLORAL SCENT RESEARCH

The number of different flower volatiles is very large (Knudsen et al., 1993) but, surprisingly, these compounds are biosynthesized by a relatively small

number of often overlapping metabolic pathways (Croteau and Karp, 1991; Croteau et al., 2000). In general, most plant volatiles are derived from three main classes of compounds—terpenoids, phenylpropanoids/benzenoids, and fatty acid derivatives—which are often greatly modified (oxidized, esterified, methylated, etc.). Mono- and sesquiterpenes belong to the terpenoids, the largest group (more than 20,000) of natural products known (Croteau et al., 2000). These terpenes are synthesized from isopentenyl diphosphate by different mono- and sesquiterpene synthases (Trapp and Croteau, 2001). Phenylpropanoids, including benzenoids, represent another biochemical class of floral fragrance compounds, which derive from L-Phe through the action of the pivotal enzyme Phe ammonia lyase. The complete biosynthetic pathway to these volatile compounds has not yet been characterized, but hydroxylation, acetylation, and methylation reactions are involved (Croteau and Karp, 1991). Other fragrance components, such as 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).

Clarkia breweri linalool synthase (LIS), which catalyzes the formation of the acyclic alcohol monoterpene linalool, was the first floral enzyme responsible for scent to be isolated and characterized (Pichersky et al., 1994), although several other enzymes responsible for the synthesis of volatiles from vegetative tissues had previously been (Alonso et al., 1992; Lewinsohn et al., 1992). The LIS protein was purified to homogeneity from thousands of stigmata, and its cDNA was cloned (Dudareva et al., 1996). Since that work, several other floral genes involved in fragrance production have been isolated and characterized in a similar way (Dudareva et al., 2000; Dudareva and Pichersky, 2000). These include *S*-adenosyl-L-Met:(iso) eugenol *O*-methyltransferase (*IEMT*), acetyl-CoA:benzylalcohol acetyltransferase (*BEAT*), *S*-adenosyl-L-Met:salicylic acid carboxyl methyltransferase (*SAMT*), and *S*-adenosyl-L-Met:benzoic acid carboxyl methyltransferase (*BAMT*). IEMT catalyzes the transfer of a methyl group to eugenol and isoeugenol; BEAT catalyzes the production of the ester benzylacetate from benzylalcohol and acetyl-CoA; SAMT catalyzes the production of methylsalicylate from salicylic acid and SAM; and BAMT catalyzes the production of methylbenzoate by transferring the methyl group of SAM to benzoic acid. All of these genes were isolated from *C. breweri*, except for *BAMT*, which was cloned from flowers of snapdragon, in which methylbenzoate is one of the major fragrance compounds (Dudareva et al., 2000). In situ hybridization and immunolocalization studies performed with *LIS*, *IEMT*, and *BAMT* have revealed specific expression of these genes in the epidermal cells of *C. breweri* and snapdragon flowers (Dudareva and Pichersky, 2000; Kolosova et al., 2001b); providing evidence that at least the volatile products of these enzymes are generated at the site of emission, thus allowing their direct access to the surroundings.

All the aforementioned enzymes are members of gene families that are found in the genomes of other species as well. For example, SAMT and BAMT were found to define a new type of methyltransferase and the Arabidopsis genome has close to 20 genes in this family (Dudareva and Pichersky, 2000). The biochemical identification of the function of SAMT in *C. breweri* has facilitated the characterization of the Arabidopsis genes, and it was recently shown that one of them, *JMT*, encodes the enzyme that catalyzes the formation of methyljasmonate in vegetative tissues (Seo et al., 2001). The JMT ortholog in *Brassica campestris* is expressed in floral nectaries (Seo et al., 2000). Likewise, BEAT from *C. breweri* is part of a new class of acyltransferases, the BAHD family (St-Pierre and De Luca, 2000), which is widely distributed throughout the plant kingdom, and some of the proteins in this family catalyze the formation of volatile esters (Dudareva et al., 1998; Aharoni et al., 2000). The terpene synthase family, to which LIS belongs, is also a rich source of enzymes for floral volatiles. Finally, IEMT is also part of a large family, the *O*-methyltransferases, and several of these enzymes catalyze the formation of volatiles (Wang et al., 1997). Overall, the data that have accumulated to date reveal that mutations that create small changes in protein sequences can lead to new enzymes that catalyze the formation of different fragrance compounds. It should thus be emphasized that the examination of various plant systems that contain such variant enzymes, which on superficial examination (sequence comparisons but not enzymatic assays) seem to be "the same" enzyme, can be extremely useful for gene discovery.

All the aforementioned floral scent genes, with the exception of *JMT*, were isolated via classical biochemical approaches. To date, no forward genetics approaches have been harnessed for the characterization of fragrance genes, mainly due to the lack of an efficient system for the identification of fragrance mutants. The novel technologies of genomics, in contrast, allow quick access to plants with poor genetic characterization, enabling the choice of a model plant system based on the trait of interest (e.g. roses with copious scent emission compared with the scent-poor Arabidopsis; Fig. 1) rather than being limited to established model systems (Fiehn et al., 2000). Indeed, several groups have recently used the high-throughput technologies to identify new fragrance genes in fruits and vegetative tissues (Aharoni et al., 2000; Lange et al., 2000; Gang et al., 2001). Aharoni et al. (2000) were the first to combine expressed sequence tag (EST) database mining with metabolic profiling and microarray expression analyses to iden-

tify an aroma-related gene, alcohol acyltransferase, responsible for the production of volatile esters in strawberry fruit.

Research into floral scent genes using high-throughput tools has recently been initiated in *C. breweri* (https://sativa.biology.lsa.umich.edu/blast/blast.html), snapdragon (N. Dudareva, Purdue University, IN, personal communication), and rose (http://agri3.huji.ac.il/~petals). These projects combine detailed fragrance analyses with the creation of petal EST databases. Integration of rose microarray expression analyses with database mining, has led to the identification of several novel genes with putative functions in floral fragrance production (http://agri3.huji.ac.il/~petals). The availability of an established *E. coli* expression system, allowing rapid functional analyses of fragrance genes (Dudareva et al., 1998), is expected to lead to smooth advances in high-throughput identification of novel scent genes. Indeed, several such genes from roses and *C. breweri*, including terpene synthases, acetyltransferases, and methyltransferases have already been functionally characterized at the protein level and shown to catalyze the formation of floral scent components (http://agri3.huji.ac.il/~petals).

MANIPULATION OF FLOWER FRAGRANCE

The manipulation of fruit aroma and flower scent would obviously have a great economic impact. Ornamental crops, a highly important economic commodity (Jensen and Malter, 1995) with a world market value of over $30 billion, naturally represent the main target for the genetic manipulation of flower fragrance level/spectrum. The lack of distinctive scent in many modern floricultural varieties, cut flowers in particular, further emphasizes the importance of ornamentals as a target (Zuker et al., 1998). Food crops can also be considered an important target, in that the manipulation of floral scent could improve seed set. Both crop types could also benefit from metabolic engineering of fragrance for increased protection against pathogens and pests (Dudareva and Pichersky, 2000).

However, the genetic engineering of ornamentals is currently lagging far behind that of main food crops. The main reason is the lack of efficient transformation systems for ornamental species; even when such procedures are available, they are generally not suited to elite varieties (Zuker et al., 1998). Although ornamentals are of high economic importance, each crop represents only a small segment of a market that consists of hundreds of varieties representing many different species. Hence, the limited economic value of each ornamental crop has prevented the massive investments, such as those spent on food crops, needed to advance ornamentals into the molecular breeding era. The high cost of registering transgenic crops is also a significant constraint in ornamentals. Nevertheless, intensive research into the micropropagation of ornamentals has led to the development of numerous regeneration procedures that can be adapted to most gene-transfer systems. As a result, the transformation of several major ornamentals, e.g. rose, carnation, and chrysanthemum has been reported in the last several years (Bajaj, 2001).

Two alternative approaches can be used to genetically engineer flower fragrance. One is based on the introduction of foreign genes encoding enzymes with activities that are missing in the target plant; these allow new branching of existing pathways or the generation of a novel one. The introduction of novel genes or the enhancement of existing genes' activities may not, in itself, be sufficient to modulate flower scent. A lack of substrate availability is one of the main limitations in volatile production. For example, it was recently shown that the level of methyl benzoate produced by snapdragon flowers is limited by the level of its precursor, benzoic acid (Dudareva et al., 2000). It may be possible to overcome the substrate shortage by enhancing the activity/level of upstream enzymes (Sandmann, 2001).

The second approach is based on modulating (down- or up-regulating) the expression of a native gene(s). Via this route, one can increase the production of the volatile through up-regulation of a gene in the pathway, or alternatively block the production of an undesirable volatile. Inhibition of the native genes' activities can also enable diversion of metabolic flow, leading to compositional modification of the fragrance spectrum. This route was recently demonstrated in carnation, in which blocking the anthocyanin biosynthetic pathway led to increased methyl benzoate production and flower scent (Zuker et al., 2001). Since both anthocyanins and methyl benzoate originate from the same phenylpropanoid pathway, it was suggested that the flower's enhanced scent production was due to diversion of metabolic flow toward benzoic acid, which is the precursor of methyl benzoate. Note that the redirection of metabolic flow may have a deleterious effect on the plant as a result of depletion in available levels of the general precursors necessary for normal plant development. The use of currently available flower-specific promoters or of those yet to be isolated from floral fragrance-related genes may be necessary to allow expression of the transgene in an adequate spatial/temporal manner.

Whereas in the last decade several groups have reported the genetic manipulation of plant volatile composition (Lewinsohn et al., 2001), attempted metabolic engineering for fragrance production in flowers was reported for the first time this year. These studies used the *C. breweri LIS* gene with the aim of generating the production of linalool in plants lacking this monoterpene. However, introduction of cauliflower mosaic virus 35S::*LIS* into petunia did not result in linalool emission; instead, the non-volatile

linalool glycoside accumulated in the transgenic plants (Lucker et al., 2001), which can happen naturally in other flowers (Watanabe et al., 1993). Introduction of a similar *LIS* construct into carnation, on the other hand, led to the emission of linalool from petals as well as from leaves (Lavy, 2001). Interestingly, transgenic carnation petals also emitted the linalool derivatives cis- and trans-linalool oxide. Although linalool and its derivatives represented almost 10% of the total volatiles emitted by the petals, no olfactorily detectable change in flower scent was observed by human subjects (Lavy, 2001). Note, however, that no experiments with these transgenic plants and insect pollinators have as yet been carried out. These two studies exemplify additional problems that can be encountered in the genetic engineering of flower fragrance: modification of the fragrance compound into a non-volatile form, e.g. glycosylation; masking by other volatiles; or the emitted amount being insufficient for olfactory detection by humans.

Metabolic engineering of odor in general and flower fragrance in particular is still in its infancy. The rapid development of analytical tools for metabolite profiling (e.g. http://www.phenomenome.com), allowing the simultaneous identification of thousands of compounds, together with ever-increasing genomic and EST databases, should be highly instrumental in deciphering the molecular nature of pathways leading to fragrance production. Using advanced metabolomics coupled with microarray techniques, it should be possible to screen for the genetic variation in fragrance production/emission with the aim of finding not only genes coding for new fragrance biosynthetic enzymes but also those regulating these pathways. Application of these high-throughput analyses to an array of plant species, including currently used model systems, should allow characterization of the unique genetic variability created in nature. Furthermore, the integration of proteomic tools into such studies, for example to design enzymes/substrates, opens up almost unlimited possibilities for the generation or manipulation of fragrance compounds.

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