Review

Metabolic engineering of aroma components in fruits

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Plants have the ability to produce a diversity of volatile metabolites, which attract pollinators and seed dispersers and strengthen plant defense responses. Selection by plant breeders of traits such as rapid growth and yield leads, in many cases, to the loss of flavor and aroma quality in crops. How the aroma can be improved without affecting other fruit attributes is a major unsolved issue. Significant advances in metabolic engineering directed at improving the set of volatiles that the fruits emit has been aided by the characterization of enzymes involved in the biosynthesis of flavor and aroma compounds in some fruits. However, before this technology can be successfully applied to modulate the production of volatiles in different crops, further basic research is needed on the mechanisms that lead to the production of these compounds in plants. Here we review the biosynthesis and function of volatile compounds in plants, and the attempts that have been made to manipulate fruit aroma biosynthesis by metabolic engineering. In addition, we discuss the possibilities that molecular breeding offers for aroma enhancement and the implications of the latest advances in biotechnological modification of fruit flavor and aroma.

Received 21 MAY 2013
Revised 16 JUL 2013
Accepted 30 JUL 2013

Keywords: Aroma and flavor biotechnology · Fruits · Metabolic engineering · Plant foods · Volatile organic compounds

1 Introduction

By manipulating plant metabolism, various technological approaches can improve a wide range of plant quality traits, such as their value as chemical feedstocks, their nutritional properties, and their defense against adverse conditions [1]. Controlling the production of plant volatiles is critical for breeders, as these compounds greatly influence pollination and fruit defense responses [2]. In edible fruits, the production of volatiles has an additional interest, as volatile emission is an indicator of quality, and favors consumer preference [3].

Modern cultivars have been continuously selected for rapid growth and enhanced yield. However, in most cases this selection negatively affects the production of secondary metabolites such as the volatile compounds emitted by plants. As a consequence, domesticated crops have lost part of their flavor and nutrient content [4], and are poorly adapted to maintain beneficial insects in agricultural systems [2].

Progress in the study of the pathways leading to the synthesis of volatile compounds, has led to the identification of numerous genes encoding biosynthetic enzymes. With this knowledge it is now technically feasible to use metabolic engineering to modify the biosynthetic pathways that produce these secondary metabolites. However, attempts to modify endogenous biosynthetic pathways to increase the flux towards a particular compound have shown not only that pathway control occurs at multiple levels, but also that the perturbation at any of these levels frequently leads to wide-ranging effects in the whole plant. On the other hand, it is possible to identify the genomic regions associated to the content of flavor and aroma compounds using linkage maps and quantitative trait loci (QTL) analyses. Therefore, using these tools for plant breeding is an important alternative to metabolic engineering.

Here we review the approaches used to modify the production of fruit volatile compounds, with a focus on the

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Abbreviations: ADH, alcohol dehydrogenase; **CCD**, carotenoid cleavage dioxygenase; **GDP**, geranyl diphosphate; **LOX**, lipoxygenase; **OMT**, O-methyltransferase; **QTL**, quantitative trait loci; **TPS**, terpene synthase

potential biotechnological benefit of improving fruit aroma. We also examine the limitations and problems associated to the use of metabolic engineering in biotechnological purposes, as well as the future prospects in this field.

2 Biosynthesis of volatile secondary metabolites in plants

The synthesis of plant volatile compounds starts from primary metabolism routes, and these compounds can be grouped into several classes on the basis of their biogenetic origin. These classes include terpenes, fatty acid derivatives, amino acid derivatives, and carbohydrate-derived aroma compounds (Fig. 1). Selected aroma volatiles of these four groups are shown in Table 1, with an indication of the fruits in which they are major contributors to the aroma. How these compounds are biosynthesized is summarized in the following sections.

2.1 Terpenoids

The terpenoid biosynthetic pathway generates more than 40 000 compounds derived from the five-carbon isoprene building units isopentenyl diphosphate (IDP) and its isomer dimethylallyl diphosphate (DMADP) [5]. Terpenoids, also called isoprenoids, are formed through two independent pathways: the cytosolic mevalonate (MVA) pathway and the plastidic methylerythritol-4-phosphate (MEP) pathway [6]. The MVA pathway starts from acetyl CoA and gives rise to sesquiterpenes (C_{15}) and triterpenes (C_{30}) (including sterols). The MEP pathway starts with the condensation of pyruvate and D-glyceraldehyde-3-phosphate to form 1-deoxy-D-xylulose 5-phosphate, producing precursors for hemiterpenes (C_5), monoterpenes (C_{10}), and diterpenes (C_{20}) (reviewed in [2, 7, 8]) (Fig. 1).

IDP and DMADP are substrates for short-chain prenyl transferases, which in condensation reactions produce prenyl diphosphate precursors such as the monoterpene precursor geranyl diphosphate (GDP), the sesquiterpene precursor farnesyl diphosphate (FDP) and the diterpene and carotenoid precursor geranylgeranyl diphosphate (GGDP). After the formation of these precursors, terpenoid scaffolds are generated by the action of terpene synthases (TPSs) and cyclases [9, 10]. Volatile terpenoids derived from carotenoids are produced after the initial dioxygenase cleavage, catalyzed by carotenoid cleavage dioxygenases (CCDs), which is followed by enzymatic transformation and conversion to volatile compounds [11].

Although many terpene volatiles are direct products of terpene synthases, terpenoid diversity is further increased by the action of other enzymes that can modify the primary structures formed by TPSs via several reactions, including hydroxylation, dehydrogenation and acylation [12].

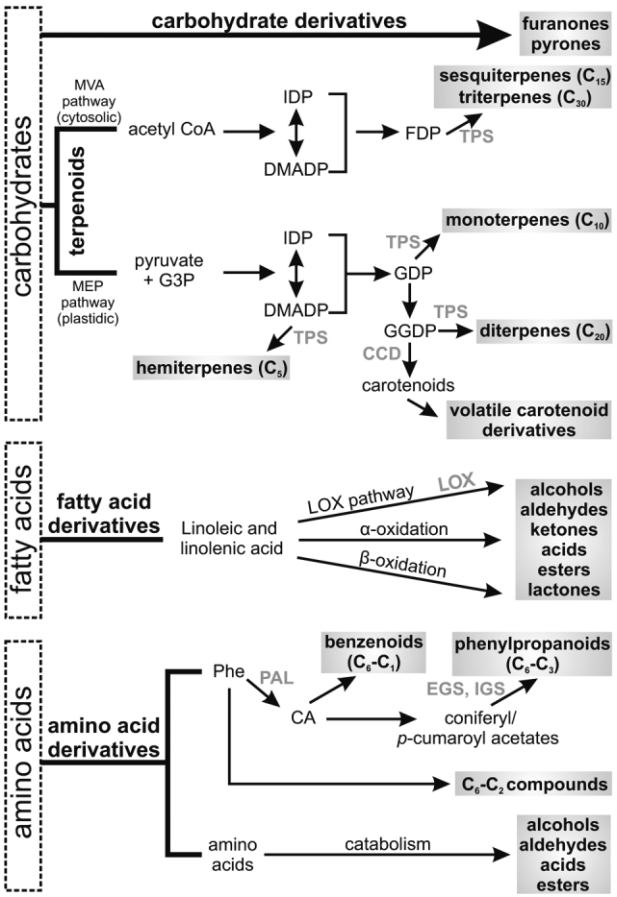

Figure 1. Pathways leading to the biosynthesis of volatile compounds in plants. A schematic overview of the biosynthesis of secondary metabolites (highlighted in gray boxes) is presented. Primary metabolism, which provides the substrates for secondary metabolism, is shown on the left (white boxes). In most cases, arrows indicate multiple enzymatic reactions. Selected enzymes are presented in gray: TPS, terpene synthase; CCD, carotenoid cleavage dioxygenase; LOX, lipoxygenase; PAL, L-phenylalanine ammonia lyase; EGS, eugenol synthase; IGS, isoeugenol synthase; MVA, mevalonate; MEP methylerythritol-4-phosphate; G3P, D-glyceraldehyde-3-phosphate; IDP, isopentenyl diphosphate; DMADP, dimethylallyl diphosphate; FDP, farnesyl diphosphate; GDP, geranyl diphosphate; GGDP, geranygeranyl diphosphate; Phe, L-phenylalanine; CA, *trans*-cinnamic acid.

2.2 Fatty acid derivatives

Volatile compounds derived from fatty acids, such as straight-chain alcohols, aldehydes, ketones, acids, esters and lactones are basically formed by three processes: the lipoxygenase (LOX), α-oxidation and β-oxidation pathways (Fig. 1). In the LOX pathway unsaturated linoleic or linolenic fatty acids are oxygenated, leading to 9-hydroperoxy and 13-hydroperoxy intermediates [13], which are further converted into volatile C_6 and C_9 aldehydes.

In plants, the fatty acid α-oxidation mechanism involves the enzymatic degradation of long-chain fatty

Table 1. Selected volatile compounds and fruits in which they are major contributors to the aroma

Volatile compound	Fruit	References
Terpenes		
R-limonene	Citrus fruit	[97, 98]
Valencene	Orange	[97]
Nerol	Grape	[99]
Nerolidol	Strawberry	[31]
S-Linalool	Strawberry, tomato, grape, peach, citrus fruit	[47, 71, 98, 99]
S-Citronellol	Citrus fruit, grape	[98, 100]
Geraniol	Grape	[99, 100]
Geranylacetone	Tomato	[47, 50]
Citral (neral + geranial)	Lemon, orange, lemon-scented herbs	[7, 12]
Geranic acid	Grape	[99]
4,5-Dihydrovomifoliol	Peach	[99]
α-Terpineol	Tomato, strawberry, citrus fruit	[31, 47, 98]
β-Ionone	Tomato	[47, 50]
3-Hydroxy-β-ionone	Peach, nectarine	[99, 101]
3-Hydroxy-5,6-epoxy-β-ionone	Peach	[99]
3-Hydroxy-5,6-epoxy-7,8-dihydro-β-ionone	Nectarine	[102]
α-Sinensal	Orange	[97]
β-Sinensal	Orange	[97]
Fatty acid derivatives		
1-*(E, Z)*-3,5-Undecatriene	Pineapple	[101]
2-Pentanol	Passion fruit	[103]
Hexanol	Strawberry, melon	[71, 99]
(Z)-3-Hexenol	Melon, tomato	[47, 99]
2-Heptanol	Strawberry, passion fruit	[71, 103]
3,7-Dimethyl-1,5-octadiene-3,7-diol	Nectarine	[102]
3-Nonen-1-ol	Watermelon	[104]
Hexanal	Tomato, peach, grape	[47, 99]
(E)-2-Hexenal	Cucumber, strawberry, apple, tomato, peach, nectarine, grape	[37, 47, 66, 71, 99, 102, 105]
(Z)-3-Hexenal	Tomato, apple	[37, 47, 99]
(E)-2-Nonenal	Cucumber, watermelon	[66, 105, 106]
(*Z*)-6-Nonenal	Watermelon	[104]
(E, Z)-2,6-Nonadienal	Cucumber, watermelon	[66, 105, 106]
(Z, Z)-3,6-Nonadienal	Cucumber	[66]
Hexanoic acid	Strawberry	[71, 99]
2-Heptyl butanoate	Passion fruit	[103]
Methyl hexanoate	Strawberry	[71, 99]
2-Heptyl hexanoate	Passion fruit	[103]
γ-Hexalactone	Strawberry, peach, nectarine	[71, 99, 102]
γ-Octalactone	Strawberry, peach	[71, 99]
γ-Decalactone	Peach	[99, 101]
γ-Jasmolactone	Peach	[99]
δ-Decalactone	Peach	[99, 101]
Ethyl (methylthio)-acetate	Melon	[39]
Ethyl 3-(methylthio)-propanoate	Melon	[39]
3-(Methylthio)-propyl acetate	Melon	[39, 99]
Ethyl 3-(methylthio)-propanoate	Melon	[39]
Amino acid derivatives		
Isoamyl alcohol	Tomato	[99]
2-Methylbutanol	Melon	[99]
3-Hydroxy-2-butanone	Melon	[99]
2-Methylbutanoic acid	Strawberry	[99]
Isoamyl acetate	Banana, melon	[107]

Table 1. (continued)

Volatile compound	Fruit	References
Amino acid derivatives		
2-Methylpropyl acetate	Melon	[99]
2-Methylbutyl acetate	Apple, melon	[37, 99]
3-Methylbutyl acetate	Banana	[104]
Butyl acetate	Apple, melon	[37, 99]
Hexyl acetate	Apple	[37]
Ethyl-2-methyl propanoate	Pineapple	[101]
Ethyl butanoate	Strawberry, melon	[99]
Methyl-2-methyl butanoate	Apple, pear, melon, pineapple	[99, 101, 108]
Ethyl-2-methyl butanoate	Melon, pineapple	[99, 101]
3-Methylbutyl butanoate	Banana	[104]
2-Methoxy-3-isobutylpyrazine	Grape, bell pepper	[109, 110]
1-Nitro-2-phenylethane	Tomato	[67]
1-Nitro-3-methylbutane	Tomato	[99]
Methyl salicylate	Tomato	[47, 99]
Benzyl alcohol	Melon	[99]
Benzyl acetate	Melon	[99]
Eugenol	Strawberry, tomato, peach	[44, 71, 73, 99]
Isoeugenol	Strawberry	[73]
3-Phenyl-1-propanol	Melon	[99]
2-Phenylacetaldehyde	Tomato, strawberry, grape	[22, 47, 99]
2-Phenylethanol	Tomato, strawberry, grape, melon	[22, 99]
Carbohydrate derivatives		
2,5-Dimethyl- 4-hydroxy-3 (2H)-furanone (furaneol)	Strawberry, pineapple	[25, 99, 101, 111]
2,5-Dimethyl- 4-methoxy-3(2H)-furanone (mesifurane)	Strawberry	[25, 71, 99, 111]
6-Pentyl α-pyrone	Peach	[99]

acids (C_{12}–C_{18}) to C_{n-1} aldehydes and CO_2 [14], while β-oxidation of a fatty acid results in successive removal of C_2 units (acetyl CoA) [15]. Alcohols and acyl CoAs are combined by the action of alcohol acyl-transferases, producing a diverse set of esters.

2.3 Amino acid derivatives

A major group of amino acid-derived flavor and aroma compounds are those synthesized from L-phenylalanine (Phe), e.g. benzenoids (C_6-C_1) and phenylpropanoids (C_6-C_3). The first step in the biosynthesis of these compounds is catalyzed by L-phenylalanine ammonia lyase (PAL), which deaminates Phe to *trans*-cinnamic acid (CA) [2] (Fig. 1). The formation of benzenoids requires shortening of the C_3 side chain of CA by two carbon units, possibly via either a CoA-dependent β-oxidative route and/or a CoA-independent non β-oxidative pathway [16, 17]. The latter finally produces free benzoic acid, a key component of different volatiles. Phenylpropenes such as eugenol, isoeugenol, chavicol and *t*-anol are biosynthesized from monolignol (phenylpropenol) acetates, e.g. coniferyl acetate or *p*-coumaryl acetate. Two of the enzymes involved in the biosynthesis of these compounds are eugenol synthase (EGS) and isoeugenol synthase (IGS) [18]. The action of *O*-methyltransferases (OMT) on phenylpropenes produces the corresponding methyl derivatives [19, 20]. Other Phe-derived aroma compounds, such as phenylacetaldehyde and 2-phenylethanol (C_6-C_2 compounds), are synthesized directly from Phe through different routes that compete with PAL for Phe utilization [21, 22] (Fig. 1).

On the other hand, catabolism of amino acids is initiated by aminotransferases and leads to the formation of α-keto acids, which can serve as substrates for decarboxylation reactions followed by reductions, oxidations and/ or esterifications. Thus, catabolism is responsible for the synthesis of several aldehydes, acids, alcohols and esters [23] (Fig. 1).

2.4 Carbohydrate derivatives

Only a few natural volatile compounds, such as furanones and pyrones (Fig. 1), are directly derived from hexoses and pentoses without prior breakdown of the carbon skeleton [24]. Although their biosynthetic pathway has not been completely elucidated in most cases, in strawberries (*Fragaria* × *ananassa*) and tomatoes (*Solanum lycopersicum*)

D-fructose-1,6-diphosphate is presumablly converted by an unknown enzyme into 4-hydroxy-5-methyl-2-methylene-3(2H)-furanone. This compound serves as a substrate for an oxidoreductase that catalyzes the biosynthesis of 4-hydroxy-2,5-dimethyl-3(2H)-furanone (furaneol) [25–27]. In strawberries, furaneol is further metabolized to 4-methoxy-2,5-dimethyl-3(2H)-furanone (mesifurane) by an *O*-methyltransferase [28].

3 Roles of volatile compounds emitted by plant tissues

Flowers are the plant organs that emit the highest and most diverse mixtures of volatiles. These compounds attract and guide pollinators, to ensure plant reproductive success. They have antimicrobial, antifungal or antiherbivore effect, and can therefore protect reproductive organs from enemies [2].

In fruits, although a few flavor components are detected throughout development, most of the volatile compounds are produced mainly at the ripe stage. The fact that these volatiles are specifically synthesized in ripe fruits, and are absent in vegetative tissues and immature fruits, indicates the role of the emitted compounds as a signal of ripeness and, therefore, as attractants for different seed-dispersing organisms, including humans [3]. Several compounds that are abundant in a number of ripe fruits have a functional role in attracting animals to the fruit, and thus in ensuring seed dispersal [2, 7]. Immature fruits also produce volatiles, but in this case their role is mainly to protect the growing organ against pathogens and predators (Aragüez et al., unpublished results and [29]).

4 Engineering fruit flavor and aroma components

Plant metabolic engineering has the potential to generate fruits that are not only more appealing to consumers, but also have additional health benefits, e.g. fruits significantly enriched in plant nutrients to improve diets. Enhancing the production of volatiles by plants is also valuable for the food industry since hundreds of tonnes of volatiles such as eugenol, limonene and various other C_6 compounds, and esters are used in making flavors [7]. In addition, metabolic engineering can help produce plants with increased defense responses, thus reducing the need for chemicals to control pest in agriculture.

Although the biochemistry of fruit flavor and aroma is still largely unexplored, over the last few years a growing number of reports have described genes involved in fruit volatile biosynthesis. Many of these genes encode enzymes that directly catalyze the formation of volatile terpenoids, phenylpropanoids or furanones. These include: a sesquiterpene synthase involved in the formation of valencene in citrus (*Citrus sinensis*) fruits [30]; a cytosolic TPS of cultivated strawberry (*F.* × *ananassa*) fruits, which produces roughly similar amounts of the sesquiterpene nerolidol and the monoterpene *S*-linalool [31]; a methylketone synthase from wild tomato (*Lycopersicum hirsutum*) [32]; a family of different genes involved in the formation of furanones in strawberry (*F.* × *ananassa*) [25–28]; tomato (*S. lycopersicum*) phenylacetaldehyde reductases that catalize the synthesis of 2-phenylethanol [22]; several alcohol acyltransferases [33–43]; CCDs controlling carotenoid and norisoprenoid volatile metabolism in peach (*Prunus persica*) [44]; a catechol-OMT that produces guaiacol in tomato [45]; and TPSs that account for the production of apple (*Malus* × *domestica*) volatile terpenes [46]. Isolation of the corresponding genes not only opens the possibility for an in-depth study of the regulation of the biosynthesis of aroma compounds, but also provides a key tool for metabolic engineering of flavor in the fruit of interest. Lately, the genome-wide sequencing of an increased number of plant species has been completed, offering the opportunity to identify other potential genes involved in volatiles production, and, therefore, increasing the possibilities for metabolic engineering to successfully improve fruit aroma.

Based on current knowledge of the biosynthesis of volatiles, several attempts have already been made to modify the blend of volatiles emitted by a fruit. The various strategies that have been followed, summarized in Table 2, are described below.

4.1 Metabolic engineering of terpenoid content in fruits

Numerous experiments have focused on improving the flavor and aroma of tomatoes (*S. lycopersicum*) using metabolic engineering methodologies. The monoterpene *S*-linalool is one of the most important compounds determining tomato aroma [47] and it is also present in many other edible fruits (Table 1). The first successful attempt to increase the concentration of *S*-linalool by genetic engineering [48] demonstrated that the expression of a *S-linalool synthase* from *C. breweri* (*CbLIS*) under the control of a late-ripening-specific promoter led to the accumulation of *S*-linalool and 8-hydroxylinalool in ripening fruit. Also, an *S-linalool/(3S)-E-nerolidol synthase* from strawberry (*F.* × *ananassa*), *FaNES1*, was constitutively overexpressed in potato (*S. tuberosum*) and *Arabidopsis thaliana*. Transformed plants produced and emitted high levels of *S*-linalool, and presented induced resistance against aphids. However, undesired effects also occurred: lines with strong transgene expression showed retarded growth and the formation of high amounts of non-volatile glycosylated derivatives [49].

Later, it was shown that tomato fruits that constitutively silenced CCDs *SlCCD1A* and *SlCCD1B* presented a

Table 2. Metabolic engineering approaches to manipulate the biosynthesis of volatile compounds in fruits

Gene source	Gene	Promoter	Up-/downregulation	Engineered fruit	Effects	Reference
C. breweri	*CbLIS*	SlE8 (late-ripening-specific)	Overexpression	Tomato (*S. lycopersicum*)	Accumulation of *S*-linalool and 8-hydroxylinalool in ripening fruit	[48]
Strawberry (*F.* × *ananassa*)	*FaNES1*	CaMV 35S (constitutive)	Overexpression	Potato (*S. tuberosum*), *Arabidopsis thaliana*	Increased emission of *S*-linalool, increased resistance against aphids, retarded growth, increased production of glycosilated non-volatile derivatives	[49]
Tomato (*S. lycopersicum*)	*SlCCD1A* *SlCCD1B*	FMV 35S (constitutive)	Silencing (antisense)	Tomato (*S. lycopersicum*)	Decreased production of β-ionone and geranyl acetone. No significant modifications in carotenoid content	[50]
Basil (*O. basilicum*)	*ObGES*	SlPG (ripening-specific)	Overexpression	Tomato (*S. lycopersicum*)	Production of geraniol, nerol, citronellol, citronellic acid, geranic acid, neric acid and other monoterpenes. Loss-of-color phenotype	[51]
Basil (*O. basilicum*)	*ObZIS*	SlPG (ripening-specific)	Overexpression	Tomato (*S. lycopersicum*)	High levels of α-zingiberene and other sesquiterpenes. Loss-of-color phenotype	[52]
Snapdragon (*A. majus*)	*AmGDPS-SSU*	SlPG (ripening-specific)	Overexpression	Tomato (*S. lycopersicum*)	Production of monoterpenes such as geraniol, geranial, neral, citronellol and citronellal. Loss-of-color phenotype	[53]
Snapdragon (*A. majus*)/ Basil (*O. basilicum*)	*AmGDPS-SSU/ ObGES*	SlPG (ripening-specific)	Overexpression	Tomato (*S. lycopersicum*)	Increased monoterpene formation	[53]
Snapdragon (*A. majus*)/ Basil (*O. basilicum*)	*AmGDPS-SSU/ ObZIS*	SlPG (ripening-specific)	Overexpression	Tomato (*S. lycopersicum*)	Increased levels of *ZIS*-derived monoterpenes compared to fruits expressing *ZIS* alone	[53]
Wild tomato (*S. habrochaites*)	*ShzFDPS/ ShZIS*	ShMKS1/ SlMTS1 (trichome-specific)	Overexpression	Tomato (*S. lycopersicum*)	Accumulation of 7-epizingiberene, increased resistance to herbivores that are pests of tomato	[54]
Satsuma mandarin (*C. unshiu*)	*CitMTSE1*	CaMV 35S (constitutive)	Silencing (antisense)	Orange (*C. sinensis*)	Reduced accumulation of limonene in the peel, increased resistance against important fungal and bacterial citrus pathogens	[55]
Rice (*O. sativa*)	*OsTPS3*	CaMV 35S (constitutive)	Overexpression	*Rice* (*O. sativa*)	Increased production of *(E)*-β-caryophyllene, increased resistance against herbivorous insects	[56]
Oregano (*O. vulgare*)	*OvEβCS*	ZmUbi (constitutive)	Overexpression	Maize (*Z. mays*)	Increased production of *(E)*-β-caryophyllene, increased resistance to insect pests	[57]
Tomato (*S. lycopersicum*)	*SlADH2*	CaMV 35S (constitutive)/SlPG (ripening-specific)	Overexpression	Tomato (*S. lycopersicum*)	Modified levels of short chain aldehydes and alcohols	[58, 59]

Table 2. (continued)

Gene source	Gene	Promoter	Up-/downregulation	Engineered fruit	Effects	Reference
Tomato (*S. lycopersicum*)	*SlscADH1*	CaMV 35S (constitutive)	Silencing (RNAi)	Tomato (*S. lycopersicum*)	Dwarf phenotype	[60]
Tomato (*S. lycopersicum*)	*SlscADH1*	Sl2A11 (fruit-specific)	Silencing (RNAi)	Tomato (*S. lycopersicum*)	Balance of aldehyde/alcohol volatiles was not altered, no morphogenetic effects, increased production of C_5 and C_6 volatile compounds of the lipoxygenase pathway	[60]
Grapevine (*V. vinifera*)	*VvADH2*	CaMV 35S (constitutive)	Overexpression	Grapevine (*V. vinifera*)	Reduction in benzyl alcohol and 2-phenylethanol in mature berries. No modifications in the levels of other free or glycosidically-bound volatiles	[61]
Grapevine (*V. vinifera*)	*VvADH2*	CaMV 35S (constitutive)	Silencing (antisense)	Grapevine (*V. vinifera*)	No modifications in the levels of free or glycosidically bound volatiles	[61]
Tomato (*S. lycopersicum*)	*TomloxC*	CaMV 35S (constitutive)	Silencing (antisense or overexpression that led to co-supression)	Tomato (*S. lycopersicum*)	Reduced levels of hexanal, *(Z)*-3-hexenal, *(Z)*-3-hexenol	[62]
Potato (*S. tuberosum*)	*StLOX H1*	CaMV 35S (constitutive)	Silencing (overexpression that led to co-supression)	Potato (*S. tuberosum*)	Decreased production of volatile aliphatic C_6 aldehydes	[63]
Yeast (*S.cerevisiae*)	*Scδ9-desaturase*	CaMV 35S (constitutive)	Overexpression	Tomato (*S. lycopersicum*)	Modified oxidation pattern of lipids, 2- to 3-fold increase of some short-chain alcohols and aldehydes, increased production of flavor compounds derived from fatty acids such as *(Z)*-3-hexenol, 1-hexanol, hexanal, and *(Z)*-3-hexenal	[64]
Canola (*B. napus*)	*BnFAD3*	CaMV 35S (constitutive)	Overexpression	Tomato (*S. lycopersicum*)	Increased linolenic/linoleic acid ratio. Increased *(Z)*-3-hexenal/ hexanal ratio. Increased chilling tolerance	[65]
Potato (*S. tuberosum*)	*StFAD7*	CaMV 35S (constitutive)	Overexpression	Tomato (*S. lycopersicum*)	Increased linolenic/linoleic acid ratio. Increased *(Z)*-3-hexenal/ hexanal ratio. Increased chilling tolerance	[65]
Thaumato-coccus daniellii	*Prepro-thaumatin II*	CaMV 35S (constitutive)	Overexpression	Cucumber (*C. sativus*)	Higher sensory perception of fruit flavor, increased levels of *(E, Z)*-2,6 nonadienal and other volatiles	[66]
Tomato (*S. lycopersicum*)	*SlAADC1A SlAADC1B*	FMV 35S (constitutive)	Overexpression	Tomato (*S. lycopersicum*)	Increased levels of 2-phenylacetaldehyde, 2-phenylethanol and 1-nitro-2-phenylethane	[67]
Tomato (*S. lycopersicum*)	*SlAADC1B*	FMV 35S (constitutive)	Silencing (antisense)	Tomato (*S. lycopersicum*)	Decreased levels of 2-phenylacetaldehyde, 2-phenylethanol and 1-nitro-2-phenylethane	[67]
Petunia (*P. × hybrida*)	*Ph ODORANT1*	SlE8 (late-ripening-specific)	Overexpression	Tomato (*S. lycopersicum*)	Enhanced flux through part of the phenylpropanoid pathway, but no increase of Phe-derived flavor and aroma compounds	[68]
Tomato (*S. lycopersicum*)	*SlSAMT1*	FMV 35S (constitutive)	Overexpression	Tomato (*S. lycopersicum*)	Increased production of methyl salicylate, delayed symptom development following infections	[69]

Table 2. (continued)

Gene source	Gene	Promoter	Up-/downregulation	Engineered fruit	Effects	Reference
Tomato (*S. lycopersicum*)	*SlCXE1-5*	CaMV 35S (constitutive)	Silencing (RNAi)	Tomato (*S. lycopersicum*)	Increased production of acetate esters	[70]
Strawberry (*F.* × *ananassa*)/ Basil (*O. basilicum*)	*FaCHS*/ *ObEGS1*	CaMV 35S (constitutive)/ CaMV 35S (constitutive)	Silencing (antisense)/ Overexpression	Strawberry (*F.* × *ananassa*)	Increased availability of precursors of the phenylpropanoid pathway, increased production of eugenol, isoeugenol, chavicol, methoxyeugenol	[73]
Strawberry (*F.* × *ananassa*)/ Petunia (*P.* × *hybrida*)	*FaCHS*/ *PhIGS1*	CaMV 35S (constitutive)/ CaMV 35S (constitutive)	Silencing (antisense)/ Overexpression	Strawberry (*F.* × *ananassa*)	Increased availability of precursors of the phenylpropanoid pathway, increased production of eugenol, isoeugenol, *t*-anol, methoxyeugenol	[73]
Strawberry (*F.* × *ananassa*)/ Petunia (*P.* × *hybrida*)	*FaOMT*	CaMV 35S (constitutive)	Silencing (antisense)	Strawberry (*F.* × *ananassa*)	Near total loss of mesifurane	[74]
Apple (*M.* × *domestica*)	*MdACS*	CaMV 35S (constitutive)	Silencing (antisense)	Apple (*M.* × *domestica*)	Decreased ethylene production, firmer fruits, increased shelf-life, dramatic decrease in ester volatiles biosynthesis	[75]
Apple (*M.* × *domestica*)	*MdACO*	CaMV 35S (constitutive)	Silencing (antisense)	Apple (*M.* × *domestica*)	Decreased ethylene production, firmer fruits, increased shelf-life, dramatic decrease in ester volatiles biosynthesis	[75]
Apple (*M.* × *domestica*)	*MdACO1*	CaMV 35S (constitutive)	Silencing (antisense)	Apple (*M.* × *domestica*)	Decreased ethylene production, increased shelf-life, decrease in ester volatiles biosynthesis	[76]
Kiwifruit (*A. chinensis*)	*AcACO1*	CaMV 35S (constitutive)	Silencing (RNAi)	Kiwifruit (*A. chinensis*)	Decreased ethylene production, increased shelf-life, dramatic decrease in ester volatiles biosynthesis	[77]

CbLIS, *C. breweri S-linalool synthase*; *FaNES1*, *F.* × *ananassa S-linalool/(3S)-E-nerolidol synthase*; *SlCCD1A* and *SlCCD1B*, *S. lycopersicum carotenoid cleavage dioxygenase 1A* and *1B*; *ObGES*, *O. basilicum geraniol synthase*; *ObZIS*, *O. basilicum α-zingiberene synthase*; *AmGDPS-SSU*, *A. majus small subunit of geraniol diphosphate synthase*; *ShzFDPS*, *S. habrochaites Z-Z farnesyl diphosphate synthase*; *ShZIS*, *S. habrochaites 7-epizingiberene synthase*; *CitMTSE1*, *C. unshiu limonene synthase 1* (*monoterpene synthase E1*); *OsTPS3*, *O. sativa (E)-β-caryophyllene synthase*; *OvEβCS*, *O. vulgare (E)-β-caryophyllene synthase*; *SlADH2*, *S. lycopersicum alcohol dehydrogenase 2*; *SlscADH1*, *S. lycopersicum short chain alcohol dehydrogenase 1*; *VvADH2*, *V. vinifera alcohol dehydrogenase 2*; *TomloxC*, *S. lycopersicum lipoxygenase C*; *StLOX H1 S. lycopersicum lipoxygenase H1*; *ScΔ9-desaturase*, *S. cerevisiae Δ9-desaturase*; *BnFAD3*, *B. napus ω-3 fatty acid desaturase 3*; *StFAD7*, *S. tuberosum ω-3 fatty acid desaturase 7*; *SlAADC1A* and *SlAADC1B*, *S. lycopersicum amino acid decarboxylase 1A* and *1B*; *ODORANT 1* is a *P.* × *hybrida MYB transcription factor*; *SlSAMT1*, *S. lycopersicum S-adenosyl-L-methionine: salicylic acid carboxylmethyltransferase*; *SlCXE1-5*, *S. lycopersicum carboxylesterase 1-5*; *FaCHS*, *F.* × *ananassa chalcone synthase*; *ObEGS1*, *O. basilicum eugenol synthase 1*; *PhIGS1*, *P.* × *hybrida isoeugenol synthase 1*; *FaOMT*, *F.* × *ananassa O-methyl transferase*; *MdACS*, *M.* × *domestica 1-aminocyclopropane-1-carboxylic acid synthase*; *MdACO*, *M.* × *domestica 1-aminocyclopropane-1-carboxylic acid oxidase*; *AcACO1*, *A. chinensis 1-aminocyclopropane-1-carboxylic acid oxidase 1*; SlE8, *S. lycopersicum* E8 promoter; SlPG, *S. lycopersicum* polygalacturonase promoter; CaMV 35S, cauliflower moisaic virus 35S promoter; FMV 35S, figwort mosaic virus 35S promoter; ShMKS1, *S. habrochaites* methyl ketone synthase promoter; SlMTS1, *S. lycopersicum* monoterpene synthase promoter; ZmUbi, *Z. mays* ubiquitin promoter; Sl2a11, *S. lycopersicum* 2A11 promoter.

decreased production of the volatiles β-ionone (a β-carotene-derived C_{13} cyclohexone) and geranylacetone (a C_{13} acyclic product likely derived from lycopene), although no significant differences in carotenoid content were found [50]. These results point to the possibility of manipulating the flavor and aroma profiles of tomato fruit through genetic manipulation of *SlCCD* genes.

In another study, the expression of a *geraniol synthase* (*GES*) from basil (*O. basilicum*) in tomato, under the control of a ripening-specific promoter, was responsible for the biosynthesis of the monoterpenes geraniol, nerol, citronellol, citronellic acid, geranic and neric acid, which were absent in control fruits [51]. In addition, the amount of other monoterpenes was increased in comparison with control fruits; all these modifications caused a general improvement of the flavor and aroma of the fruits. However, these transgenic tomatoes failed to develop the deep red color of control fruits. This was probably because the high levels of *GES* expression caused a marked depletion of the GDP pool available for lycopene and phytoene biosynthesis. A similar loss-of-color phenotype was observed when *α-zingiberene synthase* (*ZIS*) from *O. basilicum* (*ObZIS*) and the small subunit (SSU) of a snapdragon (*Antirrhinum majus*) *GDP synthase* (*AmGDPS-SSU*) were overexpressed in tomato fruits under the same ripening-specific promoter [52, 53]. Since ZIS catalyzes the formation of α-zingiberene and other sesquiterpenes from FDP, fruits that overexpressed *ObZIS* displayed high levels of these compounds. α-Zingiberene and the other sesquiterpenes were not detected, or were found only in minimal concentrations, in control fruits [52]. Fruits that overexpressed *AmGDPS-SSU* produced monoterpenes such as geraniol, geranial, neral, citronellol and citronellal, which were absent in control fruits [53]. The co-expression of *AmGDPS-SSU* and *ObGES* increased monoterpene formation more than threefold in comparison to the line that only expressed *ObGES*, thus indicating that the GDP produced can be used by plastidic monoterpene synthases (MTSs). The co-expression of *AmGDPS-SSU* with *ObZIS*, shown to possess both sesqui- and MTS activities, resulted in increased levels of *ZIS*-derived monoterpenes compared to fruits expressing *ObZIS* alone [53]. Interestingly, humans were able to perceive changes in fruit aroma caused by these genetic modifications. However, the possible effects of these genetic alterations in plant defense responses have not yet been evaluated.

In another study, wild tomato (*S. habrochaites*) *Z-Z farnesyl diphosphate synthase* (*ShzFDPS*) and *7-epizingiberene synthase* (*ShZIS*), which encodes the enzyme that synthesizes 7-epizingiberene (an estereoisomer of α-zingiberene), were expressed in cultivated tomato under the control of trichome-specific promoters. The transgenic lines containing both genes accumulated 7-epizingiberene, and the fruits gained resistance to several herbivores that are pests of tomato [54].

In citrus, the antisense downregulation of a *limonene synthase* gene from satsuma mandarin (*C. unshiu*), *CitMTSE1*, produced a reduced accumulation of limonene, a compound important for fruit aroma in the peel (Table 1). These fruits showed a marked resistance against significant fungal and bacterial citrus pathogens, and repelled a major citrus insect pest [55]. This is another example of the side effects, sometimes beneficial, that can be obtained from manipulation of volatile production in a fruit.

Terpenoid genetic engineering has also been applied to other crops. Constitutive overexpression of *(E)-β-caryophyllene synthases* from rice (*Oryza sativa*) and oregano (*Origanum vulgare*), *OsTPS3* and *OvEβCS*, in rice and maize (*Zea mays*), respectively, resulted in constitutive emissions of this sesquiterpene and improved plant defense responses [56, 57].

4.2 Metabolic engineering of fruit volatiles derived from fatty acids

The overexpression of a non-specific tomato *alcohol dehydrogenase* (*SlADH2*) gene in tomato fruit was shown to modify the levels of short-chain aldehydes and alcohols [58, 59]. On the other hand, when a tomato *short-chain ADH* (*SlscADH1*) was constitutively silenced by RNA interference (RNAi), dwarf plants were obtained. Nevertheless, when this gene was silenced specifically in fruit, there were no morphogenetic effects and the balance of aldehyde/alcohol volatiles was not altered. However, these fruits accumulated higher concentrations of C_5 and C_6 volatile compounds of the LOX pathway [60]. In contrast, when a grapevine (*Vitis vinifera*) *ADH* (*VvADH2*) was overexpressed and silenced in grape berries, the content of free volatile compounds or bound volatiles was not affected in general. The only exception was for benzyl alcohol and 2-phenylethanol, whose levels were significantly lower in overexpressing lines compared to control and silencing lines [61].

In tomato, the increase of the levels of *(Z)*-3-hexenal, which provide the highly valued fresh notes in different fruits (Table 1), has been attempted using different approaches. Apart from overexpressing *ADHs* [58, 59], one strategy was to increase the LOX activity specifically involved in the generation of short-chain aldehyde precursors. Nonetheless, in tomato the constitutive overexpression of *TomloxC* led to co-supression and consequently produced a marked reduction in the levels of hexanal, *(Z)*-3-hexenal and *(Z)*-3-hexenol [62]. Similarly, the constitutive overexpression of a potato (*S. tuberosum*) *LOX* (*StLOX H1*) in potato led to co-supression of this gene, and consequently to a decrease in the production of volatile aliphatic C_6 aldehydes [63]. A different approach was the alteration of the balance between *(Z)*-3-hexenal and hexanal by increasing the amount of fatty acid desaturases, which catalyze the conversion of linoleic acid to

linolenic acid, the precursor of *(Z)*-3-hexenal. It was reported that the constitutive overexpression of a yeast (*Saccharomyces cerevisiae*) *Δ9-desaturase* in tomato produced a modified oxidation pattern of lipids, with the result that the concentration of some short-chain alcohols and aldehydes increased two- to threefold. An increase in the production of certain flavor compounds derived from fatty acids, most notably *(Z)*-3-hexenol, 1-hexanol, hexanal, and *(Z)*-3-hexenal was also observed [64]. The overexpression of *ω-3 fatty acid desaturases* from *Brassica napus* and potato (*BnFAD3 and StFAD7*) produced an increase in linolenic/linoleic acid ratio in tomato leaves and fruits. In addition, the transgenic lines presented an increase of the *(Z)*-3-hexenal/hexanal ratio, and were more tolerant to chilling [65].

Thaumatins are monomeric alkaline sweet proteins that are of special interest as they taste sweet to humans. In cucumber (*Cucumis sativus*), the concentration of the volatile that mainly contributes to its aroma, *(E, Z)*-2,6 nonadienal (Table 1), was increased by overexpressing *preprothaumatin II* (thaumatin II is initially translated as preprothaumatin with peptides in C terminus that are cleaved off when the protein matures). Transgenic fruits presented a higher sensory perception of fruit flavor and increased levels of this aldehyde and other volatiles [66].

4.3 Metabolic engineering of amino acid-derived volatiles in fruits

The biosynthesis of amino acid-derived volatiles has also been engineered in tomato. Constitutive overexpression of *amino acid decarboxylases SlAADC1A* and *SlAADC1B*, responsible for direct decarboxylation of Phe, led to the production of fruits with up to tenfold increased levels of 2-phenylacetaldehyde, 2-phenylethanol and 1-nitro-2-phenylethane, while antisense reduction of *SlAADC1B* significantly reduced the levels of these volatiles [67]. Unfortunately, the modification of Phe-derived metabolic pathways in tomato has not always led to variations in volatile compounds: the ectopic expression of *PhODORANT1* MYB transcription factor from petunia (*P.* × *hybrida*) in cultivated tomato, under the control of a late-ripening-specific promoter, produced large increases in the levels of a specific subset of phenylpropanoid compounds, but there was no increase in the accumulation of Phe-derived aroma volatiles [68].

The levels of methyl salicylate, another important volatile derived from Phe that contributes to tomato flavor (Table 1), have been engineered in this fruit. *S-Adenosyl-L-methionine:salicylic acid carboxymethyltransferase* (*SlSAMT1*), which encodes for the protein that catalyzes the reaction of salicylic acid and the methyl donor *S*-adenosyl-L-methionine to form methyl salicylate, was constitutively overexpressed in tomato [69]. Transgenic fruits presented increased production of methyl salicylate, and also delayed symptoms after infection with a disease-causing bacterial pathogen, due to the defense role played by this compound in plants.

Recently, it was shown that the constitutive RNAi silencing of the tomato carboxylesterase *SlCXE1,* which also caused a reduction in the transcripts of the highly homologous tandem genes *SlCXE2–SlCXE5,* was responsible for an increased production of acetate esters in the fruit [70]. Interestingly, red-fruited tomato species accumulate relatively low amounts of acetate esters compared to green-fruited species. This difference is associated with the insertion of a retrotransposon adjacent to *SlCXE1* in red tomato, responsible for an increased expression of the esterase and, therefore, a reduction in the content of esters in the fruit. These results demonstrate that major differences in the volatile profile of closely related species may be caused by the alteration of a volatile-associated catabolic activity.

Strawberry (*F.* × *ananassa*) is another fruit for which attempts to modify its aroma profile have been pursued. Wild strawberry (*F. vesca*) fruit contains several important phenylpropenes, which are virtually absent in cultivated varieties [71]. One attempt to restore the wild strawberry flavor in cultivated varieties through metabolic engineering sought to increase the eugenol contribution to the flavor of the fruit by modifying the phenylpropanoid pathway. Here, chalcone synthase (FaCHS), a key enzyme for the branch pathway leading to anthocyanin biosynthesis, was downregulated with the consequent increase in precursors for the phenylpropanoid pathway [72]. Simultaneously, a basil (*O. basilicum*) *eugenol synthase* (*ObEGS*) or a petunia (*P.* × *hybrida*) *isoeugenol synthase* (*PhIGS*) were overexpressed, thereby diverting the flow of hydroxycinnamyl derivatives to phenylpropene biosynthesis. As a result, the formation of eugenol, isoeugenol, and the related compounds chavicol, *t*-anol and methoxyeugenol, was increased to concentrations that were orders of magnitude greater that their odor thresholds [73]. It has been reported that the up- and downregulation in strawberry of a strawberry *O-methyltransferase* (*FaOMT*) involved in the production of mesifurane [28], produces variations in phenylpropanoid and furanone metabolism [74]. In transformed fruit, reduced expression of *FaOMT* caused a nearly total loss of mesifurane, a key compound contributing to strawberry aroma (Table 1), whereas the levels of other volatiles remained unchanged.

The set of volatile compounds that fruits emit has also been modified by engineering genes that are not directly linked to their biosynthesis, indicating that aroma production might be influenced by several factors. For instance, in apple (*M.* × *domestica*), silencing of either *MdACS* (*ACC synthase*; *ACC*: *1-aminocyclopropane-1-carboxylic acid*) or *MdACO* (*ACC oxidase*), which encode key enzymes for ethylene biosynthesis, produced not only reduced autocatalytic ethylene production, but also a dramatic suppression of the synthesis of volatile esters. In addition, the fruits of these engineered lines

were firmer than control fruits, and displayed an increased shelf life [75]. The microarray expression analysis of a transgenic apple line in which *MdACO1* was silenced led to the identification of 17 candidate genes that are likely to be ethylene control points for aroma production in apple [76]. Fruits of this line and fruits of a kiwifruit (*Actinidia chinensis*) line in which the *AcACO1* gene was suppressed [77], presented a phenotype analogous to that described previously in apple [75].

Recently, one of the routes for the incorporation of L-methionine into sulfur-containing volatile compounds was shown to occur through L-methionine γ-lyase. Co-segregation analyses of recombinant inbred lines have shown that the expression of a melon (*Cucumis melo*) *L-methionine γ-lyase* gene (*CmMGL*) is associated with the presence of the most common sulfur volatiles in the fruit (Table 1), possibly derived by the action of the corresponding enzyme [78]. Although the function of this enzyme has been only assessed in bacteria, these results may be the basis of future attempts to modify the formation of sulfur-containing aroma volatiles in fruit.

5 Improving fruit flavor and aroma by molecular breeding

Molecular markers and marker-assisted selection have a huge potential in plant breeding because DNA markers linked to a desired trait can be used in breeding programs [79]. Populations of plants that contain variations in their volatiles are crucial tools and present the opportunity for improving their aroma by classical breeding. The generation of linkage maps and the analysis of QTLs can lead to the identification of *loci* associated to the biosynthesis of volatile compounds. The approach of linking QTLs and associated molecular markers to the content of important flavor volatiles has been followed for different fruits.

Tomato (*S. lycopersicum*) is by far the most studied fruit, and a number of QTLs associated to the production of several volatiles were identified in an intraspecific cross between a cherry tomato line with good overall aroma intensity and an inbred line with medium flavor but bigger fruits [80–82]. In this cross, major QTLs (R^2 >30%) were detected for fruit weight, diameter, color, texture and different aroma volatiles. Subsequently, using tomato lines resulting from crosses between a cherry tomato and three independent large fruit cultivars (Levovil, VilB, and VilD), a number of QTLs that determined the levels of primary metabolites and/or volatile organic components were defined [83]. In addition, several QTLs associated to the production of volatiles were identified in introgression lines generated in crosses with wild relatives such as *S. pennellii* [69, 84, 85] and *S. habrochaites* [86]. Alleles of some of these QTLs are likely to be important flavor- and aroma-associated markers that could eventually lead to improved fruit flavor and aroma.

The analysis of QTLs associated to aroma has also been made in apple (*M.* × *domestica*) [87–89], and a functional relationship between an *alcohol acyl-transferase* gene and the production of the key apple esters hexyl acetate, butyl acetate and 2-methylbuthyl acetate (Table 1), was observed [89].

In grapevine (*V. vinifera*), QTLs associated to the production of *S*-linalool, nerol and geraniol were identified [90]. In addition, it was shown that a *1-deoxy-D-xylulose 5-phosphate synthase* gene and a *deoxy-D-xylulose synthase* gene co-localize with major QTLs affecting monoterpene and terpenol content, respectively [91, 92]. Recently, a QTL approach was used to investigate the genetic basis of the biosynthesis of 2-methoxy-3-isobutylpyrazine, the major methoxypyrazine in grape berries (Table 1), and as a result two previously uncharacterized *S-adenosylmethionine-dependent O-methyltransferase* genes were identified [93].

In strawberry (*F.* × *ananassa*), a mapping population resulting from a cross between two parental lines that differed, among other traits, in fruit flavor and aroma [94], led to the identification of many flavor- and aroma-associated QTLs. Effectively, 70 QTLs covering 48 different volatiles were detected, several of which were stable over time and mapped as major QTLs. *Loci* controlling γ-decalactone and mesifurane content were mapped as qualitative traits, and, interestingly, it was shown that one homeolog of the *FaOMT* gene is the *locus* responsible for the natural variation of mesifurane content in strawberry fruit [95]. Recently, a broad set of strawberry genotypes were tested, and it was shown that the allele *FaNES1* was not present in genotypes with lower ploidy, while it was found in all the wild octoploid materials studied, with the exception of three lines. Interestingly, two of these lines continued to produce linalool. [96]. The identification of alleles of genes that result in higher or lower levels of certain aroma compounds can be used in traditional breeding to alter fruit aroma. In the context of modern strawberry breeding, this robust marker, widespread in wild materials, provides an effective means to ensure the production of the beneficial compound linalool in breeding populations, especially as wild species are introgressed into breeding programs.

By choosing the QTLs with the highest effects on the most important flavor and aroma compounds, it will be possible to characterize the genes with the largest impact on consumer preferences. The results provided by QTL and linkage analyses can be used for marker-assisted selection to transfer pleasant flavor and aroma from aromatic lines to lines with poor volatile emission, but with other interesting agronomic properties. Given that breeders need efficient and easy-to-assess selection criteria for organoleptic quality improvement, molecular breeding is a promising alternative to metabolic engineering.

6 Conclusions and future prospects

Metabolic engineering of fruit secondary metabolites is a promising strategy for manipulating the amount of specific volatiles produced *in planta,* either to omit or to increase their emission, with the consequent improvement of fruit aroma. However, it should be noted that altering a single aroma compound may not lead to an overall improvement of the global fruit aroma or flavor, as it may be necessary to modify several compounds to produce more palatable fruits. In fact, over the last few years, the manipulation of multiple, instead of single, genes has been shown to produce better outcomes in attempts to improve plant quality traits [1].

Although successful results, covered in this review, have been obtained in engineering fruit aroma and flavor, the currently poor understanding of the regulation of the pathways leading to the synthesis of volatile compounds often restricts the manipulation of their emission. In addition, the sometimes-limited knowledge about the function of these compounds in plants has caused unexpected problems, e.g. a decreased plant growth or/and an altered developmental program. Hence, there are still many questions in this field that basic research must examine and solve.

Economic and social repercussions have to be carefully considered before making novel genetically modified fruit commercially available. As a valid alternative, breeders presently have more tools to incorporate new quality traits, such as improved volatile production, into new germplasm development. This is supported by the increasing number of plant genomes sequenced, and the availability of many flavor- and aroma-associated molecular markers that can be used to track candidate genes, or the most interesting alleles. The exploration of natural diversity and the identification of the most important volatiles contributing to specific fruit aromas will provide valuable information for achieving the goal of improving the aroma profile of specific fruits, with a direct benefit for end-consumers.

The authors were financially supported by the Grant BIO2010-15630 (MICINN, Spain). IA was recipient of a FPU fellowship (MICINN, Spain). We apologize to scientists whose work is not cited due to length limitations.

The authors declare no conflict of interest.

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