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REVIEW PAPER

The expanded tomato fruit volatile landscape

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Abstract

The present review aims to synthesize our present knowledge about the mechanisms implied in the biosynthesis of volatile compounds in the ripe tomato fruit, which have a key role in tomato flavour. The difficulties in identifiying not only genes or genomic regions but also individual target compounds for plant breeding are addressed. Ample variability in the levels of almost any volatile compound exists, not only in the populations derived from interspecific crosses but also in heirloom varieties and even in commercial hybrids. Quantitative trait loci (QTLs) for all tomato aroma volatiles have been identified in collections derived from both intraspecific and interspecific crosses with different wild tomato species and they (i) fail to co-localize with structural genes in the volatile biosynthetic pathways and (ii) reveal very little coincidence in the genomic regions characterized, indicating that there is ample opportunity to reinforce the levels of the volatiles of interest. Some of the identified genes may be useful as markers or as biotechnological tools to enhance tomato aroma. Current knowledge about the major volatile biosynthetic pathways in the fruit is summarized. Finally, and based on recent reports, it is stressed that conjugation to other metabolites such as sugars seems to play a key role in the modulation of volatile release, at least in some metabolic pathways.

Key words: Aroma, conjugation, flavour, fruit, QTLs, *Solanum*, tomato, volatile organic compounds.

The quest for identification of volatiles impacting flavour in tomato

As with many fruits that are part of the human diet, tomato has been domesticated for a few centuries to satisfy human preferences, but originally evolved to attract seed dispersers. The process of fruit ripening is complex and highly coordinated, and it begins when seeds are fully developed. The variety of physical and chemical changes that occur, such as softening of the fruit, the presence of high levels of organic acids, or the conversion of starch into short-chain sugars, make the fruit more attractive to animals. Additionally, the synthesis and accumulation of carotenoids, particularly β-carotene and lycopene, produce a change in fruit colour which may act as a visual cue that the fruit is ripe.

Among the few hundreds of volatile compounds a ripe tomato fruit typically produces (Tikunov *et al.*, 2005), almost all the important volatile compounds related to flavour are derived from essential nutrients such as phenylalanine, leucine, isoleucine, or linolenic acid, a fact which has suggested a process of co-evolution between tomato and its predators. Therefore, it has been proposed that volatile compounds produced in the ripe fruit would act as sensory cues for nutritional and health value (Goff and Klee, 2006; Klee and Giovannoni, 2011).

One mechanism for the rapid release of high amounts of selected volatiles in the tomato fruit when physically damaged, for example through chewing by a feeder, relies on their previous accumulation in a conjugated non-volatile form such as glycosides. The accumulation of the appropriate glycosidase in a separate subcellular location would allow the immediate liberation of high amounts of the aglycone when the enzyme and the conjugate glycosylated form came into contact with each other. It has recently been identified in a subset of tomato varieties that a different pattern of glycosylation

is produced from the breaker stage onwards. Some volatile compounds such as guaiacol, eugenol, or methyl salicylate are glycosylated to form a diglycoside during fruit development. Endogenous glycosidases have the ability to cleave this glycosidic bond, therefore liberating the volatile aglycone upon tissue disruption. In some tomato cultivars at the onset of ripening, a recently identified glycosyltransferase adds a third sugar to the conjugate, preventing the digestion of the glycoside, which results in a sharp decrease in the release of these volatiles (Tikunov *et al*., 2010, 2013). This mechanism of volatile storage through glycosylation and wound-induced deconjugation and subsequent volatile release suggests that some volatiles may also be part of a protection strategy against predation prior to ripening, discouraging feeding on fruits with immature seeds, in a similar manner to what has been reported for other metabolites with a bitter taste such as α-tomatine, whose levels are dramatically reduced upon ripening in most varieties (Rick *et al*., 1994). According to this view, volatile compounds together with other non-volatile metabolites would have a double effect on seed dispersers: discouraging them from feeding on the fruit before the maturation of the seed, and encouraging to feed on them once seed maturation has been achieved.

From a human perspective, a relevant question is which and how many of the volatile compounds produced in the fruit are responsible for our perception of flavour and aroma. There is a great variety of volatile compounds produced in the ripe fruit, and there are differences of many orders of magnitude between their abundance levels, with concentrations ranging from several micrograms per gram of fresh weight for the most abundant such as (*Z*)-3-hexenal or hexanal to the nanogram per gram and even lower levels of β-damascenone or β-ionone (Buttery *et al*., 1988). A traditional approach for the understanding of which compounds are important in contributing to aroma and the intensity and odour quality is the application of odour thresholds and odour units. Basically, this approach consists of establishing the lowest concentration of a compound that can be perceived by the human nose. These thresholds were estimated by means of the orthonasal smell perception of decreasing concentrations of volatile compounds in water solution by panels of judges (Guadagni *et al*., 1963; Buttery *et al*., 1971, 1989). According to this approach, the compounds contributing to aroma in the tomato fruit would be those with a concentration higher than the threshold established for that particular compound. The importance of each compound to the resulting aroma perception would be estimated by means of odour units. These are calculated by dividing the concentration of each compound in the tomato samples by its odour threshold, and are usually represented in their logarithmic form. By means of this approach, a list of 16–17 compounds was produced, with the compounds arranged in decreasing order of odour contribution (Buttery *et al*., 1989).

This approach was widely accepted as a useful tool for a first attempt to identify the volatiles contributing to tomato aroma. However, it has been revealed to be too simplistic to explain the high complexity of our perception of flavour and aroma. First, odour thresholds were calculated by means of the orthonasal perception (sniffing) of volatile compounds, whilst our perception of food aroma is based on the retronasal perception of the volatile compounds released in the mouth. It has been observed that ortho- and retronasal odour thresholds for the same compound are different. Furthermore, particularly for food odours, it has been observed that each of these types of olfaction produces distinct sensory signals (Negoias *et al*., 2008; Bender *et al*., 2009). Additionally, odour thresholds were calculated based on the concentration of pure standards in water solution, not in a tomato matrix. It has been described that there is an important matrix effect on the volatility of aroma compounds, therefore affecting their access to the olfactory receptors. As a consequence, the same amount of compound in a tomato matrix or in a water solution results in a difference of up to an order of magnitude in volatile emission (Bezman *et al*., 2003).

Another fact which further complicates the making of a list of compounds contributing to aroma is the wide range of variation in their levels between cultivars, so that a particular compound may be at low levels in some cultivars and have no effect on the aroma, but in another variety with high levels could have an important effect. This has apparently been the case for guaiacol, which initially was not considered to participate in tomato aroma, but in some introgression lines derived from *Solanum lycopersicum* var. *cerasiforme* and some commercial varieties was revealed to have a relevant effect as detected by consumer panels (Causse *et al*., 2002; Zanor *et al*., 2009; Tikunov *et al*., 2013).

It has also been observed that the perception of aroma is not due to the additive effect of each individual volatile compound, but to the interaction of different volatile compounds affecting perception in different and sometimes even opposite directions. Furthermore, it has been observed that although taste and olfactory receptors are different and recognize different chemicals, there also exists an interaction in the perception between volatile and non-volatile compounds. So, it has been described that the presence of sugar or organic acids alters the taste panel perception of aromatic descriptors of samples with the same concentration of volatile compounds (Tandon *et al*., 2003; Baldwin *et al*., 2008) and, conversely, the perception of taste descriptors such as overall taste, sourness, or sweetness can be modified by the addition or naturally occurring levels of some volatiles (Baldwin *et al*., 2004; Vogel *et al*., 2010; Tieman *et al*., 2012).

Taking into consideration this complexity, efforts have been made to generate prediction models for the different descriptors of tomato flavour and consumer preference using regression analysis of both volatile and non-volatile compounds (Tandon *et al*., 2003), later enhanced by partitioning taste from aromatic flavour notes (Abegaz *et al*., 2004) and, more recently, by the integration of physicochemical, volatile, and sensory parameters in multivariate modelling (Piombino *et al*., 2013) or by means of targeted metabolomics in order to better understand the interactions between compounds leading to consumer liking (Tieman *et al*., 2012). According to the latter model, it was revealed that some compounds traditionally considered to be important for tomato aroma based on their odour units, such as phenylacetaldehyde and

particularly β-damascenone, would apparently have no contribution to cultivated tomato flavour preference (Tieman *et al.*, 2012).

An additional difficulty for the identification of targets for genetic improvement of tomato flavour is to define what consumers consider a good tomato. It has been concluded that flavour descriptors together with firmness seem to be the most important traits for improving tomato quality (Causse *et al.*, 2010), but such a thing as a perfect tomato which would be considered excellent by all consumers does not exist. On the contrary, consumer preferences are segmented, as has repeatedly been observed in French (Lê and Ledauphin, 2006; Lengard and Kermit, 2006), Italian (Sinesio *et al.*, 2010), and other European consumer studies (Causse *et al.*, 2010). Therefore, diversification of at least flavour and texture in different market varieties would be necessary to satisfy the preferences of all consumers.

Variability in volatiles: where to find it and how to harness it

To identify the genetic basis for volatile production is important since a number of surveys reveal a general dissatisfaction of consumers and complaints about the poor organoleptic quality of most commercial tomatoes (Kader *et al.*, 1977; Janse and Schols, 1995). Somewhere during the modern breeding process the aroma of traditional tomatoes has been lost (Klee and Tieman, 2013) and there is an urge to get it back. Although in many cases this loss of organoleptic quality could be due to pre- and post-harvest conditioning of the fruit, modern breeding has been focused mainly on biotic resistance, long shelf life, and productivity rather than on organoleptic/aroma quality, which in addition is a very complex and difficult trait to breed for (Klee and Tieman, 2013). Important variability in the range of volatile levels, particularly high for some branched-chain and phenolic volatiles, has been found in several experiments (see Table 1 as an example). Moreover, this variability is actually found in heirlooms (Tieman *et al.*, 2012), wild relatives (Tikunov *et al.*, 2013), and breeding populations (Causse *et al.*, 2002; Zanor *et al.*, 2009), but also in different commercial hybrids (Tikunov *et al.*, 2005; Ursem *et al.*, 2008). The assessment of such genetic variability opens up the opportunity to improve the aroma of modern tomato varieties through breeding.

Flavour is determined by a complex interaction of aroma volatiles, sugars, and organic acids. Therefore, flavour and, concomitantly, consumer perception show quantitative variation and are expected to be under complex genetic control. The first systematic attempt to analyse the genetic control of volatiles and aroma in tomato was carried out by Causse *et al.* (2002) in an intraspecific tomato mapping population, allowing the identification of some major QTLs for a number of fruit volatiles. Further studies using interspecific populations of *S. habrochaites* and *S. pennellii* with the tomato inbred lines E6203 and M82, respectively, have enlarged the volatile variation range and allowed the identification of new volatile QTLs (Tieman *et al.*, 2006a; Mathieu *et al.*, 2009).

Table 1. *Range of volatile variation (fold difference) in fruit from different tomato genotype collections*

Volatile compound	Co.1	Co.2	RILs a	RILs b	Heirloom
3-Methylbutanal	m	236	290	75	N/a
2-Methylbutanal	36	N/a	14	78	13
3-Methylbutanol	N/a	5742	344	2679	58
2-Isobutylthiazole	361	185	444	242	174
1-Penten-3-one	31	2	8	14	N/a
(*Z*)-3-Hexenal	8	3	12	180	13
Hexanal	33	3	16	52	25
(*E*)-2-Hexenal	11	5	10	16	123
(*E*)-2-Heptenal	12	3	9	12	30
(*E*,*E*)-2,4-Decadienal	152	3	40	55	211
Phenylacetaldehyde	43	112	30	106	654
Guaiacol	349	73	217	790	290
2-Phenylethanol	591	41	118	90	3142
Methyl salicylate	246	273	244	184	3354
1-Nitro-2-phenylethane	565	182	794	1920	149
Eugenol	12	36	829	1380	N/a
6-Methyl-5-hepten-2-one	150	5	13	16	120
Geranial	168	6	14	23	N/a
β-Damascenone	54	5	34	50	86
Geranylacetone	135	8	21	58	195
β-Ionone	44	4	12	44	47

Co.1, tomato breeding lines from company 1 (45 genotypes evaluated); Co.2, tomato breeding lines from company 2 (22 genotypes); RILs a, recombinant introgression lines originated from a cross *S. lycopersicum* cv. Moneymaker×*S. pimpinellifolium* accession TO-937 (Alba *et al.*, 2009), collected in the first season (169 genotypes); RILs b, the same materials collected in a different year (169 genotypes); Heirloom, *S. lycopersicum* heirloom varieties (Tieman *et al.*, 2012) (152 genotypes).
N/a, data not available.
The amplitude of variation is expressed as the fold change in the average values of each given volatile between the genotypes with the highest and lowest levels in that population.

Between 25 and 30 loci altered the volatile composition and, in most cases, each locus altered several volatiles, most often metabolically related compounds. Interestingly, while all *S. pennellii* alleles increased volatile composition in the M82 background, *S. habrochaites* alleles increased or decreased them in the E6203 background depending on the locus. Although the identification of genes involved in the regulation of biosynthetic pathways of volatile compounds is still in its infancy, volatile QTLs do not co-localize with known structural genes encoding enzymes in any of the described volatile pathways. This makes cloning of these QTLs very attractive as they may underlie important regulatory genes.

Another interesting observation is that only in a few cases is the same volatile QTL conserved among the different mapping populations (Fig. 1). This result can be attributed to multiple causes: (i) the volatile profile of the parent genotypes is quite different, indicating an important genetic variability among populations; (ii) fruit volatile composition is strongly influenced by the environment; and (iii) there are differences in sampling, methods of volatile capture, and profiling. A standardization of sampling and a large number of studies would be necessary to assess if the lack of co-localization among populations has a strong genetic basis.

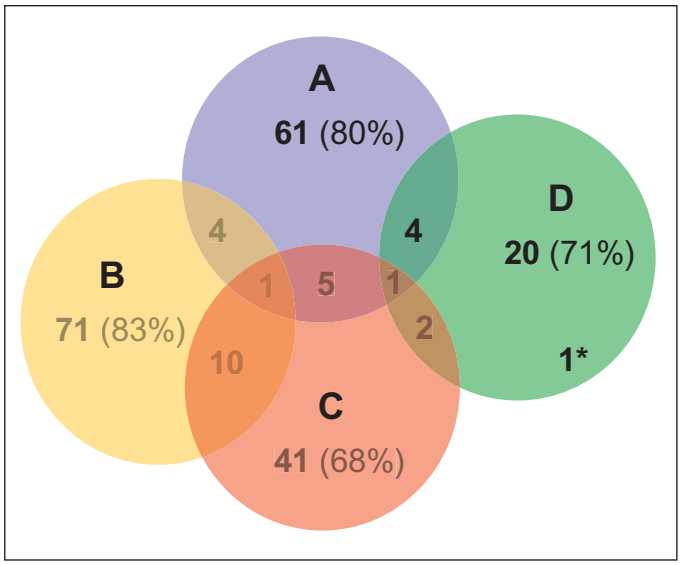

Fig. 1. Venn diagram showing the degree of overlap of the QTLs for volatiles in different introgression populations. (A) Recombinant introgression lines (RILs) from an interspecific cross *S. lycopersicum*×*S. pimpinellifolium* (own data); (B) ILs from an interspecific cross *S. lycopersicum*×*S. pennellii* (Tieman *et al.*, 2006a); (C) ILs from an interspecific cross *S. lycopersicum*×*S. habrochaites* (Mathieu *et al.*, 2009); (D) RILs from an intraspecific cross *S. lycopersicum*×*S. lycopersicum* var. *cerasiforme* (Causse *et al.*, 2002). Numbers in the overlapping areas represent the number of QTLs in common between the populations. Numbers in the non-overlapping areas represent the number of QTLs exclusive to that particular population. In this case, data are also expressed as a percentage of the total number of QTLs identified in that population. In D, 1* refers to one QTL in common between populations B and D.

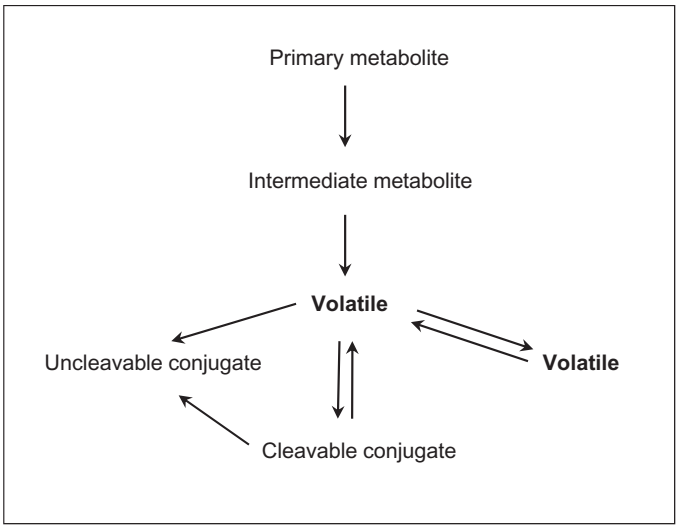

Fig. 2. General scheme of volatile biosynthesis and modifications.

Nevertheless, Zanor *et al.* (2009), studying introgression lines developed from a cherry donor into a large fruit tomato background by marker-assisted breeding, demonstrated that single QTL volatiles can be transferred between different genetic backgrounds, and a single locus can be sufficient to alter the volatile composition significantly. Therefore, the identification of QTL volatiles in introgression lines could be used both to identify and to select for genomic regions carrying genes associated with accumulation and release of the corresponding volatiles.

In summary, domesticated tomato still contains large variability for volatile accumulation in both heirloom and commercial varieties, and that variability can be increased by incorporating new loci from wild relatives. Therefore, there is ample scope to improve volatile composition in the commercial varieties which so far have been optimized for yield and other traits.

Genes involved in volatile production in tomato

Volatile compounds are secondary or specialized metabolites which, once synthesized, may undergo different modifications, either reversible or irreversible; for example, to produce a different volatile compound or a non-volatile conjugate (Fig. 2). Although many advances have been made in the last decade, many of the genes involved in volatile biosynthesis remain unknown. Figure 3 shows a scheme of the most important metabolic pathways of volatile biosynthesis in tomato fruit, which we will describe later based on the current knowledge in tomato and also supported with information obtained from other species.

Fatty acid derivatives

Volatiles derived from fatty acids constitute a class of compounds which includes the most abundant volatiles produced in the tomato fruit: the C_6 volatiles 1-hexanol, (*Z*)-3-hexenal, (*E*)-2-hexenal, or hexanal, and the C_5 volatile 1-penten-3-one. These compounds are classified as green leaf volatiles due to their characteristic 'green', fresh aroma of cut grass, since high amounts of lipid-derived C_6 aldehydes and alcohols are typically released from vegetative tissues when disrupted. In tomato fruit, the production of these compounds is increased at ripening, probably due to the loss of integrity of cellular membranes (Klee, 2010). Despite their abundance in the ripe fruit, their relevance for tomato flavour has been a matter of discussion. Although (*Z*)-3-hexenal and hexanal were originally considered among the most relevant compounds for tomato aroma in studies based on the odour units approach (Buttery *et al.*, 1989), recent studies suggest a reduced impact on tomato flavour and no effect on consumer liking (Chen *et al.*, 2004; Tieman *et al.*, 2012).

The initial step in the biosynthesis of these compounds is still not completely understood. The amount of free fatty acids available in the fruit is very limited, as plants accumulate them as acylglycerides rather than in the toxic free form. Therefore, it is believed that the catabolism of the acylglycerides by a lipase (or lypolytic acyl hydrolase, LAH), which would liberate the fatty acids, is the initial step in their biosynthesis. This has been observed in *Arabidopsis* leaves, where the production of (*Z*)-3-hexenal was associated with a decrease in the levels of galactolipids, a process which could be repressed by means of a lipase inhibitor (Matsui *et al.*, 2000*a*).

Free fatty acids are rapidly catabolysed by means of β-oxidation, α-oxidation, or the lipoxygenase pathway. The

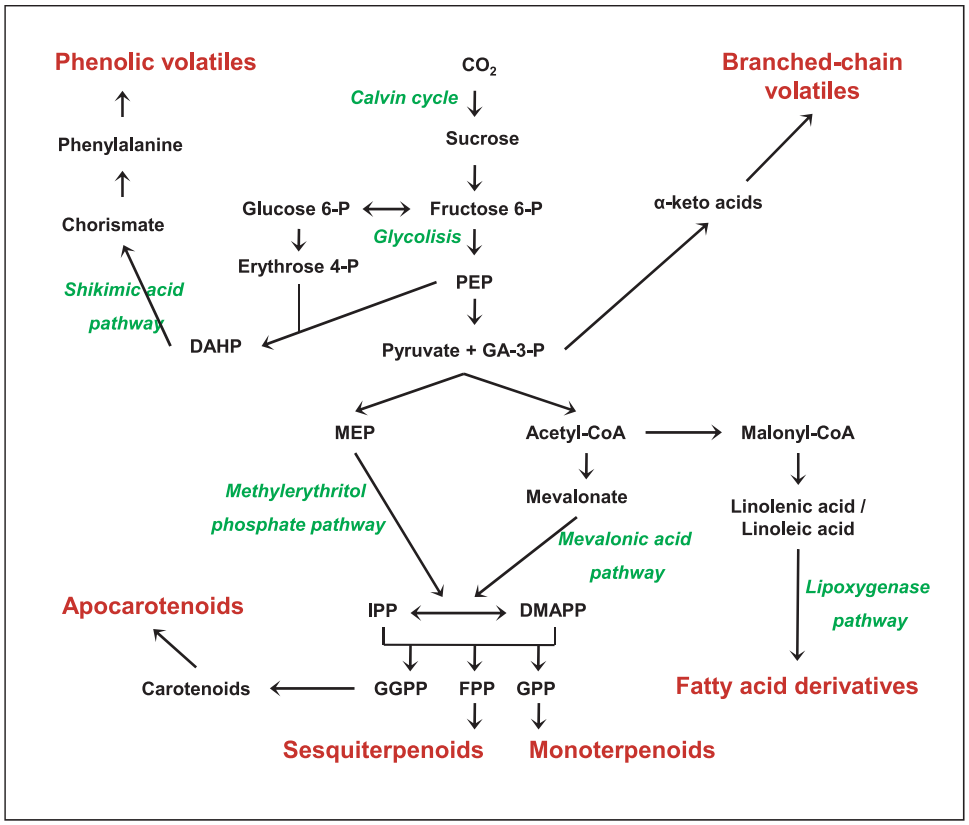

Fig. 3. Biosynthetic pathways of the most relevant classes of volatiles in the tomato fruit. Volatile classes are highlighted in bold; metabolic pathways are represented in italics. Abbreviations: DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GA-3-P, glyceraldehyde-3-phosphate; GGPP, geranyl diphosphate; IPP, isopentenyl diphosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; PEP, phosphoenolpyruvate. (This figure is available in colour at *JXB* online.)

latter is the most important for volatile production in the tomato fruit, and includes the sequential activity of lipoxygenase (LOX) and hydroperoxide lyase (HPL) enzymes. LOXs are non-haem iron-containing fatty acid dioxygenases with the ability to catalyse the regio- and stereospecific dioxygenation of polyunsutarated fatty acids with a (1*Z*,4*Z*)-pentadiene moiety, converting them into fatty acid hydroperoxides (Liavonchanka and Feussner, 2006). The most important substrates for LOX activity in tomato fruit are the C_{18} fatty acids linolenic acid and, to a lesser extent, linoleic acid.

LOXs can be divided into two groups, 13-LOX and 9-LOX, depending on the positional specificity of oxygenation, producing 13- or 9-hydroperoxides, respectively. The resulting hydroperoxides are further metabolized by HPLs, enzymes of the cytochrome P450 family which produce a volatile aldehyde and an oxoacid. These enzymes are also classified as 13- or 9-HPLs depending on the substrate they act on (Matsui, 2006). In the tomato fruit, there is an important 13-LOX activity, producing (*Z*)-3-hexenal from linolenic acid and hexanal from linoleic acid, particularly when fruits are homogenized. Five isoforms of 13-LOXs have been described in tomato, but apparently only *TomloxC* is expressed in the fruit (Chen *et al.*, 2004), similarly to what has been described in other fruits such as kiwi fruit, where the *AdLox* family is composed by six isoforms, only two of which are responsible for volatile emission in the ripe fruit (Zhang *et al.*, 2006). A 13-fatty acid HPL has been described in tomato (Howe *et al.*, 2000). Although genes encoding enzymes with 9-LOX activity have been described in the fruits of other species such as cucumber (Matsui *et al.*, 2000*b*), almond (Mita *et al.*, 2005), or rice (Kuroda *et al.*, 2005), neither *9-LOX* nor *9-HPL* gene expression has been described in tomato fruit, which is in accordance with the low levels of C_9 volatile compounds detected.

Isomeric conversion of (*Z*)-3-hexenal into (*E*)-2-hexenal occurs in the fruit, either non-enzymatically or by means of a 3*Z*,2*E*-enal isomerase (Noordermeer *et al.*, 1999), although at present this enzyme has not been identified. The aldehydes produced from this LOX pathway, like those produced by other metabolic pathways, can be reduced to alcohols by means of alcohol dehydrogenases (ADHs), enzymes catalysing their reversible interconversion. Tomato *ADH2* gene expression was observed to increase during the ripening process, particularly in the last stages, and to have an effect on the biosynthesis of hexanol and (*Z*)-3-hexenol (Speirs *et al.*, 1998). Another fruit-ripening-associated ADH, SlscADH1, has been described recently in tomato. This enzyme showed *in vitro* activity in the production of hexanol and 1-phenylethanol from hexanal and phenylacetaldehyde, respectively, but no *in vivo* effect was observed (Moummou *et al.*, 2012).

Biosynthesis of C_5 lipid volatiles, such as 1-penten-3-one, which is considered as an important contributor to tomato fruit aroma (Baldwin *et al.*, 2000), has not been investigated so far, but LOX could use linolenic acid as a substrate, producing the 13-alcoxyl radical, which is converted non-enzymatically

into the 1,3-pentene radical, which could further react with a hydroxyl radical, yielding C_5 alcohols (Gardner *et al.*, 1996). The activity of this LOX branch could be boosted by a reduction of HPL activity leading to accumulation of hydroperoxides and therefore could be considered as competing with the C_6 volatile-producing LOX pathway (Vancanneyt *et al.*, 2001).

Amino acid derivatives

A significant number of the volatile compounds considered as relevant for tomato aroma are derived from amino acids. These volatiles can be grouped into two categories: phenolic and branched-chain compounds. Their respective biosynthetic pathways are described separately below.

Phenolic volatiles

Phenolic volatiles include many compounds that are involved, either positively or negatively, in our perception of tomato flavour, and include a variety of compounds derived from the amino acid phenylalanine. In a recent study, transgenic tomatoes with enhanced levels of the phenolic volatiles 2-phenylethanol, phenylacetaldehyde, and benzaldehyde had a preferred floral aroma compared with untransformed controls, although they also had diminished levels of some positive aroma apocarotenoids such as β-ionone or geranylacetone (Tzin *et al.*, 2013). 2-Phenylethanol had been previously described to have a positive effect on tomato flavour, increasing floral aroma and the perception of sweetness (Baldwin *et al.*, 2008). Nevertheless, introgression lines harbouring the *malodorous* allele from the wild tomato species *S. pennellii*, which produces dramatically increased levels of 2-phenylethanol and its precursor phenylacetaldehyde, showed a strong undesirable flavour, probably due to the very high levels of phenylacetaldehyde produced (Tadmor *et al.*, 2002). This exemplifies the complexity of our perception of flavour based on volatile compounds and the difficulty in predicting the effect on flavour and consumer preference when considering altering a metabolic pathway.

Phenylalanine-derived compounds can be classified into different subfamilies. C_6-C_2 phenolic volatiles are probably the most important compounds for aroma, and their biosynthesis implies an initial decarboxylation of phenylalanine. A small family of genes (*AADC1A*, *AADC1B*, and *AADC2*) has been described in tomato fruit leading to the decarboxylation of phenylalanine into phenethylamine, which would then be de-aminated by means of an as yet uncharacterized amine oxidase to produce phenylacetaldehyde. Alternatively, it could be transformed into 1-nitro-2-phenylethane or benzylnitrile by means of other unknown enzymes (Tieman *et al.*, 2006*b*). 2-Phenylethanol, considered to be an important volatile for fruit aroma in many species and also in tomato, is synthesized from phenylacetaldehyde by means of phenylacetaldehyde reductases PAR1 and PAR2. These enzymes catalyse the unidirectional reduction of aldehyde into alcohol, and it is thought that they also use benzaldehyde and cinnamaldehyde as substrates for the synthesis of their respective alcohols (Tieman *et al.*, 2007).

The other group of phenolic compounds originates from the phenylpropanoid branch of phenylalanine catabolism. Biosynthetic pathways of phenylpropanoid compounds have not been completely elucidated in tomato. It is assumed that C_6-C_3 volatile synthesis would be initiated by means of a phenylalanine ammonia-lyase (PAL) producing (*E*)-cinnamic acid and would follow the pathway of lignin biosynthesis. Some of the compounds in this pathway would be substrates for enzymes producing volatile compounds, such as eugenol, which has been reported in other species to be synthesized by means of a eugenol synthase from coniferyl acetate (Koeduka *et al.*, 2006).

Shorter chain phenolic volatiles also originate from (*E*)-cinnamic acid by the shortening of their side chain and further modifications. The last steps of the biosynthesis of some of these compounds have recently been described in tomato. Methyl salicylate is produced by means of salicylic acid methyl transferase (SlSAMT), an *O*-methyltrasferase catalysing the methylation of salicylic acid (Tieman *et al.*, 2010). The synthesis of guaiacol, another important volatile compound for fruit flavour, would be produced from catechol by means of the catechol-*O*-methyltransferase COMT1 (Mageroy *et al.*, 2012). The biosynthetic pathway of the most important compounds and the identified tomato genes involved in volatile biosynthesis are summarized in Fig. 4.

Branched-chain volatiles

Another important group of volatiles related to amino acids are the branched-chain volatiles, a set of compounds with particularly low molecular weight and high volatility, some of which are considered to participate in tomato aroma, such as 3- and 2-methylbutanal, 3-methylbutanol, and 2-isobutylthiazole (Buttery *et al.*, 1989).

Biosynthesis of these compounds has not been elucidated yet in fruits, although their biosynthetic pathway has been described in yeast and bacteria. In these microorganisms, branched-chain amino acids would be the original precursors and would be converted reversibly into α-ketoacids by means of branched-chain amino acid aminotransferases (BCATs). A set of different volatile compounds can then be formed: (i) an α-hydroxyacid would be formed by the action of an α-hydroxyacid dehydrogenase; (ii) an aldehyde through the action of a decarboxylase; (iii) the latter could subsequently be reduced to an alcohol by alcohol dehydrogenases; (iv) an acyl-CoA by means of an α-ketoacid dehydrogenase; and (v) this acyl-CoA can be converted into an acid or else react with an alcohol to form an ester by the action of an alcohol acyltransferase (Marilley and Casey, 2004).

A biosynthetic pathway in fruits similar to that described in yeast and bacteria seemed reasonable after the identification in tomato of a small family of BCATs. This family is composed of six members, of which the mitochondria-located SlBCAT1 and SlBCAT2 would be implicated in the first step of amino acid catabolism (Maloney *et al.*, 2010). In this case, the amino acids leucine, isoleucine, and valine would be the precursors of branched-chain volatile compounds. Nevertheless, it was later revealed that the catabolism of amino acids by means of the BCATs is unrelated to

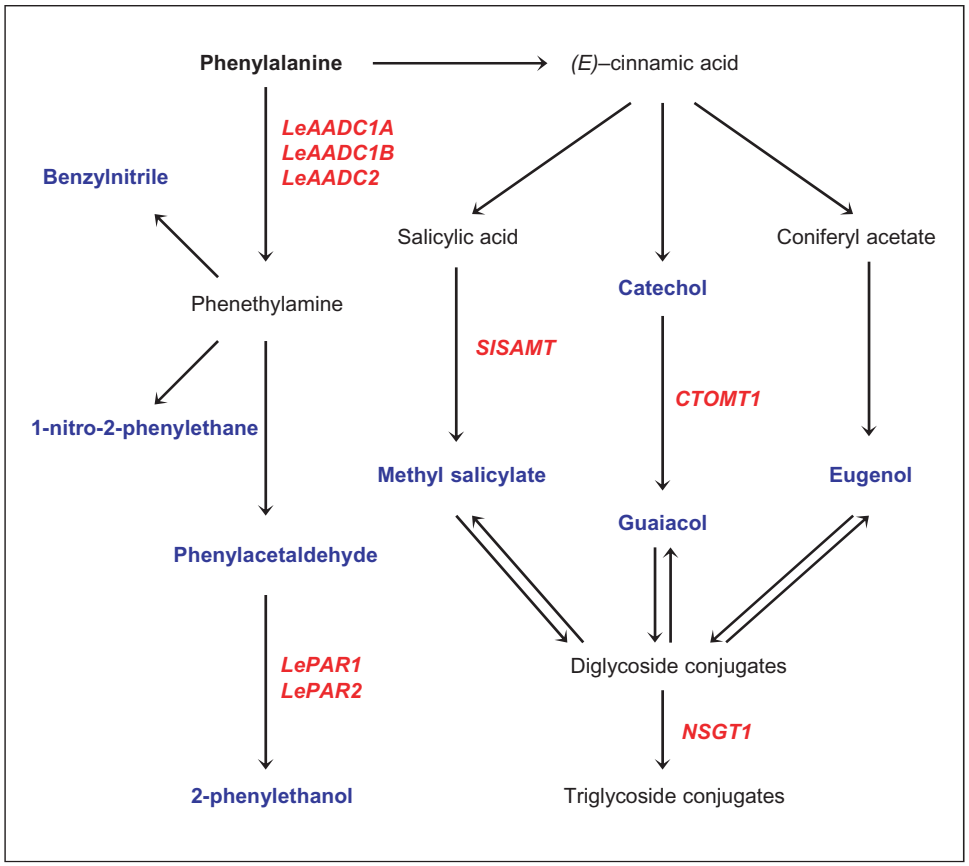

Fig. 4. Biosynthetic pathway of the phenolic volatile compounds most relevant in tomato fruit. Volatile compounds detected in the fruit are represented in bold; characterized tomato genes involved in the biosynthesis are represented in italics. Gene abbreviations are as follows: *CTOMT1*, catechol-*O*-methyltransferase 1; *LeAADC*, aromatic amino acid decarboxylases; *LePAR*, phenylacetaldehyde reductases; *NSGT1*, non-smoky glycosyltransferase 1; *SlSAMT*, salicylic acid methyl transferase. (This figure is available in colour at *JXB* online.)

the synthesis of volatiles. Therefore, it has been proposed that α-ketoacids rather than amino acids would be the direct precursors of this family of volatile compounds (Kochevenko *et al.*, 2012).

Esters

Although very abundant and extremely important for the aroma of fruit in many species such as strawberry (Zorrilla-Fontanesi *et al.*, 2012), peach (Sánchez *et al.*, 2012), or even some citrus species (González-Mas *et al.*, 2011), few esters are found in the volatile fraction of tomato, and, with the exception of the previously described phenylpropanoid ester methyl salicylate, they are not relevant for tomato flavour.

On the contrary, new evidence has revealed that this lack of esters in the cultivated species has a positive effect on tomato liking. It has been observed that green-fruited wild tomato species accumulate considerably higher levels of acetate esters compared with red-fruited species. The difference is attributed to the insertion of a retrotransposon in a position adjacent to the most enzymatically active tomato esterase, increasing gene expression in all red-fruited species including cultivated tomato. The resulting enhanced esterase activity results in a dramatic reduction in the levels of many esters that are negatively correlated with human preference, which may have provided an adaptative advantage to the ancestor of red-fruited species, such as cultivated tomato (Goulet *et al.*, 2012).

Terpenoids

Mono- and sesquiterpenoids

This class of volatiles includes an large variety of structurally complex compounds which are among the most abundant in tomato vegetative tissues and particularly in trichomes, but only a few of them, such as limonene, linalool, or α-terpineol, are present in the ripe fruit, and their impact on tomato aroma is negligible.

Volatile terpenoids can be classified into two groups: monoterpenoids (C_{10}) and sesquiterpenoids (C_{15}). They are both synthesized from the five-carbon precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). There are two alternative pathways for the biosynthesis of these precursors. The methylerythritol phosphate pathway has been described in the plastids to produce both IPP and DMAPP from pyruvate and glyceraldehyde-3-phosphate. The mevalonic acid pathway has been described in the cytosol to use acetyl-CoA to produce IPP, which can later be converted into DMAPP. Despite the different subcellular compartmentation of each of these pathways, some metabolic cross-talk

between them has been reported, particularly in the direction from the plastids to the cytosol (Hemmerlin *et al.*, 2003).

Geranyl diphosphate synthase catalyses the condensation of an IPP and a DMAPP molecule to produce geranyl diphosphate (GPP), the precursor of all monoterpenoids, while farnesyl diphoshate synthase catalyses the synthesis of farnesyl diphosphate (FPP), the precursor of all sesquiterpenoids, from two IPP molecules and one DMAPP molecule. GPP and FPP are the substrates for the diverse terpene synthases/cyclases, a large family of enzymes, to produce a variety of monoterpenoids and sesquiterpenoids, respectively (Nagegowda, 2010; Granell and Rambla, 2013).

Carotenoid-derived volatiles

Apocarotenoids can be considered as irregular terpenoids, and are synthesized from the oxidative cleavage of double bonds in carotenoids (C_{40} terpenoids), compounds which are accumulated at high levels in the ripe fruit. These volatile compounds are produced at low levels in the ripe fruit, but are important in our perception of tomato flavour due to their very low odour thresholds, particularly for some cyclic apocarotenoids such as the C_{13} ketones β-ionone or β-damascenone, which can be detected orthonasally at concentrations of 0.007 nl l^{-1} and 0.002 nl l^{-1}, respectively (Buttery *et al.*, 1989). Although recent studies have questioned the relevance of individual compounds previously considered important for the flavour of tomato, such as β-damascenone, carotenoid-derived volatiles have proved to have an important role in tomato flavour, as their levels positively correlate with tomato flavour acceptability (Vogel *et al.*, 2010).

In tomato, carotenoid cleavage dioxygenases *LeCCD1A* and LeCCD1B have been described as involved in the biosynthesis of at least some of the apocarotenoids produced in the fruit. *LeCCD1A* and *LeCCD1B* are highly expressed in the ripening fruit and their products have been proved to cleave multiple carotenoids, both linear and cyclic, producing a C_{14} dialdehyde and a variety of C_{13} volatiles such as β-ionone, geranylacetone, and pseudo-ionone (Simkin *et al.*, 2004).

Proteins of the CCD1 group have been described in other species to have the ability to cleave cyclic carotenoids at the 9,10 position and linear carotenoids at the 5,6 (5′,6′), 7,8 (7′,8′), or 9,10 (9′,10′) positions, producing many different compounds. These enzymes are located in the cytosol and show broad substrate specificity, cleaving any carotenoid after ζ–carotene in the metabolic pathway. Considering that carotenoids are accumulated in the plastids, it is still unclear how enzymes and substrates come together, although different mechanisms have been proposed (Vogel *et al.*, 2008; Ilg *et al.*, 2009; Floss and Walter, 2009; Walter *et al.*, 2010).

Apocarotenoid levels in the fruit increase dramatically during ripening, although there is significant CCD expression in the fruit during all the stages of fruit development. The coincidence of the conversion of chloroplasts into chromoplasts and the loss of membrane integrity with the increased biosynthesis of apocarotenoids suggests a key role for substrate availability in the regulation of their biosynthesis (Klee, 2010; Vogel *et al.*, 2010).

Similar processes lead to production of another class of carotenoid-derived compounds—open-chain carotenoid-derived volatiles. Eight-carbon ketone 6-methyl-5-hepten-2-one and C_{10} aldehyde α-citral (geranial) are the most abundant compounds of this class in tomato fruit and contribute to its aroma (Buttery *et al.*, 1989; Baldwin *et al.*, 2000). These two volatiles are derived from open-chain carotenoids—phytoene or phytofluene and lycopene, respectively—and the volatile products correlate strongly to the levels of the carotenoid precursors (Lewinsohn *et al.*, 2005).

Transcriptional regulation of volatile pathways

The production of volatile compounds in the fruit is the result of many interconnected metabolic pathways and a complex regulation network. The ripening of the fruit, which is a highly coordinated process, includes a dramatic change in its volatile profile (Ortiz-Serrano and Gil, 2010), for which transcriptional regulation seems to be an important aspect. Nevertheless, very little is known about the transcription factors which directly regulate volatile biosynthesis, with few possible exceptions such as the gene encoding the MYB transcription factor SlODO1 (Orzáez *et al.*, 2009).

The levels of most of the volatile compounds are increased by several orders of magnitude during the ripening process, peaking at or shortly before full ripening (Klee and Giovannoni, 2011), while a few of them remain constant or are reduced. Therefore, transcription factors which are involved in the regulation of fruit ripening, such as RIN, CNR, and NOR, have been shown to have pleiotropic effects on biosynthesis and accumulation of aroma-related volatile metabolites (Kovács *et al.*, 2009).

Conjugation and volatile management

An effective mechanism to immobilize a volatile for future use once it has been synthesized is by covalent chemical binding to a polar compound, thus producing a non-volatile molecule of higher molecular mass and increased polarity. Conjugation of volatile compounds has been known to occur for a long time in fruits of many species (Marlatt *et al.*, 1992). It usually involves *O*-glycosylation of the volatile compounds (also called aglycones) with one or more sugar moieties. The glycosyltransferase enzyme family is one of the most diverse enzyme families in plant. They lead to the production of a large variation of glycoconjugates with different structures and different biochemical properties. Such conjugation has been reported for different classes of compounds such as linear alcohols, monoterpene alcohols, apocarotenoids, and phenylpropanoids (Buttery *et al.*, 1990; Ortiz-Serrano and Gil, 2007, 2010) and the pattern of glycosidically bound volatiles in a particular fruit tends to be similar to that of the free volatiles produced (Du *et al.*, 2010). These studies show that many volatiles in tomato fruit, for example some terpenes, are exclusively present as glycoconjugates. Glycosides of other volatiles can be as equally abundant as their corresponding

free forms or can exceed their concentration. This suggests that conjugated volatiles could have a significant impact on tomato fruit aroma upon release from their glycoconjugates. Potentially, a reversible conjugation would allow the fruit to accumulate significant amounts of a volatile compound, which would otherwise be slowly released and consequently lost, and its deconjugation would enable the liberation of massive amounts of that volatile when required. On the other hand, an irreversible conjugation could be an effective way to get rid of a compound which is no longer convenient to be released. In any case, the metabolic processes leading to the formation of conjugates and their possible hydrolysis are still poorly understood. In part as a consequence of this, the relevance of conjugation of volatile compounds in the flavour of fruits has probably been underestimated.

A couple of recent papers have shed some light on part of these processes in tomato fruit. It was observed that most of the emission of the phenylpropanoids methyl salicylate, eugenol, and guaiacol, some of which are abundant compounds in tomato fruit and have an effect on flavour, relies on their liberation upon tissue disruption from their corresponding accumulated glycoconjugates. Two different patterns of glycoconjugation of these volatiles were observed in a collection of tomato cultivars which were tightly correlated with the emission of the aglycone: fruits accumulating phenylpropanoid volatile diglycosides produced high levels of the volatiles after homogenization, while those accumulating triglycosides emitted significantly reduced levels (Tikunov *et al.*, 2010). Subsequent work led to the identification of *NSGT1*, a fruit ripening-induced gene encoding a glycosyltransferase with the ability to transfer an additional glucose to a set of phenylpropanoid volatile diglycosides. The triglycosides produced cannot be cleaved by tomato glycosidases, while diglycosides can be readily hydrolysed upon fruit disruption. Consequently, NSGT1 activity produces the irreversible immobilization of these volatile phenylpropanoids from the onset of ripening, thereby preventing their emission upon tissue disruption, for example through the chewing of the fruit by a predator—or a human (Tikunov *et al.*, 2013).

Much work has yet to be done on this aspect of volatile biosynthesis, but it seems that conjugation plays an important role in the control of volatile emission in the tomato fruit.

Challenges ahead

Tomato flavour is a very complex trait in which volatile compounds play a key role, but little—if any—attention has been paid to them in plant breeding in the past, due to their high degree of complexity. Our lack of understanding not only concerns the very limited information about the genetic control of volatile levels, but even the definition of which compounds should be selected as targets for breeding.

Much progress has been made in recent years in the identification of genomic regions, genes, and enzymes involved in the biosynthesis of volatile compounds in the tomato fruit. As a result, we have been able to initiate the metabolic engineering of volatiles; however, most of the intricate inter-related biological processes leading to volatile emission still remain unclear.

The development of the different -omics technologies has provided us with a substantial amount of information about biological processes such as the ripening of the fruit. The integration of the different data obtained from these certainly increases the complexity of the data set, but also provides a deeper comprehension and a more complete vision of the biological process studied. Such integrated -omics approaches, which have already been successfully used with non-volatile metabolites (Carrari *et al.* 2006), are expected to provide a better understanding of the high complexity of the whole ripening process, and are also a powerful tool for the identification of new genes responsible for fruit volatile production. Co-localization of volatile QTLs with gene expression QTLs, and the use of modelling networks to integrate the data and predict the most interesting candidates, is also a promising approach. In this respect, the availability of the annotated genome sequence of tomato constitutes a highly valuable tool in facilitating the identification of the genes responsible for fruit volatile production in this species.

Once target genes have been selected, tools such as the visual reporter system of virus-induced gene silencing in tomato fruit (Orzáez *et al.*, 2009) are useful for a first evaluation of candidate gene function during fruit ripening and allow the selection of the most promising candidates from a larger panel. Despite the power of this platform, stable transgenic plants are required to confirm unequivocally the function of a selected candidate gene.

In conclusion, we would like to remark that, although until recently not much attention has been paid to the importance of volatiles in tomato fruit flavour, we are rapidly increasing our understanding of the metabolic pathways, key enzymes, and genes leading to flavour volatile production. This knowledge provides us with the ability to start modifying the fruit aroma through both transgenic and marker-assisted breeding approaches. Other relevant aspects which require attention are those regarding subcellular compartmentation of substrates, enzymes, and products, and the regulatory networks controlling volatile synthesis, emission, and conjugation as a developmentally regulated process coupled to fruit ripening.

The development of new technologies provides the opportunity to obtain important advances in our understanding of the whole metabolic process, which would empower breeders to modify intentionally the fruit aroma in the near future.

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