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Improving the flavor of fresh fruits: genomics, biochemistry, and biotechnology

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Summary

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It is generally accepted that the flavor quality of many fruits has significantly declined over recent decades. While some of this decline can be linked to selection for certain traits, such as firmness and postharvest shelf life, that run counter to good flavor, a major contributing factor has been the challenge of breeding for such a complex quality trait. Flavor involves integration of sugars, acids and a set of 20 or more volatile chemicals. Together, these compounds involve a large number of primary and secondary metabolic pathways, many of which have only recently been established. This review describes recent advances in the understanding of the pathways and genes controlling synthesis of the volatile components of flavor. Because of tomato's unique role as a model for fruit development, the review emphasizes advances in this fruit. In the last decade we have literally advanced from a list of chemicals known to influence flavor to a detailed understanding of how and where they are made. However, our knowledge of the regulation of the critical metabolic pathways is still limited. Nonetheless, the pieces are in place for rapid advances to be made in the manipulation of flavor chemistry in the immediate future.

I. Introduction

The term 'quality' is defined in dictionaries as the degree of excellence in a product. In the context of plant genetics,

breeding for quality means improving traits inherent in a crop that are independent of yield. Thus, quality includes flavor, nutrition, appearance and postharvest processing. Over the last 50 yr or more, important aspects of fruit

quality have been largely neglected. Indeed, much of the focus on yield that has resulted in cheaper, year-round produce availability runs counter to one important aspect of quality, namely flavor. Consumers have noticed a significant drop-off in flavor quality over the recent decades and produce flavor is a major source of consumer complaints. Happily, a growing segment of consumers is willing to pay a premium for flavor and many breeders are prioritizing this important quality attribute. However, breeding for better flavor presents many challenges. This review defines the problem and potential solutions to the unique challenges posed by quality improvement. Although this review emphasizes the work that has been done on a relatively small number of fruits, flavor quality can be considered a paradigm for improvement of many multigenic traits involving multiple metabolic pathways.

II. The importance of flavor and nutrition

Fruits contribute a large portion of vitamins, minerals, antioxidants, and fiber to the human diet. Fruits and vegetables are the major sources of micronutrients (vitamins and minerals) and phytonutrients (e.g. antioxidants) in the human diet and are integral to healthy lifestyles. The World Health Organization (2003) estimated that up to 50% of the world's population suffers from micronutrient malnutrition as a result of lack of ready access to fruits and vegetables. Approx. 100 million children suffer from vitamin A deficiency and up to 500 000 of them become blind every year. Folate deficiency can be as high as 50–70% in countries without fortification programs. Developing fruits and vegetables that are nutritionally superior can help to sustainably address these problems. Even in the developed world, fruits and vegetables with improved flavor would have a major impact on overall health. Better-tasting fruits would shift eating habits away from less healthy snack food alternatives, having a significant impact on nutrition. If we build better-tasting fruits and vegetables, the consumer will come.

While flavor improvement has clear human benefits, it is a difficult trait to modify, requiring integration of multiple primary and secondary biochemical pathways that are regulated by developmental, physiological and environmental cues. Effective manipulation requires knowledge of the pathways and the regulatory systems that control them. This review emphasizes our model of choice, tomato, but also highlights key research in other fruit crops that share many of the important metabolic and regulatory pathways. Tomato (*Solanum lycopersicum*, formerly *Lycopersicon esculentum*) is the most important vegetable crop in the USA and is an important source of essential nutrients worldwide. The molecular and genetic resources are excellent and include a draft genome sequence (<http://solgenomics.net>) as well as extensive collections of wild species accessions, cultivars, and mutants (<http://tgrc.ucdavis.edu/>). There is

an incredible diversity in chemical composition within the genus that can be readily introduced into the cultivated tomato. All of these tools have facilitated rapid progress in gene discovery and molecular breeding for a wide range of traits.

Despite the long history of tomato research and its pre-eminent status as a model for fruit development, most of the molecules detectable in a tomato fruit are still unidentified (Iijima *et al.*, 2008) and pathways for synthesis of many important secondary metabolites are not yet established. Even for defined metabolic pathways, regulatory mechanisms may not be fully understood. For example, despite having all of the genes encoding the early steps in carotenoid synthesis, we still cannot significantly increase carotenoid accumulation in a tomato fruit without negative phenotypic consequences (Sandmann *et al.*, 2006). Similarly, while the steps in folate synthesis have been elucidated, attempts at engineering the pathway indicate complex regulation (de la Garza *et al.*, 2007). As a consequence, metabolic engineering remains largely an iterative process of trial and error.

III. Defining the problem

To breed for improved flavor, we must first understand the complexity of the target. Human perception of 'flavor' involves integration of a massive amount of quantitative information from multiple sensory systems. For tomato this starts with the appearance of the fruit. Fruit color impacts the subjective evaluation of flavor, as do texture and mouth feel (Christensen, 1983; Causse *et al.*, 2001; Stommel *et al.*, 2005). Chemically, flavor is the sum of a large set of primary and secondary metabolites that are measured by the taste and olfactory systems. There are five classes of taste receptors in the mouth that recognize sweet, sour, salty, bitter and umami. The major tomato taste compounds are sugars (glucose and fructose) and acids (glutamate, citrate and malate). It is generally accepted that there must be a foundation of sufficient sugars and acids in an appropriate balance of sweet to sour for good flavor (Stevens *et al.*, 1977; Petro-Turza, 1987). But this is only the foundation. Flavor complexity is provided by the olfactory system (reviewed in Shepherd, 2006). Humans have *c.* 350 olfactory receptor genes and it is these receptors that provide the diversity of flavors we experience. As anyone who has suffered from a cold knows, olfaction is absolutely essential for flavor. More than 400 aroma volatiles have been identified in tomato, but only 15–20 are present in sufficient quantities to impact flavor (Buttery, 1993; Baldwin *et al.*, 2000). These compounds, summarized in Table 1, are derived from a diverse set of amino acid, carotenoid and fatty acid precursors. Because virtually all of these precursors are essential human nutrients, we have speculated that flavor volatiles may function as important cues for nutrient content (Goff & Klee, 2006). To further complicate flavor, there is cross-talk between the

Table 1 Volatile compounds that contribute to tomato flavor

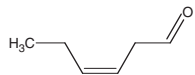
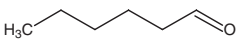
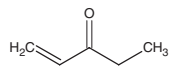
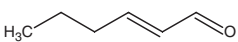
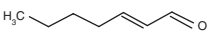
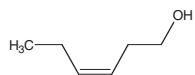
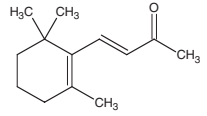
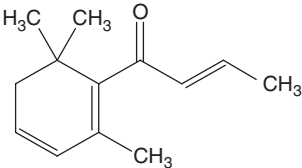
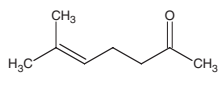
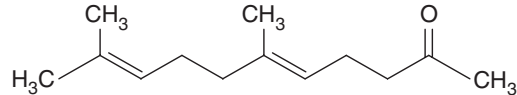
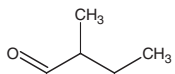
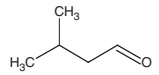
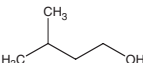
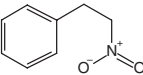
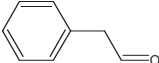
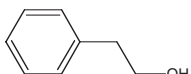
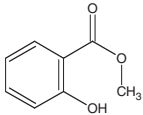
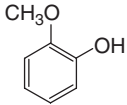
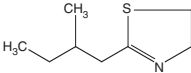
Volatile	Structure	Precursor
<i>cis</i> -3-hexenal		Fatty acid
Hexanal		Fatty acid
1-Penten-3-one		Fatty acid
<i>trans</i> -2-hexenal		Fatty acid
<i>trans</i> -2-heptenal		Fatty acid
<i>cis</i> -3-hexenol		Fatty acid
β -Ionone		Carotenoid
β -Damascenone		Carotenoid
6-Methyl-5-hepten-2-one		Carotenoid
Geranylacetone		Carotenoid
2-Methylbutanal		Isoleucine
3-Methylbutanal		Leucine

Table 1 (Continued)

Volatile	Structure	Precursor
3-Methylbutanol		Leucine
1-Nitro-2-phenylethane		Phenylalanine
Phenylacetaldehyde		Phenylalanine
2-Phenylethanol		Phenylalanine
Methyl salicylate		Phenylalanine and/or chorismate
Guaiacol (2-methoxyphenol)		Phenylalanine?
Isobutylthiazole		Unknown

Chemical structures and precursors of each are shown. The precise synthesis pathways for isobutylthiazole and guaiacol are not established. All of the volatiles except guaiacol shown are present in concentrations exceeding the average human threshold and are therefore believed to contribute to tomato aroma.

taste and olfactory systems; volatiles can impact the perception of sweetness and vice versa (Baldwin *et al.*, 2008). It is the sum of the volatiles, sugars and acids that our brains recognize as the unique flavor of a tomato. It must be noted that there are distinct individual taste preferences and there is no single, perfect tomato. There are, however, underlying commonalities that must be present for the fruit to be perceived as having good flavor.

A similar flavor constitution emerges in other fruits. In strawberry, sugars (sucrose, glucose and fructose) and acids are important for good flavor. Many of the acids in strawberries are volatile fatty acids. As in tomato, several hundred volatiles can be detected but only *c.* 15–20 are present in sufficient quantities to impact flavor (Larsen *et al.*, 1992; Schieberle & Hofmann, 1997). In contrast to tomato, a large proportion of the important volatiles in strawberry are esters and furanones. As with tomato, flavor has been largely ignored in classical strawberry breeding, with emphasis

being placed on disease resistance, yield, size, appearance and shelf life. Older varieties and species with superior flavor represent a reservoir for recapturing superior flavor (Ulrich *et al.*, 2007).

Another fruit with much flavor research is muskmelon (*Cucumis melo*). Sugars (sucrose, fructose and glucose) are the major contributors to flavor, with sweeter fruits being most desirable. Organic acids also contribute to flavor, the most abundant being succinate (Beaulieu *et al.*, 2003). There have been > 200 volatiles identified from various cultivars, the vast majority being esters, aldehydes and alcohols (Beaulieu & Grimm, 2001). Although there are varieties with outstanding taste, such as Charentais and Galia, they generally have short shelf lives, and the industry in the USA principally consists of long-shelf-life varieties with significantly less flavor.

Historically, much effort has been put into breeding varieties with higher sugar content. However, it has been

difficult to break the linkage between fruit size and sugar content; there is an inverse correlation between size and sugar. Efforts to increase sugars and solids in tomato have been described elsewhere (Klann *et al.*, 1996; Fridman *et al.*, 2004; Georgelis *et al.*, 2004) and will not be discussed further here. Varieties with sufficient sugar and acid exist, but without improvements in volatile content, flavor quality will always be perceived as inferior.

IV. Where did the flavor go?

There is tremendous heterogeneity for fruit color, size, shape and chemical composition among old, open-pollinated 'heirloom' varieties of tomato (Fig. 1). Paradoxically, DNA sequencing reveals very little polymorphism within the species (Nesbitt & Tanksley, 2002) and the molecular basis for the morphological and biochemical diversity has yet to be explained. The lack of flavor associated with modern tomato cultivars has emerged largely since the Second World War. Deterioration of flavor in the modern tomato has occurred for specific reasons. The grower is the customer of the seed company. Growers are not generally paid for good taste; they are paid for size and yield. Thus, breeding programs emphasize yield. Since 1970, USA tomato yields have increased almost threefold (<http://faostat.fao.org/>). Today's tomato must hold up to harsh treatment during harvest, shipping and storage. The modern hybrid salad tomato is large, picked too early, firm and frequently contains genes, such as *rin* or *nor* (Giovannoni, 2007), that delay ripening. Modern hybrids generally contain significantly lower amounts of sugars, acids and the important flavor volatiles (Goff & Klee, 2006). Possibly the most important factor in flavor loss, however, is that

breeders have lacked the tools to select for improvement in or even maintenance of flavor. It is extremely difficult to assay for the phenotype. Environment greatly impacts the concentrations of flavor-associated chemicals, and selection for good flavor by tasting large numbers of fruits in the field is impractical. Screening for the range of flavor chemicals is also beyond the capacity of most breeding programs. The best strategy for success is to identify markers that track flavor-associated chemicals. Rapid advances in molecular breeding tools and gene discovery promise to change the landscape. It must also be noted that postharvest handling can have a great impact on flavor quality. Selection of varieties particularly suited to withstand the postharvest handling chain would be highly desirable.

V. Identifying the important genes

The foundation for gene identification lies in our ability to measure the important flavor chemicals. Volatiles are quantified on a gas chromatograph (GC). Virtually all of the important flavor volatiles can be purchased as pure, food grade chemicals. Identities are validated by retention times and mass spectra. Concentrations of most volatiles in a sample can be determined by purge and trap headspace analysis in which the samples are heated to drive off and concentrate the volatiles (Baldwin *et al.*, 1991). Headspace analysis gives an accurate indication of the presence of the chemicals at the moment of sampling. However, some of the most important volatiles, such as β -ionone and β -damascenone, are present in very low concentrations and are almost impossible to quantify accurately. To circumvent this problem, volatiles can be captured on a resin trap (solid phase microextraction) to concentrate the less abundant

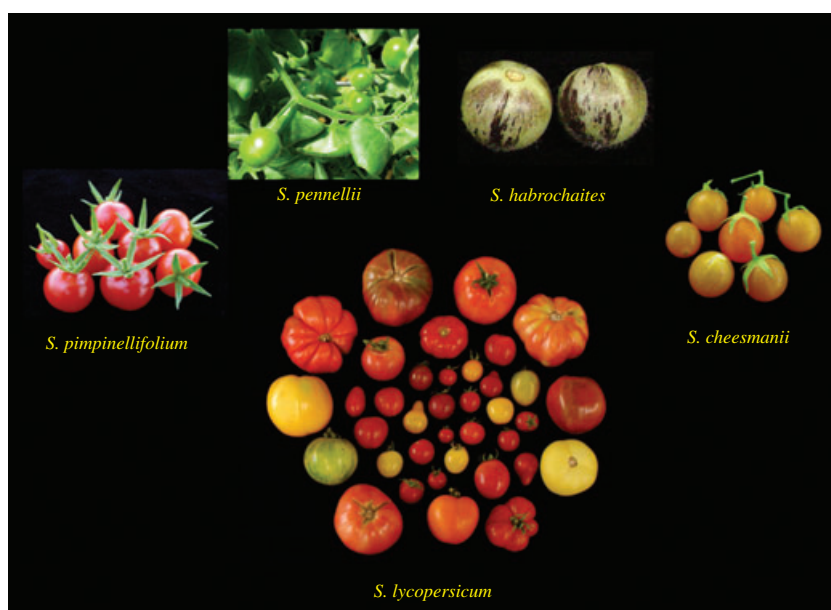


Fig. 1 Fruit diversity within *Solanum* sect. *lycopersicon*. Shown are fruits from four wild relatives of tomato: *S. pimpinellifolium*, *S. pennellii*, *S. habrochaites* and *S. cheesmanii*. Also shown are examples of the morphological diversity of fruit types within the *S. lycopersicum* species. Each *S. lycopersicum* fruit shown was taken from a different heirloom cultivar.

compounds (Buttery *et al.*, 1988). We have modified that method to collect released volatiles over an extended period (Tieman *et al.*, 2006b). For the purpose of gene identification, the most important factor is consistency in sample preparation and comparisons to appropriate control materials. Sample preparation and analysis are time- and labor-consuming and throughput is still a major issue.

The important flavor volatile genes broadly fall into two classes: those that encode enzymes responsible for synthesis of the end products and those encoding factors that regulate pathway output. Great progress has been made in defining the pathways for synthesis of flavor volatiles and identifying the genes encoding the biosynthetic enzymes. Some biosynthetic enzymes are rate-limiting to output and can thus be considered 'regulatory'. However, the regulation of metabolic pathway output is not well understood. The genetics indicate that the number of genes is likely to be quite large. In tomato, *c.* 100 quantitative trait loci (QTLs) affecting volatiles and their precursors have been identified (Saliba-Colombani *et al.*, 2001; Causse *et al.*, 2004; Schauer *et al.*, 2006; Tieman *et al.*, 2006b; Mathieu *et al.*, 2008).

1. QTL discovery

The major advantage of starting with QTLs is that they are, by definition, genes that significantly alter the target molecule. While the target is the responsible gene, a tightly linked marker is potentially valuable for breeding purposes. A necessary prerequisite to QTL discovery is the existence of substantial genetic variation in the target compound within the available germplasm. Fortunately, there is huge genetic variation within both *S. lycopersicum* accessions and the larger *Solanum* section *Lycopersicon* (Tieman *et al.*, 2006b; Mathieu *et al.*, 2008). Indeed, the contents of major flavor volatiles can differ by orders of magnitude even among heirloom varieties of *S. lycopersicum*. Similar variations occur for many fruits, most notably strawberry and melon (Jones, 1966; Ulrich *et al.*, 2007).

In contrast to efforts on sugar content, relatively little effort has been focused on flavor volatiles. High throughput assays are expensive and technically difficult. Despite the challenges, several groups have identified genes and QTLs affecting tomato volatile content as well as the key precursors of those volatiles. The genetic sources for the chemical variation are derived from either diverse germplasm within the species (Saliba-Colombani *et al.*, 2001; Causse *et al.*, 2004) or introgression lines (ILs) containing defined genomic segments of wild relatives such as *S. pennellii* (Schauer *et al.*, 2006; Tieman *et al.*, 2006b; Zhan *et al.*, 2009) or *S. habrochaites* (Mathieu *et al.*, 2008). Some of the QTLs specifically alter single volatiles while others affect larger sets of related or even unrelated volatiles. For example, there is a locus at the bottom of

chromosome 1, derived from either *S. pennellii* (Tieman *et al.*, 2006b) or *S. habrochaites* (Mathieu *et al.*, 2008), that specifically affects 2-methoxyphenol (guaiacol). By contrast, an *S. pennellii* locus at the bottom of chromosome 4 is altered in a very large set of both primary metabolites (Schauer *et al.*, 2006) and flavor volatiles (Tieman *et al.*, 2006b).

Although some flavor QTL work has been done in melon populations (Obando *et al.*, 2008; Paris *et al.*, 2008) and the important volatiles are known, flavor volatile-associated QTL analysis is limited. One paper describes QTLs in near-isogenic lines but the actual QTLs were not mapped (Obando-Ulloa *et al.*, 2008). These results indicate that the potential for QTL mapping exists in melons. In strawberry, no mapping of volatile loci has been published. Strawberry is significantly hindered by the genetics of the octoploid commercial cultivars as well as inbreeding depression. Some volatile QTLs have been identified in other fruit crops, including apple (Dunemann *et al.*, 2009) and grape (Doligez *et al.*, 2006; Duchene *et al.*, 2009).

VI. Biosynthetic gene isolation

A major limitation to progress in gene isolation has been the lack of information concerning plant secondary metabolism. Plants contain many thousands of secondary metabolites. The biosynthetic pathways and regulatory networks are known for only a small portion of the most economically important of these molecules. In the case of flavor-associated volatiles, even some of the most important ones, pathways for synthesis have only recently been established or remain to be established. Fortunately, analytical biochemistry has become significantly more powerful and cheaper in the last few years (Matsuda *et al.*, 2009) and we now have a good idea of how most of the important tomato flavor volatiles are synthesized. The current status of pathways for synthesis of the most important tomato flavor volatiles is summarized in Fig. 2.

Once the pathway for synthesis has been determined, identification of genes encoding biosynthetic enzymes can be accomplished by exploiting the extensive genome and expressed sequence tag (EST) databases for crop species. For example, the tomato database contains over 330 000 ESTs assembled into 46 849 unique sequences (<http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=tomato>). Similarly large EST sets exist for other fruit crops, including grape, orange, clementine, peach and apple. Newly available genome sequences for tomato, strawberry and melon can also be mined for genes not present as ESTs. Lists of candidate genes encoding a postulated activity can be assembled. Candidate enzymes are synthesized in *E. coli* and screened for activity. Genes that pass this stage of screening can then be validated for *in vivo* function using transgenic over- and underproduction. A major challenge to

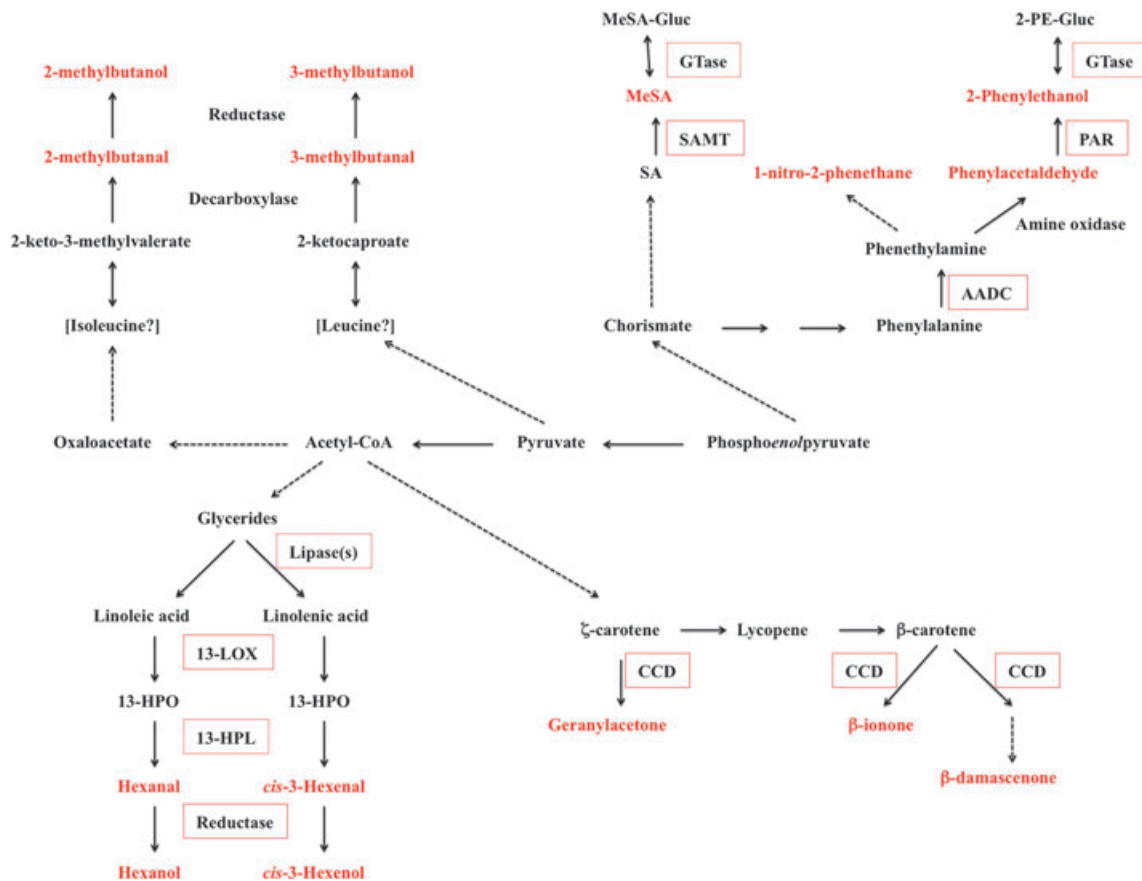


Fig. 2 Summary of metabolic pathways leading to flavor-associated volatile synthesis. The four broad classifications of volatiles include those related to branched-chain amino acids, phenylalanine, fatty acids and carotenoids. Flavor volatiles are shown in red. Dashed lines indicate multiple step reactions. Cloned genes involved in volatile synthesis are enclosed by a solid red line. Hypothesized, but as yet unidentified, enzymes are enclosed by a dashed red line. The branched-chain volatiles are synthesized from their respective α -keto acids. Since those acids are both the last intermediate in synthesis of the amino acids and the first intermediates in catabolism, it is not currently known whether the amino acids are intermediates in volatile synthesis. SAMT, salicylic acid methyltransferase; AADC, aromatic amino acid decarboxylase; PAR, phenylacetaldehyde reductase; CCD, carotenoid cleavage dioxygenase; 13-LOX, 13-lipoxygenase; 13-HPL, 13-hydroperoxide lyase.

validation is presented by gene families. In our experience, many biosynthetic activities are encoded by gene families (Tieman *et al.*, 2006a, 2007). Genetic redundancy greatly complicates the interpretation of results obtained from transgene analyses. A related challenge is that some steps in volatile synthesis are performed by enzymes with broad substrate specificities. For example, many of the important tomato flavor volatiles are aldehydes and their corresponding alcohols. Aldehyde-to-alcohol conversion is accomplished by enzymes classified as short-chain dehydrogenase/reductases (SDR). These enzymes can act upon many substrates and *in vitro* enzyme assays are not necessarily reflective of *in vivo* activity. RNAi loss-of-function experiments may be inconclusive because the targeted enzyme either does not efficiently recognize the substrate *in vivo* or there are multiple enzymes with overlapping functions that mask the loss of one. Despite these limitations, judicious combinations of *in vitro* and *in vivo* experiments usually permit assignment of gene function.

1. Synthesis of C6 volatiles

The C6 volatiles (hexanal, hexanol, *Z*-3-hexenal, *E*-2-hexenal and *Z*-3-hexenol) are among the most abundant tomato fruit volatiles. They provide the green, grassy notes associated with vegetative tissues and contribute to the flavor of many fruits and vegetables. Synthesis starts with the C18 fatty acids linoleic and linolenic acids, which are acted upon by 13-lipoxygenase to produce hydroperoxides. An anti-sense construct targeting the single tomato 13-LOX greatly reduces or eliminates synthesis of all the C6 volatiles (Chen *et al.*, 2004). The C6 aldehydes are subsequently released by hydroperoxide lyase(s). While the responsible lyases have not been identified in tomato, the gene has been characterized in potato (Vancanney *et al.*, 2001). There also appears to be a role for an as yet undefined isomerase that acts upon the C6 alkene. Finally, the first enzyme convincingly linked to tomato volatile production, ADH2, a member of the SDR family of enzymes, converts the aldehydes to alcohols

(Speirs *et al.*, 1998). Analysis of transgenic antisense plants indicates that a single activity converts both *Z*-3-hexenal and hexanal to their corresponding alcohols. Because an antisense construct utilizing the full-length cDNA was employed, the existence of highly similar redundant genes cannot be eliminated. Overexpression of *ADH2* did not affect volatile aldehyde-to-alcohol ratios, indicating that the enzyme is not generally rate-limiting in tomato fruits (Prestage *et al.*, 1999).

As has been mentioned, eliminating 13-LOX activity essentially wipes out production of the C6 volatiles. However, formation of these volatiles is regulated and LOX activity is not likely to be rate-limiting to their formation. During ripening, the C6 volatiles are synthesized at the turning stage (*c.* 50% ripe). In immature fruits or leaves, these volatiles are released only upon tissue damage where they probably act as wound signals. The 13-LOX enzyme is targeted to chloroplasts and probably does not come into contact with its substrates under normal circumstances (Chen *et al.*, 2004). Thus, the limiting step in C6 volatile production is likely to be action of an as yet undefined lipase and/or loss of integrity of membranes through cell disruption caused by chewing and/or ripening.

2. Synthesis of apocarotenoid volatiles

Apocarotenoid volatiles are among the most important contributors to flavored tomato and many economically important foods, as diverse as citrus and saffron. The cyclic apocarotenoids β -ionone and β -damascenone are characterized as fruity/floral. Humans are, for the most part, exquisitely sensitive to these molecules and they have extremely low odor thresholds – thus their importance to flavor despite their very low abundance. The linear apocarotenoids 6-methyl-5-hepten-2-one and geranylacetone are also classified as fruity/floral although their odor thresholds are significantly higher than the cyclic apocarotenoids. All but β -damascenone can be directly generated from their carotenoid precursors in tomato by the action of a pair of carotenoid cleavage dioxygenases, *LeCCD1A* and *LeCCD1B* (Simkin *et al.*, 2004). These enzymes are promiscuous, cleaving linear carotenoids at either the 5,6, the 7,8, or the 9,10 positions, and cyclic carotenoids at the 9,10 position (Vogel *et al.*, 2008; Ilg *et al.*, 2009). The enzymes recognize any carotenoid from ζ -carotene onward. Transgenic knock-down lines for *LeCCD1A* and *LeCCD1B* have significantly reduced concentrations of all apocarotenoid volatiles, but they are not eliminated (Simkin *et al.*, 2004), implicating one or more additional enzymes *in vivo*. That function may be provided by other members of the CCD gene family. *CCD4* and *CCD7* have both demonstrated abilities to cleave carotenoids at the appropriate bonds to generate these volatiles. The CCD enzymes are ubiquitous in plants. *CCD1* homologs have been identified in strawberry

(Garcia-Limones *et al.*, 2008), crocus (Rubio *et al.*, 2008), coffee (Simkin *et al.*, 2008), mandarin (Kato *et al.*, 2006), melon (Ibdah *et al.*, 2006) and grape (Mathieu *et al.*, 2005).

Much as with the C6 volatiles, synthesis of apocarotenoid volatiles is regulated in an unknown way. Apocarotenoid volatiles are synthesized only at the latest stage of ripening, even though the CCD enzymes are present throughout fruit development (Simkin *et al.*, 2004). Carotenoids are located within plastids: chloroplasts through most of fruit development and chromoplasts during ripening. The *CCD1* enzyme lacks chloroplast transit peptides, although a proteome study reported that it is associated with the outer chloroplast envelope (Joyard *et al.*, 2009). Thus a major enzyme is physically isolated from its substrates. *CCD4* and *CCD7*, on the other hand, are plastid-localized. Since the release of apocarotenoid volatiles coincides with the chloroplast-to-chromoplast conversion, substrate availability rather than enzyme synthesis appears to be limiting. Consistent with this view, all of the apocarotenoid volatile QTLs identified to date are associated with carotenoid biosynthetic enzymes, while no QTLs associate with any of the CCD-encoding genes (Lewinsohn *et al.*, 2005; Tieman *et al.*, 2006b). Thus, carotenoid content of the fruit determines the suite of synthesized volatiles.

3. Synthesis of amino acid-derived volatiles

Although the pathways are not well established, structural considerations support a metabolic linkage between branched-chain amino acids and a set of volatiles that includes 3-methylbutanal/ol, 2-methylbutanal/ol and isobutyl acetate. Since the final step in synthesis of these amino acids is also the first step in their catabolism, the volatiles could be synthesized from either the amino acid or their immediate precursors, the α -keto acids (Fig. 2). That reversible step is performed by a set of branched-chain aminotransferases (BCATs). There are six of these enzymes in tomato and they are distributed among chloroplasts, mitochondria and the cytoplasm (GS Maloney & HJ Klee, unpublished). Compartmentation is important as the biosynthetic enzymes are chloroplast-localized and the catabolic enzymes are mitochondrial. Application of amino acid to fruit pericarp segments does stimulate synthesis of the corresponding volatile and application of ^{13}C -labeled amino acid results in transfer of the label to the volatile. However, application of the corresponding α -keto acids to pericarp segments results in much higher volatile production (G. S. Maloney & H. J. Klee, unpublished). The most likely route for synthesis of the volatiles from the α -keto acids is decarboxylation by an as yet undefined enzyme to generate the aldehyde volatiles, followed by action of a SDR enzyme to generate the corresponding alcohol. Interestingly, the *ADH2* antisense transgenic lines altered in

C6 volatiles, mentioned earlier, also had a reduced capacity to convert 3-methylbutanal to 3-methylbutanol (Prestage *et al.*, 1999), suggesting that either the same or a highly homologous enzyme(s) is involved in synthesis of both sets of volatiles.

Progress has been made on elaborating the mechanisms for synthesis of the phenylalanine-derived volatiles (phenylacetaldehyde, 2-phenylethanol and 1-nitro-2-phenethane). Phenylacetaldehyde and 2-phenylethanol are important volatiles, contributing to the flavor and scent of many fruits and flowers. 2-Phenylethanol is the major constituent of rose scent and these volatiles have pleasant floral aromas that are highly desirable. In tomato, the first and rate-limiting step is performed by a family of aromatic amino acid decarboxylases (AADCs) (Tieman *et al.*, 2006a). These enzymes convert phenylalanine to phenethylamine, an unstable biogenic amine. Phenethylamine is converted to phenylacetaldehyde by an as yet unidentified amine oxidase or to 1-nitro-2-phenethane by an uncharacterized series of reactions. Interestingly, another member of the Solanaceae, *Petunia hybrida*, contains a bifunctional decarboxylase/amine oxidase enzyme that directly converts phenylalanine to phenylacetaldehyde (Kaminaga *et al.*, 2006). As would be predicted, petunia flowers do not synthesize 1-nitro-2-phenethane. The final step in the pathway, conversion of phenylacetaldehyde to 2-phenylethanol, is performed by a small family of phenylacetaldehyde reductases (Tieman *et al.*, 2007).

Methylsalicylate (MeSA) is synthesized from salicylic acid by an *O*-methyltransferase, SAMT (Ament *et al.*, 2010; Tieman *et al.*, 2010). The pathway for synthesis of salicylic acid is not fully established and there may be alternate pathways. Although the regulation of salicylic acid synthesis is not well understood, it is a known stress hormone, being induced by pathogen challenge. It appears that the tomato SAMT quantitatively affects MeSA synthesis and a QTL for MeSA synthesis derived from *S. pennellii* maps to the *SAMT* gene. Higher production of MeSA in lines containing that QTL is associated with increased *SAMT* transcript (Tieman *et al.*, 2010). MeSA is also known as oil of wintergreen and has been described as having a medicinal odor. Our work and that of others (Krumbein & Auerswald, 1998) with consumer taste panels indicate that it is negatively correlated with tomato flavor acceptability. Breeding for fruits with reduced concentrations of MeSA should be possible using markers for *SAMT* alleles.

4. Furanones

In the past few years, significant progress has been made in understanding the pathway for synthesis of an important set of furanone volatiles. 4-Hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF) has been isolated from multiple fruits, including pineapple (Rodin *et al.*, 1965) and strawberry. It

is also present in tomato but probably not at a sufficiently high concentration to impact flavor (Baldwin *et al.*, 2000). HDMF has been hypothesized to be synthesized from fructose-1,6-diphosphate (Roscher *et al.*, 1998; Schwab, 1998). The final step in HDMF synthesis from its immediate precursor, 4-hydroxy-5-methyl-2-methylene-3(2H)-furanone (HMMF), is performed by a quinone oxidoreductase (FaQR) (Raab *et al.*, 2006). HDMF is then either methylated by an *O*-methyltransferase, FaOMT, to 2,5-dimethyl-4-methoxy-3(2H)-furanone (DMMF) (Wein *et al.*, 2001) or glucosylated by one or more glucosyltransferases (Landmann *et al.*, 2007). HDMF, in particular, is an important volatile and it can be expected that identification of the genes responsible for its synthesis and subsequent metabolism will likely have an impact on breeding for improved flavor.

5. General considerations related to gene isolation

The large number of QTLs affecting flavor volatile synthesis suggests that there will be multiple points of regulation in the various metabolic pathways. Linking gene to function is the major challenge in the field. Since the regulatory networks controlling metabolic output are not well understood, the identities of many of the most important QTLs cannot be predicted. Some QTLs will certainly encode transcription factors. There are also large pools of nonvolatile sugar conjugates for many of the important flavor volatiles. Enzymes synthesizing and hydrolyzing these sugar conjugates are likely to influence the volatile pools. There are well over 1000 transcription factors and *c.* 150 functional glycosyltransferases encoded by the Arabidopsis genome (Bowles *et al.*, 2006) and there appear to be a similar number in tomato. But we can also expect that genes affecting the metabolic flux in other, as yet unidentified ways will have important influences upon pathway output. The challenge is to develop efficient methods for identifying the nonobvious genes. To accomplish this goal, we need more powerful tools.

One powerful approach involves integration of multiple datasets for correlating gene expression either temporally or spatially. For example, transcriptome profiling in wild-type vs mutant lines can provide information on functions of specific genes. Transcriptome profiling of different developmental stages is particularly useful if it can be correlated with appearance or disappearance of the target metabolite. For example, many flavor volatiles are synthesized specifically at the fully ripe stage of fruit development (Tieman *et al.*, 2006b; Schaffer *et al.*, 2007). Candidate gene lists can be prioritized by their patterns of expression across development. One of the earliest examples integrating gene expression and biochemistry to identify a flavor-associated gene involved a strawberry alcohol acyltransferase (SAAT) (Aharoni *et al.*, 2000). Using cDNA microarrays, a candidate

SAAT gene was identified based on its ripening-associated expression. Subsequent biochemical analyses demonstrated its role in synthesis of strawberry flavor volatiles.

Another application of -omics technology is the mining of transcriptome and metabolome data to identify genes whose expression is correlated with accumulation of the target metabolite across a population. Tieman *et al.* (2007) used correlations of gene expression with metabolite accumulation in a set of *S. pennellii* introgression lines to identify the phenylacetaldehyde reductases that convert phenylacetaldehyde to 2-phenylethanol. With a large set of genotypes having significant variation in the target compounds (phenylacetaldehyde and 2-phenylethanol), it was possible to identify genes whose expression tracked accumulation of the compounds. As metabolite and transcript databases become larger, this approach becomes more powerful. However, there are not established standards for data collection and formatting. And environmental variation can make comparisons between data collected at different sites impossible. Thus, transcriptome and metabolome datasets collected by different groups are not easily combined. Software for data analysis is usually written in-house (e.g. Fei *et al.*, 2006). Community standards for data collection are being developed and the future for *in silico* analysis is improving.

VII. Fixes

How do we achieve improved fruit flavor quality? There are two broad approaches to manipulating flavor chemical content: transgenes and molecular-assisted breeding. Both have advantages and disadvantages that will be addressed.

1. Transgenic approaches to flavor enhancement

A major advantage of transgenic flavor enhancement is that precise alterations in metabolic pathways can be engineered into cultivars that are already optimized for production traits such as disease resistance, yield and fruit appearance. A breeder does not have to restack essential traits or deal with linkage drag, especially if the flavor-associated trait is being introduced from a wild relative. For many fruit species, isogenic lines can be produced in months and immediately introduced into the pool of elite breeding stock. Rapid trait introduction is a major advantage for longer-lived species such as the tree crops where breeding for a specific trait like flavor would be nearly impossible. It would also be highly desirable in species with complex genetics such as the octoploid commercial strawberry.

Precise and completely novel alterations in flavor chemicals can be achieved with a transgene. For example, introduction of a basil (*Ocimum basilicum*) geraniol synthase into tomato caused large increases in volatile terpenoid compounds (Davidovich-Rikanati *et al.*, 2007). These

tomatoes were distinguishable from nontransgenic controls and 60% of panelists preferred them to the controls. Unfortunately, the diversion of isoprenoids into the terpenoid pathway resulted in lower concentrations of carotenoids and their volatile apocarotenoid derivatives. Nonetheless, the work shows that a subset of consumers respond positively to fruits with novel tastes. Similarly, we have engineered the set of phenylalanine-derived volatiles in tomato by overproduction of the first, rate-limiting step in the pathway, AADC (Tieman *et al.*, 2006a). These fruits exhibit specific increases of three- to 10-fold in 2-phenylethanol, phenylacetaldehyde and 1-nitro-2-phenethane. Consumers could distinguish them from the controls but there was no significant preference for the transgenic fruits (D. M. Tieman & H. J. Klee, unpublished). This result indicates that while it is possible to engineer volatile pathways, improving overall likeability will be more challenging.

A major hindrance to the use of transgenic materials for flavor improvement is the current regulatory environment. While the percentage of foods containing genetically modified (GM) ingredients is steadily increasing, almost all of the increase is in processed foods. There are still very few examples of unprocessed GM products on the market (http://www.aphis.usda.gov/brs/not_reg.html), the most notable being insect-resistant sweet corn, virus-resistant squash and virus-resistant papaya (Gonsalves *et al.*, 2006). Whether public resistance to unprocessed GM products is a significant barrier is arguable. What cannot be argued is that approval for GM products in the current regulatory system is expensive. Markets for most fruits and vegetables are highly fragmented and seed company product portfolios for crops like tomato and melon can be extensive. As a consequence, to introduce a trait into a product line, several to many independent transgenic events must be generated, characterized and registered. A trait would have to provide a very large return to justify the investment in transgenes. Today, it would be difficult to make that argument for flavor enhancement. The exceptions to this rule are the species with very long generation time, such as tree fruits, where it would take many years to introgress a desirable allele into a commercially suitable cultivar.

2. Molecular-assisted breeding

The foundation for breeding fruits with improved flavor is the incredible genetic and chemical diversity in the reservoir of materials available to breeders of such crops as tomato, melon and strawberry. Deterioration of flavor quality is a relatively recent event and excellent flavor exists today in many 'heirloom' cultivars of tomato, for example. But these cultivars usually fail miserably in large-scale commercial production compared with modern cultivars, since they lack many of the disease resistances of modern varieties and have

relatively poor yields. The idea of transferring complex traits controlled by many genes from a poor-yielding variety with good flavor into a modern, high-yielding cultivar would have been impossible even a few years ago. But the combination of genome and transcriptome sequencing, better understanding of the metabolic pathways, and high-throughput molecular marker screening now makes breeding for flavor much more realistic. Stacking of the multiple, independent genes likely to be needed for flavor quality improvement is now technically feasible. It is simply a matter of identifying the appropriate genes and alleles needed.

Through the use of both IL populations and crosses within the species, numerous QTLs affecting flavor constituents have been identified in tomato (Saliba-Colombani *et al.*, 2001; Causse *et al.*, 2004; Schauer *et al.*, 2006; Tieman *et al.*, 2006b; Mathieu *et al.*, 2008). Some of these QTLs have major effects on multiple primary and secondary metabolites. Some of the QTLs are mapped to well-defined regions and completion of the tomato genome sequence should lead to identification of many of these QTLs in the near future. The availability of genome sequences of tomato and closely related species will greatly accelerate the process of introgression of alleles from the wild relatives such as *S. pennellii*. Gene identification, in turn, should permit rapid introgression of these important flavor genes into breeding materials.

VIII. Conclusions

Identification of genes involved in synthesis of flavor volatiles has accelerated in the past few years, aided by rapid advances in genomics and metabolomics technologies. Most of the biosynthetic pathways for the most important flavor volatiles have been defined and many of the genes encoding the synthetic enzymes have been identified. Transgenes capable of altering many volatiles are already available. However, our knowledge of regulation of the pathways is still rudimentary. It is likely that fruits with improved flavor will require coordinate regulation of multiple biosynthetic pathways. The availability of genome sequences is already facilitating rapid advances in identification of the genes encoding the most important QTLs. It must be noted that the target concentrations for volatiles are not yet defined. While overall increases in many flavor volatiles would likely benefit taste, there will certainly be optimal concentrations that should not be exceeded. Once those optimal concentrations are defined, they should be achievable via either transgenes or introgression specific alleles.

Since many of the flavor-associated volatile compounds are common to different species, gene discovery in one organism facilitates advances in many others. The molecular toolbox is rapidly expanding. In parallel with gene discovery, we must identify appropriate germplasm containing

desirable alleles. Those alleles must be evaluated for their effects on flavor chemistry in multiple elite cultivars. As appropriate alleles are identified, progress should be very rapid and the consumer will reap the benefits of our scientific endeavor.

Finally, it must be noted that we have a rare opportunity to use the materials generated in flavor research to address fundamental questions about the very nature of human taste. What is the 'ideal' tasting tomato? How do volatiles influence taste attributes like sweetness? Transgenic plants engineered for alterations in one or a very few related chemicals permit us to ask precise questions about the contributions of specific chemicals to overall flavor, both positive and negative. While consumers certainly look forward to fruits with better flavor, the research community has the opportunity to greatly advance our understanding of the very nature of taste.

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