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**FOCUS PAPER**

# Metabolic regulation underlying tomato fruit development

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## Abstract

**The development and maturation of tomato fruits has received considerable attention because of both the uniqueness of such processes to the biology of plants and the importance of these fruits as a component of the human diet. Molecular and genetic analysis of fruit development, and especially ripening of fleshy fruits, has resulted in significant gains in knowledge over recent years. A large amount of knowledge has been gathered on ethylene biosynthesis and response, cell wall metabolism, and environmental factors, such as light, that impact ripening. Considerably less attention has been paid directly to the general metabolic shifts that underpin these responses. Given the vast complexity of fruit metabolism, the focus chosen for this review is on primary metabolites and those secondary metabolites that are important with respect to fruit quality. Here, recent advances in dissecting tomato metabolic pathways are reviewed. Also discussed are recent examples in which the combined application of metabolic and transcriptional profiling, aimed at identifying candidate genes for modifying metabolite contents, was used.**

Key words: Development, fruit metabolism, ripening, *Solanum*, tomato.

## Introduction

Fruits are not only colourful and flavoursome components of human and animal diets, but they are also an important source of minerals, vitamins, fibres, and antioxidants in food and animal feed. For this reason a fuller comprehension of the biosynthetic pathways for the production of these nutrients is of applied as well as fundamental importance. Whilst plant model systems such as *Arabidopsis* may be a suitable starting point in the search for key regulatory mechanisms acting in fruit development and ripening (Liljegren *et al*., 2004), it must be borne in mind that the term 'fruit' encompasses an enormous diversity of different kinds of organs. Thus, although fundamental development processes might be shared among different plant species, this cannot be blithely assumed. Indeed there are dramatic developmental differences across species, even in those of the same family (Fernie and Willmitzer, 2001). This fact is one of the main reasons that considerable effort is being put into genomic and post-genomic study of plant species other than *Arabidopsis* (Goff *et al.*, 2002; Carrari *et al*., 2004; Desbrosses *et al.*, 2005; Mueller *et al.*, 2005). One example of this is the use of tomato (*Solanum lycopersicum*), as a model system for plants bearing fleshy fruits. Several features of the tomato fruit make it a highly interesting system to study, all of them linked to the dramatic metabolic changes that occur during development. Tomato fruit follows a transition from partially photosynthetic to true heterotrophic metabolism during development by the parallel differentiation of chloroplasts into chromoplasts and the dominance of carotenoids and lycopene on ripening. This review will start by detailing briefly the recent advances in our understanding of the hormonal and genetic control of the ripening process that has been facilitated by the adoption of molecular genetic approaches (Vrebalov *et al*., 2002; Giovannoni, 2004), before focusing exclusively on metabolism. The rationale behind this is that whilst there are several excellent reviews in the field of genetic/hormonal control of ripening (Adams-Phillips *et al*., 2004; Giovannoni, 2001, 2004) and on the temporal regulation of specific areas of metabolism [for example, cell wall (Hadfield and Bennett, 1998; Rose *et al.*, 2004*b*) or pigments (Hirschberg, 2001)], a broad synthesis of the metabolic changes that underlie ripening has not been attempted recently. Whilst the majority of this article will

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concentrate on central carbon metabolism, since this is the subject of the majority of the authors' own research, it is also intended to document progress in the understanding of metabolic regulation of the secondary metabolites of importance to fruit quality. These include vitamins, volatiles, flavonoids, and pigments in addition to the major plant hormones. The interrelationship of these compound types is presented in Fig. 1. Given the recent development of tools that allow comprehensive phenotyping of the cell (Alba *et al*., 2004; Fei *et al*., 2004; Fernie *et al*., 2004*a*; Rose *et al*., 2004*a*), it is now possible to access vast datasets at the level of transcript abundance (Alba *et al*., 2004; Fei *et al*., 2004), protein abundance (Rose *et al*., 2004*a*), metabolite accumulation (Roessner-Tunali *et al*., 2003; Fernie *et al*., 2004*a*), and metabolic flux analysis (Roessner-Tunali *et al*., 2004). In conclusion, recent applications of multi-level phenotyping (Urbanczyk-Wochniak *et al*., 2003; Hirai *et al*., 2004) will be described and the likely outcome of taking such an approach detailed to understand better the metabolic regulation underlying tomato fruit development.

## Genetic and hormonal control of fruit development

As mentioned in the Introduction, the *Arabidopsis* model system has served as a starting point in the identification of genes influencing fruit development. In this vein, a gene family that has received considerable attention is that encoding MADS-box proteins (for a review, see Giovanonni, 2004). These floral homeotic genes are key determinants of carpel development. While many of these genes are highly represented in the large collection of the available expressed sequence tags for tomato (http://www.tigr.org/tdb/tgi/lgi and http://www.sgn.cornell.edu), only a few of them are specifically expressed at fruit development and at later stages, including ripening and senescence. An example of this is the *rin* locus, mutation at which affects all aspects of the tomato fruit ripening process. Molecular cloning of the *rin* locus revealed tandem MADS box genes separated by 2.6 kb of intervening genomic DNA but only one of them was necessary for ripening (Vrebalov *et al*., 2002). A phenotypically similar mutant in terms of responsiveness

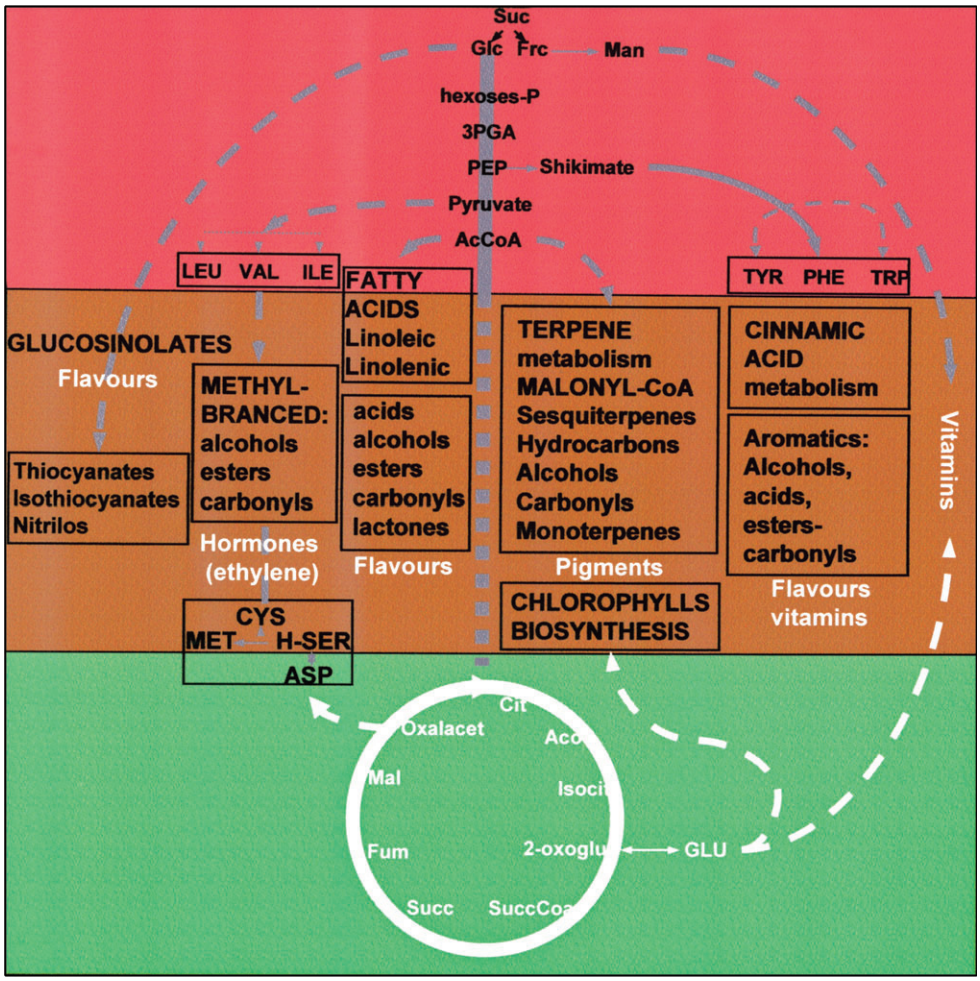


**Fig. 1.** Interrelationships of primary and secondary metabolism pathways leading to the biosynthesis of aroma volatiles, hormones, pigments, and vitamins.

to ethylene was characterized and named *nor* (non-ripening) (Lincoln and Fisher, 1988). Further genetic analysis showed that these two mutations reside in two different unlinked loci (Giovannoni *et al*., 1995); however, despite the fact that *nor* encodes a transcription factor (Giovannoni *et al*., 2001), the exact molecular mechanism of its operation remains unknown. Other fruit-ripening mutants identified on the basis of their insensitivity to ethylene are *Never ripe* (*Nr*) (Lanahan *et al.*, 1994), *green ripe* (*Gr*), and *Never ripe 2* (*Nr2*) (Barry *et al*., 2005). Since the first demonstration that the *Nr* locus encodes an ethylene receptor (Wilkinson *et al*., 1995) a broad gene family of receptors has been cloned and their expression analysed in several species (for a review, see Adams-Phillips *et al*., 2004). However, the analysis of transgenic plants with reduced *Nr* levels showed that this gene is not necessary for the ripening programme to proceed (Hackett *et al*., 2000), suggesting that the other fruit-specific member of the receptor family can compensate for its deficiency (Tieman *et al*., 2000). A further mutant worthy of mention is colourless non-ripening (*Cnr*), which results in mature fruits with colourless pericarp tissue showing excessive loss of cell adhesion (Thompson *et al*., 1999). Subsequent studies using PCR and biochemical analyses demonstrated that the expression and activity of a wide range of cell wall-degrading enzymes was altered in *cnr* during development and ripening. Further micro-array experiments demonstrated that the *Cnr* mutation had a profound effect on many aspects of ripening-related gene expression. The programme of gene expression in *Cnr* resembles, to some degree, that found in dehiscence or abscission zones, prompting speculation that there is a link between events controlling cell separation in tomato, a fleshy fruit, and those involved in the formation of dehiscence zones in dry fruits (Erickson *et al*., 2004).

Although the *Gr* and *Nr2* spontaneous mutants were identified in the early 1980s (Kerr, 1981, 1982; Jarret *et al*., 1984), it was only recently that they were physiologically characterized (Barry *et al*., 2005). As in the case of the *Nr* mutant, the reduction in the rate of ripening exhibited by *Gr* and *Nr2* also results from a decreased ethylene sensitivity of the fruits. However, as observed in *Cnr*, this phenotype was found to be extended to other unrelated ripening processes such as floral senescence, abscission, and root elongation. By genetic mapping these loci were found to be tightly linked on the long arm of chromosome 1. These results, when taken together with the similar dominant phenotypes of the mutants, suggest that they might be allelic; however, the molecular identities of *Gr* and *Nr2* genes remain unknown. As with other aspects of tomato fruit biology, the quantitative trait loci (QTL) approach has contributed to the definition of important characteristics of the tomato fruit development process. The first cloned QTL affecting tomato fruit weight (*fw2.2*) was dissected using a very well-characterized population of tomato near isogenic lines (Alpert and Tanksley, 1996). The open reading frame contained in this locus was predicted to encode a protein homologue to a human RAS oncogene (Frary *et al*., 2000), specifically expressed at pre-anthesis in floral organs. A recent analysis of transgenic tomato plants carrying an artificial gene dosage series confirmed this gene to be a negative regulator of pericarp cell division (Liu *et al*., 2003).

Climacteric fruits such as tomato are distinguished from non-climacteric by their increased respiration and ethylene biosynthesis rates during ripening. This is one of the main reasons that the majority of biochemical research has concentrated on this hormone. Initial molecular studies focused on the isolation of ethylene-regulated genes which include those encoding the ethylene biosynthesis enzymes (*S*-adenosylmethionine, SAM-synthase, 1-aminocyclopropane carboxylic acid, ACC-synthase, and ACC oxidase), and cell wall-disassembling enzymes such as endo-polygalacturonase and pectin methylesterase (PME) (reviewed by Redgwell and Fischer, 2002). It was later demonstrated, using the reverse genetic approach, that either lowering the amount of ethylene produced or delaying its production constituted a successful strategy to extend the shelf-life of fruits (Grierson, 1992). Moreover, the inhibition of ethylene biosynthesis in melon fruits by down-regulation of ACC oxidase has produced plants with improved flavour as the fruits could be left on the plants for longer before harvest (Ayub *et al*., 1996). Biochemical evidence suggests that ethylene production may well be influenced or regulated by interactions between its biosynthesis and other metabolic pathways. One such example is provided by the fact that *S*-adenosylmethionine is the substrate for both the polyamine pathway and nucleic acid methylation; the competition for substrate was demonstrated by the finding that the overexpression of a SAM hydrolase has been associated with inhibited ethylene production during ripening (Good *et al*., 1994). On the other hand, the methionine cycle directly links ethylene biosynthesis to the central pathways of primary metabolism.

A cummulative body of genetic and biochemical evidence led Klee (2002) to propose a model for ethylene perception and metabolism. As the receptor also acts as a negative regulator of downstream responses, in the absence of ethylene, receptors actively suppress expression of ethylene responsive genes. Consistent with this model, a reduction in the overall level of receptor increases ethylene responsiveness of a tissue, while higher expression of receptor decreases ethylene sensitivity. This model is supported by the fact that loss-of-function receptor mutants also exhibit a similar responsiveness level as wild-type plants, since they have lost the active suppression of response to ethylene. Recently, several further ethylene-inducible genes have been identified in tomato, including mitochondrial translation elongation factors (Benichou *et al*., 2003) and *CTR-1* (Leclercq *et al*., 2002; Adams-Philips

*et al*., 2004). It seems likely, given the development of micro-array resources for tomato, that significant advances will be made in understanding signal transduction following ethylene perception. Examples of this have already been started by using the first tomato cDNA micro-array containing 12 000 unique elements encoding 8500 genes covering a range of metabolic and developmental processes (http://bti.cornell.edu/CGEP/CGEP.html) (Fei *et al*., 2004; Baxter *et al*., 2005*b*).

In comparison with ethylene, very little is known about the role of other hormones in fruit development. The role of auxins has been extensively investigated in other fruits such as strawberry (Manning, 1994) and grape berries (Davies *et al*., 1997). In tomato, the fact that several expansins encoding genes are expressed during fruit development, and that they are regulated by auxins in other plant organs, led to the postulate that auxins are part of the hormonal signalling transduction network controlling cell expansion in tomato fruit (Catala *et al*., 2000). This hypothesis is further supported by the fact that the auxin concentration in tomato fruits peaks well before the onset of ripening [approximately at 10 d after anthesis (DAA)] coincident with a higher expression of fruit-specific expansin genes (Gillaspy *et al*., 1993). More recently, Balbi and Lomax (2003), by means of a thorough characterization of a set of the auxin-resistant mutants *dgt*, have proposed a cross-talk model of auxin responsiveness and ethylene biosynthesis at very early stages of fruit development. This finding opens a new route that merits further investigation to test whether this has implications in determining the final fruit size.

## Central carbon metabolism

To analyse metabolism proper it seems sensible to begin with the major carbohydrates, since this class of compounds comprises the most abundant and widely distributed food components derived from plants. Carbohydrate contents vary greatly in fresh tomato fruits depending on two main factors: the environmental conditions during development and ripening; and the cultivar in question. Most modern tomato varieties are derived from the domestication of the Peruvian wild cherry types, brought to Mexico by pre-Hispanic civilizations and spread over Europe in the sixteenth century (Luckwill, 1943). Since this fruit was initially used as a dessert, selection was orientated to sweetness with sugars representing up to 60% of the total dry weight. Sucrose, glucose, and fructose are the major sugars found in tomato fruits with high hexose accumulation being characteristic of domesticated tomato (*Solanum lycopersicum*), whereas some wild tomato species (i.e. *S. chmielewskii*) accumulate mostly sucrose (Yelle *et al*., 1991). Together with quinic and citric acid, these compounds are the principal quality components for 'ketchup' tomatoes, determining the soluble solid content or Brix index. The variance in relative levels of sucrose and hexoses is most likely due to the relative activities of the enzymes responsible for the degradation of sucrose – invertase and sucrose synthase. However, any discussion on sucrose metabolism in the fruit should begin by detailing the route by which carbon enters the fruit. This is, however, currently somewhat contentious since, although it has previously been suggested that unloading of sucrose occurs symplastically in the tomato pericarp until 14 DAA, following which it is unloaded apoplastically (Ruan and Patrick, 1995), recent results suggest a role for apoplastic unloading at a much earlier time point. The genetic bases of the sucrose-accumulation trait of the wild species tomato has been highly studied by means of introgressing wild germplasms into domesticated cultivars (Yelle *et al*., 1991; Fridman *et al*., 2000, 2004), and a role for an apoplastic invertase in regulating sucrose metabolism in tomato fruits has long been postulated. It was, however, not until recently that Fridman *et al*. (2004) provided conclusive evidence of the importance of this enzyme, by demonstrating that variance in its kinetic properties was the mechanistic explanation underlying a moderate QTL for Brix identified in a population derived from the cross *S. lycopersicum*×*S. pennellii* (Eshed and Zamir, 1995; Fridman *et al*., 2000). Previous to this study, map-based cloning had been used to delimit this QTL to a 484 bp region of the apoplastic invertase gene *Lin5* (Fridman *et al*., 2000). Although the expression pattern of this enzyme suggested that it was restricted to fruits and flowers (Fridman and Zamir, 2003), the exact reason for the Brix effect was unclear from these studies. Analysis of introgression lines from other wild species tomatoes, however, revealed a single nucleotide polymorphism that correlated with increased Brix (Fridman *et al*., 2004). Utilizing complementation assays in an invertase-deficient yeast strain it was possible to demonstrate that the wild allele had a far greater affinity for sucrose (Fridman *et al*., 2004), most probably due to the proximity of the single nucleotide polymorphism to the fructosyl binding site of the protein (Alberto *et al*., 2004). Other lines of evidence also support the role of this enzyme in regulating the sugar composition in tomato fruits and suggest that changes in composition contribute to alterations in fruit size. Utilizing the reverse genetic approach, Klann *et al.* (1996) reported that invertase antisense plants had increased sucrose and decreased hexose sugar concentrations in the fruits and 30% smaller fruits than those from control plants. Interestingly, a role for apoplastic invertase in the control of sink size has also been postulated previously in other species, the heterologous expression of yeast invertase in the potato tuber amyloplast resulting in dramatically increased yield, whereas the apoplastic invertase-deficient *miniature*1 mutant of maize exhibits a dramatically decreased seed size (Miller and Chourey, 1992; Sonnewald *et al*., 1997). A detailed biochemical characterization of vegetative and fruit tissues of the

introgression line carrying the *Lin5* wild allele (IL9-2-5) and harbouring the moderate Brix QTL, was recently reported by Baxter *et al*. (2005*a*). The finding in this work of an increased capacity of IL to take up sucrose from the phloem adds physiological support to the conclusions drawn by Fridman *et al.* (2004) concerning the key role played by the apoplastic invertase LIN5. Moreover, the fact that this line accumulates significantly more starch in both pericarp and columella tissues contributes new evidence on the importance of starch accumulation as a factor determining the soluble solids content of mature fruit (Dinar and Stevens, 1981; Schaffer and Petreikov, 1997).

The other enzyme with a proposed central role in developing tomato fruits is sucrose synthase (SuSy). D'Aoust *et al*. (1999), assessed the specific role of this enzyme in growing tomato fruits by means of silencing a fruit-specific isoform and found that, unlike in other sink organs [i.e. maize endosperm (Chourey and Nelson, 1978) or potato tuber (Zrenner *et al*., 1995)], SuSy activity was not essential for starch synthesis. However, its inhibition leads to a reduced unloading capacity of sucrose in the initial stages of fruit development (7 DAA) but only a small effect from 23 DAA onwards when ripening starts to take place. The influence of SuSy in the carbon metabolism of the fruit during the earliest stages of development runs in parallel with the highest demand for hexose phosphates (Roessner-Tunali *et al*., 2003), the rapid accumulation of starch and the highest levels of ADP-glucose pyrophosphorylase activity (Beckles *et al*., 2001). Thus, the reduced fruit size observed in the SuSy antisense plants may be explained, at least in part, by a reduction in starch degradation during the early stages of the fruit development. Unfortunately, however, this explanation remains speculative since no data on the starch contents in these fruits are provided during this period (D'Aoust *et al*., 1999). It should also be noted that these results were not reproduced in an independent transformation carried out by a different research group (Chengappa *et al*., 1999), who found little evidence for such an important role for SuSy in fruit metabolism and development. Furthermore, by marked contrast to the invertases, the only isoforms of sucrose synthase to have been mapped on the tomato genome to date do not co-localize with important agronomic QTL such as fruit size and total soluble solid content (Causse *et al*., 2004).

The use of introgression lines and other permanent genetic resources incorporating the diversity inherent in wild germplasm described here mirrors a broad and increasing interest in analysing the biological properties of natural genetic diversity (Maloof, 2003; Koornneef *et al*., 2004). In addition, it further highlights the enormous potential of exotic germplasm as a source for the improvement of agriculturally important traits (Zamir, 2001). Another recent example of this is provided by a comprehensive comparative analysis of the metabolite composition in leaves and fruits from six tomato species reported by Schauer *et al*. (2005). This study revealed that there is a tremendous variance in both leaves and fruits of the wild species analysed with respect to the sugar content, as well as dramatic changes in amino acid composition and secondary metabolite levels. However, somewhat surprisingly, the levels of the TCA cycle intermediates are invariant across the species. The reported changes in sugar levels are in close agreement with the above-mentioned results reporting high variability in invertase activities in fruits of wild species tomatoes (Yelle *et al*., 1991; Fridman *et al.,* 2004), whilst those in other metabolites may be explained by adaptation to the various ecological niches that the wild species are found in. Given that these species can be readily crossed, this dataset provides an interesting inventory that may eventually prove useful in the selection of breeding material as an alternative to current transgenesis-based metabolic engineering strategies (Carrari *et al*., 2003*a*).

In an ongoing project in our laboratories we are analysing the metabolite contents of the 76 introgression line population harbouring segments of the entire *S. pennellii* genome in the background of the elite processing cultivar M82 (Eshed and Zamir, 1995). Once complete, this will allow the identification of QTL for a wide range of metabolites, including those of nutritional and organoleptic importance. Such studies have been carried out previously, albeit on a smaller scale, and a handful of metabolite QTL including sugars and organic acids have been determined (Causse *et al*., 2002, 2004; Fulton *et al.*, 2002; Lecomte *et al*., 2004). It is likely, given the tomato sequencing project (Mueller *et al*., 2005*a*, *b*) and the current interest in the broad phenotyping of natural variance in crop species (European Plant Science Organisation, 2005), that such approaches will play a major role in the elucidation of key regulators of fruit metabolism.

As a first experiment in this direction, an established metabolic profiling method (Roessner *et al*., 2001) was optimized for tomato tissues and then, utilizing this method in combination with different analytical technologies (including conventional spectrophotometric and liquid chromatography) and statistical tools, the metabolite composition in developing tomato fruits was catalogued and evaluated (Roessner-Tunali *et al*., 2003). Through the analysis of over 70 primary metabolites it was possible to differentiate three developmental stages of the fruits (green, orange, and red) and follow the influence of hexose phosphorylation through fruit development by analysing transgenic plants constitutively overexpressing an *Arabidopsis* hexokinase (*At*HXK1). The changes observed in metabolite levels during ripening of the wild-type fruit were broadly similar to those previously reported for less extensive metabolic surveys (Boggio *et al*., 2000; Chen *et al*., 2001), with the major changes between green and red fruit contents summarized in Fig. 2. As illustrated, there is a large increase in the major hexoses, glucose and fructose,


**Fig. 2.** Schematic representation of the metabolic changes occurring in the transition from development to ripening processes in tomato fruits. Sugars, sugar-phosphates, sugar-alcohols, amino and organic acids, pigments, and cell wall components were determined in pericarps of tomato samples taken from 30 d until 60 d after anthesis (DAA). Names of metabolites in orange, green, and grey indicate increased, decreased, and no changes, respectively, in the levels of the corresponding metabolite at 60 DAA with respect to 30 DAA. Names in white letters indicate that the corresponding metabolite was not determined, and are included in the graph for explanatory reasons only.

in the cell wall components, and in the aromatic amino acids and aspartate, lysine, methionine, and cysteine, and, as expected, in all the pigments other than chlorophyll. By contrast, almost all of the TCA intermediates decrease in the red fruits, as well as sucrose, hexose phosphates, and most of the sugar alcohols. Although the point-by-point analysis of the changes of specific metabolites over developmental time was highly interesting, two main conclusions emerged from this study: (i) that tomato fruits of different developmental stage can be distinguished from one another on the basis of their metabolic complement alone; and (ii) that the influence of hexose phosphorylation on primary metabolism diminishes markedly over developmental time. At the same time a similar dataset was produced on the same transgenic plants (Menu *et al*., 2004) from which similar conclusions can be drawn.

Together with SuSy and HXK, fructokinase (FRK) forms the pool of hexose phosphates subsequently used as substrates for respiration and starch biosynthesis. Two different isoforms of this enzyme (FRK1 and 2) have been detected in tomato fruits exhibiting temporal and spatially distinct expression patterns (Kanayama *et al*., 1997, 1998). However, although both FRK1 and 2 enzymes have been shown to play a role in floral initiation and abortion, seed number, and stem and root growth in tomato plants (Odanaka *et al.*, 2002), their role in fruit metabolism has received far less attention to date. The regulation of the hexose content itself has recently received considerable interest following the construction of a functional linkage map of the carbohydrate metabolic pathway of the tomato fruit (Levin *et al*., 2004). This map aided the discovery of two interacting chromosomal regions introgressed from *S. habrochaites*, leading to an almost 3-fold epistatic increase in the fructose to glucose ratio in the mature fruit (Levin *et al*., 2000); however, the mechanistic reasoning for this is yet to be elucidated. Earlier work provided a study of the sucrose to starch transition in the tomato fruit and suggested that the activities of sucrose synthase, fructokinase, and AGPase are likely to share control of the rate of starch accumulation (Schaffer and Petreikov, 1997). The recent application of the theory of metabolic control analysis to the same pathway in potato tubers suggested that only AGPase exhibited considerable control of starch synthesis (Geigenberger *et al*., 2005; Davies *et al*., 2005); however, it should be noted that these studies are not directly comparable. In another study focusing on starch metabolism in the fruit the contribution of fruit photosynthesis to the total photosynthate incorporated into the fruit was assessed via transgenesis. For this purpose, the expression of the plastidial fructose bisphosphatase was inhibited in

a fruit-specific manner utilizing the antisense approach (Obiadalla-Ali *et al*., 2004*b*). The resultant transgenic lines exhibited surprisingly few changes in their carbohydrate metabolism but displayed considerably decreased fruit size. Intriguingly, the decrease in size was quantitatively similar to previous estimates of the contribution of the fruit to the production of the photosynthate, which it utilizes, made in earlier physiological studies (Guan and Janes, 1991*a*, *b*). Although clearly of central importance to the tomato fruit, relatively little is currently known concerning the regulation of glycolysis and the conversion of hexose phosphates into organic acids. Similarly, although organic acids are of fundamental importance at the cellular level for several biochemical pathways and at the whole organism level, their study has received much less attention than that of the sugars to date. Indeed the TCA cycle in plants is very poorly characterized in general and, although the structure of the pathway is well known, its regulation is not (Fernie *et al*., 2004*b*). In addition to the fundamental importance of understanding this pathway, its manipulation also has value from a metabolic engineering perspective with the organic acid to sugar ratio of particular pertinence, since it defines quality parameters at harvest time. Furthermore, especially in red fruits which contain little starch, glycolysis and respiration represent the dominant carbon fluxes in the fruit (Rontein *et al*., 2002; F Carrari and AR Fernie, unpublished results). Interestingly, the relative fluxes through the central metabolic pathways do not alter massively through the life cycle of suspension-cultured tomato cells, whilst those of anabolic pathways such as starch synthesis and the biosynthesis of amino acids and cell wall polysaccharides are low and variable (Rontein *et al*., 2002). The high-value metabolites derived from carbon skeletons provided by the central pathway encompass many more pathways than these, however, including fatty acids (Browse and Somerville, 1991), flavonoids (Dooner *et al.*, 1991; Fatland *et al*., 2002), pigments (Mann *et al*., 2000), alkaloids (Hughes and Shanks, 2002), and isoprenoids (Lange *et al.*, 2001), some of which are briefly discussed below. The exhaustive analysis of this pathway should, therefore, not only yield answers to very important fundamental biological questions but may also find useful application. Recently, a research project has been initiated focusing on organic acid metabolism in tomato. As part of this ongoing project to determine the role of the mitochondrial TCA cycle in plants, studies were first concentrated on the illuminated leaf. Comprehensive phenotyping of an aconitase mutant (*Aco*1) of *Solanum pennellii* (Carrari *et al*., 2003*b*), as well as *S. lycopersicum* plants in which the mitochondrial malate dehydrogenase (mMDH) was repressed via antisense and RNA interference techniques (Nunes-Nesi *et al*., 2005), uncovered large changes in both leaf metabolism and in plant performance. Biochemical analysis of the *Aco*1 mutant revealed that it exhibited a decreased flux through the TCA cycle, decreased levels of TCA cycle intermediates, and enhanced carbon assimilation. In addition, although it must be borne in mind that *S. pennellii* is a green-fruited species bearing very small fruits (Schauer *et al.*, 2005), these plants were characterized by a dramatically increased fruit weight. Studies in Fernie's laboratory (Nunes-Nesi *et al*., 2005) on the mMDH antisense plants revealed that decreased activity of this enzyme in the elite cultivated species *S. lycopersicum* also resulted in enhanced photosynthetic activity and in an increment in fruit dry weight. Despite the fact that much research work is needed to understand the exact reasons for the increment in the fruit dry matter, manipulation of central organic acids is clearly a promising approach to enhance tomato fruit yield. Transcript and metabolite profiling of leaf material from these lines suggests that some of the increase in photosynthetic capacity is due to an elevated expression of genes associated with photosynthesis (Urbanczyk-Wochniak *et al*., unpublished data), perhaps as a consequence of the increased levels of ascorbate found in these plants (Kiddle *et al*., 2003).

## Cell wall metabolism

The integrity of the fruit cells can be ascribed to wall-to-wall adhesion between cells and the strength of the primary wall. These traits have been described as critical factors influencing the perception of the fruit textures by the consumers (Pitt and Chen, 1983). Fleshy fruits such as tomatoes are predominantly composed of parenchyma cells enclosed by an unlignified layer of cellulose microfibrils suspended in a matrix of glycoproteins, water, and pectic and hemicellulose polysaccharides. The latter accounts for 90% of the cell wall (Redgwell and Fischer, 2002), with cell wall polysaccharides largely derived from sugars and sugar phosphates (Scheible and Pauly, 2004). Tomato fruit development is marked by significant changes in the cell wall components and a handful of polysaccharide-degrading enzymes has received much attention over the last 15 years. The activity of these enzymes is directly linked to the shelf-life of the fruits, one of the characteristics crucial to the tomato market.

Endo-polygalacturonase has been the most studied among the enzymes involved in cell-wall metabolism. Polygalacturonase catalyses the hydrolysis of the linear α-1,4-D-galacturonan backbone of pectic polysaccharides and, alongside the mRNA level, its activity increases dramatically during tomato ripening (Della Penna *et al.*, 1986). Rhamnogalacturonase and β-galactosidase (TBG) are enzymes which depolymerize branched pectins resistant to attack by endo-polygalacturonase. Rhamnogalacturonase and TBG have been purified and found to be highly active in tomato fruits (Gross *et al*., 1995). At least seven tomato *TBG* genes are expressed during fruit development (Smith and Gross, 2000); six are known to be expressed

during ripening and the products of five of them are predicted to be targeted to the cell wall. Furthermore the functionality of three of these genes (*TBG1*, *3*, and *4*) has been assessed in tomato via transgenesis. Whilst reducing the expression of *TBG1* did not result in changes in texture or cell wall composition (Carey *et al*., 2001), antisense suppression of the *TBG3* gene led to an increase in wall galactosyl content, an increased proportion of insoluble solids, and slightly increased viscosity (de Silva and Verhoeyen, 1998), and suppression of *TBG4* resulted in increased fruit cracking, reduced locular space, and a doubling in the thickness of the fruit cuticle (Moctezuma *et al*., 2003), in addition to a decrease in fruit softening (Smith *et al*., 2002).

PME catalyses the de-esterification of pectin. In tomato, PME arises from the expression of three genes (Tucker and Zhang, 1996). When a fruit-specific *PME* (*PME2*) was down-regulated in tomato, the degree of softening during ripening was unaltered, but upon storage at room temperature for 7 weeks, the transgenic fruit lost tissue integrity while the wild-types held their cohesiveness. Thus, reduced pectin depolymerization had a negative effect on shelf-life (Tieman *et al*., 1992).

Endo-β-1,4-glucanases (or cellulase, EGase) are a class of enzymes which degrade carboxymethylcellulose. Their activity is associated with softening in tomato and other fruits, suggesting a role in ripening. EGases are encoded by a seven-member gene family (Brummell *et al*., 1999) and antisense suppression of a fruit-specific member caused no change in the pattern of softening, but the abscission zones of the transgenic fruit were strengthened. In addition, xyloglucan endotransglycosylase which cleaves the xyloglucan molecules of the wall has been implicated in ripening-related changes to the fruit cell wall in tomato (Maclachlan and Brady, 1994).

Whilst the above list of enzymes is relatively extensive, it actually only constitutes a few examples of the wall-modifying proteins, with numerous new classes remaining to be discovered. It is also rather cursory due both to space limitations and the plethora of high quality reviews that characterize this area of metabolism (Pilling and Hofte, 2003; Rose *et al*., 2004*b*; Scheible and Pauly, 2004). A further complexity arises when non-enzymatic mechanisms of cell wall changes are considered. One such example are the expansins—small proteins that catalyse cell-wall extension and for which at least 10 distinct genes have been identified in the tomato. A member of this family (*LeExp2*) is expressed in several growing tissues and has been demonstrated to be induced by physiological concentrations of auxins. Moreover, during fruit development it is co-expressed with xyloglucan- endotransglycosylase-, and EGase-encoding genes (Catala *et al*., 2000), suggesting cross-talk between hormone and cell wall metabolism. Surprisingly, another α-expansin gene from tomato (*LeExp1*) was found to be specifically and abundantly expressed in ripening fruit where cell expansion was supposed not to occur (Rose *et al*., 1997). Again, this protein has been shown to be ethylene-induced in tomato fruits and other species and differentially regulated in the *rin* (ripening inhibitor) and in the ethylene receptor *Nr* (Never ripe) mutants (Rose *et al*., 2000). As cell division and ripening are physiologically distinct, the role played by expansins during these processes remains obscure (Bertin, 2005). In addition to direct studies of cell wall metabolism the recent elucidation of an alternative pathway for ascorbate biosynthesis in strawberry that utilizes glucuronic acid, presumably derived from cell wall breakdown (Valpuesta and Botella, 2004), is of high interest. Although homologues for the genes encoding the necessary enzymes have not yet been identified in tomato, the presence of this pathway currently remains an open question. Recently, efforts have begun to establish proteomics technical platforms in order to characterize the differences in wall structure and composition that occur during tomato fruit development and ripening (see Rose *et al*., 2004*a*, *b*), and the adoption of systems biology approaches to study the cell wall have been championed (Somerville *et al.*, 2004). It is thus likely that, in the coming years, our understanding both of the co-ordination of cell wall metabolism during fruit development and the consequences of temporal changes in wall metabolism on fruit metabolism, and morphology in general, will be furthered.

## Pigments and flavonoids

Pigments of ripe fruits are not only attractive to consumers, but are also beneficial for health, including protection from cancers, and it is well documented that carotenoid deficiencies may cause blindness (Mayne, 1996). They are considered essential nutrients as they cannot be synthesized *de novo* in the human body. In plants, pigments and flavonoids are derived from acetyl-CoA metabolism through conversion to mevalonic acid, and from phenylalanine metabolism through the action of the PAL (phenylalanine ammonia-lyase) enzyme, respectively. Moreover, carotenoids with a beta-ring end group are required for the synthesis of vitamin A.

Tomato fruits are the principal dietary source of carotenoids in many Western diets. Almost all the enzymes acting in the carotenoid biosynthesis pathway have been cloned, and their manipulation has been the subject of various metabolic engineering approaches aimed at enhancing pigment quantity and quality (reviewed in Hirschberg, 2001). A null mutation in the gene encoding a chromoplast-specific phytoene synthase (*Psy1*) is one of the reasons for the lack of pigmentation in the green-fruited species *Solanum pennellii* (Ronen *et al*., 2000). Up-regulation of *Psy1* in tomato fruits resulted in redirection of GGPP to the gibberellin pathway yielding dwarf plants (Fray *et al*.,

1995). However, overexpressing the *psy* gene of different species in rice caryopses has resulted in a highly successful biotechnological application—the considerable increment in the contents of pro-vitamin A in the cases of Golden Rice (Ye *et al*., 2000) and Golden Rice 2 (Paine *et al*., 2005). By contrast, the heterologous expression of a phytoene desaturase (*Pds*) from *Erwinia uredovora* in tomato resulted in a significant increment of the β-carotene levels at the expense of lycopene and the total level of carotenoids (Römer *et al*., 2000). Alterations in the pigment accumulation patterns have been observed in several spontaneously occurring tomato mutants, and two recent reports extolled the potential of these genetic tools for the manipulation of nutritional components. In the recessive mutant *high pigment* (*hp*), carotenoid levels are twice that in wild-type fruits (Yen *et al*., 1997); in addition, many other antioxidants are increased (Bino *et al.*, 2005). *Hp* carries a mutation in a tomato *UV-DAMAGED DNA-BINDING PROTEIN 1* (*DDB1*) homologue (Liu *et al*., 2004) whose *Arabidopsis* counterpart interacts with the product of *hp2* locus *DET1*. These two mutants display a similar phenotype regarding fruit pigmentation, their products being members of the same light signal cascade (Schroeder *et al*., 2002). Understanding their key control points will render the possibility of manipulating nutritional characteristics of the fruits. Recent success stories include the elevation of both lycopene and β-carotene exhibited following the fruit-specific silencing of the endogenous photomorphogenesis regulatory gene *DET1* (Davuluri *et al*., 2005). Similarly, an increase in both carotenoid and flavonoid content following the overexpression of the cryptochrome, CRY2 (Giliberto *et al*., 2005), and the production of high-flavonol tomatoes following heterologous expression of the maize transcription factor genes, LC and C1, have been reported (Bovy *et al*., 2002).

Another, non-pigment-derived vitamin of high importance is folate. It has been calculated that more than one-third of the folate in an average diet is provided by fruits and vegetables (FDA; http://www.fda.gov/fdac/features/796_fol.html). Plants synthesize folate from pteridine, but levels of this molecule are very low in tomato fruits. A novel approach reported by Diaz de la Garza *et al*. (2004) achieved a significant increment in folate levels following the overexpression of a non-regulated synthetic gene based on mammalian GTP cyclohydrolase I (Basset *et al*., 2002).

Much less attention has been placed on antioxidants with non-vitamin activity. However, recently, Giovinazzo *et al.* (2005) have produced tomato transgenic plants for a stilbene synthase from grape. The overexpression of this gene results in increased competition for substrates of the anthocyanin pathways, thus resulting in an increment in the levels of resveratrol, ascorbate, and glutathione. The above-mentioned examples constitute only a few of the recent findings utilizing transgenesis to engineer pigment and flavonoid levels in tomato fruits. Notwithstanding these successes, which are largely based on a relatively limited number of genetic manipulations, a recent report indicates that a diverse network of processes control pigment contents of tomato (Liu *et al*., 2003). Liu *et al*. (2003) utilized the *S. pennelli*×*S. lycopersicum* introgression lines described above to identify 19 QTLs for fruit colour, and analysed the co-localization of these QTLs with loci corresponding to carotenoid-related sequences. This candidate gene approach proved to be efficient for the identification of sequences that regulate fruit colour qualitatively, but not for the quantitative variation in colour or the regulation of pigment accumulation. As such, this report hints at further, as yet unidentified, factors that control the accumulation of pigments within the tomato fruit. The elucidation of these factors thus represents a significant challenge for understanding and influencing pigment content.

## Volatiles

At the onset of ripening the vast array of volatile compounds produced by tomato fruits are responsible for their flavour and aroma characteristics. These compounds are sensed orally and nasally and are the final determinant of consumers' choice of food. Plants have been reported to emit >1000 low-molecular-weight organic compounds (Knudsen *et al*., 1993). As in other areas of plant biology, major progress has recently been made in understanding plant volatiles via the application of molecular and biochemical techniques (reviewed in Dudareva *et al*., 2004). From the 400 different volatile compounds that tomato fruits are estimated to contain, the principal contributors to the ripe tomato flavour are *cis*-3-hexanal, *cis*-3-hexanol, hexanal, 3-methylbutanal, 6-methyl-5-hepten-2-one, 1-pentan-3-one, *trans*-2-hexanal, methyl salicylate, 2-isobutylthiazole, and β-ionone (Buttery and Ling, 1993). Among this group, esters are the most commonly detected in tomato fruits and their biosynthesis is catalysed by the enzyme alcohol acetyltransferase. Moreover, the availability of ester precursors may also play a role in determining the nature of the volatiles to be formed. The major source of esters is derived from the metabolism of pyruvate through its conversion to acetyl CoA by the pyruvate dehydrogenase complex or via pyruvate decarboxylase to acetaldehyde and, subsequently, to ethanol by alcohol dehydrogenase. Thus, these three enzymes are good candidates for the manipulation of fruit flavour, with modification of the alcohol dehydrogenase levels being demonstrated to be a successful strategy to modify the contents of hexanol and *cis*-3-hexenol (Speirs *et al*., 1998; Prestage *et al*., 1999). In another recent example, the *carotenoid cleavage dioxygenase 1* genes were demonstrated via transgenesis to contribute to the formation of the flavour volatiles β-ionine, psuedosonone, and geranylactone (Simkin *et al*., 2004). Another

volatile compound which influences flavour quality of tomatoes is the acyclic monoterpene alcohol, linalool (Buttery *et al*., 1990). *S*-Linalool is the product of the reaction catalysed by linalool synthase (LIS) which uses geranyldiphosphate (GPP) as substrate, and the expression of a heterologous *LIS* gene from *Clarkia breweri* in tomato under a fruit-specific promoter yielded plants displaying considerably higher levels of *S*-linalool and 8-hydroxylinalool (Lewinsohn *et al*., 2001). However, although this constitutes a clear example of the possibility of increasing the tomato fruit aroma, it remains to be tested whether the resultant fruits are actually preferred by the consumers.

A complementary approach, again utilizing broad genetic crosses, has been taken by Causse *et al*. (2002) who identified QTL for organoleptic properties of tomatoes. The lines identified as preferable by the consumer could now be comprehensively characterized with respect to volatile and non-volatile compounds alike. It is clear that not all volatile compounds will confer positive taste attributes to tomato. One such example that this is the case was provided by the identification of *malodorous* a wild species allele affecting tomato aroma that was selected against during domestication (Tadmor *et al*., 2002). However, it is perhaps not surprising that some of the chemicals emitted by plants taste bad to us, given that the plant produces many of them as protectants from pests. A combined metabolic, genomic, and biochemical analysis of glandular trichomes from the wild tomato species *S. habrochaites* recently identified a key enzyme in the biosynthesis of methylketones which serve this purpose (Fridman *et al*., 2005). To summarize this section, it seems fair to say that, in recent years, there have been dramatic improvements in the knowledge of tomato volatiles; however, there is still a great deal of work to be done before it can be claimed that the understanding of their biosynthesis is comprehensive.

## Conclusions and future perspectives

The majority of the studies detailed above were carried out exclusively on pericarp tissue or at the whole-fruit level. Whilst this provides important information, it is worth noting that it is now well accepted that the metabolism in the fruit pericarp is different from that in the placenta, and this is even different from that in the columella (Obiadalla-Ali *et al*., 2004*a*; Baxter *et al*., 2005*a*). These differences are not only evidenced by the contrasting concentrations of the major sugars and starches (Obiadalla-Ali *et al*., 2004*a*) but also by the differences observed between the tissues in the activities of several enzymes involved in glycolysis, Calvin cycle, and sucrose degradation (Obiadalla-Ali *et al*., 2004*a*). The data presented in this study also revealed an up-regulation of glycolysis just prior to the onset of ripening that, together with the increment in the ethylene biosynthesis rates, is the main feature which distinguishes climacteric fruit, such as tomato, from non-climacteric fruits. To recapitulate, whilst great progress has been made in understanding the hormonal control of fruit development and, with respect to the control of a handful of metabolic pathways during this process, compilation of the datasets is only just beginning, allowing a broader systems-orientated view of metabolism of the developing fruit. The continued establishment of even more sophisticated tools to dissect metabolism and development at both spatial and temporal levels (Rontein *et al*., 2002; Kehr, 2003; Junker *et al*., 2003, 2004) means that it will also be crucial in the future to analyse tissues independently and across a broad developmental time frame in order to allow comprehensive understanding of networks occurring within given cell types. As a first step in this direction, a study has recently been initiated in which the levels of primary metabolites in the pericarp of wild-type fruit were profiled every 7 d from 7 DAA to 70 DAA and, additionally, transcript levels in identical tissue samples were profiled for the majority of these time points. Once they are fully evaluated, it is the intention to integrate the datasets prior to correlation analysis in the same way as was previously done for the developing potato tuber (Urbanczyk-Wochniak *et al*., 2003), and to do a pathway-based analysis as recently performed for diurnal changes in potato leaf metabolism (Urbanczyk-Wochniak *et al*., 2005). It is hoped that this approach will allow a fuller understanding of the genetic and metabolic networks that govern tomato fruit metabolism and mediate the dramatic metabolic changes that occur in the life cycle of this organ.

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