**Review article**

**Regulation of ripening and opportunities for control in tomato and other fruits**

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Received 1 June 2012; revised 23 July 2012; accepted 25 July 2012.

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**Summary**

Fruits are an important part of a healthy diet. They provide essential vitamins and minerals, and their consumption is associated with a reduced risk of heart disease and certain cancers. These important plant products can, however, be expensive to purchase, may be of disappointing quality and often have a short shelf life. A major challenge for crop improvement in fleshy fruit species is the enhancement of their health-promoting attributes while improving quality and reducing postharvest waste. To achieve these aims, a sound mechanistic understanding of the processes involved in fruit development and ripening is needed. In recent years, substantial insights have been made into the mechanistic basis of ethylene biosynthesis, perception and signalling and the identity of master regulators of ripening that operate upstream of, or in concert with a regulatory pathway mediated by this plant hormone. The role of other plant hormones in the ripening process has, however, remained elusive, and the links between regulators and downstream processes are still poorly understood. In this review, we focus on tomato as a model for fleshy fruit and provide an overview of the molecular circuits known to be involved in ripening, especially those controlling pigment accumulation and texture changes. We then discuss how this information can be used to understand ripening in other fleshy fruit-bearing species. Recent developments in comparative genomics and systems biology approaches are discussed. The potential role of epigenetic changes in generating useful variation is highlighted along with opportunities for enhancing the level of metabolites that have a beneficial effect on human health.

**Keywords:** tomato, ripening, fruit, gene regulation, ethylene, crop improvement, epigenetics.

**Introduction**

Fruits are a major component of healthy diets, providing vitamins such as A, C, E and K and minerals including K and Fe. Fruits also provide a wide range of ‘bioactive’ compounds important for human health, which include antioxidant carotenoids such as lycopene and various polyphenols such as anthocyanins. There is good evidence that fruits and vegetables promote healthy ageing by protecting against heart disease, osteoporosis and likely certain cancers (Oude Griep et al., 2010; Tan et al., 2010; Tucker, 2009). In many parts of the world, the average consumer now has access to a broad range of fresh fruit products, including many imported from tropical regions or delivered from controlled atmosphere or cold storage to maintain supplies throughout the year. Health organizations typically recommend that at least five portions of fruit and vegetables are eaten every day as part of a balanced diet; however, many consumers generally do not regularly eat this quantity of fresh produce (Anon, 2012; Butelli et al., 2008). The high cost of purchasing these products and inadequate quality are a major deterrent, and 20% or more of the edible fruit purchases are discarded before being eaten (Anon, 2010). The highly perishable nature of fruit products often accounts for this postharvest waste owing to their short shelf life. In this review, we examine progress in understanding ripening with a focus initially on tomato (Solanum lycopersicum), the accepted model system for studying this developmental process in fleshy fruits. The extent to which knowledge from tomato can be used to understand ripening and associated changes in quality traits in other fleshy fruits is then discussed in the final section, along with opportunities for ripening control presented by the publication of the tomato genome sequence (The Tomato Genome Consortium, 2012) and a deeper understanding of the molecular circuits controlling this important developmental process.

**Developmental regulation of ripening in tomato and other fleshy fruits**

**Ethylene biosynthesis and perception**

Tomato belongs to the climacteric class of fruits, which includes apple (Malus domestica), banana (Musa AAA group) and pear (Pyrus communis). Climacteric fruits show a large burst of respiration known as the ‘climacteric rise’ at the onset of ripening, in concert with a substantial and autocatalytic increase in the production of the gaseous hormone ethylene. The role of the respiratory climacteric is still unknown, but ethylene acts to initiate and co-ordinate ripening in these fruits (Alexander and Grierson, 2002). The biosynthesis and perception of ethylene are highly regulated, and genes controlling these events are conserved across divergent plant taxa. Ethylene is involved in a wide range of processes in addition to fruit ripening, including abiotic and biotic stress, abscission and flowering and developmental regulation (Lin et al., 2009). Ethylene synthesis, perception and signalling events must therefore be finely controlled. During ethylene biosynthesis, S-adenosylmethionine (SAM) is converted...
to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase, and then, ACC is converted to ethylene by ACC oxidase (ACO). There are at least 12 ACS and 7 ACO genes in the tomato genome, with temporal and tissue-specific patterns of expression. ACS2, ACS4 and ACO1 are responsible for the autocatalytic ethylene production during fruit ripening (Klee and Giovannoni, 2011). These are key steps for the control of ethylene production in fruits.

Major advances have been made in understanding how ethylene is perceived by tomato and other fruits. The ethylene receptors were first cloned in the model plant Arabidopsis, but an understanding of their role in ripening has been due principally to work in tomato. These endoplasmic reticulum–associated integral membrane proteins act as negative regulators of the ethylene response pathway. In the absence of ethylene, it appears that the receptors actively suppress ethylene responses. There are 7 ethanol soluble receptor genes in tomato, and 3, NR, LeETR4 and LeETR6, are expressed in ripening fruits. The negative regulation of the ethylene signal transduction pathway indicates that receptor synthesis and degradation play a major role in this process. Current models of the mechanism of ethylene perception describe the receptors in a functionally ‘on’ state in the presence of ethylene, permitting ethylene signalling to proceed (Klee and Giovannoni, 2011). It has long been known that application of ethylene to immature fruits reduces their time to ripening, or ‘green life’. Kevany et al. (2007) demonstrated that a reduction in the levels of LeETR4 and LeETR6 caused early ripening and that in the presence of ethylene, the receptors are rapidly degraded; the receptor ‘off’ state may indeed involve the degradation of the proteins following ethylene binding (Klee and Giovannoni, 2011). The subtle control of ethylene biosynthesis, expression of receptor gene family members and receptor numbers, in combination with gene products that suppress specific signalling pathways, then ensures that the correct downstream signalling pathway is activated during a particular developmental phase.

**Transcription factors controlling the switch to the ripening phase and the ripening regulatory network**

Ethylene is only part of the ripening regulatory mechanism, and in nonclimacteric fruits, its role in ripening is still unclear. The discovery of transcription factors that are involved in controlling the switch to the ripening phase has resulted mainly from map-based cloning of genes underlying rare spontaneous nonripening mutations in tomato. These single gene mutations include rin (ripening-inhibitor), nor (nonripening) and Cnr (Colourless nonripening), and chromosome walks to these loci have identified genes encoding MADS, NAC and SBP-box transcription factors, respectively (Giovannoni, 2007; Manning et al., 2006; Vrebalov et al., 2002). These genes belong to families of transcription factors that have commonly been associated with floral development or other major phase changes. However, their association with ripening is perhaps not that surprising as flowering plants are the only group to possess true fruits. The unripe ‘leaf-like’ carpel structure then undergoes a massive metabolic phase change at the onset of ripening. It is likely that these genes operate in a network and recent evidence indicates that the CNR gene product is necessary for the action of RIN (Martel et al., 2011).

Although most of the transcription factors known to regulate ripening have been identified from tomato, there is evidence that members of orthologous gene families are involved in fruit development in many other species (Seymour et al., 2008). Comparisons can even be made to the regulatory network known to control fruit development and dehiscence in the dry fruit of the model plant Arabidopsis (Dinneny et al., 2005). Although dry fruit development does not include a ‘ripening phase’ that is analogous to that seen in most fleshy fruits, maturation usually involves a dehiscence process that is accompanied by substantial modification of the valve cell walls (Dinneny et al., 2005), the valve tissue being an equivalent structure to the tomato pericarp. Regulatory genes operating in a network to specify valve function and development of the dehiscence zone include FRUITFULL (FUL), which is a SQUAMOSA class MADS-box gene, and the SHATTERPROOF (SHP) transcription factors that are MADS-box genes that belong to the AGAMOUS subgroup (Dinneny et al., 2005). The tomato TDR4 and TAGL1 genes are likely orthologues of FUL and SHP. Both genes have ripening-related patterns of expression, and while the role of TDR4 is unknown, TAGL1 is necessary for normal ripening in tomato and also for the development of fruit ‘fleshiness’ (Itkin et al., 2009; Vrebalov et al., 2009).

Lin et al. (2008) reported that a previously uncharacterized homeobox protein, LeHB1, interacted with the ACO1 promoter and, when silenced, caused the inhibition of ethylene production and ripening. Overexpression of this gene resulted in the conversion of sepals to carpel-like structures that then grew into fruits and ripened. The precise molecular mechanism by which the gene products encoded by RIN, NOR, CNR, TAGL1 and HB1 operate in regulatory network has yet to be elucidated. It is likely, however, that the MADS-box proteins act as heterodimers or multimers (Giovannoni, 2007). However, there are undoubtedly many additional components of the regulatory complex, including transcription factors such as AP2 that act as negative regulators of the ripening (Chung et al., 2010; Karlova et al., 2011).

The HIGH-PIGMENT (hp) mutants in tomato have also revealed components that although of importance generally in plant development, may also play a role in the ripening regulation. The hp-1 and hp-2 mutations harbour lesions in the tomato orthologues of the Arabidopsis genes UV-DAMAGED DNA-BINDING PROTEIN-1 (DDB-1) and DE-ETIOLATED 1 (DET-1). Both mutants show high fruit carotenoid levels, and the gene products are involved in the suppression of light responses in the absence of light (Enfissi et al., 2010; Mustilli et al., 1999). Evidence suggests that DET-1 acts through chromatin remodelling, by causing de-condensation of DNA, thereby permitting transcription (Benvenuto et al., 2002). These gene products form a complex with Cullin4 (CUL4), a ubiquitin-conjugating E3 ligase that targets proteins for proteolysis. Down-regulation of CUL4 also leads to enhanced fruit pigmentation and other effects (Wang et al., 2008). However, the effects of the various hp mutations are not fruit specific. Up-regulation of a Golden-2-like transcription factor in tomato has also been shown to increase the levels of chlorophyll and carotenoids in tomato, and a lesion in this gene is responsible for the uniform ripening phenotype (Powell et al., 2012).

**Linking the regulatory network to the downstream effectors**

In climacteric fruit, such as tomato, the regulatory network involves the combined action of the gene products from the NOR, RIN and CNR loci, along with numerous other factors including those that are part of the ethylene signal transduction pathway.
Figure 1 Molecular regulators of ripening in tomato fruits. Ethylene biosynthesis is triggered by HB1 and other developmental cues and also promoted by ripening-related transcription factors, for example RIN and TAGL1. Ethylene perception by receptors encoded by members of the ETR family results in receptor degradation and ethylene signal transduction. This initiates and coordinates the ripening process.

Many of the ripening-related transcription factors influence ethylene synthesis, and evidence indicates that both RIN and TAGL1 do this by binding directly to the ACS2 and ACS4 promoters, while HB1 is involved in the direct regulation of ACO1 (Lin et al., 2008; Vrebalov et al., 2009).

Ethylene signalling and the role of other plant hormones

The CTR and EIN gene products are involved directly in transducing the ethylene receptor signal (Klee and Giovannoni, 2011). Additionally, a set of transcriptional regulators known as ethylene response factors (ERFs) are involved in controlling ethylene responses, although the functions of many of them are still unclear. However, new data are emerging which may link them to roles with critical importance for crop quality, such as controlling colour development and providing a method to sense environmental conditions during this and other critical stages of plant development (Gibbs et al., 2011; Lee et al., 2011a). Nonclimacteric fruit do not show the burst of ethylene production at the onset of ripening, but there is tentative evidence that they may share some elements of the downstream components normally associated with the ethylene signal transduction pathway (Lee et al., 2010).

Other plant hormones are also known to have an effect on initiating and coordinating ripening. For example, auxin signalling is involved in silique development (Sorefan et al., 2009) and in the initiation of ripening in the nonclimacteric fruit of strawberry (Manning, 1994). Abscisic acid (ABA) also plays a role in pigment accumulation, as indicated by the ABA-deficient mutant High-Pigment 3 (hp3), where low ABA levels are associated with carotenoid accumulation and increased plastid numbers (Galpaz et al., 2008).

It is apparent that our understanding of the interaction and cross-talk between hormones and their induction of transcriptional regulators controlling ripening is still at an elementary level. Additionally, the mechanisms that connect these higher-level regulatory processes to the downstream biochemical and physiological features of ripening are complex and still poorly understood. The sequenced genomes of several fleshy fruit species, including tomato (The Tomato Genome Consortium, 2012), grape (Jaillon et al., 2007), apple (Velasco et al., 2010), strawberry (Shulaev et al., 2011) and banana (D’Hont et al., 2012), provide the foundation to investigate interactions between transcription factors and regulatory sequences of downstream effectors influencing colour, texture and flavour. As additional such genome sequences are reported, new insights will be provided into molecular variation that allows for the remarkable diversity of ripening-associated phenomena that are collectively exhibited by fleshy fruits.

Pigment accumulation and texture changes

For some of the processes associated with ripening in tomato, for example carotenoid biosynthesis, the major steps in the pathway have been identified and current understanding of their role is based on more than twenty years of transgenic experiments with single ripening-related genes. More recently, advances in quantitative genetics are beginning to complement these earlier experiments and are allowing a more comprehensive approach for linking genotype and phenotype. Flavour development is discussed extensively in a recent review by Klee and Giovannoni (2011), so here we have focused on the two other key quality attributes: colour and texture.

Pigment accumulation

Chloroplast to chromoplast conversion influences not only fruit colour and associated health-promoting properties of fruits, but also their nutrient and flavour composition, as carotenoids, lipids and branched chain amino acids synthesized in the plastid can form precursors of volatile flavour compounds (Klee and Giovannoni, 2011). At the onset of ripening, the thylakoids, which contain the photosynthetic pigments, disassemble and chlorophyll degradation is one of the earliest observable signs of ripening in tomato and many other fruits. The thylakoid lipids then form plastoglobuli, where carotenoid pigments accumulate. Chromoplast metabolic activity is associated with the biogenesis of carotenoids, many other secondary metabolites, amino acids and fatty acids (Klee and Giovannoni, 2011).

Carotenoid formation has been studied extensively in tomato, and this fruit has become the model system to investigate the underlying biochemistry and molecular biology. All the major steps in the pathway have been elucidated and are reviewed elsewhere (Cazzonelli and Pogson, 2010). They can be summarized as follows. The formation of phytoene is the first committed step in carotenoid biosynthesis and is dependent on the catalytic activity of phytoene synthase (PSY). Phytoene then undergoes two desaturation reactions to form ω-carotene, catalysed by phytoene desaturase (PDS), which in turn is desaturated to neurosporene and finally lycopene. This carotenoid accumulates in tomato fruit, providing the characteristic red colour. Lycopene...
can then be cyclized at both ends of the molecule consecutively by lycopene β-cyclase (LYCβ) to form β-carotene or cyclized at one end by LYCB and at the other by lycopene α-cyclase (LYCE) to form α-carotene. These cysc carotenoids can then be converted to xanthophylls. During tomato ripening, the concentration of carotenoids increases between 10- and 14-fold, due mainly to the accumulation of lycopene (Fraser et al., 1994).

Current understanding of the regulation of carotenoid biosynthesis in fruits, and in plants in general, is incomplete. Links to light signalling and chromatin remodelling were mentioned earlier with respect to genes underlying the hp1 and hp2 mutations, but a detailed picture of the connections between the ripening regulatory network and the carotenoid biosynthetic pathway has remained elusive. However, the sequencing of the tomato and other fruit genomes, transcriptome profiling and network inference, combined with the use of chromatin immunoprecipitation experiments, is beginning to reveal aspects of these molecular circuits. For example, in tomato, the RIN gene product has been demonstrated to be associated with various ripening-related regulatory sequences upstream of PSY1 (Fujisawa et al., 2011; Martel et al., 2011). These regulatory sequences may also bind other ripening-related transcription factors, for example ERFs (Osinio et al., 2011). PSY is under strong ethylene control, as is β-cyclase and the cyclase genes (LCY-b and LCY-e) are strongly down-regulated during ripening, thus preventing lycopene cyclization. Thus, both PSY1 and β-cyclase may be under RIN/ERF control. Understanding the control of carotenoid biosynthesis is important not only for enhancing fruit colour and the associated health benefits, but also for flavour traits because carotenoids are also metabolized during ripening to flavour volatiles (Goff and Klee, 2006).

**Fruit texture and shelf life**

Texture is an important driver of consumer preferences for fruits: a consumer preference study reported that tomato fruit characteristics that were most disliked were a soft or ‘mealy’ texture, along with poor flavour characteristics such as sour, or lacking aroma (Sinesio et al., 2010). Texture not only affects consumer preference, but also has a significant impact on shelf life and transportability. Understanding the key factors that influence texture and ripening-related softening has therefore been a priority from a horticultural and commercial standpoint. However, this has proved to be far more challenging to unravel than initially expected, primarily because texture reflects many factors, including cell wall structure, cuticle properties, cellular turgor and fruit morphology (Vicente et al., 2007). The best studied of these are the multitude of cell wall changes that are apparent to varying degrees during ripening in essentially all fleshy fruit. Tomato has represented the primary experimental model to elucidate cell wall modification during ripening, as illustrated by an analysis that the authors have performed as part of the recently published work on the tomato genome sequence and ripening-related transcriptome (The Tomato Genome Consortium, 2012).

Plant cell walls are highly complex hydrated matrices, largely comprising a variety of polysaccharide networks and several types of structural glycoproteins, in addition to some minor components including ions and numerous enzymes (Cosgrove, 2005; Lee et al., 2004, 2011b). While the basic classes of wall polymers are common to most cell walls, variation is seen between cell types, such as lignin accumulation in secondary walls of vascular tissue, and structural lipid polymers of the cuticle in epidermal cells, as well as taxonomically (Bonawitz and Chapple, 2010; Burton et al., 2010; Popper et al., 2011; Schreiber, 2010). In dicots, such as tomato, current models suggest that a framework of cellulose microfibrils, coated and cross-linked with the hemicellulose xyloglucan, is embedded within a gel of pectins, including the polymers homogalacturonan (HG), rhamnogalacturonan I and rhamnogalacturonan II. The tomato fruit cell wall is probably the best studied with respect to changes during ripening (Brummell and Harpster, 2001; Seymour et al., 1990), but the precise roles of most of the polysaccharide and glycoprotein components are still poorly understood (McCann and Rose, 2010). Initial models suggested that one or two enzymes, such as polygalacturonase (PG), which can hydrolyse the pectin backbone of HG polymers de-esterified by pectin methylesterase (PME), might play the major role in controlling texture changes in tomato (Brummell, 2006; Giovannoni, 2001) because of the substantial changes in the levels of activity of these enzymes during the ripening process. However, experiments where genes encoding these and other wall remodelling proteins have been silenced in transgenic tomato fruits have not supported this hypothesis. These experiments indicated that although small effects on fruit softening can be achieved by individual gene knockdowns (Brummell and Harpster, 2001; Powell et al., 2003), substantial changes in fruit texture are likely to require the simultaneous modulation of multiple such genes.

The tomato genome sequence contains more than 700 gene models annotated as having cell wall–related functions, of which around 90 have been characterized to varying degrees (Tomato Genome Consortium, 2012; Supplementary Section). Our initial analysis showed that more than 50 of these genes show differential expression during fruit ripening and encode proteins involved in the modification of wall architecture (Figure 2). At the onset of ripening, there is a substantial decrease in the expression of at least four cellulose synthase genes and also those encoding a variety of glycosyl hydrolases. This is in contrast to genes annotated as xyloglucan endotransglucosylase hydrolases (XTHs) (Saladé et al., 2006), where ten members of this family show a ripening-related burst of expression. These new data suggest a more important role for members of the XTH family in ripening-associated cell wall changes than previously suspected.

Analyses of the tomato genome sequence and gene expression patterns in ripening fruit have supported earlier findings related to genes mainly, or exclusively, expressed in fruit tissue (Tomato Genome Consortium, 2012). Several pectin remodelling enzymes, including PMEs, show fruit-related expression, and they are all down-regulated prior to the full onset of ripening. PME enzymes act to remove methyl groups from the pectic polysaccharides in a blockwise or more random fashion, depending on the gene family member and possibly on the cell wall environment (Jolie et al., 2010). PMEs may act synergistically with PGs, with PME action resulting in the generation of HG substrates that are more susceptible to PG-mediated hydrolysis. However, PME can also potentially act to strengthen the cell wall by facilitating calcium cross-linking between adjacent pectin molecules and therefore cell-to-cell adhesion. This idea was supported by studies of the tomato PMEU1 gene, which encodes the PME1 isoform, the transcript of which is expressed highly at the mature green stage of fruit development, while levels decline substantially at the onset of ripening (Phan et al., 2007). Gene silencing experiments revealed that the loss of PMEU1 expression leads to an enhanced rate of softening during ripening, which suggests that PME action contributes to maintaining fruit firmness, although it is important to note that this possibly reflects an indirect effect. The temporal
expression of this PMEU1 gene is similar to others in that the highest levels are at MG or at least prior to ripening (Figure 2), suggesting that a family of such proteins may act in concert, although it may be that there are differences in substrate specificity or other enzyme properties.

In addition to pectin-degrading enzymes, many other cell wall–related structural genes show changes in expression during ripening (Figure 2). A ripening-related increase in α-mannosidase expression has been reported previously in tomato, and it is likely to be involved in the processing of N-glycans present in cell wall–related glycoproteins. Recent transgenic experiments (Meli et al., 2010) show that suppression of the gene enhances fruit shelf life, but the precise mechanism of action is unclear. Furthermore, five members of the arabino–galactan cell surface glycoproteins (AGPs) family showed a substantial decrease in transcript abundance just prior to the onset of ripening. The role of these glycoproteins in tomato fruit ripening is unknown, but in other tissues, they have been proposed to function in cell wall cross-linking or as pectin plasticizers (Ellis et al., 2010).

**Water relations, cuticles and fruit softening**

In addition to the structural matrices of the cell wall, another important contributor to texture and fruit firmness is cellular turgor. This in turn is governed by the water status within fruit and the relative distribution of water within the cell (the symplast) and in the cell wall (the apoplast). The factors that influence fruit turgor during development and ripening are poorly understood, and indeed, its importance as a target for extending shelf life and generally enhancing texture has long been neglected. However, some recent studies are starting to reveal some of the molecular pathways and structures that are likely involved. The most apparent of these is the cuticle, the waxy layer comprising the polyester cutin and a variety of waxes, which covers the aerial surface of land plants (Jeffree, 2006; Nawrath, 2006). A major function of the cuticle is to limit water loss and desiccation, which would also lead to a loss of cellular turgor and consequently a reduction in tissue biomechanical strength. In fruits, this would be apparent as ‘shrivelling’ during postharvest storage, or as a consequence of fruit cracking. An association between fruit firmness, shelf life and cuticle structure and composition was suggested through the characterization of the delayed fruit deterioration (DFD) tomato, whose fruit show remarkably prolonged resistance to postharvest desiccation and remain firm for many months after harvest (Saladie et al., 2007). The polysaccharide cell walls of DFD fruits appear to undergo the same extensive disassembly during ripening as is seen in normally softening fruits and loss of cell–cell adhesion also occurs to the same extent. However, analysis of the DFD cuticles revealed substantial differences in the amount of cutin as well as of some waxes. The relative contributions of cutin and waxes to resisting transpirational water loss are still not well resolved, but tomato fruit makes an excellent model to address this question as, unlike most plant cuticles, it is astomatous and far thicker than typical leaf cuticles (Buda et al., 2009; Isaacson et al., 2009). Analyses of cutin-deficient tomato mutants suggest that cutin, the major component of cuticles, does not make the most important contribution to limiting water movement across the cuticle and that waxes are relatively more important, while cutin is important in resisting microbial infection (Isaacson et al., 2009). However, the specific molecular components of the cuticle that are the primary determinants of water movement have yet to be determined.

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**Figure 2** Selection of the most highly expressed genes encoding proteins involved in cell wall remodelling and degradation in tomato. Expression at mature green (green bar), breaker (yellow bar) and ripe (red bar). Each gene is labelled with its published name where known or its Solyc chromosome address (www.solgenomics.net). Cel, cellulase; FLA, Fasciclin-like arabinogalactan protein; PL, pectate lyase; TBG, beta-galactosidase; PME, pectin methyl esterase; EXP, expansin; SIXTH, xyloglucan endotransglycosylase/hydrolase; PG, polygalacturonase; CS, cellulose synthase; LeMAN, beta-mannosidase (Data from Tomato Genome Consortium, 2012).
Many other questions remain regarding factors that influence water loss, including the potential role of aquaporin proteins during ripening in regulating the passage of water from the symplast to the apoplast, and the importance of apoplastic solutes in determining the osmolality of the apoplastic fluid, which would presumably also affect water movement out of the cell. Research into fruit water relations and cuticle biology is likely to be important in the development of strategies to extend shelf life and improve fruit texture traits, as will an improved understanding of the interplay between water relations, cell wall biology and the molecular status of the apoplast.

Opportunities for crop improvement

Tomato as a model system for understanding ripening in other fleshy fruits

A fundamental question is whether information about the mechanisms behind the ripening process gained from studying tomato can be applied not only to this and other Solanaceous crops, but also to fleshy fruit species from more distantly related taxa. Evidence is accumulating that families of genes that regulate fruit ripening have been conserved during evolution, examples of which are described here.

LeMADS-RIN is a member of the SEP4 group of the SEPALLATA (SEP) class of transcription factors, which are expressed during ripening of a wide range of fleshy-fruit-bearing species (Klee and Giovannoni, 2011; Vrebakov et al., 2002). A SEP1/2-like gene in strawberry (Fragaria × ananassa Duch.), a nonclimacteric fruit, is involved in the development and ripening of the achenes and swollen receptacle, and silencing of this gene resulted in altered receptacle development, a failure of the achenes to degreen and slow ripening in the receptacle (Seymour et al., 2011). In banana (Musa AAA group), which like tomato is a climacteric fruit, a SEP3 homologue is likely to act upstream of the increase in ethylene production in a similar way to RIN (Elizur et al., 2010). These results point to possible conservation across taxa of the families of transcription factors involved in the high-level regulatory network controlling ripening. This inference is supported by the examination of other clades of MADS-box genes, including members of the SQUAMOSA (SQU) and AGAMOUS (AG) groups. The tomato TDR4 gene, a member of the SQUA class, is highly expressed during ripening, but its function in tomato has not been established. However, studies in bilberry (Vaccinium myrtillus), a nonclimacteric fruit, indicate that a TDR4-like gene in this species is responsible for the accumulation of the anthocyanin pigments in the fruit flabby during ripening (Jaakola et al., 2010); these pigments are strong antioxidants and probably have other bioactive functions. Interestingly, TDR4-like genes are likely to be targets of SBP-box gene products such as CNR (Manning et al., 2006). The ripening of the climacteric fruit of the oil palm (Elaeis guineensis), which is a monocot and provides a major source of edible oil, has a NAC-NOR homologue that shows ripening-related expression, along with a SEP3 orthologue. This supports the hypothesis that even in these fruits, ripening is also regulated by a similar set of genes to those in tomato. These observations indicate that tomato can be used as a model to identify genes likely to regulate the aspects of ripening in distantly related flabby fruit-bearing species.

Comparative genomics and systems biology approaches

The recent publication of a range of crop genome sequences (Jaillon et al., 2007; Shulaev et al., 2011; Tomato Genome Consortium, 2012; Velasco et al., 2010) now provides the opportunity for comparative analysis to understand the evolutionary origin of gene families and will allow predictions about their likely association with specific fruit phenotypes. Genome-wide analysis also permits systems approaches to unravelling molecular networks underlying the ripening process. For example, comparison of the tomato and grape genomes reveals that regions of the tomato genome had three matching blocks to one segment of the grape genome consistent with a Solanum triplication event after tomato and grape had diverged. However, this Solanum triplication event occurred before the split between tomato and potato, estimated at around 7 Mya. Potato also shows evidence for the Solanum triplication event, but when the tomato and potato genomes are compared, there is evidence for both further gene duplication and gene loss in specific families. The Solanum triplication event and gene duplication, gene loss and neo-functionalization have created reservoir of genes, some of which have evolved specific fruit development and ripening functions that are unique to tomato (Tomato Genome Consortium, 2012). Comparative analysis of genomes and especially comparisons between a range of wild and cultivated species will likely reveal gene family expansion or loss associated with tomato domestication, and at the same time aid the identification of the gene family members associated with key fruit quality-related characteristics in this and other fruit-bearing species.

Harnessing natural genetic and epigenetic variation

The first food crops were domesticated around 10 000 years ago and selections of breeding germplasm were made based on specific desirable traits. The genetic basis of subsequent populations has been narrowed further, and a vast wealth of untapped natural variation therefore exists in crop wild species relatives (Tanksley and McCouch, 1997). Tomato is especially well positioned to allow the capture of this wild species variation because of the existence of a range of well-characterized interspecific introgression lines. Generation of these tomato lines (Eshed and Zamir, 1994; Lippman et al., 2007) has provided a marker-defined genomic library of wild species alleles in a cultivated tomato background. The Solanum pennelli introgression lines (Eshed and Zamir, 1994) were used to clone the first quantitative trait locus (QTL) for fruit size (Frary et al., 2000) and identification of a locus controlling sugar accumulation in tomato (Fridman et al., 2004) that increases sugar levels by as much as 25%. Introgessions from the S. pennelli lines provide an excellent starting point to harness natural variation for crop improvement in tomato and are now used in commercial practice (Lippman et al., 2007).

The tomato genome sequence will allow candidate genes underlying QTLs to be readily identified, and these can be functionally tested in transgenic plants at the same time as the marker-assisted selection. Current studies have now positioned hundreds of quality-related QTLs on the tomato genetic map. With respect to ethylene biosynthesis, Cin et al. (2009) investigated the natural variation across a set of well-characterized S. habrochaites introgression lines, where portions of this wild species genome were introgressed into the cultivated tomato background. A range of QTLs was discovered, many of which were likely to represent novel loci for the control of ethylene biosynthesis and ripening. These new need to be further resolved and candidate genes should be identified. Hundreds of QTL that influence primary metabolite levels have been mapped in tomato,
and in many cases, the S. pennellii allele appears to show a dominant mode of inheritance (Schauer et al., 2008). For ascorbic acid content, candidate genes have been identified associated with a range of QTLs (Stevens et al., 2007), and the S. pennellii IILs have been used to identify candidate genes linked to fruit texture (Chapman et al., 2012). The tomato genome sequence allows QTL candidate genes from this species to be nominated as candidates in related taxa, where QTL are located in syntenous genomic regions, for example pepper (Paran and Van der Knaap, 2007). This information may even extend in some cases to allow the identification of regions of synteny and candidate genes in more distantly related taxa (Zorrilla-Fontanesi et al., 2011). QTLs and their candidate genes also provide a basis for testing models of molecular circuits underlying plant development inferred from network inference studies.

Commercial breeding is currently focused on genetic polymorphisms. However, epigenetic variation is common in plant genomes and can affect phenotypes, and this provides an alternative breeding approach. The epigenome involves DNA methylation and other forms of chromatin modification that can govern gene expression and phenotype just as strongly as DNA sequence polymorphisms and, like the latter are also stably inherited, in certain circumstances (Feng et al., 2010; Martienssen and Colot, 2001). These epigenetic changes can include alterations in DNA methylation and small RNAs and chromatin remodelling events. The identification of the gene at the CNR locus has provided a new insight into the possible role of epigenetic processes in fruit ripening (Eriksson et al., 2004; Manning et al., 2006; Thompson et al., 1999). The CNR gene, discussed earlier with respect to transcription control of ripening, encodes an SBP-box transcription factor. The positional cloning of this locus was especially challenging because the mutation could not be linked with DNA sequence polymorphisms, but unexpectedly was associated with alterations in DNA methylation. Analysis of the CNR promoter revealed a region more than 2 kb upstream of the transcription start site that was heavily methylated in the Cnr mutant but unmethylated in wild-type normally ripening fruits. The hypermethylated was associated with severely reduced SBP-box gene expression. The Cnr phenotype could be induced by either virus-induced gene silencing of the SBP-box gene or transgene-induced methylation of the appropriate site on the CNR promoter (Kanazawa et al., 2011; Manning et al., 2006). This spontaneous epimutation was found to be stable over many generations, and in the cultivar Liberto, there was clear evidence of developmentally driven changes in levels of methylation in the Cnr promoter. In this case, specific nucleotides showed a loss of methylation just prior to and at the onset of ripening (Manning et al., 2006). Recent work has begun to unravel the mechanisms by which epigenetic variation may occur among individuals of the same species. Variants for both methylation and small regulatory RNA species were identified in S. pennellii IILs and hybrids (Shivaprasad et al., 2011). Naturally occurring epigenetic variation independent of selection has also been characterized in species other than tomato. Ecotypes of Arabidopsis have different patterns of methylation, which may be stable, unstable, heritable or lost (Vaughn et al., 2007). Epigenetic diversity such as methylation and sRNAs may exist within breeding populations and could be selectable for under evolutionary pressure.

Short RNAs (sRNA) are also associated with controlling gene expression and are known to guide DNA methylation, but the sRNA content of plant cells is surprisingly complex (Dalmay, 2010; Moxon et al., 2008), and our understanding of their roles in ripening and other developmental processes is at a rudimentary stage. The best characterized sRNAs are the micro-RNAs (miRNA) that guide the RNA-induced silencing complex to mRNAs containing a specific target site, and these RNAs are then cleaved. However, a range of other sRNAs are present in plant cells that appear to be associated with the induction of methylation including that in gene bodies and promoters (Moxon et al., 2008; Pilcher et al., 2007; Shivaprasad et al., 2011). Analysis of the sRNA changes in tomato indicates that miRNAs play a role in fruit development and ripening, including the regulation of CNR and genes involved in ethylene signalling (Moxon et al., 2008). Thousands of non-miRNA sRNAs are differentially expressed during fruit development and ripening (Mohorianu et al., 2011), and these changes have been analysed on a genome-wide basis using the newly published tomato genome sequence (Tomato Genome Consortium, 2012). This has revealed that sRNAs show developmental dynamics associated with ripening in tomato fruit and the mapping of sRNA to specific sites on fruit-related promoter regions. However, the function of these changes remains to be elucidated.

Bioactives

The high levels of secondary metabolites found in fruits make an important contribution to the human diet, especially in terms of their health-promoting effects. Substantial effort has been focused on enhancing levels of lycopene and other carotenoids in fruits and especially tomato, and this work has been reviewed elsewhere (Fraser and Bramley, 2004; Landrum, 2009). Carotenoids are not the only important health-promoting compounds whose levels could be enhanced, and it is possible that many important health-promoting phytochemicals may be present in cultivated tomatoes in small amounts, the identities and roles of which are yet to be revealed. Examples of additional classes of such compounds are given here.

Many berries produce high levels of the flavonoid class of polyphenols, which offer protection against cardiovascular disease and certain cancers (Visioli et al., 2011). Cultivated tomatoes lack high levels of anthocyanins, but because of the amount consumed, it is an excellent vehicle for transgenic enhancement of flavonoid content. Butelli et al. (2008) reported the expression of the Delila (Del) and Rosea1 (Ras1) transcription factors from snapdragon (Antirrhinum majus) in the fruit of transgenic tomatoes. The expression of the two transgenes enhanced the hydrophilic antioxidant content of tomato three-fold and resulted in fruit with intense purple coloration throughout the pericarp. The authors then demonstrated that the enhanced anthocyanin levels were able to offer a health protective effect in a dietary context by significantly extending the life span of lines of transgenic mice with a propensity to develop tumours and are used routinely for basic and applied cancer studies.

Other important bioactives in the human diet include compounds such as tetrahydrofolate (THF) and its derivatives (folicates), which are essential cofactors for one-carbon transfer reactions in the biosynthesis of certain amino acids, purines and thymidylate (Waller et al., 2010). Humans lack the capacity to synthesize folates and must acquire them mainly from plant foods. Folate deficiency is a worldwide health problem associated with spina bifida and other birth defects and also with cardiovascular disease. Folates are synthesized from pteridine, p-aminobenzoate (PABA) and glutamate precursors. Diaz de la Garza et al. (2007)
reported combining transgenic tomato lines engineered to generate high levels of pteridine and PABA-producing fruits with 25% more folate than the controls. The increased folate levels were engineered with very limited effects on other aspects of tomato fruit metabolism (Wallen et al., 2010) and could provide the complete adult daily requirement in less than one standard serving (Diaz de la Garza et al., 2007).

Genetic modification (GM) of tomato and other fruits offers the consumer potentially substantial benefits related to enhanced health-promoting properties, with the ultimate goal of targeting specific phytochemicals without altering other aspects of metabolism. These powerful approaches for crop improvement can be readily combined with the control of ripening, including modification of ethylene biosynthesis and perception in tomato and other climacteric fruits. However, progress with GM crops now depends on addressing the public concerns relating to the ‘controversy over GM foods’ and also the inherent regulatory costs for bringing varieties containing these modifications to market (Rommens, 2010).

Fruits and vegetables are a vital part of the human diet, but much remains to be learnt about the precise mechanisms by which these crop products provide their protective effects. Members of the Solanaceae family, including tomato, produce huge numbers of secondary products, many of which may have health benefits that are yet to be fully exploited. Wild tomato species offer a substantial and largely untapped reservoir of these compounds which demand further investigation. A combination of marker-assisted breeding, underpinned by advances in next-generation sequencing technologies and in concert with GM approaches, provides the opportunities for a step-change in breeding new varieties that combine health-promoting traits, excellent quality and long postharvest shelf life in a competitively priced product.

Acknowledgements

NHC was supported by Biotechnology and Biological Sciences Research Council of the UK ERA-PG TomQML project (BB/G02491X). Support to JKCR was provided by grants from the National Science Foundation (Plant Genome Program; DBI-0606595), the United States-Israel Binational Agricultural Research and Development Fund (IS-4234-09), U.S.-Israel Bational Science Foundation (2005-168) and CUAES-Hatch grant (NYC-184462).

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