Annual Plant Reviews (2009) **38**, 296–325
doi: 10.1002/9781444314557.ch8
www.interscience.wiley.com

Chapter 8

FACTORS INFLUENCING THE RIPENING AND QUALITY OF FLESHY FRUITS

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Abstract: Fleshy fruits have a dual function in the reproductive strategies of plants. Initially, fleshy fruits protect the developing seeds from predation and then, once the seeds are mature, they facilitate dispersal of the enclosed seeds. Plants have evolved numerous chemical and physical barriers that discourage seed predation from fleshy fruits. Similarly, the ripening of fleshy fruits occurs through a range of coordinated biochemical processes that convert an unpalatable unripe fruit into a fruit that is nutritious and desirable to seed-dispersing fauna. The biochemical changes that occur at the onset of ripening are species specific but several general processes occur that are common to many fruits, suggesting that the mechanisms that control ripening may be evolutionarily conserved. For example, fruit ripening is often accompanied by the accumulation of brightly coloured pigments, the synthesis of aroma volatiles and the conversion of complex carbohydrates into sugars. These changes facilitate seed dispersal strategies. The genetic and biochemical pathways that lead to fruit ripening are not fully understood. However, significant progress has been made in identifying some of the components of these pathways. This review highlights recent research that has contributed to the understanding of the ripening process at the molecular level and outlines the development of genomics-based resources for fleshy fruit-bearing species.

Keywords: fleshy fruits; seed dispersal; fruit ripening; hormone signalling; light signalling; aroma

8.1 Introduction

Fleshy fruits are important sources of nutrition for humans and animals providing energy, vitamins, minerals, antioxidants and fibre to the diet. In return for the energy that the plant invests in the production of the fruit, its consumption ensures that seeds are dispersed in a nutrient rich media,

 ISBN: 978-1-405-18946-0

often at a distance from the parent plant therefore facilitating survival and colonization. In order for frugivore-assisted seed dispersal to occur, dramatic biochemical changes are initiated that convert an unpalatable immature fruit into a ripe fruit that is attractive and nutritious. These ripening-associated changes are conserved in many diverse fruit species and include the conversion of starch to sugars, alterations in texture, the accumulation of brightly coloured pigments, and the synthesis of volatile aroma compounds.

Plant secondary metabolites, including aroma volatiles, appear to be key in the interaction of frugivores and ripening fruits, serving as both feeding attractants and deterrents (Tewksbury, 2002; Foley and Moore, 2005). In tomato, the precursors of many aroma volatiles are essential nutrients in animal diets including carotenoids which serve as the precursors of pro-vitamin A, essential amino acids and essential fatty acids, leading to the hypothesis that these aroma compounds serve as nutritional cues for seed dispersers (Goff and Klee, 2006). However, evidence suggests that the interaction between volatiles and frugivores may in some instances be more complex. For example, fermentation, as a result of yeast infection, can increase as fruits become overripe. Fermentation leads to the production of ethanol, which at concentrations greater than 1%, can act as a feeding deterrent to both primates and fruit bats but at lower concentrations may act as a feeding stimulant (Milton, 2004; Sanchez *et al.*, 2004, 2006). Similarly, mammalian frugivores are deterred by capsaicin in chilli peppers, yet birds, that are believed to be more efficient dispersers of the pepper seeds, show no aversion to fruits with high capsaicin content (Tewksbury and Nabhan, 2001). In addition, it has been hypothesized that the evolution of these thick fleshy fruits primarily occurred as a mechanism to prevent seed predation and that ripening may have evolved as a secondary phenomenon to aid in dispersal of the mature seeds (Mack, 2000). This hypothesis does have some credence when considering that many fleshy fruits contain toxic compounds, particularly those of the Solanaceae family that can contain high levels of glycoalkaloids which can be fatal if ingested and serve as a feeding deterrent (Cipollini and Levey, 1997).

These few examples clearly illustrate that the interaction between frugivores and plants and the mechanisms that have evolved to serve as attractants and deterrents for seed dispersal and predation are complex. However, it is well established that the ripening of fruits serves as a key positive stimulus for seed-dispersing animals. This review focuses on some of the recent advances in the biology of fruit ripening and the genetic determinants that control this process and contribute to fruit quality.

8.2 Control of fruit ripening

Fleshy fruits display a broad range of phenotypic and chemical diversity. However, despite this diversity there are several common features that accompany the onset of fruit ripening. For example, ripening often results in

changes in colour, increased softening, the conversion of starch into sugars, the production of flavour and aroma compounds and an increased susceptibility to pathogen infection. This suite of common biochemical changes suggests that diverse fruits may share common pathways that mediate ripening. Although the factors that control the onset of ripening are not fully understood, this represents an area of intense research activity. The last decade has seen tremendous progress in the molecular identification of mutant loci of tomato that alter fruit ripening and quality (Table 8.1). In addition, the development of genomics-based resources for fleshy fruit-bearing species, including the generation of large numbers of expressed sequence tags (ESTs) and gene expression profiling, coupled with functional analysis, has provided valuable insight into the identity of the genes that are expressed during fruit ripening, their regulation and contribution to the overall fruit phenotype.

8.3 Transcription factors serve as master regulators of fruit ripening

The characterization of tomato mutants with impaired fruit ripening has proven to be an effective strategy for gaining insight into the mechanisms that control ripening. The *ripening inhibitor* (*rin*), *non-ripening* (*nor*) and *Colourless non-ripening* (*Cnr*) display severe inhibition of fruit ripening manifest through inhibited ethylene synthesis, greatly reduced carotenoid synthesis and reduced fruit softening. These phenotypes cannot be alleviated by exogenous ethylene treatments although the expression of ripening-related genes can be induced by ethylene application suggesting that these loci act upstream of ethylene and control the competency of the fruit to ripen (Robinson and Tomes, 1968; Tigchelaar *et al.*, 1973, 1978; Yen *et al.*, 1995; Thompson *et al.*, 1999).

The *rin* locus maps to the long arm of tomato chromosome 5 and is tightly linked to the *macrocalyx* (*mc*) locus that causes the production of large sepals. The lack of separation between these traits led to the hypothesis that the *rin* locus may be caused by a deficiency (deletion) (Robinson and Tomes, 1968). This hypothesis was confirmed following the isolation of the *rin* locus using a positional cloning approach revealing the presence of a 1.7 kb deletion (Vrebalov *et al.*, 2002). The deletion was found to remove the last exon of the *RIN* gene and regulatory sequences upstream of the *MC* gene that are required for normal expression levels in wild-type sepals. Both *RIN* and *MC* encode members of the MADS-box transcription factor family of tomato. MADS-box proteins constitute a large family in plants that act coordinately and often redundantly to control many developmental processes including floral organ identity, meristem determinacy and flowering time (Ng and Yanofsky, 2001).

RIN is a member of the *SEPALLATA* (*SEP*) subfamily of MADS-box genes and is most similar to *Arabidopsis SEP4/AGL3* (Malcomber and Kellogg, 2005).

Table 8.1 Classical mutants of tomato displaying altered fruit phenotypes associated with ripening and quality

Locus	Gene product/ function	Fruit phenotype	Chromosome	Reference
Apricot (*at*)	Unknown	Altered carotenoids	5	Jenkins and Mackinney, 1955
Beta (β)	Lycopene β-cyclase	Altered carotenoids	6	Ronen *et al.*, 2000
Colourless epidermis (*y*)	Unknown	Reduced flavonoids in peel	1	Rick and Butler, 1956
Colourless non-ripening (*Cnr*)	SBP-box transcription factor	Severe ripening inhibition	2	Manning *et al.*, 2006
Cuticular water permeability (*Cwp*)	Novel, unknown function	Fruit cracking and shrivelling	4	Hovav *et al.*, 2007
Delayed fruit deterioration (*dfd*)	Unknown	Altered fruit cuticle properties	Unknown	Saladie *et al.*, 2007
Delta (*Del*)	Lycopene epsilon cyclase	Altered carotenoid profile	12	Ronen *et al.*, 1999
Dwarf (*d*)	Cytochrome P450, brassinosteroid biosynthesis	Delayed fruit ripening, pleiotropic effects	2	Lisso *et al.*, 2006
Green-flesh (*gf*)	STAY-GREEN homologue	Altered fruit pigmentation	8	Barry *et al.*, 2008
Green-stripe (*gs*)	Unknown	Striped fruit epidermis	7	Larsen and Pollack, 1951
Green-ripe (*Gr*)	Ethylene signalling	Reduced ethylene responsiveness	1	Barry and Giovannoni, 2006
High-pigment-1 (*hp-1*)	DDB1 homologue	Enhanced fruit pigmentation	2	Liu *et al.*, 2004
High-pigment-2 (*hp-2*)	DET1 homologue	Enhanced fruit pigmentation	1	Mustilli *et al.*, 1999
High-pigment-3 (*hp-3*)	Zeaxanthin epoxidase	Enhanced fruit pigmentation	2	Galpaz *et al.*, 1999
Lecer6	β-ketoacyl-CoA synthase	Shrivelling on the vine	Unknown	Vogg *et al.*, 2008
Never-ripe (*Nr*)	Ethylene receptor	Reduced ethylene responsiveness	9	Wilkinson *et al.*, 1995

Table 8.1 (*Continued*)

Locus	Gene product/ function	Fruit phenotype	Chromosome	Reference
Non-ripening (*nor*)	Transcription factor	Severe ripening inhibition	10	Giovannoni, 2001
Ripening-inhibitor (*rin*)	MADS-box transcription factor	Severe ripening inhibition	5	Vrebalov *et al.*, 2002
Tangerine (*t*)	Carotenoid isomerase	Altered carotenoid profile	10	Isaacson *et al.*, 2002
Yellow-flesh (*r*)	Phytoene synthase	Altered carotenoid profile	3	Fray and Grierson, 1993

In *Arabidopsis*, the four *SEP* genes act redundantly to specify floral organ identity and maintain floral meristem identity with single mutants having little or no altered phenotypes but quadruple mutants forming only leaf-like structures in place of the normal floral organs (Pelaz *et al.*, 2000; Ditta *et al.*, 2004). The severity of the *rin* mutant allele indicates that the redundancy observed in *SEP* gene function in *Arabidopsis* is not fully conserved in other species. MADS-box transcription factors regulate floral development through the formation of ternary and quaternary protein complexes suggesting that *RIN* may well function with other MADS-box genes to facilitate fruit ripening (Egea-Cortines *et al.*, 1999; Honma and Goto, 2001). Indeed, several other MADS-box genes are expressed in tomato fruit and some, like *RIN*, display a ripening-related increase in expression and are therefore good candidates for additional regulators of ripening although functional analysis to confirm this hypothesis is currently lacking (Fei *et al.*, 2004; Giovannoni, 2004; Hileman *et al.*, 2006).

The *Cnr* mutant has a particularly striking phenotype that is distinct from that observed in the *rin* and *nor* mutations (Thompson *et al.*, 1999). The fruit pericarp of mature *Cnr* fruit is white and exhibits reduced cell–cell adhesion resulting in fruits with a mealy texture. Analysis of the cell wall properties of the *Cnr* mutant revealed several changes when compared to wild type including stronger pericarp cell walls in mature *Cnr* fruit, a 50% increase in intercellular spaces, reduced calcium-binding capability of homogalacturonan in the middle lamella and altered deposition of (1 → 5)-α-L-arabinan in mutant fruit cell walls (Orfila *et al.*, 2001). Together with the altered physical and chemical properties of the cell wall in *Cnr* fruit, there is extensive alteration in the expression and activity of several enzymes involved in cell wall modification during ripening and many other ripening-related genes display reduced or altered expression in *Cnr* fruit (Eriksson *et al.*, 2004). Interestingly, there is increased expression of genes associated with stress responses and pathogen

infection in *Cnr* fruit including chitinases and PR proteins. The appearance of these classes of genes is characteristic of abscission and dehiscence zones (Roberts *et al.*, 2002). Together with the altered cell wall properties, the gene expression profile of *Cnr* fruit raises the intriguing possibility that the pericarp cells in mutant fruit are undergoing separation processes similar to those that occur during the dehiscence of dry fruits.

The *Cnr* locus was mapped to within a 13 kb interval on the long arm of chromosome 2. No sequence differences were observed between wild type and *Cnr* alleles across this region although the expression of a gene encoding a member of the SBP-box (*SQUAMOSA Promoter-Binding Protein-like*) gene family designated *LeSPL-CNR* was reduced in the *Cnr* mutant background (Manning *et al.*, 2006). Virus-induced gene silencing of this gene in fruit resulted in sectors that failed to fully ripen therefore mimicking the *Cnr* mutant phenotype. Bisulphite sequencing of the promoter region of *LeSPL-CNR* revealed hypermethylation of cytosine residues in the *Cnr* mutant background. Hypermethylation of promoter regions can lead to alteration in gene expression and has been confirmed as the cause of several higher plant epigenetic mutations affecting plant development. In addition, approximately one *Cnr* plant per thousand produced a fruit with normal ripening sectors. These rare revertants are typical of epigenetic mutations. Together these data indicate that the *Cnr* mutant phenotype is the result of a stably inherited spontaneous epigenetic mutation that leads to reduced expression of *LeSPL-CNR* during fruit development and ripening (Manning *et al.*, 2006).

LeSPL-CNR is most closely related to the *Arabidopsis SPL3* gene that has been implicated in regulating floral development *via* possible regulation of *AP1* expression. Expression profiling of *Cnr* mutant fruit revealed altered expression of several MADS-box genes compared to wild type, in particular TDR4 expression was significantly reduced in *Cnr* (Eriksson *et al.*, 2004). TDR4 is a member of the *FRUITFUL/APETALA1* lineage of MADS-box genes and is a closely related homologue of the *Arabidopsis FRUITFUL* (*FUL*) gene (Litt and Irish, 2003). Fruit of the *Arabidopsis ful* mutant displays severe growth defects due to a lack of valve cell expansion following fertilization (Gu *et al.*, 1998). *FUL* is expressed throughout the valve in wild-type fruit and acts together with *REPLUMLESS* (*REP*) to restrict the expression of four transcription factors, *SHATTERPROOF* (*SHP*) *1* and *2*, *INDEHISCENT* (*IND*) and *ALCATRAZ* (*ALC*) to the valve margin (Roeder *et al.*, 2003; Liljegren *et al.*, 2004). These four transcription factors are required to specify valve margin identity and in *ful* mutants are ectopically expressed in valve cells that subsequently appear to adopt the fate of valve margin cells including lignin deposition during the later stages of fruit development (Liljegren *et al.*, 2004). The altered phenotypes of *Cnr* fruit are indicative of a change in cell identity within the fruit pericarp and it may be possible to exploit the increasing body of knowledge on *Arabidopsis* fruit development to explain the biology of *Cnr*.

The identification of RIN and CNR as transcription factors provides a remarkable opportunity to investigate the regulation of the pathways that

control fruit ripening in more detail. Obvious experimental directions include defining the in vivo targets and interaction partners of these proteins and, given that the expression of both *RIN* and *CNR* are up-regulated during fruit ripening, it will be informative to define the factors that regulate their expression. In addition, defining the genetic interaction of *RIN* and *CNR* with each other and in the context of the *nor* mutation may also provide insight into the mechanisms that regulate fruit ripening. For example, can overexpression of *RIN* in a *nor* genetic background restore aspects of the ripening process? Of additional importance will be determining whether the action of these transcription factors is conserved across species boundaries to control ripening in other climacteric as well as non-climacteric fruits.

8.4 Hormonal control of fruit ripening

All of the major plant hormones have been shown to influence aspects of fruit development and ripening. Hormone levels change during cell division following fertilization, cell expansion during fruit growth and at the onset of ripening, influencing the expression of a multitude of genes implicated in these processes (Srivastava and Handa, 2005). Discussion of hormonal regulation in this chapter will focus purely on the effects of hormones on ripening and fruit quality.

8.4.1 Ethylene

Fleshy fruits have traditionally been classified based upon their ripening behaviour. Climacteric fruits, including tomato, avocado, apple and banana produce a burst of respiration and display increased ethylene synthesis at the onset of ripening whereas in non-climacteric fruits such as grapes, strawberries and citrus these changes are not evident (McMurchie *et al.*, 1972). The role of ethylene in regulating the ripening of climacteric fruits is well defined. Chemicals that block either ethylene synthesis or perception can inhibit fruit ripening (Hobson *et al.*, 1984; Yang and Hoffman, 1984; Watkins, 2006). Similarly, genetic control of ethylene synthesis through reduction of ACC levels or ACC oxidase activity also leads to inhibition of ripening (Klee *et al.*, 1991; Oeller *et al.*, 1991; Picton *et al.*, 1993; Ayub *et al.*, 1996; Schaffer *et al.*, 2007). Mutations or transgenic approaches that disrupt the function of genes involved in the ethylene-signalling pathway also disrupt ripening. For example, the *Never-ripe* (*Nr*) mutant of tomato displays dominant ethylene insensitivity due to an amino acid substitution in an ethylene receptor, leading to inhibition of ripening, and overexpression of a mutated Arabidopsis *ETR1* ethylene receptor in tomato also leads to inhibition of ripening (Lanahan *et al.*, 1994; Wilkinson *et al.*, 1995, 1997). Ethylene receptors are encoded by a family of at least six genes in tomato and act as negative regulators of ethylene responses (Tieman *et al.*, 2000; Kevany *et al.*, 2007). Recent evidence suggests that

ethylene exposure leads to degradation of the receptors, resulting in enhanced ethylene responsiveness and earlier fruit ripening (Kevany *et al.*, 2007).

Mutation and manipulation of ethylene receptors in tomato leads to reduced ethylene responsiveness throughout the whole plant, however, recent characterization of the *Green-ripe* (*Gr*) mutant of tomato has provided insight into factors that mediate tissue-specific control of ethylene responses. *Gr* fruit fails to fully ripen due to reduced ethylene responsiveness and also displays reduced rates of ethylene-induced floral senescence and abscission. However, *Gr* mutant seedlings retain normal ethylene responsiveness (Barry *et al.*, 2005). Using a positional cloning strategy, the *Gr* mutation was shown to result from a deletion within the promoter and 5′-untranslated region of a gene that resulted in its ectopic expression in the *Gr* mutant background (Barry and Giovannoni, 2006). Overexpression of *GR* under the control of the *CaMV35S* promoter recreated the mutant phenotype but did not lead to plants that displayed a whole-plant reduction in ethylene responsiveness, indicating that GR modulates tissue-specific ethylene signalling via an unknown mechanism. *GR* encodes a novel protein that is predicted to be membrane localized and is conserved in plants, metazoans and protozoa. In a separate study, a *GR* homologue from Arabidopsis, designated *REVERSION TO ETHYLENE SENSITIVITY 1* (*RTE1*) was isolated in a genetic screen to identify suppressors of the weak ethylene-insensitive mutant receptor allele, *etr1-2* (Resnick *et al.*, 2006). However, unlike the tissue specificity observed in the *Gr* mutant allele, *rte1* mutant alleles display ethylene-related phenotypes throughout the whole plant suggesting different ethylene signalling mechanisms operate in tomato and Arabidopsis.

In addition to targeted transgenic approaches and mutant analysis in tomato, variation in climacteric ethylene production and ethylene responsiveness has also been observed in several fleshy fruit species including apple, melon, peach, plum, pepper and, Asian pear, leading to different fruit quality and ripening characteristics (Itai *et al.*, 1999; Sunako *et al.*, 1999; Villavicencio *et al.*, 1999; Zuzunaga *et al.*, 2001; Perin *et al.*, 2002; Tatsuki *et al.*, 2006; Yamane *et al.*, 2007; Itai and Fujita, 2008). In apple, peach and Chinese pear, the reduced ethylene biosynthesis observed in cultivars with extended shelf life has been linked to reduced expression levels of ACC synthase isoforms that are typically expressed during ripening (Sunako *et al.*, 1999; Tatsuki *et al.*, 2006; Yamane *et al.*, 2007). Furthermore, in apple and Asian pear cultivars structural differences have been observed in different ACS isoforms and these correlate with ethylene production and postharvest shelf life (Itai *et al.*, 1999; Sunako *et al.*, 1999; Itai and Fujita, 2008).

Tremendous variation for fruit morphology and ripening traits also exists within the Cucurbitaceae family and in particular, melons display both climacteric and non-climacteric ripening behaviour. Cantaloupe melons display a climacteric ripening phenotype, they have a netted skin, orange flesh, are aromatic and undergo abscission as they reach maturity and ripen. In contrast, honey dew melons produce little ethylene, have low respiration rates,

have a smooth skin, reduced aroma volatiles and fail to abscise. Transgenic cantaloupe melons with reduced ethylene synthesis due to the expression of an ACC oxidase antisense transgene gene can partially suppress some of the typical 'cantaloupe-type' phenotypes resulting in plants that display phenotypes typically associated with honey dew melons (Ayub *et al.*, 1996; Guis *et al.*, 1997; Flores *et al.*, 2002). Furthermore, genetic analysis of a recombinant inbred population generated from a cross between a non-climacteric, ethylene-insensitive melon, PI161375 and a climacteric cantaloupe melon revealed that the non-climacteric character was controlled by two recessive loci (Perin *et al.*, 2002).

Fruits that have traditionally been classified as non-climacteric have recently been re-evaluated for the possible role of ethylene in regulating various aspects of the ripening process. The role of ethylene in regulating de-greening in citrus is well established but recent evidence has also been presented that suggests the possibility that citrus fruits may display climacteric-like behaviour following harvest (Goldschmidt *et al.*, 1993; Jacob-Wilk *et al.*, 1999; Katz *et al.*, 2004). Similarly, ethylene has been shown to stimulate the accumulation of anthocyanin, and the expression of ripening-related genes in grape berries and the differential expression of components of the ethylene-signalling pathway have been demonstrated in several non-climacteric fruits including strawberry, citrus and grape (El-Kereamy *et al.*, 2003; Chervin *et al.*, 2004; Tesniere *et al.*, 2004; Trainotti *et al.*, 2005; Fujii *et al.*, 2007). These experiments provide correlative evidence of a role for ethylene in different aspects of the ripening of non-climacteric fruits. The introduction of dominant ethylene receptor mutants into transgenic strawberry and grape, possibly under the control of fruit-specific promoters, is now technically feasible and should provide conclusive evidence as to the role of endogenous ethylene in these non-climacteric fruits.

8.4.2 Brassinosteroids

Although the role of ethylene in enhancing the ripening of climacteric fruits is well established, additional factors that promote ripening, particularly in non-climacteric fruits are less well defined. However, several lines of evidence point to a role for brassinosteroids (BRs) as potential promoters of fruit ripening in grape (Symons *et al.*, 2006). BRs were shown to increase in grapes at the onset of ripening and this increase is mirrored by changes in the expression of genes involved in BR biosynthesis. Furthermore, exogenous application of BRs to grape berries accelerated ripening whereas the application of the BR inhibitor, brassinazole, delayed the onset of ripening. These data provide correlative evidence for a role of BRs in stimulating ripening although a more definitive genetic or transgenic approach will be needed that disrupts either the synthesis or action of BRs in grape berries to truly verify this interesting discovery. Exogenous application of BRs has also been reported to stimulate ripening of tomato fruit discs suggesting that this phenomenon is not

restricted to grape (Vardhini and Rao, 2002). Genetic evidence of a role for BRs in mediating ripening and quality attributes in tomato has come from characterization of the *dwarf* (*dx*) mutant of tomato. The *dwarf* mutation lacks a functional cytochrome P450, required for the synthesis of castasterone and exhibits delayed fruit ripening and altered quality attributes including reduced levels of starch and sugars and elevated amino acids (Lisso *et al.*, 2006).

8.4.3 Auxin

In tomato, auxin levels increase early during fruit development during the cell division phase and subsequently decline before increasing at the onset of ripening (Srivastava and Handa, 2005). Many auxin responses are controlled by two different classes of transcription factors, the auxin response factors (ARFs) that bind to targets in the promoters of auxin-regulated genes and the Aux/IAA proteins that bind to the ARFs and act as repressors of auxin responses (Guilfoyle, 2007). Under low auxin conditions, the Aux/IAA proteins bind to the ARFs and inhibit transcription. In contrast, when auxin levels increase, binding of auxin to the receptor, an F-box protein designated SCFTIR1, targets the Aux/IAA proteins for degradation and releases their inhibitory effect on auxin-inducible gene expression (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005). The expressions of several Aux/IAA and ARF genes are induced at the onset of ripening in both tomato and peach and show regulation by ethylene, suggesting possible interplay between ethylene and auxin in mediating ripening-related gene expression (Jones *et al.*, 2002; Trainotti *et al.*, 2007). Transgenic manipulation of the ARF gene *DR12* in tomato causes a range of pleiotropic phenotypes that includes dark green immature fruits that when mature exhibit a blotchy ripening pattern. Pericarp cells also possess an unusual pattern of cell division with a higher number of small cells in the outer pericarp that contributes to an increased overall thickness of the pericarp. DR12 suppressed fruit displays enhanced firmness that may result from the increased thickness of the pericarp or subtle changes that are observed in pectin composition in the transgenic lines (Jones *et al.*, 2002; Guillon *et al.*, 2008). Evidence for auxin playing a role in early fruit development in tomato has also been obtained through transgenic manipulation of the *AUX/IAA* gene, *IAA9*, leading to cell expansion prior to fertilization and the development of parthenocarpic fruit (Wang *et al.*, 2005).

8.4.4 Polyamines

Polyamines (PAs) are ubiquitous aliphatic organic compounds that are reported to have a range of phenotypic effects including an ability to delay fruit ripening and senescence (Galston and Sawhney, 1990; Cohen, 1998). PAs are synthesized from basic amino acids. Putrescine can be formed from either arginine or ornithine by the action of either arginine or ornithine decarboxylase. Putrescine together with a decarboxylated *S*-adenosylmethionine (SAM)

moiety can then be utilized to form spermidine and subsequently spermine by the action of spermidine and spermine synthases, respectively (Cohen, 1998). In an experiment designed to assess the role of PAs in fruit ripening, Mehta *et al.* overexpressed a yeast SAM decarboxylase (ySAMdc) gene under the control of the strong ripening-specific *E8* promoter in transgenic tomatoes (Mehta *et al.*, 2002). When compared to segregating control fruit, the transgenic lines with elevated PA levels exhibited a range of beneficial phenotypes including increased vine life, higher carotenoid levels and increased juice viscosity. The transgenic fruit also produced elevated levels of ethylene. This result was unexpected given that both PAs and ethylene share SAM as a common precursor as it was anticipated that diverting SAM towards PA biosynthesis would reduce ethylene levels. However, the elevated ethylene levels in the PA overproducing lines indicate that SAM is not a limiting substrate for either pathway. Together these data suggest that the PA accumulating transgenic fruit may exhibit delayed senescence, remaining metabolically active for a longer period of time than fruits from non-transgenic controls allowing for increased synthesis of phytonutrients.

8.5 The influence of light on fruit quality

The accumulation of brightly coloured pigments in fruits is one of the most dramatic events that accompanies ripening and serves as a key signal to seed-dispersing fauna of the ripeness and palatability of the fruit. Fleshy fruits predominantly accumulate carotenoids, anthocyanins and flavonoids and the de novo synthesis of these compounds at the onset of ripening is preceded by, or occurs concomitantly with, the degradation of chlorophyll (Seymour *et al.*, 1993). An exception to this generalization occurs in banana where the degradation of chlorophyll at the onset of ripening leads to the unmasking of the yellow-pigmented xanthophylls that are already present in immature fruit (Seymour *et al.*, 1993). The biochemical pathways that lead to the synthesis of carotenoids, anthocyanins and flavonoids are well established and mutations in various steps in these pathways or regulators of these pathways lead to the colour variants witnessed in many fruit species (Hirschberg, 2001; Takos *et al.*, 2006; Bogs *et al.*, 2007; Chagne *et al.*, 2007; Espley *et al.*, 2007).

In tomato, mutants that disrupt ethylene signalling have reduced carotenoid content indicating that ethylene plays a significant role in regulating carotenoid synthesis in this species (Lanahan *et al.*, 1994; Alba *et al.*, 2005; Barry *et al.*, 2005). However, physiological and genetic studies have also implicated light-signalling pathways in influencing carotenoid, flavonoid and anthocyanin accumulation during ripening in several species including tomato, apple and grape (Alba *et al.*, 2000; Solovchenko *et al.*, 2006; Takos *et al.*, 2006; Ristic *et al.*, 2007). For example, carotenoid synthesis in tomato can be stimulated by a short red light pulse and this is reversed by a far-red light pulse indicating that this effect is mediated by phytochrome (Alba *et al.*, 2000).

Transgenic and mutant analysis in tomato has also provided several lines of evidence implicating light-signalling pathways in regulating pigment accumulation during fruit ripening. For example, overexpression of cryptochrome 2 and the oat phytochrome A gene in tomato results in plants that display light hypersensitive phenotypes characterized by shortened internodes and increased pigment accumulation including higher levels of lycopene and flavonoids in fruits (Boylan and Quail, 1989; Giliberto *et al.*, 2005). The manipulation of downstream components of the light-signalling pathway can also elicit changes in the pigment content of tomato fruit. Silencing of *LeHY5*, a positive regulator of light responses and *LeCOP1-like*, a negative regulator of light signalling, resulted in fruits that accumulated lower and higher levels of carotenoids than non-transformed controls, respectively (Liu *et al.*, 2004).

A light hypersensitive phenotype is also observed in the *high pigment 1* (*hp-1*) and *high pigment 2* (*hp-2*) mutants of tomato leading to increased levels of pigments in ripe fruits as well as altered plant morphology (Soressi, 1975; Peters *et al.*, 1989; Yen *et al.*, 1997; Cookson *et al.*, 2003). Using a combination of map-based cloning and candidate gene approaches the *hp-1* and *hp-2* loci have been identified as tomato homologues of Arabidopsis UV-*DAMAGE DNA-BINDING PROTEIN 1* (*DDB1*) and *DE-ETIOLATED 1* (*DET1*), respectively (Mustilli *et al.*, 1999; Lieberman *et al.*, 2004; Liu *et al.*, 2004). These proteins are conserved in eukaryotes and form complexes with a RING-finger protein, RBX1, CONSTITUTIVE PHOTOMORPHOGENESIS 10 (COP10) and Cullin 4 and have been implicated in a range of physiological processes including protein turnover during photomorphogenesis and modification of chromatin structure (Benvenuto *et al.*, 2002; Schroeder *et al.*, 2002; Yanagawa *et al.*, 2004; Bernhardt *et al.*, 2006). The *hp-1* and *hp-2* loci have been restricted in their use in breeding programmes because of detrimental traits associated with reduced plant vigour. However, fruit-specific expression of *HP2* in transgenic tomato led to beneficial accumulation of pigments in the transgenic fruits without the associated alteration in plant growth (Davuluri *et al.*, 2005). These diverse experiments indicate that the manipulation of light-signalling components appears to be an effective strategy to modify the phytonutrient content of fleshy fruits.

8.6 The discovery of aroma and flavour genes in fruit

The characteristic aromas imparted by fleshy fruits as they begin to ripen probably represent the most complex and species-specific aspect of the ripening process and together with colour represent one of the key attractants for frugivores. Individual species and varieties within species all have very unique aroma profiles consisting of hundreds of individual compounds that can be classified into many chemical groups including alcohols, aldehydes, ketones, esters, terpenes, furans, phenolics and sulphur-containing compounds (Buttery and Ling, 1993; Zabetakis and Holden, 1997). For

example, ripening tomato fruits have been documented to produce over 400 volatiles although a subset of approximately 30 of these compounds have been determined to define the 'characteristic aroma' of tomato (Buttery and Ling, 1993; Tieman *et al.*, 2006b). The pathways that contribute to aroma production in these diverse species are not fully understood but in recent years significant progress has been made in identifying some the genes and enzymes that synthesize aroma volatiles in fleshy fruits.

Biochemical and genetic evidence has demonstrated that volatile components are intimately linked to ripening-associated colour changes in fruits that accumulate carotenoids as the principle pigment (Lewinsohn *et al.*, 2005; Tieman *et al.*, 2006b). A number of the aroma volatiles are derived from fatty acids that are presumably formed as a result of membrane breakdown and the reorganization that occurs during the transition of the chloroplast to the chromoplast or are directly formed from the breakdown of carotenoids. Genes involved in the production of some of these compounds have been defined. For example, silencing of a plastid-targeted lipoxygenase gene, designated *TOMLOX-C*, in transgenic tomato led to a reduction in the production of C6 volatiles in ripening fruit (Chen *et al.*, 2004). Similarly, silencing of the tomato *CAROTENOID CLEAVAGE DIOXYGENASE 1* gene of tomato was found to result in a reduction of the carotenoid volatiles, β-ionone, pseudoionone and geranylacetone in ripening fruits (Simkin *et al.*, 2004).

Most domesticated fruit crops have been bred for long shelf life and tolerance to postharvest handling whereas flavour and aroma traits have been a lower priority for plant breeders. The use of wild species germplasm has proven to be an excellent source of natural variation for breeding of quality traits and for fundamental studies to assess biological phenomena (Fernie *et al.*, 2006). In tomato, a set of introgression lines (ILs) that contain defined segments of the *Solanum pennellii* genome within a cultivated, *Solanum lycopersicum*, genetic background have been utilized for identifying quantitative trait loci (QTLs) controlling a number of fruit quality traits including those contributing to aroma production (Lippman *et al.*, 2007). Utilizing this population, variation in a number of volatile compounds derived from fatty acids, amino acids and carotenoids has been identified (Tadmor *et al.*, 2002; Tieman *et al.*, 2006a, 2006b; Matsui *et al.*, 2007). The power of the IL population is that the variation detected in specific metabolites can be immediately assigned to specific chromosomal segments aiding map-based strategies for gene identification. Similarly, natural variation in aroma volatiles occurs between cultivated strawberry, *Fragaria ananassa* and wild strawberry, *Fragaria vesca* with the latter displaying increased diversity in monoterpene profiles while lacking the sesquiterpene, nerolidol (Pyysalo *et al.*, 1979; Aharoni *et al.*, 2004). This variation in terpenoid accumulation has been attributed to expression and structural differences in two genes, *NEROLIDOL SYNTHASE 1* (*NES1*) and *PINENE SYNTHASE* (*PINS*) (Aharoni *et al.*, 2004). *NES1* is highly expressed in cultivated strawberry but is expressed at low levels in *F. vesca* and is differentially localized in the two species. In contrast, *PINS* is highly

expressed in wild species but not in cultivated species and furthermore a two base pair insertion exists in the gene from the cultivated species that results in a frame shift and a corresponding premature stop codon suggesting a non-functional enzyme. These examples illustrate the utility of harnessing natural variation for elucidating biochemical pathways in ripening fruits.

8.7 Cell wall changes influence fruit quality

The softening of fleshy fruits as they ripen is one of the key determinants of quality leading to alterations in texture and is also a primary target for manipulation to enhance postharvest storage and handling characteristics. The processes that constitute the changes in cell wall structure and their contribution to fruit softening have recently and comprehensively been reviewed by Brummell (2006) and I refer readers to this article for an in depth discussion of this topic. In general terms, fruit softening is brought about by a combination of structural changes to the cell wall and also a reduction in turgor pressure. One of the initial changes in cell wall architecture at the onset of ripening is the dissolution of the pectin matrix that composes the middle lamellae that form a connective layer between adjacent cells and this is subsequently followed by alteration in the structure of the cell wall polysaccharides (Jarvis *et al.*, 2003). An interesting aspect of cell wall dissolution during fruit ripening is the diversity in polysaccharide metabolism that occurs during softening of different species. For example, pectin de-polymerization is absent in strawberry, banana, apple and pepper but occurs at moderate to high levels in tomato, peach and avocado (Brummell, 2006). Similar species variation is also apparent for other polysaccharide components highlighting the complexity of the softening process in fleshy fruits and implying that manipulation of softening by a single method is unlikely to be successful in multiple species.

Transgenic approaches to manipulate the activity of different classes of cell wall hydrolases and wall loosening enzymes, with the aim of reducing fruit softening have been undertaken with a range of success (Brummell and Harpster, 2001). In some cases, these studies have revealed a modification of polysaccharide structure following silencing or overexpression of particular enzymes, but these modifications have typically resulted in either small or no alterations in fruit softening (Giovannoni *et al.*, 1989; Brummell *et al.*, 1999; Brummell and Harpster, 2001; Smith *et al.*, 2002; Powell *et al.*, 2003). However, pyramiding of some transgenes has led to increased firmness, particularly in ripening and ripe fruit. For example, transgenic tomato lines suppressed for both polygalacturonase and expansin activity show increased firmness and were less susceptible to the postharvest pathogen *Botrytis cinerea*, suggesting that the pathogen may require certain cell wall modifications to occur within the fruit prior to being able to establish an infection (Cantu *et al.*, 2008). Further pyramiding of cell wall-associated transgenes may yield further insight into the co-operative nature of cell wall disassembly during fruit ripening.

8.8 The cuticle influences fruit quality and postharvest longevity

The plant cuticle is a layer formed by cutin and wax and is associated with the outer wall of epidermal cells. The cuticle is instrumental in maintaining water content of fleshy fruits and water content can be high for extended periods of time following harvest. The cuticle also acts as a barrier to pathogen infection and in the case of fruits can act as a reflective surface to enhance colour signals. The tomato fruit cuticle is approximately 10 μm thick and is astomatous and is composed of various waxes and cutin (Wilson and Sterling, 1976; Petracek and Bukovac, 1995; Bauer *et al.*, 2004; Bargel and Neinhuis, 2005). The genetic factors that control the composition and permeability of fruit cuticles have remained largely undefined but recent identification of loci in tomato that alter water permeability in fruit promises to provide new insight into this horticulturally important phenomenon. For example, a tomato mutant deficient in a very long chain fatty acid β-ketoacyl-CoA synthase displays enhanced water loss, cuticle permeability and fruit shrivelling due to a reduction in the content of *n*-alkanes and aldehydes with chain lengths of longer than C30 (Vogg *et al.*, 2004). Similarly, the *Cuticular water permeability 1* (*Cwp1*) locus of tomato results in microfissues on the fruit surface and causes water loss through the cuticle leading to fruit shrivelling both on and off the vine (Hovav *et al.*, 2007). The *Cwp1* locus maps to the long arm of chromosome 4 and was isolated following the introgression of a chromosomal segment from the wild species *Solanum habrochaites* into a cultivated tomato background. *CWP1* encodes a protein of unknown function that is expressed in fruit of the IL, designated *Cwphir*, but is not expressed in the wild-type control line, *Cwpesc*. Confirmation of gene identity was provided through overexpression of *CWP1* in cultivated tomato under the control of the *CaMV35S* promoter which resulted in fruits that phenocopied the *S. habrochaites* IL. Interestingly, *CWP1* is not expressed in red and orange-fruited wild species of tomato but is expressed in green-fruited species including *Solanum peruvianum*, *S. habrochaites* and *Solanum chmiliewskii*. However, the microfissure and water loss phenotype is only observed when the alleles from these green-fruited species are introgressed into cultivated tomato suggesting that there is an interaction between CWP1 and as yet unidentified components from cultivated tomato. Analysis of chemical composition of homozygous *Cwp1hir* and *Cwpesc* lines failed to reveal any differences in wax and cutin components between the genotypes and the mechanism of *CWP1* action remains unknown although it is possible that this gene may play a role in structural organization of the cuticle or in the interaction of the cuticle with the epidermal cell wall.

The *delayed fruit deterioration* (*dfd*) locus of tomato has an opposite phenotypic effect to that of *Cwp1* in that the cuticle is less permeable to water loss than wild type and had increased cell turgor leading to fruits displaying an extended shelf life (Saladie *et al.*, 2007). No visible differences were evident in the surface or thickness of *dfd* cuticles although they exhibited altered

biomechanical properties. However, large differences in wax and cutin monomers were observed in *dfd* compared with control fruits. For example, total wax content is >30% higher in *dfd* than control fruits with the largest increases observed in the content of the alkadienes. Similarly, cutin monomer content was increased by 84% in *dfd* fruits. Cloning of *dfd* should prove interesting, particularly within the context of the *CWP* gene.

8.9 Genomics resources

As in other areas of plant biology, the development of high-throughput 'omics' resources has revolutionized research in fruit biology. This has led to the generation of large data sets and the subsequent need for database infrastructure to curate, store and disseminate this data. Several family or species-specific databases now exist for many fruit crop species (Table 8.2). These databases contain a wealth of annotated data including genetic and physical maps and associated marker information, EST and genomic sequences, data sets from expression, metabolite and proteome profiling experiments and germplasm information. These databases are invaluable to researchers working on particular crops but are also extremely useful for accessing data for performing comparative genomics of fruit development and ripening. The availability of large EST collections sequenced from fruit cDNA libraries has enabled the *in silico* comparison of gene expression during fruit development and ripening and the development of microarray resources for the majority of the major fruit crop species including tomato, peach, grape, strawberry, apple and citrus. Profiling experiments using these gene expression arrays have provided insight into the identities of the genes that are expressed during fruit ripening and their regulation by hormone treatments and genetic mutations. These experiments have led to the identification of co-regulated genes that are expressed during the ripening process and have provided a platform for comparative gene expression analysis between fruit crop species (Aharoni *et al.*, 2000, 2002; Alba *et al.*, 2004, 2005; Fei *et al.*, 2004; da Silva *et al.*, 2005; Lemaire-Chamley *et al.*, 2005; Newcomb *et al.*, 2006; Park *et al.*, 2006; Trainotti *et al.*, 2006; Deluc *et al.*, 2007; Schaffer *et al.*, 2007). The availability of large EST collections has also directly led to the recent identification and characterization of small RNAs from tomato and apple fruit which will undoubtedly lead to new opportunities for examining genetic regulation of fruit development and ripening (Pilcher *et al.*, 2007; Gleave *et al.*, 2008; Itaya *et al.*, 2008).

Advances in chromatographic separation techniques coupled with sensitive mass spectrometry have facilitated the ability to simultaneously detect many primary and secondary metabolites in single small-scale extractions (Schauer and Fernie, 2006; De Vos *et al.*, 2007; Last *et al.*, 2007). The application of these metabolomic techniques to the study of fruit ripening, particularly when combined with genetic diversity is proving to be a powerful approach for identifying loci that control metabolite levels and fruit quality. A survey

Table 8.2 Databases containing genomics-based resources for fleshy-fruited crop species

Database	URL	Represented species
Vitaceae		
International grape genome programme	http://www.vitaceae.org	Grape
Vitis gene expression database	http://cropdisease.ars.usda.gov/vitis_at/mainpage.htm	Grape
Solanaceae		
Sol genomics network	http://www.sgn.cornell.edu/	Tomato, pepper, eggplant, coffee
Tomato expression database	http://ted.bti.cornell.edu/	Tomato
Tomato metabolite database	http://tomet.bti.cornell.edu/	Tomato
Metabolome tomato Database	http://appliedbioinformatics.wur.nl	Tomato
Cucurbitaceae		
Cucurbit genomics database	http://www.icugi.org/	Melon, cucumber, watermelon
Rosaceae		
Genome database for rosaceae	http://www.bioinfo.wsu.edu/gdr/	Apple, pear, peach, cherry, apricot, raspberry, strawberry
Miscellaneous		
International citrus genomics consortium	http://int-citrusgenomics.org/	Multiple citrus species
The Hawaii papaya genome project	http://cgpbr.hawaii.edu/papaya/	Papaya
Global musa genomics consortium	http://www.musagenomics.org/	Banana
PineappleDB	http://genet.imb.uq.edu.au/Pineapple/	Pineapple

of fruit from cultivated and wild tomato species revealed significant differences in the levels of several classes of primary metabolites including sugars, organic acids and amino acids, highlighting the diversity that exists between these species (Schauer *et al.*, 2005). In a refinement of this study, 74 metabolites were surveyed in an IL population of 76 individuals comprising *S. pennellii* chromosomal segments in an *S. lycopersicum* genetic background. This experimental approach localized 889 QTLs associated with fruit metabolite content onto the tomato genome (Schauer *et al.*, 2006). A number of these loci display

significant variation between the IL and the M82 parental cultivar raising the possibility that their identity may be resolved through genetic mapping and gene cloning. Liquid chromatography–mass spectrometry (LC–MS)-based metabolomics has also been utilized for identifying a range of semipolar metabolites in tomato fruit, including revealing the presence of compounds not previously identified in fruit (Moco *et al.*, 2006). Further analysis revealed distinct spatial and temporal localization of metabolites in specific tissues at different stages of tomato fruit development and ripening (Moco *et al.*, 2007). These pioneering studies open the door to incorporate large-scale analysis of metabolites into the functional genomics toolbox of biologists investigating fruit ripening.

8.9.1 Genome sequencing of fleshy-fruited species

Several genomes from higher and lower plants have now been sequenced revealing information on gene content, genome structure and evolution. Recently, draft genome sequences of two fleshy-fruited species, grape and papaya have been released (Jaillon *et al.*, 2007; Ming *et al.*, 2008). An 8.4-fold draft genome sequence has been produced for grape, revealing a 487 Mb genome containing 30 484 predicted genes (Jaillon *et al.*, 2007). Annotation of the grape genome revealed an increased prevalence of genes encoding enzymes involved in secondary metabolites associated with aroma production and wine quality. For example, the terpene synthase (TPS) family, which is important for the synthesis of oils, resins and aroma compounds, is more than twice as large in grape compared to Arabidopsis. Furthermore, monoterpene synthases make up greater than 40% of the TPS family in grape whereas in Arabidopsis they constitute approximately 15% of the total TPS complement. The monoterpene synthases are specifically involved in the formation of C10 terpenoids that are present in grape aroma volatiles such as linalool and geraniol. Similar results were found for stilbene synthases (STSs) that are involved in the synthesis of the phytoalexin resveratrol suggesting diversification and specialization of gene complements in species that produce a range of diverse secondary metabolites.

The papaya genome was sequenced to 3X coverage by whole genome shotgun sequencing, revealing 372 Mbp containing 24 746 predicted genes (Ming *et al.*, 2008). Similar to the grape genome, annotation of the papaya genome revealed an increased number of genes involved in the production of aroma volatiles compared to those present within the Arabidopsis genome. The sequencing of the grape and papaya genomes represents significant milestones in plant biology. The increased prevalence within these genomes of genes involved in aroma volatile production is congruent with the evolution of fleshy fruits and ripening processes as seed dispersal mechanisms and likely also reflects selection during crop domestication for varieties with enhanced organoleptic properties. The vast majority of the species listed in Table 8.2 have genome projects that are in progress and at various stages of completion,

and it is likely in the coming years that additional fruit crop species will be sequenced, opening the way to examine comparative fruit genomics across plant families with divergent ripening behaviours, fruit morphologies and chemical compositions.

8.10 Conclusions and future perspectives

The ripening of fleshy fruits imparts desirable characteristics on an otherwise unpalatable product. The biochemical changes that occur during fruit ripening serve as attractants and important quality determinants for both humans and other animals that ultimately aid in seed dispersal. In recent years, research on the factors that mediate fruit ripening and quality has undergone a renaissance with large advances in our knowledge of the genes involved in these processes. The sequence of the grape and papaya genomes represents a milestone in fruit biology research and the emerging sequence of the tomato genome and that of other fleshy fruit-bearing species will similarly have a tremendous impact on future research directions and will create hitherto unavailable opportunities for comparative biology of fruit crops on a genomic scale. However, functional analysis of large numbers of genes in fleshy fruit-bearing species remains a challenge due to the time and resources required to generate mutants or transgenic lines. This is particularly problematic in tree and other woody fruit-bearing crop species, although the development of transient-based assays for gene function analysis in fruits may be a useful technology to overcome this bottleneck (Fu *et al.*, 2005; Hoffmann *et al.*, 2006; Orzaez *et al.*, 2006). In addition, the generation of stable genetic populations utilizing exotic germplasm to harness natural variation will greatly enhance our future ability to isolate genes required for determining fruit ripening and quality from a variety of species and to utilize this information for breeding varieties with enhanced quality traits.

Acknowledgements

Research in the author's laboratory is supported by start-up funds from Michigan State University and the Michigan Agricultural Experiment Station.

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