Review

Regulation of tomato fruit ripening

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Abstract

Fruit ripening is a sophisticatedly orchestrated developmental process, unique to plants, that results in major physiological and metabolic changes, ultimately leading to fruit decay and seed dispersal. Because of their strong impact on fruit nutritional and sensory qualities, the ripening-associated changes have been a matter of sustained investigation aiming at unravelling the molecular and genetic basis of fruit ripening. Tomato rapidly emerged as the model of choice for fleshy fruit research and a wealth of genetic resources and genomics tools have been developed, providing new entries into the regulatory mechanisms involved in the triggering and coordination of the ripening process. Some of the key components participating in the control of tomato fruit ripening have been uncovered, but our knowledge of the network of signalling pathways engaged in this complex developmental process remains fragmentary. This review highlights the main advances and emphasizes issues still to be addressed using the rapidly developing 'omics' approaches.

Keywords: Fruit ripening, Tomato, Ethylene signalling, Genetic regulation, Climacteric, Natural mutants

Introduction

Fruit ripening is the ultimate developmental stage of the reproductive organ of higher plants from which the matured seeds are released for reproduction. In that regard, the primary role of the fruit is to provide a suitable environment for seed development and maturation. Fruit development is divided into three main phases starting with fruit set and early growth, characterized by active cell division. During the second phase, the fruit undergoes a steady increase in size, mostly through cell expansion. The last phase corresponds to fruit ripening and is characterized by dramatic changes in colour, texture and taste, which contribute to the build-up of the fruit sensory quality. Once maturation is reached, the fruit structure is continued to alter until complete decay, thus leading to seed dispersal. All biochemical, molecular, physiological and structural modifications associated with ripening are tightly orchestrated at the genetic level, enabling the control of appearance, aroma, flavour and texture so as to render the fruit appealing to a variety of seed-dispersing organisms including humans.

Given its social and economic importance, man-made selection tended to divert the fruit function from reproduction to consumption and for that reason ripening has been and continues to be extensively studied at the physiological, biochemical and genetic levels. Since the early 1980s, tomato has been recognized as a model system for studying the molecular basis of fleshy fruit development and unravelling the role of ethylene in controlling the ripening of climacteric fruit. The adaptation of a range of technological tools (e.g. microarray) and the generation of new biological resources on the tomato (e.g. EST database, TILLING resources, genetic and physical maps) have led to a step forward on the understanding of the molecular mechanisms underlying the ripening process. Tomato is an attractive model species because of the availability of a wide range of well-characterized spontaneous or induced mutants; ease of genetic transformation and manipulation and the existence of a dwarf variety

(*MicroTom*) that has a short life cycle and can be grown at high density. The status of the model system gained by the tomato has been also fostered by the genetic proximity to other species from the Solanaceae family such as potato, pepper and eggplant, all presenting important agronomical and economical interest. Taking advantage of the relatively small size of the tomato genome, major initiatives were launched by the Solanaceae Genome Network (SGN: http://www.sgn.cornell.edu/) an international consortium that includes a genome sequencing project and the generation of resources for high-throughput reverse genetics and transcriptomics [1]. Fruit research and particularly ripening research have benefited greatly from the development of these modern tools. Major progress has been made in identifying important genes that give new leads towards understanding the molecular control of the fruit ripening process.

So far, our understanding of the regulatory events controlling fruit ripening have greatly benefited from the availability of a variety of natural ripening mutants such as *rin* (*ripening inhibitor*), affected in the MADS-box [2]; *nor* (*non-ripening*), altered in a transcription factor of yet unknown function [3]; *Nr* (*Never ripe*), mutated in the ethylene receptor [4]; *Cnr* (*colourless non-ripening*), altered in the expression of Squamosa Promoter Binding Protein [5] and *Gr* (*green ripe*), affected in one component of the ethylene transduction pathway [6]. The present review will compile the most recent advances made in deciphering the molecular mechanisms regulating tomato fruit ripening. It will also emphasize new perspectives now possible in fruit research.

Biochemical Changes Associated with Ripening: The Fruit Ripening Syndrome

The majority of fruit quality attributes are elaborated during the ripening process. These traits correspond to visual, chemical and structural modifications that ultimately make fruit edible and attractive for consumption. Because these changes are crucial for the final sensory and nutritional qualities of the fruit, they have received great attention from scientists and breeders and studies have been directed toward a better understanding of their physiological, molecular and genetic basis. Among all the aspects contributing to fruit quality, changes in texture, aroma, volatile production and pigment accumulation have been most extensively studied in the tomato. Efforts in this area have first concentrated on the isolation and characterization of genes and enzymes that participate directly in the above mentioned biochemical and physiological changes. Thereafter, attempts were made to unravel the regulatory mechanisms controlling these complex processes. Studies of secondary metabolites accumulating during tomato fruit ripening were further prompted by health claims concerning these compounds, even though direct and clear evidence of their positive impact on human health is still lacking.

Shedding Light on Fruit Colour Development

Biosynthesis of a large variety of secondary metabolites is one of the most remarkable features of ripe fruit, and in the case of tomato, red pigment accumulation is emblematical of the ripening process. Carotenoid pigments, including lycopene, are key components of the sensory and nutritional quality of both fresh ripe and processed tomato fruit. The characteristic colour of ripe tomato fruit is caused by lycopene and β-carotene, which accumulate concomitantly with the decrease in chlorophyll content during the transition from chloroplast to chromoplast [7].

Carotenoid biosynthesis is a complex pathway distributed in two main steps and involving a large number of enzymes. In the early step, DOXP synthase (1-deoxy-D-xylulose-5-phosphate) catalyses the condensation of hydroxyethyl thiamine into 1-deoxy-D-xylulose 5-phosphate [8, 9]. This step, leading to the isopentenyl pyrophosphate (IPP), is also known as the non-mevalonate pathway by opposition to the mevalonic acid-dependent pathway. The later step is the isoprenoid pathway in which phytoene synthase (PSY) catalyses the condensation of two molecules of geranylgeranyl pyrophosphate (GGPP) to form phytoene [10], the immediate precursor of lycopene (Figure 1). Lycopene accumulation is correlated with the up-regulation of isoprenoid genes, notably DOXP synthase, suggesting a crucial role for the non-mevalonate pathway in lycopene biosynthesis during fruit ripening [11]. Phytoene synthase (*PSY1*) and phytoene desaturase (*PDS*) genes, which are also up-regulated during ripening [7, 12–14], encode enzymes that catalyse phytoene formation and desaturation, respectively, leading to lycopene formation. Concomitantly, lycopene cyclase genes (*LCY-b* and *LCY-e*) are strongly down-regulated during ripening [15, 16], thus preventing lycopene cyclization and so leading to its accumulation. It has been demonstrated that the inhibition of lycopene cyclization induced an increase in *PDS* and *PSY-1* expression, suggesting the existence of an autocatalytic synthesis of lycopene [13, 14].

Accumulation of lycopene is stimulated by red light treatment and is under the dependence of fruit localized phytochrome [17]. The red/far red (R/FR) regulation of the PSY activity is not reflected in *PSY1* transcript level indicating that the light-regulation of PSY occurs at the post-translational level [18]. These data suggest that light regulates at least some components of the ripening process, yet the corresponding signalling mechanisms are still unknown.

While the role of ethylene in controlling the ripening-associated colour development is well established, some data also suggest that auxin signalling is involved in the

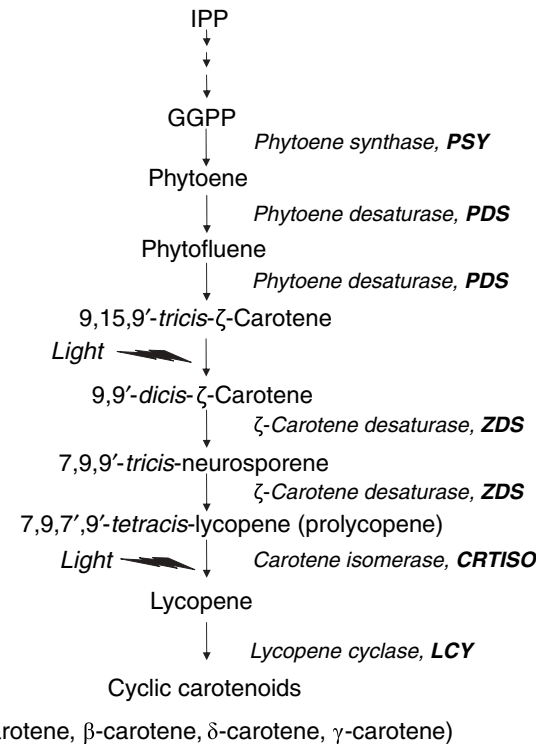

Figure 1 Simplified scheme of carotenoid biosynthesis in tomato fruit. IPP, the starting point of carotenoid biosynthesis, is produced via the plastidial mevalonate-independent pathway. IPP leads to GGPP, which is directly converted to phytoene by PSY. A cascade of desaturation reactions is then necessary to create the characteristic carotenoid chromophore including PDS and ζ-carotene desaturase (ZDS), ultimately to produce prolycopene. The last step of lycopene synthesis in fruit tissue involves carotene isomerase (CRTISO), while in photosynthetic tissues light and chlorophyll catalyse this conversion. Cyclization of lycopene by lycopene cyclase (LCY) leads to the formation of carotene. Red pigment accumulation in ripe fruit is mainly the result of lycopene accumulation resulting from restriction of lycopene cyclization

regulation of pigment accumulation. Tomato fruit under-expressing *DR12,* a gene encoding a transcriptional regulator of auxin responses (corresponding to auxin response factor 4, *ARF4*), show dark green and blotchy ripening phenotypes [19]. Because similar phenotypes have been described for transgenic lines over-producing cytokinins [20], it cannot be excluded that *ARF4* may function as a link between auxin and cytokinins signalling pathways.

AQ1 On the other hand, ABA also emerged as an important hormone involved in fruit ripening. A tomato ABA-deficient mutant (*hp3*) was shown to display higher storage capacity of carotenoid [21] and more recently ABA treatment was reported to promote tomato fruit ripening, as well as ethylene biosynthesis via the induction of *ACS2* and *ACO1* genes, whereas fluridone, the ABA synthetic inhibitor, delays ripening [22].

The Concerted Contribution of Genes with Diverse Functions to Fruit Softening

Texture change and softening are among the most striking features of fruit ripening and it has long been known that this phenomenon is caused by cell wall disassembly and reorganization. The primary cell wall is constituted of different polymers including cellulose matrix glucan, composed of neutral sugars, pectins and structural proteins [23]. During the ripening process, the pectin-rich middle lamella of the cell wall is modified and partially hydrolysed, and the structural change of this pectin gel is responsible for the loss of cohesion between cells and, at least partly, for the softening of the ripe fruit (Figure 2).

Polygalacturonase (PG) catalyses the hydrolytic cleavage of α-(1-4)-galacturonan linkages and is responsible for the change in pectin structure associated with the ripening of many fruits [24]. In ripening-impaired mutants *rin*, *nor* and *Nr*, the softening is dramatically reduced and the level of *PG* transcripts is lower than wild-type [4, 25, 26]. Consistent with the putative role of PG in the softening process, different *cis*-regulatory regions allowing the expression in the outer and inner pericarp of ripe tomato fruit have been identified in the *PG* promoter [27]. Moreover, *PG* transcripts were shown to be induced by very low levels of ethylene concentration [28]. Pectin de-methylation and de-esterification by pectin methylesterase (PME) is a prerequisite for subsequent pectin depolymerization and solubilization by PG (Figure 2). Since, the pattern of PME proteins accumulation does not correlate with the pattern of transcript accumulation; it is likely that fruit softening is also regulated at the post-translational level [29–31]. Moreover, transgenic tomato plants under-expressing a *Rab11*, a GTPase involved in the control of cellular protein trafficking, shows reduced level of PG activity and decreased fruit softening, suggesting that regulation of the trafficking of cell-wall-modifying enzymes by GTPase represents an additional point of control of texture change during fruit ripening [32].

At the beginning of the ripening process, the breakdown of polymeric galactose into free molecules of galactose is catalysed by β-galactosidase [33]. Purified tomato β-galactosidases can be classified into three forms displaying complementary activities during fruit development and ripening [34]. Forms I and III are highly active in green fruit but not at the red stage, whereas activity of form II is absent in green fruit and increases during ripening [34–36]. Tomato β–galactosidases are encoded by a gene family comprising at least seven members that show specific expression pattern throughout fruit development [33, 35, 37]. *TBG4* is up-regulated during fruit ripening and the corresponding transcripts are not detected in ripening-impaired mutants *nor*, *rin* and *Nr* [33]. It was postulated that *TBG4* may be regulated by ethylene and the reduction of its activity accounts for up to 40% decrease in fruit softening [38].

Expansin, another cell-wall protein, is responsible for the disruption of the hydrogen bonds between cellulose

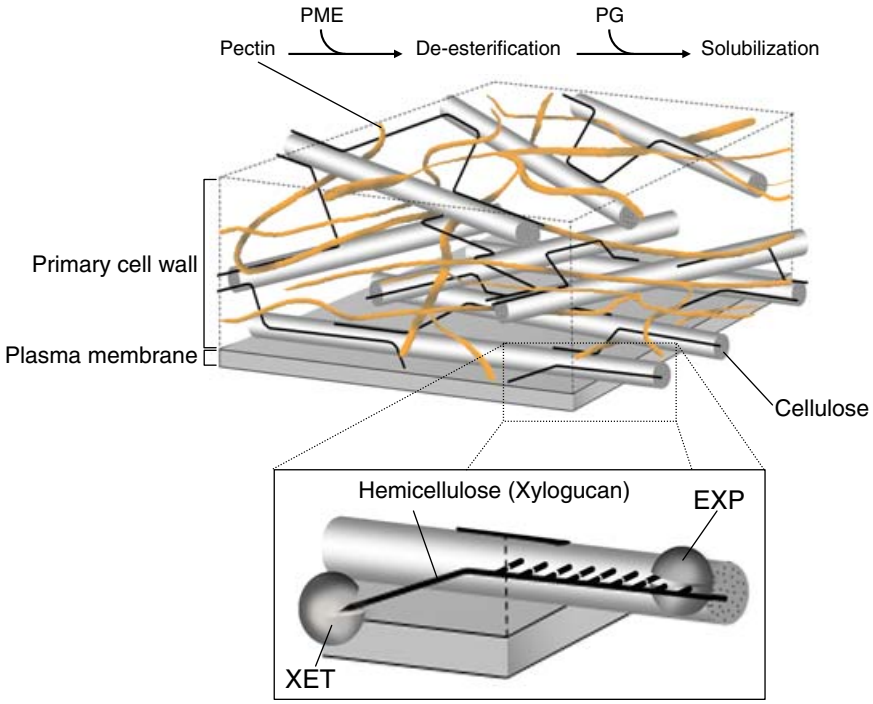

Figure 2 Schematic representation of the spatial arrangement of the primary cell wall components and the major sites leading to cell wall loosening. The primary cell wall is made up of a complex network of carbohydrates (mainly cellulose, hemicellulose and pectin) in which proteins such as expansin and extensin are embedded. The cellulose microfibrils are linked via hemicellulosic tethers to form the cellulose–hemicellulose network, which is embedded in the pectin matrix. The most common hemicellulose in the primary cell wall is xyloglucan. During fruit ripening, texture change and softening are associated with cell wall disassembly involving several enzymes. Pectin de-methylation and de-esterification by PME is required for subsequent pectin de-polymerization and solubilization by PG. Expansin is another cell wall protein responsible for the disruption of the hydrogen bonds between cellulose microfibrils and polysaccharides matrix. XET enzymes cleave xyloglucan and their activity is very high in growing tomato fruit, declines at early ripening and then increases slightly at late ripening

microfibrils and polysaccharides matrix [39]. During development and ripening of tomato fruit, at least six expansin genes show overlapping expression patterns with *EXP1* transcript being the most abundant in ripening tomato fruit. Expression of *EXP1* is ethylene-regulated and its transcripts accumulate specifically in the fruit and peaks at breaker stage [40–42]. Interestingly, down-regulation of *EXP1* results in decreased softening, whereas ectopic expression enhances fruit softening [43].

Xyloglucan endotransglycosylase (XET), also called endo-xyloglucan transferase (EXGT), is another potential actor of fruit softening and texture change [23]. The activity of XET enzymes, which cleave xyloglucan, is particularly high in growing tomato fruit, then declines at early ripening and finally increases slightly at late ripening [44–46]. In the *rin* mutant, XET activity is reduced compared with wild-type tomato, suggesting that the expression of *XET* genes is ripening-related and is possibly regulated by ethylene in climacteric fruit [23, 47]. It was reported that over-expression of *EXGT1* results in increased final fruit size and that both mRNA abundance of *EXGT1* and fruit size were inversely correlated with sugar concentration. This finding highlights the important role of *EXGT1* in final fruit size and in sugar concentration [23] but the involvement of XET in fruit softening remains unclear. Even though *rin*, *nor*, *Nr* and *Cnr* mutations have been described to affect fruit softening, little is known about the direct regulators of genes encoding cell-wall-modifying enzymes. It was, however, reported that under-expression of the auxin transcription factor *ARF4* results in altered fruit texture with enhanced firmness [19]. This phenotype was shown to result from alteration of pectin fine structure associated with changes in tissue architecture [48].

Whereas cell wall metabolism associated with fruit softening is well documented [23], the mechanism that links cell-wall-related genes to ripening-associated changes in fruit texture remains to be elucidated. Recently, the understanding of fruit softening seems to be following new leads that point at the fruit cuticle as a major actor in controlling fruit texture.

Cuticle, the Other Component of Tomato Fruit Softening

The importance of cuticle composition and architecture in maintaining fruit texture emerged from recent findings as the missing piece of the softening process. Plant cuticle is

Table 1 Metabolic origin of main volatile compounds involved in tomato fruit flavour

Pathway	Component	Enzymes	References
Fatty acids oxidation	*cis* − 3-hexenal Hexanal 1-penten-3-one Trans-2-hexenal Trans-2-pentenal pentenol 1-penten-3-ol trans-2-heptenal 2-isobutylthiazole	Phospholipase Lipoxygenase Hydroperoxyde lyase Alcohol dehydrogenase	[59–64]
Amino acids	2-phenylethanol 3-methyl-butanol 1-nitro-3-methyl-butane 2 + 3-methyl-butanal	Amino acid decarboxylases (AADC1A, AADC1B, AADC2)	[65]
Carotenoid related	6-methyl-5-hepten-2-one Geranyl-acetone Pseudoionone β-ionone	Carotenoid cleavage dioxygenase 1	[66]
Terpene pathway	Geranial Linalool Neral	Linalool synthase	[67]
Shikimate pathway	Methyl salicylate	Unknown	[60]

the first protective barrier against pathogen attacks, UV radiation and mechanical damages [49–51]. It also plays an important role in limiting transpirational water loss from the primary plant surface [52]. The cuticular layer is composed of a polymer matrix (cutin) and associated solvent-soluble lipids (cuticular waxes). The cuticle can be divided into two spatially separated layers: the epicuticular waxes coating the surface and the intracuticular waxes embedded in the cutin matrix. Epicuticular film is characterized by the presence of very long-chain aliphatic molecules, while the intracuticular compartment contains in addition large quantities of penta cyclic triterpenoids [53]. Wax composition consists of homologous series of very-long-chain aliphatic molecules, including alkanoic acids, alkanols, aldheydes, alkanes and esters, and cyclic compounds such as triterpenoids and phenylpropanoids [54, 55]. The delayed fruit deterioration (DFD) cultivar produces fruits exhibiting normal ripening but minimal softening. DFD fruits lose less water by transpiration than WT and display higher cell turgor. It was reported that the difference in water transpiration is probably the result of a higher quantity of wax and cutin that contribute to waterproofing of the cuticle [56]. These data suggest an important role for the cuticle in the ripening-associated softening of tomato fruit [56] and give new perspectives on the understanding of novel aspects underlying the ripening and post-harvest-associated modifications of fruit texture. A direct relationship between cuticular transpiration barrier properties and distinct chemical modifications in cuticular wax composition during the course of tomato fruit development was demonstrated for the cer6 mutant [57]. Indeed, a deficiency in this β-ketoacyl-coenzyme A synthase is responsible for the simultaneously occurring increase of water permeance and modification in the proportion of n-alkanes and triterpenoids composition. More recently, a combined analysis of tomato surface fruit tissue components and transcriptomic patterns of expression, allowed the identification of up to 100 candidate genes potentially involved in the cuticle formation including those belonging to a subclass of the ERF family, enoyl-CoA reductase, acyl-CoA synthetase and 3-ketoacyl-CoA synthase (CER6) [58]. Complexity of softening and texture modification during tomato ripening suggests different regulation levels: chemical, mechanical and genetic. All these control points target traits of interest for agronomist in order to modify the softening, the texture and the juiciness.

Regulation of Volatile Formation during Tomato Fruit Ripening

Though it is obvious that aroma volatiles contribute to the overall sensory quality of fruit, the most prevalent compounds that are essential for typical aroma of ripe tomato fruit are still evasive. Around 400 volatile compounds have been identified in ripe tomato but only a few have been considered to play a major role in tomato flavour [59]. Tomato volatile compounds are usually grouped into five main classes [59–67] based on their metabolic origin (Table 1). The lipid-derived volatiles represent the bulk of aroma volatiles in tomato and are generated by the lipoxygenase (LOX) pathway. This pathway appears to be located in the plastid since a natural mutation in a chloroplastic ω-3-fatty acid desaturase gene that resulted in a deficiency in linolenic acid caused profound changes in the volatile profile of tomato [61]. The pathway comprises the action of phospholipase, lipoxygenase, hydroxyperoxide

lyase and alcohol dehydrogenase. Notably, ripe tomatoes evolve very few esters, so that the involvement of alcohol acyltransferases downstream in the pathway is secondary, in contrast to many other fruit types [68]. These enzymes are encoded by multigene families and only in a few cases has the direct involvement of members of these families in aroma volatiles production been ascertained by reverse genetics approaches (for phospholipase PLD-α [62], lipoxygenase LOXC [63] and alcohol dehydrogenase, ADH2 [64]). However, down-regulation of only one of the five *LOX* of tomato, *LOXC*, did not result a significant reduction in the level of flavour volatiles such as hexanal, hexenal and hexenol [63].

The amino acid-derived volatiles are also important components of the aroma of tomato fruit and the identification of the gene encoding the enzyme responsible for the decarboxylation of phenylalanine represents a significant step forward towards the understanding of this metabolic pathway [65]. Down-regulation of this gene via antisense strategy led to reduced emissions of phenylacetaldehyde and phenylethanol in transgenic tomatoes. Conversely, its overexpression in tomato increased up to 10-fold the quantities of phenylethanol, phenylacetaldehyde, phenylacetonitrile and 1-nitro-2-phenylethane. This capacity to modulate the levels of phenylethanol and phenylacetaldehyde is important since these compounds can exert a dual effect: at low concentrations, phenylethanol and phenylacetaldehyde are associated with pleasant sweet flowery notes, while at high concentrations the pungent aroma of phenylacetaldehyde has a nauseating and unpleasant odour [69].

Carotenoid-derived volatiles are present at relatively low levels but play an important role in tomato flavour. The biosynthetic route was discovered by Simkin *et al.* [66] who demonstrated, by both heterologous expression in *Escherichia coli* and down-regulation in tomato plants, that the carotenoid cleavage dioxygenase 1 genes contribute to the formation of β-ionone, pseudoionone and geranylacetone.

Tomato produces low amounts of terpene volatiles. Expressing the *Clarkia breweri* linalool synthase gene under a fruit-specific promoter in the tomato was reported to result in a strong stimulation of the production of linalool and of 8-hydroxy-linalool, probably as a result of the presence in the tomato of a P450 enzyme capable of hydroxylating linalool [67]. These data bring new leads towards the modification of tomato fruit flavour through biotechnological approaches.

It is also important to mention that in ripe tomato many volatile compounds are present in a conjugated form, linked to glycosides to form non-volatile precursors that could be as important in quantity as the free fraction [70]. The mechanism governing, *in vivo,* the release of volatiles from the bound fraction is not very well known. It is supposed to occur by the action of endogenous β-glucosidases, preferentially upon cell disruption. Indeed, the production of aroma volatiles increases upon tissue disruption owing to tissue and cell structure destruction, which brings together enzymes and substrates that are normally sequestrated in different sub-cellular compartments. Glycoside derivatives are synthesized by glycosyltransferases (GTs). GTs are encoded by a very large gene family but so far, data on which GT genes are specifically involved in the formation of conjugated volatiles are not available.

Ethylene, a Key Hormone for Tomato Fruit Ripening

The major advances gained to date in understanding the molecular mechanisms underlying the ripening process have been achieved by the combined use of modern molecular genetics and genomic approaches. While fruit development from fruit set through ripening involves a number of plant hormones [71, 72], the phytohormone ethylene was first identified as the key regulator of tomato fruit ripening. Ethylene is a simple gaseous molecule that plays a key role in many processes, including seed germination, leaf senescence, abscission, responses to stresses and fruit ripening. Ethylene biosynthesis in higher plants originates from *S*-adenosyl-methionine (SAM) and comprises two steps catalysed by ACC synthase (ACS) and ACC oxidase (ACO), the latter converting 1-aminocyclopropane-1-carboxylic acid (ACC) into ethylene [73] (Figure 3) [74]. Genes encoding these two enzymes undergo important regulation during the process of fruit ripening. Fruits can be divided into two broad groups, known as climacteric and non-climacteric, based on their type of ripening mechanisms [75]. In contrast to non-climacteric fruit type, climacteric fruits present a peak in respiration and a concomitant burst of ethylene during maturation. This category of fruit includes tomato, banana, pears and apple; all of them need an ethylene burst for normal ripening. Corroboratively, in ethylene-suppressed transgenic plants there is no or very slow ripening [76–78]. Two distinct systems of ethylene biosynthesis have been proposed to take place during fruit development, system 1 being characterized by auto-inhibitory ethylene production, whereas system 2 is autocatalytic [79]. System 1 of ethylene production relies on the expression of *ACS1A* and *ACS6* [80] and is responsible for producing basal ethylene levels that are detected in all vegetative tissues and in pre-climacteric stages of climacteric fruit development as well as in non-climacteric fruit. During climacteric burst there is an autocatalytic production of ethylene depending on system 2, which is initiated and maintained by the ethylene-dependent *ACS2* [80]. In tomato, ACO, which catalyses the last step of ethylene biosynthesis (Figure 3), is encoded by a small gene family comprising four members *ACO1–4* [81–83]. It has been shown that *ACO* transcripts accumulate during ripening of climacteric fruits [76, 81], with *ACO1* being the most abundantly expressed during fruit ripening and more particularly after breaker stage [84, 85].

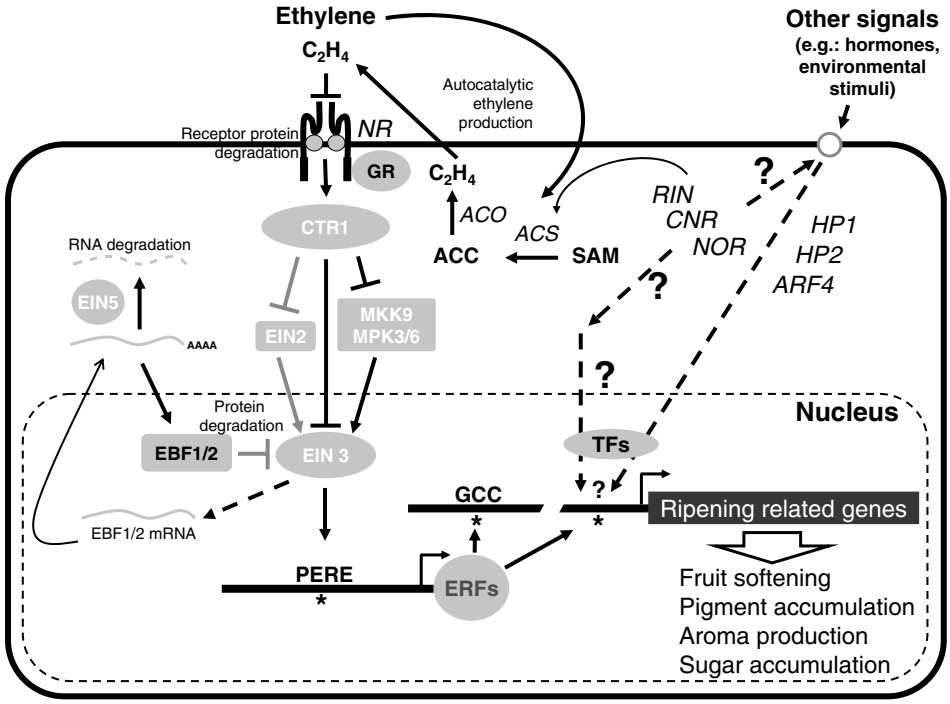

Figure 3 Schematic model depicting the ethylene-dependent and ethylene-independent components of the control of fruit ripening. Autocatalytic ethylene production associated with fruit ripening probably involves RIN MADs-box induced ACC synthase (ACS) for the production of ACC, the ethylene precursor used as substrate by ACC oxidase (ACO). Ethylene is perceived by a family of membrane-bound receptors including NR, levels of which are controlled by protein degradation. In the absence of ethylene binding, active form of the receptors acts as negative regulators of ethylene signalling. GR, a RTE-like protein, affects fruit ripening through interaction with the ethylene receptor. In the absence of ethylene, CTR1 is in its active form and negatively regulates EIN2, a positive regulator of EIN3. Subsequently, EIN3 activates ethylene responses by binding to the EIN3-binding site (PERE) in the promoter of *ERF* genes. CTR1 was also reported to inactivate MKK9-MPK3/6 in Arabidopsis but no similar data are available for the tomato [74]. *ERF*s encode transcriptional regulators that bind the GCC-box in the promoter of ethylene and ripening-regulated genes. EIN3 stability is controlled by proteasome-mediated degradation involving EBF1/2. Repression of *EBF1* and *EBF2* transcription is mediated by an exoribonuclease encoded by EIN5. RIN, NOR and CNR are ripening switches acting upstream of autocatalytic ethylene. Ripening-related genes are also controlled by other signalling pathways including auxin via ARF4 and light via HP1 and HP2. Arrows and blunt end arrows indicate positive and negative regulation, respectively. Stars indicate *cis*-elements recognized by specific transcription factors (TFs). Dashed lines indicate putative or unknown link between two components

Ripening of non-climacteric fruits such as pineapple, lemon and cherry is generally considered as an ethylene-independent process, although some recent results suggest a role of ethylene in ripening this type of fruit [86, 87].

Ethylene Signalling and the Control of Ripening Time

The molecular dissection of ethylene transduction and response pathway has been initially performed with the model plant *Arabidopsis* using genetic screens based on the well-documented triple response phenotype of ethylene-treated aetiolated seedlings [88–90]. These remarkable studies led to the identification of the first plant hormone receptor [91]. Subsequent studies revealed a simple linear signalling pathway, where ethylene is perceived by a family of membrane-bound receptors [4, 92–94] bearing similarity to two-component histidine protein kinase receptors. Ethylene binds to the N-terminal membrane-spanning domain of the receptor in the presence of a copper cofactor provided by the RAN1 copper transporter [95]. Genetically, it has been shown that the receptors are negative regulators of ethylene signalling [96, 97]. That is, in the absence of ethylene, the ETR1 and related protein receptors are active and can repress downstream ethylene response through the activation of CTR1 a Raf-like protein kinase, which also functions as a negative regulator of ethylene signalling [98]. Once ethylene binds to the receptor, ETR1 can no longer activate CTR1 and repress ethylene responses. CTR1 acts as a negative regulator of ethylene response via repressing the positive regulator EIN2 [99], which further relays the ethylene signal to the transcription factors EIN3 and EILs. These latter, activate ethylene response factor 1 (*ERF1*) the primary target transcription factor involved in the activation of secondary target ethylene-responsive genes such as *PDF1.2* [100]. The latest advances have implemented the linear model of the ethylene-signalling pathway into a more complex signalling system that

includes multiple pathways of regulators, feedback mechanisms and protein turnover. Importantly, it has been shown recently that ethylene receptor degradation controls the timing of ripening in tomato fruit [101] and that ethylene receptors ETR4 and ETR6 are rapidly degraded in the presence of ethylene via a proteasome-dependent pathway. Degradation of either of these two receptors results in early fruit ripening, suggesting that the levels of receptor in fruit tissue could be a key factor controlling the onset of ripening. It was hence suggested that receptor degradation might serve as a mechanism in fruit to measure cumulative exposure to ethylene and thus to control the timing of ripening.

The negative regulator of ethylene transduction pathway *CTR1*, is transcriptionally up-regulated during fruit ripening concomitantly with the increase in ethylene production. The accumulation of *CTR1* transcripts during fruit ripening may therefore play a role in preventing ethylene overproduction and ripening from proceeding too rapidly [102]. The *CTR* family in tomato constitutes four genes, shown to be differently regulated during ripening and in response to ethylene [103]. Among these, *CTR1* is the most induced during fruit ripening and upon ethylene treatment [103]. Another regulatory step of ethylene responses takes place at the level of *EIN3* and *EIL1* transcription factors (Figure 3). Recently, it has been shown that *EIN3* and *EIL1* are constitutively expressed in *Arabidopsis* and their levels controlled at the post-translational level through protein degradation via the 26S proteasome [104, 105]. In particular, EIN3 is degraded via two F-box proteins, EBF1 and EBF2 [106] and the expression of these two last-named genes is regulated at the post-transcriptional level via a degradation pathway controlled by the exoribonuclease EIN5 [107]. EIN5 is supposed to antagonize the negative feedback regulation on EIN3 by controlling *EBF1* and *EBF2* mRNA turnover (Figure 3). In addition, it was reported that EIN3 stability is controlled by glucose through hexokinase activity [108], suggesting that metabolite accumulation may also contribute to regulate ethylene responses and hence the ripening process. The presence of orthologues of EBF1, EBF2, EIN5 and HXK1 (GenBank accession number: AC respectively ABB89717, ABC24972, ACA05276 and AAY69841) in tomato suggests that similar mechanisms of post-transcriptional and post-translational regulation of the ethylene signalling pathway are conserved in this species and may be operating during fruit ripening.

Regulation of Ripening-related Genes via Recruitment of Selected Ethylene-responsive Genes

Ethylene is known to regulate processes as diverse as stress responses and ripening, yet the molecular mechanisms by which this hormone selects the appropriate target genes to orchestrate in a specific manner either of the two processes remain unclear. In its downstream part, the ethylene transduction pathway leads to a transcriptional cascade starting with EIN3 and EIL (EIN3-like) shown to bind the primary ethylene response element (PERE) present in the promoter of a target *ERF* [100] (Figure 3). ERFs, formerly called ethylene response element binding proteins (EREBPs), are the last known components of the ethylene transduction pathway. Because they are encoded by one of the largest plant multigene family of transcription factors, ERFs are therefore well suited to channel the ethylene signalling towards a wide diversity of responses through recruiting either ripening or stress-related genes depending on the developmental situation and the tissue taken into consideration. While ERFs are known to regulate target genes harbouring the well-conserved GCC-box-containing *cis*-regulatory element [109], some ERFs can also bind other types of *cis*-elements, such as the one described in the *E4* promoter [110]. Interestingly, it was reported that ERFs exhibit differential affinity towards the GCC-box depending on the nucleotide environment surrounding this canonical motif [111] (Pirrello *et al*., manuscript in preparation). Strikingly, so far only one ERF has been identified as direct regulator of ripening-associated genes via binding a *cis*-element present in the promoter of *E4* [110], a ripening-regulated gene [112] encoding proteins of unknown function. This *cis*-element is necessary but not sufficient to confer ethylene responsiveness to these genes since substitution of this ethylene response element abolished their ability to respond to ethylene, while its fusion to a 35S minimal promoter failed to confer ethylene response [113]. Noteworthy, the expression of *E4* is under ethylene control, whereas *E8*, another ripening-associated gene, is regulated by both ethylene and other unidentified fruit-ripening signals [114]. The case of *E8* and *E4* genes well exemplifies the complexity of the transcriptional regulations operating during fruit ripening [109]. All together these studies indicate that transcription regulation of fruit ripening-related genes involves a variety of *cis*-regulatory and *trans*-acting factors, however, to date consensus fruit-specific *cis*-elements have not been identified. It is likely that other hormones are also actively involved in the regulation of fruit ripening-related genes and that cross-talk between different signalling pathways is essential for fine tuning of this important developmental process. In keeping with this hypothesis, analysis of ripening-related gene expression in natural mutants or in transgenic plants reveals two types of gene regulation, ethylene-dependent and ethylene-independent pathways [25, 115, 116].

Genetic Control and Emerging Epigenetic Regulation of Fruit Ripening

The advances made in understanding the molecular mechanisms underlying sillique dehiscence in *Arabidopsis*

identified a series of key actors. However, this has not so far benefited the understanding of their role in fleshy fruit ripening. Instead, most of our understanding of the genetic control of tomato ripening was gained from the analysis of ripening-impaired natural mutants such as *rin*, *nor*, *Nr*, *Cnr*, *Gr*, *hp-1* and *hp-2* (*high-pigment*). Among these mutants the most commonly used, both by scientists and breeders, are *rin* and *nor*. *RIN* encodes a MADS-box protein of the SEPELATTA clade [2, 117] and loss-of-function mutation in this gene results in dramatic delay of ripening. It was shown that RIN factor acts upstream and independently from the autocatalytic ethylene production and it was suggested that RIN could be a universal ripening regulator common to both climacteric and non-climacteric fruit [118]. Recent studies indicated that RIN protein is capable of binding the *ACS2* promoter and may therefore directly regulate the expression of this *ACS* gene *in situ* [119]. The *non-ripening* (*nor*) mutant displays similar phenotypes to those exhibited by *rin* and the *NOR* gene also encodes a putative transcription factor but with uncharacterized function. It was shown that the expression of the *E8* gene is partially active in the *rin* mutant, suggesting that the expression of ripening-associated genes may be controlled by an ethylene-independent mechanism. More recently, another gene encoding a tomato *HD-Zip homoeobox* gene (*HB1*) transcription factor was also reported to result in ripening-related phenotypes in transgenic lines [120].

Tomato mutants altered in components of ethylene signalling also exhibit strong ripening-impaired phenotypes. Among these, *Nr* is mutated in the ethylene receptor gene expressed in fruit and *Gr* is altered in the gene encoding a putative membrane-bound protein with potential copper-binding activity. The *Gr* mutant is ripening-impaired, displaying reduced lycopene content and enhanced fruit firmness. Responses to ethylene are also affected in the *Gr* mutant as exemplified by the altered expression of ethylene-regulated genes such as *E4*, *E8*, *PSY1* and *PG*; however, ripening-associated ethylene production is unaffected [6]. The *hp* mutants were reported to exhibit higher fruit pigmentation because of enhanced accumulation of carotenoids and flavonoids in ripe fruit. *HP-1* gene encodes the tomato orthologue of the *Arabidopsis* UV-damaged DNA-binding protein 1 (DDB1) [121, 122], known to contribute to the initial UV damage response by stimulating nucleotide excision repair [123], while *HP-2* gene encodes the tomato orthologue of *Arabidopsis* nuclear protein DEETIOLATED (DET1), a negative regulator of photomorphogenesis [124, 125].

Epigenetic markers such as cytosine methylation alter chromatin organization, thus affecting the regulation of gene expression. The elucidation of the *Cnr* locus provided new insight into the epigenetic regulation of fruit ripening and revealed the essential role for this mechanism in controlling this developmental process [5]. Accordingly, a recent study demonstrated a link between tissue-dependent methylation and endoreduplication involved in the last step of fruit development [126]. *Cnr* is an epigenetic mutation that alters the methylation status of the promoter of an *SPB-box* (SQUAMOSA Promoter Binding Protein) gene. It was suggested that *CNR* encoded protein may target *TDR4* [127], an orthologue of the *Arabidopsis* FRUITFULL MADS-box gene involved in silique shattering [128]. These new findings demonstrate that heritable fruit quality traits can be modified without modification of the DNA sequence, and hence open new prospects for engineering fruit ripening.

Regulation of gene expression at the post-transcriptional level via small interfering RNAs (siRNAs) is an emerging theme in plant biology. However, direct evidence for siRNA-mediated regulation of developmental processes has been demonstrated so far only in *Arabidopsis*. While examples of siRNA regulation in tomato are still lacking, the identification of microRNAs that could target genes involved in fruit ripening was reported recently [129, 130], supporting the idea that this mechanism of post-transcriptional regulation is potentially important throughout fruit development and ripening. This new developing field of research is likely to shed new light on the regulation of the ripening process and to provide new leads for improving fruit quality traits.

Future Trends: From Fruit Physiology to Fruitomics

The advent of the nascent genomics revolution and the availability of new tools and biological resources on the tomato model species are already impacting fruit research and will, in the near future, further boost our knowledge of the regulatory mechanisms governing the process of fruit development and ripening. An international genome sequencing initiative targeting the gene-rich space of the tomato genome is underway [131, 132] and the expected outcome in terms of sequence information and genome organization will be implemented by several drafts from tomato eco-sequencing programmes, which will ultimately benefit to the research on fleshy fruit ripening. In keeping with this trend, the development of high throughput transcriptomics methodologies is yielding increasing amount of expression data and the accumulating microarrays studies are expected to provide new insights into the molecular basis of fruit development and ripening [133–135]. The high flow discovery of new genes arising from these transcriptomic approaches creates a need for high-speed functional identification methods of candidate genes. The construction of central TILLING (Targeting Induced Local Lesions IN Genomes) facilities for the tomato will address this issue by creating high-throughput reverse genetics technologies publicly accessible to plant biologists and breeders interested in the tomato and other genetically related Solanaceae species (http://www.evry.inra.fr/public/index.html and http://www.competences.u-bordeaux1.fr/fiche_structure.php?struct=TILLING-Tomate). Proteomics approaches have also been launched and the most

promising programmes are dedicated to the analysis of the plastidial proteome. Given that the large majority of the plastid-resident proteins are encoded by the nuclear genome, the sequencing of the plastidial genome is poorly informative regarding the proteins that mediate chromoplastic functions. Because of the prominent contribution of the chromoplast to the build up of sensory quality traits of ripe fruit, it is expected that a comprehensive analysis of the chromoplast proteome will give important leads towards understanding the mechanism of chloroplast to chromoplast transition characteristic of tomato fruit ripening [136]. Following the same line, metabolomics approaches have been developed in recent years aiming at establishing comprehensive primary and secondary metabolic profiling of tomato fruit in contrasted genotypes and various stages of fruit development [137].

The combining of high-throughput data generated by 'omics' approaches will provide important clues towards a better understanding of the molecular events underlying the ripening process and will open new avenues to uncover the signalling pathways orchestrating this genetically programmed developmental process. Indeed, some major questions related to the biology of fruit ripening still remain without clear answers, among which the following are the most important: (i) what is the molecular mechanism underlying the attainment of competence to ripen or, in other words, by what mechanism does ethylene gain its ability to selectively induce the ripening-associated genes at certain stage of fruit development but not earlier, (ii) what are the hormones that act in concert with ethylene to trigger and drive the ripening process, (iii) what signals trigger the ripening of non-climacteric fruit and are they shared between the two types of fruit, (iv) to which extend the regulation of fruit ripening involves siRNAs, and (v) do the variety of primary and secondary metabolites accumulating during fruit ripening play a role in regulating the ripening process through a feedback mechanism?

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