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Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening

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Abstract

Elucidating the mechanisms involved in ripening of climacteric fruit and the role that ethylene plays in the process are key to understanding fruit production and quality. In this review, which is based largely on research in tomato, particular attention is paid to the role of specific isoforms of ACC synthase and ACC oxidase in controlling ethylene synthesis during the initiation and subsequent autocatalytic phase of ethylene production during ripening. Recent information on the structure and role of six different putative ethylene receptors in tomato is discussed, including evidence supporting the receptor inhibition model for ripening, possible differences in histidine kinase activity between receptors, and the importance of receptor LeETR4 in ripening. A number of ethylene-regulated ripening-related genes are discussed, including those involved in ethylene synthesis, fruit texture, and aroma volatile production, as well as experiments designed to elucidate the ethylene signalling pathway from receptor through intermediate components similar to those found in *Arabidopsis*, leading to transcription factors predicted to control the expression of ethylene-regulated genes.

Key words: Carotenoid, climacteric, ethylene receptor, ethylene signal transduction, lipoxygenase, MAPKinase, tomato.

Introduction

Fruit ripening is a complex, genetically programmed process that culminates in dramatic changes in colour, texture, flavour, and aroma of the fruit flesh. Due to the economic importance of fruit crop species these processes have been, and continue to be, studied extensively at both the biochemical and genetic levels. Fruits with different ripening mechanisms can be divided into two groups; climacteric, in which ripening is accompanied by a peak in respiration and a concomitant burst of ethylene, and non-climacteric, in which respiration shows no dramatic change and ethylene production remains at a very low level. In tomato and other climacteric fruits such as apple, melon and banana the ethylene burst is required for normal fruit ripening, as illustrated by the slowing or inhibition of ripening in ethylene-suppressed transgenic plants (Oeller *et al*., 1991; Theologis *et al*., 1993; Picton *et al*., 1993; Ayub *et al*., 1996). Furthermore, it has been shown that ethylene affects the transcription and translation of many ripening-related genes (Gray *et al*., 1994; Deikman 1997; Giovannoni, 2001). However, although ethylene is the dominant trigger for ripening in climacteric fruit, it has been suggested that both ethylene-dependent and ethylene-independent gene regulation pathways coexist to co-ordinate the process in climacteric and non-climacteric fruit (Lelievre *et al*., 1997).

Two systems of ethylene regulation have been proposed to operate in climacteric plants. System 1 is functional during normal vegetative growth, is ethylene auto-inhibitory and is responsible for producing basal ethylene levels that are detected in all tissues including those of non-climacteric fruit. System 2 operates during the ripening of climacteric fruit and senescence of some petals when ethylene production is autocatalytic. Ripening usually commences in one region of a fruit, spreading to

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Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; ACS, ACC synthase; ACO, ACC oxidase; PLP, pyridoxal-5′-phosphate; MAP3K, mitogen activated kinase kinase kinase; ERE, ethylene responsive element; PG, polygalacturonase, GUS, b-glucuronidase; HPOs, unsaturated fatty acid hydroperoxides; LOX, lipoxygenase; ADH, alcohol dehyrogenase.

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neighbouring regions as ethylene diffuses freely from cell to cell and integrates the ripening process throughout the fruit.

The aim of this review is to bring together recent advances made in understanding ethylene biosynthesis and the regulatory role of the phytohormone ethylene in tomato fruit ripening (Fig. 1A). Tomato is a good model system for studying the role that ethylene plays in ripening due to its relatively small genome, well-characterized developmental mutants, ease of genetic manipulation, relatively short life cycle, and its economic importance as a crop species. As ripening progresses, fruit colour changes from green to red as chloroplasts are transformed into chromoplasts, chlorophyll is degraded and carotenoids accumulate. Fruit softening and textural changes occur as the fruit cell wall is modified and partially disassembled by enzymes and the ripe flavour develops as specific volatiles increase and the sugar–acid balance alters. Many components of biochemical pathways involved in pigmentation, cell wall metabolism, carbohydrate metabolism, ethylene biosynthesis, and signal transduction have been identified through alteration of their expression in transgenic plants (Gray *et al.*, 1994; Deikman, 1997; Giovannoni, 2001; Ciardi and Klee, 2001). Differential screening of cDNA libraries has also proved a useful tool in identifying genes that are differentially regulated during ripening in tomato and other fruit (Slater *et al.*, 1985; Dopico *et al.*, 1993; Deikman, 1997; Aggelis *et al.*, 1997; Zegzouti *et al.*, 1999). In addition to ripening, ethylene is also known to be involved in other processes such as pathogen and wounding responses, leaf senescence and abiotic and biotic stress responses; so too are other hormones such as abscisic acid (ABA) and sugar signalling (reviewed in Gazzarrini and McCourt, 2001). This cross talk between signalling pathways makes the process of unravelling the role that ethylene plays in fruit ripening

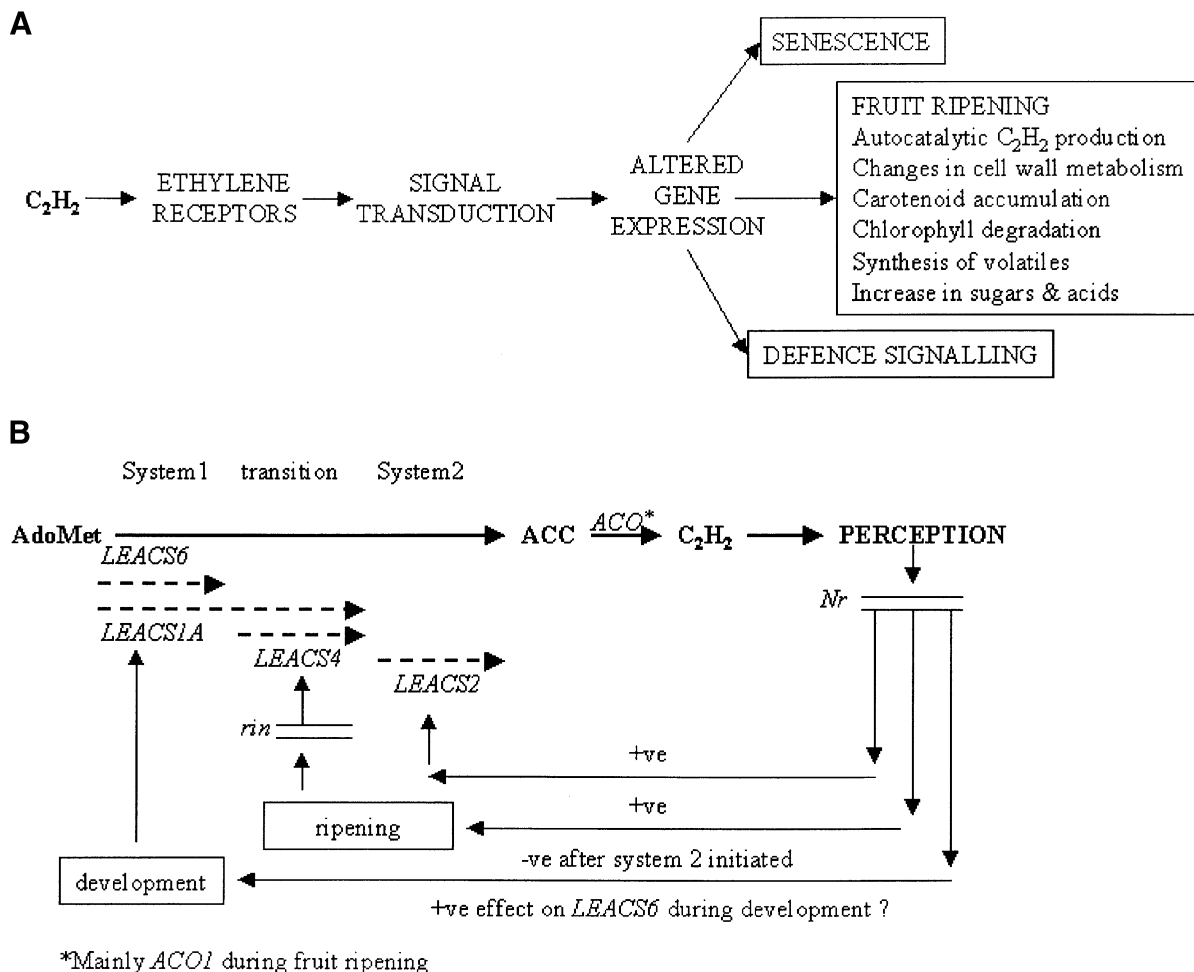

Fig. 1. (A) Schematic representation of the role that ethylene plays during tomato fruit ripening. (B) Model proposing the differential regulation of *ACS* gene expression during the transition from system 1 to system 2 ethylene synthesis in tomato. The symbols –ve (negative) and +ve (positive) refer to the action of ethylene on signalling pathways resulting in repression (–ve) or stimulation (+ve) of ACS gene expression. (Redrawn from Barry *et al.*, 2000.)

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complex, as alteration of ethylene levels may result in the unexpected or unrecognized modification of another signalling pathway.

Ethylene biosynthesis

The pathway of ethylene synthesis is well established in higher plants (reviewed in Bleecker and Kende, 2000). Ethylene is formed from methionine via *S*-adenosyl-L-methionine (AdoMet) and the cyclic non-protein amino acid 1-aminocyclopropane-1-carboxylic acid (ACC). ACC is formed from AdoMet by the action of ACC synthase (ACS) and the conversion of ACC to ethylene is carried out by ACC oxidase (ACO) (Kende, 1993). In addition to ACC, ACS produces 5′-methylthioadenosine, which is utilized for the synthesis of new methionine via a modified methionine cycle. This salvage pathway preserves the methylthio group through every revolution of the cycle at the cost of one molecule of ATP. Thus high rates of ethylene biosynthesis can be maintained even when the pool of free methionine is small. Two other ethylene-regulated genes have been identified that may play a possible role in the methionine cycle, *E4*, a putative methionine sulphoxide reductase protein and *ER69* a putative cobalamine-independent methionine synthase (Montgomery *et al*., 1993*a*; Zegzouti *et al*., 1999). In this pathway it is well known that biosynthesis is subject to both positive and negative feedback regulation (Kende, 1993). Positive feedback regulation of ethylene biosynthesis is a characteristic feature of ripening fruits and senescing flowers in which exposure to exogenous ethylene or propylene results in a large increase in ethylene production due to the induction of ACS and ACO. Both of these enzymes are encoded by small multigene families and their expression is differentially regulated by various developmental, environmental and hormonal signals (Kende, 1993; Zarembinski and Theologis 1994; Barry *et al*., 2000; Llop-Tous *et al*., 2000).

At least eight ACS genes have been identified in tomato (*LEACS1A*, *LEACS1B* and *LEACS2–7*), (Zarembinski and Theologis, 1994; Oetiker *et al*., 1997; Shiu *et al*., 1998) and many others have been identified in both climacteric and non-climacteric fruits such as melon, cucumber and citrus (Nakajima *et al*., 1990; Yamamoto *et al*., 1995; Wong *et al*., 1999). However, the role that ACS plays in ripening has been most widely studied in tomato. ACS shows homology to pyridoxal-5′-phosphate (PLP)-dependent aminotransferases and mutant complementation studies have shown that the enzyme can act as a dimer (Tarun and Theologis, 1998). Recent studies of the ACS crystal structure (Capitani *et al*., 1999) and PLP co-factor binding (Huai *et al*., 2001) have confirmed similarity between the ACS catalytic binding site and those of other PLP-dependent aminotransferases. The presence of *LEACS2* and *LEACS4* transcripts during ripening has been well documented (Rottmann *et al*., 1991; Olson *et al*., 1991; Yip *et al*., 1992; Lincoln *et al*., 1993; Barry *et al*., 2000). Recent work has also confirmed the presence of *LEACS1A* and *LEACS6* in tomato fruit before the onset of ripening and shown that each ACS in fruit has a different expression pattern (Fig. 1B) (Barry *et al*., 2000).

Analysis of ACS gene induction in mutant fruit with disrupted ethylene signalling has been used to identify which ACS gene is ethylene-regulated. The Never ripe (*Nr*) mutant cannot perceive ethylene due to a mutation in the ethylene-binding domain of the NR ethylene receptor (Lanahan *et al*., 1994; Wilkinson *et al*., 1995). Fruit from the ripening inhibitor (*rin*) mutant do not show autocatalytic ethylene production (Herner and Sink, 1973) and cannot transmit the ethylene signal downstream to ripening genes due to a mutation in the RIN transcription factor (Vrebalov *et al*., 2002). *Nr* and *rin* mutant fruit have shown that *LEACS2* expression requires ethylene whereas *LEACS1A* and *LEACS4* exhibited only slightly delayed expression in *Nr* indicating that ethylene is not responsible for regulation of these genes (Barry *et al*., 2000). All four fruit *ACS* genes showed the same expression patterns in *rin* fruit as in mature green wild-type fruit, but did not show any ripening-related changes of expression (Barry *et al*., 2000). Therefore, it has been proposed that *LEACS1A* and *LEACS6* are involved in the production of system 1 ethylene in green fruit (Barry *et al*., 2000). System 1 continues throughout fruit development until a competence to ripen is attained, whereupon a transition period is reached, during which *LEACS1A* expression increases and *LEACS4* is induced. During this transition period, system 2 ethylene synthesis (autocatalysis) is initiated and maintained by ethylene-dependent induction of *LEACS2* (Barry *et al*., 2000). Antisense inhibition of *LEACS2*, which also down-regulated *LEACS4*, reduced ripening-related synthesis of ethylene to 0.1% of control fruit. The antisense fruit displayed an abnormal pattern of ripening such as reduced lycopene accumulation, delayed softening and a much reduced climacteric peak (Oeller *et al*., 1991).

Some debate exists as to whether ACS enzymes are regulated transcriptionally, post-transcriptionally or post-translationally and whether ethylene plays a role in this regulation (Kende, 1993; Olson *et al*., 1995; Oetiker *et al*., 1997). *In vitro* analysis of LEACS2 enzyme activity has shown that the deletion of 52 amino acids from the *C*-terminus increases enzyme activity (Li and Mattoo, 1994; Li *et al*., 1996). However, it has recently been shown that LEACS2 is phosphorylated in wounded tomato fruit and is not truncated (Tatsuki and Mori, 2001). Sequence analyses have identified a conserved domain that is considered to be the phosphorylation site (F/L)RLS(F/L). Recombinant LEACS3 and LEACS2 containing this domain were phosphorylated *in vitro* whereas LEACS4 was not phosphorylated and does not contain this site (Tatsuki and

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Mori, 2001). It seems that the role of phosphorylation is not to regulate the specific activity of the enzyme but to control the rate of enzyme turnover (Spanu *et al*., 1994). The possibility that ACS phosphorylation regulates ethylene production is supported by the finding that mutation of the *C*-terminal domain of *Arabidopsis ACS5* induces the *eto2-1* mutant to overproduce ethylene (Vogel *et al*., 1998). Furthermore, observations by Ecker that the ETO1 protein bound to ACS5 *in vitro* and inhibited its activity has led to speculation that the ETO1 protein may be involved in a protein degradation pathway (Cosgrove *et al*., 2000; Tatsuki and Mori, 2001). Therefore; it is possible that phosphorylation of ACS protects the protein from degradation, which in turn could cause ACS to accumulate and ACS activity to increase, accounting for the burst of ethylene produced by ripening fruit (Tatsuki and Mori, 2001).

Initially it was thought that ACS activity was the key step in controlling the production of ethylene and that ACO activity was constitutive (Yang and Hoffman, 1984; Theologis *et al*., 1993). However, the role that ACO activity plays in the regulation of ethylene biosynthesis has become apparent in recent years. The rise in ACO activity precedes ACS activity in preclimacteric fruit in response to ethylene, indicating that ACO activity is important for controlling ethylene production (Lui *et al*., 1985). Examination of *ACO* mRNA expression patterns in various tissues and different developmental stages provided further evidence for the regulatory role that ACO plays in ethylene production during fruit ripening (Holdsworth *et al*., 1987; Hamilton *et al*., 1990; Balague *et al*., 1993; Barry *et al*., 1996). Historically, studying ACO has proved to be problematic due to the lack of an *in vitro* assay and difficulties encountered during purification (Kende, 1993). The first *ACO* gene was identified through antisense expression of a clone, pTOM13, then of unknown function (Holdsworth *et al*., 1987). mRNA expression patterns showed the pTOM13 gene was expressed in both ripening tomatoes and wounded leaves and down-regulation of this gene produced transgenic tomato plants with reduced levels of ethylene synthesis and ACO activity (Hamilton *et al*., 1990). The role of this enzyme in ethylene biosynthesis from ACC was confirmed by expression of pTOM13 in yeast and *Xenopus* oocytes, where the pTOM13-encoded protein was shown to convert ACC to ethylene, with the correct stereospecificity (Hamilton *et al*., 1991; Spanu *et al*., 1991). A further three *ACO* genes have been identified in tomato in response to wounding and during flower development, leaf and flower senescence and fruit ripening (Holdsworth *et al*., 1988; Barry *et al*., 1996; Blume and Grierson, 1997; Nakatsuka *et al*., 1998; Llop-Tous *et al*., 2000). *ACO* genes have also been identified in petunia, mung bean and other climacteric fruit such as melon, avocado, apples, and bananas (reviewed in Jiang and Fu, 2000).

ACO enzymes are members of the Fe(II-dependent family of oxidases/oxygenases (Hamilton *et al*., 1990; Prescott, 1993). *In vitro* ACO activity requires ascorbate as a substrate and the CO_2 produced during the climacteric peak is thought to activate the enzyme *in vivo* (Dong *et al*., 1992; Smith and John, 1993). Two models have been proposed for the production of ethylene from ACC. In the first model the ascorbate association with the Fe(II) ion activates a bound O_2 to yield high-valent iron-oxo species that oxidizes ACC to release ethylene (Zhang *et al*., 1997). More recently, it has been suggested that the role of the Fe(II) ion is to bind ACC and O_2 simultaneously and promote electron transfer, which initiates catalysis of ACC to ethylene (Rocklin *et al*., 1999).

Analysis of *ACO* gene expression patterns in ripening fruit shows that each gene is highly regulated with transcripts of individual members accumulating to varying degrees at distinct developmental stages (Barry *et al*., 1996). *LEACO1* and, at a lower level *LEACO3*, are expressed at the onset of fruit ripening. *LEACO1* transcripts peak at breaker +3 and then fall back to levels observed at breaker, whereas *LEACO3* transcripts are only transiently expressed at breaker before disappearing. Therefore it is likely that the first step in catalytic ethylene biosynthesis is the *de novo* synthesis of ACO1, the ethylene produced induces ACS gene expression, which in turn produces more ACC.

Ethylene perception and signal transduction in ripening fruit

During climacteric fruit ripening, the burst of autocatalytic ethylene co-ordinates and accelerates the ripening process. Although ethylene cannot induce immature tomato fruit to ripen rapidly, exposure will hasten its onset by shortening the 'green life', as in banana. The exact mechanisms of ethylene signal transduction are not yet fully understood, however, seedlings that show disruption of the normal triple response phenotype have given valuable insights into ethylene perception and signalling. Analysis of *Arabidopsis* mutants that display either etiolated growth in the presence of ethylene in the dark or show a constitutive triple response in the absence of the hormone, have led to the identification of five ethylene receptors and a number of components of the signal transduction pathway.

Ethylene receptors have homology to bacterial two-component receptors, which consist of a sensor protein, and a separate response regulator protein that function together, allowing bacteria to respond to different environmental conditions (Chang and Stewart, 1998). All ethylene receptors have a sensor domain that can be subdivided into a transmembrane domain and a GAF domain (found in cGMP phosphodiesterases, adenylate cyclases and Fh1a transcription factors), a histidine kinase

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domain and a response domain. The GAF domain binds cyclic nucleotides in a number of bacterial proteins, and the chromophore in the plant photoreceptor phytochrome (Aravind and Ponting, 1997), however, the function of this domain in the ethylene receptors is unknown. The binding of ethylene to the receptor is mediated by a copper co-factor (Rodriguez *et al*., 1999) and comparison with bacterial two component systems suggests a phosphorelay may pass the signal downstream. The presence of ethylene has been shown transiently to increase polypeptide phosphorylation *in vivo* (Raz and Fluhr, 1993). CTR1, a protein kinase with homology to the Raf family of mitogen-activated protein kinase kinase kinase (MAP3K) has been identified as the next component of the signal transduction pathway (Kieber *et al*., 1993). CTR1 is a negative regulator of the signal transduction pathway as CTR1 mutants constitutively respond to ethylene even in its absence. The N-terminus of CTR1 interacts with two ethylene receptors, ETR1 and ERS1 (Clark *et al*., 1998) and the current model of ethylene action suggests that, in the absence of the hormone, receptors signal to the negative regulator CTR1 and the response pathway is blocked. Binding of ethylene by the receptors releases the negative regulator allowing ethylene responses to occur (Bleecker *et al*., 1998). However, ethylene promotes MAPK activity, suggesting that both negative and positive ethylene signal transduction may be mediated through MAP kinase cascades (Novikova *et al*., 2000). Several excellent reviews concerning ethylene-mediated responses at the level of the ethylene receptor and down-stream signalling components have recently been published (Stepanova and Ecker, 2000; Bleecker and Kende, 2000; Ciardi and Klee, 2001; Chang and Stadler, 2001), therefore, this review will concentrate only on ripening-related ethylene perception and signal transduction.

A family of at least six putative ethylene receptors, *LeETR1*, *LeETR2* (Zhou *et al*., 1996; Lashbrook *et al*., 1998), *NR* (Wilkinson *et al*., 1995; Payton *et al*., 1996), *LeERT4*, *LeETR5* (Tieman and Klee, 1999), and *LeETR6* (Ciardi and Klee, 2001) and two *CTR1* homologues, *TCTR2* (Lin *et al*., 1998) and *ER50* (Zegzouti *et al*., 1999), have been identified in tomato. Each ethylene receptor is expressed in different temporal and spatial patterns dependent on developmental stage and external stimuli. *LeETR1* and *LeETR2* are expressed constitutively in all tissues throughout development, *NR* is up-regulated at anthesis and both *NR* and *LeETR4* are up-regulated during ripening, senecence, abscission (Payton *et al*., 1996; Tieman *et al*., 2000) and pathogen infection (Ciardi *et al*., 2000). *LeETR5* is also expressed in fruit, flowers and during pathogen infection (Tieman and Klee, 1999). Five consensus motifs named, H, N, G1 F, and G2, which are key features of bacterial histidine kinases are present in LeETR1, LeETR2 and NR, although NR lacks the response domain as in *Arabidopsis* ERS1 type receptors (Hua *et al*., 1995, 1998). In LeETR4 and LeETR5 the third and fourth motifs are highly divergent and the putative autophosphorylated HIS residue in the histidine kinase domain of LeETR5 is absent (Tieman and Klee, 1999), indicating that one or several ethylene receptors do not have histidine kinase activity although they all bind ethylene *in vitro* (Ciardi and Klee, 2001). Homodimerization of ETR1 is known to occur (Schaller *et al*., 1995; Rodriguez *et al*., 1999) although heterodimerization has not yet been proven. It has been proposed that the response regulator of ETR1 forms a homodimer when unphosphorylated (Muller-Dieckmann *et al*., 1999) and that phosphorylation of the receptor in the presence of ethylene causes monomerization of the response regulator that may, in turn, inactivate CTR1 (Chang and Stadler, 2001).

Ethylene receptors are, at least in some cases, ethylene-inducible and it has been demonstrated that increased levels of receptor reduces ethylene sensitivity, supporting the negative regulation of ethylene receptors model (Ciardi *et al*., 2000). As *NR* and *LeETR4* genes are up-regulated during ripening, they have both been targeted for antisense experiments. However, down-regulation of *NR* had no obvious effects on ethylene signalling and ripening apart from elevated expression of *LeETR4*, indicating that *LeETR4* could be compensating for *NR* (Tieman *et al*., 2000). By contrast, antisense repression of the mutant *NR* gene in the *Nr* background produced fruit that ripened normally indicating that the *NR* ethylene receptor is not required for ripening and confirming the receptor inhibition model of ethylene signalling (Fig. 2A, B) (Hackett *et al*., 2000). It is now becoming apparent that *LeETR4* plays an important role in ripening. Uniquely, down-regulation of *LeETR4* produces an ethylene hypersensitive phenotype that includes accelerated ripening (Tieman *et al*., 2000), whereas in *Arabidopsis* increased ethylene sensitivity is not observed until three receptors have been knocked out (Hua and Meyerowitz, 1998). This difference suggests that another level of ethylene signal transduction may be required in climacteric plants in contrast to *Arabidopsis*. Over-expression of *NR* in tomato plants with reduced levels of *LeETR4* eliminates ethylene sensitivity, further indicating that despite differences in protein structure, ethylene receptors are functionally redundant (Tieman *et al*., 2000). These observations have led to the hypothesis that *LeETR4* may monitor the levels of receptor and initiate the synthesis of new receptors as an ethylene response occurs, thus maintaining homeostasis in the ethylene response (Tieman *et al*., 2000).

While receptor genes have been isolated from tomato, few putative ethylene signal transduction components from this species have been described. A gene, *TCTR2*, for which the encoded protein is 41% identical to CTR1, was recently reported (Lin *et al*., 1998). Identification of another *CTR1*-like protein, *ER50* (Zegzouti *et al*., 1999)

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suggests that there may be multiple MAP3Ks regulating different pathways in which ethylene is involved. *TCTR2* mRNA is constitutively expressed and expression is not up-regulated by exposure to exogenous ethylene (Z Lin, Santalla, RM Hackett, D Grierson, unpublished results). However, in fruit, the *CTR1*-like *ER50* mRNA is up-regulated by exogenous ethylene and during ripening, which is intriguing as CTR1 is thought to act as a negative regulator of the ethylene response (Zegzouti *et al*., 1999).

Analysis of *Arabidopsis* ethylene triple response signalling mutants has identified other proteins, EIN2, EIN3, EIN5, and EIN6, which act as positive regulators downstream in the ethylene-signalling pathway. EIN2, an unknown membrane protein with N-terminal sequence similarity to mammalian metal-ion transport NRAMP proteins, is required for ethylene signalling (Alonso *et al*., 1999). Overexpression of the *C*-terminal domain in the *ein2* mutant will partially restore downstream responses to

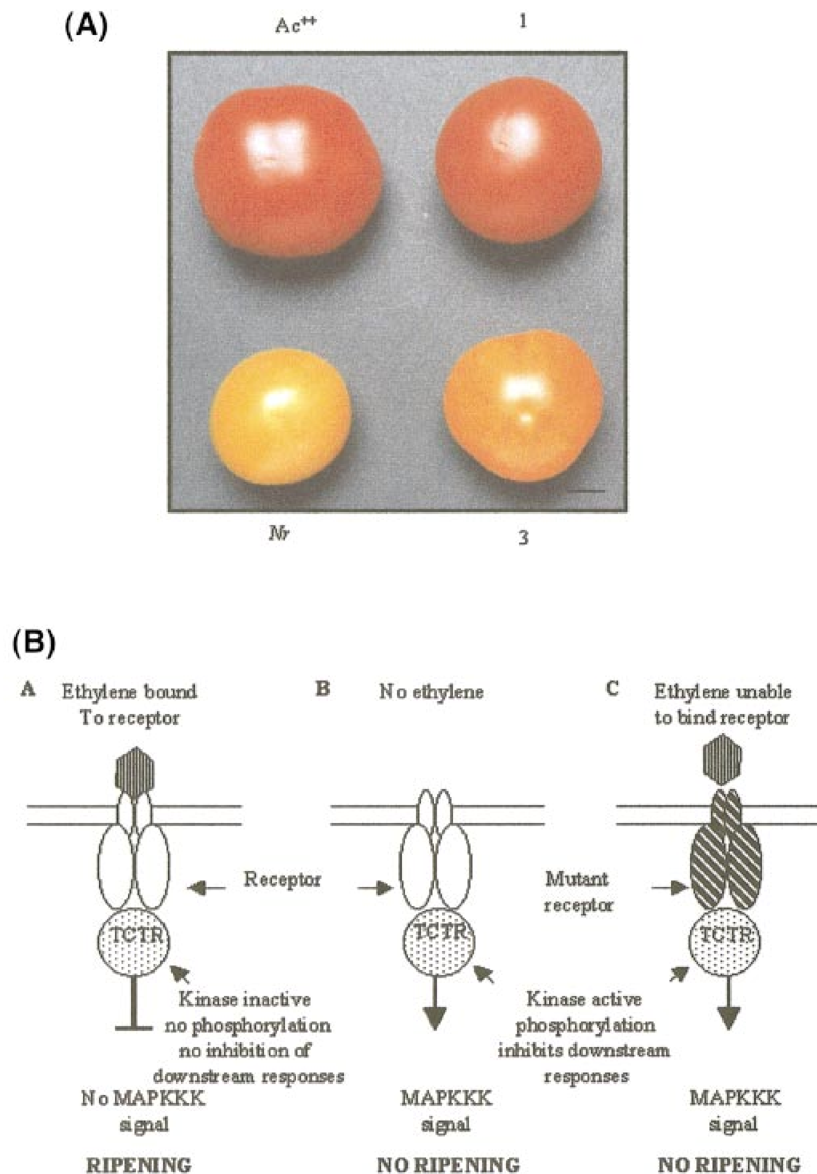

Fig. 2. (A) Antisense repression of the mutant *NR* gene in the *Nr* background restored normal ripening. The phenotype of fruit from T1 progeny of *Nr* transformants: Top row, left to right: non-transformed wild type, (1) T1 plant with antisense inhibition of mutant *Nr* mRNA (red fruit); lower row, left to right: non-transformed *Nr*, (3) T1 plant with partial antisense inhibition of antisense *NR* mRNA (orange fruit). Fruit were harvested at 7 d post-breaker and allowed to ripen for a further 16 d before photographing (Hackett *et al*., 2000). (B) Model of ethylene signalling from receptors to TCTR2. Ethylene receptors and TCTR2 are negative regulators of the ethylene signal transduction pathway. When ethylene binds the receptor (A) the receptors and TCTR are inactivated allowing signal transduction. In the absence of ethylene (B) or with a loss of function receptor mutant unable to bind ethylene (C) the receptors activate TCTR, possibly by phosphorylation, which results in an inhibition of ethylene signal transduction.

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jasmonic acid and paraquat, but not ethylene, indicating that this protein functions in at least two different signalling pathways (Alonso *et al.*, 1999). EIN3 and EIN3-like (EIL) proteins belong to a family of transcription factors that work downstream from EIN2 (Chao *et al.*, 1997; Alonso *et al.*, 1999). EIN3, EIL1 and EIL2 bind in a sequence-specific manner to the primary ethylene-response element (PERE) of *ERF1*, an ethylene inducible gene that belongs to the Ethylene Response Element Binding Protein (EREBP) family of DNA binding proteins (Solano *et al.*, 1998). ERF1 directly activates transcription of a wide variety of ethylene-responsive pathogenesis-related genes by binding to the GCC-box, indicating that a transcriptional cascade is involved in ethylene signalling. ERN1, a nuclear localized negative regulator of ethylene responses that acts downstream of EIN3 has also been identified recently (Trentmann, 2000).

Three genes with homology to the *Arabidopsis EIN3* have been cloned from tomato, *LeEIL1–3*, all of which were shown to be functional by mutant complementation in *Arabidopsis*, and have been shown to play a role in regulating ethylene responses (Tieman *et al.*, 2001). Transgenic tomato plants with reduced levels of either one or more *LeEIL* genes showed that LeEILs are functionaly redundant positive regulators of many ethylene responses (Tieman *et al.*, 2001). The LeEIL transcription factors are believed to regulate the ERF1 transcription factors, and a possible signal transduction pathway from the receptor to ERF1 is illustrated in Fig. 3. However,

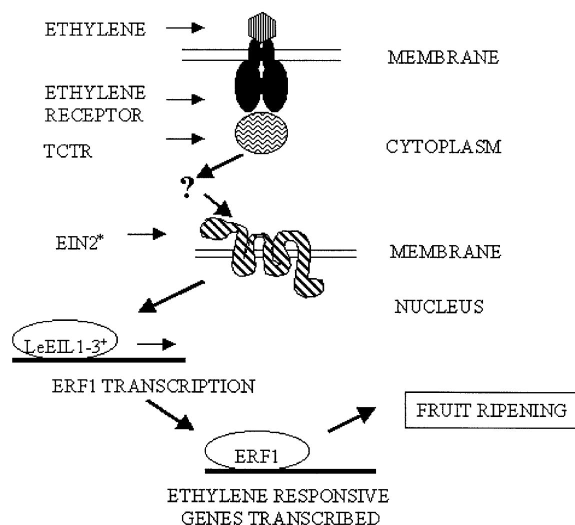

Fig. 3. Schematic diagram of the putative ethylene signal transduction pathway downstream of TCTR2 in ripening tomato fruit. TCTR has homology to MAP3K and therefore probably acts as the first component in a kinase signalling cascade. The components of this cascade have yet to be identified as has the tomato counterpart of EIN2. Synthesis of LeEILs, with homology to the *Arabidopsis* EIN3 transcription factor, are then thought to be activated by EIN2, which in turn activate ERF1. ERF1 transcription factors are though to activate ethylene-dependent ripening genes.

although an ethylene-related signal-transduction pathway from EIN3 to ERF1 to pathogenesis related (PR) proteins has been established in *Arabidopsis* (reviewed in Deikman, 1997; Solano *et al.*, 1998) and from EREBPs to PR proteins in tomato (Gu *et al.*, 2000), a similar route from EIN3 to fruit ripening has yet to be elucidated in tomato.

Gene regulation during ripening

As ripening progresses, the expression of many genes has been shown to be initiated or up-regulated (Table 1). Analysis of ripening-related gene expression in mutant or transgenic plants has indicated the presence of two types of gene regulation, ethylene-dependent gene regulation and ethylene-independent gene regulation. (DellaPenna *et al.*, 1989; Oeller *et al.*, 1991; Theologis *et al.*, 1993; Picton *et al.*, 1993). These phenomena are both illustrated by examination of expression patterns of ripening-related cDNA clones in antisense *ACO* tomato fruit (Picton *et al.*, 1993). However it has been found that certain ripening-related genes are more sensitive to low levels of ethylene than others (Lincoln and Fischer, 1988*b*; Sitrit and Bennett, 1998), and that the residual ethylene levels in low ethylene transgenic fruit may affect the pattern of ripening gene expression (Theologis *et al.*, 1993; Klee, 1993; Picton *et al.*, 1993; Sitrit and Bennett, 1998). Molecular characterization of the promoter regions of ripening-related genes has begun to unravel the mechanisms by which genes are regulated and the role that ethylene plays. One of the motifs identified has been the ethylene-responsive element (ERE), an 8 bp motif, A(A/T)TTCAAA (Montgomery *et al.*, 1993*b*; Itzhaki *et al.*, 1994) The transcription of *E4* and *E8* in fruit is stimulated by ethylene (Lincoln *et al.*, 1987). However, *E4* expression in leaves is also induced by ethylene but *E8* expression is not, suggesting that ethylene regulation of these genes is tissue specific or developmentally regulated (Lincoln and Fischer, 1988*a*). Analysis of the *E8* gene promoter has shown that the DNA sequences required for ethylene-regulated transcription, organ specificity and ethylene-independent ripening-related transcription are distinct (Deikman *et al.*, 1992, 1998). Although the functions of E4 and E8 during ripening are unknown, the predicted peptides encoded by these genes show significant similarity to methionine sulphoxide reductase protein and a dioxygenase with similarity to ACC oxidase, respectively (Montgomery *et al.*, 1993*a*; Deikman *et al.*, 1998).

Antisense suppression of *E8* in transgenic tomato fruit results in increased ethylene production indicating that E8 participates in feedback regulation of ethylene during ripening (Kneissl and Deikman, 1996). Analysis of the *E4* promoter has shown that ethylene responsiveness requires a minimum of two co-operative *cis* elements, an upstream regulatory element and a downstream regulatory element (Xu *et al.*, 1996). An EREBP that interacts with one of the

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Table 1. *Some examples of ripening related genes that exhibit ethylene-enhanced expression*

Gene	Homology	Function	Reference
LEACS2	ACC synthase	Catalyses system 2 ACC formation	Barry *et al*., 2000
LEACS6	ACC synthase	Catalyses system 1 ACC formation	Barry *et al*., 2000
LEACO1, 3	Dioxygenase, ACC oxidase	Catalyses C_2H_4 formation	Barry *et al*., 1996
E4	Methionine sulphoxide reductase protein	Unknown	Lincoln *et al*., 1987
E8	Dioxygenase	Unknown	Lincoln *et al*., 1987
ER24	Transcriptional co-activator MBF1	Link EREBPs to TATA box binding protein	Zegzouti *et al*., 1999
ER49	Translation elongation factor EF-Ts	Post-transcriptional regulation	Zegzouti *et al*., 1999
ER68	RNA helicase DBP2	Post-transcriptional regulation	Zegzouti *et al*., 1999
ER50	*Arabidopsis CTR1*	Negative regulator of ethylene signal transduction	Zegzouti *et al*., 1999
LeRab11a	Rab GTPase	Trafficking of cell-wall modifying enzymes	Lu *et al*., 2001
TomloxA, B, C	Lipoxygenase	Hydroperoxidation of polyunsaturated fatty acids	Griffiths *et al.,* 1999*a*
PG	Polygalacturonase	Depolymerizes cell wall pectins	Nicholass *et al*., 1995; Sitrit and Bennett, 1998
PME	Pectin methylesterase	Maintains tissue integrity in senescent fruit	Hall *et al*., 1994
LeEXP1	Expansin	Disrupts hydrogen bonds in wall matrix	Rose *et al*., 1997

elements is present in unripe fruit, indicating that this may act as a repressor of *E4* transcription and may be inactivated by ethylene (Montgomery *et al*., 1993*a*). Another DNA binding protein, E4/E8BP, has been identified that interacts with both *E4* and *E8* promoter sequences (Cordes *et al*., 1989). Mutational analysis of the *E4/E8BP* DNA binding sequence of *E4* has shown that it is involved in modulating *E4* transcription and it was proposed that this sequence may function as part of an ethylene response complex (Xu *et al*., 1996). Subsequently, a cDNA with similar DNA-binding specificity was cloned and expression rates were found to be higher in fruit and increased during ripening, suggesting that E4/E8BP-1 may play a role in ripening (Coupe and Deikman, 1997). Polygalacturonase (PG) promoter analysis has also revealed the presence of ethylene-inducible elements with similarity to the promoters of *E4* and *E8* (Nicholass *et al*., 1995). *LEACO1* promoter GUS fusions have identified that the region between –396 and –1825 upstream of the *LEACO1* sequence is sufficient to confer strong and specific induction of GUS expression in situations known to be accompanied by strong ethylene production (Blume and Grierson, 1997). Sequence analysis of the *LEACO1* promoter (Blume *et al*., 1997) has identified several regions of homology to the promoters of the ripening specific genes *2A11* and *E4* between –1722 and –590 (Cordes *et al*., 1989; Pear *et al*., 1989), as well as a TCA motif present in stress and pathogen genes (Goldsbrough *et al*., 1993) and ethylene responsive elements (ERE) found in carnation and tomato *E4* (Fig. 4) (Itzhaki *et al*., 1994; Blume and Grierson, 1997). The –396 region of the *LEACO1* promoter–GUS fusion induces the same GUS expression pattern, but with 330-fold lower GUS activities than observed with the –1825 promoter. However, expression of this GUS fusion is not ethylene inducible, indicating that *LEACO1* is also regulated by ethylene-independent factors (Itzhaki *et al*., 1994; Blume and Grierson, 1997). Inconsistencies in transcript accumulation observed during *LEACO1* promoter–GUS fusion analyses suggest that during autocatalytic ethylene production, *LEACO1* transcription rate and mRNA stability may be enhanced, indicating a level of post-transcriptional control (Blume and Grierson, 1997).

Differential display of mRNA has recently been used to isolate novel early ethylene-regulated genes from ethylene-treated late immature green tomato (Zegzouti *et al*., 1999). On the basis of sequence homology, many of the isolated clones appear to be regulatory proteins involved in signal transduction pathways. Three clones show homology to proteins involved in transcriptional and post-transcriptional regulation of gene expression. *ER24*, shows 47% identity with a transcriptional co-activator, multi-protein bridging factor 1 (MFB1), that forms part of the TAF (TATA box–binding protein association factors) complex required for transcription activation and, therefore, may form a complex with EREBPs to activate genes

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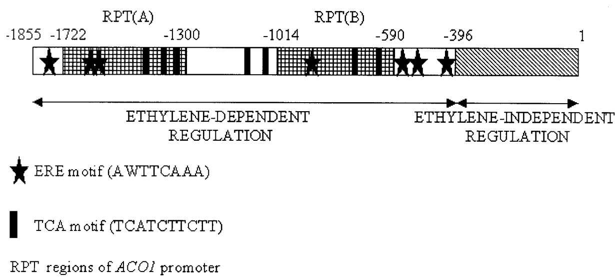

Fig. 4. Structure of the tomato *ACO1* promoter. The position of the two repeat regions (RPT) which contains sequences with homology to the ethylene responsive promoters of *2A11* and *E4* are shown. The position of the ethylene responsive (ERE) regions and stress related (TCA) motifs are also shown. The –1855 to –396 region of the promoter confers ethylene-dependent expression whereas the –396 region confers ethylene-independent expression of *GUSACO1* promoter fusions.

such as *E4* and *E8*. *ER49* shows similarity to a mitochondrial translational elongation factor. *ER68* shows homology to the DEAD (containing the Asp, Ala, Glu, Asp type motif) box ATPase/RNA helicases that facilitates gene expression by unwinding RNA molecules (Zegzouti *et al*., 1999).

Novel ethylene-regulated ripening-related cDNA clones have recently been isolated from melon using differential display (Hadfield *et al*., 2000). Furthermore, analysis of *ACO* antisense melon has revealed that some ripening-related events such as degreening of the rind and cell separation in the peduncular zone are totally dependent on ethylene, whereas other ripening-related events, such as softening and membrane deterioration, are dependent only partially on ethylene (Flores *et al*., 2001). Thus it appears that some aspects of ethylene-dependent gene expression are conserved between climacteric fruits such as melon and tomato. However, there are examples of ethylene-dependant gene regulation in non-climateric fruits. Degreening of the rind in citrus fruit is prevented by ethylene antagonists (Goldschmit *et al*., 1993) whereas expression of *chlorophyllase* (*Chlase 1*), the enzyme catalysing the first step in the chlorophyll degradation pathway in oranges is enhanced by exogenous application of ethylene (Jacob-Wilk *et al*., 1999).

Colour development

The characteristic pigmentation of red ripe tomato fruit is due to the deposition of lycopene, the predominant carotenoid found in tomato fruit, and β-carotene, which are associated with the change from green to red as chloroplasts are transformed to chromoplasts. At the breaker stage of tomato ripening, i.e. when a red/orange coloration becomes apparent to the human eye, lycopene begins to accumulate and its concentration increases 500-fold in ripe fruits (Fraser *et al*., 1994). Although genes encoding the majority of the carotenoid biosynthesis enzymes have been cloned, the regulation of this pathway is poorly understood. Therefore, the analysis of ripening mutants is a useful tool for exploring the regulation of this process. Carotenoids are formed by the condensation of two molecules of geranylgeranyl pyrophosphate (GGPP), the ubiquitous isoprenoid precursor, to produce phytoene, which is catalysed by the enzyme phytoene synthase (PSY). A series of dehydrogenation reactions, catalysed by phytoene and ζ-carotene desaturases, converts phytoene into lycopene. The function of the *Psy* gene was confirmed by the production of transgenic plants with altered levels of *Psy* expression. Antisense suppression of a ripening-related clone pTOM5 (Psy1) resulted in yellow tomatoes with disrupted lycopene accumulation (Bird *et al*., 1991) whereas over-expression of pTOM5 in the mutant *yellow flesh* tomato restored lycopene synthesis (Fray and Grierson, 1993). Due to the beneficial anti-oxidant properties of lycopene, researchers have tried transgenic approaches to increase tomato fruit lycopene content. However, this has resulted in unwanted pleiotropic effects, such as co-suppression of endogenous *Psy* genes and dwarfism due to adverse changes in metabolism (Fray *et al*., 1995). Recently a bacterial *Psy* has been over-expressed successfully in tomato resulting in 1.8-fold and 2.2-fold increases in tomato lycopene and β-carotene, respectively (Fraser *et al*., 2002).

Biochemical data obtained from analysis of the colourless non-ripening mutant *Cnr* suggests that carotenoid formation is dependent on an accessible pool of GGPP (Fraser *et al*., 2001). The *Cnr* mutation is the result of a lesion in a single gene that has pleiotropic effects on ripening, including a lack of pigmentation and minimal softening, indicating that the *Cnr* gene is required for normal ripening (Thompson *et al*., 1999). The *Cnr* mutant exhibits reduced levels of GGDP, low levels of total carotenoids and undectectable levels of phytoene and lycopene. As levels of other related isoprenoids were similar to that of wild type it is possible that an as yet unidentified GGPP synthase (two have been identified from *Arabidopsis*: Scolnik and Bartley, 1996), is responsible for the production of GGPP that is committed to the carotenoid pathway. Cloning of *Cnr* may thus provide valuable insight into ripening-related carotenoid formation. However, the characterization of other colour mutant phenotypes has contributed to the understanding of carotenoid biosynthesis. Map-based cloning of two mutant genes *Beta* (*B*), a single dominant mutation that increases β-carotene in the fruit, and *old-gold* (*og*), a recessive mutation that abolishes β-carotene and increases lycopene, has revealed that *B* encodes a novel type of lycopene β-cyclase, an enzyme that converts lycopene to β-carotene. Null mutations in the *B* gene are responsible for the phenotype in *og* mutants (Ronen *et al*., 2000).

The high-pigment mutant (*hp*) exhibits twice the normal level of carotenoids (Bramley, 1997), increased plastid

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number (Yen *et al*., 1997) and an exaggerated photomorphogenic de-etiolation response, indicating that *hp-1* may influence phytochrome signalling (Peters *et al*., 1992). Analysis of phytochrome levels in mutant and wild-type seedlings has indicated that the wild-type HP-1 acts as a negative regulator of phytochrome signal transduction (Peters *et al*., 1992). The *hp-2* mutant, which is non-allelic to *hp-1* but exhibits a similar phenotype, is homologous to *Arabidopsis DE-ETIOLATED 1* (Pepper *et al*., 1994; Mustilli *et al*., 1999) indicating that light signalling plays an important role in fruit pigment accumulation. Recent work (Alba *et al*., 2000) has further confirmed the role that light plays in phytochrome mediated carotenoid biosynthesis (Khudairi and Arboleda, 1971). Alba *et al*. (2000) have shown that red light treatment of mature green fruit resulted in increased lycopene accumulation that could be reversed by exposure to far red light. Continued research in this area is necessary to establish if crosstalk occurs between ethylene and phytochrome signal transduction pathways during fruit ripening. However, crosstalk between phytochrome signalling and PR gene expression has been observed in *Arabidopsis* (Genoud and Metraux, 1999)

Cell wall softening

Due to the economic importance of post-harvest deterioration of fruit crops, enzymes that are implicated in cell wall softening have been examined by transgenic manipulation and *in vitro* assays. A comprehensive review of this topic has recently been published (Brummell and Harpster, 2001) therefore this review will not cover all the data concerning cell wall softening enzymes but rather draw attention to enzymes that exhibit ethylene regulation. During ripening partial disassembly of the fruit cell wall is largely responsible for softening and textural changes. As ripening progresses, the cell wall becomes increasingly hydrated as the pectin rich middle lamella is modified and partially hydrolysed. The changes in cohesion of the pectin gel governs the ease with which one cell can be separated from another, which in turn affects the final texture of the ripe fruit. In soft fruit such as tomato this process occurs early in ripening (Crookes and Grierson, 1983) whereas in crisp fruits such as apple it is a late-ripening process.

The tomato fruit PG is a major cell wall polyuronide degrading enzyme. It is transcriptionally activated during ripening (DellaPenna *et al*., 1989; Montgomery *et al*., 1993*b*) and the PG promoter sequence contains ethylene-dependent ripening-specific control elements (Nicholass *et al*., 1995). Analysis of low ethylene transgenic tomato plants has shown that induction of *PG* mRNA occurs at very low ethylene levels (Sitrit and Bennett, 1998). The observations that PG is synthesized *de novo* during the onset of ripening (Tucker and Grierson, 1982) and purified extracts were able to break down fruit cell walls *in vitro* (Crookes and Grierson, 1983) made this enzyme a good target for antisense suppression (Sheehy *et al*., 1988; Smith *et al*., 1988). Although PG activity in homozygous transgenic plant lines was reduced to 1% of the normal value, the ripe fruit were only slightly firmer leading to the conclusion that PG is not the major determinant of tomato fruit softening (Grierson and Schuch, 1993). However, low PG fruit are more resistant to splitting, mechanical damage and pathogen infection (Gray *et al*., 1994; Cooper *et al*., 1998). Analysis of low PG fruit cell walls showed reduced amounts of water-soluble polyuronides, matched by an increase in calcium carbonate-soluble polyuronides suggesting that PG depolymerizes covalently bound pectin allowing it to solublize into an aqueous fraction (Smith *et al*., 1990; Carrington *et al*., 1993). Identification of tomato lines in which the *PG* gene has been functionally inactivated by the insertion of a transposable element show a similar phenotype to the transgenic low PG tomatoes, although polyuronide depolymerization was not examined (Cooley and Yoder, 1998). PG extracted from cell walls may be associated with a 38 kDa glycoprotein, known as the PG β-subunit or converter, that has not been shown to possess any intrinsic enzyme activity (reviewed in Brummell and Harpster, 2001). However, transgenic plants suppressed for this protein exhibit increased fruit softening during ripening, higher extractable PG activity and more polyuronide solublization, indicating that this protein may be distributed throughout the cell wall to control PG diffusion or action during ripening (Chun and Huber, 2000).

During ripening, pectin methylesterase (PME) is responsible for de-esterification of the highly methyl-esterified polygalacturonans in the cell wall. Esterification drops from 90% in mature green fruit to 35% in red ripe fruit and this makes the polyuronides susceptible to degradation by PG (Koch and Nevins, 1989; Carpita and Gibeaut, 1993). *PME* is present as a small gene family in tomato, some members of which are highly homologous. PME protein is found in most plant tissues with three isoforms being specific to fruit, one of which, PME1, peaks at breaker (Hall *et al*., 1993, 1994; Gaffe *et al*., 1994, 1997). PME-suppressed transgenic plants did not exhibit altered fruit softening during ripening, although pectin fragments extracted from cell walls showed an increase in fragment size and methyl esterification (Tieman *et al*., 1992; Hall *et al*., 1993). However, suppression of PME in over-ripe fruit caused an almost complete loss of tissue integrity, therefore PME plays little role in ripening but does affect fruit senescence (Tieman and Handa, 1994). Recent cloning of two ripening-related Rab GTPases has provided evidence that ethylene may regulate vesicular transport between different cellular compartments (Zegzouti *et al*., 1999; Lu *et al*., 2001). Interestingly, tomato plants expressing an antisense *Rab11 GTPase* gene show reduced levels of PG and PME and reduced fruit softening,

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indicating that *Rab11 GTPase* plays a role in trafficking of cell-wall modifying enzymes (Lu *et al*., 2001).

Early in ripening polymeric galactose within the wall begins to be broken down into free galactose and the rise in free galactose continues throughout ripening. The enzyme responsible for this is β-galactosidase, which is encoded by a gene family of at least seven members, all of which display different patterns of expression during fruit ripening (Smith and Gross, 2000). Analysis of β-galactosidase-suppressed transgenics and ripening mutants (Carey *et al*., 1995; Smith and Gross, 2000) has shown that one gene family member, *TBG4*, may be regulated by ethylene and that strong suppression of β-galactosidase activity early in ripening can reduce fruit softening by up to 40% (DL Smith, KC Gross, unpublished results, cited in Brummell and Harpster, 2001).

Expansins are cell wall localized enzymes that are thought to cause cell wall loosening by reversibly disrupting the hydrogen bonds between cellulose microfibrils and matrix polysaccharides (Cosgrove, 2000). At least six different expansin genes are expressed during tomato fruit development (Brummell *et al*., 1999), one of which, EXP1, is ethylene-regulated and is specific for fruit with mRNA transcripts accumulating either just before or at breaker stage (Rose *et al*., 1997). These results indicate that several enzymes are regulated by ethylene during ripening, but their precise role in fruit softening remains to be elucidated.

Volatile production

The concentration of organic acids and sugars has an important influence on the taste of ripening fruits. The characteristic flavour of edible fruits results from the aroma volatiles produced within the fruit during ripening and on maceration. The volatile profile of fruits determined by gas chromatography and mass spectroscopy is complex, including many alcohols, aldehydes and esters. Previous studies indicate that the differences in flavour between tomato varieties is due, at least in part, to variation in aroma volatile production (Brauss *et al*., 1998). Over 400 volatile compounds are detected in tomato fruit (Hobson and Grierson, 1993), although a group of seven including hexanal, hexenal, hexenol, 3-methylbutanal, 3-methylbutanol, methylnitrobutane, and isobutylthiazole are amongst the most important contributors to fruit aroma. These flavour volatiles are formed by several different pathways: 3-methylbutanal and 3-methylbutanol are formed by the deamination and decarboxylation of amino acids whereas hexanal, hexenal and hexenol are formed by lipid oxidation of unsaturated fatty acids on the maceration of fruit. Hexanal and hexenal arise through the activity of lipoxygenases (LOX), which catalyse the hydroperoxidation of polyunsaturated fatty acids containing a *cis*,*cis*-pentadiene structure. In plants, reaction intermediates are unsaturated fatty acid hydroperoxides (HPOs), which give rise to the C6 aldehydes through the action of hydroperoxide lyases (HPO-lyase). Two groups of HPO-lyases cleave either 9-(*S*)-HPOs or 13-(*S*)-HPOs, generating two C9 fragments, or a C6 and a C12 fragment, respectively (Hatanaka, 1993).

In tomato fruit, linoleic and linolenic acid are the main LOX substrates, and the majority of HPOs found in tomato fruit, however, are 9-isomers. It appears that the 13-isomers, which are produced in a much smaller proportion, are metabolized further to produce flavour volatiles and compounds involved in defence, such as jasmonic acid (JA) (Galliard and Matthew, 1977; Regdel *et al*., 1994; Smith *et al*., 1997). The main aldehydes produced are hexanal and hexenal (Galliard and Matthew, 1977), and these aldehydes can then be further transformed into hexenol and hexanol by the action of alcohol dehydrogenase (ADH).

Tomato LOX consists of a family of at least five genes, *TomloxA* and *TomloxB* (Ferrie *et al*., 1994), *TomloxC* and *Tomlox D* (Heitz *et al*., 1997), and *TomloxE* (NCBI Accession AY008278). Analysis of the role that ethylene plays in the regulation of *TomloxA*, *TomloxB* and *TomloxC* during tomato ripening has shown that the individual isoforms are differentially regulated and may have different functions (Griffiths *et al*., 1999*a*). Levels of *TomloxA* mRNA decrease as ripening progresses and this is delayed in the mutants, *Nr* and *rin* as well as sense suppressed *ACO1* (low ethylene) fruit, indicating that this gene is regulated by both ethylene and developmental factors (Griffiths *et al*., 1999*a*). *TomloxB* expression increases during ripening and is regulated by ethylene, since the mutant and low ethylene transgenic fruit show reduced expression. *TomloxC* transcripts increase in response to ethylene, however, ethylene treatment of mature green fruit does not induce expression. This would indicate the presence of a developmental pathway that initiates expression and an ethylene component that enhances mRNA levels once expression has been initiated by the developmental pathway (Griffiths *et al*., 1999*a*).

Silencing of *TomloxA* and *TomloxB*, by antisense gene knockout, failed to reduce flavour volatiles in ripening fruit and did not alter the levels of *TomloxC* mRNA (Griffiths *et al*., 1999*b*), suggesting that *TomloxC* may encode the major fruit lipoxygenase involved in flavour volatile production. TomloxC and the mainly leaf expressed TomloxD differ from the other LOX enzymes in that they are chloroplast targeted (Heitz *et al*., 1997). It therefore seems likely that during ripening TomloxC utilizes the polyunsaturated fatty acids from the redundant thylakoid structures as a substrate to produce the aroma volatiles hexanal and hexanol. TomloxD is thought to function in the octadecanoid defence signalling pathway which is activated in response to herbivore and pathogen attack (Heitz *et al*., 1997). Suppression of the *Arabidopsis*

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chloroplast *atLOX2* gene, which is most similar to *TomloxD*, resulted in the absence of wound-inducible jasmonic acid (JA) accumulation and reduced expression of the wound- and JA-inducible *vsp* gene (Bell *et al*., 1995). Therefore, it appears that LOX has a dual role in both volatile production and defence signalling. Recent work with *Arabidopsis* has provided evidence that atLOX2 is a translation initiation factor-4e-binding protein and that this interaction may play a regulatory role given the numerous examples of products of LOX activity, such as JA, found to be involved in translational activation (Freire *et al*., 2000). Therefore the ethylene-dependent and independent regulation of LOX genes may orchestrate many aspects of fruit ripening and defence against pathogens.

Alcohol dehydrogenase (ADH) has also been shown to play a role in hexanol and hexenol accumulation in ripening tomato fruit (Speirs *et al*., 1998). Two ADHs have been indentified in tomato, ADH1 which is found only in pollen, seeds and young seedlings (Tanksley, 1979) and ADH2 which accumulates during the later stages in ripening commitant with the accumulation of flavour volatiles (Chen and Chase, 1993; Longhurst *et al*., 1994). Genetic manipulation of ADH2 levels in ripening tomato fruit has been shown to affect the balance of some flavour aldehydes and alcohols and fruits with increased ADH2 levels had a more intense 'ripe fruit' flavour (Speirs *et al*., 1998). Tomato ADH2 has not been identified as ethylene inducible. However, it is induced by low oxygen stress and it is likely that increasing ADH2 activity during ripening is a function of decreasing oxygen concentration within ripening fruit (Speirs *et al*., 2002).

Ethylene has also been shown to be important in the production of aroma volatiles in Charentais melon fruit, as antisense suppression of ethylene production results in strong inhibition of aroma (Ayub *et al*., 1996; Bauchot *et al*., 1998). The melon aroma volatile profile mainly consists of volatile esters and, although little information exists regarding the biosynthetic pathways involved, the last step in their formation is catalysed by acyl-transferases (AAT) (Fellman *et al*., 2000). AATs are a super family of multifunctional AATs and are implicated in diverse biochemical pathways such as fruit ripening, the production of epicuticular waxes and benzoyltransfer reactions (St-Pierre *et al*., 1998). A ripening-related gene, *MEL2*, isolated from melon fruit (Aggelis *et al*., 1997), has been identified as an ATT by expression in yeast (Yahyaoui *et al*., 2002). Recent analysis of antisense *ACO* melon has shown that fruit treated with the ethylene antagonist 1-methylcyclopropane have a 50% reduction in AAT activity. This indicates that the last step of alcohol acetylation comprises ethylene-independent and ethylene-dependent AATs (Flores *et al*., 2002). *MEL2* also shows similarity to pTOM36 isolated from a tomato fruit-ripening library (Davies and Grierson, 1989). Although the function of this gene has not been fully investigated, ripening tomato does produces aromatic esters. An AAT gene that plays a crucial role in flavour biogenesis has also been recently cloned from strawberry, a non-climacteric fruit, indicating that AATs are not exclusively regulated by ethylene (Aharoni *et al*., 2000).

Concluding remarks

It can be seen that fruit ripening and the role that ethylene plays in its regulation is complex. Identification of additional components involved in ethylene signal transduction, the further characterization of ripening mutants, and additional studies on the biochemistry of ripening are essential for complete understanding of the ripening process. There are similarities between non-climacteric and climacteric fruit ripening and certain ethylene-dependent events in climacteric fruit are observed, apparently in the absence of or with extremely low levels of ethylene, in non-climacteric fruits. Therefore, understanding what controls these processes in non-climacteric ripening may prove pertinent to gaining full understanding of climacteric fruit ripening and vice versa.

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