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Ethylene and Fruit Ripening

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Abstract

The ripening of fleshy fruits represents the unique coordination of developmental and biochemical pathways leading to changes in color, texture, aroma, and nutritional quality of mature seed-bearing plant organs. The gaseous plant hormone ethylene plays a key regulatory role in ripening of many fruits, including some representing important contributors of nutrition and fiber to the diets of humans. Examples include banana, apple, pear, most stone fruits, melons, squash, and tomato. Molecular exploration of the role of ethylene in fruit ripening has led to the affirmation that mechanisms of ethylene perception and response defined in the model system *Arabidopsis thaliana* are largely conserved in fruit crop species, although sometimes with modifications in gene family size and regulation. Positional cloning of genes defined by ripening defect mutations in the model fruit system tomato have recently led to the identification of both novel components of ethylene signal transduction and unique transcription factor functions influencing ripening-related ethylene production. Here we summarize recent developments in the regulation of fruit ripening with an emphasis on the regulation of ethylene synthesis, perception, and response.

Key words: Ethylene; Fruit; Tomato; Ripening; Climacteric; Signal transduction

Introduction

Fruits of different plant species are highly diverse, ranging from dry seed capsules that burst to allow seed dispersal, to relatively large complex fleshy fruits that have evolved bright colors and complex aromas to attract seed-dispersing birds and animals. Fleshy fruits in themselves are botanically diverse with some such as tomato and grape being true berries derived from the ovary and others such as strawberry, pineapple, and apple derived from the receptacle tissues or from expansion of the sepals. Fleshy fruits also come in a wide range of sizes, shapes, and colors, and each species possesses its own very unique flavor characteristics. Ripening programs can also be diverse. For example, avocado do not ripen until after harvest, whereas the majority of studied fruits ripen on the plant. Despite this great diversity, aspects of the ripening of fleshy fruits are often conserved between species. For example, the onset of ripening is often associated with color changes, altered sugar metabolism, fruit softening and alterations in texture, the synthesis of aroma volatiles, and an increased susceptibility to pathogen infection. These common events suggest that the underlying genetic mechanisms that regulate fruit ripening may well be conserved between fruits of different species (Adams-Phillips and others 2004a, b; Giovannoni 2004).

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Tomato is the most genetically tractable plant system for studying fruit ripening because it has simple diploid genetics and a relatively short generation time and small habit compared to many other fruit crop species that are either polyploids or trees. The ripening phenotype is easy to score and there is a large collection of germplasm resources, including monogenic mutants with inhibited or altered ripening phenotypes (http://www.tgrc.ucdavis.edu/, http://www.zamir.sgn.cornell.edu/mutants/). There is also a long history and a wealth of biochemical and molecular data relative to the processes that are involved during ripening, and a large platform of tools for functional genomics is continually being developed, including an emerging genome sequence (Fei and others 2006; Mueller and others 2005).

Differential screens, candidate gene analysis, gene expression profiling, and digital gene expression analysis have led to the identification of hundreds of genes whose expression profiles change during the course of fruit development and ripening (Alba and others 2005; Fei and others 2004; Picton and others 1993a, b; Slater and others 1985; Zegzouti and others 1999). Through a combination of approaches many of the downstream components that mediate the biochemical changes associated with ripening have been defined. For example, cell wall hydrolases, the enzymes involved in carotenoid synthesis and sugar metabolism, and some of the enzymes involved in the generation of flavor and aroma compounds have been characterized (Chen and others 2004a, b; Fridman and others 2004; Hirschberg 2001; Rose and Bennett 1999; Tieman and others 2006). The pathways that determine the competency of a fruit to ripen or the signals that initiate the ripening program are less well defined, although the molecular identification of mutants that are impaired in fruit ripening are beginning to yield valuable insight into some of these genetic pathways, and multiple hormones, including jasmonates, auxin, and brassinosteroids, have all been implicated in the promotion of ripening in various species (Fan and others 1998; Given and others 1988; Manning and others 2006; Symons and others 2006; Vardhini and Rao 2002; Vrebalov and others 2002). Signaling through the plant hormone ethylene, however, remains the most well-defined pathway that mediates the phenotypic changes that occur during ripening. Treatment of various fruits with inhibitors that block ethylene synthesis or action or the manipulation of these processes by transgenic or mutant approaches have revealed the essential role of this hormone in regulating fruit ripening (Hobson and others 1984; Klee and others 1991; Lanahan and others 1994; Oeller and others 1991; Picton and others 1993a, b; Yang and Hoffman 1984). In this review we summarize our current understanding of ethylene biosynthesis and signaling pathways in relation to fruit ripening. Major emphasis is placed on knowledge obtained using the tomato model system, although where appropriate we highlight discoveries and novel findings in other fruit crop species.

The Regulation of Ethylene Biosynthesis During Fruit Ripening

Fruits have classically been categorized based upon their abilities to undergo a program of enhanced ethylene production and an associated increase in respiration rate at the onset of ripening. Fruits that undergo this transition are referred to as climacteric and include tomato, apple, peach, and banana, whereas fruits that do not produce elevated levels of ethylene are known as nonclimacteric and include citrus, grape, and strawberry. However, these distinctions are not absolute, as closely related melon and capsicum species can be both climacteric and nonclimacteric (see below for further discussion) and some so-called nonclimacteric fruits display enhanced ripening phenotypes in response to exogenous ethylene (see below for further discussion). Nevertheless, increased ethylene synthesis at the onset of ripening is required for the normal ripening of many fruits.

Two systems of ethylene production have been defined in plants. System 1 functions during normal growth and development and during stress responses, whereas system 2 operates during floral senescence and fruit ripening. System 1 is autoinhibitory, such that exogenous ethylene inhibits synthesis, and inhibitors of ethylene action can stimulate ethylene production (Figure 1). In contrast, system 2 is stimulated by ethylene and is therefore autocatalytic, and inhibitors of ethylene action inhibit ethylene production (McMurchie and others 1972).

The biochemical features of the ethylene biosynthesis pathway in higher plants are well defined and have been reviewed previously (Bleecker and Kende 2000). Briefly, ethylene is synthesized from methionine in three steps: (1) conversion of methionine to S-adenosyl-L-methionine (SAM) catalyzed by the enzyme SAM synthetase, (2) formation of 1-aminocyclopropane-1-carboxylic acid (ACC) from SAM via ACC synthase (ACS) activity, and (3) the conversion of ACC to ethylene, which is catalyzed by ACC oxidase (ACO). The formation of ACC also leads to the production of 5′-methylthioadenosine

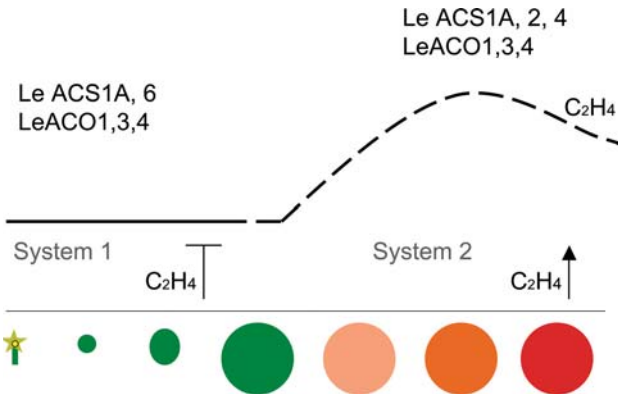

Figure 1. Differential expression of *ACS* and *ACO* genes associated with system 1 and system 2 ethylene synthesis during fruit development and ripening in tomato. Autoinhibition of ethylene synthesis during system 1 ethylene production is mediated by a reduction in *LeACS1A* and *6* expression. Autocatalytic ethylene synthesis at the onset of fruit ripening is mediated through ethylene-stimulated expression of *LeACS2* and *4* and *LeACO1* and *4* (see text for details).

(MTA), which is recycled via the methionine cycle to yield a new molecule of methionine. Increased respiration provides the ATP required for the methionine cycle and can lead to high rates of ethylene production without high levels of intracellular methionine. SAM is an important methyl donor and is involved in multiple aspects of cellular metabolism. Consequently, the two committed steps in the synthesis of ethylene are the formation of ACC and its conversion to ethylene. The genes encoding ACS and ACO have thus been studied in more detail than other enzymes in the pathway, although there is evidence that several other genes involved in methionine synthesis and the methionine salvage pathway are differentially expressed during ripening and in response to ethylene (Alba and others 2005; Zegzouti and others 1999).

ACS and ACO are encoded by multigene families in higher plants, with tomato possessing at least nine *ACS* (*LEACS1A*, *LEACS1B*, and *LEACS2-8*) and five *ACO* (*LEACO1-5*) genes (Barry and others 1996; Nakatsuka and others 1998; Oetiker and others 1997; Van-der-Hoeven and others 2002; Zarembinski and Theologis 1994). Expression analysis has revealed that at least four *ACS* (*LEACS1A*, *LEACS2*, *LEACS4*, *LEACS6*) and three *ACO* (*LEACO1*, *LEACO3*, *LEACO4*) genes are differentially expressed in tomato fruit (Barry and others 1996, 2000; Nakatsuka and others 1998; Rottmann and others 1991). *LEACO1*, *LEACO3*, and *LEACO4* are expressed at low levels in green fruit that are in a system 1 mode of ethylene synthesis, but the transcripts of each increase at the onset of ripening as the fruit transition to system 2 ethylene production and response. During ripening, *LEACO1* and *LEACO4* are sustained in expression, whereas the increase in *LEACO3* expression is transient (Barry and others 1996; Nakatsuka and others 1998). In the case of *LEACO1* and *LEACO4*, ripening-related increases in transcript abundance are largely blocked by 1-MCP treatment, indicating that these genes are positively regulated by ethylene. The regulation of *ACS* gene expression during fruit ripening has been investigated using a combination of ethylene and inhibitor studies together with expression analysis in various ripening mutants (Barry and others 2000; Nakatsuka and others 1998). The *ripening-inhibitor* (*rin*) and *non-ripening* (*nor*) mutants fail to undergo the typical ripening-related increase in ethylene synthesis (system 2) and respiration that occurs in wild-type fruit and, as such, maintain a low-level system 1-type ethylene production as they mature (Tigchelaar and others 1978). *LEACS6* is expressed in wild-type green fruit but rapidly declines at the onset of ripening during the transition to system 2 ethylene synthesis. In contrast, *LEACS6* transcripts persist throughout development and ripening in the *rin* mutant (Barry and others 2000). Ethylene and 1-MCP treatments indicated that this ripening-related decline was mediated by ethylene, suggesting that *LEACS6* is responsible for low-level ethylene production in preclimacteric fruit (Barry and others 2000; Nakatsuka and others 1998). *LEACS1A* is also expressed in preclimacteric fruits and declines upon ethylene treatment, but transcripts show a transient increase at the onset of ripening that is *rin* dependent, suggesting that this gene may be important in regulating ethylene synthesis during the transition from system 1 to system 2 ethylene synthesis (Barry and others 2000). *LEACS4* is not expressed in green fruit but is induced at the onset of ripening. This induction is dependent on *rin* and is stimulated by ethylene. *LEACS2* expression is also induced at the onset of ripening; this induction requires ethylene but is independent of *rin* (Barry and others 2000; Nakatsuka and others 1998). Therefore, it seems likely that *LEACS1A* and *LEACS4* are responsible for initiating system 2 ethylene synthesis and that this is maintained by a combination of *LEACS2* and *LEACS4*. The specific transcription factors that mediate the changes in *ACS* and *ACO* gene expression at the onset of ripening remain to be determined. In a recent study, a 40-bp promoter fragment of *LEACS2* was identified that is required for ethylene-inducing-xylanase (EIX) responsiveness. Both *in vitro* and *in vivo* studies indicated that a novel cysteine protease, designated *LeCP*, was bound to this region and was capable of inducing *LEACS2* expression when

Figure 2. Fruit ripening mutants of tomato. From left to right, ripe fruit of wild type (cultivar Ailsa Craig) and near isogenic lines homozygous for the *ripening-inhibitor* (*rin*), *non-ripening* (*nor*), *Never-ripe* (*Nr*), and *Green-ripe* (*Gr*) loci. Note association of the *macrocalyx* (*mc*) (large sepal) phenotype with the *rin* mutation. The *rin* and *nor* loci act upstream in the ripening regulatory pathway and are required for system 2 ethylene synthesis during fruit ripening. The nonripening phenotypes of *Nr* and *Gr* are caused by reduced ethylene responsiveness (see text for details).

overexpressed in tomato leaves, suggesting that this protein may possibly have dual functions as a protease and a transcriptional regulator (Matarasso and others 2005). However, it remains to be determined whether the same promoter region of *LEACS2* and *LeCP* are required for ethylene-regulated ripening induction. Although regulation of *ACS* at the level of expression and transcript abundance is clearly important for ripening-related ethylene synthesis, there is considerable evidence that regulation of ACS activity, through protein phosphorylation and turnover, also plays a critical role in the function of this enzyme (for review, see Argueso and others, this issue).

The physiologic and molecular pathways that act to initiate the transition from a system 1 to a system 2 mode of ethylene synthesis at the onset of ripening remain undefined. However, a recent study performed on detached persimmon (*Diospyros kaki* Thunb.) fruit indicated that ripening-related ethylene synthesis in the fruit was initiated by a burst of drought-induced ethylene synthesis from the fruit calyx following harvest (Nakano and others 2003). Detached persimmon fruit initiated ethylene production and associated loss of firmness within two days after harvest. Treatment of fruit parts with the ethylene action inhibitor 1-methylcyclopropene (1-MCP) inhibited ethylene synthesis in all tissues except the calyx, indicating that the ethylene produced from the calyx was independent of ethylene itself. Induction of an ACC synthase gene, *DkACS2*, correlated with ethylene synthesis and was unaffected by 1-MCP. The calyx of fruits stored at high humidity initiated ethylene production and *DkACS2* expression four days later than fruits stored at low humidity. Similar delays were also observed in the pulp under high-humidity conditions. These data clearly show a role for water loss in regulating the onset of ethylene synthesis in detached persimmon fruit. Although this mechanism has been described only in persimmon fruit to date, certain fruits, including avocado and the wild species of tomato *Solanum chilense* and *Solanum peruvianum*, are known to initiate ripening once abscission has occurred from the parent plant. It is possible that water loss from these fruits following detachment could be a possible mechanism to initiate ethylene synthesis and ripening (Grumet and others 1981; Dopico and others 1993).

Distinct Transcription Factors Act Upstream of Ethylene Synthesis to Regulate Fruit Ripening

With the exception of ethylene, the signaling pathways that regulate fruit ripening remain largely undefined. In tomato, three pleiotropic nonripening mutants, *ripening-inhibitor* (*rin*), *non-ripening* (*nor*), and *Colorless non-ripening* (*Cnr*), have been described in which virtually all aspects of the ripening process are inhibited, including ethylene synthesis, increased respiration, carotenoid accumulation, softening, and aroma production (Thompson and others 1999; Tigchelaar and others 1978) (Figure 2). In these three mutants, the typical ripening-associated rise in autocatalytic ethylene synthesis is blocked due to abnormal regulation of *ACS* expression (see above). Although ethylene synthesis is blocked in these mutants, studies using *rin* and *nor* fruits have indicated that they retain the capacity to synthesize wound ethylene, indicating that the mutations are not simply the result of a general block in ethylene synthesis (Lincoln and Fischer 1988; Yokotani and others 2004). Similarly, exogenous ethylene does not restore ripening in these mutants, although ethylene-regulated gene expression can be partially restored, indicating that *rin*, *nor*, and *Cnr* fruits do not display ethylene insensitivity (Barry and others 2000; Griffiths and

others 1999; Thompson and others 1999; Yen and others 1995; Yokotani and others 2004). Together these data suggest that *rin*, *nor*, and *Cnr* act upstream of ethylene in the ripening cascade and determine the competency of the fruit to ripen.

The molecular identities of the *rin* and *Cnr* loci have been determined using positional cloning strategies, and both encode different classes of transcription factor (Manning and others 2006; Vrebalov and others 2002). The *rin* locus harbors a deletion occurring between two adjacent MADS-box genes. Genetic complementation and antisense experiments confirmed that one of these genes, termed *LEMADS-RIN*, was responsible for conferring the nonripening phenotype of the *rin* mutant, whereas the associated *macrocalyx* (*mc*) phenotype was the result of a promoter deletion in a second gene, termed *LEMADS-MC* (Vrebalov and others 2002). *RIN* is a member of the *SEPALLATA* subfamily of MADS-box genes, whereas *MC* is a member of the *APETALA 1* subfamily (Litt and Irish 2003; Malcomber and Kellogg 2005). As MADS-box proteins have been shown to act together in multimeric complexes, it is possible that other MADS-box genes act together with *RIN* to regulate fruit ripening in tomato. Indeed, expression analysis and data mining of EST collections have revealed several possible candidates to fulfill this role (Fei and others 2004; Giovannoni 2004). The *Cnr* mutation is the result of an epigenetic mutation that causes hypermethylation and reduced expression of a *SQUAMOSA PROMOTER BINDING PROTEIN* (*SBP*) gene (Manning and others 2006). SBP-box proteins have been shown to directly regulate the expression of MADS-box genes, raising the possibility that *CNR* may act to directly influence the expression of *RIN* or other MADS-box genes during fruit ripening. The characterization of the *RIN* and *CNR* transcription factors is currently the subject of intense research. It will be particularly interesting to identify the direct targets of these proteins and determine how they are able to regulate ethylene synthesis during ripening.

Ethylene Signaling in Tomato: Conservation and Diversity

Much of our knowledge concerning the mode of action of ethylene in plants has been generated from the use of the triple-response screen in *Arabidopsis* to identify mutants that are either insensitive to ethylene or show enhanced ethylene responses in the absence of exogenous ethylene. The power of *Arabidopsis* molecular genetics has facilitated the rapid identification of many components of the signaling pathway from an initial mutant phenotype. The components of ethylene signaling and their mechanisms of action in *Arabidopsis* are the subject of two additional reviews by Hall and others and Li and Guo, in this special issue. We focus our discussions on ethylene signaling research in fruit crop species, primarily reviewing research on tomato and how findings differ from the *Arabidopsis* model.

The Ethylene Receptors

The development of the triple-response screen in *Arabidopsis* (Bleecker and others 1988; Guzman and Ecker 1990), together with the identification of *ETR1* as an ethylene receptor (Chang and others 1993), led directly to the identification of an ethylene-insensitive mutant of tomato and the cloning of the family of ethylene receptors to which it belonged. The *Never-ripe* (*Nr*) mutant was initially described as a nonripening mutant 50 years ago (Rick 1956). Although *Nr* clearly displayed a dramatic inhibition of fruit ripening (Figure 2), other phenotypes associated with *Nr* had been overlooked. However, in light of the findings from *Arabidopsis*, Lanahan and coworkers (1994) showed that *Nr* displayed a range of phenotypes that could be directly attributed to reduced ethylene sensitivity. The semidominant ethylene-insensitive phenotype of *Nr* is reminiscent of the *etr1* ethylene receptor mutant, and with the availability of the *ETR1* gene for use as a heterologous probe, several ethylene receptor homologs were identified in tomato, one of which was found to cosegregate with the *Nr* phenotype on chromosome 9 (Yen and others 1995). Subsequent molecular analysis revealed a single C > T base change that results in conversion of a conserved proline residue into leucine (Wilkinson and others 1995). This proline residue lies within the N-terminal ethylene binding domain of the NR protein at a similar location to dominant ethylene-insensitive alleles of *ETR1* that disrupt ethylene binding (Chang and others 1993; Hall and others 1999).

To date, a total of six ethylene receptors have been identified in tomato: *LeETR1*, *LeETR2*, *NR* (also referred to as *LeETR3*), and *LeETR4*, *5*, and *6* (Klee 2004; Lashbrook and others 1998; Payton and others 1996; Tieman and Klee 1999; Wilkinson and others 1995; Zhou and others 1996). Based on structural similarity, the *Arabidopsis* ethylene receptors have been classified into two subfamilies (Guo and Ecker 2004). Subfamily-1 consists of ETR1 and ERS1 that share three N-terminal membrane-spanning

domains and a conserved carboxy terminus histidine (His) kinase domain. *LeETR1*, *LeETR2*, and *NR* possess a structure that is consistent with the subfamily-1 receptors. Subfamily-2 receptors in *Arabidopsis* (ERS2, ETR2, and EIN4) lack a complete His kinase domain and possess an additional transmembrane-spanning domain at the N terminus. The tomato receptors *LeETR4*, *5*, and *6* can be classified as subfamily-2 receptors. In addition, receptor structure differs with regard to the presence or absence of a receiver domain at the carboxy terminus. *Arabidopsis* ETR1, ETR2, and EIN4 all possess a receiver domain, as do all of the tomato receptors with the exception of NR. Thus, the receptor complement varies slightly between *Arabidopsis* and tomato. Tomato contains an additional subfamily-1 receptor compared with *Arabidopsis*, but contains only a single receptor lacking a receiver domain (NR), whereas *Arabidopsis* has two receptors (ERS1 and ERS2) that lack this domain. It is evident that the ethylene receptor family shows a high degree of structural divergence in plants, but despite this diversity all of the receptors thus far examined have the capacity to bind ethylene when expressed in yeast (O'Malley and others 2005).

The tomato ethylene receptors are differentially expressed in organs and tissues at various stages of development and in response to exogenous stimuli (Ciardi and others 2001; Lashbrook and others 1998; Moeder and others 2002; Tieman and Klee 1999). The changes in receptor profiles appear to be quantitative rather than qualitative, with expression of receptors detected in all tissues so far examined, implying that all tissues have the potential to respond to ethylene. However, specific receptors appear to be more prevalent in certain tissues; for example, *NR* and *LeETR4* are highly expressed in reproductive tissues and transcript abundance is enhanced during fruit ripening (Tieman and Klee 1999; Wilkinson and others 1995). Different expression levels of receptors may potentially lead to different pools of ethylene receptors that may act to regulate specific responses.

Characterization of the individual functions of members of the ethylene receptor gene family is subject to ongoing investigation. Experiments designed to downregulate specific receptor isoforms using antisense suppression have been reported for *LeETR1*, *NR*, and *LeETR4* (Hackett and others 2000; Tieman and others 2000; Whitelaw and others 2002). Downregulation of *LeETR1* expression in transgenic plants did not alter fruit ripening but resulted in plants with shorter internodes and reduced rates of floral abscission (Whitelaw and others 2002). Downregulation of *NR* expression in a wild-type background did not result in any dramatic phenotypes but did result in subtle changes indicative of slightly delayed fruit ripening, that is, reduced rates of ethylene synthesis and slower carotenoid accumulation (Tieman and others 2000). Elevated expression of *LeETR4* was detected in the *NR* antisense lines, suggesting that this receptor may compensate for loss of *NR*. Reduction of *LeETR4* expression using an antisense transgene resulted in plants with enhanced ethylene sensitivity manifested through extreme epinasty, increased floral abscission, enhanced triple response, and accelerated fruit ripening, confirming that *LeETR4* acts as a negative regulator of ethylene responses in tomato (Tieman and others 2000). Interestingly, these phenotypes could be complemented by overexpression of a *NR* transgene, indicating that these two receptors are functionally redundant, a phenomenon that is unexpected when one considers that they are extremely divergent (see above). Although studies of individual receptor function in tomato requires additional experimentation, an obvious difference between the tomato and *Arabidopsis* systems is evident. Reduction of the subfamily-2 receptor *LeETR4* in transgenic plants leads to strong phenotypic effects throughout the plant, whereas single loss-of-function mutants in type 2 receptors of *Arabidopsis* do not show dramatic phenotypic changes (Hua and Meyerowitz 1998).

GREEN-RIPE Encodes a Novel Regulator of Ethylene Responses

The dominant *Green-ripe* (*Gr*) and *Never-ripe 2* (*Nr-2*) mutants of tomato fail to fully ripen and possess a fruit phenotype very similar to that of *Nr* (Jarret and others 1984; Kerr 1958, 1982) (Figure 2). This similarity prompted an examination of the ethylene physiology of *Gr* and *Nr-2*. Examination of ethylene synthesis and responses in *Gr* and *Nr-2* fruits indicated that reduced ethylene responsiveness was the basis for ripening inhibition in these mutants (Barry and others 2005). However, unlike *Nr*, which shows reduced ethylene sensitivity throughout the whole plant, *Gr* and *Nr-2* show reduced ethylene sensitivity predominantly in fruit and floral tissues with weak ethylene insensitivity evident in roots. Dark-grown *Gr* and *Nr2* hypocotyls and petioles maintain normal ethylene responsiveness. High-resolution genetic and physical mapping of the *Gr* and *Nr-2* loci revealed that they were both linked to a 38-kb interval of the long arm of chromosome 1, suggesting that they may be allelic. This hypothesis was confirmed when sequence analysis of this region

identified a 334-bp deletion in both *Gr* and *Nr-2* mutants compared to wild type. The deletion occurs at the junction between the 5′-UTR and the promoter of a gene of unknown function and causes ectopic expression of the gene in *Gr* and *Nr-2* mutant backgrounds, consistent with a dominant gain-of-function mutation (Barry and Giovannoni 2006). To avoid confusion with the *NR* ethylene receptor, the gene residing at the *Gr* and *Nr-2* locus was designated *GREEN-RIPE* (*GR*).

Ectopic expression of *GR* under the control of the CaMV35S promoter recreated the *Gr* mutant phenotype but did not lead to plants that displayed whole-plant ethylene insensitivity despite high levels of transgene expression. This suggests that GR is able to selectively modify ethylene responsiveness in a tissue-dependent manner indicating that components of the ethylene signaling pathway must be distinct in different tissues of tomato (Barry and Giovannoni 2006). These differences have yet to be defined, but in a separate study a homolog of *GR*, *REVERSION TO ETHYLENE SENSITIVITY 1* (*RTE1*), was identified as a specific suppressor of ethylene insensitivity mediated by the *etr1-2* mutant allele of *Arabidopsis*, suggesting that RTE1 and, therefore, possibly GR act at the level of the ethylene receptors (Resnick and others 2006). It is tempting to speculate that the specificity of GR action in tomato may be linked to different pools of ethylene receptors that are present in different tissues in tomato (Lashbrook and others 1998; Tieman and Klee 1999).

GR and *RTE1* encode predicted transmembrane proteins of unknown function that are conserved in plants, animals, and protists but are not present in bacterial or fungal genomes. Higher plants typically contain two or three *GR* homologs, whereas animal and protist genomes possess a single copy of this gene. In plants there appears to be two phylogenetically distinct clades. One clade contains *GR*, *RTE1*, and a closely related tomato gene designated *GREEN-RIPE LIKE 1* (*GRL1*). The second clade contains distant homologs of *GR* and *RTE1* designated *GRL2* and *RTE1 HOMOLOG* (*RTH*). *GR* and *RTE1* have clear impacts on ethylene responses in plants, but it remains to be determined if *GRL1*, *GRL2*, and *RTH* also function in the ethylene response pathway or some other aspect of cellular metabolism. The second scenario seems more likely, particularly for the more distinct *GRL2* and *RTH* genes and in light of the fact that animals and protists are not known to signal using ethylene. The cloning of *GR* and *RTE1* has identified new proteins that can influence the ethylene response pathway in plants; however, determining how these proteins interact and function within the context of other pathway components is required.

Multiple CTR Kinases are Present in Crop Plants

The *constitutive triple response* (*ctr1*) mutant of *Arabidopsis* was identified in a genetic screen designed to identify dark-grown seedlings that possess the triple-response phenotype in the absence of exogenous ethylene (Guzman and Ecker 1990). The *CTR1* gene encodes a protein with high similarity to the mammalian RAF serine/threonine MAP kinase kinase kinase (MAP3K) and acts as a negative regulator of ethylene responses (Kieber and others 1993). CTR1 localizes to the endoplasmic reticulum, the same location as the ETR1 ethylene receptor, and interacts preferentially with the type I ethylene receptors ETR1 and ERS1 (Clark and others 1998; Gao and others 2003). The identification of a MAP3K that functions within the ethylene signaling pathway has led to the speculation that a MAP kinase cascade may mediate ethylene responses in *Arabidopsis*, although this hypothesis awaits experimental validation.

To date, four *CTR1* homologs have been isolated from tomato: *tCTR1* (also known as ER50), *tCTR2*, *tCTR3*, and *tCTR4* (Adams-Phillips and others 2004a, b; Leclercq and others 2002; Lin and others 1998; Zegzouti and others 1999). These genes were identified either using differential display (ER50) or through heterologous hybridization using the *Arabidopsis CTR1* gene as a probe. Phylogenetic analysis has indicated that *tCTR1*, *tCTR3*, and *tCTR4* are closely related to *Arabidopsis CTR1*. In addition, these three genes are all able to complement, at least partially, the weak *ctr1-8* allele of *Arabidopsis*, suggesting that the tomato genes are functionally equivalent to *CTR1*. Interestingly, the efficacy of complementation follows the phylogenetic relationship of the tomato genes to *Arabidopsis CTR1* such that *tCTR3* is able to fully complement *ctr1-8*, whereas *tCTR1* and *tCTR4* display only partial complementation (Adams-Phillips and others 2004a, b; Leclercq and others 2002). This may be indicative of slightly different signaling specificities of these proteins. *tCTR1*, *3*, and *4* show differential expression in various plant tissues, but as in the case of receptor gene expression, all tissues express *CTR-like* genes (Adams-Phillips and others 2004a, b; Leclercq and others 2002). *tCTR2* is more divergent and is the likely ortholog of the *ENHANCED DISEASE RESISTANCE 1* (*EDR1*) gene of *Arabidopsis* that has been implicated in disease resistance, stress responses,

and ethylene-induced leaf senescence and may act at the interface between the ethylene and salicylic acid signaling pathways (Adams-Phillips and others 2004a, b; Frye and others 2001; Tang and others 2005).

These data indicate that tomato possesses at least three *CTR* genes, whereas *Arabidopsis* possesses only a single gene. Subsequent analysis of EST and genomic sequence repositories has uncovered evidence for multiple *CTR-like* genes in a number of species, suggesting that the single gene found in *Arabidopsis* may be the exception (Adams-Phillips and others 2004a, b). The presence of multiple *CTRs* raises questions as to the individual functions of these genes and the level of redundancy that operates within this gene family. These questions will need to be addressed through the use of RNAi targeted at individual members of the family and by gain-of-function analysis. However, virus-induced gene silencing (VIGS) of a generic tCTR sequence led to tomato plants that showed severe epinasty and upregulation of ethylene-induced gene expression, indicating that silencing of *tCTR* genes can mimic the *Arabidopsis ctr1* mutant phenotype (Liu and others 2002). Because CTR1 is able to interact with at least some of the *Arabidopsis* ethylene receptors, it is likely that the tCTR proteins will also interact with the tomato ethylene receptors. The presence of larger gene families for both *CTRs* and the ethylene receptors in tomato compared to *Arabidopsis* indicates that the possible interactions between these two families is potentially more complex and that localization experiments will need to be performed together with interaction studies to confirm that any interactions are physiologically meaningful.

Transcriptional Regulation of Ethylene Responses During Fruit Ripening

In *Arabidopsis*, downstream ethylene responses are mediated by two classes of transcription factors encoded by *EIN3* and *ERF* gene families. *ETHYLENE-INSENSITIVE 3* (*EIN3*) also includes the *EIN3-LIKE* (*EIL*) family members and the *ETHYLENE RESPONSE FACTOR* (*ERF*) family is inclusive of genes referred to as *ETHYLENE-RESPONSIVE ELEMENT BINDING PROTEIN* (*EREBP*). *EIN3* is a positive regulator of ethylene responses with loss of function resulting in ethylene insensitivity, whereas overexpression results in a constitutive triple-response phenotype (Chao and others 1997). Emerging evidence suggests that the ethylene response pathway is regulated at least in part by turnover of the EIN3 protein. In the absence of ethylene, two partially redundant F-box proteins, EIN3-binding F-box 1 and 2 (EBF1 and EBF2), target EIN3 for degradation. A negative feedback loop exists whereby EIN3 is self-regulating through directly influencing the accumulation of EBF1 and EBF2 (Gagne and others 2004; Guo and Ecker 2003; Potuschak and others 2003; Yanagisawa and others 2003). In a recent study, the EIN5 protein was identified as the XRN4 $5' \rightarrow 3'$ exoribonuclease (Olmedo and others 2006). Both EBF1 and EBF2 mRNAs are significantly more abundant in the *ein5* mutant background than in wild type, suggesting that the wild-type function of EIN5 is to regulate the accumulation of EIN3 via turnover of *EBF1/2* transcripts.

EIN3 binds a conserved motif known as the primary ethylene responsive element (PERE) that is present within the promoters of *ERF1* and several senescence and ripening-related genes, including *E4*, *GST1*, and *LeACO1* (Solano and others 1998). *ERF1* expression is rapidly induced by ethylene, and overexpression of *ERF1* confers a constitutive ethylene response phenotype. ERFs in turn bind to the GCC-box that is present in the promoters of several stress- and pathogen-responsive genes, including chitinases and *PDF1.2* (Solano and others 1998). ERF1 and related genes form a subgroup of the large *APETALA2* (*AP2*) family of DNA-binding proteins that consists of 145 members in *Arabidopsis* (Gutterson and Reuber 2004). These transcription factors act to both positively and negatively regulate transcription and are involved in a wide range of developmental processes, responses to environmental challenges, and pathogen infections.

To date, four *EIN3-LIKE* genes have been described in tomato, *LeEIL1-4* (Tieman and others 2001; Yokotani and others 2003). *LeEIL1-3* are each able to complement the *Arabidopsis ein3-1* mutant allele, indicating that they are able to function in the ethylene signaling pathway. Antisense suppression of *EIL1-3* in tomato revealed that this gene family is functionally redundant (Tieman and others 2001). However, overexpression of a GFP-tagged *EIL1* in the *Nr* mutant restored normal fruit ripening and the expression of a subset of ethylene-inducible genes in transgenic fruit. In addition, petiole epinasty was restored in the *35S:EIL1:GFP* transgenic lines, but seedling responses remained unaltered, suggesting that individual members of the tomato *EIL* family may perform specific functions *in vivo* (Chen and others 2004a, b). The observation that sequences related to the PERE are contained within the promoters of the ethylene-regulated and ripening-related genes *E4* and *LeACO1*

suggests that EIN3 proteins may directly regulate their transcription and that of other coregulated genes (Solano and others 1998).

Thirty-six *ERF1-like* genes have been built into an *Arabidopsis* and rice phylogeny and it is likely that as more ESTs and tomato genomic sequence become available, this number will increase (Gutterson and Reuber 2004). Given the high copy number of this gene family, it is not surprising that several members have been isolated from fruit and show differential expression patterns during fruit development and ripening (Alba and others 2005; Tournier and others 2003). Because of the large size of this gene family, assigning functions to individual family members will be a daunting task, although the use of phylogeny coupled to phenotypic information from *Arabidopsis* may help guide studies in tomato (Gutterson and Reuber 2004). Furthermore, the promoters of many of the genes that have been associated with ripening do not contain the GCC-box that forms the binding site of the ERF protein, suggesting that these factors may have a limited role in the regulation of ripening. Interestingly, a number of stress- and defense-associated genes whose expression can be enhanced by ethylene have been shown to be induced during fruit ripening (Alba and others 2005; Picton and others 1993a, b; Zegzouti and others 1999). Stress-related genes often contain the GCC-box within their promoter regions that can be directly targeted by ERF proteins. Therefore, it is possible that the ERF proteins that are present in tomato fruit may regulate the expression of this subset of ripening-related genes, a hypothesis that will become open to testing as the tomato genome sequence becomes available in the next few years (Mueller and others 2005).

Clearly finding the immediate targets and *in vivo* function of both the EIN3-LIKE and ERF protein families during ripening will be important if a transcriptional network of ethylene-regulated gene expression is to be elucidated. A recent study examining the comparative transcriptome of tomato fruit during development and ripening in wild type and the *Nr* mutant revealed that 37% of the gene expression changes observed were influenced by ethylene (Alba and others 2005). Furthermore, clustering of gene expression profiles revealed that many of these changes were coordinated, implying that large groups of genes are coregulated. Although this approach addressed only steady-state mRNA levels and therefore did not distinguish between transcriptional and post-transcriptional events, it is highly likely that a large proportion of these changes involve at least partial ethylene-regulated transcriptional control. Dissecting specific signaling modules within this vast sea of ethylene-regulated events will be challenging. One approach that may be useful in this regard is a scaled-up version of the experimental system used by Chen and others (2004a, b) to study *EIL1* function in tomato (described above). A series of *EIL* or *ERF* constructs under the control of an inducible promoter could be introduced into the *Nr* mutant, possibly using transient expression systems, and early changes in gene expression could be monitored by microarrays following induction. This approach could reveal the identities of subsets of coregulated genes or pathways and provide information about potential targets of EILs and ERFs in tomato that could then be addressed by *in vitro* and *in vivo* DNA-binding studies.

Climacteric, Nonclimacteric, and Somewhere in Between: Variation in the Ethylene Physiology of Ripening Fruits

Fruits have been classically categorized into climacteric and nonclimacteric based on increased ethylene synthesis and a concomitant rise in the rate of respiration during ripening (Lelievre and others 1997). The role of ethylene as the ''ripening hormone'' in climacteric fruits such as tomato, apple, and banana has been firmly established. However, there is an increasing body of experimental evidence that implicates ethylene in the ripening of fruits that have been classically thought of as nonclimacteric. There are also a number of species in which the fruits of different varieties and cultivars exhibit both climacteric and nonclimacteric behavior.

A Role for Ethylene in the Ripening of ''Nonclimacteric'' Fruit

The highly sensitive technique of laser photoacoustic spectroscopy coupled with the development of specific apparatus to determine *in planta* ethylene production in fruits and flowers of strawberry during development and ripening has revealed an increase in ethylene production and a concomitant rise in respiration rate in red ripe strawberry fruits (Iannetta and others 2006). Furthermore, experiments with the ethylene action inhibitor silver thiosulfate revealed that this increased ethylene production was under the control of a positive feedback mechanism in ripe fruits, suggesting that a form of autocatalytic ethylene production is operational during ripening in strawberry. The timing of

this ripening-related increase in ethylene production is distinct from the patterns of ethylene production typically associated with the ripening of fruits such as tomato. For example, ethylene production increases at the onset of ripening in tomato, and ripening is severely disrupted in transgenic fruit where this phenomenon is blocked (Oeller and others 1991). In contrast, the increase observed in strawberry was not detected until 24 h after the fruits had developed full red pigmentation. Therefore, the physiologic role of the increased ethylene synthesis in strawberry remains to be determined. Nevertheless, in support of a role for ethylene in the ripening of strawberry, a body of molecular evidence concerning ethylene-related changes in gene expression is becoming available. For example, increased expression of cystathionine-gamma-synthase (CGS) and ACC oxidase genes has been reported during ripening of strawberry (Aharoni and others 2002; Marty and others 2000; Trainotti and others 2005). Similarly, increased expression of ethylene receptor homologs in strawberry fruit was also observed with increasing ripeness. In particular, increased expression of *FaETR2*, a type II receptor homolog that is closely related to the ripening-related *LeETR4*, was associated with ripening (Trainotti and others 2005). In addition, the isolation and characterization of a peptide methionine sulfoxide reductase (PMSR) gene that is expressed late in strawberry ripening were also recently described (Pedraza-Lopez and others 2006). This gene is homologous to the tomato ripening-related gene *E4*, whose expression is regulated by ethylene in tomato and may be involved in the methionine salvage pathway that operates during increased ethylene synthesis (Lincoln and others 1987). Although currently untested, it may be pertinent to examine the relationship of ethylene and the expression of FaPMSR and other genes whose expression is induced at late stages of ripening of strawberry fruit. The emerging data could be consistent with either a regulatory role for ethylene in as-yet defined aspects of ripening in strawberry fruit or a response to the dramatic cellular changes associated with ripening and the concomitant loss in cellular integrity and senescence characteristic of this developmental process. Either answer would be interesting because the former would demonstrate a fundamental role of ethylene in ripening of most if not all fruits and the other might point to a more primal developmental response that may indeed have been recruited through evolution as a signaling system to catalyze the ripening process in a subset of species. The creation of transgenic strawberry with reduced ethylene responsiveness, possibly by expression of a dominant mutant allele of an ethylene receptor, may help clarify this role.

Small but significant increases in ethylene synthesis at the onset of ripening have also been detected in grape berries. Chervin and others (2004) demonstrated the presence of a transient peak of ethylene production in grapes just prior to the onset of ripening, and experiments with 1-MCP indicated that ethylene was required at this stage for the onset of anthocyanin accumulation, fruit swelling, and the decrease in acidity that is associated with ripening. Concomitant with an ethylene-stimulated rise in anthocyanin production, the abundance of four transcripts encoding enzymes involved in anthocyanin synthesis also increased following ethylene treatment (El-Kereamy and others 2003). In an additional study, 1-MCP treatment of grape berries was found to partially repress the ripening-induced expression of the *VvADH2* gene that encodes an alcohol dehydrogenase (Tesniere and others 2004). These studies suggest that ethylene may influence multiple aspects of ripening in grape. A similar strategy as described for strawberry above could clarify the role of ethylene in grape berry ripening.

Citrus is also classified as a nonclimacteric fruit, but studies with inhibitors of ethylene action revealed that ripening-related color changes in the flavedo are regulated by endogenous ethylene and that ethylene treatments can stimulate both chlorophyll breakdown and carotenoid accumulation (Goldschmidt and others 1993; Purvis and Barmore 1981; Stewart and Wheaton 1972). Furthermore, studies have shown some genes, including chlorophyllase, to be ethylene regulated in citrus fruit (Alonso and others 1995; Jacob-Wilk and others 1999). Recently, as in the case of strawberry, autocatalytic ethylene production has also been described in citrus fruit, but again with altered timing relative to typical ripening climacteric fruit. Whereas mature fruits exhibited no increased ethylene production associated with ripening, harvested immature fruits produce high levels of ethylene that can be further stimulated by ethylene and propylene treatments and inhibited by 1-MCP, indicating the autocatalytic nature of this phenomenon (Katz and others 2004). This study clearly showed that citrus fruit have the capability to produce autocatalytic ethylene, although there may be little significance of this phenomenon in relation to the ripening process. Citrus and other fruit trees undergo a continual process of fruit drop or selective abscission to ensure that resources are available to allow the development of mature fruits. It is possible that the climacteric behavior of young citrus fruitlets

may be linked to this abscission process or may be the result of stress during detachment as was witnessed in persimmon fruit (see above).

Climacteric and Nonclimacteric: Ethylene-Mediated Ripening Inhibition Through Natural Allelic Variation

Apple cultivars are highly heterozygous and their pedigrees are often poorly defined. In addition, they possess different ripening rates leading to varied storage properties ranging from rapid postharvest deterioration to cultivars that can be stored for up to a year under optimal conditions. A clear positive correlation exists between ethylene production during storage and softening, which in turn is tightly associated with postharvest deterioration (Gussman and others 1993). Sunako and others (1999) identified two allelic forms of the *Malus domestica ACS1* gene from the Golden Delicious cultivar, *MdACS1-1* and *MdACS1-2*. *MdACS1-1* is highly expressed at the onset of apple fruit ripening, whereas *MdACS1-2* transcripts are absent from ripening fruits. Sequence analysis of these alleles revealed only seven nucleotide substitutions within the protein-coding region of *MdACS1* and six of these encoded silent mutations. However, more sequence divergence was evident in the 5′-flanking region, including the presence of a short interspersed DNA element (SINE) in the *MdACS1-2* allele. Thirty-five apple cultivars were tested for the presence or the absence of the SINE element and results indicated that cultivars were either homozygous for either *MdACS1-1* or *MdACS1-2* or heterozygous for each allele. In subsequent studies, cultivars that were homozygous for the *MdACS1-2* allele had significantly lower internal ethylene concentrations than *MdACS1-1* homozygotes or *MdACS1-1*/*MdACS1-2* heterozygous individuals and possessed enhanced storage capabilities and reduced rates of fruit drop (Harada and others 2000; Oraguzie and others 2004; Sato and others 2004). These data indicate that the insertion of a SINE into the promoter of the *MdACS1* gene is directly responsible for reduced gene expression, lower ethylene production, and enhanced storage properties of certain apple cultivars. This discovery provides a useful tool for selecting new apple varieties with optimal storage properties.

Reduction in *ACS* gene expression and ethylene production also seems to be responsible for the nonripening phenotype in peach cultivars that carry the recessive *stony hard* (*hd*) mutation. *hd* fruit fail to soften on the tree or postharvest, although other ripening traits such as color development, soluble solids, and flavor characteristics are fairly normal (Haji and others 2001). Furthermore, the phenotype of *hd* can be reversed by ethylene treatment (Haji and others 2003). Expression of a ripening-related *ACS* gene, *PpACS1*, is eliminated in *hd* backgrounds during ripening, although this gene remains wound-inducible in both leaves and fruits (Tatsuki and others 2006). The mechanism of the reduction of *PpACS1* expression in *hd* is currently unknown. Southern analysis failed to reveal any significant structural differences in *PpACS1* between *hd* and a normal-ripening cultivar, indicating that disruption of the promoter by a transposable element, as in the case of the *MdACS1-2* allele, is unlikely. It is possible that a SNP or small deletion or insertion may disrupt a ripening-specific transcription factor binding site in the *PpACS1*promoter or that the *hd* phenotype may be caused by a mutation in a ripening-specific transcription factor.

Melon is also a fruit crop that exhibits great phenotypic diversity. Fruits of the cantaloupe type often have netted skin and orange flesh, are susceptible to abscission, and produce large quantities of ethylene. In contrast, melons of the ''honey dew'' type are often smooth skinned, have green or yellow flesh, do not abscise, and produce little or no ethylene during ripening. Therefore, melons behave as both climacteric and nonclimacteric fruit, and the level of ethylene production produced by melon fruit is directly proportional to postharvest rates of decay (Zheng and Wolff 2000). Perin and others (2002) examined the ethylene physiology of a smooth-fruited, nonabscising melon designated PI161375. They found that exogenous ethylene failed to induce abscission, fruit ethylene production, or the expression of ethylene-regulated genes, suggesting that PI161375 fruit is ethylene insensitive. However, the seedling triple response in this line was normal. Genetic analysis of a recombinant inbred population generated from a charentais (cantaloupe) × PI161375 cross indicated that fruit abscission and ethylene production were controlled by two independent loci designated *Abscission layer* (*Al*)*-3* and *Al-4*. F_1 progeny of the charentais × PI161375 cross produced fruits that abscised and produced a climacteric ethylene peak, indicating that the alleles from the PI161375 parent are recessive. The fruit and abscission zone-specific responses in PI161375 share similarities to, but are distinct from, tomato mutants that display inhibited fruit ripening. For example, like the PI161375 line, the *rin*, *nor*, and *Cnr* mutants can be classified as nonclimacteric; however, they retain the capacity to respond to exogenous ethylene at the level of gene expression (Tigchelaar and others 1978; Thompson and others 1999; Yen and others 1995; Yokotani and others 2004). In contrast, the *Gr* mutant of tomato

displays tissue-specific ethylene insensitivity associated with fruit ripening and abscission, but unlike PI161375, *Gr* fruit are capable of synthesizing large quantities of ethylene (Barry and others 2005).

Pepper fruit also exhibits a wide range of ethylene production rates and respiratory behavior during ripening (Villavicencio and others 1999). Although a role for endogenous ethylene in mediating the phenotypic changes associated with ripening in pepper has not been determined, exogenous ethylene treatments can lead to enhanced carotenoid accumulation (Fox and others 2005). From the data generated by studies on apple and melon, it seems that large differences in fruit ethylene production appear to be controlled by one or two genetic loci. Although still unproven, it is possible that a similar situation may be occurring between closely related pepper species. QTL analysis on segregating populations generated from crosses between high and low ethylene-producing parents should be able to resolve these issues and address whether differing ripening mechanisms truly exist within these species or (as is more likely) nonclimacteric varieties represent allelic variants in ethylene synthesis, response, or more general ripening genes within what are normally climacteric species.

Controlling Ethylene Responses for Horticultural Crop Improvement

Clearly, ethylene is required for the ripening of many fruits and in its absence the ripening process fails to proceed to completion, rendering the product unpalatable. However, once initiated, ripening is a one-way process and the beneficial aspects of ethylene for generating a high-quality product can soon be outweighed by its propensity to stimulate over-ripening and decay. This is particularly true under postharvest storage conditions where considerable effort is expended to control ethylene effects not only in fruits but also in vegetables and ornamental crops. Depending on the commodity, specialized harvesting, packaging, shipping, temperature, and controlled atmospheres may be required, adding to the cost of production through additional labor and energy use. Control of ethylene responses is therefore important for the agricultural and food industries.

Chemical Control of Ethylene Responsiveness

Several compounds have been developed that can block ethylene action in fruits, vegetables, and floral crops and these are thought to act through binding to the ethylene receptors (Sisler 2006). 1-Methylcyclopropene (1-MCP) is a potent inhibitor of ethylene responses and under the tradenames EthylBloc® and SmartFresh™ it has been approved for commercial use on ornamental and edible horticultural products, respectively. A whole research field has evolved to test the efficacy and physiologic effects of 1-MCP on fruit crops and a wide range of effects are observed that vary between species and even between cultivars (Watkins 2006). It appears that this compound does have limitations in many species, but major successes have been reported for prolonging the storage life of apples leading to widespread commercial utilization within the apple industry.

Genetic Control of Ethylene Synthesis and Responsiveness

The role of ethylene in regulating fruit ripening in tomato has been unambiguously determined through the genetic manipulation of ethylene synthesis and perception (Klee and others 1991; Oeller and others 1991; Picton and others 1993a, b; Wilkinson and others 1997). Transgenic manipulation of ethylene synthesis through downregulation of *ACS* and *ACO* expression has also been achieved in melon and apple (Ayub and others 1996; Dandekar and others 2004). Transgenic melon expressing an *ACO* antisense gene displayed inhibition of ripening, including a reduction in external pigmentation and fruit softening. The transgenic lines also failed to develop a peduncular abscission zone and therefore did not abscise from the plant at maturity, leading to an enhanced accumulation of sugars (Guis and others 1997). The production of volatile esters that represent important flavor components in ripe melon fruit are also greatly inhibited in the *ACO* antisense lines (Bauchot and others 1998; Flores and others 2002). In addition, fruit of the antisense lines are less susceptible to chilling injury than those of wild type (Flores and others 2004). Similarly, ethylene-suppressed apples were firmer, had an extended shelf life, altered sugar and organic acid profiles, and reduced volatile ester formation that was accompanied by ethylene-dependent reductions in alcohol acyl-CoA transferase (AAT) activity (Dandekar and others 2004; Defilippi and others 2004, 2005).

The natural variation seen in ethylene production and responsiveness in apples, peaches, melons, and peppers indicate that there is considerable scope for the generation of new cultivars within these species with differing ethylene physiology and ripening characteristics. The development of introgression lines of tomato harboring defined chromosomal

segments of wild species within the cultivated *Solanum lycopersicum* genome is providing unprecedented quantities of information about loci that control fruit quality traits (Gur and others 2004; Liu and others 2003; Schauer and others 2006). Considerable variation in ethylene synthesis and ripening characteristics of wild species of tomato have been reported (Grumet and others 1981). It may be possible to harness the power of the introgression lines for uncovering QTL that alter ethylene-related phenotypes during fruit development and ripening.

Conclusions

From utilizing the wealth of information generated using the *Arabidopsis* model and from many fundamental studies on fruit crop species, we now have a basic framework of ethylene biosynthesis and signaling during fruit ripening. This knowledge has increased our ability to modify ethylene synthesis and responses in fruit crops to lessen the effects of postharvest deterioration. However many fundamental biological questions remain to be addressed:

How does the transition from system 1 to system 2 modes of ethylene synthesis occur at the onset of ripening, what is the involvement of the *rin*, *nor*, and *Cnr* loci in mediating this transition, and how is the system 2 ethylene synthesis perpetuated?

What are the functions of the individual ethylene signaling components and how is this network of proteins assembled *in vivo*? Is the expansion of gene families in tomato, and perhaps other fleshy fruit-bearing species, linked to special ethylene signaling requirements in these species? What are the downstream targets of the EILs and ERFs in ripening fruits and how do these transcription factors activate or repress specific ripening-related pathways? How are tissue-specific ethylene signaling effects, such as those mediated by the GR protein and the *Al3* and *Al4* loci, achieved?

What role does ethylene play in the ripening of nonclimacteric fruits? What is the significance, if any, of small transient increases in ethylene production in species such as grape and strawberry? In nonclimacteric fruits, do changes in ethylene sensitivity rather than ethylene synthesis mediate physiologic changes during ripening? Could nonclimacteric fruits carry mutations within components of the ethylene synthesis or signaling pathways? Within closely related species such as capsicums and melons, what is the molecular identity of loci that control climacteric ethylene production, how widespread is this natural variation in fruit crop species, and can it be harnessed for generating new varieties with broad consumer appeal but with enhanced shipping and storage capability?

Clearly there is still much to discover within the field of ethylene biology as it relates to both the biological and practical aspects of fruit ripening. The questions outlined above are complex and will require multidisciplinary experimental approaches to resolve but will ultimately provide important information regarding the mechanism by which this hormone functions to regulate fruit ripening.

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