The Regulation of 1-Aminocyclopropane-1-Carboxylic Acid Synthase Gene Expression during the Transition from System-1 to System-2 Ethylene Synthesis in Tomato[1]

Cornelius S. Barry[2], M. Immaculada Llop-Tous[3], and Donald Grierson*

Plant Science Division, School of Biological Sciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, United Kingdom

1-Aminocyclopropane-1-carboxylic acid synthase (ACS) is one of the key regulatory enzymes involved in the synthesis of the hormone ethylene and is encoded by a multigene family containing at least eight members in tomato (*Lycopersicon esculentum*). Increased ethylene production accompanies ripening in tomato, and this coincides with a change in the regulation of ethylene synthesis from auto-inhibitory to autostimulatory. The signaling pathways that operate to bring about this transition from so-called system-1 to system-2 ethylene production are unknown, and we have begun to address these by investigating the regulation of *ACS* expression during ripening. Transcripts corresponding to four *ACS* genes, *LEACS1A*, *LEACS2*, *LEACS4*, and *LEACS6*, were detected in tomato fruit, and expression analysis using the *ripening inhibitor* (*rin*) mutant in combination with ethylene treatments and the *Never-ripe* (*Nr*) mutant has demonstrated that each is regulated in a unique way. A proposed model suggests that system-1 ethylene is regulated by the expression of *LEACS1A* and *LEACS6*. In fruit a transition period occurs in which the *RIN* gene plays a pivotal role leading to increased expression of *LEACS1A* and induction of *LEACS4*. System-2 ethylene synthesis is subsequently initiated and maintained by ethylene-dependent induction of *LEACS2*.

The plant hormone ethylene mediates plant responses to many developmental signals and environmental stimuli (Abeles et al., 1992). Tomato (*Lycopersicon esculentum*) fruit ripening represents just one example in plant development where ethylene synthesis and perception have been shown to be essential for the full completion of the ripening process (Oeller et al., 1991; Picton et al., 1993; Lanahan et al., 1994; Wilkinson et al., 1997). The pathway of ethylene synthesis is well established in higher plants (Yang and Hoffman, 1984), and regulatory control is achieved at two steps: the formation of 1-aminocyclopropane-1-carboxylic acid (ACC) from *S*-adenosyl-L-Met and the conversion of this intermediate to ethylene (Kende, 1993). The first step is catalyzed by the enzyme ACC synthase (ACS) and the second by ACC oxidase (ACO). In higher plants both of the enzymes are encoded by multigene families (Zarembinski and Theologis, 1994) generating the option of multiple control points at which ethylene synthesis may be regulated. Eight ACC-synthase genes have been identified in tomato (Rottmann et al., 1991; Yip et al., 1992; Lincoln et al., 1993; Olson et al., 1995; Nakatsuka et al., 1998; Shiu et al., 1998) along with four ACO genes (Holdsworth et al., 1988; Blume et al., 1997; Nakatsuka et al., 1998). Complexity in the regulation of ethylene synthesis in tomato fruit is emerging with no fewer than five *ACS* and three *ACO* genes reportedly expressed. The ripening-related expression of two *ACS* genes, *LEACS2* and *LEACS4*, has been well documented in tomato (Olson et al., 1991; Rottmann et al., 1991; Yip et al., 1992; Lincoln et al., 1993). Antisense inhibition of *LEACS2* in transgenic plants caused down-regulation of endogenous *LEACS2* and *LEACS4* expression and reduced ripening-related ethylene synthesis to 0.1% of that produced by control fruit, thus demonstrating the importance of these isoforms during ripening (Oeller et al., 1991). In addition, in a more recent study, the expression of *LEACS1A*, *LEACS3*, and *LEACS6* in tomato fruit has also been described (Nakatsuka et al., 1998). The importance of ACO in regulating ethylene synthesis was also demonstrated using antisense technology (Hamilton et al., 1990; Picton et al., 1993). Down-regulation of endogenous *LEACO1* expression in transgenic plants caused reduced ethylene synthesis in transgenic fruit and retarded ripening (Picton et al., 1993). *LEACO1* appears to be the major *ACO* gene expressed in tomato fruit (Barry et al., 1996; Blume and Grierson, 1997) but ripening-related expression of *LEACO3* and *LEACO4* has also been reported (Barry et al., 1996; Nakatsuka et al., 1998).

Two systems of ethylene regulation have been proposed to operate in higher plants (for review, see Lelièvre et al., 1998). System 1 is functional during

[1] This work was supported by the European Union Fair Program (grant no. CT95 0225 to D.G.).

[2] Present address: Department of Horticultural Sciences, Texas A&M University, College Station, TX 77843–2133.

[3] Present address: Consejo Superior de Investigaciones Científicas, Jordi Girona 18–26, 08034 Barcelona, Spain.

* Corresponding author; e-mail donald.grierson@nottingham.ac.uk; fax 44–0–115–951–6334.

normal vegetative growth, is ethylene auto-inhibitory, and is responsible for producing the basal levels of ethylene detectable in all of the tissues including non-ripening fruit. System 2 operates during the ripening of climacteric fruit and during petal senescence when ethylene is autostimulatory and requires the induction of both of the ACS and ACO. The signaling pathways that bring about the induction of these two enzymes through co-ordinated regulation of *ACS* and *ACO* gene families remain unknown, although a large amount of evidence is available that indicates that a combination of both ethylene and developmental factors are required. For example, analysis of tomato and melon fruit expressing an ACO antisense transgene showed that ACC accumulated despite greatly reduced ethylene synthesis indicating that ACC synthesis is under developmental regulation (Picton et al., 1993; Guis et al., 1997). Additionally, treatment of immature green fruit with ethylene is insufficient to induce ACS activity, indicating that ACS induction is dependent on the developmental stage of the fruit (Liu et al., 1985). However, ethylene has also been shown to stimulate *ACS* and *ACO* expression in tomato and other fruit (Maunders et al., 1987; Dong et al., 1992; Lincoln et al., 1993; Lasserre et al., 1996; Blume and Grierson, 1997). These results suggest that different *ACS* genes may be regulated by different mechanisms in tomato fruit.

In this study we have examined the regulation of the tomato *ACS* gene family during fruit ripening using a combination of ripening mutants and ethylene treatments. Four transcripts corresponding to *LEACS1A*, *LEACS2*, *LEACS4*, and *LEACS6* were identified in tomato fruit, and we demonstrate that the corresponding genes are each regulated in a unique way. The results are discussed in terms of system-1 and -2 ethylene synthesis and a model of *ACS* gene regulation proposed.

RESULTS

ACS Gene Expression during Ripening of Wild-Type and *Ripening Inhibitor* (*rin*) Tomato Fruit

The expression of eight members of the tomato *ACS* gene family was analyzed during ripening of wild-type cv Ailsa Craig fruit. Transcripts from *LEACS1A*, *LEACS2*, *LEACS4*, and *LEACS6* were detected in tomato fruit (Fig. 1), however no expression of *LEACS1B*, *LEACS3*, *LEACS5*, and *LEACS7* was evident (data not shown). Three of the genes, *LEACS1A*, *LEACS2*, and *LEACS4* showed a ripening-related increase in expression. Transcripts of these genes were low or undetectable in mature green fruit and increased at the breaker stage. As ripening progressed the expression levels of both of the *LEACS2* and *LEACS4* continued to rise, whereas the increase in *LEACS1A* transcripts was only transient with maximum abundance detected at the breaker stage. In contrast to the other three genes, *LEACS6* transcripts were present in mature green fruit but declined rapidly as ripening was initiated. Based on relative exposure times to x-ray film (see legend to Fig. 1), both of the *LEACS1A* and *LEACS6* represent lower abundance transcripts than *LEACS2* and *LEACS4*.

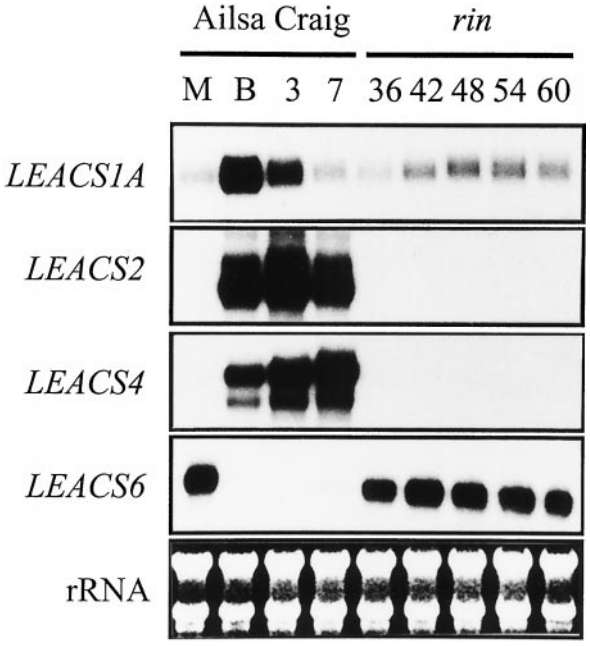

Figure 1. ACS gene expression in wild-type cv Ailsa Craig and *rin* fruit. Twenty-five micrograms of total RNA from cv Ailsa Craig mature green (M), breaker (B), breaker +3 (3), and breaker +7 (7) and *rin* fruit harvested at 36, 42, 48, 54, and 60 DPA was hybridized to radiolabeled RNA probes specific for tomato *ACS* genes and RPA analysis was performed as described in "Materials and Methods." Results are shown only for the genes that showed a positive hybridization signal. Exposure times to x-ray film were as follows: *LEACS1A*, 7 d; *LEACS2*, 20 h; *LEACS4*, 20 h; and *LEACS6*, 16 d.

The expression of the ACS gene family was also examined in the *rin* mutant of tomato (Tigchelaar et al., 1978). The *rin* mutant produces fruit with severely reduced ripening, and one characteristic of these fruit is that the burst of ethylene production normally associated with the ripening of climacteric fruit (system 2) is absent. However, *rin* fruit still produce a low basal level of ethylene production (system 1), similar to wild-type green fruit, throughout their development (Herner and Sink, 1973; Lincoln and Fischer, 1988). Therefore, *rin* fruit represent an ideal system for identifying individual genes that are involved in either system-1 or -2 ethylene synthesis. All of the four *ACS* genes showed the same expression pattern in *rin* fruit throughout development as was observed in mature green cv Ailsa Craig wild-type fruit. Both of the *LEACS1A* and *LEACS6* transcripts were present in *rin* fruit at comparable levels with those seen in wild-type mature green fruit. However, the ripening-related changes in ex-

pression of the four genes observed in wild-type fruit did not occur in *rin*.

ACS Gene Expression in Wild-Type and *rin* Fruit Treated with Ethylene

The role of ethylene in inducing ripening-related changes in *ACS* gene expression was investigated by incubating mature green wild-type fruit in 10 μL L^{-1} ethylene over 24 h (Fig. 2). Following the 1st h after ethylene application *LEACS1A* transcripts began to decline and remained low for the duration of the experiment. *LEACS6* expression was also reduced by ethylene exposure, although kinetically these changes occurred slightly slower than for *LEACS1A* between 2 and 4 h after treatment. *LEACS2* transcripts were undetectable until 4 h and were highly up-regulated by 12 h of incubation. No *LEACS4* expression was detected throughout the duration of the experiment.

An identical experiment was performed using *rin* fruit to ascertain whether the expression patterns detected during *rin*-fruit development (Fig. 1) were the result of the mutation per se or an indirect consequence brought about by reduced-ethylene synthesis. The changes in *ACS* gene expression that occurred in ethylene-treated wild-type fruit were also seen in *rin* with almost identical kinetics (Fig. 2). Both of the *LEACS1A* and *LEACS6* transcripts declined throughout the duration of the experiment. *LEACS2* expression was induced by ethylene treatment, and no induction of *LEACS4* expression was evident.

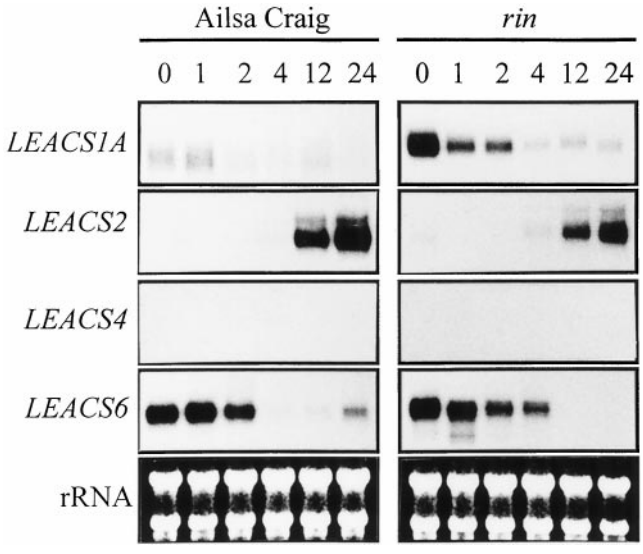

Figure 2. ACS gene expression in response to ethylene. Mature green wild-type and *rin* tomato fruit (37 DPA) were treated with 10 μL L^{-1} ethylene as described in "Materials and Methods" and harvested at time zero (0), 1 h (1), 2 h (2), 4 h (4), 12 h (12), and 24 h (24) after treatment. Twenty-five micrograms of total RNA was hybridized with radiolabeled RNA probes specific for *LEACS1A*, *LEACS2*, *LEACS4*, and *LEACS6* and subjected to RPA analysis as described in the "Materials and Methods." Exposure times to x-ray film were as follows: *LEACS1A*, 20 d; *LEACS2*, 4 d; *LEACS4*, 4 d; *LEACS6*, 14 d (cv Ailsa Craig); *LEACS1A*, 4 d; *LEACS2*, 3 d; *LEACS4*, 4 d; and *LEACS6*, 2 d (*rin*).

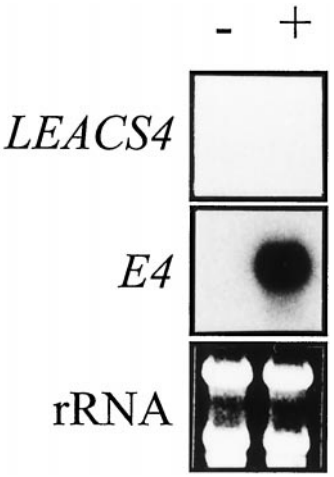

Figure 3. LEACS4 expression in 60-DPA *rin* fruit treated with ethylene. Sixty-DPA *rin* fruit were held in air (−) or in 10 μL L^{-1} ethylene (+) for a further 4 d. Twenty-five micrograms of total RNA was hybridized with a radiolabeled RNA probe specific for *LEACS4* and subjected to RPA analysis as described in the "Materials and Methods." RNA gel-blot analysis of *E4* expression is included as a positive control. Exposure times to x-ray film were as follows: *LEACS4*, 4 d; and *E4*, 16 h.

The lack of *LEACS4* expression in wild-type and *rin* fruit treated with ethylene may be due to a lack of maturity and, therefore, competence to respond to ethylene. To test this possibility an additional experiment was performed using 60-d-old *rin* fruit that were incubated in either air or 10 μL L^{-1} ethylene for 4 d (Fig. 3). Following ethylene exposure, transcripts of *E4*, an ethylene-regulated gene (Lincoln et al., 1987), were induced in *rin* fruit, whereas no induction of *LEACS4* was observed.

ACS Expression in Tomato Seedlings

In fruit, ethylene negatively regulated *LEACS1A* and *LEACS6* expression, whereas it induced *LEACS2* expression (Fig. 2). To investigate ethylene regulation of *ACS* gene expression in a non-climacteric tissue, expression was analyzed in 10-d-old light-grown seedlings held in air or treated with 10 μL L^{-1} ethylene for 12 h (Fig. 4). Of the eight *ACS* genes only transcripts corresponding to *LEACS1A*, *LEACS1B*, and *LEACS6* were detectable in air- and ethylene-treated tomato seedlings. Ethylene treatment caused a reduction in transcript abundance of *LEACS1A* and *LEACS6* but had no effect on *LEACS1B* expression. *E4* expression was measured as a positive control for ethylene response and showed clear ethylene induction in tomato seedlings.

ACS Gene Expression Is Altered in Never-Ripe (*Nr*) Fruit

To investigate the role of ethylene in regulating *ACS* gene expression throughout ripening we have used the *Nr* mutant of tomato, which displays ethylene insensitivity due to a dominant mutation in a member of the ethylene receptor gene family (Lanahan et al., 1994; Wilkinson et al., 1995). The expression of each *ACS* gene followed the expected expression pattern in the cv Pearson control fruit (Fig. 5) except that the decline in *LEACS6* expression took slightly longer than in cv Ailsa Craig fruit (Fig. 1). In *Nr* fruit the four genes displayed altered expression patterns. The transient increase in *LEACS1A* expression that occurred at the breaker stage of ripening in wild-type fruit was delayed in the *Nr* mutant and persisted for longer. Transcript levels increased at the onset of ripening and continued to rise, reaching a maximum 7 d after the onset of color change before declining slightly at 10-d post-breaker. Thus, although the *Nr* mutation appeared to affect the temporal expression of *LEACS1A*, transcript abundance ultimately reached the same levels as in wild-type fruit. The same effect was observed for *LEACS4* expression. However, in contrast, *LEACS2* transcript abundance was greatly reduced in *Nr* fruit and did not reach the levels seen in wild-type samples. In wild-type fruit *LEACS6* transcripts declined as ripening progressed from a maximum abundance in mature green fruit, whereas in *Nr* fruit, transcript levels increased throughout fruit development, reaching the levels of wild-type mature green fruit at around breaker +7.

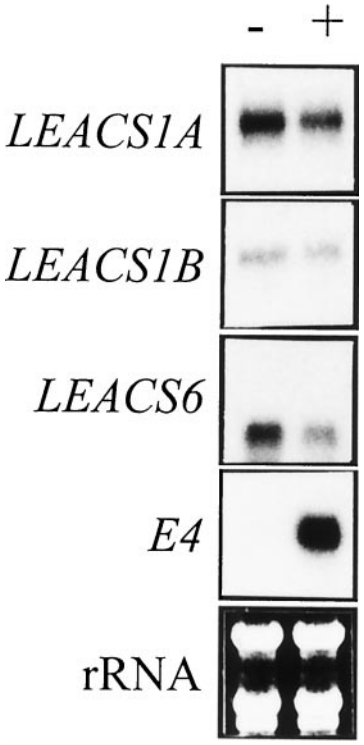

Figure 4. *ACS* expression in tomato seedlings. The expression of eight *ACS* genes was examined in 10-d-old light-grown tomato seedlings grown in air (−) or treated with 10 μL L^{-1} ethylene (+) for 12 h. Protection assays were carried out according to the "Materials and Methods" using 50 μg of total RNA per assay. RNA gel-blot analysis of *E4* expression is included as a positive control. Exposure times to x-ray film were as follows: *LEACS1A*, 7 d; *LEACS1B*, 5d; *LEACS6*, 3 d; and *E4*, 16 h.

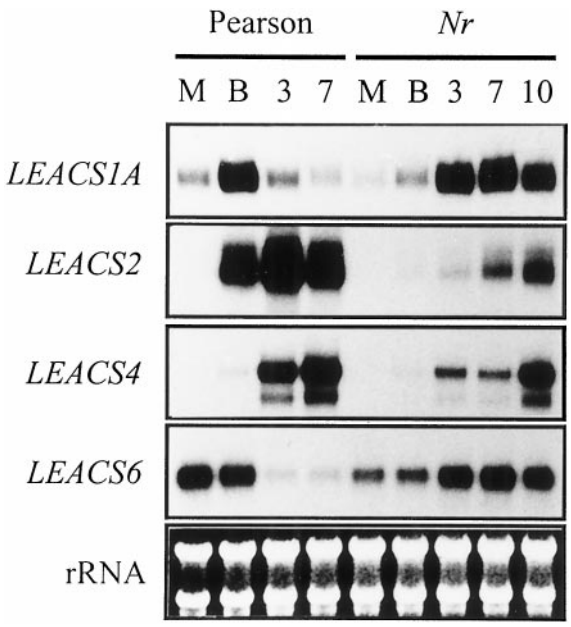

Figure 5. ACS gene expression in *Nr* fruit. Total RNA was extracted from cv Pearson mature green (M), breaker (B), breaker +3 d (3), and breaker +7 d (7) and an isogenic line of the *Nr* mutant at mature green (M), breaker (B), breaker +3 d (3), breaker +7 d (7), and breaker +10 (10). The RNA was hybridized with radiolabeled RNA probes specific for *LEACS1A*, *LEACS2*, *LEACS4*, and *LEACS6* and subjected to RPA analysis as described in the "Materials and Methods." Exposure times to x-ray film were as follows: *LEACS1A*, 7 d; *LEACS2*, 20 h; *LEACS4*, 20 h; and *LEACS6*, 14 d.

DISCUSSION

Expression of *ACS* Gene Family Members in Tomato Fruit

We have undertaken a detailed examination of the regulation of *ACS* gene expression during tomato fruit ripening using a combination of ripening mutants and ethylene treatments. The results indicate that four *ACS* genes, *LEACS1A*, *LEACS2*, *LEACS4*, and *LEACS6*, are expressed in tomato fruit (Fig. 1), and our data suggest that each shows distinct regulation that until now has not been detected. Increased *LEACS2* and *LEACS4* expression during fruit ripening has been extensively characterized (Olson et al., 1991; Rottmann et al., 1991; Yip et al., 1992; Lincoln et al., 1993; Nakatsuka et al., 1998). However, earlier studies have failed to distinguish differences in regulation of expression of these two genes, which

were simply classed as ethylene- or ripening-related. In a more recent study the presence of *LEACS1A*, *LEACS3*, and *LEACS6* transcripts in tomato fruit has been reported (Nakatsuka et al., 1998). These authors found that *LEACS6* expression was negatively regulated by ethylene during ripening, which corresponds well with the findings of the present study. However, our data for *LEACS1A* and *LEACS3* expression are clearly different. Nakatsuka et al. found that *LEACS1A* was expressed at a low constitutive level throughout fruit ripening, whereas our data suggests that it is highly regulated. Furthermore, in three independent experiments we failed to detect any *LEACS3* transcripts in tomato fruit (data not shown), whereas data presented by Nakatsuka et al. described the expression of this gene as constitutive during fruit ripening. These discrepancies may be caused by the use of different cultivars or sampling techniques or alternatively may be due to the fact that we have used ribonuclease protection assays (RPA) as our preferred method for determining gene expression compared with RNA gel-blot analysis used by Nakatsuka et al. (1998). RPA analysis has the advantage of being more sensitive and offers a higher degree of specificity when analyzing transcripts of individual members of a gene family.

Differential *ACS* Expression during System-1 and System-2 Ethylene Synthesis

The use of the *rin* mutant has allowed us to gain new insights into *ACS* gene regulation. Throughout *rin* fruit development the expression profile of the *ACS* genes remained the same as in a wild-type mature green fruit with no ripening-related changes occurring (Fig. 1). This confirms, at the molecular level, previous findings that *rin* fruit are in a perpetual state of system-1 ethylene synthesis (Lincoln and Fischer, 1988) and indicates that *LEACS6* together with the low levels of *LEACS1A* expression are likely to be responsible for system-1 ethylene synthesis in fruit. By analogy it follows that the ripening-related increases in *LEACS1A*, *LEACS2*, and *LEACS4* expression, which occur only in wild-type fruit, are important for the transition to system-2 ethylene synthesis.

It has been proposed that system-1 ethylene synthesis is under negative regulation by ethylene (Lelièvre et al., 1998), and other studies have confirmed that ACS enzyme activity and gene expression in vegetative tissue can be negatively regulated by ethylene (Yoon et al., 1997; Peck and Kende, 1998). Treatment of mature green tomato fruit and seedlings with ethylene (Figs. 2 and 4) resulted in a decline in *LEACS1A* and *LEACS6* transcript abundance, indicating that high ethylene levels exert a negative effect on regulation of these two genes. This suggests that at the molecular level system-1 ethylene synthesis involves the same two *ACS* genes in both of the vegetative and fruit tissues of tomato. Additionally, low levels of *LEACS1B* transcripts were also detected in seedlings, although the abundance was not influenced by ethylene. From this analysis it is not clear whether *LEACS1B* is also functioning in system-1 ethylene synthesis or in a separate developmental process associated with seedling development.

Differences between *ACS* Gene Regulation in Tomato Fruit

Our data clearly show that increased ethylene synthesis at the start of ripening, i.e. the transition to system 2, is correlated with increased *LEACS1A*, *LEACS2*, and *LEACS4* expression (Fig. 1). Previous work has shown that *LEACS2* and *LEACS4* expression can be stimulated by ethylene (Lincoln et al., 1993; Nakatsuka et al., 1998), although differences between the regulation of these two genes were not highlighted. Ethylene treatment of *rin* fruit (Figs. 2 and 3) indicates differences are apparent between *LEACS2* and *LEACS4* regulation. The induction of *LEACS4* expression has a strict requirement for the *RIN* gene product and is not influenced by ethylene treatment even in older mutant *rin* fruit treated for extended time periods. In contrast, the expression of *LEACS2* could be induced purely by ethylene even in the *rin* background. This finding suggests that, whereas *LEACS4* expression is directly influenced by RIN, the effect on *LEACS2* expression is an indirect one brought about as a result of reduced ethylene synthesis in the mutant background. However, general induction of *LEACS2* by ethylene throughout the plant does not occur as expression was not detected in ethylene-treated seedlings. This suggests that ethylene signal transduction, with respect to *LEACS2* expression, differs between fruit and vegetative tissues.

Further differences between *LEACS2* and *LEACS1A* and *4* were identified by expression analysis in *Nr* fruit (Fig. 5). *LEACS2* transcripts show severely delayed accumulation in *Nr* fruit, indicating that *LEACS2* expression has a strong ethylene requirement, which was confirmed by the results of the experiment shown in Figure 2. In contrast, only a slight delay to maximal expression of *LEACS1A* and *LEACS4* was observed in *Nr* fruit, implying that ethylene may not have such an essential role in regulation of these genes. The regulation of *LEACS6* expression by ethylene appears complex. Transcript levels declined rapidly in response to treatment with high levels of ethylene (Figs. 2 and 4) and during ripening when ethylene levels peak (Fig. 1). However, expression analysis in *Nr* fruit suggests that ethylene perception is important for maintaining low-level *LEACS6* expression as transcript abundance was lower in *Nr* mature green fruit than in cv Pearson control fruit (Fig. 5). Furthermore *LEACS6* expression increased as ripening was initiated in *Nr* fruit, and transcript levels eventually reached the same level as observed in cv Pearson mature green fruit. The sig-

nificance of this is unclear but one possibility is that a critical level of *LEACS6* expression may need to be achieved for ripening to be initiated and that this requires ethylene perception.

Model of *ACS* Gene Regulation during the Transition from System-1 to System-2 Ethylene Synthesis

A model for the proposed interactions that regulate *ACS* gene expression to initiate the transition to autocatalytic ethylene synthesis in tomato fruit is shown in Figure 6. In green fruit and vegetative tissue, system-1 ethylene is regulated by developmental pathways with unknown components via *LEACS1A* and *6* expression. System 1 continues throughout fruit development until a competence to ripen is achieved at which point a transition occurs. This transition may be brought about by a change in ethylene sensitivity due to continual system-1 ethylene production. This is supported by the observation that the transition period, as evidenced by delayed *LEACS6*, *LEACS1A*, and *LEACS4* expression, is extended in *Nr* fruit. The RIN protein plays an integral role within this transition period to cause an increase in *LEACS1A* expression and induce *LEACS4*. As a result of increased ethylene synthesis due to *LEACS1A* and *LEACS4* activation, *LEACS2* expression is induced and autocatalysis is initiated. High ethylene production occurs, resulting in negative feedback on the system-1 developmental pathway, resulting in reduced *LEACS1A* and *LEACS6* expression.

CONCLUSIONS

The regulation of four members of the tomato *ACS* gene family during fruit ripening has been investigated. We have extended previous studies to show that each has a unique expression pattern. Additionally we have described for the first time the expression pattern of *ACS* gene family members in vegetative tissues of tomato. The use of ripening mutants and ethylene treatments has allowed us to propose a model that begins to explain system-1 and -2 ethylene synthesis at the molecular level. This model will be used as a basis to unravel the signaling pathways that cause changes in *ACS* gene expression during ripening.

MATERIALS AND METHODS

Plant Material and Treatments

Tomato (*Lycopersicon esculentum* Mill. cv Ailsa Craig and cv Pearson) plants were grown and maintained under standard greenhouse conditions. Fruit from wild type and mutants from the near-isogenic lines of cv Ailsa Craig homozygous for *rin* and near-isogenic lines of cv Pearson homozygous for *Nr* were picked at distinct stages of ripening or number of DPA. Ethylene measurements were performed as described below to confirm the developmental stage of fruit. Mature green fruit were classified as having well-formed locular gel but with no internal lycopene formation and producing less than 0.2 nL g^{-1} h^{-1} of ethylene. Breaker fruit showed a loss of chlorophyll and the first signs of lycopene accumulation and control fruit produced around 0.5 to 1.0 nL g^{-1} h^{-1} of ethylene. All of the harvested pericarp tissue was frozen immediately in liquid nitrogen and then stored at −70°C until required. Ethylene treatments were performed by incubating fruit in sealed jars of known volume and injecting ethylene to a final concentration of 10 μL L^{-1}.

Experiments using tomato seedlings were performed as follows. Seeds were surface sterilized by soaking in 70% (v/v) ethanol for 10 min followed by 50% (v/v) bleach for a further 10 min. Bleach residues were removed by rinsing several times using sterile distilled water. Seeds were sown under sterile conditions on the surface of 1% (v/v) water agar containing Murashige and Skoog salts at a concentration of 3.8 g L^{-1} and were grown in controlled-environment conditions under a 16-h photoperiod at 23°C followed by an 8-h dark cycle at 16°C. Ten days after sowing, the seedlings were transferred to chambers of known volume and incubated in air or 10 μL L^{-1} ethylene for 12 h. The roots were

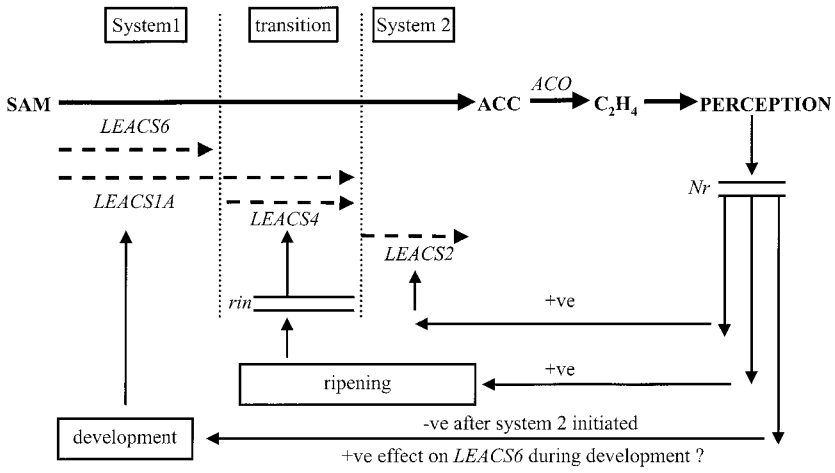

Figure 6. Model proposing the regulation of *ACS* gene expression during the transition from system-1 to system-2 ethylene synthesis in tomato. The symbols −ve (negative) and +ve (positive) refer to the action of ethylene on signaling pathways resulting in repression (−ve) or stimulation (+ve) of *ACS* gene expression.

excised, and the remaining tissue was frozen in liquid nitrogen and stored at −70°C.

Ethylene Measurements

Fruit were harvested and stored in open jars of known volume for 2 h to reduce the effect of wound ethylene caused by picking. The jars were then sealed for 2 h, and a 1-mL gas sample was withdrawn from the jar via a Subaseal. Ethylene concentration in the gas sample was measured by gas chromatography using an ATI UNICAM 610 series gas chromatograph linked to a PC with UNICAM 4880 chromatography data handling software. Column specifications were as follows: 150-mm length, 6-mm outer dimension, 4-mm inside dimension, and alumina F_1 mesh-range 80 to 100 support. Temperatures were as follows: 110°C oven/column, 108°C injector, and 160°C detector.

RNA Isolation and Gel-Blot Analysis

RNA was extracted from 10 g of fruit pericarp tissue from a mixed pool of at least three individual fruit as previously described (Griffiths et al., 1999). Seedling RNA was extracted using the same protocol but from 2 g of frozen material. The RNA was quantified spectrophotometrically and 10 μg was fractionated on 1% (w/v) agarose gels containing 7.5% (v/v) formaldehyde to check for integrity and to compare sample concentration. Northern-blot analysis was performed as described by Griffiths et al. (1999).

ACS Probes and RNase Protection Analysis

Gene-specific probes for *LEACS1A*, *LEACS1B*, *LEACS5*, and *LEACS6* were PCR amplified using primers previously described by Oetiker et al. (1997). The products were cloned into the pCR2.1 vector (Invitrogen, San Diego). *LEACS2*, *LEACS3*, *LEACS4*, and *LEACS7* (Rottmann et al., 1991; Lincoln et al., 1993; Olson et al., 1995; Shiu et al., 1998) gene-specific probes were designed from the 3′ end of each gene based on sequences deposited within databases. Primer pairs were as follows: *LEACS2*, ACS2F 5′-ttaaaagggaagaatttaatt-3′ and ACS2R 5′-taacaatataatcgagaaag-3′ generating a probe from nucleotides 2,702 through 2,957; *LEACS3*, ACS3F 5′-gtcattctccaagtgggttt-3′ and ACS3R 5′-gtagtagtttgaacatttcaag-3′ generating a probe from nucleotides 4,073 through 4,377; *LEACS4*, ACS4F 5′-ggagtcatgaagaacaagcac-3′ and ACS4R 5′-aactatgttgggcccgtgct-3′ generating a probe from nucleotides 2,624 through 2,855; *LEACS7*, ACS7F 5′-gtctagtcatgtgaaagt-3′ and ACS7R2 5′-gcacttgtgcggtcacct-3′ generating a probe from nucleotides 4,066 through 4,335. PCR products were cloned into *Sma*I cut pBluescript II SK$^+$ (Stratagene, La Jolla, CA) or pGEM-T Easy (Promega, Madison, WI). The identity of all of the clones was confirmed by DNA sequence analysis.

Single-strand-specific radiolabeled RNA probes were prepared by in vitro transcription from linearized plasmid template using either T3 or T7 RNA polymerase (Promega) according to the manufacturer's instructions. RNase-protection assays were performed as described by Barry et al. (1996) using 25 μg of total RNA for fruit samples and 50 μg for seedlings. Digestions with RNase ONE (Promega) were performed at 28°C for 3 h using 3 units of enzyme per reaction. Protection assays were performed at least twice to confirm reproducibility.

ACKNOWLEDGMENTS

The authors would like to thank Paul Kasprzak for technical assistance and Dr. Jeremy Roberts for comments on the manuscript.

Received October 29, 1999; accepted April 6, 2000.

LITERATURE CITED

Abeles FB, Morgan PW, Saltveit ME (1992) Ethylene in Plant Biology. Academic Press, San Diego

Barry CS, Blume B, Bouzayen M, Cooper W, Hamilton AJ, Grierson D (1996) Differential expression of the 1-aminocyclopropane-1-carboxylate oxidase gene family of tomato. Plant J **9:** 525–535

Blume B, Barry CS, Hamilton AJ, Bouzayen M, Grierson D (1997) Identification of transposon-like elements in non-coding regions of tomato ACC oxidase genes. Mol Gen Genet **254:** 297–303

Blume B, Grierson D (1997) Expression of ACC oxidase promoter-GUS fusions in tomato and *Nicotiana plumbaginifolia* regulated by developmental and environmental stimuli. Plant J **12:** 731–746

Dong JG, Fernández-Maculet JC, Yang SF (1992) Purification and characterization of 1-aminocyclopropane-1-carboxylate oxidase from apple fruit. Proc Natl Acad Sci USA **89:** 9789–9793

Griffiths A, Barry C, Alpuche-Solis AG, Grierson D (1999) Ethylene and developmental signals regulate expression of lipoxygenase genes during tomato fruit ripening. J Exp Bot **50:** 793–798

Guis M, Bouquin T, Zegzouti H, Ayub R, Ben Amor M, Lasserre E, Botondi R, Raynal J, Latché A, Bouzayen M, Balagué C, Pech, JC (1997) Differential expression of ACC oxidase genes in melon and physiological characterization of fruit expressing an antisense ACC oxidase gene. *In* A Kanellis, C Chang, H Kender, D Grierson, eds, Biology and Biotechnology of the Plant Hormone Ethylene. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 327–338

Hamilton AJ, Lycett GW, Grierson D (1990) Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants. Nature **346:** 284–287

Herner RC, Sink KC (1973) Ethylene production and respiratory behavior of the *rin* tomato mutant. Plant Physiol **52:** 38–42

Holdsworth MJ, Schuch W, Grierson D (1988) Organisation and expression of a wound/ripening-related small multigene family from tomato. Plant Mol Biol **11:** 81–88

Kende H (1993) Ethylene biosynthesis. Annu Rev Plant Physiol Plant Mol Biol **44:** 283–307

Lanahan MB, Yen H-C, Giovannoni JJ, Klee HJ (1994) The *Never ripe* mutation blocks ethylene perception in tomato. Plant Cell **6:** 521–530

Lasserre E, Bouquin T, Hernandez JA, Bull J, Pech JC, Balagué C (1996) Structure and expression of three genes encoding ACC oxidase homologs from melon (*Cucumis melo* L.). Mol Gen Genet **251:** 81–90

Lelièvre JM, Latché A, Jones B, Bouzayen M, Pech JC (1998) Ethylene and fruit ripening. Physiol Plant **101:** 727–739

Lincoln JE, Campbell AD, Oetiker J, Rottmann WH, Oeller PW, Shen NF, Theologis A (1993) LE-ACS4, a fruit ripening and wound-induced 1-aminocyclopropane-1-carboxylate synthase gene of tomato (*Lycopersicon esculentum*). J Biol Chem **268:** 19422–19430

Lincoln JE, Cordes S, Read E, Fischer RL (1987) Regulation of gene expression by ethylene during *Lycopersicon esculentum* (tomato) fruit development. Proc Natl Acad Sci USA **84:** 2793–2797

Lincoln JE, Fischer RL (1988) Regulation of gene expression by ethylene in wild-type and *rin* tomato (*Lycopersicon esculentum*) fruit. Plant Physiol **88:** 370–374

Liu Y, Hoffman NE, Yang SF (1985) Promotion by ethylene of the capability to convert 1-aminocyclopropane-1-carboxylic acid to ethylene in preclimacteric tomato and cantaloupe fruit. Plant Physiol **77:** 407–411

Maunders MJ, Holdsworth MJ, Slater A, Knapp JE, Bird CR, Schuch W, Grierson D (1987) Ethylene stimulates the accumulation of ripening-related mRNAs in tomatoes. Plant Cell Environ **10:** 177–184

Nakatsuka A, Murachi S, Okunishi H, Shiomi S, Nakano R, Kubo Y, Inaba A (1998) Differential expression and internal feedback regulation of 1-aminocyclopropane-1-carboxylate synthase, of 1-aminocyclopropane-1-carboxylateoxidase, and ethylene receptor genes in tomato fruit during development and ripening. Plant Physiol **118:** 1295–1305

Oeller PW, Min-Wong L, Taylor LP, Pike DA, Theologis A (1991) Reversible inhibition of tomato fruit senescence by antisense RNA. Science **254:** 437–439

Oetiker JH, Olson DC, Shiu OY, Yang SF (1997) Differential induction of seven 1-aminocyclopropane-1-carboxylate synthase genes by elicitor in suspension cultures of tomato (*Lycopersicon esculentum*). Plant Mol Biol **34:** 275–286

Olson DC, Oetiker JH, Yang SF (1995) Analysis of *LE-ACS3*, a 1-aminocyclopropane-1-carboxylic acid synthase gene expressed during flooding in the roots of tomato plants. J Biol Chem **270:** 14056–14061

Olson DC, White JA, Edelman L, Harkins RN, Kende H (1991) Differential expression of two genes for 1-aminocyclopropane-1-carboxylic acid synthase in tomato fruits. Proc Natl Acad Sci USA **88:** 5340–5344

Peck SC, Kende H (1998) Differential regulation of genes encoding 1-aminocyclopropane-1-carboxylate (ACC) synthase in etiolated pea seedlings: effects of indole-3-acetic acid, wounding and ethylene. Plant Mol Biol **38:** 977–982

Picton S, Barton SL, Bouzayen M, Hamilton AJ, Grierson D (1993) Altered fruit ripening and leaf senescence in tomatoes expressing an antisense ethylene-forming enzyme transgene. Plant J **3:** 469–481

Rottmann WH, Peter GF, Oeller PW, Keller JA, Shen NF, Nagy BP, Taylor LP, Campbell AD, Theologis A (1991) 1-Aminocyclopropane-1-carboxylate synthase in tomato is encoded by a multigene family whose transcription is induced during fruit and floral senescence. J Mol Biol **222:** 937–961

Shiu OI, Oetiker JH, Yip WK, Yang SF (1998) The promoter of *LE-ACS7*, an early flooding-induced 1-aminocyclopropane-1-carboxylate synthase gene of the tomato, is tagged by a *Sol3* transposon. Proc Natl Acad Sci USA **95:** 10334–10339

Tigchelaar EC, McGlasson WB, Buescher RW (1978) Genetic regulation of tomato fruit ripening. Hortscience **13:** 508–513

Wilkinson JQ, Lanahan MB, Clark DG, Bleecker AB, Chang C, Meyerowitz EM, Klee HJ (1997) A dominant mutant receptor from *Arabidopsis* confers ethylene insensitivity in heterologous plants. Nat Biotech **15:** 444–447

Wilkinson JQ, Lanahan MB, Yen H-C, Giovannoni JJ, Klee HJ (1995) An ethylene-inducible component of signal transduction encoded by Never-ripe. Science **270:** 1807–1809

Yang SF, Hoffman NE (1984) Ethylene biosynthesis and its regulation in higher plants. Annu Rev Plant Physiol **35:** 155–189

Yip WK, Moore T, Yang SF (1992) Differential accumulation of transcripts for four tomato 1-aminocyclopropane-1-carboxylate synthase homologs under various conditions. Proc Natl Acad Sci USA **89:** 2475–2479

Yoon IS, Mori H, Kim JH, Kang BG, Imaseki H (1997) *VR-ACS6* is an auxin-inducible 1-aminocyclopropane-1-carboxylate synthase gene in mung bean (*Vigna radiata*). Plant Cell Physiol **38:** 217–224

Zarembinski T, Theologis A (1994) Ethylene biosynthesis and action: a case of conservation. Plant Mol Biol **26:** 1579–1597