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Ripening Physiology of Fruit from Transgenic Tomato (*Lycopersicon esculentum*) Plants with Reduced Ethylene Synthesis

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The physiological effects of reduced ethylene synthesis in a transgenic tomato (*Lycopersicon esculentum*) line expressing 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase enzyme have been examined. Fruit from the transgenic line 5673 ripen significantly slower than control fruit when removed from the vine early in ripening. In contrast, fruit that remain attached to the plants ripen much more rapidly, exhibiting little delay relative to the control. Ethylene determinations on attached fruit revealed that there was significantly more internal ethylene in attached than detached fruit. The higher ethylene content can fully account for the observed faster on-the-vine ripening. All of the data are consistent with a catalytic role for ethylene in promoting many, although not all, aspects of fruit ripening. Biochemical analyses of transgenic fruit indicated no significant differences from controls in the levels of ACC oxidase or polygalacturonase. Because transgenic fruit are significantly firmer than controls, this last result indicates that other enzymes may have a significant role in fruit softening.

Tomato (*Lycopersicon esculentum*) fruit ripening is a highly regulated developmental process requiring expression of a large number of gene products (Grierson and Kader, 1986). Enzymes involved in the degradation of cell walls, complex carbohydrates, Chl, and other macromolecules must be coordinately expressed with enzymes that make the fruit desirable nutritionally and aesthetically. Although mutants blocked in aspects of tomato fruit ripening are available (Tigchelaar et al., 1978) and numerous ripening-related genes have been described (Slater et al., 1985; Lincoln and Fischer, 1988), many aspects of ripening are not fully understood.

It is now well established that ethylene functions to promote many aspects of ripening in climacteric fruits. Ethylene is synthesized from *S*-adenosylmethionine by way of ACC (McKeon and Yang, 1987). Synthesis of ethylene has been greatly reduced in transgenic tomato plants by expression of antisense gene constructs of ACC oxidase (Hamilton et al., 1990) or ACC synthase (Oeller et al., 1991) or by expression of ACC deaminase (Klee et al., 1991). In all of these cases, significant delays in ripening have resulted.

The availability of transgenic tomato plants that synthesize reproducibly reduced levels of ethylene permits a critical assessment of the role of ethylene in ripening. The evidence to date with transgenic plants indicates that there is a quantitative correlation between ethylene synthesis and the rate of fruit ripening; as ethylene synthesis is lowered, ripening is progressively delayed. There is also evidence that many aspects of ripening are ethylene independent. For example, PG mRNA accumulates to a high level even when ethylene synthesis has been reduced greater than 100-fold (Oeller et al., 1991). A significant problem with all of the results obtained to date, however, is that a low level of ethylene synthesis persists and as little as 0.1 parts per million of ethylene can lead to a biological response (Reid, 1987). One particularly interesting and unexplained observation from transgenic plants with reduced ethylene concerns ripening behavior of detached versus attached fruit. Transgenic fruit attached to the vine ripen at a significantly faster rate than fruit detached at the MG or breaker stage (Hamilton et al., 1990; Klee et al., 1991). These observations suggest that the plant could synthesize factors that are translocated to fruit and are promotive to ripening. The work described here addresses the role of ethylene in several aspects of ripening, including the different behavior of attached and detached ripening fruit.

MATERIALS AND METHODS

Plant Materials

The ACC deaminase-containing transgenic tomato (*Lycopersicon esculentum*) line 5673 has been described previously (Klee et al., 1991). The isogenic UC82B variety was used as a control. Plants were grown in a greenhouse supplemented with 16 h of supplemental artificial lighting. Plants were fertilized with Peters fertilizer (20–19–18 alternated with 15–16–17) once a week.

Ethylene Measurements

Ethylene was measured by enclosing fruit in sealed containers and withdrawing 2.0-mL gas samples within 1 h. Ethylene was quantified on a gas chromatograph equipped with an alumina column and flame ionization detector as described by Ward et al. (1978). For measurement of ethylene release versus time, 13 fruit between ripening stages 3 and 5 were picked and immediately placed in sealed jars. A gas

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Abbreviations: "a, b," chromaticity coordinates on green-red and yellow-blue axes, respectively; "L," lightness variable; MG, mature green; PG, polygalacturonase.

sample was removed at 15 min, the jar was flushed with air and immediately resealed, and the gas was sampled again at 30 min.

Fruit Color Determinations

Color development of fruit was measured with a Minolta CR-300 Chroma Meter. Measurements were made using the L*a*b color system. The chromameter measures color and intensity of reflected light. "L" is the lightness variable; "a" and "b" are chromaticity coordinates on a green-red and yellow-blue axis, respectively. Fruits were harvested at all stages of ripening from MG through pink. The fruits were grouped into maturity stages, and the "a" value was averaged within each group. The "a" value was observed to most closely follow the progression of ripening. MG fruit typically have an "a" value of −12 and proceed to a value of 30 or greater when fully red. A minimum of six readings around the diameter of each fruit were averaged. Fruit was evaluated for stage of ripening using U.S. Department of Agriculture standards for ripening. Stage 1 is defined as MG with no external color change. Stage 2 is breaker and indicates a definite color change covering not more than 10% of the surface of the fruit. Stage 3 is defined as turning and indicates that 10 to 30% of the fruit surface has changed color. Stage 4 is defined as pink and indicates that 30 to 60% of the fruit surface area is pink or red.

ACC Oxidase Determinations

Antibody against the ACC oxidase (pTOM13) protein was prepared essentially as previously described by Klee et al. (1991). A full-length cDNA clone homologous to pTOM13 (Slater et al., 1985) was generated by polymerase chain reaction of reverse-transcribed RNA from ripening tomato fruit. This cDNA was cloned into pBSSK+ (Stratagene) in an orientation that would allow expression from the bacteriophage T7 promoter. This plasmid, pMON11043, was then introduced into *Escherichia coli* containing pGP1–4 (Tabor and Richardson, 1985). The gene was then induced as described by Tabor and Richardson (1985), and the protein was purified for antibody production in a goat. Fruit samples were harvested at the indicated stages and immediately processed. Each sample consisted of between 7 and 11 pooled fruit. Protein gel blotting was then performed as previously described (Klee et al., 1991).

PG Measurements

PG enzyme activity was measured as described by Sheehy et al. (1988). Fruit were harvested at stages 2 to 3 and allowed to ripen at room temperature. The day that fruits were scored as fully red on the L*a*b scale, samples were frozen. Each assay included pooled samples from at least eight independent fruit.

Fruit Firmness Measurements

Fruit firmness was measured with an Instron model 3110 pressure-testing device. The firmness was expressed as kg of force ± SE required to compress the fruit by 5 mm. Fruit were sampled either on the day that they were scored as reaching full red or harvested at full red and stored at 20°C for 2 weeks before being assayed. Statistical analyses were performed using Student's *t* test.

RESULTS

We have previously described the isolation of transgenic tomato plants expressing ACC deaminase (Klee et al., 1991). Fruit from the best line, 5673, when picked at MG (stage 1) never developed a full-red color. This transgenic line synthesizes only 10% of the ethylene produced by UC82B control fruit when grown in a greenhouse or growth chamber. In all of the transgenic lines, fruits were observed to ripen faster when they were attached to the plant than when they were detached at either the MG or breaker stage. To understand more fully the relationship between ethylene inhibition and ripening, the ripening process in 5673 and control UC82B fruit was further characterized.

Maturation of Detached Fruit

The progression of ripening of detached fruit was monitored daily by following color development with a color analyzer using an L*a*b scale. Figure 1A shows the progression of ripening in wild-type UC82B fruit picked at various stages of maturity. UC82B fruit picked at all stages from MG onward eventually reached a full-red stage, with fruit picked at MG taking approximately 5 d. In contrast, as shown in Figure 1B, many transgenic fruit never developed full-red color. There was a direct correlation between the stage at which fruit were harvested and the amount of subsequent color development. Fruit harvested at MG or breaker never developed full color. Fruit picked early in the turning stage did eventually achieve red color but took 16 d, whereas fruit harvested late in the turning stage took 5 d to reach full red.

Because color development was clearly slowed in transgenic fruit, other aspects of the ripening process were examined for delays. We were particularly interested in expression of PG, an enzyme that has been proposed to have a major role in fruit softening. Enzyme assays on transgenic and control fruit revealed similar levels of activity in both sets. In one experiment, the UC82B fruit contained 600 ± 66 nM min^{-1} g^{-1} ($n = 4$) and the 5673 fruit contained 672 ± 42 nM min^{-1} g^{-1} ($n = 3$) of PG activity (Table I). In a second independent experiment, the control fruit contained 620 nM min^{-1} g^{-1} fresh weight and the 5673 fruit contained 657 nM min^{-1} g^{-1} of PG activity. Thus, transgenic fruit contained essentially the same levels of PG enzyme as control fruit. This result is consistent with the results of Oeller et al. (1991), who reported accumulation of PG mRNA in transgenic fruit expressing an antisense ACC synthase gene.

Maturation of Attached Fruit

We have previously observed little difference between the time that transgenic and control fruit take to achieve a red color when they are attached to the vine (Klee et al., 1991). As described above, this is in contrast to the behavior of

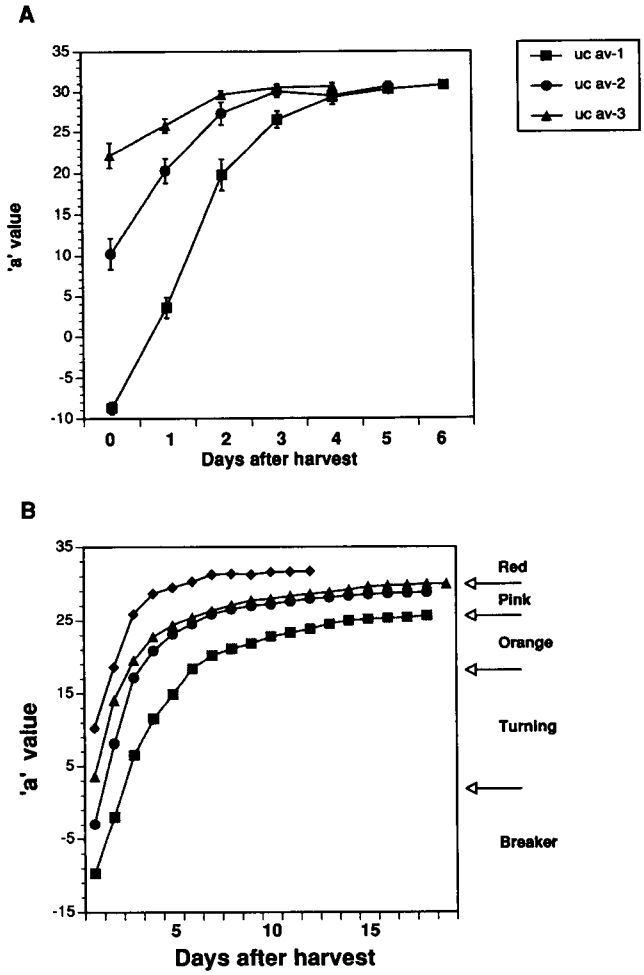

Figure 1. Development of color in fruit picked at various stages of ripening. Fruit from UC82B (A) or 5673 (B) transgenic plants were harvested, staged, and pooled according to color value and allowed to ripen at 25°C. The progression of red color development ("a" value) for each fruit was monitored daily with a color analyzer. Fruit was initially grouped into the following categories: early breaker, late breaker, early turning, late turning, and orange. Each point consisted of between five and eight fruit.

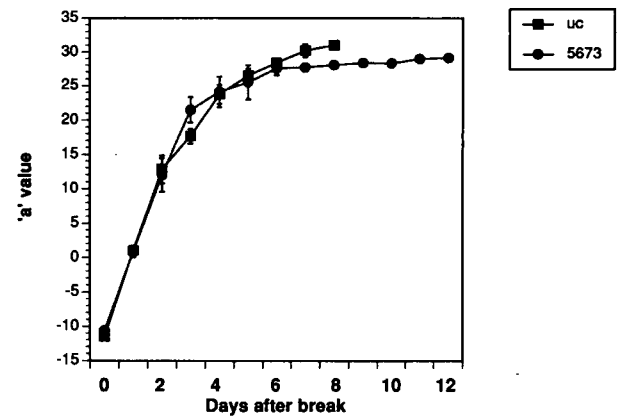

Figure 2. Development of color in attached UC82B (uc) and 5673 fruit. Individual fruit were tagged at the breaker stage and monitored daily for color development with a color analyzer.

detached fruit, i.e. 5673 fruit picked early in ripening never achieve a full-red color. To quantitate this phenomenon, the rate of color development was monitored on individual attached fruit. The results are summarized in Figure 2. Although there is little observed difference in the rate of ripening during the first 6 d when the fruit ripen from a value of −12 to approximately 28, color development in the transgenic fruit slows down from this point on. Although the fruit are visibly red, they are not as fully red as the UC82B controls as measured with the L*a*b scale.

The slowdown in color development is indicative of a more general halt in the overall ripening process. Following full color development ("a" value >31), fruit were harvested and stored at 21°C. Typically, the UC82B fruit started to show signs of overripening within 14 d of picking. The term overripening is used here to indicate a loss of marketable quality, principally consisting of softening and shriveling, indicative of water loss. Compression measurements indicated that there was no statistical difference in firmness of fruit on the day that they were scored as fully ripe, but 14 d after harvest, transgenic fruit were significantly firmer than control fruit (Table II). The transgenic fruit usually showed no signs of overripening for at least 6 weeks. Figure 3 shows a comparison of control and transgenic fruit 50 d following harvest at the full-red stage.

Table I. *PG activity in transgenic (5673) and control (UC82B) red fruit*

Two independent sets of fruit were extracted and assayed for PG enzyme activity. In experiment 1, each sample was independently assayed the indicated number of times. Units of PG activity are expressed as nM min^{-1} g^{-1} fresh weight of reducing sugar as described in "Materials and Methods."

Experiment	Line	Units PG	Replicates
1	UC82B	600 + 66	4
	5673	672 ± 42	3
2	UC82B	620	
	5673	657	

Table II. *Comparison of firmness between nontransgenic (UC82B) and transgenic (5673) fruit*

Firmness was determined by crushing fruit with an Instron model 3110 pressure tester either on the day fruit reached full-red color or 2 weeks after full color. The numbers of fruit for each treatment are indicated in parentheses. Values are given as mean kg of force ± SE required to compress fruit a distance of 5 mm. The red fruit were not significantly different. Red plus 14 d fruit were significantly different (P = 0.017).

Stage	UC82B	5673
Red	25.4 ± 1.1 (7)	23.7 ± 1.4 (8)
Red + 14 d	15.5 ± 1.5 (10)	19.8 ± 1.0 (12)

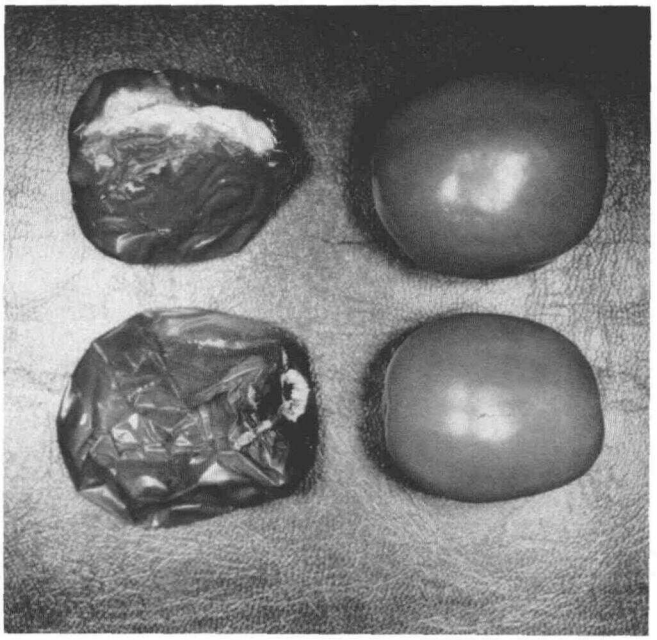

Figure 3. Transgenic and control fruit picked at the full-red stage and stored at 21°C for 45 d. The control UC82B fruit are on the left and transgenic fruit are on the right.

The level of another ripening-associated enzyme, ACC oxidase, was also examined during ripening of the attached fruit. Fruit were detached at various stages of ripening, and soluble proteins were immediately extracted. Proteins were separated on polyacrylamide gels, transferred to nitrocellulose membranes, and detected with an antibody prepared against the pTOM13 protein. The results of this analysis are shown in Figure 4. The ACC oxidase enzyme can be detected in immature green fruit in the transgenic line. Fruit at this stage exhibited no softening of the locular tissue and were at least 5 d before the respiratory climacteric. The enzyme is also present in MG fruit and increases through the breaker and turning stages before decreasing in transgenic pink fruit. The higher level of ACC oxidase enzyme in control pink fruit has been observed in two separate experiments, but its significance remains to be determined. Despite the 90% reduction in ethylene in transgenic fruit, there is no apparent difference in the level of ACC oxidase relative to similar-staged control fruit with the exception of the pink stage.

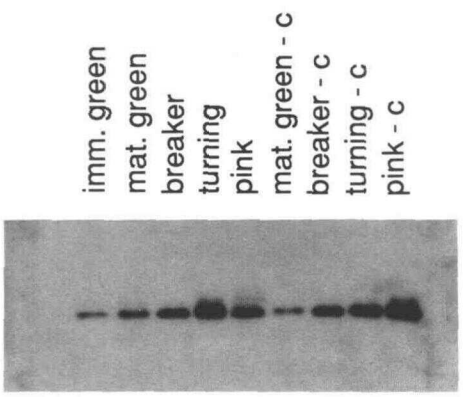

Figure 4. Expression of ACC oxidase in transgenic and control fruit at different stages of ripening. Fruit were harvested at the indicated stages of ripening and proteins were extracted as described in "Materials and Methods." ACC oxidase was detected with an antibody prepared against tomato ACC oxidase. C, Control.

Ethylene Production by Attached Tomatoes

Transgenic tomatoes ripened significantly faster and to a greater extent when left attached to the vine than when removed. One explanation for the more rapid ripening of attached tomatoes is that the fruit have a higher level of ethylene than detached fruit. To examine this possibility, the ethylene content of attached tomatoes was determined. Fruit from all stages of ripening were picked and immediately placed in sealed containers for ethylene determinations. The results are shown in Figure 5. Our previous work indicated that detached fruit show a defined peak of ethylene production at approximately the transition from breaker to turning and that ethylene production rapidly decreases through the rest of ripening (Klee et al., 1991).

In contrast, attached fruit continue to have very high levels of ethylene through the red ripe stage. The transgenic fruit, although containing levels of ethylene reduced by 85% relative to controls, also have higher levels of ethylene than when they are detached. The higher ethylene content of attached fruit could be due to either a higher rate of synthesis or the presence of a diffusion barrier. It has been previously demonstrated that tomato skin provides an effective barrier to ethylene, being approximately 1000-fold less permeable to gas exchange than the stem scar (Cameron and Yang, 1982). To distinguish between these two possibilities, fruit were harvested and immediately placed in sealed jars. Ethylene release was measured during 0 to 15 min and compared with release during the period from 15 to 30 min. Because the half-life for ethylene diffusion is 15 min (Cameron and Yang,

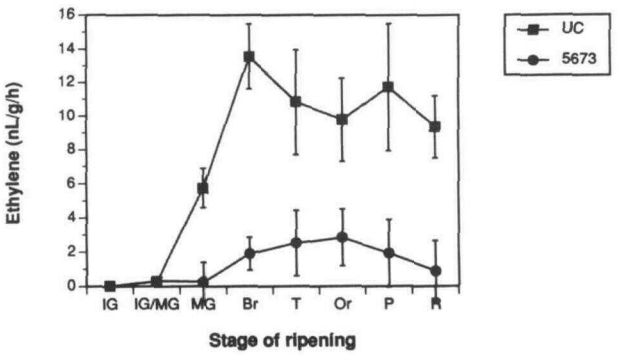

Figure 5. Ethylene production by attached transgenic and control fruit during ripening. Fruit was harvested, immediately placed in sealed containers, and assayed for ethylene as described in "Materials and Methods." At least 50 fruit for each genotype were measured. Each value is expressed as nL g^{-1} fresh weight h^{-1}, mean ± SE, and represents the average of at least five fruit at the indicated stage of ripening. IG, Immature green; MG, mature green; Br, breaker; T, turning; Or, orange; P, pink; R, red.

1982), the amount of ethylene released during the 15- to 30-min period would be expected to be significantly lower than that released from 0 to 15 min if the skin is providing a diffusion barrier that leads to a higher internal concentration of ethylene. The result of this experiment is shown in Table III. In every sample, the ethylene content decreased significantly in the second sampling with an average reduction of 32 ± 12%. It can be concluded that attached fruit have significantly higher ethylene content than detached fruit from turning to the red ripe stage due to the presence of an effective diffusion barrier. Therefore, the likely explanation for the more rapid ripening of attached transgenic fruit is a higher effective ethylene concentration in the fruit.

DISCUSSION

Independent observations indicate that transgenic tomatoes producing reduced levels of ethylene ripen more rapidly when attached to the vine than when they are detached. This difference has not been observed with wild-type tomatoes. These results would indicate that ethylene is normally present in excess of what is needed for achieving a maximal ripening rate. When ethylene production is lowered, it becomes rate limiting. Under these circumstances, the influences of other factors on ripening physiology can be more critically examined.

In the experiments described here, we examined the role of ethylene in determining the rate of tomato fruit ripening; higher ethylene content results in faster ripening. We have generated many transgenic tomato lines with ACC deaminase, and there is a direct correlation between the degree of ethylene inhibition and the rate of ripening (H. Klee and K. Kretzmer, unpublished data). The concept of ethylene as a catalyst of ripening is illustrated by the experiment in which fruit are detached at different stages of ripening. When fruit are removed from the vine, the internal ethylene content decreases rapidly. As a consequence, fruit are exposed to less total ethylene during the course of ripening, and transgenic fruit ripen less rapidly. With nontransgenic fruit, there is sufficient ethylene that it does not become rate limiting. Ethylene can be considered as a catalyst for progression through ripening. Transgenic fruit have sufficient ethylene to proceed rapidly through development for 2 to 3 d following removal from the vine before slowing down. Transgenic fruit that are picked at the turning stage will thus proceed to the ripe stage, whereas fruit picked at the breaker stage never achieve the red ripe stage. Nontransgenic picked fruit, although their ripening is slowed relative to attached fruit, still produce sufficient ethylene to ripen fully.

Table III. *Release of ethylene following picking*

Ethylene release was measured from individual fruit during the 0- to 15- and 15- to 30-min period. Thirteen fruit were harvested at stages 3 to 5 for analysis. Every fruit showed a decrease in ethylene, with a range between 17 and 54%.

Average Ethylene Release		Average Decrease
0–15 min	15–30 min	
nL g^{-1} fresh wt		%
2.5 ± 1.2	1.7 ± 0.8	32.4 ± 12.3

A faster rate of ripening of attached fruit has been observed with plants transformed with ACC deaminase (Klee et al., 1991), antisense ACC synthase (H. Klee, unpublished data), and antisense ACC oxidase (Hamilton et al., 1990). The observation that fruit attached to the vine contain more ethylene than detached fruit can explain the observation that transgenic fruit ripen more quickly. The rapid decrease in ethylene content following picking indicates that the higher ethylene concentration is due to the existence of a diffusion barrier leading to accumulation of ethylene rather than a higher rate of synthesis. Measurements of ethylene diffusion out of tomato fruit have shown that the skin provides an effective barrier. In detached fruit, up to 97% of ethylene diffusion occurs through the stem scar with a half-life of only 15 min (Cameron and Yang, 1982). Because there is no stem scar in attached fruit, ethylene accumulates to higher internal levels without the necessity for higher synthesis.

It is interesting to note that there are no differences in softness between transgenic and control fruit at the point that they achieve full-red color. However, there is a major difference in degradation of fruit that occurs following ripening that is apparently dependent on ethylene content. Even transgenic fruit that are picked after achieving full-red color do not undergo the biochemical processes that lead to disintegration of the fruit to the same extent as control fruit. This result suggests that overripening is mediated by the high internal content of ethylene in fruit during the later stages of ripening. Because the attached 5673 fruit contain only slightly more ethylene than control fruit at the immature green stage, overripening does not proceed to nearly the same degree in transgenic fruit as in control fruit. The lack of overripening should cause the transgenic tomatoes to have a greatly enhanced shelf life.

The results presented here, taken together with the knowledge of mutants defective in ripening, suggest the complexity of the entire ripening process. It has been previously suggested that tomato fruit ripening is not a single process triggered by ethylene (Tigchelaar et al., 1978). Many aspects of ripening proceed normally, even in tomatoes with greatly reduced ethylene or in the ethylene-insensitive Never-Ripe mutant (M. Lanahan and H. Klee, unpublished data). As has been suggested by Tigchelaar et al. (1978), the earliest events may be blocked in the *rin* and *nor* mutants. Experiments with transgenic plants synthesizing greatly reduced ethylene levels begin to address the early events that are either ethylene independent or require very low levels of ethylene at a molecular level. For example, induction of ACC oxidase appears to precede the respiratory climacteric and would be necessary for rapid and quantitative conversion of ACC to ethylene. PG also appears to be unaffected by ethylene concentration.

It is impossible at this time to rule out a crucial role for ethylene in the induction of ACC oxidase or PG. But any such linkage would have to be qualitative and not quantitative in nature, because fruit that are reduced in ethylene content by 90% have similar levels of these enzymes relative to controls. After fruit become competent to respond to ethylene, many aspects of ripening are clearly modulated by

ethylene. Our results further suggest that overripening may represent a biochemically distinct phase of fruit development. This last stage is also mediated by ethylene but can be distinguished from ripening in the transgenic plants. Finally, the lack of difference in PG in the transgenic fruit indicates that another unidentified activity must be critical for the softening that ultimately leads to spoilage.

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