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An Antisense Gene Stimulates Ethylene Hormone Production during Tomato Fruit Ripening

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The ripening of many fruits is controlled by an increase in ethylene hormone concentration. E8 is a fruit ripening protein that is related to the enzyme that catalyzes the last step in the ethylene biosynthesis pathway, 1-aminocyclopropane-1-carboxylic (ACC) oxidase. To determine the function of E8, we have transformed tomato plants with an E8 antisense gene. We show here that the antisense gene inhibits the accumulation of E8 protein during ripening. Whereas others have shown that reduction of ACC oxidase results in reduced levels of ethylene biosynthesis, we find that reduction of the related E8 protein produces the opposite effect, an increase in ethylene evolution specifically during the ripening of detached fruit. Thus, E8 has a negative effect on ethylene production in fruit.

INTRODUCTION

The hormone ethylene influences many aspects of plant development (Mattoo and Suttle, 1991). It plays an important role in shoot and root growth, leaf abscission, fruit ripening, and flower senescence. Moreover, ethylene is involved in response of plants to wounding, pathogen attack, and environmental stress. Ethylene production is influenced by plant hormones, plant metabolites, and a wide variety of environmental stresses.

In fruits such as tomato, the onset of ripening is controlled by an increase in ethylene production (Yang and Hoffman, 1984). As shown in Figure 1, the pathway for ethylene biosynthesis has been elucidated, with the first committed step being the conversion of *S*-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid (ACC) by the ACC synthase enzyme (Adams and Yang, 1979). At the onset of tomato fruit ripening, expression of multiple ACC synthase genes is activated, resulting in increased production of ACC (Van der Straeten et al., 1990; Olson et al., 1991; Rottmann et al., 1991; Yip et al., 1992). ACC is then oxidized to ethylene by ACC oxidase, also called the ethylene-forming enzyme (Liu et al., 1985a). Inhibition of ethylene biosynthesis by production of antisense RNA to ACC synthase (Oeller et al., 1991) and ACC oxidase (Hamilton et al., 1990), or by deamination of ACC (Klee et al., 1991) represses tomato fruit ripening. Thus, ethylene plays an essential role in tomato fruit ripening.

Ethylene regulates its own biosynthesis (Yang and Hoffman, 1984). In ripening the feedback is positive, whereas in wound-induced ethylene production the feedback is negative. In most cases, it is thought that ACC synthase activity determines the rate of ethylene biosynthesis, although there are competing reactions that may serve to reduce ethylene production (Figure 1). In certain situations during plant development, ACC is removed by malonylation (Liu et al., 1985b) and ethylene is oxidized to a variety of products (Hall, 1991).

Fruit ripening involves an array of biochemical changes, including increased respiration, chlorophyll degradation, carotenoid biosynthesis, production of essential oils and flavor components, increased activity of cell wall–degrading enzymes, and a transitory increase in ethylene production (Rhodes, 1980). To begin to understand the mechanism of ethylene action during ripening, we have cloned and analyzed a variety of ethylene-responsive genes (Lincoln et al., 1987). One gene, designated E8, is transcriptionally activated at the onset of ripening, coincident with the increase in ethylene biosynthesis (Lincoln and Fischer, 1988). The E8 predicted polypeptide is a member of a family of dioxygenases found in plants and microorganisms (McGarvey et al., 1992) and is related to ACC oxidase, sharing 34% amino acid sequence identity over 295 residues (Deikman and Fischer, 1988). We have inhibited E8 gene expression by producing E8 antisense RNA in transgenic tomato plants. We find that a reduced level of E8 protein results in overproduction of ethylene during the ripening of detached tomato fruit.

RESULTS

E8 Antisense Gene Inhibits E8 Protein Accumulation

As shown in Figure 2, the E8 antisense gene consisted of a full-length E8 cDNA clone ligated in reverse orientation to a cauliflower mosaic virus 35S promoter. Ten primary

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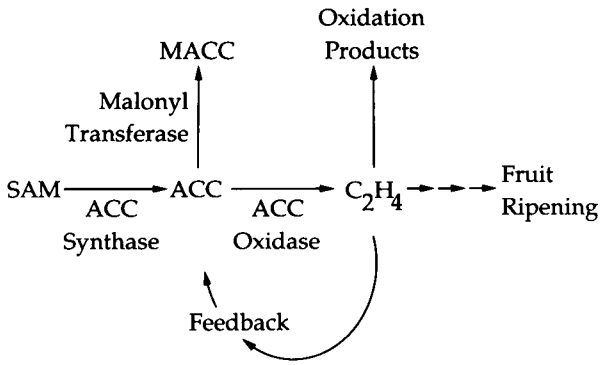

Figure 1. Pathway for Ethylene Synthesis and Metabolism.

SAM, *S*-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylic acid; MACC, malonyl-ACC; Feedback, positive and negative effect of ethylene on its own biosynthesis. The three short arrows represent the activation of ripening by ethylene.

transformed plants, designated T_1, were obtained, and two independently transformed plants, designated line 125 and line 191, showed greatly reduced levels of E8 mRNA in fruit (data not shown). The effect of the E8 antisense gene was specific and did not reduce the concentration of the related (53% nucleotide sequence identity) ACC oxidase mRNA (data not shown). All experiments reported here were carried out with detached fruit from T_2 progeny of line 125 and 191 primary transgenic plants.

To determine the effect of the E8 antisense gene on E8 protein production, tomato fruit pericarp proteins were isolated and analyzed by immunoblotting. As shown in Figure 3, in fruit from untransformed plants the E8 protein is produced specifically during ripening. That is, E8 protein was not observed in immature fruit, was detected at the onset of color change coincident with the increase in ethylene biosynthesis (day 0), and accumulated to significant levels in ripening fruit. This result is consistent with previous experiments showing that E8 gene transcription is activated at the onset of ripening (Lincoln and Fischer, 1988). By contrast, E8 protein was not detected in fruit of equivalent age from transgenic lines 125 or 191. Analysis of the intensity of the immunoblot reaction to dilutions of E8 protein indicated that the level of E8 protein in fruit from transgenic plants (day 3) was less than 1% of fruit from untransformed plants (day 3).

Ethylene Overproduction in Fruit from Transgenic Plants

To determine if E8, like the related ACC oxidase, plays a role in ethylene production, we measured the rate of ethylene evolution in fruit from transgenic plants. As shown in Figure 4A, at the onset of ripening (day 0), fruit from transgenic (line 125) and untransformed plants evolved equivalent rates of ethylene. However, 2 days later, the rate of ethylene evolution was approximately sixfold higher in fruit from transgenic plants compared with untransformed controls. The effect of the antisense gene was transitory, and by the fifth day the rate of ethylene evolution was essentially the same in fruit from transgenic and untransformed plants. A similar pattern, although less extreme, was observed in progeny from an independently transformed plant (line 191; Figure 4B). That is, 2 days after the color change, the rate of ethylene evolution in line 191 fruit was approximately twofold greater than untransformed controls. These results suggest that inhibition of E8 protein accumulation in detached ripening fruit results in ethylene overproduction.

Cosegregation of Ethylene Overproduction and Suppression of E8 Protein Accumulation

DNA gel blot analysis indicated that a cluster of approximately four E8 antisense genes segregated as a single locus in the T_2 population obtained by selfing a hemizygous primary transformant (line 125; data not shown). To investigate further whether the E8 antisense gene was responsible for ethylene overproduction, we analyzed 14 T_2 progeny that had not inherited the E8 antisense locus. As shown in Figure 5, fruit from these progeny produced normal levels of ethylene and E8 protein. These results are consistent with the hypothesis that the E8 antisense gene is responsible for suppression of E8 protein accumulation and overproduction of ethylene.

Analysis of progeny that inherited the E8 antisense locus revealed that not all continued to suppress E8 protein accumulation during fruit ripening. Among 10 progeny that inherited the E8 antisense locus, fruit from six progeny (125-08, 125-09,

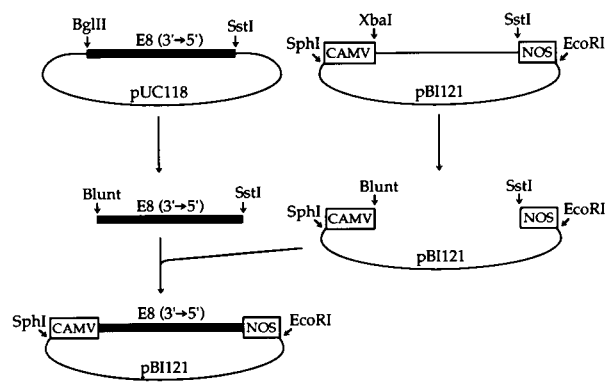

Figure 2. Construction of an E8 Antisense Gene.

DNA fragments were generated and joined by the indicated restriction endonuclease and ligase reactions, respectively. pBI121, plasmid vector (Jefferson et al., 1987) containing the cauliflower mosaic virus 35S promoter (CAMV) and the nopaline transcription termination site (NOS); pUC118, plasmid vector (Vieira and Messing, 1987); E8 (3'→5'), full-length E8 cDNA clone (Deikman and Fischer, 1988) in the reverse orientation relative to the transcription initiation site in the cauliflower mosaic virus promoter; Blunt, addition of nucleotides to fill in the single-stranded end of a restriction endonuclease site.

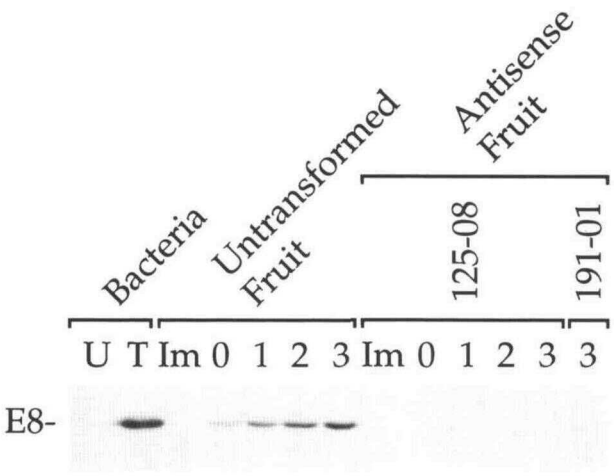

Figure 3. E8 Protein Accumulation in Fruit from Transgenic and Untransformed Plants.

For immature fruit, protein was extracted from green, half-size fruit. For all other fruit, protein was extracted from fruit harvested at the onset of color change and stored the indicated number of days. The time from anthesis to the onset of color development was the same in transformed and untransformed plants, 41 ± 2 days. Fifty micrograms of protein from fruit or 5 μg of protein from bacteria was subjected to electrophoresis, blotted, and reacted with anti-E8 antibodies. 191-01, fruit from progeny number 01 from line 191; 125-08, fruit from progeny 08 from line 125; U, untransformed bacteria; T, bacteria transformed with E8 cDNA; Im, immature fruit. Molecular mass of the E8 protein is 42 kD.

125-19, 125-21, 125-25, and 125-22) had no detectable E8 protein, whereas fruit from four "revertant" progeny (125-26, 125-28, 125-29, and 125-23) accumulated E8 protein (Figure 5B). Based on the intensity of the immunoblot reaction, the level of E8 protein in the four revertant progeny was approximately 10% (125-23 and 125-26) to 25% (125-28 and 125-29) of that found in fruit that had not inherited the E8 antisense locus. Analysis of ethylene evolution rates revealed that the six progeny that did not accumulate detectable levels of E8 protein overproduced ethylene, whereas the four revertant progeny produced normal levels of ethylene (Figure 5A). Taken together, these experiments demonstrate that ethylene overproduction correlates strictly with reduction of E8 protein below a threshold level of 10%. This correlation and the fact that ethylene overproduction was observed in two independently transformed lines, 125 and 191 (Figure 4B), make it highly unlikely that ethylene overproduction is the result of somaclonal variation.

Ethylene Overproduction Occurs Specifically during Fruit Ripening

Many plant tissues, including fruit, that are exposed to abiotic or biotic stress rapidly produce high levels of ethylene (Hyodo, 1991). To begin to distinguish between fruit-specific and stress-induced ethylene overproduction, fruit were harvested well before the onset of ripening and the ethylene evolution rate was analyzed daily. As shown in Figure 6, the antisense gene had no effect on ethylene production in unripe fruit. That is, the ethylene evolution rate for fruit from both transgenic and untransformed plants was the same, 0.1 nL/(g-hr), for 4 days before the onset of ripening. However, after the onset of ripening, ethylene production in fruit from transgenic plants was approximately sixfold higher than untransformed controls. As with fruit harvested at the onset of ripening (Figure 4A), ethylene overproduction in fruit from transgenic plants was transitory, lasting approximately 2 to 3 days. These results suggest that the effect of the E8 antisense locus on ethylene biosynthesis is specific to fruit ripening and does not represent a generalized response to stress associated with harvest and/or storage.

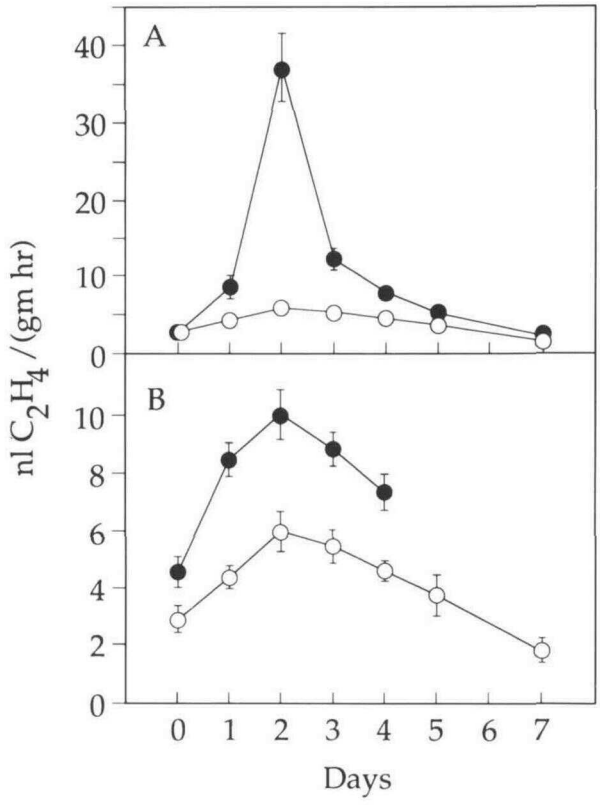

Figure 4. Ethylene Evolution Rate of Fruit from Independently Transformed Lines.

Fruit were harvested at the onset of color change, and the rate of ethylene evolution was measured daily.

(A) Line 125. ●, ethylene evolution rate of fruit from 125-08, 125-09, and 125-19 transgenic plants (n = 77); ○, ethylene evolution rate of fruit from untransformed plants (n = 15).

(B) Line 191. ●, ethylene evolution rate of fruit from 191-01, 191-02, and 191-03 transgenic plants (n = 32); ○, ethylene evolution rate of fruit from untransformed plants (n = 15).

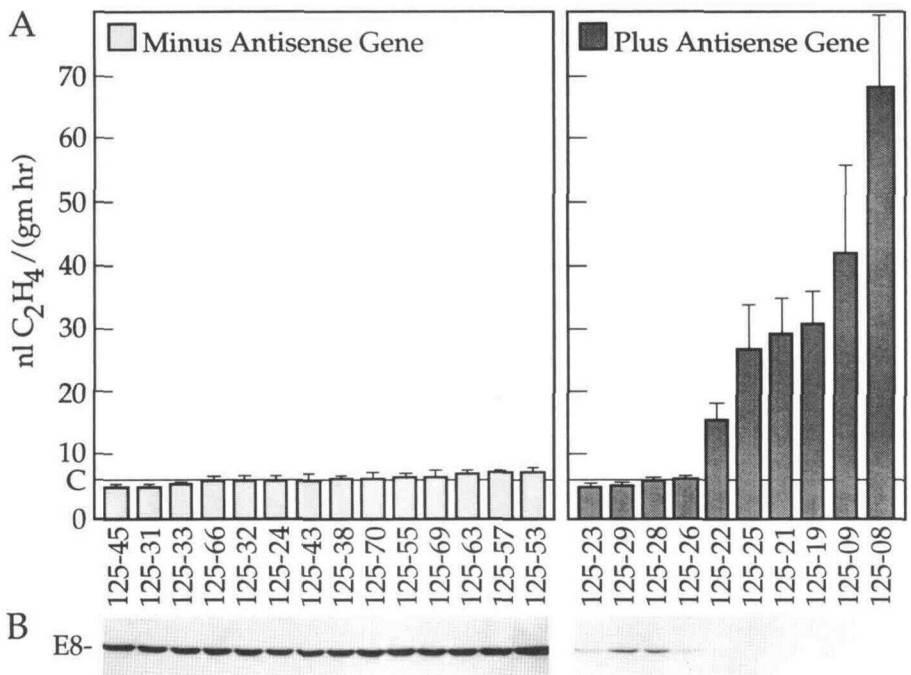

Figure 5. Segregation of the E8 Antisense Locus, E8 Protein Accumulation, and Ethylene Evolution Rate.

Data are presented for T_2 progeny of line 125 (e.g., 125-19 is progeny number 19 from line 125).

(A) Maximum ethylene evolution rate. From five to 10 fruit from each plant were harvested at the onset of color development. The maximum ethylene evolution rate during ripening was determined by measuring ethylene production daily for each fruit. C, maximum level of ethylene evolution in fruit from untransformed plants, 6.0 nL/(g-hr).

(B) E8 protein accumulation. Fruit were harvested at the onset of color change and stored 3 days. Proteins were extracted, and 50 μg was subjected to electrophoresis, blotted, and reacted with anti-E8 antibodies.

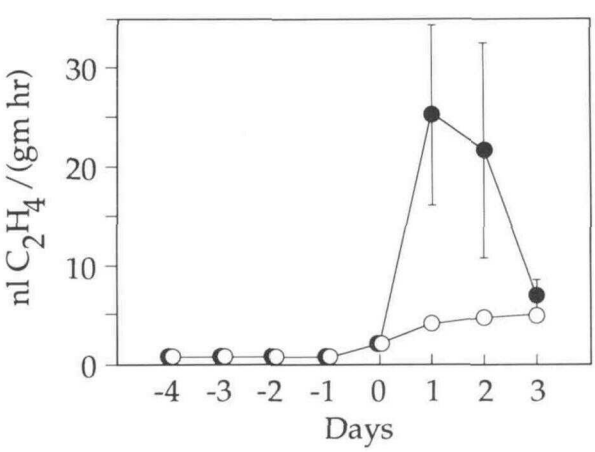

Figure 6. Ethylene Evolution Rate of Fruit from Transgenic Plants Harvested Prior to the Onset of Ripening.

Unripe fruit were harvested approximately 4 days before the onset of color development, and the ethylene evolution rate was measured daily. ●, Ethylene evolution rate of fruit from 125-08, 125-09, and 125-19 transgenic plants (n = 11); ○, ethylene evolution rate of fruit from untransformed plants (n = 5).

DISCUSSION

Relationship between E8 and Dioxygenases

Although we have not yet analyzed the E8 protein biochemically, DNA sequence analysis indicates that E8, like ACC oxidase, is a member of a family of dioxygenase enzymes (McGarvey et al., 1992). Members of this oxidase family all require Fe^{II} and a reductant, usually ascorbate, for catalytic activity. Three histidine residues and one cysteine residue are conserved throughout the family and may interact with the Fe^{II} cofactor. The structural similarities of this extended family, and in particular the relationship between E8 and ACC oxidase, suggest that the E8 protein may have a metal cofactor and the ability to react with molecular oxygen, ACC, and/or ethylene.

Hamilton et al. (1990) elucidated the function of an ACC oxidase gene by analyzing the effect of an ACC oxidase antisense gene on ethylene production. We have utilized the same strategy to elucidate the function of the E8 gene. In the experiments presented here, we show that an E8 antisense gene suppresses the accumulation of E8 protein in fruit harvested from transgenic plants (Figure 3). Whereas reduction of ACC

oxidase activity results in reduced levels of ethylene biosynthesis (Hamilton et al., 1990), we find that reduction of the related E8 protein below a threshold value produces the opposite effect, an increase in ethylene evolution during the ripening of detached fruit (Figures 4 to 6). Thus, although E8 and ACC oxidase are structurally related, they have evolved different functions during fruit ripening.

Variable Penetrance and Expressivity of an E8 Antisense Locus

The ability of the E8 antisense locus to stimulate ethylene overproduction to any degree is called penetrance. The fact that some progeny with an antisense locus do not overproduce ethylene (Figure 5A) suggests that penetrance of the antisense locus is partial. However, progeny that fail to overproduce ethylene also fail to suppress effectively E8 protein accumulation (Figure 5B). Thus, penetrance of the ethylene overproduction phenotype correlates well with the degree of suppression of E8 protein accumulation. Why E8 protein accumulates in certain progeny with the E8 antisense locus is not known. One possibility is aberrant transmission of the E8 antisense locus. However, DNA gel blot experiments have not revealed gross DNA deletions or rearrangements in T_2 progeny. Alternatively, inactivation of the E8 antisense locus may be epigenetic, similar to the inactivation of other transformed genes (Scheid et al., 1991) and to the variable penetrance of phenotypes associated with cosuppression of homologous genes (Mol et al., 1990; Matzke and Matzke, 1991; Napoli et al., 1991).

The degree of ethylene overproduction by a plant with a penetrant E8 antisense locus is called expressivity. The fact that certain progeny (e.g., 125-08; Figure 5A) tend to overproduce ethylene to a greater extent than other progeny from the same line (e.g., 125-22; Figure 5A) or an independently transformed line (e.g., 191-01; data not shown) shows that expressivity of the E8 antisense locus is variable. One possibility is that expressivity, like penetrance, correlates with the degree of suppression of E8 protein accumulation. In the future, this hypothesis will be tested by measuring residual E8 protein in penetrant progeny using a more sensitive immunological assay. We have also observed that the ripening of detached fruit from transgenic plants (line 125) is delayed, but, like ethylene overproduction, this aspect of the phenotype is subject to variable penetrance and expressivity (L. Peñarrubia and R. L. Fischer, unpublished results).

Possible Mechanisms for E8 Action

The effect of E8 is specific to the burst of ethylene produced during ripening (Figure 6). The onset of ethylene overproduction in fruit from transgenic plants is coincident with the smaller increase of ethylene produced in fruit from untransformed plants. Moreover, the burst of ethylene overproduction in fruit from transgenic plants and the smaller burst observed in fruit from untransformed plants are transitory. The fact that a decrease in E8 protein level results in ethylene overproduction indicates that E8 has a negative effect on ethylene production during fruit ripening. One possibility is that reduction in E8 protein results in a metabolic imbalance leading to stress-inducible ethylene production specifically during fruit ripening. Alternatively, E8 protein could be involved in negative feedback regulation of ethylene biosynthesis during ripening.

It is well known that ethylene stimulates its own biosynthesis at the onset of ripening (Yang and Hoffman, 1984). However, in a variety of fruit and vegetative tissues, ethylene also negatively controls its own production, a process termed autoinhibition (Yang and Hoffman, 1984). For example, wound-inducible ethylene production is greatly reduced by exogenous ethylene and/or ethylene analogs (Riov and Yang, 1982), and inhibition of ethylene perception either biochemically (Atta-Aly et al., 1987; Chi et al., 1991) or by mutation (Guzmán and Ecker, 1990) can result in ethylene overproduction. Although the mechanisms of autoinhibition are not fully understood, ethylene has been shown to inhibit its production by reducing the level of ACC, the precursor of ethylene (Riov and Yang, 1982). This may occur by reducing ACC synthase activity (Yoshi and Imaseki, 1982) and/or by promoting the malonylation of ACC to an inactive form, malonyl-ACC (Liu et al., 1985b). In addition, ethylene is oxidized to a variety of products, although recent experiments suggest that in most plants ethylene oxidation does not have a significant effect on ethylene concentration or the mode of action of ethylene (Hall, 1991). The mechanism of E8 action may be to suppress ethylene production during fruit ripening by limiting the amount of ACC, either by inhibiting synthesis of ACC or by inactivation of ACC (Figure 1). This could occur by the direct involvement of E8 in the biochemical reactions of ACC and/or ethylene metabolism. Alternatively, the effect of E8 could be indirect, constituting a part of a signal transduction pathway by which ethylene controls its level of production.

METHODS

Plant Material

Tomato seed (*Lycopersicon esculentum* cv Ailsa Craig) were obtained from the Glasshouse Crops Research Institute (Little Hampton, West Sussex, England), and plants were grown under standard greenhouse conditions. Fruit were harvested at the indicated stage and stored at 28°C.

Plant Transformation

The E8 antisense gene, subcloned in the intermediate vector pMLJ1, was transferred to the disarmed *Agrobacterium tumefaciens* pGV3850 Ti plasmid vector, as described previously (Van Haute et al., 1983). Sterile cotyledon pieces were incubated on tobacco feeder cells, infected with Agrobacterium with the pGV3850:pMLJ1:antisenseE8-9

co-integrate plasmid, and transformants were selected with 50 μg/mL kanamycin by the procedure of Fillatti et al. (1987). The presence of T-DNA in primary transformed plants and their progeny was determined by DNA gel blot hybridization (Deikman and Fischer, 1988) and/or by detection of neomycin phosphotransferase II activity (McDonnell et al., 1987).

Determination of Ethylene Evolution Rate

Individual fruits were placed in 500-mL containers that were sealed and incubated 1 hr at room temperature. A 1-mL sample from the closed atmosphere was removed, and the ethylene content was determined by gas chromatography (Hach/Carle, Loveland, CO). In figures, error bars represent standard errors. Where error bars are not shown, the standard error was no greater than the size of the symbol.

Protein Gel Blotting

A full-length cDNA clone, pE8-9 (Deikman and Fischer, 1988), was inserted into the pT7-7 expression vector (Tabor, 1990), and bacterial protein extracts were prepared as described previously (Studier et al., 1990). E8 fusion protein (E8 plus eight amino acids at the amino terminus encoded by the vector) was purified by $NaDodSO_4$/polyacrylamide (10%; 29:1 acrylamide/bisacrylamide) gel electrophoresis (Laemmli, 1970), and anti-E8 polyclonal antibodies were prepared in mice (Harlow and Lane, 1988). To prepare protein extracts from fruit pericarp, tissue was ground in liquid nitrogen and boiled for 15 min in three volumes of Laemmli buffer (Laemmli, 1970), and debris was removed by centrifugation (12,000*g*) at room temperature. Proteins were subjected to electrophoresis on 10% $NaDodSO_4$/polyacrylamide gels, transferred to nitrocellulose membranes, and reacted with mouse polyclonal anti-E8 antibody/anti-mouse IgG alkaline phosphatase conjugate (Harlow and Lane, 1988). Protein concentration of extracts was determined as described by Schaffner and Weissmann (1973).

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