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Organization of Ripening and Ethylene Regulatory Regions in a Fruit-Specific Promoter from Tomato (*Lycopersicon esculentum*)[1]

Jill Deikman[2], Randy Kline, and Robert L. Fischer*

Plant Biology Department, 111 Genetics and Plant Biology Building, University of California, Berkeley, California 94720

ABSTRACT

Tomato (*Lycopersicon esculentum*) fruit ripening is initiated by an increase in ethylene hormone concentration. E8 gene transcription is fruit-specific and is activated at the onset of ripening and in unripe fruit treated with exogenous ethylene. To understand how E8 gene transcription is controlled during ripening, we analyzed the effect of deletions of flanking DNA sequences on E8 gene expression in transgenic tomato fruit. We found that a minimum of three 5′ and one 3′ regions influence E8 gene expression during fruit ripening. DNA sequences that confer responsiveness to exogenous ethylene in unripe fruit are distinct from DNA sequences that are sufficient for expression during fruit ripening.

Ethylene is one of five classical plant hormones that control plant growth and development. Ethylene affects shoot and root growth and differentiation, promotes leaf abscission, induces fruit ripening and flower senescence, and is an important part of the plant's response to wounding and pathogen attack (13). Experiments have shown that ethylene exerts its effect on plant growth and development, at least in part, by controlling the transcription of specific genes (1, 11, 18). In tomato (*Lycopersicon esculentum*) fruits, ripening is controlled by an increase in ethylene hormone production (6, 9, 19, 23).

The E8 predicted polypeptide is a member of a family of dioxygenases found in plants and microorganisms (15) and is related to the enzyme that catalyzes the last step in the ethylene biosynthesis pathway, ACC[3] oxidase, sharing 34% amino acid sequence identity over 295 residues (20). Although E8 does not participate in the oxidation of ACC to ethylene, it does influence the level of ethylene produced during ripening. Reduction of E8 protein by antisense RNA results in overproduction of ethylene specifically during the ripening of detached tomato fruit (20).

Ethylene plays an important role in the regulation of E8 gene expression during tomato fruit ripening. E8 is transcriptionally activated at the onset of ripening, coincident with the increase in ethylene biosynthesis. Moreover, treatment of unripe fruit with 10 μL/L of ethylene rapidly activates E8 transcription (11). To understand the regulation of gene expression during fruit ripening, we have studied the effect of promoter deletions on E8 gene transcription in transgenic tomato plants.

MATERIALS AND METHODS

Plant Material and Transformation

Tomato (*Lycopersicon esculentum* cv Ailsa Craig) plants were grown under standard greenhouse conditions. Fruit maturity stage was determined as described (10). To treat with ethylene, unripe fruit were placed in a 25-L chamber and exposed for 6 h to 4.5 L/min of ethylene (10 μL/L) in humidified air.

Construction of Chimeric Genes and Plant Transformation

The E8-Tag construct (4) was used to generate a nested set of 5′ deletions as described by Henikoff (7). Deletion end points were determined by DNA sequencing. For plant transformation, genes bearing 5′ deletions were subcloned into the *Eco*RI site of shuttle vector pMLJ1 using *Eco*RI/*Hin*dIII adapters (New England Biolabs). The 3′ deletion was constructed using a unique *Hga*I restriction endonuclease site. This deletion removed 337 bp of 3′-flanking DNA and left 185 bp of DNA 3′ of the poly(A) addition site. The E8-Tag plasmid was digested with *Hga*I, and DNA polymerase was used to fill in the single-stranded ends of the *Hga*I restriction endonuclease site. *Eco*RI linkers (New England Biolabs) were ligated to the fragments, which were then digested with *Eco*RI. The resulting 4.2-kb E8-Tag fragment was purified by agarose gel electrophoresis and ligated into the *Eco*RI site of pMLJ1. Plant transformation was carried out as described (4) and was verified by DNA gel blot hybridization analysis.

S1 Nuclease Protection

DNA was labeled with T4 polynucleotide kinase using the 5′-Terminus Labeling Kit from Bethesda Research Laboratories. Total RNA (25 μg) was hybridized with a 3-fold molar excess of probe in 0.5 M NaCl, 0.04 M Pipes, pH 6.4, 1 mM EDTA, and 80% formamide at 37°C overnight. Digestion with S1 nuclease (Sigma) was in 30 mM sodium acetate, pH

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[2] Present address: Department of Biology, The Pennsylvania State University, University Park, PA 16802.

[3] Abbreviation: ACC, 1-aminocyclopropane-1-carboxylic acid.

5, 0.25 M NaCl, 1 mM $ZnSO_4$, 5% glycerol, and 1 unit of S1/μL at 30°C for 30 min. Samples were then extracted with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with ethanol, resuspended in DNA sequencing loading buffer, denatured by heating to 80°C, and analyzed by electrophoresis on an 8% polyacrylamide/7 M urea gel.

Gel Blot Hybridization

Total plant RNA was isolated from fruit pericarp tissue as described by DellaPenna et al. (5). Poly(A)$^+$ mRNA (1.3 μg) was purified, denatured with formaldehyde, analyzed by electrophoresis on agarose gels, blotted onto Genescreen membranes (New England Nuclear), and hybridized with ^{32}P-labeled DNA prepared as described (4). The extent of hybridization was measured by scanning autoradiograms with a densitometer.

Determination of DNA Sequences

Nucleotide sequences were determined using the dideoxy chain-termination method (22). Chemically synthesized oligonucleotides were used as primers using procedures and enzymes provided by Sequenase (United States Biochemical). DNA sequence analysis was carried out using Intelligenetics computer programs.

RESULTS

Construction of Deletions

We previously demonstrated that an E8 gene tagged with 125 bp of bacteriophage λ DNA, designated E8-Tag, was expressed in ripening tomato fruit to levels similar to those of the native E8 gene and was ethylene inducible in unripe fruit (4). The E8-Tag gene included 2181 bp of 5′-flanking sequence and 522 bp of DNA 3′ to the poly(A) addition site. To identify DNA regulatory sequences, a nested set of 5′ promoter deletions and a 3′ deletion were constructed (Fig. 1). These genes were introduced into the tomato genome using modified Ti-plasmid vectors from *Agrobacterium tumefaciens* and their expression was measured in mature transgenic tomato plants as described in "Materials and Methods."

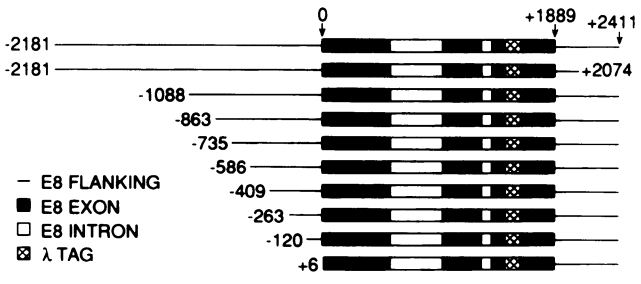

Figure 1. Structure of E8-Tag genes. 5′ and 3′ deletion end points relative to the start of transcription are indicated in bp. Intron and exon regions were determined by comparison of the E8 gene and cDNA sequences (4). 0, Transcription initiation site; +1889, poly(A) addition site; λ Tag, 125 bp of bacteriophage λ DNA.

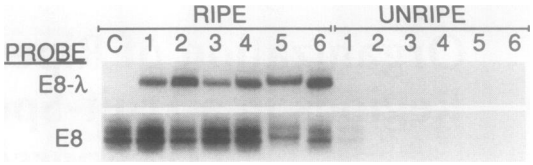

Figure 2. E8-Tag mRNA concentration in independently transformed tomato plants. Tomato plants were transformed with an E8-Tag gene with 2181 bp of 5′ flanking and 522 bp of 3′ flanking DNA sequences. S1 nuclease analysis was performed as described in "Materials and Methods." The 5′ end-labeled DNA probe contains 133 nucleotides of E8 coding sequence followed by 125 nucleotides of bacteriophage λ DNA (4) plus pUC118 vector DNA. A doublet is observed due to limited S1 nuclease digestion at the end of the DNA/RNA hybrid molecule. E8-λ, 258 (125 + 133) nucleotide fragment protected by E8-Tag mRNA; E8, 133 nucleotide fragment protected by native E8 mRNA; C, RNA from untransformed 50% red fruit; 1 through 6, RNA from six independently transformed plants. The approximate E8-Tag gene copy number for each transgenic plant (1–6) is 8, 2, 1, 1, 1, and 2, respectively.

Analysis of Independently Transformed Plants

We determined the degree of variability of chimeric gene expression due to position effects (8) and/or cosuppression effects (14, 16, 17). To this end, we measured E8-Tag mRNA concentration by S1 nuclease analysis in six plants independently transformed with the same E8-Tag gene. The E8-Tag gene with 2181 bp of 5′ flanking and 522 bp of 3′ flanking sequence was developmentally regulated during fruit ripening, verifying that the chimeric gene spans the appropriate control regions (Fig. 2). In ripening fruit, less than 2-fold differences in the level of E8-Tag gene expression were observed. In addition, the number of E8-Tag genes, determined by DNA gel blot hybridization (see Fig. 2 legend for values), had little effect on the amount of E8-Tag mRNA present in ripe fruit. Thus, the variability of E8-Tag gene expression in these six independently transformed plants proved to be minimal. In subsequent experiments, RNA was isolated from equal amounts of tissue from multiple independently transformed plants bearing the same E8-Tag chimeric gene.

Effect of 5′ Deletions on E8-Tag Gene Expression in Fruit

The level of E8-Tag mRNA was determined by hybridizing RNA gel blots to the labeled Tag, bacteriophage λ DNA. RNA gel blot hybridization (Figs. 3 and 4) was more sensitive than S1 nuclease analysis (Fig. 2), making it possible to observe basal levels of E8-Tag mRNA in unripe fruit. Removal of DNA sequences from −2181 to −1088 resulted in a 10-fold decrease in E8-Tag mRNA concentration in 50% red fruit. Further deletion of sequences to −863 caused an additional 7-fold decrease in E8-Tag mRNA concentration in 50% red fruit. Deletion of sequences from −409 to −263 reduced the level of E8-Tag mRNA to below the level of detection. Control duplicate blots hybridized with a different fruit-ripening cDNA, E4 (10), demonstrated that equal amounts of RNA were analyzed. These results show that a minimum of three 5′ regions control E8 gene expression during fruit ripening.

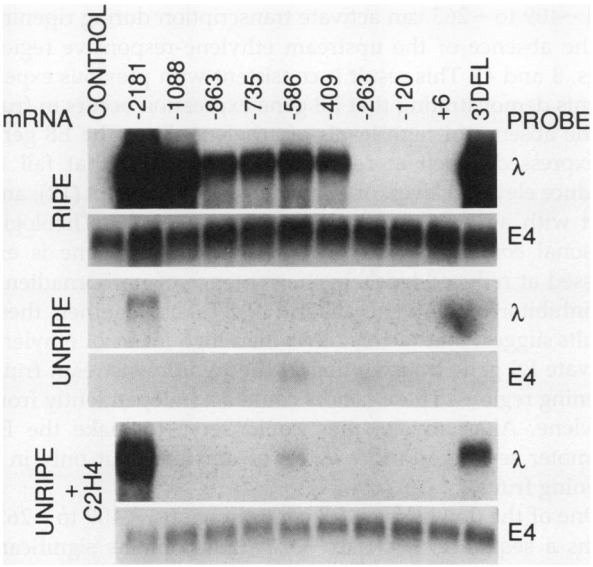

Figure 3. Effect of deletions on E8-Tag mRNA concentration in fruit. Gel blot analysis was performed as described in "Materials and Methods." 5′ deletion end points are indicated. 3′-DEL, Chimeric gene with a full-length promoter containing a 337-bp deletion at the 3′ end (+2074 to +2411, Fig. 1); λ, hybridization with the λ Tag DNA; E4, hybridization with E4 cDNA; ripe, 50% red fruit; unripe, early mature green fruit treated with air for 6 h; unripe + C_2H_4, early mature green fruit treated with 10 μL/L of ethylene for 6 h; control, mRNA from untransformed fruit. The number of independently transformed plants analyzed is given in parentheses in the following order: unripe + air, unripe + C_2H_4, ripe; −2181 (6, 5, 6); −1088 (6, 5, 6); −863 (6, 6, 6); −735 (6, 5, 7); −586 (5, 5, 6); −409 (6, 5, 7); −263 (4, 3, 5); −120 (7, 6, 6); +6 (5, 4, 5); 3′ deletion (5, 4, 7).

Unripe fruit bearing an E8-Tag gene with 2181 bp of 5′ flanking sequences responded to the brief ethylene treatment by producing E8-Tag mRNA (Figs. 3 and 4). A deletion to −1088 eliminated this response in unripe fruit, reducing the level of E8-Tag mRNA more than 70-fold. Examination of autoradiograms exposed for longer times revealed that the very low level of E8-Tag mRNA present in ethylene-treated −1088 fruit was equivalent to that found in nonethylene-treated controls (data not shown). Taken together, these results indicate that DNA sequences necessary for ethylene responsiveness in unripe fruit reside in the promoter region between −2181 and −1088.

We detected a small increase in E8-Tag mRNA concentration in ethylene-treated fruit bearing the −586 deletion (Figs. 3 and 4). It is possible that this construct has regained ethylene responsiveness. However, an alternative explanation is that the population of fruit contained individuals that had advanced into later stages of fruit ripening.

Effect of a 3′ Deletion on E8-Tag Gene Expression in Fruit

We have also investigated the role of 3′ flanking sequences in the regulation of E8-Tag gene expression. To this end, we constructed a chimeric gene with a full-length promoter containing a 337-bp deletion at the 3′ end (+2074 to +2411, Fig. 1). Even though the full-length promoter was intact, the 3′ deletion reduced the level of E8-Tag gene expression. E8-Tag mRNA concentration was approximately 4-fold lower in unripe fruit, unripe fruit treated with ethylene, and 50% red fruit (Fig. 3). Thus, the 3′ region appears to influence quantitatively the overall level of E8-Tag gene expression in fruit, and is not involved in the response of the E8-Tag gene to ethylene and/or ripening.

DNA Sequence of the Region that Confers Ethylene-Responsive E8 Gene Expression

Because sequences important for ethylene regulation are located between −2181 and −1088, we determined the DNA sequence of this region (Fig. 5). The 1093 bp in this region were compared to the region −193 to −86 that is required for the ethylene-regulated expression of another tomato fruit-ripening gene, E4 (J. Montgomery, J. Deikman, R. Fischer, manuscript in preparation). Several short regions that are at least 70% identical are indicated, and these may constitute DNA sequences that regulate gene expression in response to ethylene during tomato fruit ripening.

DISCUSSION

Regulation of E8 Gene Expression during Fruit Ripening

The rate of E8 gene transcription is low in unripe fruit and dramatically increases during ripening (11). Deletions in the promoter region serve to reduce the level of E8-Tag mRNA in ethylene-treated unripe fruit and in ripe fruit (Figs. 3 and 4). These results indicate that the E8 gene is positively regulated. E8 gene expression is controlled by transcription factors that interact with sites in the promoter region to activate E8 gene transcription at the onset of ripening.

Inhibition of E8 gene expression by antisense RNA results in overproduction of ethylene specifically during the ripening of detached tomato fruit (20). We have shown that ethylene plays a role in the activation of E8 gene transcription (11,

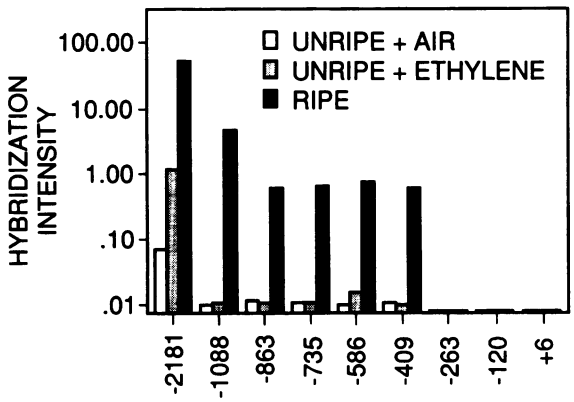

Figure 4. Quantitative analysis of promoter deletions on E8-Tag gene expression. Kodak X-Omat film was exposed to the gel blots in Figure 3 for 1 to 14 d. Autoradiograms were scanned using a densitometer. 5′ deletion end points are indicated. Hybridization intensity is the signal obtained from densitometer multiplied by the exposure time.

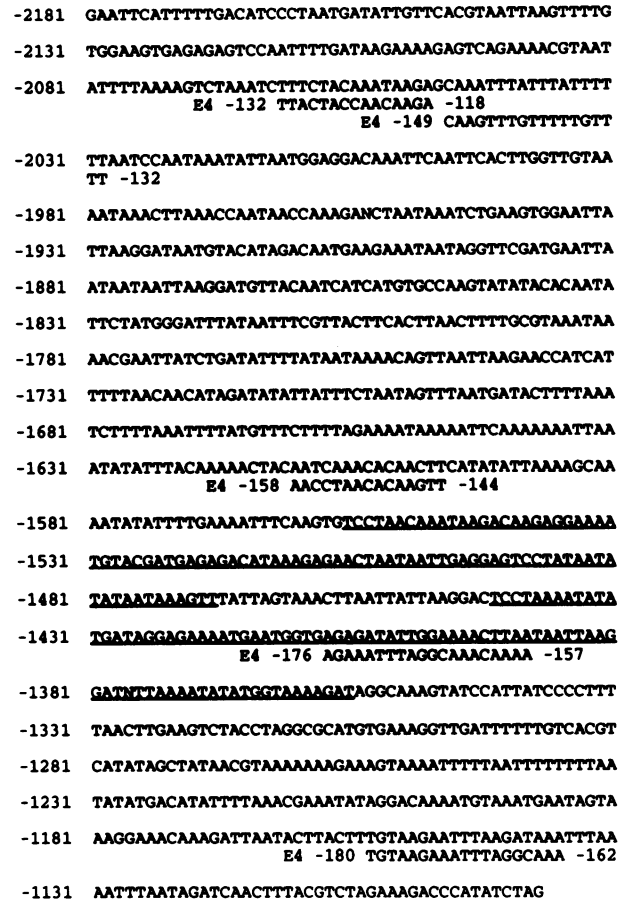

Figure 5. DNA sequence from −2181 to −1088 that influences ethylene-responsive E8 gene expression. Homologous DNA sequences from the E4 promoter are shown. An imperfect direct repeat (66 of 91 bp are identical) is underlined.

Figs. 3 and 4). Taken together, these results suggest that the E8 gene may participate in feedback inhibition of ethylene production during fruit ripening (20).

Organization of Ethylene-Responsive and Fruit-Ripening Regulatory Regions

The E8 gene has multiple promoter regions that influence its transcription. One region, −2181 to −1088, is required for the activation of E8 gene transcription in unripe fruit by ethylene, and also contributes to the overall level of E8-Tag mRNA during ripening (Figs. 3 and 4). These results indicate that the −2181 to −1088 region contains DNA sequences that confer ethylene responsiveness in both unripe and ripe fruit. Further deletion of sequences revealed two additional regions, −1088 to −863 and −409 to −263, that are unable to confer ethylene responsiveness in unripe fruit but are sufficient for E8 gene expression during ripening (Fig. 3). Thus, the ethylene-response region located between −2181 and −1088 is distinct from two downstream fruit-ripening regions located at −1088 to −863 and −409 to −263, respectively.

Two downstream fruit-ripening regions at −1088 to −863 and −409 to −263 can activate transcription during ripening in the absence of the upstream ethylene-responsive region (Figs. 3 and 4). This result is consistent with previous experiments demonstrating that E8 gene expression occurs in fruit in the absence of high levels of ethylene. First, the E8 gene is expressed, albeit at reduced levels, in fruit that fail to produce elevated levels of ethylene, *rin* mutant fruit (12), and fruit with an ACC synthase antisense gene (A. Theologis, personal communication). In addition, the E8 gene is expressed at reduced levels in the presence of norbornadiene, an inhibitor of ethylene action (10). Taken together, these results suggest that factors other than high levels of ethylene activate E8 gene transcription at the two downstream fruit-ripening regions. These factors could act independently from ethylene. Alternatively, they could serve to make the E8 promoter sensitive to low levels of ethylene, but only in a ripening fruit.

One of the downstream E8 control regions, −409 to −263, spans a sequence, −371 to −328, that contains significant sequence identity (74% over 43 bp) with a region in the cellulase promoter that is transcriptionally activated during avocado fruit ripening (2). Thus, it is possible that this promoter region that may control transcription during fruit ripening has been conserved during evolution of these two plant species.

DNA-Binding Factors that Interact with the E8 Promoter

Previously, we showed that a DNA-binding factor that is more active in nuclear extracts from ripe fruit than from unripe fruit binds with E8 promoter DNA sequences at −936 to −920 (3, 4). This site is also present in an ethylene-regulated gene expressed in petals during flower senescence (21). We find that a deletion extending from −1088 to −863 that removes the binding site results in a significant decrease in E8-Tag mRNA in ripening fruit (Fig. 4). This correlation suggests that the factor is important for high-level E8 gene expression during fruit ripening. However, because the factor does not bind in the region responsible for ethylene responsiveness (−2181 to −1088), it is probably not involved in an ethylene signal transduction pathway in tomato fruit. This is consistent with the observation that the DNA-binding activity was not detected in nuclear extracts from ethylene-treated fruit (4). An additional fruit-ripening-specific DNA-binding activity was shown to bind to E8 sequences located between −631 and −349 (4). This region overlaps with the DNA sequences (−409 to −263) that, when deleted, results in a dramatic loss of E8 gene expression in ripening fruit (Fig. 4). Thus, both DNA-binding factors identified previously may play a role in controlling E8 gene expression during tomato fruit ripening.

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