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Interaction of a DNA binding factor with the 5′-flanking region of an ethylene-responsive fruit ripening gene from tomato

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To understand how fruit development is controlled, we have begun experiments to identify DNA sequences and proteins that regulate gene expression during tomato (*Lycopersicon esculentum*) fruit ripening. We have focused on the E8 gene because its transcription is responsive to ethylene hormone and is activated at the onset of fruit ripening. We report here that sequences required for ethylene-responsive and developmentally regulated E8 gene expression in transgenic tomato plants are contained on a 4.4 kb restriction fragment which includes sequences 2 kb 5′ and 0.5 kb 3′ to the gene. In addition, we have identified a DNA-binding factor that specifically interacts with DNA sequences that flank the E8 gene. This DNA-binding activity is low in unripe fruit and increases during fruit ripening. This factor also binds to the 5′-flanking region of another ethylene-responsive gene which is coordinately expressed during tomato fruit ripening. These data suggest that the DNA binding-factor may be involved in the regulation of gene expression during fruit ripening.

Key words: gene regulation/*Lycopersicon esculentum*/transgenic plants

Introduction

Ripening is the final phase in the development of the fruit organ. It is associated with a sharp change in metabolism involving the promotion of both anabolic and catabolic processes. Specific events correlated with ripening include increased respiration, autocatalytic ethylene production, chlorophyll degradation, carotenoid synthesis, conversion of starch to sugars, production of essential oils and other flavor components, and increased activity of cell wall degrading enzymes (Lyons and Pratt, 1964; Rick, 1978; Rhodes, 1980; Grierson, 1985). The mechanisms that control these processes are undoubtedly complex; however it has been shown that the plant hormone ethylene plays an important direct role in regulating the ripening of climacteric fruits such as tomato (Rhodes, 1980; Biale and Young, 1981; Yang, 1985). Ethylene biosynthesis increases dramatically at the onset of tomato fruit ripening. Furthermore, ripening of these fruits can be caused to occur prematurely by exposure to ethylene, while exposure to inhibitors of ethylene biosynthesis or removal of ethylene from the fruit prevents ripening.

One hypothesis is that activation of diverse physiological and biochemical processes at the onset of fruit ripening is the result of changes in gene expression. To investigate this issue, mRNAs that increase in concentration during fruit ripening, and when unripe fruit are exposed to exogenous ethylene, have been identified and cloned (Grierson, 1985; Mansson *et al.*, 1985; Lincoln *et al.*, 1987). Previously, we have shown that the expression of one such gene of unknown function, designated E8, is both responsive to ethylene and under strict developmental control (Lincoln *et al.*, 1987; Lincoln and Fischer, 1988a,b). That is (i) E8 gene expression is organ specific. E8 mRNA is abundant in ripe tomato fruit, but is not detected in leaf, root or stem organs. (ii) E8 gene expression is temporally regulated by ethylene during fruit ripening. At the onset of fruit ripening, E8 mRNA concentration and the level of ethylene increase concurrently. Exposing fruit to an inhibitor of ethylene action reduces E8 gene expression, as does a mutation that blocks the increase in ethylene biosynthesis. (iii) The E8 gene is ethylene responsive, but in an organ-specific fashion. Exposing unripe tomato fruit to exogenous ethylene results in the rapid accumulation of E8 mRNA. In contrast, treating leaves with ethylene has little effect on E8 gene expression. (iv) E8 gene expression is regulated at the level of gene transcription. Nuclear run-on transcription experiments indicate that increases in E8 mRNA concentration are associated with increases in the relative rate of E8 gene transcription. In addition, we have shown that a second gene, E4, is coordinately expressed during tomato fruit ripening. As is the case for the E8 gene, E4 gene transcription is activated during fruit ripening and when unripe fruit is exposed to ethylene (Lincoln and Fischer, 1988a).

To begin to understand how gene expression is regulated during fruit ripening and by ethylene, we have initiated experiments designed to identify DNA sequences and cellular factors that control E8 gene expression. Here we show that the sequences required for ethylene-responsive and developmentally regulated E8 gene expression in transgenic tomato plants are contained on a restriction fragment which includes the E8 transcribed sequences, 2 kb of 5′-flanking sequence and 0.5 kb of 3′-flanking sequence. In addition, we have identified a DNA-binding factor which specifically interacts with 5′-flanking sequences of both the E8 and E4 genes and which may coordinate expression of these genes in ripening tomato fruit.

Results

E8 gene organization

Previously, we described the isolation of a cDNA clone, pE8, representing a 1.4 kb mRNA that accumulates during tomato fruit ripening and when unripe fruit are exposed to ethylene (Lincoln *et al.*, 1987). In order to estimate the number of genes homologous to pE8, tomato genomic DNA was digested with *Eco*RI restriction endonuclease and hybridized with labeled pE8 DNA. pE8 hybridized with three

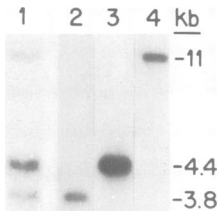


Fig. 1. Representation of E8 genes in the tomato genome. Ten micrograms of tomato genomic DNA (**lane 1**) or ~0.2 μg of λE8-10 (**lane 2**), λE8-3 (**lane 3**), and λE8-1 (**lane 4**) were digested with *Eco*RI, fractionated by agarose gel electrophoresis, blotted, and hybridized with labeled pE8 DNA. Autoradiography was carried out at −80°C with intensifying screen for 20 h (lane 1) or 2 h (lanes 2−4).

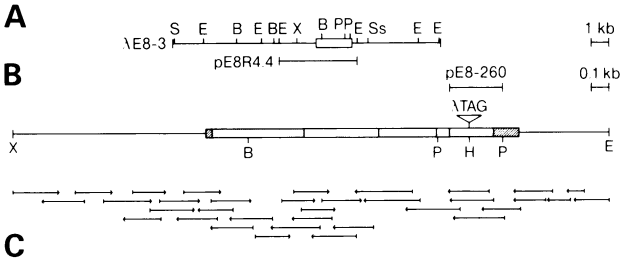
*Eco*RI fragments, 11, 4.4 and 3.8 kb in length (Figure 1, lane 1). To investigate E8 gene organization further, a library of tomato leaf nuclear DNA was screened by hybridizing plaques with labeled pE8 DNA. Three genomic clones were isolated, λE8-1, λE8-3, and λE8-10, that contained the 11, 4.4 and 3.8 kb restriction fragments, respectively (Figure 1, lanes 2−4). Determination of restriction endonuclease sites showed that the three clones represented non-overlapping regions of the tomato genome (data not shown). Comparison of hybridization intensity of tomato nuclear DNA and a single-copy equivalent of λE8-3 DNA with the labeled pE8 probe indicated that the 4.4 kb restriction fragment was present approximately once per haploid tomato genome (data not shown). Taken together, these results suggest that approximately three different E8 genes are present in the tomato genome.

E8 gene structure

To investigate the structure of the E8 gene, we determined the DNA sequence of both genomic and cDNA clones. Because the 4.4 kb *Eco*RI restriction fragment of genomic DNA hybridized most intensely with the labeled pE8 cDNA (Figure 1), the corresponding fragment in λE8-3 was subcloned (designated pE8R4.4; Figure 2A), and the sequence of 3360 base pairs was determined (Figure 2C). In addition, DNA sequences from two full-length E8 cDNA clones, pE8-9 and pE8-21, were determined.

Comparison of pE8-9 cDNA and genomic DNA sequences revealed that the E8 gene is composed of three exons interrupted by two introns that display consensus splice junction sequences (Brown, 1986). A consensus poly (A) addition signal precedes the end of the mature message. The longest open reading frame encodes a polypeptide of 41 081 daltons, which agrees with the result of *in vitro* translation of E8-selected mRNA (Lincoln *et al.*, 1987). The transcription start site was defined by S1 nuclease protection and primer extension experiments (data not shown) and the location determined using these techniques coincided with the 5′ end of the cDNA clone pE8-9 (Figure 2C). TATA and plant −80 consensus sequences (Heidecker and Messing, 1986) are present 32 and 85 bp, respectively, 5′ to this start site.

The DNA sequence of cDNA clone pE8-21 revealed that an alternative splicing pattern may be employed in tomato fruit. Like the DNA sequence of pE8-9, the sequence of pE8-21 is identical to the genomic sequence indicating that both cDNAs are derived from the cloned E8 gene. Clone pE8-21, however, encodes a 21 kd polypeptide which was



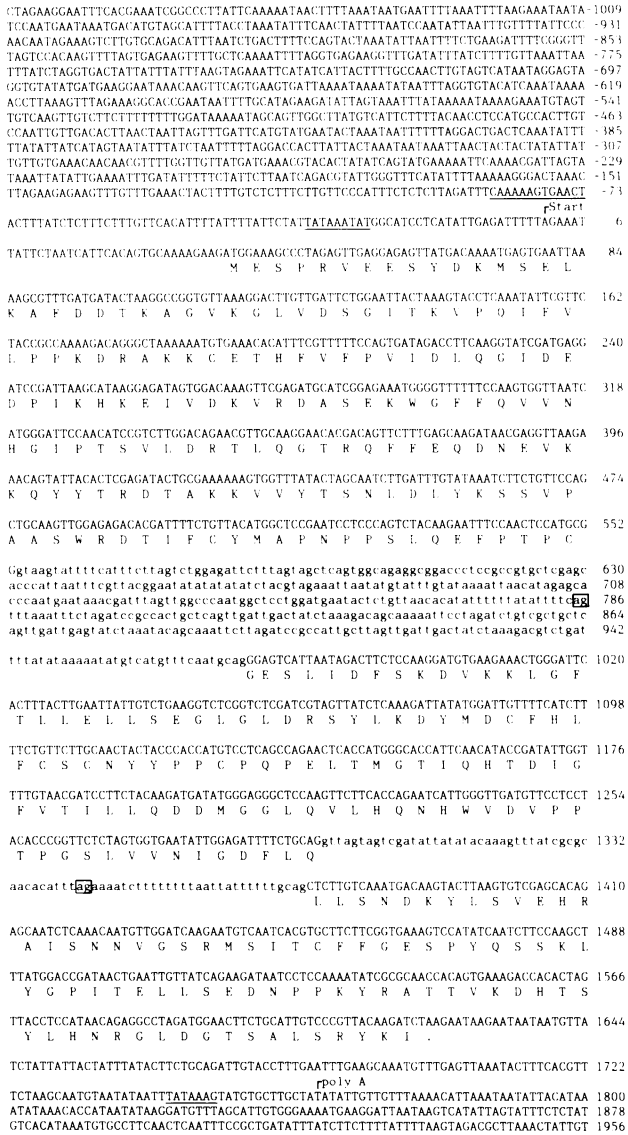


Fig. 2. E8 gene structure. (**A**) Restriction endonuclease site map of λE8-3, a genomic clone containing an E8 gene. Sites were deduced from the results of single and double digestions. S, *Sal*I; E, *Eco*RI; B, *Bam*HI; X, *Xba*I; P, *Pst*I; Ss, *Sst*I; H, *Hin*dIII. (**B**) Strategy for determining DNA sequences. pE8R4.4 was subcloned from λE8-3 and the DNA sequence from the *Xba*I to *Eco*RI site was determined. Horizontal arrows indicate the extent and direction of sequence determinations. Structure of E8-9 transcript: cross-hatched, untranslated exon sequences; stippled, translated exon sequences; open, intron sequences. pE8-260, a 260 bp probe from an E8 cDNA clone; λtag, a 125 bp *Hin*dIII restriction fragment was inserted into the indicated *Hin*dIII site in the E8 gene. (**C**) DNA sequence of the E8 gene and predicted amino acid sequence of the polypeptide encoded by cDNA clone pE8-9. TATA, plant −80 and poly(A) addition consensus sequences are underlined. Lower case DNA sequences represent introns. Alternative acceptor splice sites used by cDNA clone pE8-21 are boxed. Start, transcription start as determined by S1 protection and primer extension experiments and by pE8-9 sequence data. poly A, the poly-adenylation site. Amino acid sequences are shown in single-letter code.

Fig. 3. Alignment of amino acid sequences predicted from pE8 and pTOM13 DNA sequences. Amino acids 74−363 of E8 and the entire 295 amino acid pTOM13 polypeptide are shown. Regions of high sequence similarity are boxed. **Box 1**, 17 of 29 (59%) amino acids are identical; **Box 2**, 17 of 33 (52%) amino acids are identical; **Box 3**, 44 of 85 (52%) amino acids are identical.

not detected by *in vitro* translation of hybrid-selected mRNA (Lincoln *et al.*, 1987). The 5′ end of this cDNA extends 70 bp upstream from the major transcription start site. Furthermore, restriction analysis and partial sequencing of 10 additional cDNA clones (data not shown) indicated that pE8-9 represents the most common E8 mRNA. Therefore, we believe that clone pE8-21 represents a rare E8 transcript.

The E8 gene and predicted polypeptide sequences were compared to sequences compiled in the National Institutes of Health GenBank, the European Molecular Biology Laboratory Nucleotide Sequence Library and the National Biomedical Research Foundation Protein Identification Resource. The homology search indicated a distant relationship with another gene expressed during tomato fruit ripening and also in wounded leaf, pTOM13 (Holdsworth *et al.*, 1987). Optimal alignment of 1174 bp revealed 53% sequence identity. Alignment of amino acids showed three domains of 29−85 amino acids with 52−59% sequence identity. In the 295 amino acid overlap there was 34% amino acid identity overall (Figure 3). However, the function of the polypeptide encoded by the E8 gene remains to be elucidated.

Expression of the E8 gene in transformed tomato plants

In order to verify that the cloned E8 gene was expressed in ripening fruit and to determine whether sequences sufficient for its normal expression were contained on the 4.4 kb *Eco*RI restriction fragment, this DNA was inserted into the tomato genome. So that expression of the re-introduced E8 gene could be distinguished from that of the endogenous E8 gene, a tag consisting of the 125 bp *Hin*dIII fragment from bacteriophage lambda was inserted at the *Hin*dIII site in the third exon of the E8 gene (Figure 2B). Tomato plants were transformed with the tagged gene as described in Materials and methods. RNA was isolated from two independent transformants that displayed single-copy insertions of the tagged E8 gene in DNA gel blot experiments (data not shown). RNA encoded by the tagged E8 gene was detected on RNA gel blots by hybridization with labeled lambda tag DNA (Figure 4, lanes 1−5). The tag DNA did not hybridize with RNA isolated from untransformed tomato fruit (Figure 4, lane 1). RNA encoded by both the

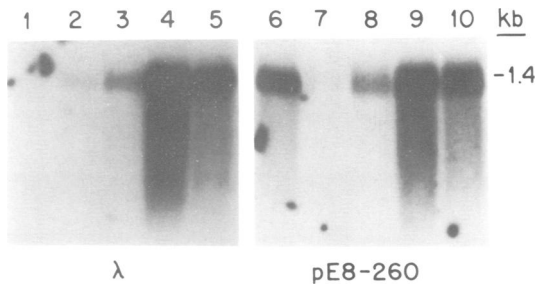


Fig. 4. Expression of a tagged E8 gene in transformed tomato plants. RNA was isolated from fruit, subjected to electrophoresis, and blotted. After hybridization with either the 125 bp labeled lambda tag or the 260 bp E8 cDNA clone (pE8-260, Figure 2B), the blots were exposed to X-ray film for 19 and 4 h, respectively. **Lanes 1** and **6**, ripe untransformed fruit; **lanes 2** and **7**, unripe transformed fruit; **lanes 3** and **8**, unripe transformed fruit exposed to ethylene; **lanes 4** and **9**, ripe transformed fruit; **lanes 5** and **10**, ripe fruit from another independently transformed plant.

endogenous and tagged genes was detected by hybridization with labeled E8 cDNA sequences (Figure 4, lanes 6−10). Expression of the tagged gene in unripe fruit (Figure 4, lane 2) was similar to the low level of endogenous E8 expression in unripe fruit (Figure 4, lane 7; Lincoln and Fischer, 1988a). Expression of the tagged E8 gene in ripening fruit from the two transformants was similar (Figure 4, lanes 4 and 5), and the concentration of mRNA derived from the tagged gene was close to that from the endogenous gene (Figure 4, lane 6). In addition, expression of the tagged gene was inducible by ethylene (Figure 4, lane 3). These results indicate that the DNA sequences that regulate E8 gene expression during fruit ripening and in response to exogenous ethylene are present on the 4.4 kb *Eco*RI restriction fragment.

Binding of nuclear proteins to E8 5′-flanking sequences

In order to begin to characterize *trans*-acting factors required for E8 gene expression, nuclear proteins were extracted from unripe and ripe tomato fruit and reacted with a series of labeled overlapping DNA fragments spanning 1.1 kb of E8 5′-flanking sequences. As described in Materials and methods, all reactions included poly(dI-dC)·poly(dI-dC) duplex DNA to eliminate nonspecific protein−DNA interactions. The presence of DNA-binding factors was assayed by the DNA gel electrophoresis mobility retardation assay (Singh *et al.*, 1986).

As shown in Figure 5, specific fragments formed protein−DNA complexes when reacted with nuclear extracts from ripe fruit. Interaction of these extracts with fragment 1 (−1088 to −682), fragment 2 (−863 to −432), and fragment 3 (−631 to −349) resulted in slowly migrating complexes designated C1, C2 and C3, respectively. In each case DNA-binding activity was less when nuclear proteins were isolated from unripe fruit (Figure 5). It is unlikely that the unripe fruit extract was inactivated or degraded during isolation, because we detected equal levels of a DNA-binding protein activity when unripe and ripe fruit extracts were reacted with 5′-flanking sequences from another fruit ripening gene (J.Montgomery and R.Fischer, unpublished results). Thus, activation of E8 gene transcription during fruit ripening is correlated with the accumulation of nuclear proteins that bind *in vitro* to multiple sites flanking the E8 gene. In addition, constitutive activities were detected in

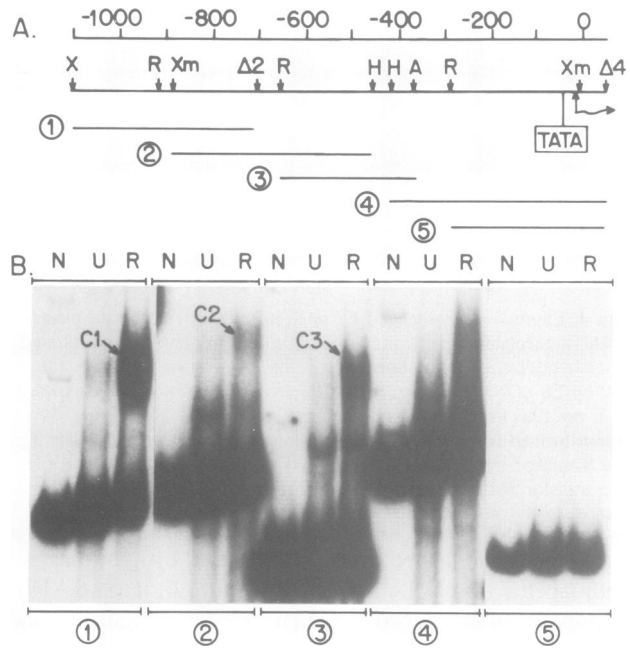


Fig. 5. E8 DNA binding activities during fruit ripening. (**A**) Schematic representation of the 5′-flanking region of the E8 gene. The indicated labeled DNA fragments representing specific 5′-regions were gel-purified and were used in protein–DNA binding reactions shown in panel B. Fragment 1, −1088 to −682; fragment 2, −863 to −432; fragment 3, −631 to −349; fragment 4, −396 to +60; fragment 5, −265 to +60; ~>, transcription initiation site; X, *Xba*I; R, *Rsa*I; H, *Hin*fI; A, *Ava*II; Xm, *Xmn*I; Δ2 and Δ4, end points of deletions. (**B**) Interaction of nuclear proteins from ripe and unripe fruit with 5′-E8 gene fragments. N, control reaction without proteins (the faint slowly migrating restriction fragments in N lanes for fragments 1, 3 and 4 are contaminants); U, nuclear proteins from unripe fruit; R, nuclear proteins from ripe fruit. C1, C2 and C3 refer to nuclear protein bound to fragments 1, 2 and 3, respectively.

unripe and ripe fruit extracts that reacted with restriction fragments 2 and 3 (Figure 5B).

Because the level of C1 DNA-binding activity was the greatest (Figure 5), we analyzed the specificity of this interaction in greater detail. To this end, nuclear extracts from ripe fruit were subjected to heat treatment or to incubation with proteinase K or RNase A for 10 min before the binding reactions were carried out. As shown in Figure 6, heat (68°C and 100°C, lanes 3 and 4) and proteinase K (lane 5) completely abolished binding. In contrast, treatment with RNase A (lane 6) did not affect binding. These results verify that protein is required for C1 complex formation.

To determine if the C1 protein–DNA complex was specific for fragment 1 of the E8 gene, we added unlabeled competitor DNAs to the binding reactions (Figure 7). Whereas unlabeled fragment 1 (designated E8-1) eliminated the protein–DNA complex formation, addition of pUC18 plasmid DNA had no effect on binding. A restriction fragment spanning 5′-flanking sequences (−463 to +61) of a light-regulated tomato gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase (designated SSU-2A) also failed to alter binding to E8 fragment 1. It is not known whether the 5′-flanking sequences of the tomato small subunit of ribulose-1,5-bisphosphate carboxylase gene are sufficient for controlling light-regulated gene expression. However, for a pea small subunit gene, this region has been shown to contain all the DNA regulatory sequences for high-level light regulated gene expression (Kuhlemeier *et al.*, 1987).

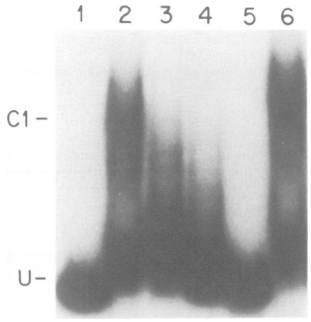


Fig. 6. Stability of the C1 complex. Nuclear protein from ripe fruit was treated with heat, proteinase K or RNase A for 10 min and reacted with fragment 1 (−1088 to −682). **Lane 1**, control reaction without proteins; **lane 2**, control reaction reacted with nuclear proteins from ripe fruit; **lane 3**, 68°C heat treatment; **lane 4**, 100°C heat treatment; **lane 5**, 0.1 μg/μl proteinase K; **lane 6**, 0.1 μg/μl RNase A. U, unbound DNA; C1, nuclear protein-E8 fragment 1 complex.

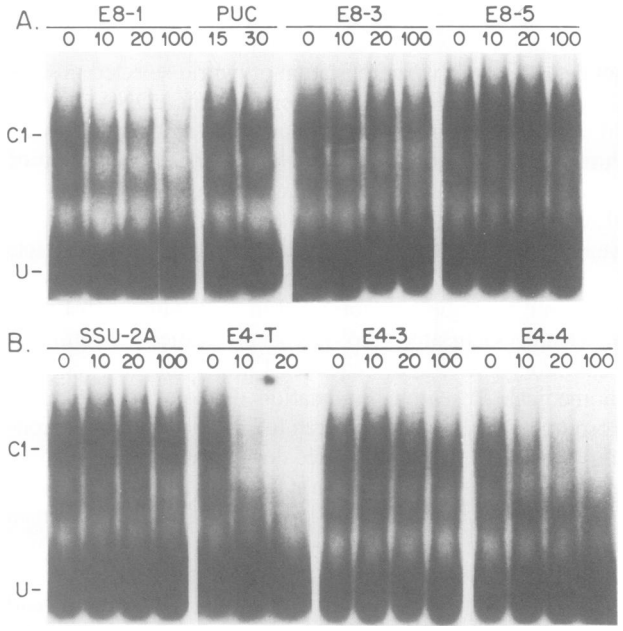


Fig. 7. Influence of unlabeled homologous and heterologous DNAs on C1 complex formation. E8 gene fragment 1 (−1088 to −682) was reacted with ripe fruit nuclear protein in the presence of the following unlabeled restriction fragments: E8-1, homologous E8 fragment 1; pUC, pUC18 plasmid DNA digested with *Hin*fI and *Xmn*I; E8-3, E8 gene fragment 3 (−631 to −349); E8-5, E8 gene fragment 5 (−265 to +60); SSU-2A, flanking sequences (−463 to +61) of a tomato gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase; E4-T, E4 gene flanking sequences (−1421 to +65); E4-3, E4 gene fragment 3 (−703 to −353); E4-4, E4 gene fragment 4 (−403 to +65). Numbers directly above lanes refer to the molar ratio of unlabeled to labeled DNAs in the binding reaction. 15- and 30-fold molar excess of pUC18 DNA is equivalent to 100- and 200-fold mass excess, respectively. U, unbound DNA; C1, nuclear protein-E8 fragment 1 complex. In addition to the C1 complex, radioactivity was detected at a position between the C1 complex and unbound DNA. However, its appearance was variable from experiment to experiment and its significance is unknown.

Taken together, these data show that ripe fruit nuclear protein forms a specific complex with fragment 1 of the E8 gene.

In theory, the C1 and C3 complexes (Figure 5) could represent two related sites that react, albeit with different affinities, with the same DNA-binding protein. Alternatively, they might represent two unique sites that each react with

a distinct DNA-binding protein. To distinguish between these possibilities, unlabeled adjacent E8 fragments were added to binding reactions containing labeled E8 fragment 1. Neither E8-3 nor E8-5 (Figure 7) eliminated the binding to labeled fragment 1, suggesting that DNA sequences required for formation of the C1 complex are unique to fragment 1.

To determine if the C1 DNA-binding activity associated with the E8 gene reacts with DNA from a gene that is coordinately expressed during fruit ripening, we added unlabeled E4 gene competitor DNAs to the binding reaction (Figure 7). E4 gene transcription, like E8, is activated during fruit ripening and when unripe fruit is exposed to ethylene (Lincoln *et al.*, 1987; Lincoln and Fischer, 1988a). An E4 gene fragment (designated E4-T in Figure 7), extending −1421 to +65 nucleotides from the site of E4 gene transcription initiation (S.Cordes and R.Fischer, unpublished results), competed with labeled E8 fragment 1 for binding with the C1 factor. To localize more precisely the competing E4 DNA sequences, overlapping unlabeled DNA fragments spanning 1.4 kb of E4 5′-flanking sequences were individually added to the binding reaction. E4 fragment 4 (designated E4-4; −353 to +65) eliminated the formation of the C1 complex while others did not (E4-3 in Figure 7 and data not shown). We also found that labeled E4 fragment 4 reacted with a nuclear protein in ripe fruit extracts, and that addition of unlabeled E8 fragment 1 eliminated the binding (S.Cordes and R.Fischer, unpublished results). These results suggest that DNA sequences of two coordinately transcribed fruit ripening genes, E8 fragment 1 and E4 fragment 4, bind to the same factor.

Discussion

E8 gene transcripiton is both responsive to ethylene and under strict developmental control during fruit ripening (Lincoln *et al.*, 1987). To begin to elucidate the mechanisms that coordinate gene expression during fruit ripening, we have begun studies to identify *cis*-acting DNA sequences and *trans*-acting protein factors that may regulate E8 gene expression.

cis-acting DNA sequences

We have identified a family of genes that are homologous to the E8 mRNA (Figure 1) and have isolated and determined the structure of an E8 gene (Figure 2). We report here that a tagged E8 gene is expressed at the proper time during fruit development, and in response to ethylene hormone in transgenic plants (Figure 4). This result verifies that the cloned E8 gene is a functional gene whose expression is strictly controlled during plant development. Furthermore, it shows that the DNA sequences that regulate E8 gene expression are present on a 4.4 kb restriction fragment that includes 2 kb of 5′-flanking sequence, 1.8 kb of transcribed sequence, and 0.5 kb of 3′-flanking sequence. To our knowledge, the correct expression of a hormonally regulated gene in transgenic plants has not been reported previously. The precise location of regulatory sequences will be determined in the future by monitoring the expression of a series of *in vitro* mutagenized tagged E8 genes in transformed tomato plants.

trans-acting protein factors

We have shown that ripening tomato fruit nuclei contain DNA-binding activities that interact *in vitro* with multiple sites flanking the E8 gene (Figure 5). Analysis of the strongest activity, C1, indicated that a nuclear protein is involved in the binding reaction (Figure 6). Competition experiments showed that the interaction of the C1 protein with E8 gene fragment 1 is highly specific, and that the C1 complex is distinct from the C3 complex (Figure 7).

Whether the C1 complex regulates E8 gene transcription during fruit ripening is not known; however, three results suggest that it may play a role. First, the binding site is located within the broad region shown to be required for developmentally regulated and ethylene-inducible E8 gene expression (Figure 4). Second, the level of C1 DNA-binding protein activity is correlated with the level of E8 gene expression during fruit ripening. That is, ripe fruit extracts contain significantly more activity that unripe fruit extracts (Figure 5). Third, as shown in Figure 7, the C1 DNA-binding protein appears to interact with sequences flanking two coordinately transcribed (Lincoln and Fischer, 1988a) heterologous fruit ripening genes, E8 gene fragment 1 (−1088 to −682) and E4 gene fragment 4 (−403 to +65).

Recently, it has been shown that plant genes are flanked by enhancer regions that are required for high level expression (Kaulen *et al.*, 1986; Simpson *et al.*, 1986; Baumann *et al.*, 1987; Stougaard *et al.*, 1987). These regulatory regions, like enhancers from other eukaryotic systems, are often relatively distant from the site of transcription initiation, and function when moved to other sites. The considerable distance between the E8 C1 binding site and E8 transcription initiation site, and the fact that the spatial separation is significantly compressed in the E4 gene, is consistent with the hypothesis that the common binding sites represent enhancer-like regions required for high level gene expression. Comparing DNA sequences of E8 fragment 1 and E4 fragment 4 revealed several regions of 12−20 bp that had 85% or greater sequence identity (data not shown). DNase footprint analysis will be used to precisely delineate the common binding sites.

The role played by the C1 complex in the regulation of gene expression by ethylene is not known. Although treating unripe fruit for 2 h with ethylene induced E8 gene transcription (Lincoln and Fischer, 1988a), we did not detect formation of the C1 complex when labeled E8 fragment 1 DNA was reacted with nuclear extracts from ethylene-treated fruit (data not shown). A more slowly migrating complex, C1*, was generated which, however, also binds to competitor pUC18 DNA. Thus, the specificity of the C1* complex, and its relationship to the C1 complex, are unknown. In this regard it is important to note that the correlation between E8 gene expression and high levels of ethylene is not absolute. That is, there are instances during wild-type (Lincoln and Fischer, 1988a) and mutant (Lincoln and Fischer, 1988b) fruit development where one observes partial activation of E8 gene transcription in the absence of elevated levels of ethylene, suggesting that E8 gene expression may be regulated by several different mechanisms. In the future the role of C1 and other factors (Figure 5) will be elucidated by mutagenizing their respective *in vitro* binding sites and assaying the effect of the lesion on E8 gene expression in transgenic tomato plants.

Materials and methods

Plant material

Tomato (*L.esculentum* cv. VFNT Cherry) plants were grown under standard greenhouse conditions. Fruit maturity stage was determined as described

in Lincoln *et al.* (1987). Unripe fruit were green, full size, and evolved low levels of ethylene (0.6 ± 0.2 nl/g h). Ripening fruit were 20% red, full size, and evolved elevated levels of ethylene (3.5 ± 1.0 nl/g h). To treat plant material 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.

Isolation of clones

A library of tomato (*L.esculentum* cv. VFNT Cherry) genomic DNA in the Charon 35 vector was screened by plaque hybridization with labeled pE8 to obtain genomic clones containing an E8 gene. A cDNA library enriched for full-length cDNA clones of tomato ripe fruit mRNAs (DellaPenna *et al.*, 1986), was screened by colony hybridization with labeled pE8 to obtain full-length E8 cDNA clones.

Isolation of plant nucleic acids

Polysomal poly(A)$^+$ mRNA was isolated as described in Lincoln *et al.* (1987) and tomato leaf genomic DNA was isolated by procedures described in Fischer and Goldberg (1982).

Gel blot hybridization experiments

RNA was denatured with formaldehyde, fractionated by electrophoresis on agarose gels, blotted to nitrocellulose paper, and hybridized to labeled DNAs as described by Maniatis *et al.* (1982). DNA was digested with restriction endonucleases, subjected to agarose gel electrophoresis, blotted to nitrocellulose paper, and hybridized to ^{32}P-labeled DNAs as described by Fischer and Goldberg (1982).

Determination of DNA sequences

The 4.4 kb *Eco*RI restriction fragment from λE8-3 that spans the E8 gene was subcloned in both orientations into pUC118 to enable both sense and anti-sense single-strand template preparation (Vieira and Messing, 1987). Deletions were generated using exonuclease III (Henikoff, 1984). Nucleotide sequences were determined using the dideoxy chain-termination method (Sanger *et al.*, 1977). DNA sequence analysis and searches of the NIH GenBank, EMBL Nucleotide Sequence Library and NBRF Protein Identification Resource were performed using the Bionet National Computer Resource for Molecular Biology.

S1 nuclease protection and primer extension experiments

S1 nuclease protection assays were carried out using the procedure of Favaloro *et al.* (1980). Primer extension analysis was performed using methods described by Dunsmuir *et al.* (1988).

Plant transformation

The strategy of Zambryski *et al.* (1983) was used. In brief, the 4.4 kb *Eco*RI restriction fragment containing the tagged E8 gene (designated pE8R4.4T) was subcloned into the intermediate vector pMLJ1 (de Block *et al.*, 1984) and transferred into the disarmed *Agrobacterium* pGV3850 Ti-plasmid vector by the procedure of Van Haute *et al.* (1983). Sterile tomato (*L.esculentum* cv. Pearson Improved) cotyledon pieces were incubated on tobacco feeder cells and infected with *Agrobacterium* with the pGV3850:pMLJ1E8R4.4T co-integrate plasmid by the procedure of Fillatti *et al.* (1987). Transformants were selected with 50 mg/l kanamycin on the regeneration medium of Shahin (1985).

Preparation of nuclei and nuclear extracts

Nuclei were isolated as described by Walling *et al.* (1986) except that all buffers were adjusted to 0.1 mM phenylmethylsulfonylfluoride immediately before use. Proteins were extracted from the nuclei by the procedure of Miskimins *et al.* (1985).

DNA gel electrophoresis mobility retardation assay

DNA restriction fragments used in the binding reactions were isolated from low-melting-point agarose gels. Binding was carried out in 15 μl reactions containing 0.25 ng of labeled DNA restriction fragment, 1.5 μg nuclear protein, 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 10% glycerol at 30°C for 30 min. In addition, 4.4 μg of poly(dI-dC)·poly(dI-dC) duplex DNA was included in all reactions. Titration experiments with labeled E8 restriction fragments indicated that this amount of poly(dI-dC)·poly(dI-dC) was sufficient to eliminate nonspecific protein–DNA interactions. The DNA–protein complex was separated from unbound DNA by electrophoresis on 4% acrylamide gels as described by Singh *et al.* (1986). Following electrophoresis, the gel was dried and exposed to X-ray film with an intensifying screen for 12–24 h.

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References

Baumann,G., Raschke,E., Bevan,M. and Schoffl,F. (1987) *EMBO J.*, **6**, 1161–1166.

Biale,J.B. and Young,R.E. (1981) In Friend,J. and Rhodes,M.J.C. (eds), *Recent Advances in the Biochemistry of Fruit and Vegetables*. Academic Press, London.

Brown,J.W.S. (1986) *Nucleic Acids Res.*, **14**, 9549–9559.

De Block,M., Herrera-Estrella,L., Van Montagu,M., Schell,J. and Zambryski,P. (1984) *EMBO J.*, **3**, 1681–1689.

DellaPenna,D., Alexander,D.C. and Bennett,A.B. (1986) *Proc. Natl. Acad. Sci. USA.*, **83**, 6420–6424.

Dunsmuir,P., Bond,D., Lee,K., Gidoni,D. and Townsend,J. (1988) In Gelvin,S. and Schilperoot,R. (eds) *Plant Molecular Biology Manual*. Kluwer Academic Publishers, Dordrecht, pp. C1/1–17.

Favaloro,J., Treisman,R. and Kamen,R. (1980) *Methods Enzymol.*, **65**, 718–749.

Fillatti,J.J., Kiser,J., Rose,B. and Comai,L. (1987) In Nevins,D.J. and Jones,R.A. (eds) *Tomato Biotechnology*. Alan R. Liss, New York, pp. 199–210.

Fischer,R.L. and Goldberg,R.B. (1982) *Cell*, **29**, 651–660.

Grierson,D. (1985) *CRC Crit. Rev. Plant Sci.*, **3**, 113–132.

Heidecker,G. and Messing,J. (1986) *Annu. Rev. Plant Physiol.*, **37**, 439–466.

Henikoff,S. (1984) *Gene*, **28**, 351–359.

Holdsworth,M.J., Bird,C.R., Ray,J. and Grierson,D. (1987) *Nucleic Acids Res.*, **15**, 732–739.

Kaulen,H., Schell,J. and Kreuzaler,F. (1986) *EMBO J.*, **5**, 1–8.

Kuhlemeier,C., Green,P.J. and Chua,N.-H. (1987) *Annu. Rev. Plant Physiol.*, **38**, 221–257.

Lincoln,J.E., Cordes,S., Read,E. and Fischer,R.L. (1987) *Proc. Natl. Acad. Sci. USA.*, **84**, 2793–2797.

Lincoln,J.E. and Fischer,R.L. (1988a) *Mol. Gen. Genet.*, **212**, 71–75.

Lincoln,J.E. and Fischer,R.L. (1988b) *Plant Physiol., in press.*

Lyons,J.M. and Pratt,H.K. (1964) *Proc. Am. Soc. Hortic. Sci.*, **84**, 491–500.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 202–203.

Mansson,P.-E., Hsu,D. and Stalker,D. (1985) *Mol. Gen. Genet.*, **200**, 356–361.

Miskimins,W.K., Roberts,M.P., McClelland,A. and Ruddle,F.H. (1985) *Proc. Natl. Acad. Sci. USA.*, **82**, 6741–6744.

Rhodes,M.J.C. (1980) In Thimann,K.V. (ed.) *Senescence in Plants*. CRC Press, Florida, pp. 157–205.

Rick,C. (1978) *Sci. Amer.*, **239**, 77–87.

Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA.*, **74**, 5463–5467.

Shahin,E.A. (1985) *Theor. Appl. Gen.*, **69**, 235–240.

Simpson,J., Van Montagu,M. and Herrera-Estrella,L. (1986) *Science*, **233**, 34–38.

Singh,H., Sen,R., Baltimore,D. and Sharp,P.A. (1986) *Nature*, **319**, 154–158.

Stougaard,J., Sandal,N.N., Gron,A., Kuhle,A. and Marcker,K.A. (1987) *EMBO J.*, **6**, 3565–3569.

Van Haute,E., Joos,H., Maes,M., Warren,G., Van Montagu,M. and Schell,J. (1983) *EMBO J.*, **3**, 411–417.

Vieira,J. and Messing,J. (1987) *Methods Enzymol.*, **153**, 3–11.

Walling,L., Drews,G.N. and Goldberg,R.B. (1986) *Proc. Natl. Acad. Sci. USA.*, **83**, 2123–2127.

Yang,S.F. (1985) *Hort. Sci.*, **20**, 41–45.

Zambryski,P., Joos,H., Genetello,C., Leemans,J., Van Montagu,M. and Schell,J. (1983) *EMBO J.*, **2**, 2143–2150.

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