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Ethylene control of E4 transcription during tomato fruit ripening involves two cooperative *cis* elements

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Abstract

E4 gene transcription is controlled by ethylene during tomato fruit ripening. To define the ethylene-responsive promoter elements, we have tested the activity of mutations of the E4 promoter, and of chimeric genes in transient assay. Using a set of linker scan mutations of the region from −160 to −91, we determined that sequences located between −150 and −121 bp from the transcription start site are required for normal levels of ethylene-regulated transcription. However, E4 sequences from −193 to −40 were not able to confer ethylene-responsiveness to the minimal (−46) 35S promoter. The E4/E8 binding protein (E4/E8 BP) interacts with sequences in the 5′-flanking regions of both E4 and the coordinately regulated E8 gene, and its role in regulation of E4 transcription was investigated. The E4 binding site spans the E4 TATA box, and so mutations of this site were limited to those that did not disrupt the E4 TATA box. Mutations of this site which reduced affinity for the E4/E8 BP also resulted in reduced activity in transient assay, supporting a role for this element in normal regulation of the gene. Fusion of the 35S enhancer to E4 sequences from −85 to +65 did not result in an ethylene-responsive promoter, indicating that the E4/E8 BP-binding site is not sufficient for ethylene response. We conclude that at least two *cis* elements are required for ethylene-responsive transcription of the E4 gene during fruit ripening, one between −150 and −121 and the other between −40 and +65.

Introduction

Ethylene has many diverse functions in normal plant growth and development, including regulation of growth, seed germination, leaf abscission, senescence and fruit ripening [1]. In addition, ethylene is produced in response to many stresses [1]. The role of ethylene in controlling fruit ripening is supported by extensive physiological data and postharvest practices [3]. More recently, ethylene was unequivocally shown to be required for the initiation and maintenance of tomato fruit ripening by antisense inhibition of 1-aminocyclopropane-1-carboxylate (ACC) synthase, the gene encoding the enzyme that carries out a key regulatory step in ethylene biosynthesis [20]. When ethylene biosynthesis was inhibited in these transgenic plants, fruit ripening was inhibited, and ripening could be restored by treatment of the fruit with exogenous ethylene. Ethylene has been shown to activate transcription of specific genes during tomato fruit ripening [14], and the products of these genes are thought to carry out the various processes that occur during fruit ripening. The regulation of the E4 gene by ethylene has been extensively characterized. Although its function during fruit ripening is not yet known, the predicted polypeptide encoded by E4 has significant sequence identity with a peptide methionine sulfoxide reductase protein from *Escherichia coli* [24]. Transcription of E4 is rapidly activated by ethylene in both leaves and fruit, and E4 is not expressed in mutant fruit which do not produce ethylene and do not ripen [15, 16]. Treatment of the mutant fruit with ethylene results in normal levels of E4 mRNA accumulation [16]. An inhibitor of

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ethylene action, norbornadiene, also inhibits E4 gene expression during fruit ripening [14]. Thus, the expression of E4 appears to have an absolute requirement for elevated levels of ethylene. We are interested in the molecular mechanism of activation of E4 gene transcription by ethylene.

DNA sequences required for ethylene-responsive transcription of the E4 gene during tomato fruit ripening were shown to be present within 161 bp 5′ to the transcription start site [18]. An internal deletion of sequences from −140 to −85 eliminated ethylene-responsive transcription in transient assay. A nuclear protein was shown to interact with sequences from −142 to −110, but its DNA-binding activity did not correlate with E4 gene activity. This DNA-binding protein had greatest activity in nuclear extracts from unripe fruit, but its activity was reduced in extracts from ethylene-treated unripe fruit, and was absent in extracts from ripening fruit [18]. This protein could either be a repressor that interacts with sequences close to those of the binding site of a positive regulator of E4 transcription, or its affinity for the DNA could be reduced in its activated state [18].

A second DNA-binding protein has been studied that interacts with sequences of both the E4 promoter and sequences in the 5′-flanking region of E8, a gene that is regulated coordinately with E4 during fruit ripening, and which is also responsive to ethylene [5, 4]. The binding activity of this protein correlates with the expression of E4 and E8. That is, it has greater DNA-binding activity in extracts from ripening fruit than in extracts from unripe fruit [4]. The binding site for this protein spans the E4 TATA box but is located from position −936 to −920 upstream of the E8 transcription start [4]. We will refer to this protein as the E4/E8-binding protein (E4/E8 BP).

Ethylene has also been shown to activate transcription of genes encoding pathogenesis-related proteins such as chitinase [29], glucanase [31], and basic PR proteins [28]. A similar *cis*-element, which contains a GCC motif, is found upstream of many of these pathogenesis-related genes [29]. In some cases, the GCC box has been shown to be necessary for ethylene-responsive transcription [29], and a 47 bp fragment containing two copies of this motif was shown to be sufficient to confer ethylene responsiveness to a neutral promoter [21]. A family of genes encoding DNA-binding proteins that interact with the GCC box were cloned, and their products were called the ethylene-responsive element binding proteins (EREBP; [21]). Interestingly, the accumulation of mRNAs for these genes is induced by ethylene. No GCC box motif is present within the 5′-flanking regions of the E4 or E8 genes. Therefore, different proteins are likely to be involved in ethylene activation of genes during fruit ripening and in response to pathogens.

The induction of genes by ethylene during carnation flower petal senescence has also been studied, and this work has focused on the regulation of the glutathione *S*-transferase (GST1) gene [10]. A 126 bp sequence from the 5′-flanking region of the GST1 gene was shown to be both necessary and sufficient for ethylene regulation in carnation petals in transient assay [10]. A protein that interacts with this sequence was identified, and part of its binding site resembles part of the binding site for the protein that interacts with E4 sequences from − 142 to −110. It is possible that a related protein could be involved in regulating gene transcription during fruit ripening and flower petal senescence.

We have more closely examined the sequences required for ethylene responsiveness of the E4 gene by measuring the activity of various mutations in transient assay. Our results indicate that a single element is not sufficient for ethylene responsive transcription of E4, but that at least two elements are necessary. Using site-specific mutagenesis techniques we have identified two *cis*-elements that play a role in activation of E4 transcription by ethylene.

Materials and methods

Plant material

Tomato plants (*Lycopersicon esculentum* cv. VFNT Cherry) were grown under standard greenhouse conditions. The developmental stage of the fruit was determined as described [14].

Construction of chimeric genes and mutations

The wild-type E4 promoter consisted of sequences from −193 to +65 fused to the luciferase (LUC)-coding sequence in pUC119 [18].

Linker scan mutagenesis and construction of the TATA1 and PGTATA + 2 bp shift mutations was accomplished using the method of Kunkel *et al.* [13]. For the linker scan mutations, the sequence GCGC-CGGCGC, which contains a site for the restriction endonuclease *Not*I, was substituted for 10 bp of E4 sequence. A series of constructs was made that had

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                -160      -150      -140      -130      -120      -110      -100      -90
wild type  CAAAACCTAACACAAGTTTGTTTTTGTTTTTACTACCAACAAGAAATTCAAATGGCAAATGTATAACGCATCT

           -160     -151
pΔ160      -GCGCCGGCGC--------------------------------------------------------------

                     -150     -141
pΔ150      -----------GCGCCGGCGC----------------------------------------------------

                               -140     -131
pΔ140      ---------------------GCGCCGGCGC------------------------------------------

                                         -130     -121
pΔ130      -------------------------------GCGCCGGCGC--------------------------------

                                                   -120     -111
pΔ120      -----------------------------------------GCGCCGGCGC----------------------

                                                             -110     -101
pΔ110      ---------------------------------------------------GCGCCGGCGC------------

                                                                       -100     -91
pΔ100      -------------------------------------------------------------GCGCCGGCGC--
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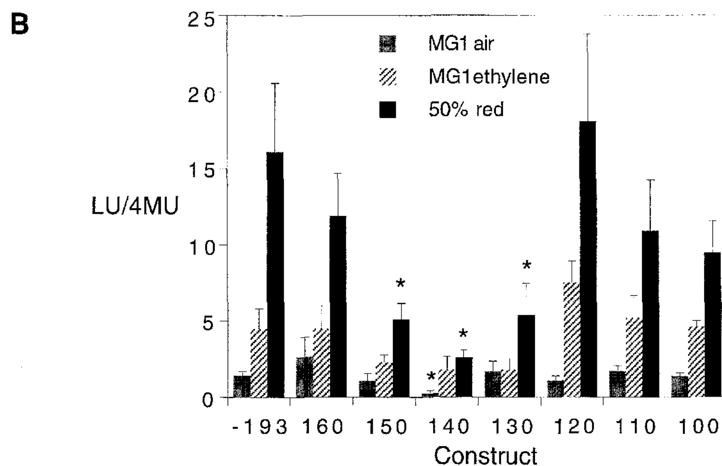
B



C

Positive element (linker scan result)	-150 ACAAGTTTGTTTTTGTTTTTACTACCAACA -121	
Footprint with unripe fruit extract	-142 GTTTTTGTTTTTACTACCAACAAGAAATTCAAA -110	

Figure 1. A (top). Sequence of the wild type E4 promoter from −161 to −90 bp, and of the linker scan mutations. B. Expression of linker scanning mutants in transient assay. −193, wild-type E4 promoter; MG1 air, mature green stage 1 (unripe) fruit treated with air; MG1 ethylene, mature green stage 1 fruit treated with ethylene; 50% red, 50% red (ripening) fruit. The level of gene expression is presented as the ratio of light emission events per minute per mg protein (LU) to GUS activity, which was monitored by formation of the product 4-methylumbelliferone (4MU), and expressed as picomole 4MU per mg protein per minute. Error bars represent the standard error, and a minimum of four independent determinations were completed for each construct. An asterisk (*) indicates that the data point was determined to be different from wild type of the same stage or treatment with $P > 0.95$. C. Comparison of sequences identified by the linker scanning mutations to be required for normal gene expression with the binding site for a nuclear protein that has high DNA-binding activity in extracts from unripe fruit, and low activity in extracts from ethylene-treated or ripening fruit [18]. The sequences most important for activity in transient assay are underlined.

this substitution covering the region from −160 to −91 (see Fig. 1A).

Construction of the −46 35S-LUC construct required several steps. First, pSP72 (Promega) was digested with *Eco*RI and *Sst*I, and ligated with the

NOS terminator isolated from pBI121 (Clontech) with the same restriction enzymes. Then, the LUC-coding sequence [22] modified as described [18] was cloned into this plasmid using the restriction sites *Sst*I and *Bam*HI. The 35S TATA sequences were obtained from the plasmid pMBL GUS-46 (gift of Dr Nam-Hai Chua) by digestion with *Bgl*II and *Hin*dIII, and the ends of the fragment were filled in with T4 DNA polymerase. This fragment was ligated with the promoterless LUC-NOS plasmid, which was digested with *Sal*I and *Bam*HI and filled in with T4 DNA polymerase. The *Bgl*II site at the 3′ end of the pSP72 polylinker was destroyed by partial digestion, filling-in with T4 DNA polymerase and re-ligation.

To make the E4(−193 to −85)-35S(−46)-LUC construct, the sequences from −193 to −85 were isolated from the E4 promoter with the enzymes *Dra*I and *Alu*I. The ends of the fragment were filled in with Klenow, ligated with *Hin*dIII linkers, and digested with *Hin*dIII. After gel purification, the fragment was ligated with *Hin*dIII-digested −46 35S-LUC vector. A construct with 3 copies of the E4(−193 to −85) fused to the −46 35S-LUC vector was also isolated. To make the construct including E4 sequences from −193 to −40, the E4 sequences were isolated from a subclone including this region using an *Eco*RI site in the pBS KS- polylinker and an *Nla*III site within the E4 promoter. The fragment was subcloned into pUC118 using the restriction sites *Eco*RI and *Sph*I. The 150 bp E4 fragment was finally fused to the 35S(−46) promoter as an *Eco*RI (filled-in)/*Hin*dIII fragment using the *Pvu*II and *Hin*dIII sites of the 35S(−46)-LUC vector. The 35S(−800 to −90)-E4(−85 to +65) construct was assembled in a three way ligation using the promoterless LUC coding sequences in pUC119 digested with *Bam*HI and *Hin*dIII, the −85 to +65 E4 fragment excised with *Bam*HI and *Alu*I, and the 35S enhancer region [8] removed from pBI221 (Clontech) with *Hin*dIII and *Eco*RV.

Other site-specific mutations of the E4 promoter were produced using the overlap extension PCR mutagenesis method [9].

The sequences of all constructs were verified by double-stranded DNA sequence analysis. Plasmid DNAs were purified either by CsCl gradient centrifugation or on Qiagen columns (Qiagen, Chatsworth, CA).

Transient gene expression assay

The E4 promoter has been shown to have similar activity in transient assay as in stably transformed plants [18]. Using both gene introduction methods, the 193 bp promoter fragment was ethylene responsive in unripe fruit, and was active in ripening fruit. Therefore, we used this previously characterized transient assay system for a finer mutational analysis of the E4 promoter. Tomato fruit was prepared for particle bombardment as described [18]. Particle bombardment was either performed as described ([18], Figs. 1 and 4), or by using a Biolistic PDS 1000/HE particle delivery system (BioRad, Figs. 2 and 3). In both cases, the test E4-LUC plasmid was co-precipitated onto the particles with a 35S promoter-GUS plasmid, which served as a control for transformation efficiency. In the case of the helium gun, 2.5 μg 35S-GUS DNA and 1.65 μg E4-LUC DNA was precipitated onto 3 mg of 1.6 μm gold particles using the procedure recommended by BioRad. The amounts of test (LUC) plasmids was adjusted so that equal molar amounts were compared. The helium bombardment pressure was 1828 kPa, the gap distance was 32 mm, and the traveling distance of the particles to the fruit was 6 cm. A coarse stopping screen, made of stainless steel mesh of 2 mm × 2 mm, was used, and tomato fruit were restrained during bombardment with a galvanized steel grid of 12 mm × 12 mm.

Bombarded tissue was incubated for 2 days at room temperature. Ethylene-treated tissue was exposed to 30–40 μl/l ethylene during the 2-day incubation in a 35 l plexiglass chamber at 100% relative humidity.

After incubation, fruit was frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. For Figs. 1B and 4, a protein extract was prepared, and LUC and GUS activities were measured as described [18]. For Figs. 2 and 3, the frozen fruit powder was homogenized in Promega Luciferase Assay Buffer (Promega, Madison, WI) which was modified by the addition of 0.3 M Tris phosphate buffer pH 7.8 [17], and insoluble material was removed by centrifugation at 4°C. Luciferase (LUC) activity was immediately assayed using the Promega Luciferase Assay System, and a scintillation counter (Beckman model LS 5000TA) equipped with a single photon counter. β-glucuronidase (GUS) activity was measured in the same protein extract, using the procedure described by Jefferson *et al.* [11]. The level of gene expression was determined by normalizing LUC activity with respect to GUS activity. Differences between data points were analyzed by Student's t-test.

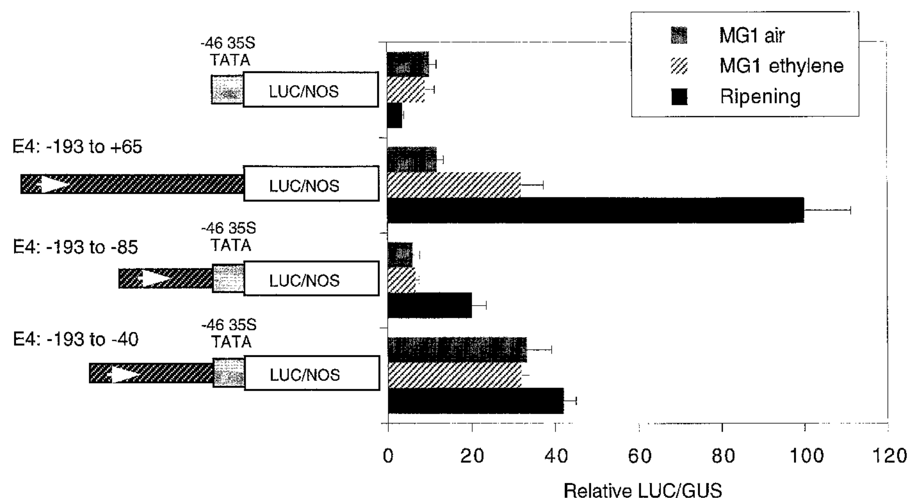


Figure 2. Activity of upstream E4 sequences in transient assay. The white arrow within the E4 sequences represents the upstream element identified by linker scan mutant analysis to be necessary for normal transcriptional activity. Fruit stages and treatments are the same as in Fig. 1. Expression is relative to wild type E4 (−193 to +65) in ripening fruit. Error bars represent the standard error, $n = 3$ to 12.

Gel shift assay

Isolation of nuclear proteins from tomato fruit and gel shift assays were performed as described [5]. Probe and competitor DNAs were isolated from gels using DEAE membranes (Schleicher and Schuell, Inc., Keene, NH), and the probe was end-labeled with Klenow [27].

Results

Identification of sequences required for E4 gene transcription in response to ethylene and during fruit ripening by linker-scan analysis

A *cis* element required for ethylene responsive expression of E4 was localized to the region between −140 and −85 bp from the E4 transcription start by an internal deletion of those sequences [18]. In addition, sequences between −161 and −140 were shown to be important for full activity of the E4 promoter. In order to more precisely define the critical sequences in this region, a linker scan mutation series of the E4-LUC gene was constructed in which a 10 bp sequence composed of Gs and Cs was substituted for E4 sequences every 10 bp from −160 to −91 (Fig. 1A). Expression of these constructs in unripe (mature green 1) fruit treated with air or ethylene, and in ripening (50% red) fruit, was analyzed in transient assay (Fig. 1B). The results showed that the sequences from −140 to −131 were the most critical for expression of the gene. Substitution of this region reduced the overall level of expression in every fruit stage, with a 6-fold reduction of expression in ripening fruit, but it did not completely eliminate ethylene-responsive expression. The sequences between −150 to −141 and −130 to −121 were also important for activity. Each of these mutations reduced expression in ripening fruit about 3-fold. Substitution of sequences from −130 to −121 eliminated ethylene-induced expression in unripe fruit, but still allowed an increase in expression during fruit ripening. The sequences shown by the linker scan analysis to be most important for gene expression overlap with the sequences identified by DNAse I footprinting, from − 142 to −110, that interact with a nuclear protein that is present in extracts from unripe fruit but significantly reduced in extracts from ethylene-treated or ripening fruit ([18]; Fig. 1C).

No significant matches with the GTTTTTGTTTTT sequence, which forms the core of the region that is critical for gene expression, was found in the 5′-flanking regions of other genes that are regulated by ethylene including E8 [6], chitinases from bean and tobacco (GenBank accession numbers S43926, X51599, S54701, X64519), or the carnation GST1 (L05915) and SR12 (X57171) genes, or within the 5′-flanking regions of other genes expressed during tomato fruit ripening such as polygalacturonase (M37304), ACC synthase (L34171, M88487), or ACC oxidase (X58273, Y00478).

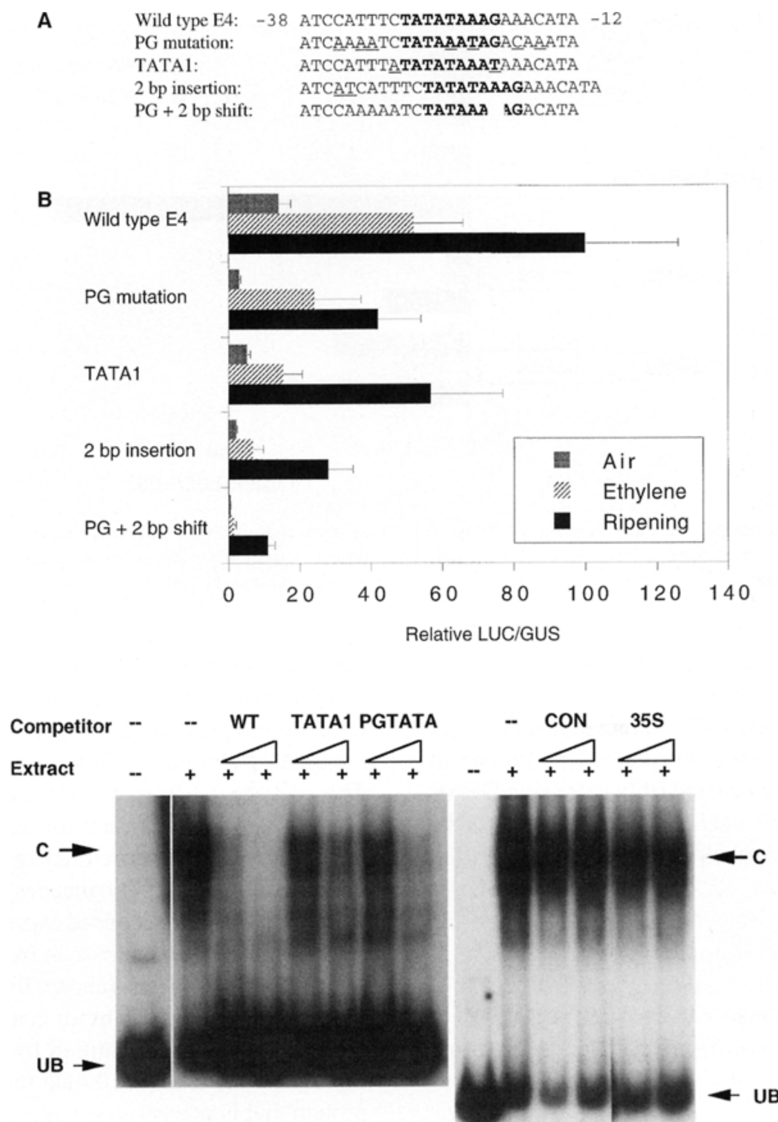


Figure 3. Activity of downstream E4 sequences in transient assay. A. Sequences of the wild type E4 TATA region and the various site-specific mutations. The TATA boxes are in bold, PG sequences [2] different from E4 are underlined, the two base changes in the TATA1 mutation are underlined, and the 2 bp insertion is underlined. B. Expression of specific mutants in transient assay. Fruit stages and treatments are the same as in Fig. 1. Expression is relative to wild-type E4 in ripening fruit. Error bars represent the standard error, $n = 3$. C (bottom). Gel shift assay and competitions. E4 sequences from -193 to $+65$ were labeled and reacted with nuclear extracts from 50% red fruit. Competitors were added as a $10\times$ (small point of triangle), or $50\times$ molar excess (large side of triangle). C, protein/DNA complex; UB, unbound DNA. The competitors used are as follows: WT, wild-type E4 sequences; TATA1, TATA1 mutation; PG TATA, E4 TATA converted to PG sequences; CON, non-specific competitor consisting of sequences from the PG gene from 7192 to 7218 [2]; 35S, sequences from the 35S promoter extending to -46 bp and including the 35S TATA box.

Activity of the -193 to -40 region of the E4 promoter

To determine whether the sequences of the upstream region shown to be required for ethylene-responsiveness are sufficient for ethylene response, several constructs were made in which these sequences were fused to the minimal promoter from the 35S gene from Cauliflower Mosaic Virus (CaMV), which included 35S sequences to -46 bp [8]. The construct containing E4 sequences from -193 to -85 fused to the 35S promoter was not responsive to ethylene (Fig. 2). It had significantly higher expression in ripen-

ing fruit than in unripe fruit, and had greater expression than the 35S(−46) promoter alone only in ripening fruit. When inverted, the E4 sequences from −193 to −85 were inactive in every fruit stage (data not shown). When these sequences were placed in three copies upstream of the minimal 35S promoter, the construct was significantly more active than the construct with one copy in every fruit stage, but it was still not ethylene responsive (data not shown). These results suggest that E4 sequences within the −193 to −85 region contain *cis* elements that interact with factors that increase in abundance during fruit ripening and are capable of stimulating transcription. However, although these sequences are necessary for ethylene response, they do not contain sufficient information to respond to ethylene. E4 sequences from −193 to −40 were also fused to the −46 35S minimal promoter, and these sequences were also able to stimulate transcription of the −46 35S promoter in both unripe and ripening fruit, but this construct was also not ethylene-responsive (Fig. 2). The E4(−193 to −40) region was able to stimulate greater levels of expression than the E4(−193 to −85) construct, indicating that an element with constitutive activity is present between −85 and −40. Presumably, in the wild-type E4 promoter the activity of this element is repressed in unripe fruit. More significantly, these experiments indicate that sequences between −193 and −40 are not sufficient for ethylene response, and suggest that sequences downstream from −40 are also required for E4 response to ethylene.

Activity of the E4 TATA region in controlling gene transcription

A candidate *cis* element involved in regulation of E4 was the site which interacted with the E4/E8 BP [4]. The binding site of this protein spans the E4 TATA box, and so mutations to test the activity of this *cis* element were limited to those that preserve the TATA box. We made two site-specific mutations of the E4/E8 BP binding site. For the first one, we mutated the E4 TATA box to be identical to the TATA box for polygalacturonase (PG), a gene that is expressed during fruit ripening but that is not rapidly responsive to ethylene ([14], Fig. 3A). The second mutation (TATA1) changed the two guanines involved in binding with the E4/E8 BP [4] to thymine residues (Fig. 3A). Each of these mutations of the E4/E8 BP-binding site resulted in a decrease in activity in transient assay of about two-fold in each fruit stage examined (Fig. 3B). To learn the effect of these mutations on interaction with the E4/E8 BP, we carried out competitive gel shift reactions. The wild-type E4 promoter region was labeled with ^{32}P and interacted with nuclear extracts from 50% red fruit (Fig. 3C). The ability of both wild-type and mutated sequences to compete for binding was tested by adding unlabeled fragments containing these sequences to the reaction. These experiments indicated that both the PG TATA and the TATA1 mutations of the E4 promoter had significant interaction with the E4/E8 BP, but with decreased affinity. Comparison of the competitions of wild-type E4 sequences and the mutations at two different competitor concentrations indicated that the decrease in affinity of the mutations for the E4/E8 BP was about 5-fold (Fig. 3C). A control competitor consisting of sequences from 7192 to 7218 of the PG 3′-untranslated sequences [2] was not able to compete for binding with the E4/E8 BP. Competition with the TATA region of the 35S gene indicated that the 35S TATA box was not able to compete with the E4 fragment, and suggests that this sequence does not interact with the E4/E8 BP. This result is consistent with the fact that the consensus sequence for the E4/E8 BP-binding site does not include the E4 TATA sequences, but consists of the sequences that flank the E4 TATA box [4], and the sequences flanking the 35S TATA box are quite different from those that flank the E4 TATA box. These results indicate that the E4/E8 BP is important for normal regulation of the E4 gene, since mutations causing reduced affinity for the E4/E8 BP resulted in reduced activity in transient assay.

As a second approach to examine the role of the E4 TATA region in regulation of the E4 gene, we created a mutation in which a 2 bp sequence was inserted at position −35 within the wild-type E4 promoter (Fig. 3A). This mutation caused a significant decrease in transient expression in each fruit stage (Fig. 3B). There was a 3.5-fold decrease in ripening fruit, 7.6-fold decrease in ethylene-treated fruit, and a 6.4-fold decrease in air-treated unripe fruit compared to wild type. To ensure that we had not inactivated an unidentified *cis* element by insertion of the 2 bp, we also created a comparable mutation within the PG TATA mutation. That is, we shifted the PG TATA box downstream 2 bp by adding back 2 bp of E4 sequence at the 5′ end of the PG sequence (PGTATA + 2 bp shift). The addition of this 2 bp sequence caused a further reduction in expression of the PG TATA mutation. The double-mutation had 3.8-fold less activity than the PG TATA mutation in ripening fruit, and 17-fold less activity in ethylene-treated fruit. Both of the mutations with the

additional 2 bp were still responsive to ethylene, but had decreased activity in every fruit stage.

It was possible that the sequences from −85 to +65 encoded qualitative information for ethylene-responsive expression, but that no ethylene-responsive expression was detected from a construct containing only these promoter sequences because of the absence of a general enhancer-type element. To determine whether a heterologous enhancer could provide a general stimulatory role to render the E4 TATA region ethylene-responsive, we fused the strong enhancer of the 35S gene including sequences from −800 to −90 [8] to the E4 sequences from −85 to +65. Although this construct had very high activity in every fruit stage examined, the construct was not ethylene-responsive (Fig. 4). This result indicates that not all of the information necessary for ethylene response is present in the E4 promoter from −85 to +65.

Discussion

We have found that a minimum of two cis-elements is required for ethylene-responsive transcription of the E4 gene. Previous work indicated that sequences from −140 to −85 are required for ethylene-responsive expression [18], yet E4 sequences from −193 to −40 were not able to confer ethylene-responsiveness to the minimal promoter from the 35S gene (Fig. 2). This result implies that a downstream element must exist that is also required for ethylene responsiveness.

The upstream element was further defined by a linker scan analysis of the region from −160 to −91, which indicated that sequences between −150 and −121 are critical for E4 transcription (Fig. 1). Substitution of sequences from −140 to −131 had the greatest effect on expression in every fruit stage, but this construct was still ethylene-responsive. Only substitution of sequences from −130 to −121 completely eliminated ethylene responsiveness, but this construct was expressed at a greater level in ripening fruit than in unripe fruit. These results suggest that *cis* elements within the E4 promoter that are responsive to ethylene can be separated from those responsive to ethylene-independent fruit ripening signals. However, analysis of E4 expression in ripening-impaired and ethylene-insensitive mutants [7], and in fruit treated with an inhibitor of ethylene action [14], indicated that E4 transcription is dependent on ethylene, and is not responsive to ethylene-independent fruit ripening signals. It is possible that the expression of the linker scan mutants observed in ripening fruit is a result of endogenous ethylene production. For most of the constructs, greater expression was observed in ripening fruit than in ethylene-treated fruit, but this increase could be due to the increase in ethylene sensitivity that is thought to occur during fruit ripening [32]. Thus, the exact location of the ethylene-response element within the region from −150 to −121 will require a more detailed analysis. The fact that no single linker scan mutation completely eliminated E4 transcription suggests that more than one *cis* element is present in this region. It is interesting to note that E4 sequences required for ethylene responsiveness include a similar sequence to one that may be involved in ethylene-regulated transcription of the carnation GST1 gene [10]. The sequence TACCAAC from −128 to −122 resembles the GST1 sequence TACCACC that was identified by DNAse I footprinting of a part of the GST1 promoter that contains an ethylene-response element [10].

A candidate for the downstream element involved in activation of E4 transcription by ethylene is the binding site for the E4/E8-binding protein. This binding site (from −34 to −18 bp) spans the E4 TATA box. Therefore, mutagenesis of this site was limited to mutations that did not destroy the E4 TATA box. Two such mutations decreased affinity for the E4/E8 BP, and resulted in a decrease in activity of the gene in each fruit stage examined (Fig. 3). A second line of evidence implicating the coordinated action of two *cis*-elements is that when we inserted a 2 bp sequence at position −35, activity of the promoter was decreased in each fruit stage examined (Fig. 3). A 2 bp insertion would cause a twist in the DNA of about 70°, which could disrupt the interaction of proteins binding to elements on either side of the insertion. For both the site-specific mutations of the E4/E8 BP binding site, and the 2 bp insertion mutations, the fact that expression in every fruit stage was affected, and not just ethylene-responsive expression, does not necessarily mean that the elements involved in ethylene response were not affected. These mutations would reduce the efficiency of binding of the putative transcription factors, or the assembly of a transcription factor complex, but once the factors were bound, and the complex assembled, these factors could carry out their normal activities. Alternatively, it is possible that the upstream element contains all the information necessary for the ethylene response, but its spatial orientation with respect to the basal transcription factors is so critical that no activity was possible from the constructs in which we fused the E4 sequences from −193 to −85 or −193 to

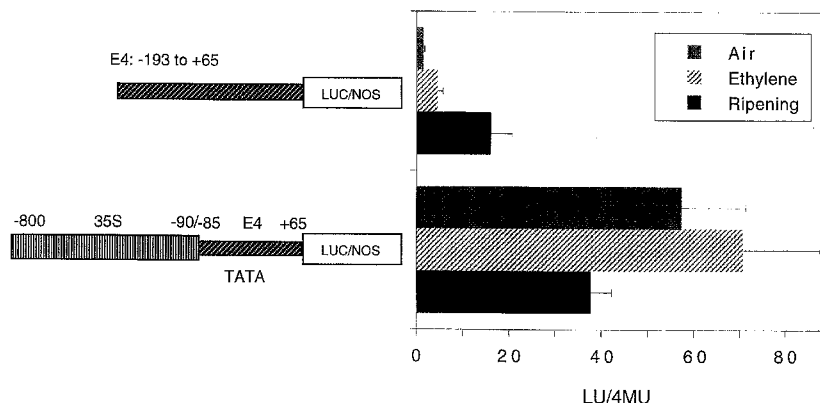


Figure 4. Effect of the 35S enhancer on the activity of E4 sequences from −85 to +65. Units, fruit stages, and treatments are as for Fig. 1. Error bars represent standard errors, $n = 4$.

−40 to the 35S(−46) TATA region. For the E4(−193 to −85)−35S(−46)-LUC construct, the difference in spacing between the upstream element and the TATA box was exactly 10 bp less than in the wild-type E4 promoter. A 10 bp difference would result in approximately one turn of the DNA helix, and should thus have a minimal effect on protein-protein interactions. Furthermore, this construct did have significant activity in ripening fruit, indicating that transcription factors recognizing *cis* elements on this fragment were able to contact the basal transcription factors. We showed that the 35S TATA region does not interact with the E4/E8 BP (Fig. 3C), which may be why it cannot substitute for the E4 TATA region as part of an ethylene-response complex.

Finally, we showed that the downstream element, and sequences from −85 to +65, also do not contain sufficient information to confer ethylene responsiveness to a neutral enhancer (Fig. 4). Taken together, all of the above evidence supports the requirement for at least two *cis* elements for ethylene-responsive transcription. A summary of these findings, and the binding sites for proteins previously shown to interact with the E4 promoter [4, 18], are diagrammed in Fig. 5A.

Our findings suggest that the molecular mechanism of ethylene-responsive transcription of the E4 gene during tomato fruit ripening may differ fundamentally from that of ethylene-regulated transcription of the pathogenesis-related glucanase gene [21], or the flower petal senescence-related GST1 gene of carnation [10]. In each of those cases, a relatively short segment of the 5′-flanking regions of those genes was able to confer ethylene responsiveness to the minimal 35S promoter. However, the minimal fragments from

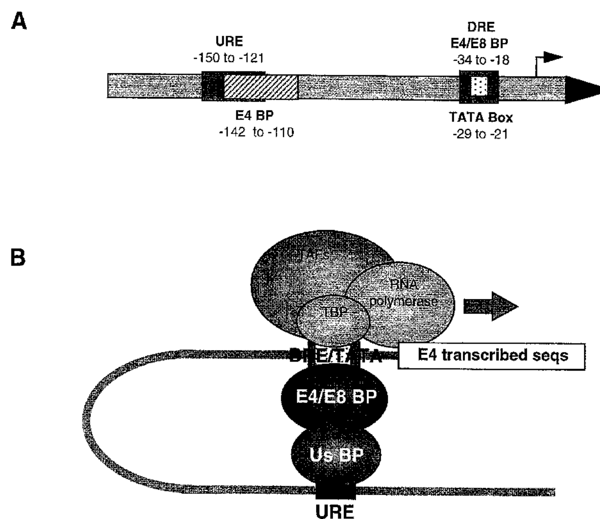


Figure 5. A. Diagram indicating the relative locations of the sequences important in regulation of the E4 gene in response to ethylene, and proteins that have been identified that interact with the E4 promoter. URE, upstream regulatory element; DRE, downstream regulatory element. Arrow on bent line is start of transcription, and numbers refer to bases from the transcription start. B. Model for interaction of proteins in ethylene-responsive transcription factor complex. TBP, TATA binding protein; TAFs, TBP-associated factors; Us BP, upstream binding protein.

these genes shown to have ethylene responsiveness are still large enough to contain binding sites for multiple proteins, and the requirement for multiple elements specific to ethylene response has not been entirely ruled out.

The requirement for the cooperation of two or more *cis* elements for regulation of gene transcription is emerging as a common theme. It has been documented in the case of gibberellic acid (GA) regulation of α-

amylase gene transcription in barley aleurone [25], and is the rule for regulation of gene transcription in response to light [30]. The orientation and spacing of elements within the GA-response complex have been shown to be critical to the level of expression of the promoter [26]. We have also found that the orientation and position of the *cis* elements within the E4 ethylene-responsive promoter are important (Fig. 3, and data not shown).

A model that suggests how the two elements we have identified interact with previously studied DNA-binding proteins to activate gene transcription in response to ethylene is shown in Fig. 5B. We hypothesize that the E4/E8 BP interacts with sequences spanning the E4 TATA box and does not interfere with the binding of TATA-binding protein (TBP). This simultaneous interaction could occur because TBP interacts with the minor groove of the DNA double helix while most transcription factors interact with the major groove [23, 12]. Furthermore, the E4/E8 BP may straddle TBP since the consensus sequence is actually composed of the sequences flanking the E4 TATA box [4]. The model further suggests that a protein that acts as a positive regulator binds at the upstream element and contacts the E4/E8 BP.

Two alternative mechanisms may explain how ethylene stimulates this proposed transcription factor complex to activate E4 gene transcription. The first hypothesis is that a repressor binds to the upstream element in the absence of ethylene, preventing the binding of the positive regulator. A component of ethylene signal transduction would then modify the repressor so that it would no longer bind the upstream element, and the constitutively expressed positive regulator could then bind. This model is suggested by the DNA-binding protein that interacts with the upstream element, and has greater activity in extracts from unripe fruit than in extracts from ethylene-treated fruit [18]. Our linker scan analysis did not provide evidence for a negative regulatory element, but it is possible that positive and negative regulatory proteins could interact with overlapping or even identical sequences, so that mutation of the site would eliminate binding of both negative and positive regulatory proteins, and only destruction of the positive element would be noticed. Also, a DNA-binding protein that interacts with the upstream element with greater activity in ripening fruit was not detected by Montgomery et al. [18]. However, *in vitro* methods of analysis of DNA-binding proteins do not reveal every DNA-binding activity. It is possible that a positive regulator with greater activity in ripening fruit interacts with the upstream element but is not stable when purified by the methods previously used.

The second hypothesis is that a component of ethylene signal transduction modifies the synthesis or activity of one of the positive regulators shown in Fig. 5B that interact with either the upstream or the downstream element. This modification would allow the specific interaction with the proteins binding to the two cooperative elements, and allow them to activate gene transcription. In this model, the protein previously identified that interacts with the upstream element would bind to the upstream element in the inactivated state, but would have decreased affinity for the DNA after activation, and when interacting with the other proteins of the transcriptional apparatus.

The models proposed for ethylene activation of the E4 gene may also be applicable to the E8 gene. The sequences shown to be required for ethylene responsive transcription of E8 are located upstream of −1088 bp [6], and the E8 binding site for E4/E8 BP is at −936 to −920 [4]. Thus, both the E4 and E8 genes share a similar organization in which sequences required for ethylene-responsive transcription are located upstream of the binding site for the E4/E8 BP.

It has recently been pointed out that the E4/E8 BP consensus sequence is found surrounding the TATA box for the PG gene [19]. We have shown that the PG TATA sequences can interact with the E4/E8 BP, though with lower affinity than the E4 TATA region (Fig. 3C). It is possible that the E4/E8 DNA-binding protein, which has greater activity in extracts of ripening fruit than in extracts of unripe fruit, may participate in regulation of many genes during fruit ripening.

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