Journal of Experimental Botany, Vol. 55, No. 402, pp. 1519–1528, July 2004
DOI: 10.1093/jxb/erh162 Advance Access publication 18 June, 2004
This paper is available online free of all access charges (see http://jxb.oupjournals.org/open_access.html for further details)

Journal of Experimental Botany

RESEARCH PAPER

Developmental regulation of peach ACC oxidase promoter–GUS fusions in transgenic tomato fruits

Hangsik Moon* and Ann M. Callahan†

USDA-ARS Appalachian Fruit Research Station, 2217 Wiltshire Road, Kearneysville, WV 25430, USA

Received 25 March 2004; Accepted 1 April 2004

Abstract

A genomic DNA sequence (*PpACO1*) encoding 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) from peach (*Prunus persica* L. Batsch cv. Loring) was isolated. It has four exons interrupted by three introns and 2.9 kb of flanking region 5′ of the translational start codon. Previous work with the cDNA demonstrated that accumulation of the peach ACO message correlated with increasing amounts of ethylene synthesized by the fruit as they ripened. To identify regulatory elements in the peach ACC oxidase gene, chimeric fusions between 403, 610, 901, 1319, 2141, and 2919 bp of the 5′ flanking region of the *PpACO1* sequence and the β-glucuronidase (GUS) coding sequence were constructed and used to transform tomato (*Lycopersicon esculentum* [Mill] cv. Pixie). Fruits from the various promoter lines were analysed for GUS expression by histochemical GUS staining, GUS quantitative enzyme activity determination, and measuring the relative amounts of GUS mRNA. Constructs with the smallest promoter of 403 bp had significant GUS expression in fruit, but not in other tissues, indicating the presence of a region that affects tissue-specific expression. An increase in GUS expression was observed with promoters longer than 901 bp, indicating an enhancer region between −1319 and −901. The full-length promoter of 2919 bp directed GUS expression in the green stage of fruit development, and increased GUS expression as fruit matured, indicating a regulatory region between −2919 and −2141 that controls the temporal expression of the gene in fruit. Only the full-length promoter sequence demonstrated responsiveness to ethylene.

Key words: Peach ACC oxidase, promoter analysis, promoter–GUS fusions, transgenic tomato.

Introduction

The phytohormone ethylene is involved in multiple aspects of plant growth, development, and stress responses including seed germination, cell elongation, fruit ripening, senescence, abscission, wounding, and defence against pathogen attack (Abeles *et al.*, 1992; Klee and Tieman, 2002). The role of ethylene in fruit development and maturation has been intensively studied (Giovannoni, 2001). In climacteric fruits such as peaches and tomato, ripening is associated with a characteristic burst of respiration which correlates with an increase in ethylene production. Ethylene is synthesized from *S*-adenosyl-L-methionine (SAM) via the intermediate 1-aminocyclopropane-1-carboxylic acid (ACC). The conversion of SAM to ACC is catalysed by ACC synthase, and the subsequent oxidation of ACC to ethylene is catalysed by ACC oxidase (Yang and Hoffman, 1984; Ververidis and John, 1991).

The gene encoding ACC oxidase was first isolated as a cDNA from tomato based on its increased expression during fruit ripening and following mechanical wounding of leaves (Holdsworth *et al.*, 1987). Homologous sequences have since been isolated from many other plants including avocado (McGarvey *et al.*, 1990), peach (Callahan *et al.*, 1992), apple (Ross *et al.*, 1992), petunia (Wang and Woodson, 1992), kiwifruit (MacDiarmid and Gardner, 1993), and melon (Lasserre *et al.*, 1996). Expression of the tomato ACC oxidase in the antisense orientation in transgenic plants resulted in the reduction of ethylene biosynthesis, which in turn delayed ripening of the transgenic fruits and extended their storage life (Hamilton *et al.*, 1990; Ayub *et al.*, 1996).

The regulation of the softening process in peach fruit is a key to obtaining higher quality fruit with adequate shelf-life. The longer the fruit is left on the tree to ripen, the better the flavor (Do *et al.*, 1969); however, shelf-life is reduced. Delaying the softening process in peach fruits may allow

* Present address: Department of Genetics, Development and Cell Biology, 353 Bessey Hall, Iowa State University, Ames Iowa 50011, USA.
† To whom correspondence should be addressed. Fax: +1 304 728 2340. E-mail: acallaha@afrs.ars.usda.gov

 © Society for Experimental Biology 2004; all rights reserved

them to be harvested at a time nearer to optimum quality. Efforts to genetically engineer peach and other fruit crops in this manner will benefit from the availability of tissue-specific promoters that control the expression of a transgene in a tissue- and/or temporal-specific manner. As part of a project to manipulate the peach ripening process, a peach cDNA clone encoding an ACC oxidase had previously been isolated (Callahan *et al.*, 1992). RNA gel blot analyses demonstrated that ACC oxidase transcripts greatly increased at late stages of fruit development and were induced in the leaves at lower levels after wounding. In this work, the isolation of a corresponding genomic clone, *PpACO1*, and partial characterization of its 2.9 kb 5′ upstream, non-coding sequence through fusions of various lengths to the coding sequence of *uidA* (β-glucuronidase [GUS]) and subsequent expression in tomatoes are reported. The goal of this investigation was to identify regions of the *PpACO1* promoter that affected fruit expression and developmental regulation in order to design specific promoters in the future.

Materials and methods

Isolation of a peach ACC oxidase genomic clone

Peach (*Prunus persica* L. Batsch cv. Loring) genomic DNA was isolated from leaf nuclei using a modification of the Oncor (Gaithersburg, MD) non-organic DNA extraction kit (Scorza *et al.*, 1990). A combination of *Sau*3AI and *Bam*HI partially digested DNA was size fractionated and cloned into the Lambda vector, λDASHII (Stratagene, La Jolla, CA). Pools of primary plaques, ranging from 2000 to 30 000 plaques were screened by PCR for the presence of ACO-related sequences using a pair of consensus ACO primers EFE4-5′and EFE8-3′ (sequences are shown in Table 1). The screening was done as described by Amaravadi and King (1994). The final screening for individual clones used a phage blot assay where plaques were hybridized with a labelled ACO probe as described earlier (Callahan *et al.*, 1993). Screening of 87 000 primary clones yielded eight clones that had ACO-related sequences. These clones could be grouped into two major classes based on the sizes of the PCR products using primers EFE4-5′ and EFE8-3′, the patterns of restriction endonuclease digestions with *Eco*RI and *Bgl*II, and DNA blot analyses (data not shown). One clone was chosen from each class for subcloning. The Lambda DNA clones containing ACO-related sequences were digested with *Eco*RI, and subcloned into pBluescript SK(+) (Stratagene) that had been digested with *Eco*RI and phosphatased. The resulting plasmid DNA was used to transform *E. coli* DH5α cells (Invitrogen, Carlsbad, CA) and random clones were isolated. One isolate containing an insert for each of the predicted size fragments was kept after confirmation by restriction enzyme digestion of the plasmid DNA. Inserts containing the ACO coding sequence were identified by DNA blot hybridizations with the labelled ACO probe. The positive ACO clone inserts were sequenced in both directions by a combination of a manual sequencing method using Sequenase 2 (USB, Cleveland, OH) and an automated DNA sequencer using BigDye (ABI, Foster City, CA). Sequences were analysed with MacVector (Accelyrs, San Diego, CA) and PCGENE (Intelligenetics, Mountain View, CA).

Peach ACC oxidase promoter–GUS fusion constructs

The entire 2919 bp of the 5′ flanking region of the *PpACOI* was considered the full-length promoter in this study and was used to generate smaller segments, all of which were inserted upstream of the coding region of the *uidA* gene in pBI101 (Clontech, Palo Alto, CA) to make the promoter–GUS fusion constructs. The 2919 bp promoter was amplified using the high-fidelity Platinum *Pfx* DNA polymerase (Life Technologies) with the 2919-5′ primer which contained a *Sal*I site and the 5′ end of the 2919 bp promoter, −2919 to −2908, and the PhACOIR1 primer that represented bases −23 to −1 upstream to the translational start, ATG, of *PpACO1,* and a *Sma*I site (primer sequences are in Table 1). The amplified PCR product was digested with *Sal*I and *Sma*I and inserted upstream of the *uidA* gene in pBI101 (Clontech) cut with *Sal*I and *Sma*I. The 2141 bp promoter fragment was cloned in a similar way using the 2141-5′ primer and PhACO1R1 primer. The 1319 bp promoter–GUS fusion construct was generated by removing 1.6 kb of the 5′ region of the promoter from the 2919 bp promoter–GUS cassette by digestion with *Hin*dIII, which cut in the pBI101 multi-cloning site just upstream from the original *Sal*I site, and once in the 2919 bp *PpACO*1 promoter, followed by religation of the plasmid. The 901 bp fragment of the promoter and the 610 bp fragment were amplified using Herculase Enhanced DNA polymerase (Stratagene) with the 901-5′ primer and PhACOIR1, and the 610-5′ primer and PhACO1R1 respectively, digested with *Sal*I and *Sma*I and inserted into pBI101 predigested with *Sal*I and *Sma*I. The 403 bp fragment was amplified using the high-fidelity Platinum *Pfx* DNA polymerase (Life Technologies) with 403-5′ primer and the PhACOIR1 primer and similarly cloned into pBI101. All primer sequences are presented in Table 1. The ACO promoter–GUS fusions were sequenced to verify the accuracy of the constructs (data not shown). The constructs were introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation and were selected for resistance to kanamycin (50 mg l^{-1}).

Tomato transformation

Transformation of tomato (*Lycopersicon esculentum* Mill. cv. Pixie) cotyledons was performed following the method of Diana Medrano, Cornell University (personal communication). Cotyledons were cut into two or three pieces, precultured for 2 d, inoculated with *Agrobacterium* for 15 min, cocultivated for 2–3 d, and regenerated under selection. Transgenic shoots were selected and rooted on media containing 100 mg l^{-1} kanamycin before being transferred to the greenhouse.

Table 1. *Primer sequences*

Primer name	Sequence
EFE4-5′	5′-GA(C/T)TGGGA(A/G)AGCAC(C/T)TTCT-3′
EFE8-3′	5′-ACCTTGTCATC(C/T)TGGAA-3′
PhACOIR1	5′-AATCCCGGGCTCTCTCTCTCTCTTTGTG TGTG-3′
2919 bp 5′	5′-TTTAAAGCATGCTCGAGGTCGACGGTATC GATAAG-3′
2141 bp 5′	5′-CCAAGTCGACCTACGTAGACATCTTTCTC AAAAGCG-3′
901 bp 5′	5′-TTAAGTCGACAATCAAGCATGGAAAGCCG CCC-3′
610 bp 5′	5′-TATAGTCGACATTTTGTGGTGGAGAGATGC CGC-3′
403 bp 5′	5′-TTAAGTCGACAAGACACCAAGATGGTGGTG GTC-3′
GUS forward	5′-GATCAGCGTTGGTGGGAAAGCGCG-3′
GUS reverse	5′-CTACACTCCCCTCACACCGAGGAA-3′
26S rRNA5′	5′-GCAGCCAAGCCTTCATAGCG-5′
26S rRNA3′	5′-GTGCGAATCAACGGTTCCTC-3′
Tom 541-5′	5′-GGCTGAGGAGTTACTTGACT-3′
Tom 693-3′	5′-TCCTGCGTCTGTATGAGCGC-3′
UidSau-fwd	5′-TCAGCGTTGAACTGCGTGAT-3′
UidSau-rev	5′-TAGTGCCTTGTCCAGTTGCAA-3′

The presence of the promoter–GUS fusions in the transgenic plants was confirmed by PCR using GUS forward and reverse primers (Scorza *et al.*, 1994; see Table 1) and promoter-specific primers (used in cloning the sequences) with genomic DNA extracted from young leaves as the templates. Only those plants with expected PCR products (GUS and ACO promoter) were used in the promoter analyses. Approximately 20 plants were analysed for each construct. Fruit from 20 of each construct were all tested for GUS expression by histochemical analyses, and subsets (2–6) of those were analysed quantitatively by the MUG assay, relative amounts of *PpACO1* mRNA, and non-fruit tissue responses.

Extraction of plant DNA and determination of copy number of insert

Genomic DNA used for PCR was prepared from young leaves of the transgenic tomato plants using the FastDNA Kit and a FastPrep 101 bead beater (Qbiogene, Inc., Carlsbad, CA) according to the manufacturer's protocol with the addition of 1% (w/v) polyvinylpyrrolidone-40 and 1% (v/v) β-mercaptoethanol to the extraction buffer.

Genomic DNA used for copy number determination was extracted as previously described (Callahan *et al.*, 1993) using a CTAB method (Doyle and Doyle, 1990). The DNA was subjected to Real-Time PCR using primer pairs for *uidA*, UidSau-fwd and UidSau-rev, and for *LeACO1*, Tom 541-5′ and Tom 693-3′ (see Table 1) using SYBR Green Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's directions. The ratio of transgene *uidA* to native gene *LeACO*1 was determined for each in order to determine the number of insertion events (Ingham *et al.*, 2001).

GUS assay of the transgenic tomato tissues

Fruit development was divided into six stages based on colour according to the United States Standards for Grades of Fresh Tomatoes (CFR51.1855-51.1877). 'Green', the surface of the tomato is completely green; 'Breaker', a definite break in colour from green to yellow, pink or red but not more than 10% of the surface; 'Turning', 10–30% of the surface shows a definite change in colour; 'Pink', 30–60% of the surface has changed colour to yellow, pink or red; 'Light red', 60–90% of the surface is red colour; and 'Red', more than 90% of the surface shows red colour. Those fruit picked at the Green stage were mature green as judged by size and the amount of locular material around the seeds, and those fruit picked at the Red stage were not over-ripe as defined by softness. Tomato fruits at each stage of development were harvested and sliced longitudinally. Only the middle sections were used for GUS staining and GUS enzyme activity assay. The whole fruit was processed for RNA extraction.

The GUS histochemical assay was performed by immersing tissues in the GUS staining solution (Jefferson, 1987) at 37 °C. Following staining, the samples were fixed in 70% (v/v) ethanol. When the fruit was incubated for more than 2.5 or 3 h in the GUS staining solution, blue staining could be observed in fruits from plants transformed with pBI101 (*uidA* without a promoter) and non-transgenic plants (data not shown). To avoid this false-positive response, the fruit were stained for only 2 h at 37 °C.

GUS activity was determined fluorometrically as described by Jefferson (1987) with a modification in the extraction buffer. Due to the acidity of tomato fruits, 100 mM sodium phosphate, pH 8.0 was used for protein extraction instead of the 50 mM, pH 7.0. The production of 4-methylumbelliferone (4-MU) was measured using a fluorometer (CytoFluor, Applied Biosystems). The amount of 4-MU was determined from a standard curve. Protein concentrations of the samples were determined using Bradford reagent (Bio-Rad, Hercules, CA) and BSA as a standard. GUS activity was expressed as pmol 4-MU min^{-1} μg^{-1} protein.

Ethylene induction and wounding

Leaflets were harvested and dipped in either a control solution containing 0.05% Triton X-100 (v/v) or an ethrel (Rhone-Poulanc, EPA number 264-267) solution containing 0.05% Triton X-100 and 0.282% ethrel (v/v). The control leaves were placed on damp Whatman 3MM paper in a large vacuum jar and sealed, and the ethrel-treated leaves were likewise placed in a separate sealed vacuum jar. The vacuum jars were placed in the greenhouse for 24 h. A 1 ml sample of the head space was analysed for ethylene prior to removing the leaves to verify that only the ethrel treatment initiated ethylene production. Approximately 10 ppm of ethylene was present. The leaves were then stained with GUS solution for 24 h and the reaction stopped by 70% ethanol.

For the wounding experiments, old and young leaves were detached from each plant and wounded by cutting them into 1 cm^2 pieces, which were then placed on damp paper towels and incubated at room temperature. At various time points, leaves were placed into the GUS histochemical stain for 24 h as described above, or proteins were extracted and MUG assays were performed as above. Ethylene measurements were also recorded by placing the leaves in a stoppered flask and sampling headspace at various times in order to verify that the wounding had been enough to generate wound response ethylene.

RNA extraction and Real-Time PCR

RNA was extracted from lyophilized tissue as described earlier (Callahan *et al.*, 1992). Prior to Real-Time PCR, the RNA was DNased using DNA-free kit (Ambion, Austin, TX) according to the manufacturer's directions. RNAs were subjected to Real-Time PCR using the SYBR Green Master Mix kit and reverse transcriptase (Applied Biosystems, Foster City, CA) according to the manufacturer's directions using 1–3 μl of RNA in a 10 μl reaction. RNA was diluted to 0.0005 μg μl^{-1} for the 26S rRNA detection and RNA for determining *uidA* and tomato ACO1 amounts was used at 0.1 μg μl^{-1}. The reactions were run on an ABI 7900 sequence detection machine (Applied Biosystems) programmed to heat for 30 min at 45 °C then 10 min at 95 °C, followed by 40 cycles of 1 min at 60 °C and 30 s at 95 °C. This was followed by a denaturation step to determine the melting point of the products formed to verify that a single identical product was formed. All reactions were done in triplicate and the results were averaged. Standard curves for each primer pair were derived from dilution series, and the relative amount of RNA in each sample was determined from the standard curves. The amounts of the *uidA* and tomato ACO1 RNA were adjusted by the differences in the amount of 26S rRNA to account for any variation in concentrations of the total RNA in each reaction.

Results

Genomic sequence of PpACO1

A 5201 bp cloned fragment of the peach ACO1 gene (*PpACO1*) was sequenced and found to have a 2919 bp 5′ flanking region, a 1273 bp open reading frame interspersed with introns, and a 1009 bp 3′ flanking region (Fig. 1A). The *PpACO1* coding sequence matched the original ACO cDNA sequence (Callahan *et al.*, 1992), and the first 798 bases of sequence 5′ to the ATG translational start were nearly identical to the 798 bases of an existing peach ACO1 promoter sequence (Ruperti *et al.*, 2001) with only 20 base differences (data not presented). The nucleotide sequence of the clone was deposited in GenBank as accession number AF532976.

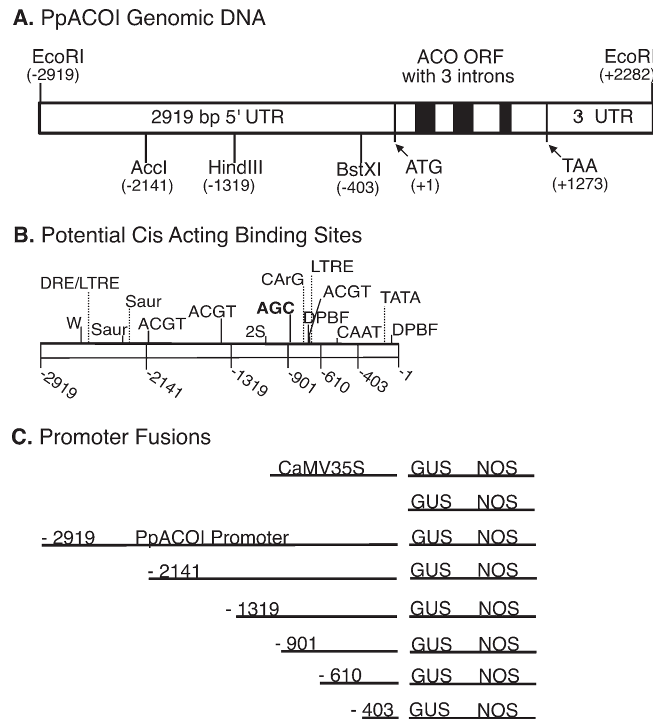

Fig. 1. Schematic representation of the *PpACO1* genomic clone and the subsequent promoter fusions. (A) Presentation of the organization of the *PpACO1* gene based on sequence comparisons with a cDNA clone of ACO. The numbers in parentheses represent the distance from the translation start of the coding sequence. The shaded areas represent intron sequences. Restriction sites are indicated when they were used in cloning different promoter lengths. (B) Schematic drawing of the promoter region of *PpACO1* with potential *cis*-acting factor binding sites (as defined in Higo *et al.*, 1999) marked on the segments used in the promoter analyses. W represents part of a WRKY transcription factor binding site (Rushton *et al.*, 2002); DRE/LTRE represents a binding site for factors involved in the response to drought and low temperature (Baker *et al.*, 1994; Dunn *et al.*, 1998); Saur represents a binding site for factors involved in auxin responses (Xu *et al.*, 1997); ACGT represents a binding site for factors involved in sugar repression (Toyofuku *et al.*, 1998); 2S represents a site found in 2S protein storage genes that enhances their transcription (Stalberg *et al.*, 1996); AGC is a binding site for factors responding to pathogen attacks (Ohme-Takagi *et al.*, 2000); CArG represents a binding site for MADS transcription factors involved in flowering (Tilly *et al.*, 1998); DPBF represents a binding site for a ZIP transcription factor involved in ABA responses (Kim *et al.*, 1997); and CAAT and TATA represent consensus boxes for eukaryotic promoters. (C) Schematic of the different promoter fusions that were used to analyse the activity of different regions of the *PpACO1* promoter. The first line represents the promoter region and the number indicates the base that the promoter region begins at. All the constructs contain the sequence from −403 to −1, which does not include the translational start. The GUS–NOS line represents the complete coding sequence of the *uidA* gene beginning at the translational start ATG, and the nopaline synthase terminator sequence (Jefferson, 1987). CaMV35S is the pBI121 plasmid containing a 35S-containing promoter, and the no promoter line is the pBI101 plasmid (Jefferson, 1987).

A sequence comparison of the genomic clone with its cDNA (Callahan *et al.*, 1992) revealed four exons interrupted by three introns of 118 bp, 117bp, and 78 bp. The 5′ 2919 bases upstream of the start codon were analysed for potential *cis*-acting transcription factor binding sites using the PLACE program (http://www.dna.affrc.go.jp/htdocs/PLACE/) (Higo *et al.*, 1999). An AGC box (AGCCGCC) was found between 886 and 892 bp upstream of the translation start site which has been shown in other ethylene-responsive PR genes to be a binding site for ethylene-responsive binding factor proteins (ERF proteins) (Ohme-Takagi and Shinshi, 1995; Sato *et al.*, 1996; Jia and Martin, 1999; Fujimoto *et al.*, 2000). No other ethylene-associated sequences were found, although there were areas of similarity to binding sites for different types of ERFs as well as similarities to other ethylene-induced gene and fruit-related gene promoter sequences. A summary of potential sites is shown in Fig. 1B.

In a comparison of ACO promoter sequences, the only region of high similarity was that of a predicted TATA box as well as the predicted transcriptional and translation start sites (data not shown). Those promoter sequences were also analysed with the PLACE program (Higo *et al.*, 1999) and, again, there was no pattern of similar sites with the exception of at least one ERE or ethylene-responsive element site (AWTTCAAA) found in all the promoters except *PpACO1* (Montgomery *et al.*, 1993; Itzhaki *et al.*, 1994) (data not shown). There was no similarity in the location of those sites.

GUS staining of ripening transgenic tomato fruit

Tomato fruit from plants transformed with progressively longer lengths of the *PpACO1* gene promoter fused to the GUS gene (Fig. 1C) were subjected to GUS histochemical staining. GUS staining in fruit first appeared in vascular bundles, regardless of developmental stage. In the low expression lines, staining was confined to the vascular bundles and the collumella, whereas in the high expressers, staining was extended to the placental tissue and the pericarp. The locular tissue was never stained as much as the rest of the fruit tissues and no staining was visible in the epidermis. There were no differences in the localization of GUS staining related to the different length promoters.

There were variations in terms of the intensity of the GUS staining among individual transgenic plants (i.e. high and low expressers). The number of insertion events was approximated to determine if the higher levels of GUS staining resulted from higher copy number. Three lines for each construct were chosen, one high expresser, one medium and one low expressor. Most of the lines tested had approximately one copy of the GUS gene for every two copies of the tomato ACO1 gene (17/21), which would be equivalent to one insertion event (data not shown). There was no correlation of expression level with copy number.

There were differences in the amount of GUS staining resulting from the different lengths of promoters in fruit ripening series. The GUS expression patterns of the different lines suggested that there were three regions of the promoter that had specific properties in fruit ripening. The first region was contained within the first 403 bases 5′

to the translational start. This promoter length was sufficient to direct GUS expression in fruit tissue although the levels of the expression were generally low (Fig. 2A). Fourteen of the 22 lines analysed had detectable GUS histochemical expression. Among them, two lines showed GUS expression significantly higher than the rest of the −403 lines.

The next significant region was between 901 and 1319 bases 5′ to the translational start. The lines with the longer promoter showed elevated GUS expression suggesting either that there was an enhancer element in the 418 bases in between, or that some type of negative regulation was overcome by those 418 bases (Fig. 2B).

The last significant change in GUS expression came in the longest promoter length, 2919 bases. This was the only promoter length that resulted in a consistent increase in expression from the green to the red fruit stages, as demonstrated by histochemical staining of GUS (Fig. 2C). There was lower expression in the green fruit than in the ripe fruit for all the −2919 lines.

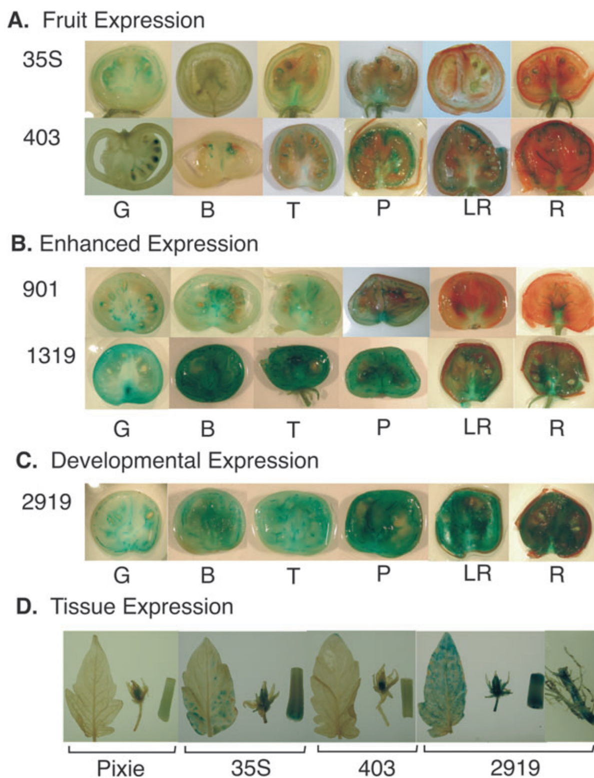

Fig. 2. GUS histochemical staining identified three segments of the *PpACO1* promoter that affected expression in ripening tomato fruit and the relative tissue specificity of expression. (A) Fruit expression: fruit from the constitutive promoter lines, 35S, is compared with fruit from the shortest promoter, 403 bp. (B) Enhanced expression: fruit from a 901 bp promoter line is compared with fruit from the 1319 promoter line. (C) Developmental expression: fruit from the longest promoter, 2919 bp, had a consistent increase of GUS expression during tomato fruit ripening. (D) Tissue expression: leaf, flower, stem, and roots were stained for 24 h to detect GUS expression. The full-length promoter, 2929 bp, had significant staining detected, but at apparently lower levels than in the fruit tissue. G, B, T, P, LR, and R represent the different stages of ripeness: green, breaker, turning, pink, light-red, and red, respectively. Pixie is the untransformed control line that does not contain a gene expressing GUS. The lines with representative patterns of expression and the highest levels of expression are presented.

GUS staining in non-fruit tissue

GUS histochemical staining of vegetative, root, and floral tissue was done for 24 h because of the lower level of staining in non-fruit tissue. At 24 h, staining was detected in leaves, stems, petals, sepals, and stigmas from most of the plants tested and in the roots of only the highest expressing lines (data summarized in Table 2). There was nearly undetectable staining in all non-fruit tissues of the −403 lines, including the two with high expression in fruit (Fig. 2D). Low levels of staining were detected in the −610 lines and −901 lines, and slightly higher levels were detected in the −1319 and −2141 lines and still higher in the −2919 full-length lines (Table 2; Fig. 2D).

No increase in GUS histochemical staining was detected from any leaf tissue 1, 2, 3, 4, or 5 h following wounding (data summarized in Table 2). In an independent series of woundings, the GUS activity was measured using the MUG assay and, again, no increase in activity was detected (data not shown). When leaf tissues were exposed to ethrel-induced ethylene for 24 h, only the full-length promoter lines consistently had enhanced GUS staining following exposure to ethylene (Table 2).

GUS activity in ripening fruit

The histochemical staining results were corroborated by activity data. Figure 3 presents the activity of the GUS enzyme from three to six lines at varying stages of fruit ripening for each promoter line. As expected, the untransformed 'Pixie' and the no-promoter lines, 101 (pBI101),

Table 2. *Relative amount of GUS expression as determined histochemically in various tissues and in response to induction*

Promoter	Fruit[a]	Leaf	Stem	Flower	Wounded leaf	Ethrel-treated leaf	Roots
None	−(4)[b]	−(2)	−(2)	−(2)	−(2)	−(4)	−(4)
35S	+(5)	+(3)	+(4)	++(4)	−(2)	−(3)	−(3)
403 bp	+(22)	−(4)	−(3)	−(3)	−(2)	−(4)	−(4)
610 bp	+(10)	Faint(4)	+(4)	++(5)	−(2)	−(5)	+[c](5)
901 bp	+(15)	Faint(5)	+(5)	++(5)	−(2)	−[d](5)	+[c](5)
1319 bp	+++(27)	Faint(5)	+(5)	+(5)	−(2)	−(5)	+[c](5)
2141 bp	+++(21)	+(5)	+(5)	+(5)	−(2)	−(5)	+[c](5)
2919 bp	+++(11)	+(3)	+(3)	++(3)	−(2)	+(3)	+[e](3)

[a] Fruit tissue was stained for 2 h while all the other tissues were stained for 24 h.
[b] Number in parentheses represents the number of independent lines tested.
[c] Detected only in the highest expressing lines.
[d] Variable by lines; most were negative, some are induced, while some are repressed.
[e] Detected in all lines.

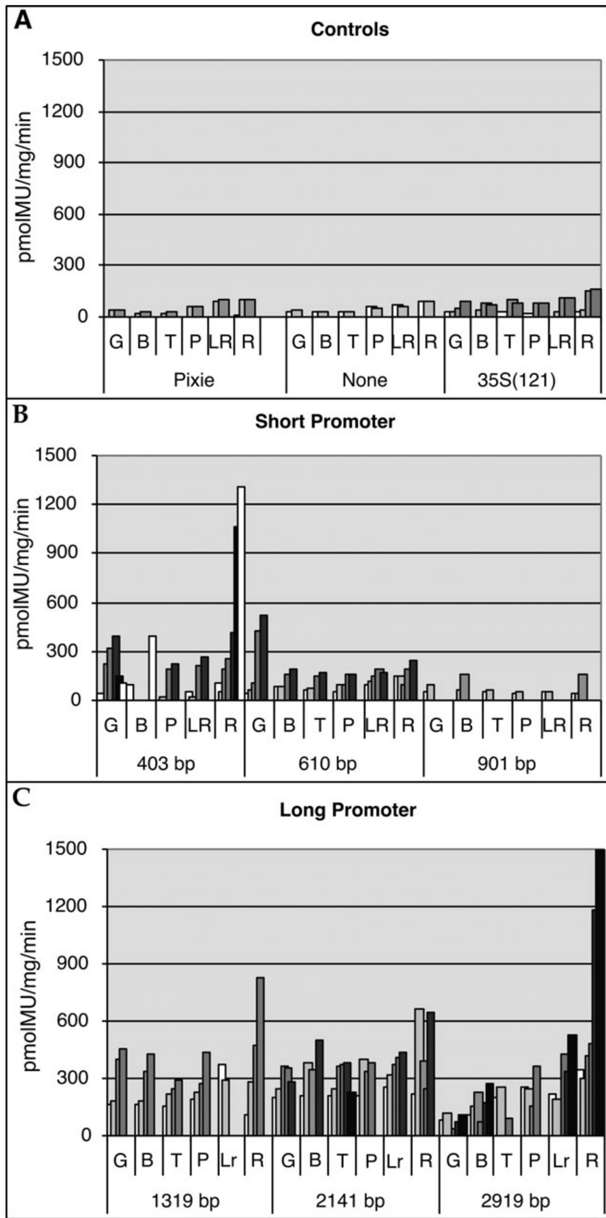

Fig. 3. Quantitative measurement of the GUS enzyme activity in the fruit of different transgenic lines carrying different lengths of the *PpACO1* promoter fused to GUS. Each bar represents the value of a single fruit. Fruit from the same line have the same shading. The different stages of fruit ripening are as in Fig. 2 except that Lr now represents the light red stage. 'Pixie' is an untransformed cultivar, None represents a promoterless line, 121 is the 35S promoter line and the rest, 403, 610, 901, 1319, 2149, and 2919 are the different lengths of PpACO1 promoter fused to the *uidA* gene (GUS).

had nearly undetectable activity (Fig. 3A). The shortest promoter lines, −403, had significant activities and the −901 lines had a decrease in overall activity (Fig. 3B). As in the histochemical assays an increase in activity can be seen with promoters longer than 901 bp and again only the longest length, 2919 bp, had a developmentally regulated pattern of the GUS enzyme activity (Fig. 3C).

RNA expression during tomato fruit development

Real-Time PCR was used to analyse the amount of native tomato ACO RNA and transgenic GUS RNA in fruit of different stages from each length promoter. The relative levels of tomato ACO RNA were lowest in green fruit for all the transgenic lines and increased during the 'Turning', 'Pink' and 'Light Red' ripening periods (Fig. 4A). By comparison, the levels of the transgene GUS RNA were very low in the transgenic plants with the three shortest promoter lengths, with only a slight increase detected in the −610 line (Fig. 4B). There was a significant increase in the amount of mRNA detected in the transgenic plants with the three longer promoters (Fig. 4B), but only the full-length promoter line, −2919, had an accumulation pattern similar to that of the native tomato ACO RNA. It was lowest in the green fruit, highest in the 'Turning' to 'Pink' stages, and decreased in the ripest stages.

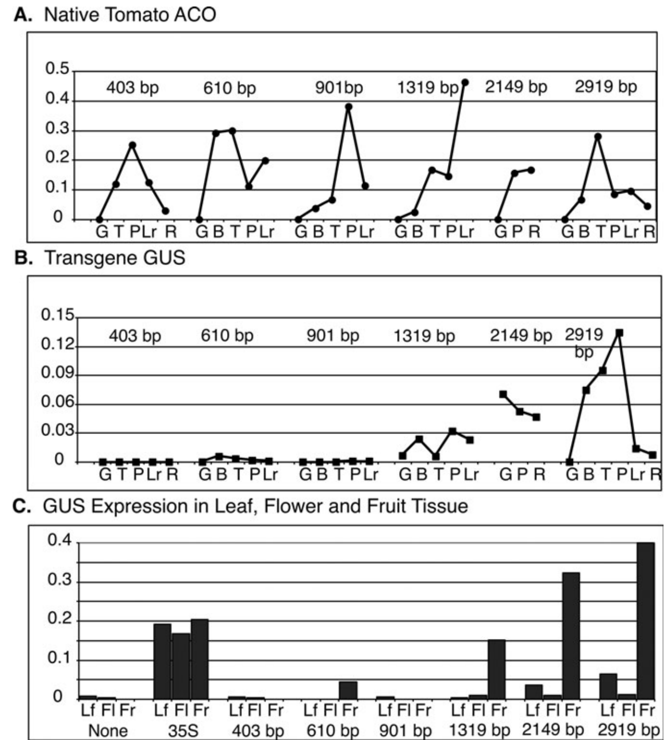

Fig. 4. Relative quantification of transgene RNA (GUS) and native tomato ACO1 RNA during fruit ripening in lines containing different lengths of peach ACO promoter–GUS fusions, and comparative amounts of transgene RNA in leaf, flower, and fruit from the same lines. A series from one line from each promoter length was analysed. (A) Relative amount of native tomato ACO1 RNA present in a ripening series from each of the transgenic lines. A significant increase in the amount of tomato ACOI RNA is detected during fruit ripening as expected. (B) Relative amount of transgene (GUS) RNA detected in each the lines carrying different lengths of *PpACO1* promoter fused to the GUS gene. The RNA analysed is the same RNA as in (A). The stages of fruit ripening are represented by G, B, T, P, Lr, and R, which are described in the Fig. 2 legend. (C) Relative amount of transgene (GUS) RNA detected in each line in leaf, flower, and the fruit stage with the highest level. Lf, Fl, and Fr represent the leaf, flower, and fruit tissue, respectively.

Comparative levels of RNA expression of leaf, flower and fruit

Real-Time PCR was used to determine relative amounts of transgene expressed RNA in leaf, flower, and fruit tissue relative to the amount of 26S rRNA. As expected from the histochemical staining, expression in leaves and flowers was much lower than in fruit. In most lines it was undetectable (Fig. 4C). Only in the longest length promoters, as well as the constitutive 35S promoter, were RNA levels above the background levels.

Discussion

Promoter sequences that confer tissue specificity, temporal or developmental specificity, inducibility, and alternative levels of activity are needed to regulate transgene expression finely. A number of promoters have been characterized that are related to the fruit ripening process, such as the E4 and E8 genes from tomato (Deikman and Fischer, 1988; Cordes *et al.*, 1989), the polygalacturonase promoter from tomato (Bird *et al.*, 1988), the 2A11 promoter from tomato (Van Haaren and Houck, 1993), and a number of ACO promoters from tomato, apple, and melon (Blume and Grierson, 1997; Bouquin *et al.*, 1997; Atkinson *et al.*, 1998). Earlier work with peach fruit on the accumulation of *ACO1* mRNA showed they were detectable at low levels in immature fruit and increased during the fruit softening process (Callahan *et al.*, 1992; Tonutti *et al.*, 1997). A similar transcript detected in leaf tissue at low levels was observed to increase following wounding (Callahan *et al.*, 1992). This ACO gene, *PpACO1*, provided the template for isolating a fruit-regulated peach promoter.

The genomic ACO1 clone isolated from peach was nearly identical to the sequence for an ACO1 clone isolated by Ruperti *et al.* (2001); except that the clone reported here has 2919 bp of sequence upstream of the translational start for ACO1 compared with 798 bp in the Ruperti *et al.* (2001) clone. By analysing various lengths of this 2919 upstream sequence fused to the promoterless *uidA* sequence, three major regulating regions were detected by differences in GUS expression. The stability of GUS protein is well known and can often give misleading results when examining expression over time because of the long turn-over time (Taylor, 1997). For this reason the levels of GUS expression were also measured by quantifying the mRNA with 'Real-Time' PCR to confirm the initial enzyme activity measurements and histochemical staining. The sequence from −2919 to −2141 is apparently needed to regulate the increase in expression from green to ripening fruit (Figs 2C, 3C, 4B) and to respond to ethylene (Table 2); the sequence from −1319 to −901 has a positive effect on the amount of expression in fruit (Figs 2B, 3C, 4B); and the first 403 bases are enough to drive GUS expression in fruit with little or no expression in other tissues (Figs 2A, D, 3B, Table 2). All the *PpACO1* promoter sequences, from 403 bp to 2919 bp, resulted in higher expression in fruit tissue (which only needed a 2 h stain) compared with leaf, stem, or flower (Figs 2, 4C; Table 2), and none of the promoters responded to wounding in leaf tissue (Table 2). The 901 bp promoter sequence resulted in variable responses to the ethrel-induced ethylene treatment suggesting those responses were more dependent upon where the construct had inserted into the genome. As this was the minimal fragment containing an ethylene-responsive AGC box, it may be that the preceding upstream region affects whether there will be a negative effect or a positive effect in response to ethylene.

There are suggestions that other areas of the promoter sequence are important. The −403 high expressing line has less expression in leaf, flower or stem than any of the other lines suggesting a very strong fruit tissue element or lacking a sequence for expression in other tissues. The −610 lines have very high expression in flower parts relative to all the other lines and even relative to other tissues (Table 2). This suggests that somewhere between −610 and −403 there may be a strong flower-specific enhancer. The levels of expression in the ripest fruit of the −901 lines decrease relative to the shorter promoter lines. This suggests there may be some negative regulatory elements between −901 and −610, even though the −901 lines have a putative ethylene responsive site (AGC box).

Deletion analyses of other ACO promoters have been reported (Blume and Grierson, 1997; Bouquin *et al.*, 1997; Atkinson *et al.*, 1998). They looked primarily for regions that respond to fruit ripening, wounding, ethylene, and senescence. The proximal upstream 450 bp of an apple ACO1 were able to direct fruit GUS expression in transformed tomato, but 1159 or 1966 bp of the upstream sequence were needed for ripening specificity (Atkinson *et al.*, 1998). In tomato, the upstream 221 bp from the translational start of *LeACO1* were enough to confer fruit ripening GUS activity. Longer sequences had stronger expression and conferred responsiveness to ethylene and senescence (Blume and Grierson, 1997). Bouquin *et al.* (1997), showed that when tested in tobacco, an upstream 726 bp of melon ACO1 conferred wound-inducible expression and ethylene responsiveness while the first 476 bp sequence had only ethylene responsiveness.

To understand what sequences might be involved in these responses, the ACO promoter sequences were subjected to analyses for potential *cis*-regulatory regions as predicted by the PLACE web site (Higo *et al.*, 1999) with special interest in ethylene-responsive element (ERE) binding sites due to the autocatalytic nature of ethylene (Abeles *et al.*, 1992). All the ACO promoters except *PpACO1* have at least one perfect copy of the ethylene-responsive element (ERE, consensus AT/ATTCAA) binding site that is found in the promoters of both the GST1 and E4 genes, and is assumed to be a binding site for ethylene-responsive transcription factors involved in fruit ripening and senescence (Montgomery *et al.*, 1993; Itzhaki *et al.*,

1994). There are, however, numerous partial consensus ERE sites in *PpACO1* fairly evenly distributed throughout the 2919 bases upstream to the translational start site. The peach ACO1 does have an AGC box that has been found to bind ethylene responsive elements in response to pathogen infections (Ohme-Takagi *et al.*, 2000; Rushton *et al.*, 2002). Only the apple ACO1 also contains this sequence. In addition, both *PpACO1* and the apple ACO1 have a MADS box transcription factor binding site (CarG) (Tilly *et al.*, 1998), but none of the other ACO genes do. These are the only *cis*-acting elements in *PpACO1* predicted to show some relationship with expression in fruit.

In trying to relate the placement of these *cis*-regulatory regions with the activity and responsiveness of the different promoter lengths, there was very little sequence information found in common among the various ACO promoters. One of the strongest determinants in this analysis was the region between −2919 and −2141 that appeared to confer ripening enhanced expression. In that region, there were sequences identified by the PLACE web site predicted to be responsive to auxin regulation and abiotic stress responses such as low temperature and drought responses (W, Saur, DRE/LTRE, Fig. 1B). These binding site sequences are found in some of the other ACO genes, but not in the regions defined by the GUS fusion experiments as conferring fruit ripening specificity. The relationship of those stresses with fruit ripening is not thoroughly understood even though many of the genes induced during abiotic stress responses are members of families of genes also induced during fruit ripening (Frenkel and Callahan, 1996/97).

The second region identified in these studies, −1319 to −901, enhances expression. The only transcription factor binding sequence identified is that with homology to the 2S promoter for seed storage protein expression, which has been associated with increased expression (Stalberg *et al.*, 1996). None of the other ACO genes contain a homologous region.

The region with an ERE binding site AGC, from −901 to −610, appears to have a negative expression when added to the minimal promoter. This region contains the MADS box transcription factor site, as well as a low temperature inducible site, a Zip factor binding site (DPBF) and an ACGT-sugar repression site (Baker *et al.*, 1994; Kim *et al.*, 1997; Dunn *et al.*, 1998; Tilly *et al.*, 1998; Toyofuku *et al.*, 1998). The minimal promoter length of 403 bp that is able to confer GUS expression in a fruit-specific manner contains only a consensus TATA box and a potential ZIP factor binding site, which again is not shared by other ACO promoter regions.

The four different plant systems with dissection of the ACO promoters appear to have certain elements in common, in that a short upstream region of the promoter is able to confer GUS expression in fruit, but that longer lengths are needed for stronger expression and for fruit developmental or ethylene responsiveness. The regions having already defined ERE binding sites do not appear to correlate with these expression patterns. This underlines the lack of knowledge that exists on the particular regulation of the ethylene biosynthetic pathway. More careful dissections of the promoter regions are needed rather than sequence comparisons, to elucidate the exact regulatory sequences. The comparison of potential *cis*-acting sites with the activity of the different promoter regions only suggests some areas of importance, and the comparison with other ACO promoters yielded no regions in common.

In plant systems that have long generation times such as apple and peach, it is hard to test promoter fusions that have expected expression in fruit because of the extensive time to fruiting. By using an alternate system such as tomato, problems may arise due to differences in the systems such as in *trans*-acting transcription factors and developmental processes. Yet, the longest peach ACO1 promoter sequence, 2919 bp, was able to express GUS in a very similar pattern in ripening tomato fruit to that of the native tomato ACO1 promoter, rather than the ACO1 pattern seen in peach. In tomato, as in peach, the accumulation of the *LeACO1*, the major ACC oxidase gene expressed during fruit ripening in tomato, was very low in green fruit (Barry *et al.*, 1996). However, unlike in peach where the accumulation of the *ACO1* transcript occurred at mature stages and continued to increase as the fruit softened, the *LeACO1* message in tomato peaked at turning stage (breaker +3) and then declined subsequently (Barry *et al.*, 1996). This suggests that although the overall ripening process is different in tomato and peach, specific aspects regarding regulation of the two ACO genes could still be very similar.

In conclusion, a genomic DNA encoding an ACC oxidase (*PpACO1*) has been isolated from peach. Its sequence is comprised of a 2.9 kb 5′ flanking region, a 1.3 kb open reading frame, and a 1 kb 3′ flanking sequence. The open reading frame has 4 exons interrupted by 3 introns. The 2.9 kb promoter of the *PpACO1* drove the expression of a GUS reporter gene in transgenic tomato fruit in a manner similar to the native tomato ACO gene. Promoter deletion analysis revealed three putative positive regulatory regions (−2919 to −2141, −1319 to −901, and the first 403 bp) that affect gene expression during fruit ripening in transgenic tomato.

Acknowledgements

The authors would like to thank Linda Dunn and Kristy Anderson for considerable technical help in assaying the plants, Dr C Srinivasan for advice on transforming tomatoes and tissue culture, Genevieve Byrne for help in tissue culture, and Dr Ralph Scorza for overall support and guidance.

References

Abeles FB, Morgan PW, Saltveit Jr ME. 1992. *Ethylene in plant biology*. San Diego: Academic Press Inc.

Amaravadi L, King MW. 1994. A rapid and efficient nonradiactive method for screening recombinant DNA libraries. *Biotechniques* **16,** 99–103.

Atkinson RG, Bolitho KM, Wright MA, Iturriagagoitia-Bueno T, Reid SJ, Ross GS. 1998. Apple ACC-oxidase and polygalacturonase: ripening specific gene expression and promoter analysis in transgenic tomato. *Plant Molecular Biology* **38,** 449–460.

Ayub R, Guis M, Ben Amor M, Gillot L, Roustan JP, Latche A, Bouzayen M, Pech JC. 1996. Expression of ACC oxidase antisense gene inhibits ripening of cantaloupe melon fruits. *Nature Biotechnology* **14,** 862–866.

Baker SS, Wilhelm KS, Thomashow MF. 1994. The 5′-region of *Arabidopsis thaliana* cor15a has *cis*-acting elements that confer cold-, drought-, and ABA-regulated gene expression. *Plant Molecular Biology* **24,** 701–713.

Barry CS, Blume B, Bouzayen M, Cooper W, Hamilton AJ, Grierson D. 1996. Differential expression of the 1-aminocyclopropane-1-carboxylate oxidase gene family of tomato. *The Plant Journal* **9,** 525–535.

Bird CR, Smith CJS, Ray JA, Moureau P, Bevan MW, Bird AS, Hughes S, Morris PC, Grierson D, Schuch W. 1988. The tomato polygalacturonase gene and ripening-specific expression in transgenic plants. *Plant Molecular Biology* **11,** 651–662.

Blume B, Grierson D. 1997. Expression of ACC oxidase promoter–GUS fusions in tomato and *Nicotiana plumbaginifolia* regulated by developmental and environmental stimuli. *The Plant Journal* **12,** 731–746.

Bouquin T, Lasserre E, Pradier J, Pech JC, Balaque C. 1997. Wound and ethylene induction of the ACC oxidase melon gene CM-ACO1 occurs via two direct and independent transduction pathways. *Plant Molecular Biology* **35,** 1029–1035.

Callahan AM, Morgens PH, Cohen RA. 1993. Isolation and initial characterization of cDNAs for mRNAs regulated during peach fruit development. *Journal of the American Society of Horticultural Science* **118,** 531–537.

Callahan AM, Morgens PH, Wright P, Nichols Jr KE. 1992. Comparison of pch313 (pTOM13 homolog) RNA accumulation during fruit softening and wounding of two phenotypically different peach cultivars. *Plant Physiology* **100,** 482–488.

Cordes S, Deikman J, Margossian LJ, Fischer RL. 1989. Interaction of a developmentally regulated DNA binding factor with sites flanking two different fruit-ripening genes from tomato. *The Plant Cell* **1,** 1025–1034.

Deikman J, Fischer RL. 1988. Interaction of a DNA binding factor with the 5′ flanking region of an ethylene-responsive fruit ripening gene from tomato. *EMBO Journal* **7,** 3315–3320.

Do JY, Salunkhe DK, Olson LE. 1969. Isolation, identification and comparison of the volatiles of peach fruit as related to harvest, maturity and artificial ripening. *Journal of Food Science* **34,** 618–621.

Doyle JJ, Doyle JL. 1990. Isolation of plant DNA from fresh tissue. *Focus* **12,** 13–15.

Dunn MA, White AJ, Vural S, Hughes MA. 1998. Identification of promoter elements in a low-temperature-responsive gene (*blt4.9*) from barley (*Hordeum vulgare* L.) *Plant Molecular Biology* **38,** 551–564

Frenkel C, Callahan AM. 1996/97. Fruit ripening: a stress acclimation phenomenon? In: Purohit SS, ed. *Agro's annual review of plant physiology*. Bikaner, India: Agro Botanical Publishers, 37–82.

Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohme-Takagi M. 2000. *Arabidopsis* ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *The Plant Cell* **12,** 393–404.

Giovannoni J. 2001. Molecular biology of fruit maturation and ripening. *Annual Review of Plant Physiology and Plant Molecular Biology* **52,** 725–749.

Hamilton AG, Lycett GW, Grierson D. 1990. Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants. *Nature* **346,** 284–286.

Higo K, Ugawa Y, Iwamoto M, Iorenaga T. 1999. Plant *cis*-acting regulatory DNA elements (PLACE) database. *Nucleic Acids Research* **27,** 297–300. http://www/dna.affrc.go.jp/sigscan/signal1.pl

Holdsworth MJ, Bird CR, Ray J, Schuch W, Grierson D. 1987. Structure and expression of an ethylene-related mRNA from tomato. *Nucleic Acids Research* **15,** 731–739.

Ingham DJ, Beer S, Money S, Hansen G. 2001. Quantitative real-time PCR assay for determining transgene copy number in transformed plants. *BioTechniques* **31,** 132–140.

Itzhaki H, Maxson JM, Woodson WR. 1994. An ethylene-responsive enhancer element is involved in the senescence-related expression of the carnation glutathione-*S*-transferase (GST1) gene. *Proceedings of the National Academy of Sciences, USA* **91,** 8925–8929.

Jefferson RA. 1987. Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Molecular Biology Reporter* **5,** 387–405.

Jia Y, Martin GB. 1999. Rapid transcript accumulation of pathogenesis-related genes during an incompatible interaction in bacterial speck disease-resistant tomato plants. *Plant Molecular Biology* **40,** 455–465.

Kim SY, Chung HJ, Thomas TL. 1997. Isolation of a novel class of bZIP transcription factors that interact with ABA-responsive and embryo-specification elements in the Dc3 promoter using a modified yeast one-hybrid system. *The Plant Journal* **11,** 1237–1251.

Klee H, Tieman D. 2002. The tomato ethylene receptor gene family: form and function. *Physiologia Plantarum* **115,** 336–341.

Lasserre E, Bouquin T, Hernandez JA, Bull J, Pech J-C, Balagué C. 1996. Structure and expression of three genes encoding ACC oxidase homologs from melon (*Cucumis melo* L.). *Molecular and General Genetics* **251,** 81–90.

MacDiarmid CWB, Gardner RC. 1993. A cDNA sequence from kiwifruit homologous to 1-aminocyclopropane-1-carboxylic acid oxidase. *Plant Physiology* **101,** 691–692.

McGarvey DJ, Yu H, Christofferson RE. 1990. Nucleotide sequence of a ripening-related cDNA from avocado fruit. *Plant Molecular Biology* **15,** 165–167.

Montgomery J, Goldman S, Deikman J, Margossian L, Fischer RL. 1993. Identification of an ethylene-responsive region in the promoter of a fruit ripening gene. *Proceedings of the National Academy of Sciences, USA* **90,** 5939–5943.

Ohme-Takagi M, Shinshi H. 1995. Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. *The Plant Cell* **7,** 173–182.

Ohme-Takagi M, Suzuki K, Shinshi H. 2000. Regulation of ethylene-induced transcription of defence genes. *Plant Cell Physiology* **41,** 1189–1192.

Ross GS, Knighton ML, Lay-Yee M. 1992. An ethylene-related cDNA from ripening apples. *Plant Molecular Biology* **19,** 231–238.

Ruperti B, Bonghi C, Rasori A, Ramina A, Tonutti P. 2001. Characterization and expression of two members of the peach 1-aminocyclopropane-1-carboxylate oxidase gene family. *Physiologia Plantarum* **111,** 336–344.

Rushton PJ, Reinstadler A, Lipka V, Lippok B, Somssich IE. 2002. Synthetic plant promoters containing defined regulatory elements provide novel insights into pathogen-and wound-induced signaling. *The Plant Cell* **14,** 749–762.

Sato F, Kitajima S, Koyama T, Yamada Y. 1996. Ethylene-induced gene expression of osmotin-like protein, a neutral isoform of tobacco PR-5, is mediated by the AGCCGCC *cis*-sequence. *Plant Cell Physiology* **37,** 249–255.

Scorza R, Morgens PH, Mante S, Cordts J, Callahan AM. 1990. *Agrobacterium*-mediated transformation of peach (*Prunus persica* L. Batsch) leaf segments, immature embryos, and long-term embryogenic calli. In Vitro *Cellular and Developmental Biology* **26,** 829–834.

Scorza R, Ravelonandro M, Callahan AM, Cordts J, Fuchs M, Dunez J, Gonsalves D. 1994. Transgenic plums (*Prunus domestica* L.) express the plum pox virus coat protein gene. *Plant Cell Reports* **14,** 18–22.

Stalberg K, Ellerstom M, Ezcurra I, Ablov S, Rask L. 1996. Disruption of an overlapping E-box/ABRE motif abolished high transcription of the napA storage-protein promoter in transgenic *Brassica napus* seeds. *Planta* **199,** 515–519.

Taylor CB. 1997. Promoter fusion analysis: an insufficient measure of gene expression. *The Plant Cell* **9,** 273–275.

Tilly JJ, Allen DW, Jack T. 1998. The CArG boxes in the promoter of the *Arabidopsis* floral organ identity gene *APETALA3* mediate diverse regulatory effects. *Development* **125,** 1647–1657.

Tonutti P, Bonghi C, Ruperti B, Tornielli GB, Ramina A. 1997. Ethylene evolution and 1-aminocyclopropane-1-carboxylate oxidase gene expression during early development and ripening of peach fruit. *Journal of the American Society of Horticultural Science* **122,** 642–647.

Toyofuku K, Umemura T, Yamaguchi J. 1998. Promoter elements required for sugar-repression of the RAmy3D gene for alpha-amylase in rice. *FEBS Letters* **428,** 275–280.

Van Haaren MJJ, Houck CM. 1993. A functional map of the fruit-specific promoter of the tomato 2A11 gene. *Plant Molecular Biology* **21,** 625–640.

Ververidis P, John P. 1991. Complete recovery *in vitro* of ethylene forming enzymes activity *Phytochemistry* **30,** 725–727.

Wang H, Woodson WR. 1992. Nucleotide sequence of a cDNA encoding the ethylene-forming enzyme from petunia corollas. *Plant Physiology* **100,** 535–536.

Xu N, Hagen G, Guilfoyle T. 1997. Multiple auxin response modules in the soybean SAUR 15A promoter. *Plant Science* **126,** 193–201.

Yang SF, Hoffman NE. 1984. Ethylene biosynthesis and its regulation in higher plants. *Annual Review of Plant Physiology* **35,** 155–189.