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Short communication

Analysis of *Arabidopsis* cDNA that shows homology to the tomato E8 cDNA

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Abstract

The negative regulatory protein of ethylene synthesis in ripening tomato fruit, E8, is structurally related to the enzyme that catalyzes the last step in ethylene synthesis,1-aminocyclopropane-1-carboxylate (ACC) oxidase, and to a large family of 2-oxoglutarate-dependent dioxygenases (2-ODD). A cDNA with structural homology to the tomato E8 was isolated from a cDNA library of *Arabidopsis thaliana*. Sequence analysis showed that this cDNA, 2A6, encodes a protein of 361 amino acids. Southern blot analysis indicated that the corresponding gene is unique in the *Arabidopsis* genome. The level of the 2A6 transcript was not increased by ethylene in siliques of *Arabidopsis*, as was E8 in tomato fruits, and was also expressed in etiolated seedlings, leaves, stems and flowers. The 2A6 protein shows three domains that are highly conserved among E8, ACC oxidases, and 2-ODDs.

The production of ethylene, a potent plant growth regulator, is induced during certain stages of plant development such as germination, leaf abscission, flower senescence, and fruit ripening [1]. Ethylene is synthesized in higher plants from methionine via *S*-adenosyl-L-methionine (AdoMet) and 1-aminocyclopropane-1-carboxylic acid (ACC). The enzymes catalyzing the three steps of this pathway are AdoMet synthetase, ACC synthase, and ACC oxidase [for review see 9]. Although the pathway of ethylene biosynthesis is well understood, the knowledge of the molecular mechanisms underlying the regulation of ethylene synthesis is limited. Ethylene biosynthesis is subject to positive and negative feedback regulation [9]. Positive feedback regulation occurs in climacteric fruits and other senescing tissues in which an increase in ethylene synthesis can be triggered by exposure to ethylene. Negative feedback regulation (autoinhibition) has been observed in a number of fruits and vegetative tissues. The first cloned gene expressing a protein that mediates negative feedback regulation of ethylene biosynthesis in tomato fruits is called E8 [5]. Transcription of E8 is activated by ethylene at the onset of fruit ripening. E8 mRNA is abundant in ripening tomato fruits, is not detectable in leaves, roots, stems or unripe fruits, and its expression is promoted by

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X83096.

ethylene only in the fruit [2]. Inhibitors of ethylene action reduce E8 expression at the level of transcription. Inhibition of E8 gene expression by production of antisense mRNA in transgenic tomato plants led to a reduced level of E8 protein and overproduction of ethylene in ripening, detached tomato fruits [14]. In this communication, we describe a cDNA clone, 2A6, from *Arabidopsis thaliana* that shows high homology to the E8 cDNA.

The large-scale sequencing program of *Arabidopsis thaliana* ecotype Columbia cDNAs at the Plant Research Laboratory at Michigan State University yielded several cDNA clones with significant homologies to cDNAs encoding dioxygenases, including E8 of tomato and ACC oxidases. These clones were isolated from a cDNA library constructed from mRNA representing leaves, roots, etiolated seedlings and shoots (including stems, flowers, and developing seeds) [12], using the lambda ZipLox vector [3]. Computer analysis of the 122 bp 5′-terminal sequence of the cDNA clone 2A6, which is deposited in the Arabidopsis Resource Center at Ohio State University under the code 2A6T7P, showed, on the amino acid level, 60% identity and 90% similarity to the ethylene-responsive fruit-ripening protein E8. The nucleotide sequence of the entire 2A6 cDNA was obtained by sequencing ordered deletion clones from the 5′ and 3′ ends, using the modified T7 DNA polymerase Sequenase according to the manufacturer's instructions (USB, Cleveland, OH). The 1466 bp cDNA clone contains an open reading frame of 1086 bp encoding a protein of 361 amino acids. Because this is only two amino acids shorter than the tomato E8 protein, it appears likely that 2A6 includes the entire coding region of the gene, a 5′-untranslated region of 18 bp, and a 3′-untranslated region of 362 bp. The nucleotide sequence of 2A6 was found to have significant homology with E8 (62%), ACC oxidases [e.g. the tomato ACC oxidase pRC13 (59%) and the *Arabidopsis* ACC oxidase pEAT1 (61%)], and with a large family of 2-oxoglutarate-dependent dioxygenases (2-ODD). ACC oxidases require ferrous iron as cofactor and ascorbate and molecular oxygen, but not 2-oxoglutarate, as co-substrates [16, 17].

Table 1. Amino acid sequence comparison of the cDNA clones 2A6 and pEAT1 from *Arabidopsis* and E8 and pRC13 from tomato.

	E8 (tomato)	pRC13 (tomato)	pEAT1 (*Arabidopsis*)
2A6	50.4[1] (70.5)	31.5 (55.1)	29.8 (56.3)
E8		32.8 (55.4)	34.5 (57.2)
pRC13			76.8 (88.6)

[1] The numbers are given in % identity and in % similarity (in parenthesis).

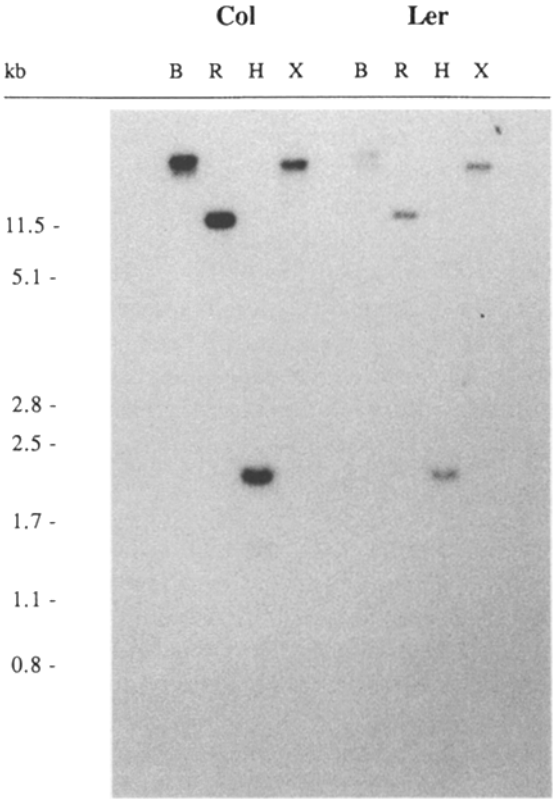

Fig. 1. Representation of the gene corresponding to the 2A6 cDNA in the *Arabidopsis* genome. Genomic DNA of the *Arabidopsis thaliana* ecotypes Columbia (Col = 10 μg) and Landsberg erecta (Ler = 5 μg) was digested with *Bam* HI (B), *Eco* RI (E), *Hind* III (H) and *Xba* I (X), fractionated by agarose gel electrophoresis, blotted, and hybridized with random prime-labeled 2A6 cDNA. The blots were washed two times under non-stringent conditions in 2× SSC, 0.1% SDS at 65 °C. Autoradiography was carried out at −80 °C with intensifying screens for 20 h.

Therefore, ACC oxidases cannot be considered to be true 2-ODDs despite the high degree of sequence homology to these dioxygenases [4, 15]. It remains to be shown whether E8 or 2A6 requires any of the co-substrates mentioned above.

Table 1 shows that both, the 2A6 and E8 proteins, have nearly equal homology to the ACC oxidases pRC13 from tomato [7, 8] and pEAT1 from *Arabidopsis* [6]. The 2A6 and E8 proteins are less similar to each other than are ACC oxidases, which are known to be highly homologous even between different species. However, the nucleotide sequence identity of 62% and the amino acid identity of 50% between E8 and 2A6 are quite significant for two cDNA clones from different species. This made 2A6 a candidate for a negative regulatory protein of ethylene biosynthesis in *Arabidopsis*. In contrast to about three different E8-like genes in the genome of tomato [5], genomic Southern blot analysis showed that

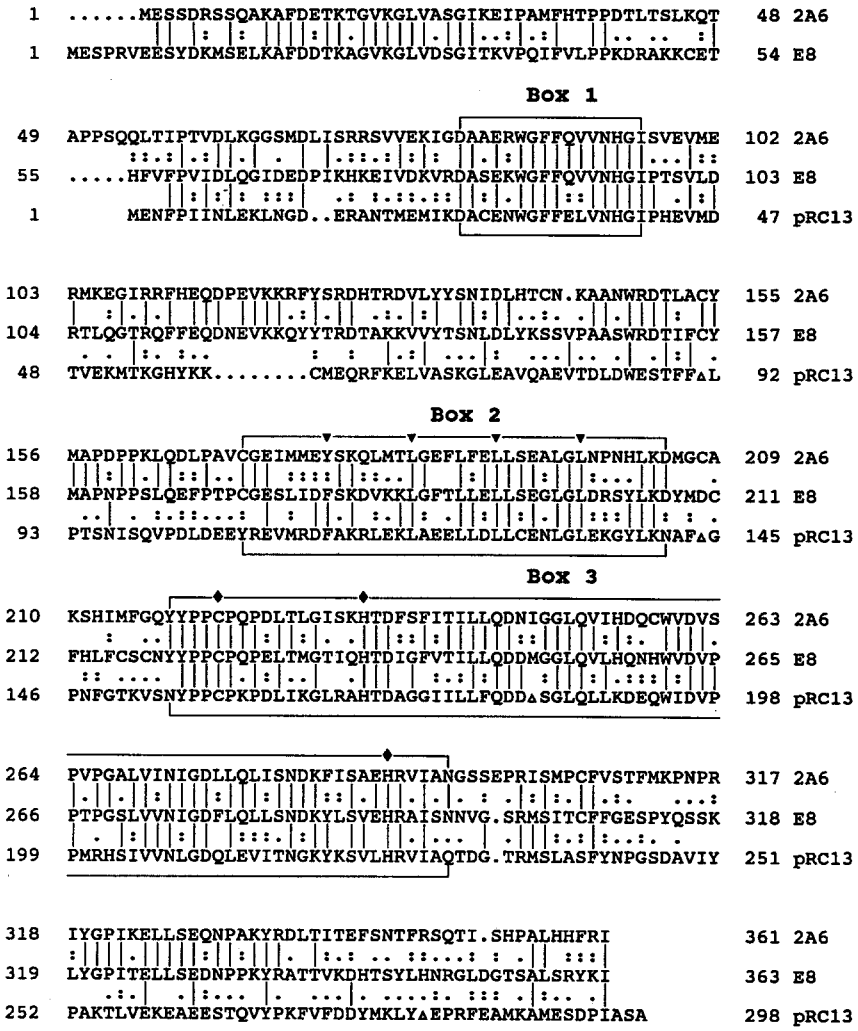

Fig. 2. Alignment of the amino acid sequence of the cDNA clone 2A6 from *Arabidopsis thaliana* with the proteins E8 and pRC13 from tomato (*Lycopersicon esculentum*). Gaps in the amino acid sequences, indicated by periods, are based on computer alignment. Identical amino acids are indicated by vertical bars, similar amino acids by dots. Conserved regions among the three clones are boxed (boxes 1 to 3). The potential leucine repeat in box 2 is indicated by solid triangles. Diamonds indicate a conserved cysteine residue and two conserved histidine residues in box 3 that may be involved in binding of ferrous iron.

2A6 is unique in the *Arabidopsis* genome of both ecotypes, Columbia and Landsberg erecta (Fig. 1). Digestion of genomic DNA with *Bam* HI, *Eco* RI, or *Xba* I yielded a single band after hybridization to the labeled full-length 2A6 cDNA. Digestion with *Hin*d III gave one main signal and also a second, weakly hybridizing band of 1.5 kb when 10 μg of genomic DNA were used. This weak band was due to a *Hin*d III restriction site located 28 bp downstream from the ATG codon of the 2A6 DNA.

In Fig. 2, the amino acid sequence of 2A6 is aligned with those of the tomato E8 and pRC13 proteins. This comparison shows that the 2A6 protein has three distinct domains in common with the E8 protein, with ACC oxidases, and with a large group of dioxygenases [15]. One of these domains (Fig. 2, Box 2) contains a putative leucine zipper, consisting of one phenylalanine or tyrosine and three leucine residues, indicating that these proteins have the potential to dimerize [13, 18]. Since ACC oxidase and the dioxygenases require Fe^{2+} for their enzymatic function, one of the other two domains is thought to be involved in binding of this co-factor [10, 15, 16]. One cysteine residue (in clone 2A6, Cys-223) and two histidine residues (in clone 2A6, His-235 and His-291) are well conserved in the third domain sequence of the above proteins (Fig. 2, Box 3), raising the possibility that these residues may be involved in iron binding [10]. The function of domain one (Fig. 2, Box 1) could involve the binding of ascorbate.

The mRNA of 2A6 is of low abundance in etiolated seedlings, leaves, stems, flowers, siliques, and roots. The expression of 2A6 was detected in young leaves on a northern blot of 4 μg of poly(A)$^+$ RNA and was not induced by ethylene (results not shown). In analogy to E8, the expression of 2A6 was more likely to be increased by ethylene in siliques than in any other tissue. However, the 2A6 mRNA could not be detected in siliques by northern blot analysis. Therefore, the expression of 2A6 was examined by reverse-transcription polymerase chain reaction (RT-PCR, Fig. 3). The primers used for PCR amplified a 490 bp cDNA fragment and span a ca. 500 bp intron in the 5′-terminal region of the

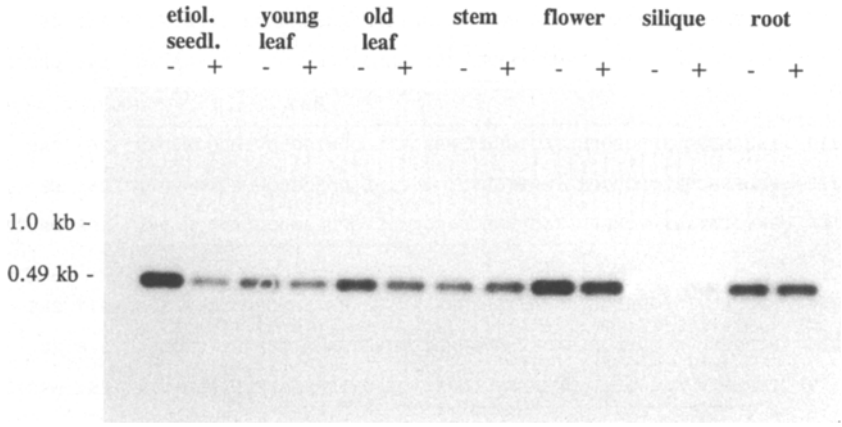

Fig. 3. Expression analysis of the cDNA clone 2A6 by RT-PCR in ethylene-treated (+) and untreated (−) tissue of *Arabidopsis thaliana*. The ethylene-treated plants grew under a continuous flow of 10 μl/l ethylene for 14 h before harvest. DNase I-treated total RNA (2 μg) from etiolated seedlings, young and old leaves, stems, flowers, siliques, and roots were reverse transcribed using M-MLV reverse transcriptase. The PCR reactions contained 1/10 of the RT-reaction mixture and were carried out with 5 pmol each of the following two primers: 5′-18-mer 5′ > CGCTCGCCTGTTACATGG < 3′, 3′-19-mer 5′ > CGGCTTCATGAACGTGCTG < 3′ in a total volume of 20 μl. The PCR was stopped after 20 cycles of 1 min at 94 °C; 1 min at 52 °C; 1 min at 72 °C; the rates of amplification were exponential between 16 and 24 cycles (results not shown). Five μl of the PCR products were separated on a 1.5% agarose gel, blotted, and hybridized with random prime-labeled 2A6 cDNA. Autoradiography was carried out at −80 °C with intensifying screens for 6 h. This experiment was repeated three times with different RT preparations giving the same results.

2A6 genomic clone. Therefore, a contamination of the RNA used in the RT-PCR with genomic DNA would have become apparent through the appearance of a 1 kb PCR fragment. The mRNA corresponding to clone 2A6 was not induced by ethylene in siliques, as was E8 in tomato fruits, nor in any of the other tissues investigated (Fig. 3). Therefore, 2A6 is probably not a functional analogue of E8 in *Arabidopsis*, despite the striking homology between these two proteins.

The negative feedback inhibition of ethylene biosynthesis in ripening fruit apparently involves mechanisms other than an end-product inhibition because suppression has been shown to continue even after the end of treatment with ethylene [11]. The high homology between the negative regulatory protein E8 to known dioxygenases and ACC oxidases led to speculations about the possible mechanism of negative feedback regulation of ethylene biosynthesis by E8. The increase in ethylene synthesis in E8 antisense tomato plants during fruit ripening can be the result of a metabolic imbalance leading to stress-induced ethylene production [14]. The E8 protein could also mediate negative feedback regulation of ethylene synthesis during ripening by a direct involvement in the biochemical reactions of ACC or ethylene synthesis [14], affecting either the synthesis of ACC synthase and ACC oxidase or their enzymatic function. Because of the three highly conserved domains of these proteins, it is possible that E8 and similar proteins in Arabidopsis and other plants affect ACC oxidase activity by reducing the cellular level of Fe^{2+} and/or ascorbate or by forming heterodimers with ACC oxidases via the leucine zipper. Alternatively, E8 may be a dioxygenase whose product inhibits ethylene biosynthesis by activating a signal transduction pathway that controls the level of ethylene formation.

Acknowledgements

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