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Reversible Inhibition of Tomato Fruit Senescence by Antisense RNA

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Ethylene controls fruit ripening. Expression of antisense RNA to the rate-limiting enzyme in the biosynthetic pathway of ethylene, 1-aminocyclopropane-1-carboxylate synthase, inhibits fruit ripening in tomato plants. Administration of exogenous ethylene or propylene reverses the inhibitory effect. This result demonstrates that ethylene is the trigger and not the by-product of ripening and raises the prospect that the life-span of plant tissues can be extended, thereby preventing spoilage.

IN PLANTS, RIPENING OF A FRUIT IS the prelude to senescence, the final phase of development and differentiation (*1, 2*). During ripening, climacteric fruits, such as tomato and banana, undergo marked changes in composition and texture and have a burst of respiration (climacteric rise) with a concomitant increase in ethylene (C_2H_4) production (*1, 3*). Climacteric fruits can be induced to ripen by treatment with C_2H_4 at concentrations above 0.1 μl of C_2H_4 per liter of air. Once ripening is initiated, the endogenous C_2H_4 production rises autocatalytically (*4*). Ethylene affects gene transcription in a variety of tissues (*5*), and physiological evidence indicates that C_2H_4 is the natural ripening hormone (*1, 6,* 7). We now use antisense RNA to inactivate the rate-limiting enzyme in the C_2H_4 biosynthetic pathway and show that C_2H_4 triggers ripening and senescence of tomato (*Lycopersicon esculentum*) fruit.

The rate-limiting step in the synthesis of C_2H_4 is the conversion of S-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of C_2H_4, a process that is catalyzed by the enzyme ACC synthase (*4, 8*). Induction of C_2H_4 production requires de novo synthesis of this enzyme (*8*). ACC synthase is encoded in tomato by a divergent multigene family, two members of which are expressed during fruit ripening (*9*). Full-length cDNAs from the two genes, *LE-ACC2* and *LE-ACC4*, have been isolated and structurally characterized (*9*).

We expressed antisense RNA derived from the tACC2 cDNA of the *LE-ACC2* gene constitutively in transgenic plants using the cauliflower mosaic virus (CaMV) 35*S* promoter (Fig. 1A). Thirty-four independent transgenic tomato plants were obtained, and three of them, A11.1, A11.4, and A2, showed a marked inhibition in C_2H_4 production and delay in the onset of fruit ripening. The strongest phenotype was observed with fruits from the A11.1 transformant, which was chosen for further analysis. All of the experiments reported here have been carried out with homozygous A11.1 fruits from the second (R1) or third (R2) generation of transgenic plants. Southern (DNA) blot analysis showed that A11.1 plants from the R1 generation (Fig. 1B) contained an additional 1.7-kb DNA fragment that segregated as a single locus (3:1 ratio). Comparison of the hybridization intensities between the endogenous single-copy *LE-ACC2* synthase gene (*9*) and the antisense gene indicates the presence of ten antisense insertions per plant (Fig. 1B).

Control fruits kept in air begin to produce C_2H_4 48 to 50 days after pollination, then undergo a respiratory burst (*10*), and fully ripen after ten more days (Figs. 2A and 3). Ethylene production was inhibited by 99.5% in antisense fruits, which fail to ripen (Fig. 2A). The basal level of C_2H_4 evolution in antisense fruits is below 0.1 nl of C_2H_4 per gram of fruit mass per hour. The red coloration resulting from chlorophyll degradation and lycopene biosynthesis is also inhibited in antisense fruits (*11*). A progressive loss of chlorophyll from antisense fruits is seen 10 to 20 days later than the loss is seen in the control fruits, resulting in a yellow color. Antisense fruits kept in air or on the plants for 90 to 120 days eventually develop an orange color but never turn red and soft (*12*) or develop an aroma. Antisense fruits in air do not show the respiratory

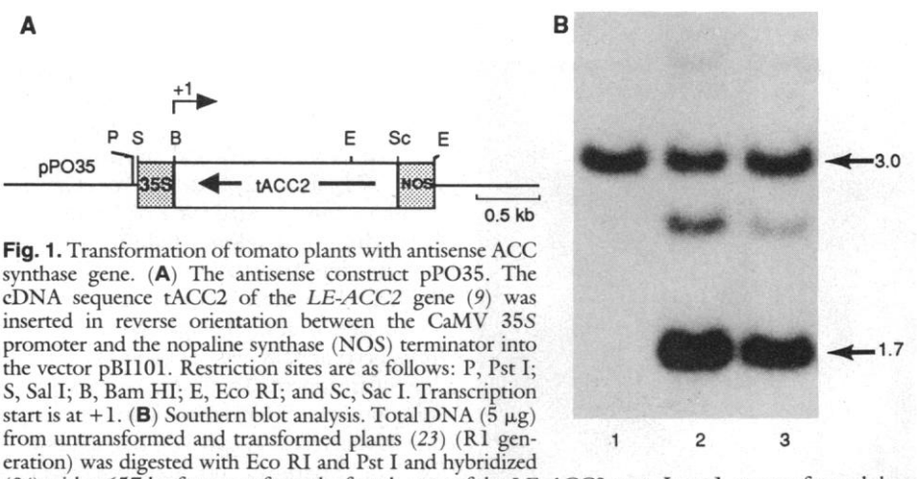

Fig. 1. Transformation of tomato plants with antisense ACC synthase gene. (**A**) The antisense construct pPO35. The cDNA sequence tACC2 of the *LE-ACC2* gene (*9*) was inserted in reverse orientation between the CaMV 35*S* promoter and the nopaline synthase (NOS) terminator into the vector pBI101. Restriction sites are as follows: P, Pst I; S, Sal I; B, Bam HI; E, Eco RI; and Sc, Sac I. Transcription start is at +1. (**B**) Southern blot analysis. Total DNA (5 μg) from untransformed and transformed plants (*23*) (R1 generation) was digested with Eco RI and Pst I and hybridized (*24*) with a 657-bp fragment from the fourth exon of the *LE-ACC2* gene. Lane 1, untransformed; lane 2, homozygous antisense; and lane 3, heterozygous antisense plants. Hybridizing fragments correspond to the endogenous (*LE-ACC2*) gene at 3.0 kb and the antisense (PO35) gene at 1.7 kb. The 2.5-kb hybridizing fragment in lanes 2 and 3 is due to partial digestion.

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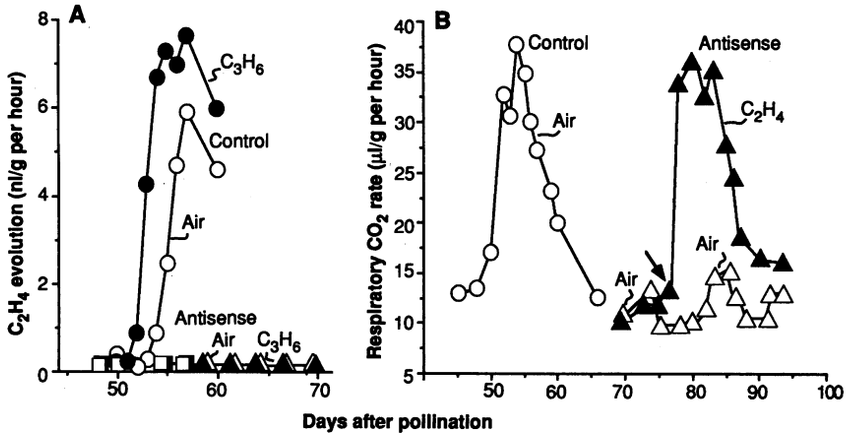

Fig. 2. (**A**) Inhibition of C_2H_4 production in antisense fruits during ripening, and C_2H_4 evolution in air- and C_3H_6-treated fruits. Mature green fruits from control (○, ●), heterozygous (□, ■), and homozygous (△, ▲) A11.1 antisense plants were treated with air (open symbols) or with 1000 µl of C_3H_6 per liter of air (solid symbols). Ethylene production was monitored daily (*25*). (**B**) Inhibition of the respiratory burst in antisense fruits. Respiratory CO_2 production was monitored in a control (45-day-old) and two homozygous antisense (70-day-old) fruits with CO_2-free air. At the arrow, one of the antisense fruits was placed in a stream of air containing 10 µl of C_2H_4 per liter of air as described (*25*). CO_2 production was monitored with a Beckman infrared analyzer.

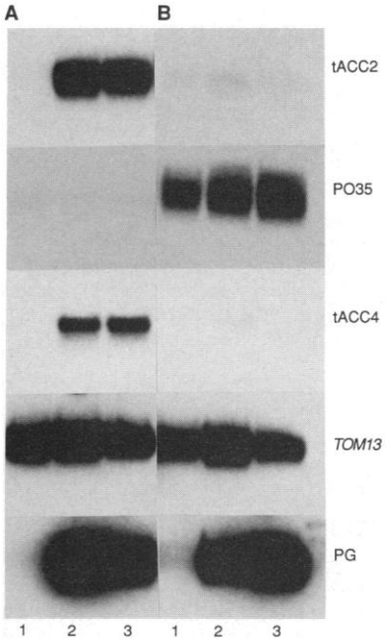

Fig. 4. RNA hybridization analysis (*26*) of control (**A**) and homozygous antisense fruits (**B**). The source of the RNAs were the fruits shown in Fig. 3. Lane 1, RNA isolated from control (48-day-old) and antisense (55-day-old), mature, green fruits. Lane 2, RNA from control and antisense fruits kept in air for 11 days. Lane 3, RNA from control and antisense fruits treated with 1000 µl of C_3H_6 per liter of air for 14 days. The hybridizations were carried out with probes specific for sense tACC2 RNA and antisense RNA (PO35) and with tACC4, *TOM13*, and PG double-stranded probes (*9, 14, 15*).

burst even when they are 95 days old (Fig. 2B). Treatment with propylene (C_3H_6) or C_2H_4 (*13*), which accelerates normal ripening, reverses the antisense phenotype in the absence of endogenous C_2H_4 production (Fig. 2A), and induced the respiratory rise (Fig. 2B) and the ripening process. Propylene- or C_2H_4-ripened antisense fruits are indistinguishable from naturally ripened fruits with respect to texture, color, aroma, and compressibility (*11, 12*).

Mature, 57-day-old, green, antisense fruits express tACC2 antisense RNA (Fig. 4), whereas control fruits do not. Treatment with air or C_3H_6 for 14 days does not alter the amount of antisense RNA. The expression of mRNAs from both ripening-induced ACC synthase genes, *LE-ACC2* and *LE-ACC4* (*9*), is inhibited in antisense fruits treated with air or C_3H_6 (Fig. 4).

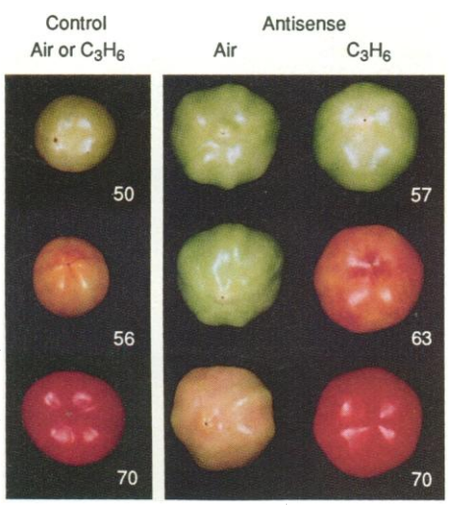

Fig. 3. Phenotype of the fruits used in Fig. 2A. The numbers indicate the age of the fruits in days.

The expression of two other genes, *TOM13* and that encoding polygalacturonase (PG) (*14, 15*), was also analyzed (Fig. 4). *TOM13* mRNA is first detected in control fruits at about 48 days, before ACC synthase mRNA is detectable, and expression remains the same in air- or C_3H_6-treated control fruits. In antisense fruits, *TOM13* and PG mRNA expression is similar to that observed in control fruits, demonstrating that expression of both genes during ripening is C_2H_4-independent. PG may therefore not be solely responsible for tomato fruit softening or may require the induction of a C_2H_4-inducible factor or factors. Antisense RNA to PG does not prevent tomato fruit softening (*16*), and expression of active PG polypeptide in the tomato-ripening mutant *rin* does not result in fruit softening (*17*).

To determine the duration of C_2H_4 treatment required to reverse the antisense phenotype, we treated mature, green fruits (49 days old) from control and antisense plants with C_2H_4 for 1, 2, and 15 days (Fig. 5). Antisense fruits treated for 1 or 2 days with C_2H_4 did not develop a fully ripe phenotype, as compared to control fruits treated similarly. However, antisense fruits treated for 15 days with C_2H_4 ripen normally. The fruits become fully red (*18*) and soft after 7 days of treatment. The C_2H_4-mediated ripening process requires continuous transcription of the necessary genes (C_2H_4 treatment for 1 or 2 days was not sufficient), which may reflect a short half-life of the induced mRNAs or polypeptides.

The short half-life of the ACC synthase polypeptide (25 min) (*19*) is probably an important factor for successful gene inactivation by antisense RNA. Genes whose encoded polypeptides turn over rapidly may be inactivated by their antisense RNA more effectively. The inability to inhibit tomato fruit senescence by antisense RNA of two other ripening-induced genes might be due to the longer half-life of the encoded polypeptides (*14, 16*).

These results demonstrate that C_2H_4 controls the climacteric rise of respiration during fruit ripening and the biochemical changes associated with it, such as softening, color, and aroma development. The respiratory burst is viewed as a consequence of the ripening process, which requires more energy due to the necessary enhanced transcription and protein synthesis (*20*). Ethylene production during ripening is indeed autocatalytically regulated. However, the developmental signals that initially induce ACC synthase gene expression remain to be elucidated. The mode of C_2H_4 action also remains a mystery. It has been suggested that because C_2H_4 is an olefin, its receptor may be a metalloprotein (*21*), a viable proposition in view of the fact that some mam-

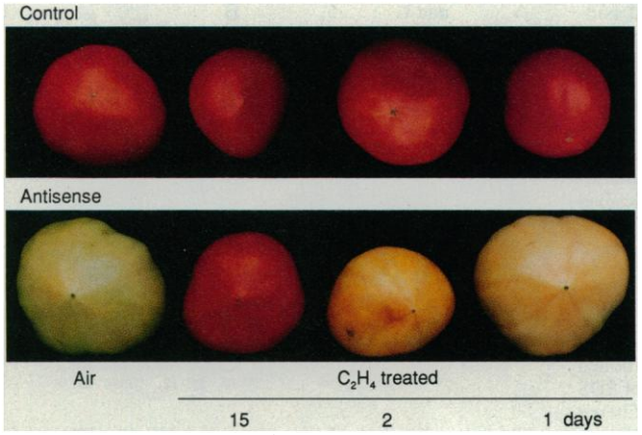

Fig. 5. Effect of C_2H_4 treatment on the antisense phenotype. Mature green fruits from control and homozygous antisense plants were harvested 49 days after pollination and treated with air for 15 days or with 10 μl of C_2H_4 per liter of air for 1, 2, or 15 days and then returned to air (*25*). Fruits were photographed on day 16 after harvesting.

malian hormone receptors are both transcriptional activators and Zn^{2+} metalloproteins (*22*). Expression of antisense RNA to ACC synthase may ameliorate losses due to overripening of fruits and vegetables during transportation or because of lack of refrigeration.

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11. Fruit pigments were extracted with methylene chloride and separated by high-performance liquid chromatography on a Microsorb C_{18} reverse-phase column (Rainin Instruments, Emeryville, CA). The lycopene peak was quantified with the use of external standards with absorbance readings taken at 450 nm. The lycopene content in micrograms per gram of fruit mass of the fruits including those shown in Fig. 3 was the following: control in air at 50 days (green), <0.02; control in air at 59 days (red), 19; control in C_3H_6 at 59 days (red), 25; antisense in air at 57 days (green), <0.02; antisense in air at 70 days (yellow), <0.02; and antisense in C_3H_6 at 70 days (red), 17. The numbers are the average of three measurements.
12. The compressibility was measured in pounds per square inch with a 5/16-inch Magness-Taylor puncture probe (D. Bellauf Manufacturing, Washington, DC). The compressibility data was the following: control in air at 50 days (green), 184; control in air at 59 days (red), 52; control in C_3H_6 at 59 days (red), 52; antisense in air at 57 days (green), 196; antisense in air at 70 days (yellow), 156; and antisense in C_3H_6 at 70 days (red), 52. The numbers are the average of four measurements.
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18. The lycopene content in micrograms per gram of fruit mass of the fruits shown in Fig. 5 is as follows: control green fruit at 49 days, <0.02; control in air for 15 days, 38; control in C_2H_4 for 15 days, 76; control in C_2H_4 for 2 days, 35; control in C_2H_4 for 1 day, 32; antisense green fruit at 49 days, <0.02; antisense in air for 15 days, <0.02; antisense in C_2H_4 for 15 days, 29; antisense in C_2H_4 for 2 days, 1.1; and antisense in C_2H_4 for 1 day, 0.03. The numbers are the average of two measurements.
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23. We ligated the 1789-bp insert of the cDNA clone ptACC2 (*9*) into the Sma I site of pUC19 to yield ptACC2A. A 302-bp CaMV 35*S* promoter fragment was isolated from Hind III–digested pJ024d [D. W. Ow *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4870 (1987)]. The ends were filled with Klenow polymerase and digested with Bam HI [J. Sambrook *et al.*, *Molecular Cloning* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989)]. The promoter fragment was inserted into ptACC2A digested with Xba I, made blunt-ended with Klenow polymerase, and digested with Bam HI. The resulting plasmid ptACC2B contains the 302-bp CaMV 35*S* promoter, followed by the tACC2 ACC synthase cDNA in an antisense orientation. The 2.1-kb antisense gene (CaMV-tACC2) was excised by partial Sac I and complete Sal I digestion and ligated into pBI101 vector (Clontech, Palo Alto, CA), which yields pP035 (Fig. 1A). The pP035 plasmid was transferred from *Escherichia coli* DH5α to *Agrobacterium tumefaciens* LBA4404 by triparental mating with *E. coli* HB101 harboring pRK2013. The tomato cultivar VF36 was transformed with 14-day-old cotyledons as described [S. McCormick *et al.*, *Plant Cell Rep.* **5**, 81 (1986)].
24. Genomic DNA was extracted from frozen leaf tissue, digested with Eco RI and Pst I, and hybridized with a ^{32}P-labeled 657-bp Sac I–Hind III fragment from the fourth exon of the *LE-ACC2* gene (4642 to 5299 bp) (*9*).
25. Fruits were placed in 4-liter glass jars and flushed with a stream of water-saturated air with or without 1000 μl of C_2H_6 or 10 μl of C_2H_4 per liter of air. We monitored C_2H_4 production by sealing individual fruits in glass containers of known volume for 1 hour. Gas samples (1 ml) were analyzed with a Hewlett-Packard gas chromatograph.
26. Total nucleic acids were extracted from tomato fruit and analyzed as described (*9*). Sense and antisense ACC synthase RNA were detected with strand-specific ^{32}P-labeled riboprobes transcribed by T7 polymerase containing the tACC2 cDNA (*9*). The washing was at 75°C instead of 60°C. Other hybridization probes were a random-primed, ^{32}P-labeled, 1.2-kb Eco RI fragment of tACC4 (*9*); a 1.6-kb Eco RI fragment of a PG cDNA (*15*); and a 0.9-kb fragment obtained with the polymerase chain reaction on the *TOM13* cDNA (228 to 1122 bp) (*14*).
27. This paper is dedicated to G. G. Laties and J. E. Varner on their retirement as professors of biology at the University of California at Los Angeles and Washington University, respectively. We thank M. Johnston, L. Giudice, and R. A. Wells for help with the manuscript; B. Baker and S. McCormick for seeds of the cultivar VF36; K. Corr and M. Boylan for technical advice; and J. Fink, N. Shen, K. Fong, N. Mahoney, A. Spelletich, and B. Lamb for technical and greenhouse assistance. Supported by grants from the NSF (DCB-8645952, DCB-8819129, DCB-8916286), the NIH (GM-35447), and the United States Department of Agriculture (5835-23410-D002) (to A.T.).

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A 32-kD GTP-Binding Protein Associated with the CD4-p56^{lck} and CD8-p56^{lck} T Cell Receptor Complexes

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The guanosine triphosphate (GTP)–binding proteins include signal-transducing heterotrimeric G proteins (for example, G_s, G_i), smaller GTP-binding proteins that function in protein sorting, and the oncogenic protein p21^{ras}. The T cell receptor complexes CD4-p56^{lck} and CD8-p56^{lck} were found to include a 32- to 33-kilodalton phosphoprotein (p32) that was recognized by an antiserum to a consensus GTP-binding region in G proteins. Immunoprecipitated CD4 and CD8 complexes bound GTP and hydrolyzed it to guanosine diphosphate (GDP). The p32 protein was covalently linked to [α-^{32}P]GTP by ultraviolet photoaffinity labeling. These results demonstrate an interaction between T cell receptor complexes and an intracellular GTP-binding protein.

GTP-BINDING PROTEINS AFFECT the enzymatic activity of adenyl cyclase, retinal cyclic guanosine monophosphate phosphodiesterase (GMP),

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