Plant Physiol. (1988) 88, 370–374
0032-0889/88/88/0370/05/$01.00/0

Regulation of Gene Expression by Ethylene in Wild-Type and *rin* Tomato (*Lycopersicon esculentum*) Fruit[1]

Received for publication March 8, 1988 and in revised form May 23, 1988

JAMES E. LINCOLN AND ROBERT L. FISCHER*
Division of Molecular Plant Biology, University of California, Berkeley, California 94720

ABSTRACT

Levels of ethylene biosynthesis and ethylene-inducible gene expression in wild-type tomato (*Lycopersicon esculentum*) fruit and in nonripening fruit from the tomato mutant *rin* (ripening inhibitor) were compared in order to investigate the mechanism of ethylene action. Whereas wild-type tomato fruit dramatically increase the rate of ethylene biosynthesis at the onset of ripening, *rin* fruit constitutively produce ethylene at a low basal level. We have compared the mRNA levels and transcription rates of four cloned ethylene-inducible genes (JE Lincoln, S Cordes, E Read, RL Fischer 1987 Proc Natl Acad Sci USA 84: 2793–2797) during wild-type and *rin* fruit development. In wild-type fruit, both mRNA levels and transcription rates of these genes increase. The effect of the *rin* mutation on gene expression is different for each ethylene-inducible gene. In one case expression is completely suppressed, while in other instances it is either partially inhibited or relatively unaffected by the mutation. The mRNA levels of each of these genes in response to exogenous ethylene in *rin* fruit was also measured. The mRNAs for all four genes accumulate to similar levels in both ethylene treated *rin* and ethylene treated wild-type fruit. These results are discussed with regard to the response of plants to ethylene hormone at the level of gene expression.

The plant hormone ethylene affects many aspects of plant development (2, 10) and is thought to regulate the ripening of many climacteric fruits, such as tomato (3, 13, 21, 27). For example, the onset of tomato fruit ripening is associated with an increase in ethylene biosynthesis, the onset is hastened when unripe fruit are exposed to exogenous ethylene, and the removal of ethylene from fruit or exposure of fruit to specific inhibitors of ethylene biosynthesis greatly retard ripening. Thus, ethylene influences the developmental processes associated with tomato fruit ripening.

One hypothesis for the mechanism of ethylene action is that it controls the expression of specific genes. Exposure of unripe tomato fruit to exogenous ethylene has been shown to increase both the concentration of specific mRNAs (11, 15) and the transcription rate of specific genes (12) which are expressed during ripening. We have previously shown that the activation of expression of certain ethylene-inducible genes during tomato fruit development coincides with the increase in endogenous ethylene levels, while the induction of others precedes the increase in ethylene levels. These results suggested that both levels of ethylene and sensitivity to basal levels of ethylene play a role in the regulation of gene expression (11).

[1] This research was supported by National Institutes of Health grant (GM33856).

The tomato fruit ripening mutant *rin*[2] has been an important tool in comparative biochemical and physiological studies with wild-type ripening genotypes (4, 6, 16, 17, 22). Normal ripening processes (*e.g.* chlorophyll degradation, synthesis of carotenoid pigments, breakdown of cell wall components, climacteric respiration, increased ethylene biosynthesis) are all inhibited by the *rin* mutation. All other aspects of *rin* plant growth and development with the exception of calyx size appear to be normal. We report here the mRNA levels and relative transcription rates of ethylene-inducible genes during *rin* fruit development and upon exposure of *rin* fruit to exogenous ethylene. We discuss these results with regard to the regulation of gene expression by ethylene levels and sensitivity.

MATERIALS AND METHODS

Plant Material. Wild-type (*Lycopersicon esculentum* cv Ailsa Craig) and isogenic *rin* (ripening inhibitor) (14) tomato plants were grown under standard greenhouse conditions. Wild-type and *rin* fruit appear similar during the early stages of fruit development before the onset of ripening. Wild-type and *rin* immature fruit were 50% full size. Mature green stages MG1, MG2, and MG3 of wild-type and *rin* fruit were identified by the extent of locular tissue breakdown (11). In wild-type fruit the onset of ripening is thought to occur at the MG4 stage, when the level of ethylene biosynthesis increases and traces of yellow and red carotenoid pigments accumulate in the interior of the fruit. Because ripening is inhibited by the *rin* mutation, later stages were defined by fruit age (DPA). Forty-two DPA wild-type fruit were MG3 to 50% red. Forty-two DPA *rin* fruit were mature green, although traces of yellow pigment could be detected on the exterior of some fruits. Sixty-two DPA fruit were red ripe for wild-type and light yellow for *rin* fruit.

Exposure of Tissue to Ethylene. Approximately 1 kg of mature green fruit was placed in a 25-L chamber and exposed to 4.5 L per min of 10 μL/L ethylene in humidified air.

Wounding of Tissue. MG1 fruit pericarp was cut into slices 2 mm thick using a razor blade. The slices were placed on filter paper moistened with distilled water in a 50-mL container which was then sealed. One-mL samples of gas were removed, and the ethylene content was determined by gas chromatography (Varian 500).

mRNA Isolation. Fruit pericarp was frozen in liquid nitrogen and stored at −80°C. Polysomal, poly(A)$^+$ mRNA was isolated using procedures described elsewhere (11).

***In Vitro* Nuclear RNA Synthesis and [^{32}P]nRNA Isolation.** Nuclei isolation, [^{32}P]nRNA synthesis, and [^{32}P]nRNA isolation were carried out as described elsewhere (12, 26). Under

[2] Abbreviations: *rin*, ripening inhibitor tomato mutant; MG, mature green; DPA, days post-anthesis; [^{32}P]nRNA, *in vitro* ^{32}P-labeled nuclear RNA transcripts.

these conditions [^{32}P]UTP incorporation was reduced by 45% in the presence of 2 μg/ml of α-amanitin, transcript sizes ranged from 0.1 to 4.0 kb, and transcription was asymmetric.

DNA Gel-Blot Hybridization. Five μg of plasmid DNA was digested with appropriate restriction endonucleases, and the cDNA insert was separated from the vector DNA by agarose gel electrophoresis. The DNA was then blotted onto nitrocellulose as described by Southern (23), and hybridized with [^{32}P]nRNA for 48 hr at 42°C. The hybridization buffer contained 10 mM Tes (pH 7.4), 1 mM EDTA, 0.2% SDS, 300 mM NaCl, 30% formamide, and 0.2 mg/ml polyadenylic acid.

RNA Dot-Blot Hybridization. RNAs were bound to nitrocellulose and then hybridized with a mass excess of [^{32}P]-labeled plasmid DNA as described by Thomas (24).

Plasmids. pE4, pE8, pE17, and pJ49 are cDNA clones of ethylene-inducible genes from wild-type tomato fruit, *Lycopersicon esculentum* cv VFNT Cherry (11, 12). pD21-3 is a control cDNA clone. D21-3 mRNA concentration is constant (approximately 0.5% mRNA mass) during wild type tomato fruit ripening and does not change in response to ethylene treatment (12).

RESULTS

Ethylene Production in Wild-Type and *rin* Fruit. The ethylene production rates during wild-type and *rin* tomato fruit development were measured. Unripe wild-type fruit (immature, MG1, and MG2) evolved ethylene at a low, basal rate (Fig. 1A). At the MG3 stage a small increase in ethylene evolution rate was detected, and by the MG4 stage when overt signs of ripening occur, the ethylene evolution rate sharply increased. In contrast, *rin* fruit did not exhibit a burst of ethylene production during development characteristic of wild-type fruit (6), but did produce ethylene at a low basal level throughout fruit development (Fig. 1A). Though ethylene production is inhibited during *rin* fruit development, the fruit are capable of producing large amounts of ethylene in response to wounding (6, 17). The rate of ethylene production in wounded unripe *rin* fruit was found to be identical to that of wounded unripe wild-type fruit (Fig. 1B).

Changes in Gene Expression during Wild-Type and *rin* Fruit Development. We have previously analyzed the expression of four ethylene-inducible genes (*E4*, *E8*, *E17*, and *J49*) from wild-type tomato fruit (11, 12). Several results demonstrated regulation by ethylene levels for all four genes. (a) Messenger RNA encoded by each gene rapidly accumulated in response to exogenous ethylene. (b) Norbornadiene, a specific competitive inhibitor of ethylene, inhibited expression of each gene. (c) During fruit ripening, the activation of *E4* and *E8* gene transcription coincided with the increase in endogenous ethylene levels. However, we also found evidence suggesting that changes in sensitivity to ethylene also played a role in the regulation of *E17* and *J49* gene expression. That is, activation of *E17* and *J49* gene transcription preceded the burst in ethylene biosynthesis.

To continue our investigation of the role of ethylene in the expression of these genes, the levels of E4, E8, E17, and J49 mRNAs were compared during wild-type and *rin* fruit development. E4 and E17 mRNA levels remain very low throughout *rin* fruit development (Fig. 2). The level of E8 mRNA is significantly suppressed, and accumulates to only 30% of the level attained during wild-type fruit development. J49 mRNA accumulation during fruit development is only slightly depressed by the *rin* mutation. From these results we conclude that the *rin* mutation inhibits the accumulation of some but not all ethylene-inducible mRNAs during fruit development.

Transcriptional Regulation of Gene Expression during Wild-Type and *rin* Fruit Development. To determine whether transcriptional processes were responsible for the observed changes in mRNA concentration, we isolated nuclei from MG1 and 42 DPA fruit harvested from wild-type and *rin* plants and used the nuclei to synthesize [^{32}P]nRNA. The [^{32}P]nRNA was purified and hybridized to blotted cDNA clones. Others have shown that the extent of hybridization estimates [^{32}P]nRNA concentration, which is proportional to the rate of gene transcription (7, 8). The degree of hybridization to each of the cDNA inserts is shown in Figure 3. Our results show that transcription of the *E4* gene is significantly suppressed in *rin* fruit compared to wild-type fruit. *E8* and *J49* gene transcription, which greatly increase in 42 DPA wild-type fruit, are substantially reduced in 42 DPA *rin* fruit. In contrast, the *E17* gene transcription rate is not significantly affected by the *rin* mutation. *E17* gene transcription is low in wild-type and *rin* MG1 fruit and increases approximately twofold in both wild-type and *rin* 42 DPA fruit. The transcription rate of a control clone, D21-3, is high and does not change significantly during development in both wild-type and *rin* fruit. We conclude from these data that the *rin* mutation inhibits the transcriptional activation of some, but not all ethylene-inducible genes during fruit development.

E17 gene transcription during both wild-type and *rin* fruit development increases twofold but the mRNA level (Fig. 2) increases 20-fold in wild-type fruit and remains basal in *rin* fruit.

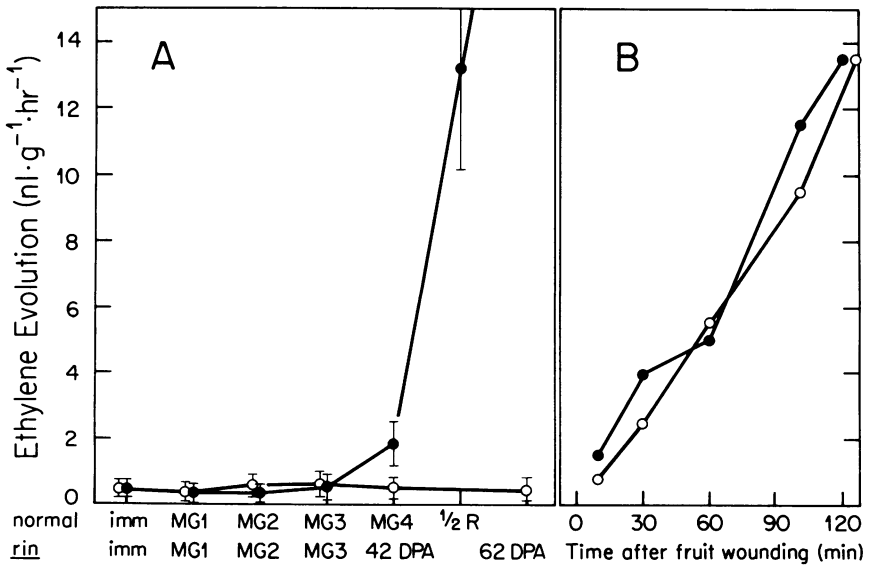

FIG. 1. Ethylene evolution during fruit development and upon wounding of fruit pericarp. (A) Individual wild-type fruit (●) or *rin* fruit (○) were placed in 100-mL containers that were sealed and incubated 1 h. A 1-mL sample from the closed atmosphere was removed and the ethylene content was determined by gas chromatography (Varian 500). Results represent the mean ± standard deviation. (B) MG1 pericarp of wild-type fruit (●) or *rin* fruit (○) were wounded and the rate of ethylene evolution determined as described in the "Materials and Methods."

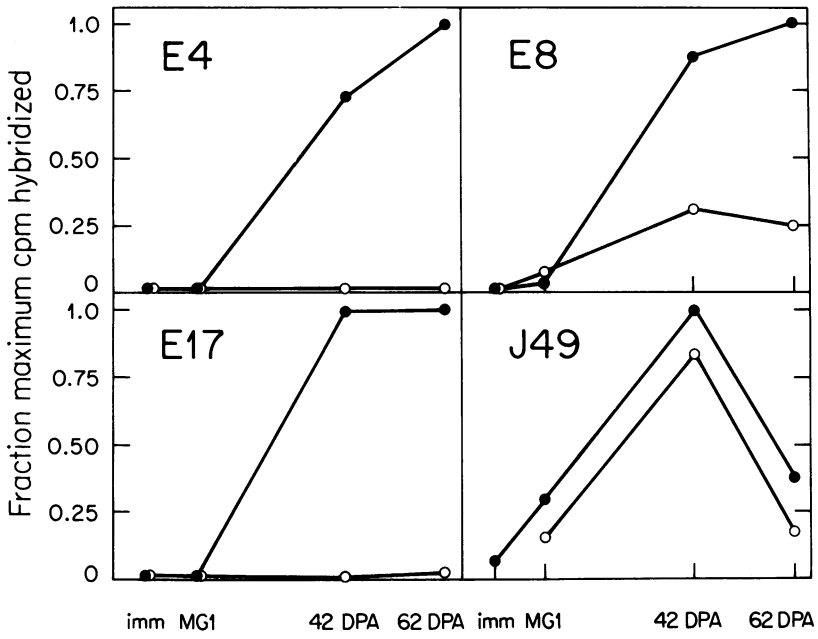

FIG. 2. Accumulation of specific mRNAs during fruit development. One microgram of mRNA from wild-type fruit (●) or *rin* fruit (○) at the indicated stages was dotted onto nitrocellulose filters and hybridized with the cloned ^{32}P-labeled DNA probes. Following autoradiography, each dot was excised, and the extent of hybridization was determined by liquid scintillation spectrometry. Maximum cpm hybridized for each ^{32}P-labeled DNA probe was 6652 cpm for E4, 2692 cpm for E8, 2311 cpm for E17, and 3884 cpm for J49.

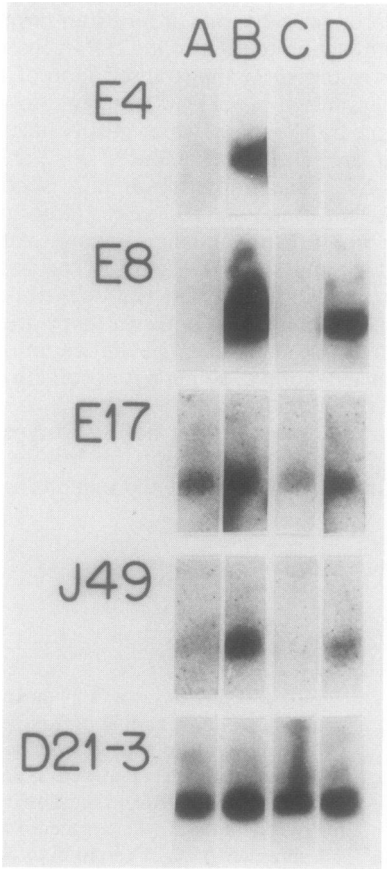

FIG. 3. Relative rate of gene transcription during fruit development. Nuclei were isolated from wild-type MG1 fruit (lane A), wild-type 42 DPA fruit (lane B), *rin* MG1 fruit (lane C), *rin* 42 DPA fruit (lane D). [^{32}P]nRNA was synthesized and hybridized with DNA gel blots of the designated cDNA clones. Blots were then treated with RNAse A according to Walling *et al.* (26) and autoradiographed for 2 d (E4, E8, and D21-3) or 9 d (E17 and J49).

This result suggests that the *rin* mutation inhibits the accumulation of E17 mRNA at the post-transcriptional level.

Induction of Gene Expression by Exogenous Ethylene in *rin*. One possibility is that low ethylene levels are responsible for the inhibition of E4, E8, and E17 mRNA accumulation in *rin* fruit. To test this hypothesis, *rin* fruit were exposed to exogenous ethylene and the mRNA levels were measured. We isolated mRNA from 35, 42, and 62 DPA *rin* tomato fruit exposed to 10 μL/L ethylene for 10 h and found that exposure of *rin* fruit to exogenous ethylene results in the accumulation of the E4, E8, and E17 mRNAs. In addition, we found that J49 mRNA concentration also increased in response to exogenous ethylene (Fig. 4). The degree to which exogenous ethylene affects these specific mRNA concentrations varies depending on the age of the fruit. E4 and E8 mRNA levels accumulate to higher levels in 62 DPA fruit than in 35 DPA fruit. The reverse is true for E17 and J49, where exogenous ethylene leads to higher mRNA concentrations in 35 DPA fruit than in 62 DPA fruit. We conclude that these genes are capable of responding to exogenous ethylene by rapid changes in mRNA levels in both wild-type (11) and *rin* tomato fruit.

DISCUSSION

Ethylene and the *rin* Mutation. The *rin* mutation affects many aspects of tomato fruit ripening. However, one of the most striking characteristics of *rin* fruit is that they synthesize basal levels of ethylene throughout development and lack the large increase in ethylene production characteristic of wild type fruit (Fig. 1A). Others have attempted to induce ripening by treating *rin* fruit with exogenous ethylene (5, 6, 20). Ethylene treated *rin* fruit exhibit an increase in respiration and lycopene pigment levels, but do not exhibit autocatalytic ethylene production and do not ripen completely. It has been suggested that *rin* fruit are lacking certain ripening specific ethylene receptors (19). We found that *rin* fruit exposed to exogenous ethylene expressed ethylene-inducible genes (Fig. 4). This suggests that for the regulation of these genes, *rin* fruit have the capacity to interact with elevated levels of ethylene in a manner similar to that of wild-type fruit. However, measurements of ethylene-inducible gene expression in response to various ethylene concentrations (dose

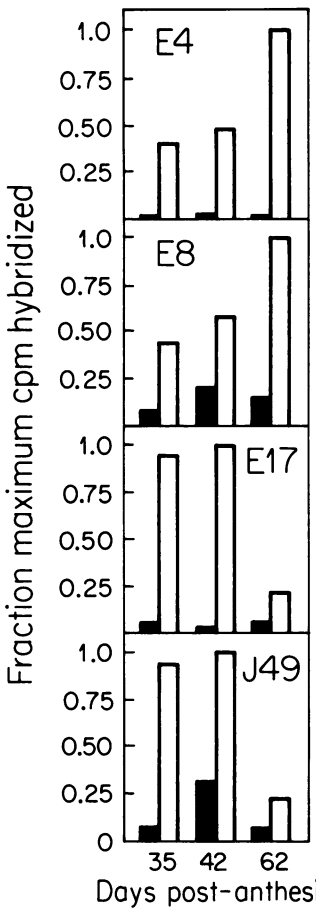

FIG. 4. Accumulation of specific mRNAs in ethylene treated *rin* fruit. RNA was isolated from *rin* fruit of the indicated ages either untreated (■) or treated with 10 μL/L ethylene in air for 10 h (□). RNA was dotted onto nitrocellulose filters and hybridized with the indicated ^{32}P-labeled DNA probes. Following autoradiography, each dot was excised, and the extent of hybridization was determined by liquid scintillation spectrometry. Maximum cpm hybridized for each ^{32}P-labeled DNA probe was 1143 cpm for E4, 1958 cpm for E8, 438 cpm for E17, and 1328 cpm for J49.

response curves) may reveal more subtle differences in ethylene perception between wild-type and *rin* fruit.

Regulation of Gene Expression by Ethylene. It has been proposed that cellular responses to plant hormones are mediated by changes in hormone concentration and by changes in sensitivity to hormones (25). Physiological evidence indicates that ethylene-mediated processes such as leaf abscission (1), flower senescence (9), and fruit ripening (18, 27) involve changes in both ethylene levels and sensitivity to ethylene. We have previously reported molecular evidence that ethylene levels and sensitivity play a role in regulating the expression of four ethylene-inducible genes (*E4*, *E8*, *E17*, and *J49*) during fruit ripening (11, 12). We have shown that *rin* fruit synthesize constitutive basal ethylene levels (Fig. 1A) and respond to ethylene by activating *E4*, *E8*, *E17*, and *J49* gene expression (Fig. 4). These results suggest that by comparing the expression of ethylene-inducible gene expression during *rin* and wild-type fruit development, it may be possible to distinguish between the regulation of gene expression by changes in ethylene concentration from changes in sensitivity to ethylene. That is, one would expect the former to be inhibited in *rin* fruit, whereas the latter might be unaffected by this mutation.

During *rin* fruit development we find that E4 and E8 mRNA levels (Fig. 2) and relative rates of gene transcription (Fig. 3) are substantially inhibited. However, exposure of *rin* fruit to ethylene activates their expression (Fig. 4). These data are consistent with our hypothesis that *E4* and *E8* gene expression is regulated by changes in ethylene concentration (11, 12). Since *E8* gene expression still occurs at 30% of wild-type levels, this result suggests that during fruit development, *E8* gene expression may also be influenced by changes in ethylene sensitivity or by factors other than ethylene. Although *J49* gene transcription (Fig. 3) is partially suppressed, the fact that the J49 mRNA levels (Fig. 2) are relatively unaffected by the *rin* mutation suggests that changes in ethylene sensitivity, or possibly factors other than ethylene, regulates *J49* gene expression during *rin* fruit development.

The regulation of *E17* gene expression is perhaps the most complex. Although *E17* gene transcription (Fig. 3) is not significantly affected by the *rin* mutation, E17 mRNA does not accumulate (Fig. 2). This result suggests that *E17* gene transcription is activated by increased sensitivity to the basal levels of ethylene in *rin* fruit, but that post-transcriptional processes, essential for the accumulation of E17 mRNA, require increased levels of ethylene. This result is consistent with previous experiments that indicated that ethylene regulates *E17* gene expression in wild type fruit at both transcriptional and post-transcriptional levels (12). However, these results are not consistent with the fact that the accumulation of E17 mRNA during wild-type fruit development occurs before the onset of elevated ethylene biosynthesis (11). Thus, processes that allow the E17 mRNA to accumulate in wild-type fruit when the level of ethylene is basal are not functioning in the *rin* fruit. Identification of the processes involved will require a greater understanding of the biochemical defects associated with the *rin* genetic lesion.

In summary, we have investigated the regulation of gene expression in wild-type and *rin* fruit. We have found that the *rin* mutation causes ethylene levels to remain at basal levels throughout fruit development. We have shown that these reduced levels of ethylene inhibit transcriptional activation of the *E4* gene and significantly reduces *E8* and *J49* gene transcription, although J49 mRNA shows normal patterns of accumulation. In contrast, the *E17* gene exhibits relatively normal patterns of gene transcription but E17 mRNA accumulation is inhibited. These results suggest that during fruit ripening, gene expression is regulated by both ethylene levels and sensitivity to ethylene, at both transcriptional and post-transcriptional levels. The molecular basis for these kinds of regulation by ethylene will be better understood when the nucleotide sequences and cellular factors that regulate ethylene-inducible gene expression are isolated and analyzed.

Acknowledgment—We express our appreciation to Evan B. Read for expert technical assistance during the early stages of this investigation.

LITERATURE CITED

1. ABELES FB 1967 Mechanism of action of abscission accelerators. Physiol Plant 20: 442–454
2. ABELES FB 1973 Ethylene in Plant Biology. Academic Press, New York, pp 1–302
3. BIALE JB, RE YOUNG 1981 Respiration and ripening in fruits—retrospect and prospect. *In* J Friend, MJC Rhodes, eds, Recent Advances in the Biochemistry of Fruits and Vegetables. Academic Press, London, pp 1–39
4. DELLAPENNA D, DS KATES, AB BENNETT 1987 Polygalacturonase gene expression in Rutgers, *rin*, *nor*, and *Nr* tomato fruits. Plant Physiol 85: 502–507
5. FRENKEL C, SA GARRISON 1976 Initiation of lycopene synthesis in the tomato mutant *rin* as influenced by oxygen and ethylene interactions. HortScience 11: 20–21
6. HERNER RC, KC SINK 1973 Ethylene production and respiratory behavior of the *rin* tomato mutant. Plant Physiol 52: 38–42
7. HOFER E, JE DARNELL 1981 The primary transcription unit of the mouse Beta-major globin gene. Cell 23: 585–593
8. HOFER E, R HOFER-WARBINEK, JE DARNELL 1982 Globin RNA transcription: a possible termination site and demonstration of transcriptional control correlated with altered chromatin structure. Cell 29: 887–893
9. KENDE H, AD HANSON 1976 Relationship between ethylene evolution and senescence in morning-glory flower tissue. Plant Physiol 57: 523–527

10. LIEBERMAN M 1979 Biosynthesis and action of ethylene. Annu Rev Plant Physiol 30: 533–591
11. LINCOLN JE, S CORDES, E READ, RL FISCHER 1987 Regulation of gene expression by ethylene during *Lycopersicon esculentum* (tomato) fruit development. Proc Natl Acad Sci USA 84: 2793–2797
12. LINCOLN JE, RL FISCHER 1988 Diverse mechanisms for the regulation of ethylene-inducible gene expression. Mol Gen Genet 212: 71–75
13. LYONS JM, HK PRATT 1964 Effect of stage of maturity and ethylene treatment on respiration and ripening of tomato fruits. Proc Am Soc Hortic Sci 84: 491–500
14. MAXON SMITH J W, DB RITCHIE 1983 A collection of near-isogenic lines of tomato. Plant Mol Biol Rep 1: 41–45
15. MAUNDERS MJ, MJ HOLDSWORTH, A SLATER, JE KNAPP, CR BIRD, W SCHUCH, D GRIERSON 1987 Ethylene stimulates the accumulation of ripening-related mRNAs in tomatoes. Plant Cell Environ 10: 177–184
16. MCGLASSON WB, HC DOSTAL, EC TIGCHELAAR 1975 Comparison of propylene-induced responses of immature fruit of normal and *rin* mutant tomatoes. Plant Physiol 55: 218–222
17. MCGLASSON WB, BW POOVAIAH, HC DOSTAL 1975 Ethylene production and respiration in aging leaf segments and in disks of fruit tissue of normal and mutant tomatoes. Plant Physiol 56: 547–549
18. MCGLASSON WB 1978 Phytohormones and fruit ripening. *In* DS Letham, PB Goodwin, TJ Higgens, eds, Phytohormones and Related Compounds: A Comprehensive Treatise. Elsevier, Amsterdam, pp 447–493
19. MCGLASSON WB 1985 Ethylene and fruit ripening. HortScience 20: 51–54
20. MIZRAHI Y, HC DOSTAL, JH CHERRY 1975 Ethylene-induced ripening in attached *rin* fruits, a non-ripening mutant of tomato. HortScience 10: 414–415
21. RHODES MJC 1980 The maturation and ripening of fruits. *In* Thimann KV, ed, Senescence in Plants. CRC Press, Boca Raton, pp 157–205
22. ROBINSON RW, ML TOMES 1968 Ripening inhibitor: a gene with multiple effects on ripening. Tomato Genet Coop Rep 18: 36–37
23. SOUTHERN EM 1975 Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98: 503–517
24. THOMAS PS 1983 Hybridization of denatured RNA transferred or dotted to nitrocellulose paper. Methods Enzymol 100: 255–266
25. TREWAVAS AJ, RE CLELAND 1983 Is plant development regulated by changes in the concentration of growth substances or by changes in the sensitivity to growth substances. Trends Biochem Sci 8: 354–357
26. WALLING L, GN DREWS, RB GOLDBERG 1986 Transcriptional and posttranscriptional regulation of soybean seed protein mRNA levels. Proc Natl Acad Sci USA 83: 2123–2127
27. YANG SF 1985 Biosynthesis and action ethylene. HortScience 20: 41–45