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# Diverse mechanisms for the regulation of ethylene-inducible gene expression

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**Summary.** We have investigated the mechanism of action of the plant hormone ethylene by analyzing the expression of ethylene-inducible genes isolated from tomato (*Lycopersicon esculentum*). We have found that the expression of each cloned gene is regulated by ethylene in a unique manner. That is, for certain genes ethylene affects transcriptional processes, while for another gene it affects both transcriptional and post-transcriptional processes. Furthermore, induction of gene transcription by ethylene is organ specific for one gene, while for others it is not. In addition, we have measured gene expression as a function of ethylene concentration and have found that each gene displays a unique ethylene dose-response curve. Our results suggest that ethylene modulates gene expression by a variety of mechanisms.

**Key words:** Ethylene – *Lycopersicon esculentum* – Fruit ripening – Gene transcription

## Introduction

The plant hormone ethylene affects many different aspects of plant development, including seed germination, seedling growth, root and leaf growth, certain stress phenomena, such as wounding, and fruit development (Abeles 1973; Lieberman 1979). The involvement of ethylene in the ripening of many climacteric fruits, such as tomato, has much experimental support (Lyons and Pratt 1964; Rhodes 1980; Biale and Young 1981; Yang 1985). First, the onset of tomato fruit ripening is associated with an increase in ethylene biosynthesis. Second, the onset is hastened when unripe fruit are exposed to exogenous ethylene. Third, the removal of ethylene from fruit or exposure of fruit to specific inhibitors of ethylene biosynthesis greatly retards ripening. Thus, many different processes associated with fruit ripening including the accumulation of carotenoid pigments, the conversion of chloroplasts to chromoplasts, and the breakdown of cell wall components may be influenced by ethylene.

One hypothesis for the diversity of physiological and biochemical responses to ethylene is that ethylene regulates the expression of many genes. Changes in gene expression involving both protein and mRNA accumulation have been shown to occur during tomato fruit ripening (Rhodes 1980; Biale and Young 1981; Su et al. 1984; Grierson 1985; Biggs et al. 1986; Lincoln et al. 1987). In some cases, the induction of gene expression coincides with the increase in endogenous ethylene levels (Lincoln et al. 1987). In addition, exposure of unripe fruit to exogenous ethylene has been shown to affect changes in the accumulation of specific mRNAs which are expressed during ripening (Lincoln et al. 1987; Maunders et al. 1987).

To understand the mechanism of ethylene action, we have studied how the expression of four cloned genes is regulated by endogenous ethylene during tomato fruit ripening, and by exogenous ethylene applied to unripe fruit and leaves. Specifically, we have determined the extent to which ethylene regulates transcription of specific genes, and the effect of ethylene concentration on gene expression. Our results suggest that ethylene modulates gene expression by a variety of mechanisms.

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*Abbreviations:* MG, mature green; [$^{32}$P]nRNA, in vitro $^{32}$P-labeled nuclear RNA transcripts

## Materials and methods

*Plant material. Lycopersicon esculentum* cv. VFNT Cherry plants were grown under standard green house conditions. Fruit maturity stage and ethylene evolution rates were determined as described previously (Lincoln et al. 1987). In immature, MG1, and MG2 stage fruit the ethylene evolution rate was basal ($0.6 \pm 0.2$ nl/g per h). In MG3 fruit the ethylene production rate increased ($1.0 \pm 0.4$ nl/g per h). In MG4 fruit, carotenoid pigments were observed in the interior of the fruit and a sharp rise in ethylene production was detected ($3.5 \pm 1.0$ nl/g per h). Ethylene production rate ($9.5 \pm 2.5$ nl/g per h) and carotenoid pigment levels increased further in 50% red fruit.

*Exposure of tissue to gases.* One kilogram of mature green fruit or a 10 in. tall potted plant was placed in a 25 l chamber and exposed for 2 h to 4.5 l per min of ethylene (10 µl per l unless specified otherwise) in humidified air. Control tissue was exposed to humidified air alone.

*mRNA isolation.* Fruit pericarp or leaf tissue was frozen in liquid nitrogen and stored at −80° C. Polysomal, poly(A)$^+$ mRNA was isolated using procedures described elsewhere (Lincoln et al. 1987; Goldberg et al. 1981).

*In vitro nuclear RNA synthesis.* Nuclei were isolated as described by Luthe and Quatrano (1980a; b) except buffers were adjusted to pH 8.5. [$^{32}$P]nRNA synthesis was carried

out as described by Walling et al. (1986). Under these conditions [$^{32}$P]-UTP incorporation was reduced by 45% in the presence of 2 μg/ml of α-amanitin, transcript sizes ranged from 0.1–4.0 kb, and transcription was asymmetric.

*[$^{32}$P]nRNA isolation.* [$^{32}$P]nRNAs were isolated according to the procedure of Groudine et al. (1981) with the modifications introduced by Walling et al. (1986), except the CsCl gradient step was omitted.

*DNA gel-blot hybridization.* Five micrograms of plasmid DNA was digested with the appropriate restriction endonuclease to separate recombinant DNA sequences from vector DNA sequences. The digested DNA was subjected to agarose gel electrophoresis, blotted onto nitrocellulose as described by Southern (1975), and hybridized with [$^{32}$P]nRNA for 48 h at 42° C. The hybridization buffer contained 10 mM TES (N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid) 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.* mRNAs were bound to nitrocellulose and then hybridized with a mass excess of [$^{32}$P]-labeled plasmid DNA as described by Thomas (1983).

*Plasmids.* pE4, pE8, pE17, and pJ49 are cDNA clones to ethylene-inducible genes from tomato (Lincoln et al. 1987). pD21-3 is a control cDNA clone. D21-3 mRNA concentration is constant (approximately 0.5% mRNA mass) during fruit ripening and does not change in response to ethylene treatment.

## Results

### *Transcriptional control of gene expression during fruit development*

Previously, we have shown that during tomato fruit development there is an increase in the levels of four mRNAs represented by the E4, E8, E17, and J49 cDNA clones (Lincoln et al. 1987). Based on gene cloning experiments, the E4, E8, E17 and J49 mRNAs are encoded by small families of 1, 3, 1, and 5 genes, respectively (R. Fischer et al., in preparation). To determine whether transcriptional activation of these genes was responsible for the observed increase in mRNA concentration, we isolated nuclei from fruits at different developmental stages and used them 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 the [$^{32}$P]nRNA concentration which is proportional to the rate of gene transcription (Hofer and Darnell 1981; Hofer et al. 1982). Figure 1 shows the degree of hybridization of [$^{32}$P]nRNA to each of the cDNA inserts. *E4, E8, E17,* and *J49* gene transcription rates increased markedly during fruit development. In contrast, the transcription rate of a constitutively expressed gene, represented by the cDNA clone D21-3, was constant. These results indicate that changes in gene transcription rate during fruit development play an important role in regulating E4, E8, E17, and J49 mRNA levels.

The exposure of unripe MG1 tomato fruit to ethylene leads to an increase in E4, E8, E17 and J49 mRNA levels (Lincoln et al. 1987). To investigate the mechanism of gene activation, we measured the relative transcription rates of these ethylene-inducible genes in MG1 tomato fruit treated with ethylene or with air for 2 h. As shown in Fig. 2, the level of [$^{32}$P]nRNA hybridizing with the E4, E8, and J49 cDNA clones was low in the control fruit, but dramatically increased when MG1 fruit were exposed to ethylene. This result indicates that ethylene increased the relative transcription rate of the *E4, E8,* and *J49* genes. In contrast, *E17* gene transcription did not significantly increase in response to exogenous ethylene, although the concentration of E17 mRNA increased sixfold. Since the rate of *E17* gene transcription at the MG1 stages is already approximately 60% of the maximum level it attains during fruit ripening (Figs. 1 and 5), it is not unexpected that exogenous ethylene did not elevate the transcription rate of this gene further. This data indicates that post-transcriptional processes regulate the increase in E17 mRNA concentration observed in response to exogenous ethylene.

To determine whether the rapid accumulation of specific mRNAs in response to ethylene is organ specific, whole plants were treated with ethylene or with air for 2 h. The leaves were harvested, and the mRNA level and the relative transcription rate for each cloned gene was determined. As shown in Fig. 3, E4, E17 and J49 mRNA levels and relative rates of gene transcription increased in response to the ethylene treatment in leaves. This shows that ethylene-induc-

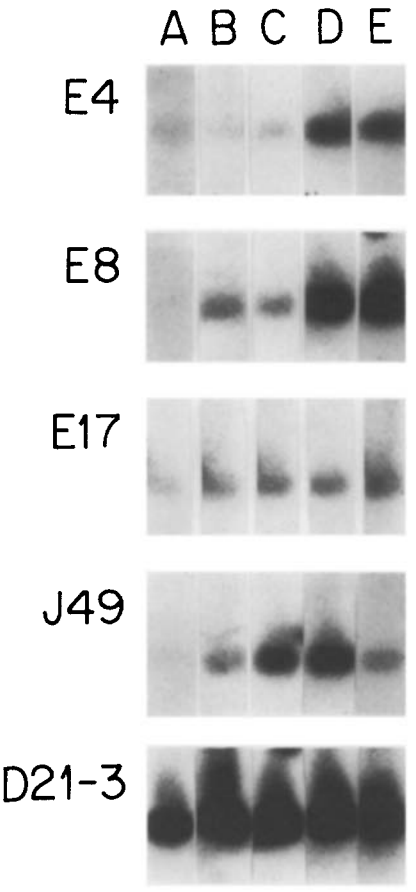


**Fig. 1.** Hybridization of developing tomato fruit [$^{32}$P]nRNAs with cDNA clones. [$^{32}$P]nRNAs were isolated from fruit staged as immature (lane A), MG1 (lane B), MG2 (lane C), MG3 (lane D), MG4 (lane E) and hybridized with DNA gel blots of the designated cDNA clones

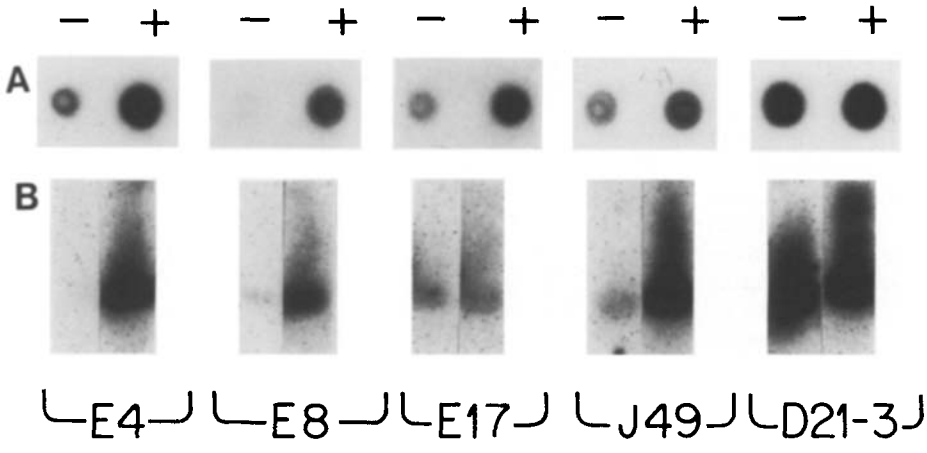


**Fig. 2A, B.** Messenger RNA accumulation and relative rate of gene transcription in ethylene treated fruit. **A** Messenger RNA was isolated from MG1 fruit treated with 10 µl ethylene/l (+) or air (−) for 2 h, dot-blotted, and hybridized with the indicated $^{32}$P-labeled cDNA clones. **B** [$^{32}$P]nRNA was isolated from MG1 fruit treated as in **A** and hybridized with DNA gel blots of the designated cDNA clones

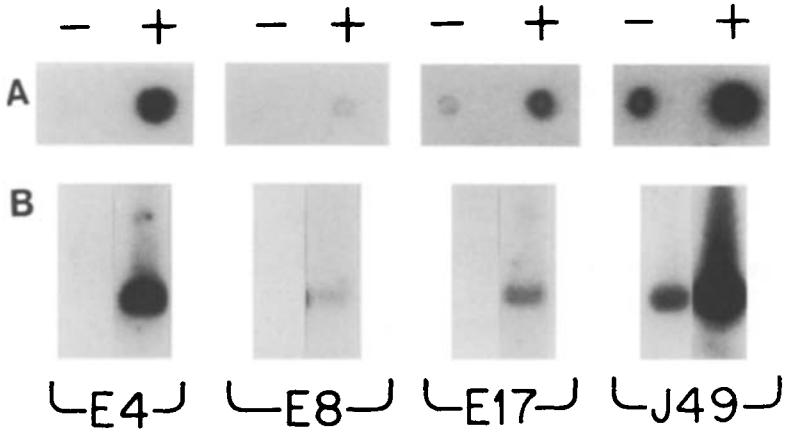


**Fig. 3A, B.** Messenger RNA accumulation and relative rate of gene transcription in ethylene treated leaf. **A** Messenger RNA was isolated from leaves of intact plants treated with 10 µl ethylene/l (+) or air (−) for 2 h, dot-blotted, and hybridized with the indicated $^{32}$P-labeled cDNA clones. **B** [$^{32}$P]nRNA was isolated from leaves treated as in **A** and hybridized with DNA gel blots of the designated cDNA clones

ible expression of these genes is not organ specific. In contrast, only a slight increase in *E8* gene transcription rate or E8 mRNA concentration was detected when leaves were exposed to ethylene (Fig. 3). Thus, the ethylene-inducible *E8* gene transcription is fruit organ specific.

*Gene expression as a function of ethylene concentration*

One possibility for the mechanism of action of the plant hormone ethylene is that it acts in a manner similar to that of animal hormones, where the hormone binds to a receptor, and the resulting receptor hormone complex mediates changes in gene transcription by interaction with the genome (Payvar et al. 1983; Scheidereit and Beato 1984; von der Ahe et al. 1985). As pointed out by Kende and Gardner (1976), this model predicts saturation of a hormone inducible response within two orders of magnitude of hormone concentration. To address this issue, we examined the effect of ethylene concentration on the regulation of cloned gene expression. Messenger RNA was isolated from MG1 fruit treated for 2 h with different concentrations of ethylene and the level of mRNA was determined by RNA dot-blot analysis. Figure 4 shows that for each cloned gene, the concentration range of ethylene that elicits an increasing level of mRNA was narrow, spanning approximately two orders of magnitude. Specifically, the threshold of response to ethylene ranged from 0.24–0.75 µl/l. The half-maximum response varied from 0.3 µl/l for *J49* gene expression to 2.5 µl/l for *E8* gene expression. The concentration which saturates the response ranged from 0.75 µl/l for *J49* gene expression to 23 µl/l for *E4* and *E8* gene expression. We conclude from these results that each gene displays a unique, narrow dose response curve in response to exogenous ethylene.

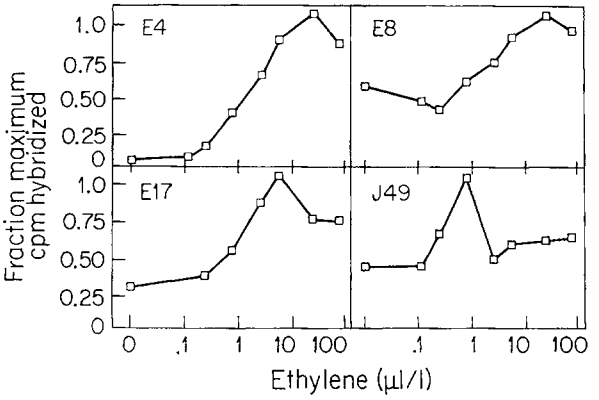


**Fig. 4.** Ethylene dose response curves. Messenger RNA was prepared from MG1 stage tomato fruit treated for 2 h, with 0, 0.11, 0.24, 0.75, 2.5, 5.4, 23, or 72 µl ethylene/l. mRNA was dotted onto nitrocellulose and hybridized with the indicated $^{32}$P-labeled cDNA clones. The dots were excised and the extent of hybridization determined by liquid scintillation spectrometry. The maximum cpm for each $^{32}$P-labeled cDNA probe was 1229 cpm for E4, 932 cpm for E8, 361 cpm for E17, and 1493 cpm for J49

## Discussion

### *Regulation of gene expression during fruit development*

Previously we showed that the increase in E4 and E8 mRNA concentration coincided with the increase in ethylene concentration, while the increase in E17 and J49 mRNA concentration preceded the increase in ethylene concentration during fruit development (Fig. 5). We also showed that a specific competitive inhibitor of ethylene action, norbornadiene, inhibited expression of these cloned genes during tomato fruit ripening (Lincoln et al. 1987). From these results we proposed that increased ethylene concentration activated *E4* and *E8* gene expression, while an increased capacity of tissue to respond to low levels of ethylene was responsible for the early activation of *E17* and *J49* gene expression (Lincoln et al. 1987). As shown in Fig. 5, we observe the same phenomenon at the level of gene transcription. That is, the onset of *E4* and *E8* gene transcription coincides with increase in ethylene concentration, while the onset of *E17* and *J49* gene transcription precedes the increase in ethylene concentration. These results support the hypothesis that in some cases gene transcription during ripening is primarily activated by increased sensitivity to basal ethylene levels, while in other instances it is regulated by an increase in ethylene concentration.

We have also found that, in certain instances, an excess of ethylene is not sufficient for induction of cloned gene expression. As shown in Fig. 5, the rate of *J49* gene transcription and mRNA concentration decreases at the MG4 and 50% red stages, respectively, when the ethylene concentration is elevated. A similar result was observed in the ethylene dose-response curve for *J49* gene expressions. That is, treating unripe fruit with increasingly higher concentrations of ethylene inhibited *J49* gene expression (Fig. 4). Thus, although we have shown ethylene is required for maximal *J49* gene expression during fruit ripening (Lincoln et al. 1987), ethylene concentration cannot be the simple limiting factor for *J49* gene expression.

### *Regulation of gene expression by exogenous ethylene*

We have investigated the extent to which transcriptional and post-transcriptional processes regulate gene expression when different plant tissues are exposed to exogenous ethylene. As shown in Fig. 2, *E4*, *E8*, and *J49* gene transcription significantly increased when fruit tissue was exposed to ethylene for 2 h. Furthermore, others have shown that ethylene activates the transcription of specific genes in carrot roots (Nichols and Laties 1984). Adjusting for the time (15–30 min) it takes for ethylene gas to diffuse into fruit (Cameron and Yang 1982), ethylene-induced gene transcription is nearly as rapid as auxin-induced gene transcription in soybean (Hagen and Guilfoyle 1985; Key et al. 1986). These results suggest that the plant hormones, auxin and ethylene, are capable of inducing changes in plant physiology by rapidly altering patterns of gene transcription.

We have found that the transcriptional activation of specific genes by ethylene can depend on the particular plant organ that is exposed to the exogenous ethylene. *E4* and *J49* gene transcription is activated by ethylene in both leaves and fruit (Figs. 2 and 3). In contrast, *E8* gene transcription is strongly activated in fruit, but not in leaves. These results suggest that the cellular factors that activate

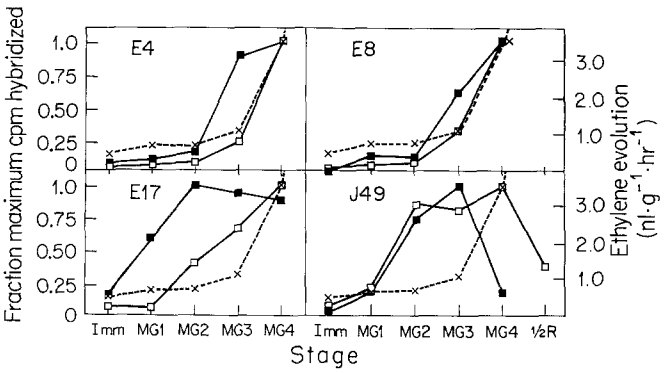


**Fig. 5.** Ethylene production rate, accumulation of cloned mRNAs, and relative rates of cloned gene transcription during fruit development. Percent maximum hybridization of [$^{32}$P]nRNAs with cDNA clones (■) was determined by tracing autoradiograms with a densitometer and determining the area of each peak. Ethylene evolution rates (x) and messenger RNA levels (□) were determined as described previously (Lincoln et al. 1987). Imm, immature fruit; $^1/_2$R, 50% red fruit

*E4* and *J49* gene transcription in leaf tissue are not sufficient to activate *E8* gene transcription.

Ethylene appears to control *E17* gene expression by several mechanisms. Transcriptional activation is evident during fruit ripening (Fig. 1) and when leaves are exposed to exogenous ethylene (Fig. 3). Furthermore, two experimental results suggest that post-transcriptional processes, such as cytoplasmic entry rates and/or differential mRNA stabilities (Tobin 1979), also regulate *E17* gene expression. First, as shown in Fig. 5, during fruit development the increase in *E17* gene transcription (MG1 stage) precedes the increase in E17 mRNA accumulation (MG2 stage). Second, exposing MG1 stage fruit to exogenous ethylene did not further increase the *E17* gene transcription rate, but resulted in a sixfold increase in E17 mRNA concentration (Fig. 2). We conclude from these experiments that ethylene regulates *E17* gene expression transcriptionally and post-transcriptionally.

### *Gene expression as a function of ethylene concentration*

To investigate further how the plant hormone ethylene influences gene expression, we measured expression of specific genes as a function of ethylene concentration. For certain effects of ethylene on plant development, such as stem elongation and root growth, the dose response curves show approximate half-maximum responses at 0.1–1 µl ethylene/l (Abeles 1973; Burg and Burg 1967). The results of our dose-response measurements (Fig. 4) indicate that the half-maximum values of ethylene concentration leading to specific mRNA accumulation in fruit are similar to these physiologically determined values. However, we find that the dose-response curves for *E4, E8, E17* and *J49* gene expression saturate within two orders of magnitude of ethylene concentration (Fig. 4). This result contrasts with physiological and molecular examples of plant hormone action. For example, in some cases where application of plant hormones, including ethylene, affects plant physiology, the concentration range which elicits an increased response may span three to four orders of magnitude (Abeles 1973; Kende and Gardner 1976). Furthermore, in studying auxin-induc-

ible gene expression, others have demonstrated responses over a wide range of auxin concentrations (Theologis et al. 1985; Guilfoyle 1986). The dose response curves that we observe for *E4, E8,* and *E17* ethylene-inducible gene expression closely resemble those observed with certain animal hormones, in that they saturate within two orders of magnitude of hormone concentration (Katzenellenbogen and Gorski 1972; Palmiter et al. 1977; Yamamoto et al. 1977).

In summary, we have investigated the mechanisms of action of the plant hormone ethylene by isolating and analyzing a set of ethylene-inducible genes. We have found that the expression of each cloned gene is regulated by ethylene in a unique fashion. These results suggest there are multiple mechanisms for the regulation of gene expression by ethylene. The molecular basis for this diversity will be better understood when the DNA sequences and cellular factors that regulate ethylene-inducible gene expression are isolated and analyzed.

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