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Differential Expression and Internal Feedback Regulation of 1-Aminocyclopropane-1-Carboxylate Synthase, 1-Aminocyclopropane-1-Carboxylate Oxidase, and Ethylene Receptor Genes in Tomato Fruit during Development and Ripening[1]

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We investigated the feedback regulation of ethylene biosynthesis in tomato (*Lycopersicon esculentum*) fruit with respect to the transition from system 1 to system 2 ethylene production. The abundance of *LE-ACS2*, *LE-ACS4*, and *NR* mRNAs increased in the ripening fruit concomitant with a burst in ethylene production. These increases in mRNAs with ripening were prevented to a large extent by treatment with 1-methylcyclopropene (MCP), an ethylene action inhibitor. Transcripts for the *LE-ACS6* gene, which accumulated in preclimacteric fruit but not in untreated ripening fruit, did accumulate in ripening fruit treated with MCP. Treatment of young fruit with propylene prevented the accumulation of transcripts for this gene. *LE-ACS1A*, *LE-ACS3*, and *TAE1* genes were expressed constitutively in the fruit throughout development and ripening irrespective of whether the fruit was treated with MCP or propylene. The transcripts for *LE-ACO1* and *LE-ACO4* genes already existed in preclimacteric fruit and increased greatly when ripening commenced. These increases in *LE-ACO* mRNA with ripening were also prevented by treatment with MCP. The results suggest that in tomato fruit the preclimacteric system 1 ethylene is possibly mediated via constitutively expressed *LE-ACS1A* and *LE-ACS3* and negatively feedback-regulated *LE-ACS6* genes with preexisting *LE-ACO1* and *LE-ACO4* mRNAs. At the onset of the climacteric stage, it shifts to system 2 ethylene, with a large accumulation of *LE-ACS2*, *LE-ACS4*, *LE-ACO1*, and *LE-ACO4* mRNAs as a result of a positive feedback regulation. This transition from system 1 to system 2 ethylene production might be related to the accumulated level of *NR* mRNA.

Fruits can be classified as climacteric or nonclimacteric depending on the presence or absence of massive ethylene production during ripening and on their response to exogenous ethylene (Biale and Young, 1981). Even in climacteric fruit, ethylene production is generally very low until the commencement of ripening. At the onset of ripening, fruit exhibit a climacteric increase in respiration, with a concomitant burst of ethylene production. Based on the level of ethylene production during fruit development, McMurchie et al. (1972) introduced the concept of system 1 and system 2 ethylene. System 1 is the basal low rate of ethylene production present in preclimacteric fruits. The basal level of ethylene produced by vegetative tissues and nonclimacteric fruits can be classified as system 1 (Oetiker and Yang, 1995). System 2 is the high rate of ethylene production observed during ripening in climacteric fruits and in certain senescent flowers (Oetiker and Yang, 1995).

In the ethylene-biosynthetic pathway, ACC synthase and ACC oxidase catalyze the reaction from *S*-adenosylmethionine to ACC and from ACC to ethylene, respectively (Yang, 1987). In this pathway it is well known that biosynthesis is subject to both positive and negative feedback regulation (Kende, 1993). Positive feedback regulation of ethylene biosynthesis is a characteristic feature of ripening fruits and senescing flowers. In tomato (*Lycopersicon esculentum*) and cantaloupe fruits (Liu et al., 1985), banana fruit (Inaba and Nakamura, 1986), and carnation flowers (Wang and Woodson, 1989), a large increase in ethylene production is triggered by exposure to exogenous ethylene, with activation of ACC synthase and/or ACC oxidase. From these observations system 2 ethylene was thought to be regulated by a positive feedback mechanism. A significant amount of ethylene is also induced by auxin or stress in a number of plant tissues, and in many cases it has been shown to be under negative feedback regulation (Yang and Hoffman, 1984). Therefore, since there are two types of large ethylene production regulated in opposite

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Abbreviations: MCP, 1-methylcyclopropene; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase.

feedback directions, the term system 2 ethylene should be limited to the ethylene produced from ripening fruits.

In recent molecular studies it has been demonstrated that both ACC synthase and ACC oxidase are encoded by multigene families in various plants (Kende, 1993; Zarembinski and Theologis, 1994; Fluhr and Mattoo, 1996). These genes have been isolated and structurally characterized and are differentially expressed in various tissues at different stages of development and in response to internal or external stimuli such as ripening, senescence, wounding, and auxin (Fluhr and Mattoo, 1996). In tomato fruit a large body of evidence demonstrates that massive ethylene production is responsible for increases in *LE-ACS2*, *LE-ACS4*, and *LE-ACO1* transcripts (Van Der Straeten et al., 1990; Olson et al., 1991; Rottmann et al., 1991; Yip et al., 1992; Lincoln et al., 1993; Barry et al., 1996). Expression of these genes in preclimacteric tomato fruit is rapidly induced and/or enhanced by treatment with ethylene (Maunders et al., 1987; Rottmann et al., 1991; Lincoln et al., 1993). Therefore, the expression of the genes related to system 2 ethylene may be under a positive feedback regulation mechanism in tomato fruit, at least at the initiation of ripening.

We previously demonstrated the involvement of a strong positive feedback regulation mechanism in tomato fruit even at the stage with a burst of ethylene production (Nakatsuka et al., 1997). The increases in the abundance of *LE-ACS2*, *LE-ACS4*, and *LE-ACO1* mRNAs in ripening fruit were prevented to a large extent by treatment with MCP, an inhibitor of ethylene action. However, ethylene production, ACC content, and the activities of ACC synthase and ACC oxidase in the fruit were not inhibited to the expected level with respect to suppression of the expression of the ACC synthase and ACC oxidase genes, suggesting an involvement of a negatively regulated gene(s) in ethylene biosynthesis in tomato fruit.

The involvement of positive feedback regulation in ethylene biosynthesis has been elucidated at the molecular level for ACC synthase and/or ACC oxidase in plants such as carnation (Jones and Woodson, 1997), orchid (O'Neill et al., 1993), and petunia (Tang and Woodson, 1996) flowers and mung bean (Kim and Yang, 1994) and pea (Peck and Kende, 1995) seedlings. The negative feedback regulation of ethylene biosynthesis at the molecular level has been reported in winter squash fruit (Nakajima et al., 1990), mung bean seedlings (Kim et al., 1997; Yoon et al., 1997), transgenic petunia flowers (Wilkinson et al., 1997), and leaves of the tomato cv *Never ripe* (Lund et al., 1998). Although it has been suggested that different ACC synthases may be involved in the two systems of ethylene production (McGlasson, 1985), it has not been clarified which members of the ACC synthase and/or ACC oxidase gene families are responsible for system 1 ethylene synthesis.

We demonstrate the involvement of positive and negative feedback regulated and constitutively expressed ACC synthase genes in tomato fruit, in which system 1 and system 2 ethylene production are regulated toward opposite directions of feedback, with differential expression of some members of the ACC synthase gene family.

MATERIALS AND METHODS

Plant Material and Treatments

Greenhouse-grown tomato (*Lycopersicon esculentum* Mill. cv Momotaro) fruit were harvested from a commercial farm at the following stages: immature green (about 2 weeks after flowering), mature green (pale-green color on fruit surface), turning (first appearance of pink color at blossom end), pink (pink color in approximately one-third of fruit surface), red (red color in approximately two-thirds of fruit surface), and full ripe (red color on entire fruit surface). Ethylene production by the fruit was measured at 22°C. Turning and pink fruits were treated with 10 to 20 nL L^{-1} MCP for 6 h and then ripened at 22°C. Ripening stages of MCP-treated fruit were monitored with reference to the color development of control fruit. Immature green fruit were treated with 5000 μL L^{-1} propylene for 2 and 4 d at 22°C. Respiration and ethylene production rates, ACC content, and in vivo ACC oxidase activity were measured in the fruit treated with propylene. Mature green fruit were divided into three stages based on the basal level of ethylene production: MG1, MG2, and MG3. After the determination of ethylene production, pericarp tissues from the fruit equatorial region were frozen in liquid nitrogen and stored at −80°C until RNA extraction. All experiments except RNA extraction were repeated at least three times. MCP synthesis and treatment were carried out as described previously (Nakatsuka et al., 1997).

Determination of Ethylene Biosynthesis and CO_2 Production

Ethylene and CO_2 production from fruit were measured by enclosing samples in an airtight chamber for 1 h at 22°C, withdrawing for each determination 1 mL of headspace gas from the chamber, and injecting into a gas chromatograph (model GC-4CMPF, Shimadzu, Kyoto, Japan) fitted with a flame-ionization detector and an activated alumina column for ethylene and into another gas chromatograph (model GC-3BT, Shimadzu) fitted with a thermal conductivity detector and a Porapack Q column for CO_2. For immature and mature green fruits, the basal level of ethylene production was measured using the mercuric perchloride method described by Akamine and Goo (1978). ACC content was measured by the method of Lizada and Yang (1979), with 80% ethanol extracts from pericarp tissues. In vivo ACC oxidase activity was assayed by the method of Moya-Leon and John (1994), with minor modifications. Enzyme activity was expressed as the amount of ethylene (in nanomoles) produced per gram per hour.

RNA Extraction and RT-PCR

RNA was extracted by the hot borate method (Wan and Wilkins, 1994). Poly(A)$^+$ RNA was isolated using Oligotex-dT30 (Takara, Kyoto, Japan) according to the manufacturer's protocol. The first-strand cDNAs synthesized by RT from 2 μg of the poly(A)$^+$ RNA isolated from ripe tomato fruit with or without MCP treatment were used as a

template for RT-PCR with degenerated primers A and B for ACC synthase (*LE-ACS1A*, *LE-ACS2*, *LE-ACS4*, and *LE-ACS6*), primers C and D for ACC oxidase (*LE-ACO1* and *LE-ACO4*), and primers E and F for the ethylene receptor (Table I). These primers were designed with reference to the conserved amino acid sequences of ACC synthase and ACC oxidase (Kende, 1993) with restriction site sequences of *Bam*HI or *Pst*I (indicated in parentheses in Table I). Primers for the ethylene receptor were designed with reference to the nucleotide sequences of *NR* (accession no. U38666) and *eTAE1* (accession no. U41103) registered in the nucleotide sequence databases with restriction site sequences of *Bam*HI. Reactions for the RT-PCR mentioned above were subjected to 30 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. For amplification of the cDNA fragment of *LE-ACS3*, we used specific primers K (bp 175–201) and L (bp 822–848) designed from the given nucleotide sequences registered on the database (accession no. U17972) with restriction site sequences of *Bam*HI and *Kpn*I. Reactions were subjected to 30 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min.

Amplification of Full-Length cDNA by RACE-PCR

To determine the full-length nucleotide sequences for *LE-ACS6* and *LE-ACO4*, RACE-PCR was performed using a cDNA amplification kit (Marathon, Clontech, Palo Alto, CA) according to the manufacturer's protocol. The 5′-end fragments were amplified using specific primers N and P for *LE-ACS6* and *LE-ACO4*, respectively (Table I). To amplify 3′-end fragments, specific primers M and O were used for *LE-ACS6* and *LE-ACO4*, respectively (Table I). Each primer was designed based on the nucleotide sequences of the cDNA fragments for *LE-ACS6* and *LE-ACO4* obtained from the RT-PCR described above.

Cloning and DNA Sequencing

The PCR products were either ligated into vector pUC118 (Takara, Kyoto, Japan) or TA-cloned in pCR (Invitrogen, Carlsbad, CA) and then introduced into *Escherichia coli* JM109. After screening, target cDNAs were sequenced using a DNA sequencer (model DSQ-1000, Shimadzu) with either the −21M13 or the M13 sequencing primers according to the manufacturer's instructions (Amersham).

Confirmation of *LE-ACS1A* and *LE-ACS1B* Expression

To determine whether *LE-ACS1A* and *LE-ACS1B*, which have very high sequence similarity, were expressed in fruit tissue, a cDNA fragment was amplified on RT-PCR with a template of the combined single-strand cDNAs prepared from preclimacteric and ripening fruits in a ratio of 1:1 using specific primer pairs of G and H and I and J for *LE-ACS1A* and *LE-ACS1B*, respectively. These primers were synthesized with reference to the nucleotide sequences registered in the database (primers G and H, bp 958–985 and bp 1311–1334 for *LE-ACS1A* [accession no. U72389]; primers I and J, bp 958–985 and bp 1311–1337 for *LE-ACS1B* [accession no. U72390]). Competence of primers was confirmed by PCR with a template of genomic DNA extracted from tomato leaves. The PCR products were ligated into a plasmid, introduced into *E. coli*, and sequenced as described above. The resulting plasmids inserted with the fragments of *LE-ACS1A* or *LE-ACS1B* were used as a template to ascertain the specificity of each primer pair in PCR. Reactions were subjected to 25 cycles of 94°C for 1 min, 63°C for 2 min, and 72°C for 3 min.

RNA Blotting and Hybridization

Three-microgram samples of mRNA isolated from pericarp tissues were separated by electrophoresis on 1% agarose gels containing 0.66 M formaldehyde, blotted onto nylon membranes (Hybond N, Amersham), and fixed with a UV cross-linker (Amersham). The membranes were hybridized with ^{32}P-labeled cDNA probes obtained from the RT-PCR products mentioned above and hybridized as described previously (Nakatsuka et al., 1997). Following hy-

Table I. *Oligonucleotide primers used for amplification of cDNAs by RT-PCR or RACE-PCR*

	Name	DNA Sequence	Gene
A	ACS-F	cccc(ggatcc)atgggyytngcdgaraaycag	Degenerate
B	ACS-R	cccc(ggatcc)acnarncyraarcthgacat	Degenerate
C	ACO-F	cgc(ggatcc)gcntgysaraantggggntt	Degenerate
D	ACO-R	aaa(ctgcag)nggytcyttngcytgraaytt	Degenerate
E	ETR-F	gcg(ggatcc)gartgtgcwttrtggatgcca	Degenerate
F	ETR-R	gcg(ggatcc)gctctggagttarrtcwgtttc	Degenerate
G	LEACS1AF	gcatcaatgttgtctgatgaagtattca	*LE-ACS1A*
H	LEACS1AR	gcaatgttgttaagtccctttggc	*LE-ACS1A*
I	LEACS1BF	gcatcaatgttgtctgatgagatatttg	*LE-ACS1B*
J	LEACS1BR	gcagcaatgttgttaagtccctttgtt	*LE-ACS1B*
K	LEACS3F	gg(ggtacc)ctagcacaaaatccagacgcagctggg	*LE-ACS3*
L	LEACS3R	cg(ggatcc)gcaccaatgcgaaaaccggggagaccg	*LE-ACS3*
M	LEACS6RACE3	gtatctcagaagtcaagagtgaagttgttgg	*LE-ACS6*
N	LEACS6RACE5	gcatccaacaacttcactcttgacttctgag	*LE-ACS6*
O	LEACO4RACE3	cactgaagctagagaaactagctgaaaatc	*LE-ACO4*
P	LEACO4RACE5	ggatacttcaatttgatgtcctcttctgtc	*LE-ACO4*

bridization, membranes were washed once at 60°C in 2× SSPE (1× SSPE = 0.15 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.4) and 0.1% SDS for 30 min, in 0.5× SSPE and 0.1% SDS for 30 min, and in 0.2× SSPE and 0.1% SDS for 30 min. cDNA probes were labeled with a randomly primed DNA-labeling kit (Boehringer Mannheim) with [^{32}P]dCTP. The membranes were then exposed to an imaging plate (Fuji Photo Film, Tokyo, Japan) at room temperature. Equal reactivity and amount of RNA in all samples were verified by hybridization with ^{32}P-labeled actin (Nakatsuka et al., 1997).

RESULTS

Isolation and Identification of cDNA Clones

Using degenerate and specific oligonucleotide primers (Table I), we cloned nine fragments from ripe tomato fruit without or treated with MCP, including five different cDNAs for ACC synthase (*LE-ACS1A*, *LE-ACS2*, *LE-ACS3*, *LE-ACS4*, and *LE-ACS6*), two for ACC oxidase (*LE-ACO1* and *LE-ACO4*), and two for the ethylene receptor (*NR* and *TAE1*). Nucleotide sequences of each fragment except *LE-ACO4* were more than 99.6% identical to those of corresponding cDNA previously registered in the databases: *LE-ACS1A*; *LE-ACS2* (accession no. X59145); *LE-ACS3* (accession no. U17972); *LE-ACS4* (accession no. X59146); *LE-ACS6* (accession no. U74461); *LE-ACO1* (accession no. X58273); *NR*; and *TAE1*. The mismatch of sequences between fragments and the registered cDNAs were probably due to PCR errors or differences in tomato cultivars. One fragment for ACC oxidase cloned in this study had low sequence similarity compared with other genes encoding ACC oxidase already known in tomato (Barry et al., 1996), with 76% to 77% and 80% to 84% at the nucleotide and deduced amino acid levels, respectively (Table II). Therefore, we considered this fragment as a new member of the ACC oxidase gene family in tomato and registered it in the database as *LE-ACO4* (accession no. AB013101).

The full-length cDNA of *LE-ACO4*, which was obtained by RACE-PCR, contained an open reading frame of 960 bp encoding a sequence of 320 amino acids. The amino acid sequence comparison among the four tomato ACC oxidase proteins is shown in Figure 1. The *LE-ACS6* fragment cloned in this study had a completely identical sequence to an already registered ACC synthase gene (Oetiker et al., 1997; accession no. U74461) except for the degenerate primer regions. The registered sequence length is limited to 308 bp and we determined full-length sequences of its cDNA using the RACE-PCR method. The full-length cDNA of *LE-ACS6* contained an open reading frame of 1431 bp encoding a sequence of 477 amino acids.

Table II. *Percentage sequence identity between ACC oxidases encoded by multigene families in tomato plant*

Deduced Amino Acid Sequence	Nucleotide Sequence			
	LE-ACO1	LE-ACO2	LE-ACO3	LE-ACO4
LE-ACO1	—	84.6	95.9	82.8
LE-ACO2	84.6	—	85.9	80.0
LE-ACO3	92.7	82.1	—	83.6
LE-ACO4	77.5	76.2	77.5	—

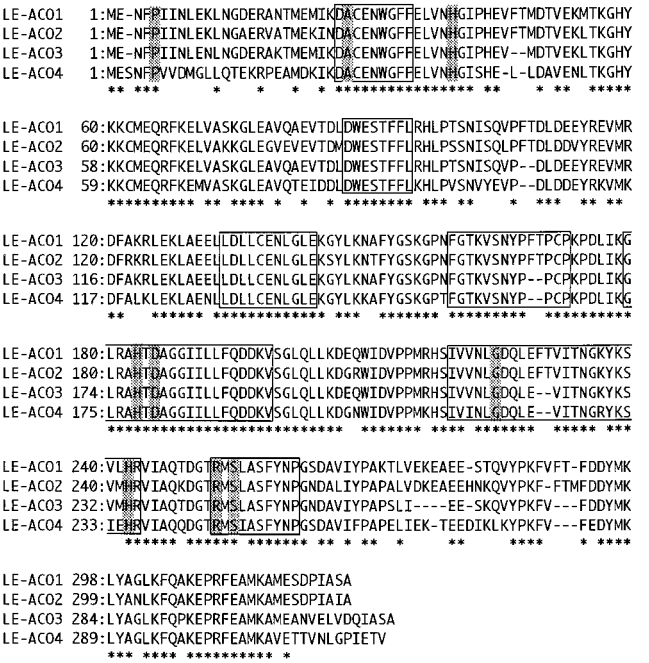

Figure 1. Comparison of the deduced amino acid sequences among the four tomato ACC oxidase proteins (*LE-ACO1*, *LE-ACO2* [accession no. Y00478], *LE-ACO3* [accession no. Z54199], and *LE-ACO4*). The asterisks indicate sequence identity. Highly conserved regions for ACC oxidase are boxed, and the nine shaded amino acid residues are conserved in all members of the Fe(II) ascorbate family of dioxygenases (Lasserre et al., 1996).

Ethylene Production during Fruit Development and Ripening and Effect of MCP

Figure 2 shows the rate of ethylene production by the fruit immediately after harvest at the indicated stages and by the fruit treated with MCP at the turning or pink stages. In the control fruit ethylene production was very low at the basal level at the preclimacteric stage and increased during ripening, reaching a peak at the red stage and declining slightly thereafter. This increase in ethylene production was inhibited by about 66% and 75% 2 d after MCP treatment at the turning and pink stages, respectively. Thereafter, ethylene production recovered slowly without any decline to the basal level, contrary to the expectation from the action of MCP (Sisler and Serek, 1997).

Confirmation of *LE-ACS1A* Expression in Fruit Tissue

Since the twin *LE-ACS1* cDNAs *LE-ACS1A* and *LE-ACS1B*, which share very high sequence similarity, have been cloned from a tomato genomic library (Oetiker et al., 1997), we determined whether both were expressed in the fruit. As shown in Figure 3, only the *LE-ACS1A* cDNA fragment with the expected length of 377 bp was amplified

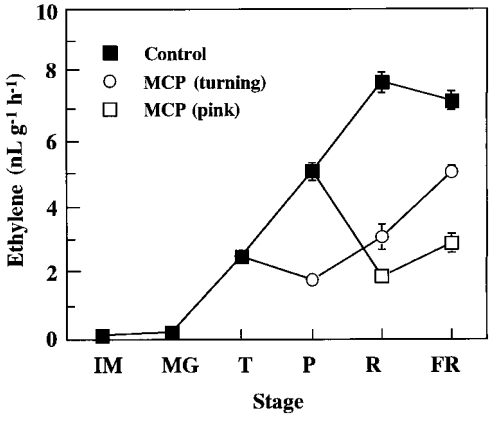

Figure 2. Changes in the rate of ethylene production in tomato fruit during development and ripening and the effect of MCP. Fruit were harvested at six stages: immature green (IM), mature green (MG), turning (T), pink (P), red (R), and full ripe (FR), based on the observations described in "Materials and Methods." Fruit harvested at the turning and pink stages were treated with 10 to 20 nL L^{-1} MCP for 6 h and then ripened at 22°C. The ripening stages of MCP-treated fruit corresponding to the control fruit were determined as described in "Materials and Methods." Vertical bars are the SE of three replications; missing error bars are smaller than the symbols.

by RT-PCR when the specific primers designed to have a 2-base mismatch at 3′ ends in both upstream and downstream primers (compare lanes 2 and 6) were used. The *LE-ACS1A* and *LE-ACS1B* genomic DNA fragments were amplified by PCR using each primer pair (Fig. 3, lanes 3 and 7), ligated into a plasmid, and then introduced into *E. coli*. The nucleotide sequences of each fragment were completely identical to those of the corresponding regions for each cDNA (data not shown). When these plasmids inserted with the *LE-ACS1A* or *LE-ACS1B* fragments were used as templates for PCR, the *LE-ACS1A* primer amplified the *LE-ACS1A* fragment but not the *LE-ACS1B* fragment (Fig. 3, compare lanes 4 and 9) and vice versa (Fig. 3, compare lanes 5 and 8). These experiments confirmed that, among the twin *LE-ACS1* genes, only *LE-ACS1A* mRNA was expressed in the fruit tissue.

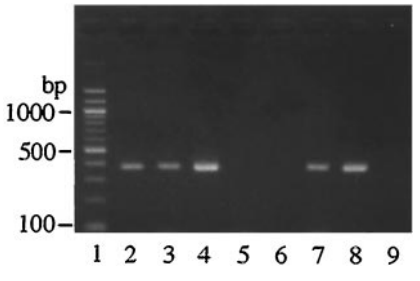

Figure 3. Agarose/ethidium bromide gel image of RT-PCR products amplified using specific primers for *LE-ACS1A* and *LE-ACS1B*. Each primer was designed to amplify the corresponding region in *LE-ACS1A* and *LE-ACS1B* but with two different nucleotides at the 3′ ends either upstream or downstream set to avoid cross-amplification. The *LE-ACS1A* primers were used for the reaction of lanes 2, 3, 4, and 9, and the *LE-ACS1B* primers were used for lanes 5 to 8. Templates used for RT-PCR were the combined single-strand cDNAs prepared from preclimacteric and ripening fruits in a ratio of 1:1 (lanes 2 and 6), the genomic DNA extracted from tomato leaves (lanes 3 and 7), and the plasmid inserted with the *LE-ACS1A* (lanes 4 and 5) or *LE-ACS1B* (lanes 8 and 9) fragment. Lane 1 shows a 100-bp DNA ladder as a size marker.

Gene Expression during Fruit Development and Ripening and Effect of MCP

Figure 4 shows the expression of members of the gene families for ACC synthase, ACC oxidase, and the ethylene receptor in tomato fruit during development and ripening and in the fruit treated with MCP. Among the five members of the *LE-ACS* gene family, the abundance of *LE-ACS2* and *LE-ACS4* mRNAs in the fruit was undetectable in fruit at the preclimacteric stage, increased from the turning to pink stages, and thereafter slightly declined (Fig. 4, lanes

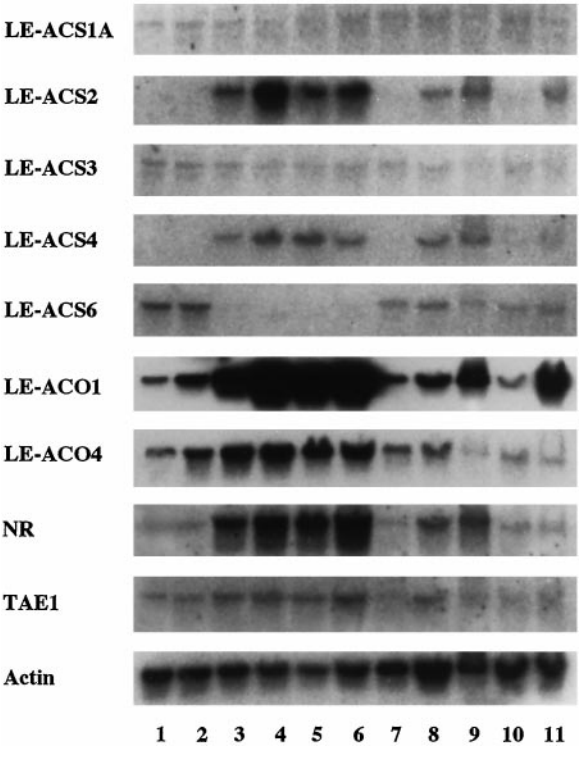

Figure 4. Expression of *LE-ACS*, *LE-ACO*, and ethylene receptor genes in tomato fruit during development and ripening and effect of MCP. mRNAs were prepared from the fruit immediately after the determination of ethylene levels as shown in Figure 2. Lane 1, Control fruit at the immature stage; lane 2, control fruit at the mature green stage; lane 3, control fruit at the turning stage; lane 4, control fruit at the pink stage; lane 5, control fruit at the red stage; lane 6, control fruit at the full-ripe stage; lane 7, turning-stage fruit 2 d after MCP treatment; lane 8, turning-stage fruit 4 d after MCP treatment; lane 9, turning-stage fruit 6 d after MCP treatment; lane 10, pink-stage fruit 2 d after MCP treatment; and lane 11, pink-stage fruit 4 d after MCP treatment. Each lane contained 3 μg of mRNA. Actin was used as an internal control to normalize the amount of mRNA loaded.

Table III. *Effect of propylene on the rates of respiration and ethylene production, ACC content, and in vivo ACC oxidase activity in immature green fruit*

Fruit were harvested about 2 weeks after flowering and then treated with 5000 μL L^{-1} propylene for 2 and 4 d at 22°C. The values are the means ± SE of three replications.

Treatment Time	Respiration		Ethylene		ACC		ACC Oxidase	
	Control	Propylene	Control	Propylene	Control	Propylene	Control	Propylene
d	*μL CO_2 g^{-1} h^{-1}*		*pmol g^{-1} h^{-1}*		*nmol g^{-1}*		*nmol ethylene g^{-1} h^{-1}*	
0	46.7 ± 4.6		7.9 ± 3.1		0.32 ± 0.04		0.48 ± 0.15	
2	20.8 ± 1.9	23.0 ± 4.0	8.8 ± 2.5	6.9 ± 2.0	0.32 ± 0.06	0.35 ± 0.05	0.42 ± 0.08	1.02 ± 0.18
4	24.1 ± 2.8	24.4 ± 1.4	8.8 ± 1.3	7.0 ± 3.0	0.32 ± 0.05	0.40 ± 0.03	0.55 ± 0.09	1.67 ± 0.35

1–6). These increases in the mRNA abundance associated with ripening were prevented to a large extent by treatment of fruit with MCP at both the turning (Fig. 4, lanes 7–9) and pink (Fig. 4, lanes 10 and 11) stages. In particular, 2 d after MCP treatment, the abundance of mRNA that hybridized with the *LE-ACS2* and *LE-ACS4* probes was almost completely eliminated (Fig. 4, compare lanes 4 and 5 with 7 and 10, respectively). This elimination recovered gradually in 2 and 4 d (lanes 8, 9, and 11).

In contrast, the *LE-ACS6* gene was expressed in the fruit at the immature green and mature green stages (Fig. 4, lanes 1 and 2), whereas no signals for this gene were detected in the ripening fruit (Fig. 4, lanes 3–6). However, accumulation of *LE-ACS6* mRNA was detected in the fruit treated with MCP at both the turning and pink stages (Fig. 4, lanes 7–11). *LE-ACS1A* and *LE-ACS3* genes were expressed weakly in the fruit throughout development and ripening, and the abundance of their mRNAs was less influenced by treatment with MCP. Although two *LE-ACO* genes were expressed in immature green and mature green fruit (Fig. 4, lanes 1 and 2), the abundance increased further upon commencement of ripening (Fig. 4, lanes 3–6), particularly in *LE-ACO1*. The increases in accumulation of the *LE-ACO* mRNAs with ripening were prevented considerably by treatment of fruit with MCP at both the turning and pink stages (Fig. 4, lanes 7–11). Of the two members of the ethylene receptor gene family, the abundance of *NR* mRNA in the fruit was at a very low level at the preclimacteric stage (Fig. 4, lanes 1 and 2), increased suddenly at the turning stage, and maintained its strong signals during ripening (Fig. 4, lanes 3–6). This increase of *NR* mRNA associated with ripening was also lowered by MCP treatment in a manner similar to that observed for *LE-ACS2* (Fig. 4, lanes 7–11). Signals for the *TAE1* gene in the fruit were detected at the preclimacteric stage (Fig. 4, lanes 1 and 2) and increased slightly during ripening (Fig. 4, lanes 3–6). MCP decreased the abundance of *TAE1* mRNA in ripening fruit (Fig. 4, lanes 7–11).

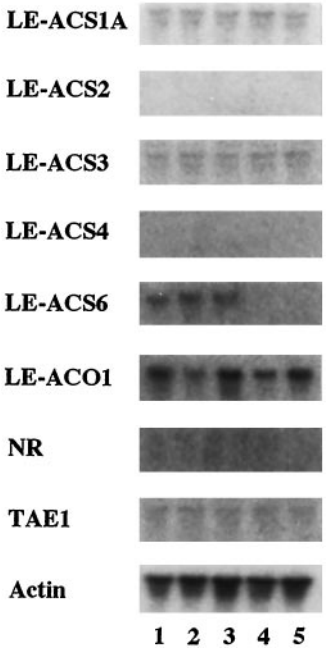

Figure 5. Effect of propylene on the accumulation of mRNAs corresponding to *LE-ACS* and ethylene receptor gene families and the *LE-ACO1* gene in immature green fruit. mRNAs were isolated from the same fruit sample shown in Table III. Lane 1, Control fruit at harvest; lane 2, control fruit 2 d after harvest; lane 3, control fruit 4 d after harvest; lane 4, propylene-treated fruit for 2 d; lane 5, propylene-treated fruit for 4 d. Each lane contained 3 μg of mRNA. Actin was used as an internal control to normalize the amounts of mRNAs loaded.

Effect of Propylene on Gene Expression in Immature Green Fruit

The results presented above suggest that the expression of the *LE-ACS6* gene may be under negative feedback regulation in tomato fruit. To test this hypothesis, immature green fruit were treated with 5000 μL L^{-1} propylene for 2 and 4 d. Neither autocatalytic ethylene production nor increases in respiration rate and ACC content was induced by propylene in these young fruit, whereas ACC oxidase was activated more than 2- to 3-fold (Table III). The results of northern analysis for mRNAs from these fruit are shown in Figure 5. The accumulation of *LE-ACS6* transcript in the control fruit (Fig. 5, lanes 1–3) was strongly prevented by treatment with propylene for 2 and 4 d (Fig. 5, lanes 4 and 5, respectively). Since there were no increases in ethylene production or ACC content in the fruit, propylene did not induce the accumulation of transcripts for *LE-ACS2* and *LE-ACS4*. *LE-ACS1A* and *LE-ACS3* were expressed constitutively in the fruit irrespective of propylene treatment.

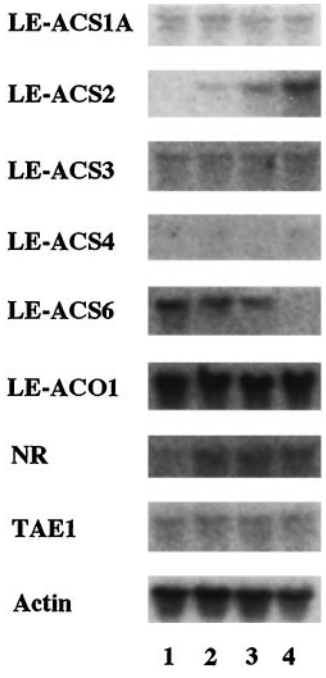

Figure 6. Changes in the accumulation of mRNAs corresponding to *LE-ACS* and ethylene receptor gene families and the *LE-ACO1* gene in fruit with different rates of ethylene production from the mature green stage to the turning stage. Lane 1, MG1 fruit (0.18 nL g^{-1} h^{-1} ethylene production); lane 2, MG2 fruit (0.36 nL g^{-1} h^{-1} ethylene production); lane 3, MG3 fruit (0.96 nL g^{-1} h^{-1} ethylene production); and lane 4, turning fruit (1.46 nL g^{-1} h^{-1} ethylene production). Each lane contained 3 μg of mRNA. Actin was used as an internal control to normalize the amounts of mRNAs loaded.

Although in vivo activity of ACC oxidase in the fruit was increased by propylene treatment, we did not observe an enhancement of the accumulation of *LE-ACO1* mRNA. Signals for the *NR* and *TAE1* genes were weak in the control fruit and were less influenced by treatment with propylene.

Transition of Expression of Genes at Ripening Onset

It is possible that the elimination of *LE-ACS6* and the appearance of *LE-ACS2* transcripts may have been responsible for the transition from system 1 to system 2 ethylene production. To examine this concept, northern analysis was performed in fruit at stages from mature green to turning, all of which had different levels of basal ethylene production (Fig. 6). The rates of ethylene production in the fruit were 0.18, 0.36, 0.96, and 1.46 nL g^{-1} h^{-1} at the MG1, MG2, MG3, and turning stages, respectively. The abundance of *LE-ACS6* mRNA in the fruit decreased gradually with ripening, reaching undetectable levels at the turning stage. In contrast, the *LE-ACS2* transcript, which was undetectable at the MG1 stage, increased gradually when the rate of ethylene production was increased. Signals for the *NR* gene at the MG1 stage were very weak, increasing from the MG2 stage to the turning stage. Signals for the *LE-ACS1A* and *LE-ACS3* genes changed little from the MG1 stage to the turning stage. The abundance of *LE-ACO1* and *TAE1* mRNAs was also unchanged from the MG1 stage to the turning stage. No signal for the *LE-ACS4* gene was detected in the turning fruit, which had a lower ethylene level (1.46 nL g^{-1} h^{-1}) than that used in the fruit shown in Figures 2 and 4 (2.35 nL g^{-1} h^{-1}).

DISCUSSION

The climacteric life of fruits is divided into preclimacteric and climacteric stages depending on whether a massive production of ethylene has commenced. In tomato fruit ethylene production during the climacteric stage has been demonstrated to be due to the accumulation of transcripts of two ACC synthase genes, *LE-ACS2* and *LE-ACS4* (Rottmann et al., 1991; Lincoln et al., 1993), and one ACC oxidase gene, *LE-ACO1* (Barry et al., 1996). Using MCP, an ethylene action inhibitor, we previously demonstrated that the expression of all three of these genes is highly regulated through a positive feedback mechanism in ripening tomato fruit (Nakatsuka et al., 1997). In that study we suggested the possible existence of a gene(s) under negative feedback regulation, because the inhibitory effects of MCP on the expression of the genes were not correlated with those on ethylene biosynthesis. To provide experimental evidence to support our hypothesis, we cloned nine cDNA fragments, including five members of the ACC synthase gene family, two of the ACC oxidase family, and two of the ethylene receptor family. Among the seven previously cloned genes for ACC synthase (Rottmann et al., 1991; Yip et al., 1992; Lincoln et al., 1993; Spanu et al., 1993; Olson et al., 1995; Oetiker et al., 1997), fragments of *LE-ACS1B* and *LE-ACS5* could not be amplified by RT-PCR used in this study, even by the use of specific primers. Although the transcription of these two genes has been demonstrated in tomato roots and suspension cultures (Yip et al., 1992; Spanu et al., 1993; Oetiker et al., 1997), there is no evidence demonstrating their expression in the fruit. Therefore, we concluded that their transcripts were absent in the fruit tissue.

In the present study we observed large ethylene production in the fruit from the turning stage with further increases toward the red stage (Fig. 2). This increase in ethylene production was prevented to a large extent by treatment with MCP at both the turning and pink stages. Using mRNAs extracted from these fruit, we performed northern analysis with the probes prepared from cDNA fragments cloned in this study (Fig. 4). Among five members of the *LE-ACS* gene family, the abundance of *LE-ACS2* and *LE-ACS4* mRNAs in the fruit increased beginning at the turning stage, and MCP greatly suppressed this increase in a manner similar to that observed in our previous study (Nakatsuka et al., 1997). In mature green fruit the transcripts of these genes were absent but were inducible by treatment with ethylene through a positive feedback mechanism, resulting in the induction of ripening (Lincoln et al., 1993).

Expression of *LE-ACS2* during the natural progress of ripening first appeared in MG2 fruit, the stage showing the first elevation of ethylene production from the basal level (Fig. 6). However, propylene did not induce the accumu-

lation of *LE-ACS2* and *LE-ACS4* transcripts in immature green fruit within 4 d (Fig. 5) but did by 8 d of treatment (data not shown), indicating a possible lack of a rapid, autocatalytic system for ethylene biosynthesis in young fruit. This lack of a rapid response to applied ethylene has been reported in young tomato fruit, in which fruits harvested as early as 17 d after pollination required 12 to 15 d of continuous treatment with 1000 μL L^{-1} ethylene to develop red color (Lyons and Pratt, 1964). Although expression of the *LE-ACS2* and *LE-ACS4* genes is also inducible by wounding (Lincoln et al., 1993), these are probably the major genes responsible for the system 2 ethylene production during ripening in tomato fruit. More direct evidence for this is shown in transgenic tomatoes in which the *LE-ACS2* antisense fruits produce less ethylene and fail to ripen, with complete inhibition of the *LE-ACS2* and *LE-ACS4* genes during ripening (Oeller et al., 1991).

In contrast to *LE-ACS2* and *LE-ACS4*, the *LE-ACS6* gene was expressed in fruit from the immature green to the mature green stages, whereas no signals for this gene were detected in the ripening fruit. Signals for this gene were detected in the ripening fruit treated with MCP (Fig. 4), strongly suggesting that the expression of the *LE-ACS6* gene is regulated by a negative feedback mechanism. This concept was clearly demonstrated in immature green fruit, in which the previously detected signals for the *LE-ACS6* gene were eliminated by treatment with propylene, an ethylene analog (Fig. 5). Furthermore, the abundance of this mRNA in the fruit during the natural onset of ripening decreased gradually to an undetectable level at the turning stage (Fig. 6).

Oetiker et al. (1997) isolated *LE-ACS6* cDNA from tomato roots, but theirs is the only available information concerning its expression, suggesting that it exhibits an elicitor-inducible feature. Lincoln et al. (1993) also previously described the cloning of *LE-ACS6* cDNA and suggested the possible expression of this gene in ripe tomato fruit. However, their suggestion differs from our present observation with respect to the characteristic features of the *LE-ACS6* gene. Therefore, *LE-ACS6* reported by Lincoln et al. (1993) may have been a different cDNA from that cloned by Oetiker et al. (1997) and that obtained in the present study. Mori (1995) described an expression pattern of *LE-ACS6* in tomato fruit that is similar to ours, with an elimination of its transcripts in ripe fruit, but to our knowledge, no further information is available for this observation (in particular the gene sequences). The present results clearly demonstrate the existence of an ethylene-biosynthetic gene, the expression of which is regulated under a negative feedback mechanism in fruit. The possible involvement of a negative feedback regulation at the ethylene-production level has been suggested in fruits such as banana (Vendrell and MacGlasson, 1971), citrus (Riov and Yang, 1982), and winter squash (Hyodo et al., 1985).

LE-ACS1A and *LE-ACS3* genes were expressed in the fruit throughout development and ripening (Figs. 4 and 6). Furthermore, the abundance of their mRNAs was not influenced by treatment with either MCP (Fig. 4) or propylene (Fig. 5), indicating that the expression of these genes is independent of ethylene action. Although these two genes resembled each other closely in expression pattern, *LE-ACS3* had low sequence similarities (less than 62%) among the *LE-ACS* gene family (data not shown). This may exclude a possibility that the probe for *LE-ACS3* could hybridize to other transcripts encoding tomato ACC synthase. The full-length sequence of *LE-ACS1A* mRNA together with its twin of *LE-ACS1B* was previously registered on the database (accession nos. U72389 and U72390), and their expression was first examined in cultured cells using the RNase-protection assay, in which *LE-ACS1B* was strongly and constitutively expressed but no signals for *LE-ACS1A* were detectable (Oetiker et al., 1997). However, only the *LE-ACS1A* cDNA fragment was amplified on RT-PCR. *LE-ACS5* was not amplified in the present study, suggesting a tissue-specific expression of each ACC synthase gene family. The transcript of *LE-ACS3* has been detected in fruits (Yip et al., 1992) and suspension cultures (Oetiker et al., 1997). Among the members of the *LE-ACS* gene family studied, *LE-ACS1A*, *LE-ACS3*, and *LE-ACS6* genes were expressed in the preclimacteric fruit, suggesting that system 1 ethylene in tomato fruit may be mediated via these three genes.

In tomato at least three genes encode ACC oxidase (Barry et al., 1996): *LE-ACO1* is the main gene expressed in ripening tomato fruit, *LE-ACO2* expression is mainly restricted to the tissues associated with the anther cone, and *LE-ACO3* transcripts accumulate in floral organs and transiently appear with a weak signal in fruit at the breaker stage (Barry et al., 1996). In the present study we cloned a novel ACC oxidase gene and named it *LE-ACO4*. Both *LE-ACO1* and *LE-ACO4* transcripts accumulated in preclimacteric fruit, and this accumulation increased in ripening fruit. This increase was prevented to a large extent by MCP treatment in a manner similar to that of the *LE-ACS2* and *LE-ACS4* genes (Fig. 4).

Although feedback regulation of the ACC oxidase genes has not yet been clarified, there is evidence that accumulation of the transcripts is enhanced with increases in ethylene production and by exogenously applied ethylene in fruits such as tomato (Barry et al., 1996), apple (Ross et al., 1992), melon (Lasserre et al., 1996), banana (Huang et al., 1997), kiwifruit (Whittaker et al., 1997), and pear (Lelievre et al., 1997). In vegetative tissues ACC oxidase mRNA has also been shown to be regulated by ethylene; the transcript for an ACC oxidase gene in excised mung bean hypocotyls was enhanced by exogenous ethylene and suppressed by aminooxyacetic acid, an ACC synthase inhibitor, with a reduction of endogenous ethylene to the basal level (Kim and Yang, 1994). From these observations, it may be reasonable to assume that a positive feedback regulation is involved in the expression of ACC oxidase gene in a manner similar to that in ACC synthase. However, since propylene did not enhance the already-accumulated *LE-ACO1* transcript in immature green fruit (Fig. 5), the responsiveness of *LE-ACO1* to ethylene may be less than that of *LE-ACS6*.

Since the *ETR1* gene in Arabidopsis was cloned and sequenced as the gene related to ethylene receptors (Chang et al., 1993), five homologs have been isolated from tomato (Lashbrook et al., 1998). We cloned cDNA fragments cor-

responding to the *NR* (Wilkinson et al., 1995) and *TAE1* (Zhou et al., 1996) genes based on their reported sequences. Expression of the *NR* gene was extremely low in immature and mature green fruit but suddenly increased greatly at the turning stage (Fig. 4). Investigations at the onset of ripening revealed that this increase commenced in MG2 fruit, the stage of the first increase in ethylene production from the basal level (Fig. 6). Wilkinson et al. (1995) indicated that *NR* mRNA in tomato fruit is positively regulated by ethylene in a development-specific manner from observations that the amount of *NR* mRNA increases in ripening fruit and ethylene-treated mature green fruit but not in *Nr* mutant tomato.

A strong induction of *NR* mRNA at the onset of ripening has also been demonstrated in tomato fruit (Lashbrook et al., 1998). In the present study this accumulation of *NR* mRNA associated with ripening was prevented in the fruit treated with MCP (Fig. 4). It has been proved that MCP is an ethylene-action inhibitor that binds to the receptor site competitively, thereby preventing tissue response to ethylene (Sisler and Serek, 1997). The present results demonstrate that MCP prevents the accumulation of *LE-ACS2*, *LE-ACS4*, *LE-ACO1*, and *LE-ACO4* mRNAs in the ripening fruit with an almost complete elimination of *NR* transcripts (Fig. 4). Furthermore, inhibition of the accumulation of *LE-ACS* and *LE-ACO* transcripts recovered after 2 to 4 d concomitantly with the recovery of *NR* transcripts. A similar observation has been reported for tomato fruit using diazocyclopentadiene, another inhibitor of ethylene action (Tian et al., 1997).

The above observations, together with the results presented here, suggest that the NR protein may be synthesized successively in tomato fruit during ripening, leading to the recovery of the gene transcripts that are regulated under positive feedback. The present results also suggest that this successive synthesis of NR protein might be under the control of a positive feedback mechanism. However, the expression of this gene was not inducible in immature green fruit by exposure to ethylene for 1 d (Wilkinson et al., 1995) or propylene for 4 d (Fig. 5). These differences in *NR* gene expression in response to ethylene treatment between immature and ripening fruits may modulate the differential sensitivity to ethylene in maturing tomato fruits (Wilkinson et al., 1995). McGlasson (1985) previously pointed out that most fruit become increasingly sensitive to ethylene with time after anthesis. The abundance of *TAE1* mRNA accumulated constitutively throughout development and ripening irrespective of treatment with either MCP or propylene. Similar results have been reported for tomato leaf, flower, and fruit tissues, in which expression was unaffected by ethylene, silver ions, an ethylene-action inhibitor, or auxin in leaf-abscission zones (Zhou et al., 1996). Using the RNase-protection assay, Lashbrook et al. (1998) recently demonstrated that the signals for three members of *ETR1* homologs, including *NR* and *TAE1*, were detectable in tomato fruit throughout preclimacteric stages. Therefore, the presence of one or more *ETR1* homologs prior to ripening may contribute ripening-independent ethylene perception processes in immature fruit, in which propylene eliminated the *LE-ACS6* transcript but did not induce the *LE-ACS2* transcript (Fig. 5).

In conclusion, the results presented here suggest that ethylene biosynthesis in tomato fruit is regulated by the three different members of the ACC synthase gene family: (a) *LE-ACS2* and *LE-ACS4* are the dominant genes responsible for system 2 ethylene production in ripening fruit and their expression is regulated by a positive feedback mechanism, (b) the *LE-ACS6* gene is responsible for the low rates of system 1 ethylene production and is negatively regulated in preclimacteric fruit, and (c) the *LE-ACS1A* and *LE-ACS3* genes are also responsible for the preclimacteric system 1 ethylene production, and their transcripts accumulate constitutively throughout fruit development irrespective of the mode of feedback regulation.

In tomato fruit the preclimacteric system 1 ethylene production is mediated by the *LE-ACS1A*, *LE-ACS3*, and *LE-ACS6* genes, together with *LE-ACO1* and *LE-ACO4*. Ethylene production shifts to system 2 at the climacteric stage, with a burst in the accumulation of *LE-ACS2*, *LE-ACS4*, *LE-ACO1*, and *LE-ACO4* mRNAs as a result of positive feedback regulation. This transition from system 1 to system 2 ethylene production may be controlled by the accumulated level of NR protein from the mature green stage to the turning stage. Considering the existence of multiple *ETR1* homologs in tomato (Yen et al., 1995), further work is needed to clarify the induction mechanism of fruit ripening, especially whether the expression of *LE-ACS2* gene induces *NR* transcript or vice versa.

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