Ethylene-regulated gene expression in tomato fruit: characterization of novel ethylene-responsive and ripening-related genes isolated by differential display

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Summary

Differential display was used to isolate early ethylene-regulated genes from late immature green tomato fruit in order to obtain a broader understanding of the molecular basis by which ethylene coordinates the ripening process. Nineteen novel ethylene-responsive (ER) cDNA clones were isolated that fell into three classes: (i) ethylene up-regulated (ii) ethylene down-regulated, and (iii) transiently induced. Expression analysis revealed that ethylene-dependent changes in mRNA accumulation occurred rapidly (15 min) for most of the ER clones. The predicted proteins encoded by the ER genes are putatively involved in processes as diverse as primary metabolism, hormone signalling and stress responses. Although a number of the isolated ER clones correspond to genes already documented in other species, their responsiveness to ethylene is described here for the first time. Among the ER clones sharing high homology with regulatory genes, *ER43*, a putative GTP-binding protein, and *ER50*, a *CTR1*-like clone, are potentially involved in signal transduction. *ER24* is homologous to the multi-protein bridging factor MBF1 involved in transcriptional activation, and finally, two clones are homologous to genes involved in post-transcriptional regulation: *ER49*, a putative translational elongation factor, and *ER68*, a mRNA helicase-like gene. Six ER clones correspond to as yet unidentified genes. The expression studies indicated that all the ER genes are ripening-regulated, and, depending on the clone, show changes in transcript accumulation either at the breaker, turning, or red stage. Analysis of transcript accumulation in different organs indicated a strong bias towards expression in the fruit for many of the clones. The potential roles for some of the ER clones in propagating the ethylene response and regulating fruit ripening are discussed.

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Introduction

The phytohormone ethylene orchestrates a variety of physiological processes in plants, including senescence, fruit ripening and abscission (Abeles *et al.*, 1992; Lelièvre *et al.*, 1997). It also plays an important role in physiological responses to environmental stresses such as water deficit, mechanical wounding and pathogen attack (Abeles *et al.*, 1992). As well as being wide-ranging, the physiological responses to ethylene are complex and may vary depending on tissue type and interactions with other effectors. For example, it has been shown that in *Arabidopsis thaliana*, ethylene stimulates hypocotyl elongation in light-grown seedlings (Smalle *et al.*, 1997) but induces the opposite effect in the dark (Guzmàn and Ecker, 1990). The tremendous progress made in elucidating the mechanisms involved in the perception and transduction of the ethylene signal in recent years (Bleecker and Schaller, 1996; Ecker, 1995) is not sufficient to explain the diversity of plant responses to the hormone. The identification of early ethylene-regulated genes is another important strategy in gaining further understanding of the molecular mechanisms of ethylene action and in defining the role of this hormone in physiological processes. To this end, previous studies have resulted in the isolation of a number of ethylene-regulated genes through differential screening techniques (reviewed in Deikman, 1997). For example, a set of genes including *E4*, *E8* and a protease inhibitor were isolated by screening ethylene-treated mature green tomato fruit (Lincoln *et al.*, 1987). Others include a chitinase which is ethylene-regulated in bean leaves (Boller *et al.*, 1983; Broglie *et al.*, 1986) and a glutathione-*S*-transferase which responds to ethylene in senescing carnation petals (Meyer *et al.*, 1991). While these genes offer the potential for insights into the molecular basis of ethylene action, the limited number isolated so far cannot account for the wide range of biochemical and physiological responses to the hormone. In an attempt to identify novel genes involved in the ethylene response, we screened for early ethylene-regulated genes in late immature green tomato fruit using the mRNA differential display approach.

In this paper, we describe the isolation of 19 cDNA clones, corresponding to novel ethylene-responsive genes

(ER clones) that show differential regulation during the ripening process. Among the isolated ER clones, several display homology to regulatory genes that may participate in the ethylene response at the levels of signal transduction, transcription and translation.

Results and discussion

The capacity of tomato fruit to synthesize and respond to ethylene

In an attempt to select the optimal plant material for the characterization of ethylene-regulated gene expression in tomato fruit, we tested three pre-ripening stages (early immature green, late immature green and mature green) for their capacity to produce and respond to ethylene. Figure 1 shows that tomato fruit harvested at the mature green stage and stored for 7 days at 22°C were able to ripen without exogenous ethylene treatment. In contrast, both late and early immature green fruit could not initiate the ripening process autonomously (Figure 1b) because at these stages the fruit are unable to synthesize ethylene. However, in the late immature green fruit, the ripening process was able to be induced by 12 h exogenous ethylene treatment (Figure 1c), indicating that at this stage the fruit have gained the capacity to respond to ethylene and to undergo the ripening process. Late immature green tomato fruit, unable to autonomously produce ripening-related ethylene (data not shown), were therefore used to identify genes responsive to either 15 min or 5 h ethylene treatment (50 $\mu l\, l^{-1}$).

Isolation of early ethylene-regulated cDNA clones by differential display

RNA from ethylene-treated and untreated tomatoes was used in an improved differential display screening procedure (Zegzouti *et al.*, 1997a) to isolate 19 partial cDNA clones corresponding to novel early ethylene-regulated genes. With the exception of clones that displayed strong homology to known genes, sequence extension was performed for all the cDNA fragments using PCR-based screening of a tomato fruit cDNA library as described in Experimental procedures. Full-length clones were obtained for *ER5*, *ER24*, *ER43*, *ER49*, *ER50* and *ER68*, and the sequences of *ER6*, *ER33* and *ER66* were significantly extended.

An interesting observation was that genes already known to be highly regulated by ethylene and abundantly present in tomato fruit, such as *LE–ACO* (Barry *et al.*, 1996), were not isolated by our screening procedure. One explanation for this could be that the high number of PCR cycles used in the differential display procedure yielded saturating signals for abundant mRNA species resulting on the differential display gel in an apparent constitutive expression pattern for transcripts that were initially differential. Nevertheless, clones such as *E4*, *E8* and *LE-ACS*, already described in the literature as ethylene-responsive (Lincoln *et al.*, 1987, 1993), were isolated but disregarded in this study. However, the *ER* clone which corresponds to the tomato *E4* gene was used as a control to validate the effectiveness of the ethylene treatment and the appropriateness of the plant material chosen for the study. Northern analysis (data not shown) showed that *E4* is not expressed in untreated fruit but strongly induced upon 5 h of ethylene treatment and this induction is totally suppressed in the presence of 1-methylcyclopropene, a potent inhibitor of ethylene action (Sisler *et al.*, 1995).

Ethylene-dependent expression of the ER clones in tomato fruit

Many of the isolated clones correspond to weakly expressed messengers and could not be analysed by Northern blots. In order to standardize the expression study of the ER genes, we used an RT–PCR technique suitable for the quantification of rare transcripts. In each PCR amplification, the tomato ubiquitin cDNA (*ubi3*, Hoffman *et al.*, 1991) was co-amplified with the ER cDNA as an internal standard for comparative assessment of ER mRNA accumulation in the various samples (Figure 2). The constitutive accumulation of ubiquitin mRNA in the various samples used in this study was initially verified by Northern analysis (data not shown). The RT–PCR analysis identified three classes of ethylene-regulated genes in tomato fruit: up-regulated (Figure 2a), transiently induced (Figure 2b) and down-regulated (Figure 2c). For some clones requiring a low number of PCR cycles, the pattern of expression in response to ethylene was confirmed by Northern analysis. Ethylene-dependent changes in mRNA accumulation occur rapidly (15 min) for most of the ER clones. The data presented in Figure 2 also indicate that the ethylene-dependent alteration of gene expression ranged from strong (*ER5*, *ER21*, *ER24*, *ER27*, *ER28*, *ER31*, *ER33*, *ER34*, *ER50*, *ER55*, *ER60* and *ER69*) to moderate (*ER6*, *ER15*, *ER35*, *ER43*, *ER49*, *ER66*, *ER68*).

Expression study of the ER clones during ripening of tomato fruit

As the ER clones were isolated from fruit tissue on the basis of their responsiveness to ethylene, a plant hormone known to play an important role in the ripening process, we also analysed the accumulation of the corresponding transcripts during tomato fruit ripening. Even though a weak basal level of expression

Figure 1. Evaluation of the capacity of the tomato fruit to synthesize and respond to ethylene.
Tomato fruits harvested during the pre-climacteric period of the ripening process were classified as early immature green, late immature green or mature green fruit. The fruit were treated or not with ethylene (50 $\mu l\, l^{-1}$ for 12 h) and stored 7 days at 22°C. (a) Harvest day, (b) 7 days after harvest at 22°C without ethylene treatment, and (c) ethylene-treated and stored for 7 days at 22°C.

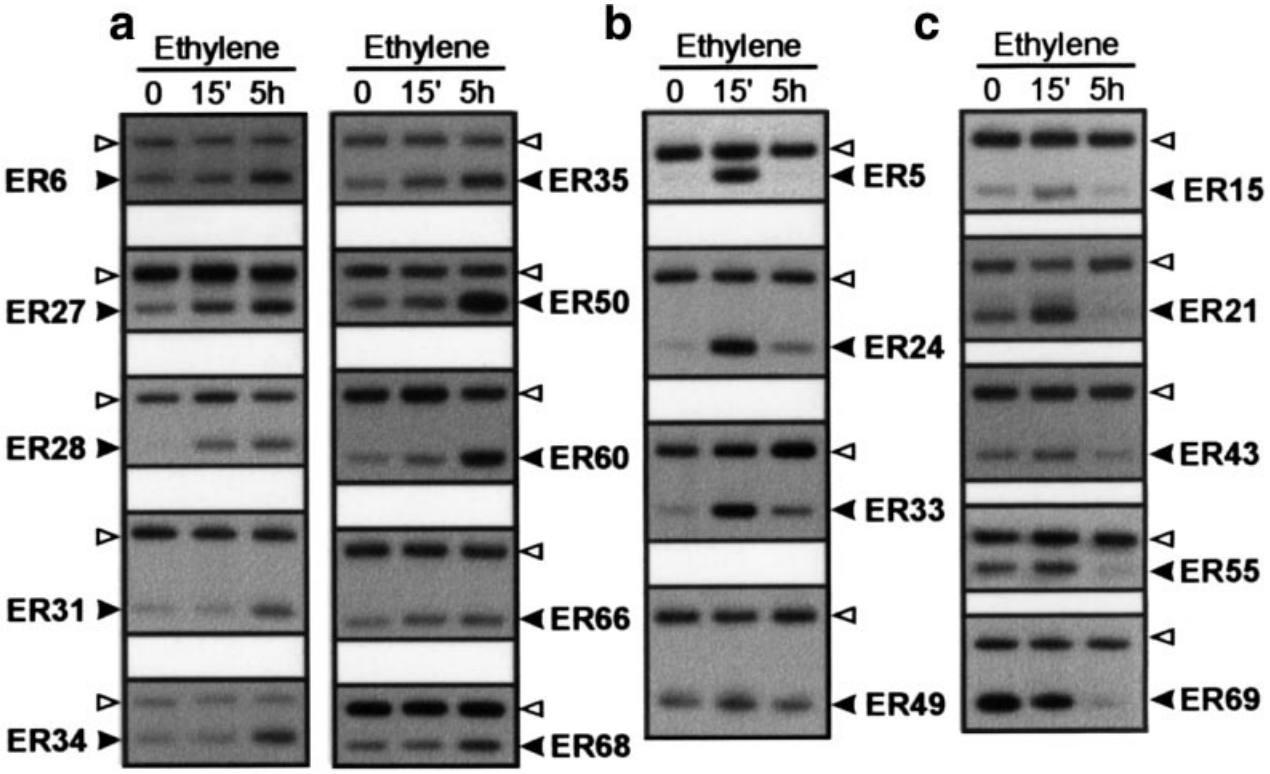

Figure 2. RT–PCR analysis of the ethylene-dependent expression of the ER clones in tomato fruit.
Total RNA from untreated fruit (0) and from fruit treated with ethylene for 15 min (15′) or 5 h (5 h) were reverse-transcribed in the presence of an oligo-$d(T)_{21}$ primer. The PCR amplification was performed using ER cDNA-specific primers. As an internal control, an endogenous tomato ubiquitin cDNA (*ubi3*) was amplified concomitantly with the ER cDNA by adding *ubi3*-specific primers to the PCR amplification. The PCR products were separated on a 1.4% agarose gel, transfered to a nylon membrane and hybridized with a mixture of equal counts min^{-1} of the ER clone and *Ubi3* probes. The membranes were exposed to Amersham Hyperfilm-MP at –80°C for 30–60 min.
(a) Ethylene up-regulated clones, (b) transiently induced clones, and (c) ethylene down-regulated clones. Open arrowheads indicate the position of the *ubi3* cDNA fragment representing the internal control of the PCR amplification. The data are representative of three independent experiments.

was detected at the mature green stage for the majority of the clones, all the ER genes undergo significant changes in their expression during tomato fruit ripening (Figure 3). The high sensitivity of the RT–PCR technique used in this study partially accounts for the signal detected at the mature green stage. During ripening, transcripts for most of the ER clones accumulate to higher levels beginning at either the breaker (*ER15*, *ER31*, *ER34*, *ER35*, *ER50* and *ER60*), the turning (*ER21*, *ER24*, *ER49*) or the red stage (*ER5*, *ER6*, *ER33*). Two clones (*ER27* and *ER55*) showed a transient induction during ripening. However, while increased accumulation of *ER27* mRNA occurred only at the turning stage, *ER55* transcripts were first induced at the breaker stage and continued to accumulate until the turning stage. Thereafter, mRNA levels for both genes underwent a dramatic decline at the red stage. *ER43* and *ER68* displayed an unusual pattern of expression during ripening, with high levels of mRNA accumulation at the breaker and red stages interspaced by minimal levels at the turning stage. While unusual, this pattern of expression is similar to that found for other developmentally regulated genes in kiwi fruit (Ledger and Gardner, 1994). Interestingly, the induction of *ER15*, *ER21*, *ER43* and *ER55* (Figure 3) during ripening contrasts with the ethylene-dependent inhibition showed by these clones at the late immature green stage (Figure 2). This discrepancy raises the possibility that in tomato fruit the regulation of these genes involves interactions between ethylene and other developmentally regulated factors. *ER69* was the only clone that showed down-regulation in response to exogenous ethylene treatment (Figure 2) and during tomato fruit ripening (Figure 3).

Expression of the ER clones in tomato leaf and root tissues

RT–PCR was used to investigate the expression of the ER clones in leaf and root tissues. The use of

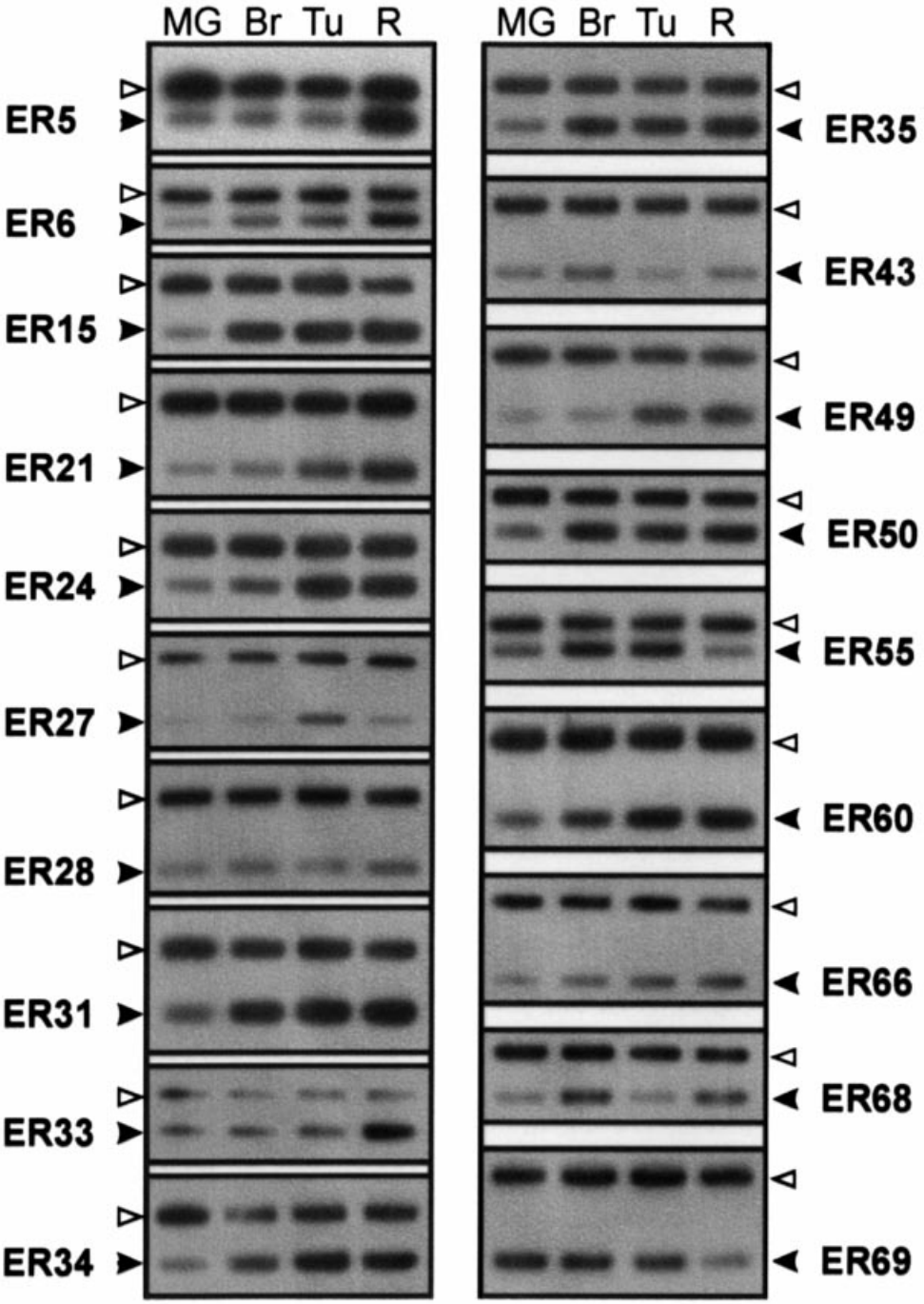

Figure 3. Expression analysis of the ER clones during ripening of tomato fruit.
Total RNA from tomato fruit stages mature green (MG), breaker (Br), turning (Tu) and red (R) were used in the RT–PCR experiment as described in Figure 2. Open arrowheads indicate the position of the *ubi3* cDNA fragment representing the internal control of the PCR amplification.

*ubi*3 as an internal reference was initially verified and showed equal amplification in the three tissues given identical RT–PCR conditions. In order to facilitate comparison of ER clone expression in the different organs, RT–PCR analysis in leaves and roots was first performed using a number of cycles shown to be sufficient to detect the ER trancripts in the fruit tissue. Under these conditions, several ER clones displayed levels of transcript accumulation in leaves similar to that found in fruit (Figure 4a). In contrast, Figure 4(a)

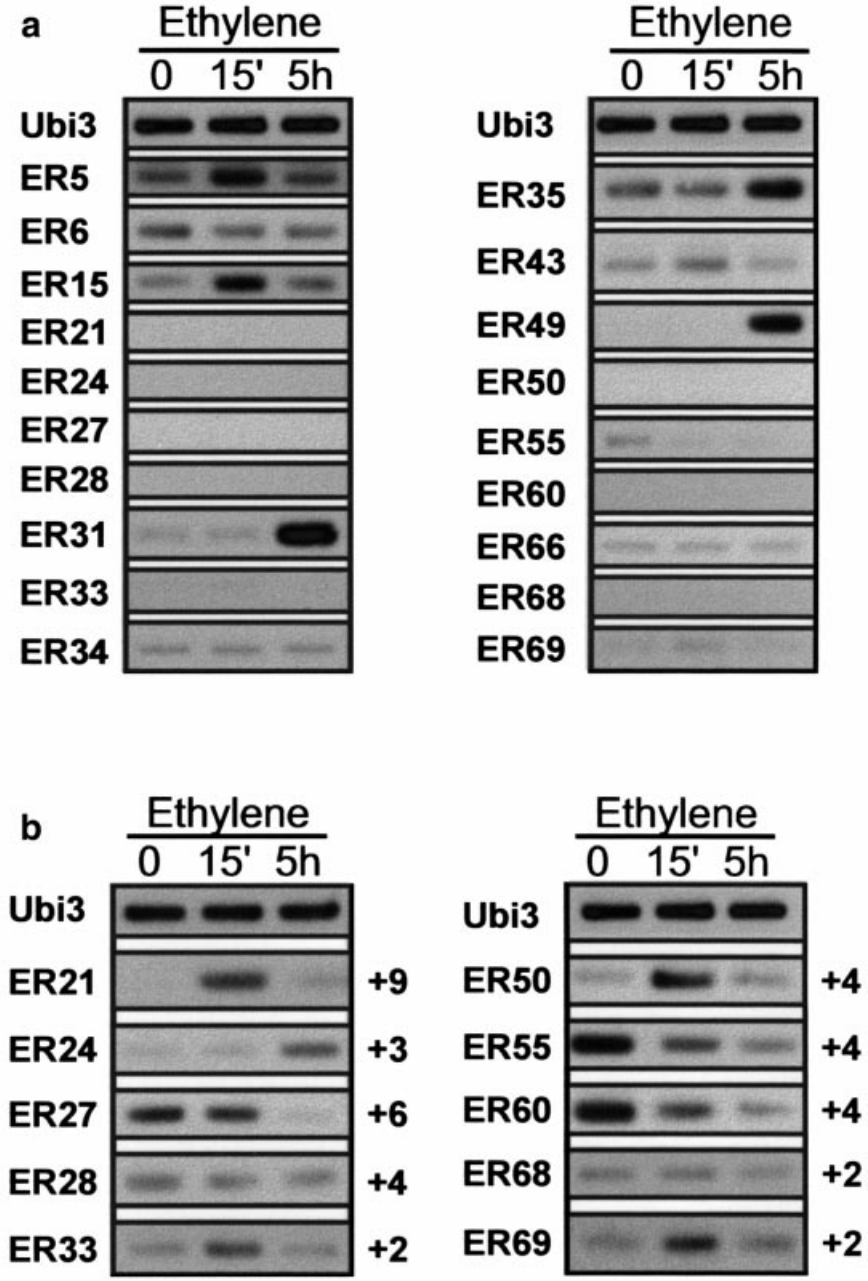

Figure 4. RT–PCR analysis of ethylene-dependent transcript accumulation of the ER clones in tomato leaves.
Total RNA from untreated leaves (0) and leaves treated with ethylene for 15 min (15′) or 5 h (5 h) were used in the RT–PCR experiment as described in Figure 2. In each sample, the internal reference *ubi3* was co-amplified with the ER cDNA as described in Experimental procedures.
(a) Transcript accumulation of the ER clones in leaves using the same number of PCR cycles as used in fruit. (b) RT–PCR data obtained after increasing the number of PCR cycles to reach a detectable level of transcript amplification. The figures on the right represent the number of additional cycles used in the PCR.

shows that transcripts corresponding to clones *ER21*, *ER24*, *ER27*, *ER28*, *ER33*, *ER50*, *ER55*, *ER60*, *ER68* and *ER69* could not be detected in the leaves either before or after ethylene treatment. In order to ascertain the ethylene responsiveness of these latter ER clones and to assess the level of their expression in leaves, the number of PCR cycles was increased until the amplification product reached a detectable level. The least abundant transcripts in leaves were *ER21* and *ER27* which required an additional nine and six PCR cycles,

respectively, in order to be detected (Figure 4b). This indicates a strong bias towards expression in the fruit for these two clones. *ER28*, *ER50*, *ER55* and *ER60* also showed significantly lower expression in leaves than in fruit as their transcripts only became visible after four more cycles than required to detect their transcripts in fruit. Finally, clones such as *ER24*, *ER33*, *ER68* and *ER69* can be considered as preferentially expressed in fruit since the amplification of their transcripts in leaves reached a detectable level only after two to three additional cycles.

The RT–PCR analysis shown in Figure 5(a) indicates that if the same number of PCR cycles is used, most of the ER clones display similar levels of expression in roots and in fruit. In contrast, 13 additional PCR cycles failed to reveal any expression of *ER27* in roots (Figure 5b). The expression of *ER21* and *ER24* in roots is also very low as suggested by the high number of additional cycles required for detection of their transcripts. The analysis also revealed that *ER28*, *ER33*, *ER60* and *ER68* are preferentially expressed in fruit as compared to roots.

While all of the ER clones were isolated on the basis of their ethylene responsivness in fruit, *ER6* and *ER66* showed no ethylene response in leaves and roots (Figures 4 and 5). Some of the clones displayed no ethylene responsiveness in leaves (*ER28*, *ER34* and *ER68*) and others failed to respond to ethylene only in the roots (*ER35* and *ER50*). However, the ethylene-dependent transcript accumulation in leaves and roots of many clones matched the pattern seen in the fruit. Interestingly, some of the clones, such as *ER60* and *ER27*, showed an opposite response to ethylene in fruit and in roots or leaves.

Sequence analysis of the ER genes

For six of the ER clones (*ER15*, *ER27*, *ER31*, *ER34*, *ER35* and *ER55*), database searches failed to provide significant homology with listed sequences, suggesting that these clones correspond to as yet unidentified genes (Table 1). In addition, *ER6*, *ER33* and *ER66* display only limited nucleotide homology with *Arabidopsis thaliana* transcribed sequences for which no function has yet been determined. For the remaining ER clones, significant homology was found with genes of known or suggested function, enabelling interpretation of their putative role in the ethylene response. Based on sequence homology, many of the encoded ER proteins are putative regulatory proteins involved in transduction pathways and in control of gene expression at the transcriptional and post-transcriptional levels (Table 1).

ER clones involved in transcriptional and post-transcriptional regulation of gene expression

ER24, an early and transiently induced clone in ethylene-treated late immature green fruit (Figure 2), displays 47% amino acid identity with a transcriptional co-activator known as multi-protein bridging factor 1 (MBF1) isolated from the silkworm *Bombyx mori* (Takemaru *et al.*, 1997). MBF1 type proteins contribute with other proteins to the formation of the TAF complex (TBP-associated factors) required for transcription initiation (Li *et al.*, 1994; Takemaru *et al.*, 1997). *ER24* is the first gene encoding a TAF component that shows regulation by ethylene. In the case of the *E4* gene, responsiveness to ethylene has been shown to require the presence of *cis* elements and their as yet unidentified ethylene-responsive element binding proteins (EREBP) (Coupe and Deikman, 1997; Xu *et al.*, 1996). Based on its high regulation by ethylene, ER24 may be one of the bridging proteins that link EREBPs to the TATA box-binding protein allowing transcription to proceed.

The ethylene responsiveness of *ER49* and *ER68* suggests that the hormone also controls gene expression at the post-transcriptional level. *ER49*, which bears a putative mitochondrial localization sequence at its N-terminal, encodes a mitochondrial protein that shows homology to both the prokaryotic translation elongation factor EF-Ts (Blank *et al.*, 1996) and the bovine mitochondrial EF-Ts_{mt} (Xin *et al.*, 1995). While it is well known that in climacteric fruits such as the tomato a sharp increase in respiration occurs at the onset of ripening (Abeles *et al.*, 1992), the regulation of genes encoding mitochondrial proteins is still poorly understood. Here, we show that *ER49* mRNA accumulates in response to ethylene and during ripening which raises the question of its potential role in the increase of mitochondrial metabolic activity during tomato fruit ripening.

The *ER68* gene product is another candidate for a role in post-transcriptional regulation based on its strong homology with the DEAD box ATPase/RNA helicases. Members of this class of RNA helicases facilitate gene expression by structurally modulating RNA molecules (unwinding) during mRNA maturation or translation (Iggo *et al.*, 1991; Okanami *et al.*, 1998).

ER genes encoding components of signal transduction pathways

Many components of the ethylene signal transduction pathway have been isolated and characterized in recent years, mainly through the use of the model plant *Arabidopsis thaliana* (Bleecker and Schaller, 1996; Ecker, 1995). In tomato fruit, ethylene has been shown to regulate its own transduction pathway through the induction of the

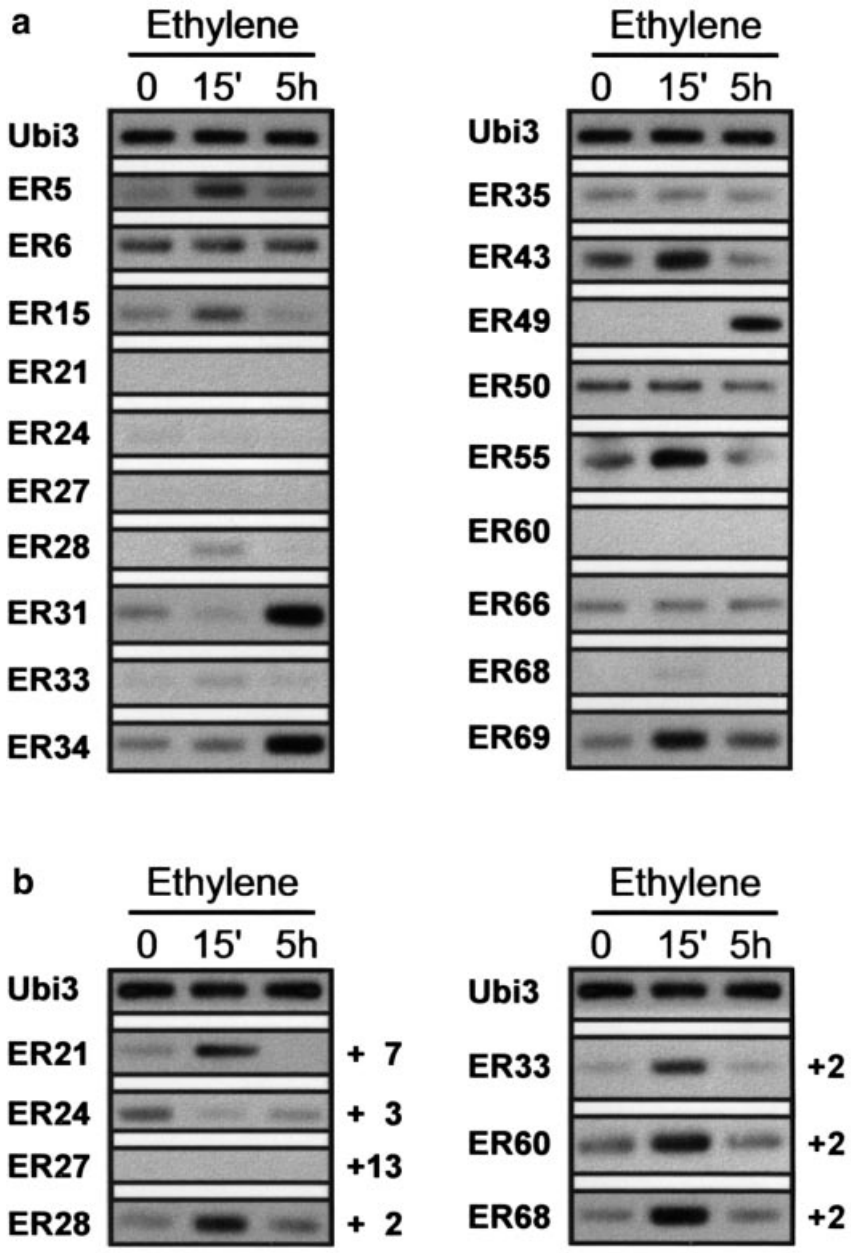

Figure 5. RT–PCR analysis of ethylene-dependent transcript accumulation of the ER clones in tomato roots.
Total RNA from untreated roots (0) and from roots treated with ethylene for 15 min (15′) or 5 h (5 h) were used in the RT–PCR experiment as described in Figure 2. The *ubi3* clone was used as an internal reference under the conditions described in Experimental procedures.
(a) Transcript accumulation of the ER clones in roots using the same number of PCR cycles as used in fruit.
(b) RT–PCR data obtained after increasing the number of PCR cycles to reach a detectable level of transcript amplification. The figures on the right represent the number of additional cycles used in the PCR.

Nr gene which encodes an ethylene receptor (Payton *et al.*, 1996; Wilkinson *et al.*, 1995). Another component of the ethylene signal transduction pathway isolated in *A. thaliana* and situated downstream of the receptor is the *CTR1* gene which has been shown to be constitutively expressed and to negatively regulate the ethylene response in *A. thaliana* (Kieber *et al.*, 1993). We have isolated *ER50* which is identical to a serine/threonine kinase clone from tomato (Wang and Li, 1997) and shows strong homology to the *Arabidopsis CTR1* gene. However, unlike *CTR1*, the transcript accumulation of *ER50* was found to be strongly up-regulated by ethylene in fruit (Figure 2) and during ripening (Figure 3). Like CTR1, the ER50 predicted protein shows typical characteristics of the Raf protein kinase family known to be involved in the transduction of regulatory signals through MAP kinase cascades (Camp-

Table 1. Tomato ethylene-regulated cDNA clones isolated by differential display

Clone	Sequence homology[a]	% amino acid identity	Accession number[c]
Transcriptional and post-transcriptional regulation			
ER24[b]	Transcriptional co-activator MBF1 (*B. mori*)	47%	AB001078
ER49[b]	Elongation factor EF-Ts *(T. thermophilus)*	46%	X83598
ER68[b]	RNA helicase DBP2 (*S. cerevisiae*)	61%	X52649
Signal transduction components			
ER43[b]	Small GTP-binding protein (*Pisum sativum*)	83%	Z49901
ER50[b]	*CTR1*-like kinase (*L. esculentum*)	100%	Y13273
Stress-related proteins			
ER5[b]	*lea*-like gene (*L. esculentum*)	67%	Z46654
ER21	*Hsc70* gene (*L. esculentum*)	100%	L41253
ER60	Catalase (*N. plumbaginifolia*)	90%	Z36975
Primary metabolism			
ER28	Enolase (*L. esculentum*)	97%	X58108
ER69	Methionine synthase (*C. roseus*)	92%	X83499
Unidentified function			
ER6	Sequence *F*21M12.12 (*A. thaliana*)	71%	AC000132
ER33	Putative protein (*A. thaliana*)	40%	AL022198
ER66	F1N21.9 similar to extensin (*A. thaliana*)	42%	AC002130
ER15	Unknown	–	–
ER27	Unknown	–	–
ER31	Unknown	–	–
ER34	Unknown	–	–
ER35	Unknown	–	–
ER55	Unknown	–	–

[a]Determined using the BLAST program. [b]Full-length size. [c]GenBank accession number of the homologous gene.

bell *et al.*, 1995). To investigate the putative role of ER50 in the ethylene signal transduction pathway, and to address the apparent paradox of a negative regulator being up-regulated during the onset of ripening, transgenic tomato plants under- and over-expressing the gene have been generated and are currently being analysed.

The ER43 predicted protein is highly homologous to the rab/ypt-related small GTP-binding protein family (Roehl *et al.*, 1995) which has been shown to play a role in vesicular transport between different compartments of eukaryotic cells (Zerial and Stenmark, 1993). *ER43* is another ethylene-regulated gene potentially involved in signal transduction.

Stress-related ER clones

The *ER5* clone shares strong homology with members of the *lea*-like gene family (Hughes and Galau, 1991). Previous characterization has indicated that in addition to its responsiveness to ethylene, this gene is regulated by ABA, drought stress and wounding (Zegzouti *et al.*, 1997b) suggesting the existence of cross-talk between hormone signalling pathways.

Sequence analysis revealed 95% homology between the *ER21* partial cDNA and the tomato heat-shock cognate 70 gene (*hsc70*) (Sun *et al.*, 1996). This is the first time that regulation by ethylene has been reported for a member of the Hsc70 family. It has been previously reported that the Hsc70 protein is down-regulated during mung bean seed germination (Wang and Lin, 1993). *ER21* mRNA accumulation is repressed by ethylene in the fruit tissue, suggesting that the increase in ethylene production during seed germination (Abeles *et al.*, 1992) may account for the inhibition of *hsc70* in mung bean seeds. Another clone, *ER60*, which is up-regulated in the fruit by ethylene, shares 90% amino acid identity with the tobacco catalase (Willekens *et al.*, 1994) and is likely to encode an ethylene-responsive tomato catalase.

Ethylene-regulated expression of primary metabolism-related genes

Some of the ER clones have homology to genes encoding enzymes involved in primary metabolism, indicating that in addition to regulating ripening-specific pathways, the plant hormone ethylene may also modulate primary metabolic pathways during fruit ripening. ER28, which is up-regulated by ethylene, is nearly 100% identical at the amino acid level to the tomato enolase. Although the corresponding gene was first isolated as a ripening-related gene (Van der Straeten *et al.*, 1991), in our study it showed strong induction by ethylene but

only slight up-regulation during ripening. However, in the experiments performed by Van der Straeten *et al.* (1991), ripening was artificially induced by treating tomato fruit with LiCl and ethylene. Hence, it is possible that the enolase gene was induced by exogenous ethylene independently from the ripening process. Finally, based on its strong homology, ER69 is likely to be a tomato cobalamine-independent methionine synthase (Eichel *et al.*, 1995), indicating that ethylene is involved in regulating methionine biosynthesis.

The isolation of these ethylene-regulated genes broadens the scope for research into the mechanisms by which ethylene coordinates developmental processes such as fruit ripening. The opposite responses to ethylene displayed by some ER clones in the different organs is in agreement with the observation that this hormone can have opposite physiological effects depending on tissue type and environmental conditions (Smalle *et al.*, 1997). Our study shows that the predicted proteins encoded by the ER genes show a wide diversity of functions indicating the complexity of cellular responses to ethylene signalling. We have chosen the ER clones encoding putative components of signal transduction, transcription and post-transcriptional regulation as priority targets for in-depth characterization. *Agrobaterium*-mediated generation of transgenic plants over- or under-expressing the genes is now underway.

Experimental procedures

Plant materials

Lycopersicon esculentum (Cherry tomato, cv Evita) plants were grown in soil under standard greenhouse conditions. Late immature green fruit, unable to produce ripening-related ethylene, were harvested and treated in 10-litre sealed jars with 50 $\mu l\, l^{-1}$ of ethylene for 15 min or 5 h. The control green fruit were exposed to air alone. The ripening stages were harvested at mature green (MG), breaker (Br), turning (Tu) and red (R) according to the levels of pigment development. The whole-plant ethylene treatment was as follows. Tomato plants of about 30 cm high were treated with 50 $\mu l\, l^{-1}$ of ethylene for either 1 h or 5 h while control plants were exposed to air alone. For the 1-methylcyclopropene (1-MCP) (Sisler *et al.*, 1995) treatment, tomato fruit were treated for 2 h with 5 $\mu l\, l^{-1}$ 1-MCP prior to ethylene treatment. After the treatments, whole fruit, leaves and roots were immediately frozen in liquid nitrogen and stored at –80°C until RNA extraction. RNA was extracted according to Hamilton *et al.* (1990).

Differential display screening

The differential display step (DDRT–PCR) was performed as previously described (Liang and Pardee, 1992; Liang *et al.*, 1993) and the screening for true positives was performed by reverse Northern blots (Zegzouti *et al.*, 1997a). The differential cDNA fragments were then cloned into the pGEM-T vector (Promega, France) according to the manufacturer's protocol.

RT–PCR analysis

The RT–PCR analysis was carried out as previously described (Zegzouti *et al.*, 1997a). As an internal control, a fragment of the endogenous tomato ubiquitin cDNA *ubi3 (Hoffman et al.*, 1991) was amplified concomitantly with the ER clone by adding 2 μM of *ubi3*-specific primers to the PCR reaction after *n* cycles from the beginning of the amplification of the ER clone. To maintain the amplification of the internal control and the ER clone within the exponential phase, the number of PCR cycles was adjusted to 20 for the ubiquitin and to $n+20$ cycles for the ER clones. The PCR products were separated on a 1.4% agarose gel, transferred to a nylon membrane and hybridized with a mixture of [α-^{32}P]dCTP-labelled ER and *ubi3* probes. The RT–PCR data presented are representative of three independent experiments.

Extension of the cDNA fragments by PCR-based screening of cDNA library

A tomato fruit cDNA library (0.5 μl) was screened by PCR using a 2 μM of specific primer for each clone with combination to M13 universal primer which hybridizes to the λZap pBluescript sequence. The longest fragments corresponding to each ER cDNA were cloned and sequenced.

DNA sequence analysis

The ER clones were sequenced from both sides with universal primers using the Thermosequenase^{TM} cycle sequencing kit (Amersham) according to the supplier's instructions. The reaction was carried out in the presence of [α-^{35}S]dATP. The following cycling parameters were used: 50 cycles of 95°C for 20 sec and 60°C for 30 sec (labelling step) and 60 cycles of 95°C for 30 sec and 72°C for 1 min (termination step). Database searches and comparison with published sequences were carried out using the BLAST program (Altschul *et al.*, 1990).

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