*Proc. Natl. Acad. Sci. USA*
Vol. 85, pp. 8012–8016, November 1988
Botany

# Ethylene-regulated expression of a tomato fruit ripening gene encoding a proteinase inhibitor I with a glutamic residue at the reactive site

(DNA sequence/gene expression/*Lycopersicon esculentum*/*Staphylococcus aureus* V8 proteinase)

LINDA J. MARGOSSIAN, ALEX D. FEDERMAN, JAMES J. GIOVANNONI, AND ROBERT L. FISCHER*

Division of Molecular Plant Biology, University of California, Berkeley, CA 94720

*Communicated by Clarence A. Ryan, July 28, 1988 (received for review May 12, 1988)*

ABSTRACT **We report the isolation from tomato (*Lycopersicon esculentum*) of an ethylene-responsive member of the proteinase inhibitor gene family. DNA sequence analysis of a full-length cDNA clone indicates that the ethylene-responsive gene is distantly related to the tomato proteinase inhibitor I gene, having 53% sequence identity. The predicted amino acid sequence reveals 47% and 45% sequence identity with the tomato and potato proteinase inhibitor I polypeptides, respectively. Additionally, the ethylene-responsive inhibitor has evolved a completely different pattern of gene expression and inhibitory specificity than other members of the inhibitor I family. Gel blot hybridization experiments show that, unlike the tomato proteinase inhibitor I gene, it is not induced in wounded leaves. In contrast, it is activated by the plant hormone ethylene in leaves and during fruit ripening. Furthermore, the ethylene-responsive inhibitor exhibits a novel reactive site, having glutamic acid as the $P_1$ residue. This suggests that the ethylene-responsive proteinase inhibitor does not react with chymotrypsin, as does proteinase inhibitor I, but that it reacts with proteolytic enzymes that cleave at glutamic residues, such as the *Staphylococcus aureus* V8 proteinase, for which no inhibitors are known. Finally, isolation and analysis of a genomic clone reveals that the ethylene-responsive proteinase inhibitor gene is tightly linked to another, yet unidentified, coordinately expressed gene. We discuss these results with regard to the function and evolution of proteinase inhibitor genes in tomato.**

The serine proteinase inhibitors are a family of proteins whose function is to prevent unwanted proteolysis in the tissues of both animals and plants (see refs. 1 and 2 for reviews). In plants, proteinase inhibitors reduce the nutritional quality of plant organs, and their presence is thought to represent a defense against herbivorous insects (3–5). Tomato proteinase inhibitors I and II accumulate in tomato leaves that have been wounded either mechanically or by chewing insects (6, 7). They are also developmentally regulated in potato tubers and in fruit of wild tomato species, but not in modern tomato fruit (8, 9). Although the proteinase inhibitor I and II genes are nonhomologous, some are tightly linked in the tomato genome (10).

The inhibitory specificities of serine proteinase inhibitors are, to a great extent, determined by the amino acid at the reactive site (designated $P_1$), and replacements of the $P_1$ residue have been shown to alter the specificities of proteinase inhibitors (1, 2, 11–14). It is thought that the ability to tolerate mutational replacements at the $P_1$ residue is responsible, at least in part, for the hypervariability of the reactive site that has occurred during the evolution of proteinase inhibitors (13, 14).

We now report the isolation and characterization of a gene that is related to the tomato proteinase inhibitor I gene. Although we have not yet analyzed the protein encoded by this gene, DNA sequence analysis indicates that it is a member of the proteinase inhibitor I gene family, and for this reason we refer to it as a proteinase inhibitor gene.† However, we find that it has evolved a completely different pattern of gene expression than other members of the inhibitor I family and it exhibits a novel $P_1$ reactive site. We discuss these results with regard to proteinase inhibitor gene evolution and function.

## MATERIALS AND METHODS

**Plant Material.** Tomato (*L. esculentum* cv. VFNT Cherry) plants were grown under standard greenhouse conditions. Fruit maturity stage was determined as follows. Immature fruits were 50% full-size. Mature green stage 1 (MG1) fruit were green, full-size, and evolved low levels of ethylene (0.6 $\pm$ 0.2 $nl \cdot g^{-1} \cdot hr^{-1}$). Mature green stage 4 (MG4) fruit were 10% red, full-size, and evolved elevated levels of ethylene (3.5 $\pm$ 1.0 $nl \cdot g^{-1} \cdot hr^{-1}$). To treat plant material with specific gases, 1 kg of mature green fruit or a 25-cm-tall potted plant was placed in a 25-liter chamber and exposed to 4.5 liters of humidified ethylene (10 $\mu$l per liter) per minute or to air alone for 8 hr.

**Isolation of Plant Nucleic Acids.** Polysomal poly(A)$^+$ RNA was isolated as described (15), except that poly(A)$^+$ RNA from wounded leaves was isolated as described by Graham *et al.* (6) and was a generous gift from C. Ryan. Tomato leaf genomic DNA was isolated by procedures described previously (16).

**Isolation of Clones.** An incomplete ethylene-responsive proteinase inhibitor cDNA clone (designated pE17) was selected from a cDNA library of tomato (*L. esculentum* cv. VFNT Cherry) ripe-fruit mRNAs as described (15). A cDNA library enriched for full-length cDNA clones of tomato (ref. 17; *L. esculentum* cv. Castlemart) ripe-fruit mRNAs was provided by A. Bennett. $^{32}$P-labeled pE17 DNA was used to screen this library for a full-length cDNA clone (designated pERI, for ethylene responsive inhibitor). A proteinase inhibitor I cDNA clone (ref. 6; designated pTI-24) was a gift from C. Ryan. The pJ49 cDNA clone encodes an mRNA that accumulates during tomato fruit ripening and when plant tissues are exposed to ethylene (15). A library of tomato (*L. esculentum* cv. T6) genomic DNA cloned in the Charon 4 vector was a gift from R. Breidenbach (University of California at Davis). Plaque hybridization was used to screen this

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: MG*n*, mature green stage *n*.

*To whom reprint requests should be addressed.

†The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J04099).

library for a genomic clone (designated λERI) encoding the ethylene-responsive proteinase inhibitor gene.

**Gel Blot Hybridization Experiments.** RNA was denatured with glyoxal, fractionated by electrophoresis in agarose gels, blotted to *o*-diazophenyl thioether (DPT)-paper, and hybridized with $^{32}$P-labeled DNAs as described by Alwine *et al.* (18). DNA was digested with restriction endonucleases, subjected to agarose gel electrophoresis, blotted to nitrocellulose paper, and hybridized with $^{32}$P-labeled DNAs as described (16).

**Nucleotide and Amino Acid Sequence Analysis.** The complete sense and antisense DNA sequence of the full-length cDNA clone, pERI, was determined as follows. pERI was subcloned into pUC118 and pUC119 to enable sense and anti-sense single-strand DNA template preparation. Deletions were generated by the procedures of Henikoff (19) to serve as start points for systematic, directional DNA sequencing. Nucleotide sequences were determined by the dideoxy chain-termination method (20). Computer analysis of DNA and amino acid sequences was performed with the GEL, GENED, PEP, and SEQ programs of the BIONET National Computer Resource for Molecular Biology.

## RESULTS

**DNA Sequence Analysis.** Previous analysis of cloned mRNAs from tomato fruit identified an ethylene-responsive proteinase inhibitor gene related to proteinase inhibitor I (15). Specifically, the 3′ portion of an incomplete cDNA clone, pE17, was shown to exhibit ≈70% nucleotide sequence identity with a proteinase inhibitor I cDNA clone, pTI-24. In order to analyze further the ethylene-responsive proteinase inhibitor gene and polypeptide, it was necessary to determine the DNA sequence of a full-length cDNA clone. To this end, a library enriched for full-length cDNA clones was screened with $^{32}$P-labeled pE17 DNA. The insert size of each clone was determined by digestion with the appropriate restriction endonuclease followed by agarose gel electrophoresis (data not shown), and the DNA sequence of the clone with the largest insert size, pERI (for ethylene-responsive inhibitor), was determined. As shown in Fig. 1, the pERI nucleotide sequence is 524 base pairs long and exhibits 53% sequence identity with the DNA sequence of the tomato proteinase inhibitor I cDNA clone pTI-24. This result suggests that the proteinase inhibitor I and ethylene-responsive proteinase inhibitor genes are distantly related members of a gene family. It should be noted that the preliminary report of the partial pE17 DNA sequence (15) was incorrect in several details, and that further analysis indicates that the pE17 sequence is identical to pERI.

**Predicted Amino Acid Sequence.** As shown in Fig. 2, the predicted amino acid sequence (119 residues) of the ethylene-responsive proteinase inhibitor polypeptide has 47% and 45% sequence identity with proteinase inhibitor I polypeptides from tomato and potato, respectively. Moreover, 17 of 20 amino acids that are relatively conserved during the evolution of inhibitor I polypeptides are present in the ethylene-responsive proteinase inhibitor. The remaining three that are not conserved represent conservative substitutions (i.e., isoleucine to leucine, isoleucine to valine, and isoleucine to phenylalanine at residues 63, 84, and 93, respectively). These results suggest that the ethylene-responsive proteinase inhibitor is part of the divergent family of proteinase inhibitor I polypeptides.

After they are translated, the potato and tomato inhibitors are processed by the removal of signal sequences and pro sequences (21). As shown in Fig. 2, the 27 amino acid sequence at the amino terminus of the ethylene-responsive inhibitor is typical of many eukaryotic signal sequences (22, 23). It consists of a positively charged lysine residue (position 5) adjacent to a highly hydrophobic core region that terminates in alanine (position 27). From these results we predict that the ethylene-responsive inhibitor has a peptidase signal cleavage site at alanine-glutamine (position 27–28). The pro sequences in the tomato and potato inhibitor I polypeptides are highly charged sequences that terminate in asparagine and glutamine, respectively (21). Similarly, the ethylene-responsive inhibitor has an analogous highly charged domain (residues 28–48) terminating in glutamine (position 48). From these results we predict that the ethylene-responsive inhibitor has a propeptide processing site at glutamine-serine (position 48–49). Taken together, these results suggest that the ethylene-responsive proteinase inhibitor has sites for posttranslational modifications that are analogous to those found in the tomato and potato inhibitor I polypeptides.

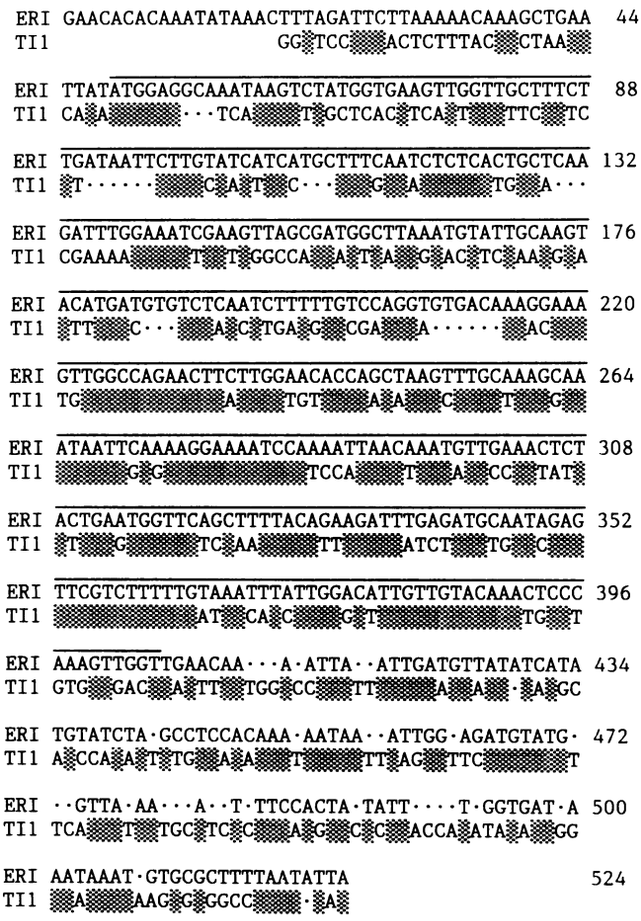


FIG. 1. DNA sequence of the tomato ethylene-responsive proteinase inhibitor and the proteinase inhibitor I cDNA clones. The tomato ethylene-responsive inhibitor sequence (ERI) was determined as described in *Materials and Methods*. The tomato inhibitor I cDNA sequence (TI1) is from Graham *et al.* (6). Optimal alignment of cDNA sequences was achieved with the GENALIGN program. Inhibitor I nucleotides that are identical to those in ethylene-responsive inhibitor are indicated by stippled boxes, and gaps are represented by dots. The open reading frame for both sequences is indicated by a horizontal line.

**Expression of Proteinase Inhibitor Genes.** A previous report (15) showed that the ethylene-responsive proteinase inhibitor mRNA accumulates in unripe fruit exposed to exogenous ethylene and in ripening fruit producing elevated levels of endogenous ethylene. In addition, Graham *et al.* (6) demonstrated that the tomato inhibitor I mRNA accumulates in response to oligosaccharides released during wounding. Because ethylene hormone is produced by wounded plant tissues (24) and numerous oligosaccharides are released by the action of cell wall-degrading enzymes associated with

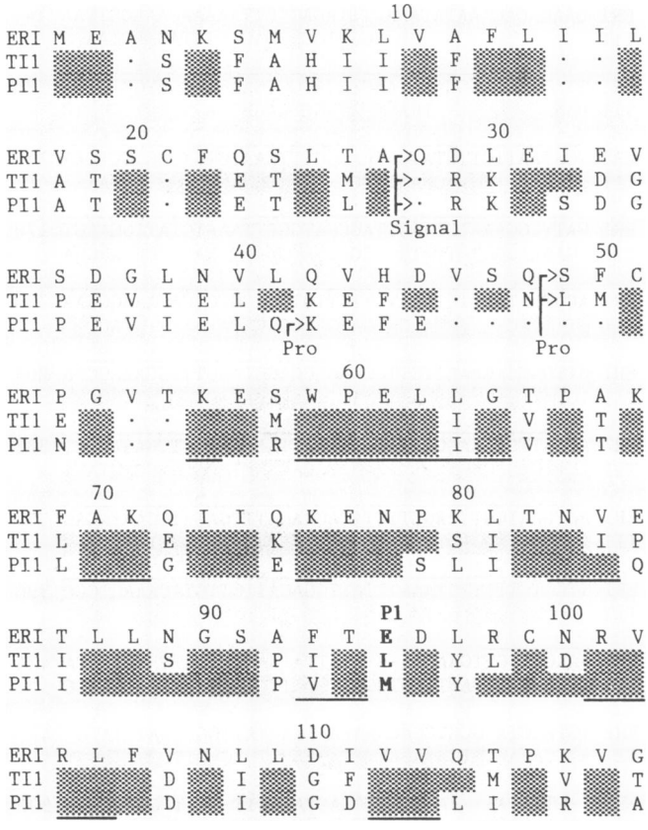


FIG. 2. Comparison of predicted amino acid sequences of the ethylene-responsive inhibitor, tomato inhibitor I, and potato inhibitor I. The GENALIGN program was used to obtain the optimal alignment of amino acid sequences, which are shown in single-letter code. Amino acids identical to those in the ethylene-responsive proteinase inhibitor are indicated by stippled boxes, and gaps are represented by dots. Amino acids conserved during the evolution of inhibitor I proteins (6) are underlined. Amino acids at the putative $P_1$ reactive center (6) are designated P1. Processing sites for the potato and tomato inhibitor I polypeptides are from Cleveland *et al.* (21). Processing sites for the ethylene-responsive inhibitor are by analogy to the potato and tomato inhibitor I sites (see text). ERI, tomato ethylene-responsive inhibitor; TI1, tomato inhibitor I sequence from Graham *et al.* (6); PI1, potato inhibitor I sequence from Cleveland *et al.* (21). Signal, cleavage site for removal of signal peptide; Pro, cleavage site for removal of propeptide.

fruit ripening (25, 26), it was of interest to compare the expression of these two tomato inhibitor genes. To this end, mRNA was isolated from wounded leaves, from ethylene-treated leaf and unripe fruit, and from fruit at different stages of ripening and was hybridized with $^{32}$P-labeled inhibitor I (pTI-24) and ethylene-responsive inhibitor (pERI) cDNA clones. As shown in Fig. 3, the 0.6-kilobase (kb) inhibitor I mRNA accumulates in wounded leaf but is not detected in ethylene-treated leaf or fruit, nor during fruit development. In contrast, the 0.6-kb ethylene-responsive inhibitor mRNA is not detected in wounded leaf but accumulates in ethylene-treated leaf and fruit, and also at the onset of fruit ripening. These results indicate that these divergent members of the proteinase inhibitor gene family have evolved different patterns of gene expression.

**Organization of the Ethylene-Responsive Proteinase Inhibitor Gene.** Lee *et al.* (10) have shown that the tomato inhibitor I gene was tightly linked, within 13.1 kb, to a nonhomologous wound-inducible inhibitor II gene. To investigate further the organization of inhibitor genes in the tomato genome, a genomic clone encoding an ethylene-responsive proteinase inhibitor gene was isolated (designated λERI). Fig. 4 *A* and

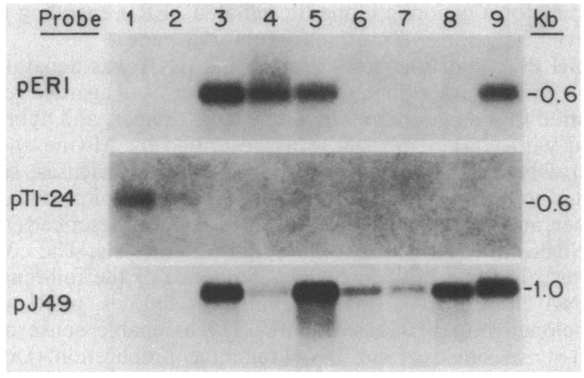


FIG. 3. Accumulation of cloned mRNAs in response to ethylene, in response to wounding, and during fruit development. mRNA was isolated from the following organs: wounded leaf (lane 1), untreated leaf (lane 2), leaf exposed to ethylene (lane 3), leaf exposed to air (lane 4), MG1 fruit exposed to ethylene (lane 5), MG1 fruit exposed to air (lane 6), untreated immature fruit (lane 7), untreated MG1 fruit (lane 8), and untreated MG4 fruit (lane 9). One microgram of each mRNA was denatured with glyoxal, fractionated by electrophoresis in an agarose gel, blotted, and hybridized with the indicated $^{32}$P-labeled DNA probes.

*B* shows the position of relevant restriction endonuclease sites and subcloned restriction fragments. The ethylene-responsive inhibitor gene was localized to the 2-kb *Hin*dIII restriction fragment represented in subclone pHH2.0. That is, this restriction fragment hybridized with the ethylene-responsive inhibitor cDNA clone pERI (Fig. 4*C*, lane 2) and with a 0.6-kb mRNA that accumulates during fruit ripening and when unripe fruit are exposed to ethylene (Fig. 4*A*). λERI DNA adjacent to the ethylene-responsive inhibitor gene, 3.7 and 7.9 kb of flanking sequences (Fig. 4*B*), does not hybridize with the proteinase inhibitor I cDNA clone pTI-24 (data not shown). Thus, the inhibitor I gene must be separated from the ethylene-responsive inhibitor by at least 3.7 kb. However, restriction fragments represented by the subclones pHK1.1, pKL0.8, and pLR4.9 hybridize with a 1.0-kb mRNA that accumulates during fruit ripening and when unripe fruit are exposed to ethylene (Fig. 4*A*). Furthermore, these restriction fragments hybridize (Fig. 4*C*, lane 1) with a cDNA clone, pJ49, that was shown previously to encode a 1.0-kb mRNA (15). Expression of the gene represented by the pJ49 cDNA clone is ethylene-responsive and developmentally regulated during fruit ripening (Fig. 3). Thus, the ethylene-responsive inhibitor gene is tightly linked to a coordinately expressed gene.

To determine whether the recombinant phage, λERI, described in Fig. 4 contained DNA segments representative of those in the genome, we hybridized $^{32}$P-labeled pERI and pJ49 plasmid probes with the same leaf DNA used to construct the library and with single-copy equivalents of λERI DNA. As shown in Fig. 4*D*, a 13.6-kb *Eco*RI restriction fragment from the tomato genome hybridizes with both the pERI and pJ49 probes (lanes 1 and 3). The 13.6-kb restriction fragment is identical in size and hybridization intensity with that produced with *Eco*RI-digested λERI DNA (Fig. 4*D*, lanes 2 and 4). These results indicate that λERI clone is an accurate copy of this region of the tomato genome and confirm that the genes represented by the pERI and pJ49 cDNA clones are tightly linked. That additional 6.5- and 4.9-kb genomic restriction fragments hybridize with the pERI (Fig. 4*D*, lane 3) and pJ49 (Fig. 4*D*, lane 1) cDNA clones suggests that other members of the ethylene-responsive inhibitor gene family may be tightly linked to other members of the J49 gene family.

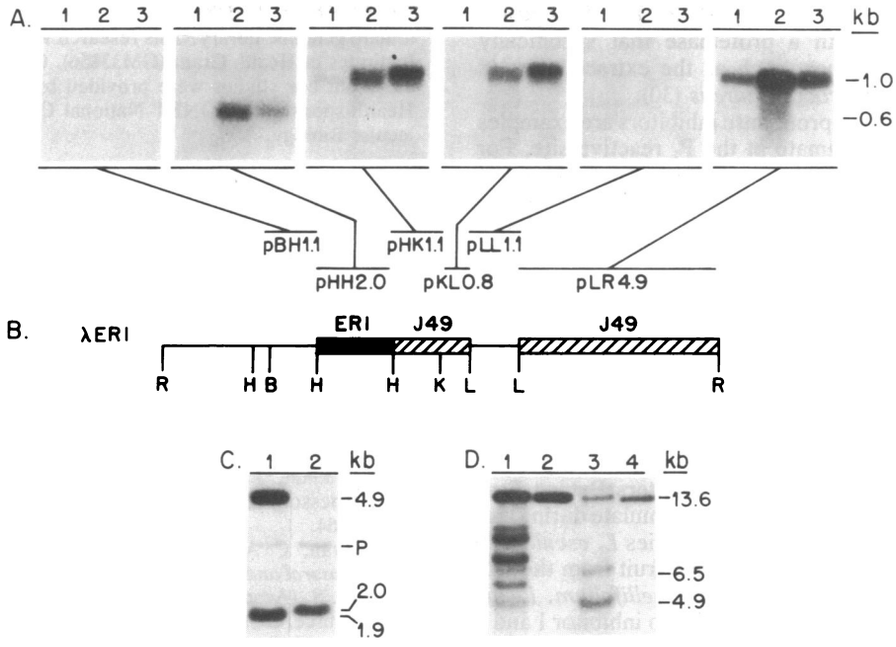


FIG. 4. Organization of the ethylene-responsive proteinase inhibitor gene region. (*A*) Molecular size of mRNAs encoded in the ethylene-responsive proteinase inhibitor gene region. Fruits used for mRNA isolation were untreated MG1 (lanes 1), MG1 treated with ethylene (lanes 2), and untreated MG4 (lanes 3). One microgram of each mRNA was denatured with glyoxal, fractionated by electrophoresis in an agarose gel, blotted, and hybridized with the indicated $^{32}$P-labeled DNA probes. (*B*) Map of restriction endonuclease sites within the ethylene-responsive proteinase inhibitor gene region. Sites were deduced from the results of single and double digestions. Restriction fragments that hybridize with ethylene-responsive proteinase inhibitor (pERI) and J49 cDNA clones as described below are indicated by the solid box and the hatched boxes, respectively. The lines above the restriction map represent subcloned probes used in the RNA gel blot studies. The name of each subclone indicates its flanking restriction sites and its molecular size (in kb). B, *Bam*HI; H, *Hin*dIII; K, *Kpn* I; L, *Bgl* II; R, *Eco*RI. (*C*) Localization of the ethylene-responsive proteinase inhibitor and J49 genes. λERI DNA digested with *Eco*RI, *Hin*dIII, and *Bgl* II was fractionated by electrophoresis in an agarose gel, blotted, and hybridized with $^{32}$P-labeled pJ49 (lane 1) or pERI (lane 2) DNA. P, restriction fragment resulting from partial digestion of the DNA. (*D*) Organization of the ethylene-responsive gene region in the tomato genome. Five micrograms of tomato genomic DNA (lanes 1 and 3) or 112 pg of λERI DNA (lanes 2 and 4) was digested with *Eco*RI, fractionated by electrophoresis in an agarose gel, blotted, and hybridized with $^{32}$P-labeled pJ49 (lanes 1 and 2) or pERI (lanes 3 and 4). Since the tomato genome and recombinant phage genome sizes are $\approx 2 \times 10^6$ and 45 kb, respectively, lanes 2 and 4 contain a single-copy equivalent of phage DNA.

## DISCUSSION

**The Ethylene-Responsive Gene Is Related to a Family of Proteinase Inhibitors.** We have isolated a gene that displays significant DNA sequence identity with the tomato proteinase inhibitor I gene (Fig. 1). Comparison of amino acid sequences that span the putative mature portion of the ethylene-responsive inhibitor (i.e., serine at position 47 to glycine at position 119) to inhibitor polypeptides from tomato, potato, barley, broad bean, and the leech reveals 57%, 58%, 33%, 27%, and 36% sequence identity, respectively (6, 21). Statistically, this level of sequence identity is highly significant (27). Thus, the ethylene-responsive proteinase inhibitor is part of a family of homologous proteinase inhibitors that has members in both the animal and the plant kingdom.

**Multiple Duplications of the Ethylene-Responsive Gene Region Have Probably Occurred.** The potato and tomato inhibitor I cDNA clones (21) exhibit more sequence identity (>90%) than the tomato inhibitor I and tomato ethylene-responsive inhibitor (53%; Fig. 1), suggesting that the duplication event that generated the inhibitor I and ethylene-responsive inhibitor genes occurred before tomato and potato divergence. We find that the ethylene-responsive inhibitor gene is tightly linked to a coordinately expressed gene of unknown function represented by the pJ49 cDNA clone (Fig. 4*B*). DNA blot hybridization experiments (Fig. 4*D*) suggest that there are multiple ethylene-responsive inhibitor and J49 genes per haploid tomato genome and that some may also be tightly linked. These results suggest that the region spanning the ethylene-responsive inhibitor and J49 genes has undergone further duplications within the tomato genome.

**Differential Expression of Related Tomato Inhibitor Genes.** Although many sequences of the tomato inhibitor I and ethylene-responsive inhibitor genes have been conserved, important aspects about the regulation of their expression have undergone significant changes. Tomato inhibitor I mRNA accumulates when leaves are wounded but not in response to ethylene (Fig. 3). In contrast, we do not detect the accumulation of ethylene-responsive inhibitor mRNA in wounded leaf (Fig. 3) or wounded fruit (data not shown). However, ethylene-responsive inhibitor mRNA accumulates when *L. esculentum* fruit ripen, and this activation of gene expression most likely occurs in response to increases in ethylene levels and sensitivity associated with fruit ripening (15). Taken together, these results indicate that the two homologous tomato inhibitor genes have evolved completely different patterns of gene expression.

**Predicted Inhibitory Specificity of the Ethylene-Responsive Inhibitor.** Within its family of homologous proteinase inhibitors, the ethylene-responsive polypeptide appears to have evolved a unique inhibitory specificity. As shown in Fig. 2, the $P_1$ amino acids for potato (28) and tomato (6) inhibitor I are methionine and leucine, respectively. These $P_1$ amino acids are consistent with the fact that the potato and tomato inhibitor I polypeptides are chymotrypsin inhibitors (29). Similarly, the homologous inhibitors from barley (methionine, leucine), broad bean (alanine), and leech (leucine) have uncharged, hydrophobic $P_1$ amino acids (6). In contrast, the amino acid at the same relative position in the ethylene-responsive proteinase inhibitor, and therefore the putative $P_1$ reactive site, is a negatively charged amino acid, glutamic acid. This result makes it unlikely that the ethylene-responsive

inhibitor reacts with chymotrypsin (1). Rather, it suggests that it might interact with a proteinase that specifically cleaves at glutamic residues, such as the extracellular V8 proteinase from *Staphylococcus aureus* (30).

Among other families of proteinase inhibitors are examples of polypeptides with glutamate at the $P_1$ reactive site. For example, $P_1$ glutamate is often found in the first domain of ovomucoid proteinase inhibitors that are prevalent polypeptides in avian egg whites (31). However, it has been shown these ovomucoids do not inhibit the action of V8 proteinase. Rather, the $P_1$ glutamate peptide bonds of these ovomucoids are hydrolyzed preferentially by the V8 proteinase (14). These results suggest that the $P_1$ glutamate is in the optimal configuration for hydrolysis by the V8 proteinase but that the ovomucoid inhibitor–V8 proteinase complex is not stable. This information underscores the importance of isolating the ethylene-responsive proteinase inhibitor polypeptide and determining its inhibitory specificity directly.

**Possible Functions for Proteinase Inhibitors During Fruit Ripening.** Tomato inhibitor I does not accumulate during fruit development of the cultivated tomato species *L. esculentum* (ref. 9; Fig. 3), but it is present in unripe fruit from the wild tomato species *L. peruvianum*, *L. pimpinellifolium*, *L. hirsutum*, and *L. parvaflorum* (9). The tomato inhibitor I and II polypeptides are thought to defend the plant by inhibiting insect gut trypsin and chymotrypsin enzyme activities, resulting in pernicious hyperproduction of gut proteases. This, in turn, leads to inhibition of insect growth due to insufficient dietary availability of sulfur-containing amino acids (3–5). As fruit from these wild tomato species ripen, the inhibitor I activity decays, making the fruit edible to small animals and birds, which facilitates seed dispersal.

In contrast, ethylene-responsive inhibitor gene expression is activated by the increased levels of ethylene that are present in ripening *L. esculentum* fruit (ref. 15; Fig. 3). However, because of the its $P_1$ glutamic acid reactive site, the ethylene-responsive inhibitor probably does not interact with trypsin or chymotrypsin and may not lead to the chronic stress on the digestive-enzyme-producing system that proves detrimental to insect growth. Thus, if the ethylene-responsive inhibitor does not interact with the digestive physiology of animals or birds, its presence in ripening fruit would not inhibit this important mechanism for seed dispersal. Rather, we speculate that its unusual inhibitor specificity and pattern of gene expression may have evolved either to regulate an endogenous tomato proteinase activity or to protect the ripening fruit and seeds from a secreted proteinase produced by bacteria or fungi upon infection. Recent results may support the latter hypothesis. That is, it has been shown that the amino acid residues of proteinase inhibitors that make contact with the proteinase, including the $P_1$ amino acid, evolve at a significantly higher rate than other amino acids. From this result, others (13, 14) have speculated that the nucleotide substitutions that generate proteinase inhibitors with diverse reactive centers, and therefore different specificities, provide the host organism with a defense against rapidly evolving proteinases brought in by bacteria or parasites.

We express our gratitude to Prof. Clarence Ryan for the tomato inhibitor I cDNA clone and for wounded-leaf mRNA, Alan Bennett for the ripe-fruit cDNA library, and R. W. Breidenbach for the tomato genomic library. This research was supported by a National Institutes of Health Grant (GM33856). Computer resources used to carry out our studies were provided by the National Institutes of Health-sponsored BIONET National Computer Resource for Molecular Biology.

1. Laskowski, M., Jr., & Kato, I. (1980) *Annu. Rev. Biochem.* **49,** 593–626.
2. Ryan, C. A. (1981) *Biochem. Plants* **6,** 351–370.
3. Broadway, R. M., Duffey, S. S., Pearce, G. & Ryan, C. A. (1986) *Entomol. Exp. Appl.* **41,** 33–38.
4. Broadway, R. M. & Duffey, S. S. (1986) *J. Insect Physiol.* **32,** 673–680.
5. Hilder, V. A., Gatehouse, A. M. R., Sheerman, S. E., Barker, R. F. & Boulter, D. (1987) *Nature (London)* **330,** 160–163.
6. Graham, J. S., Pearce, G., Merryweather, J., Titani, K., Ericsson, L. & Ryan, C. A. (1985) *J. Biol. Chem.* **260,** 6555–6560.
7. Graham, J. S., Pearce, G., Merryweather, J., Titani, K., Ericsson, L. H. & Ryan, C. A. (1985) *J. Biol. Chem.* **260,** 6561–6564.
8. Ryan, C. A. (1983) in *Variable Plants and Herbivores in Natural and Managed Systems*, eds. Denno, R. F. & McClure, M. S. (Academic, New York), pp. 43–60.
9. Pearce, G., Liljegren, D. & Ryan, C. A. (1988) *Planta*, in press.
10. Lee, J. S., Brown, W. E., Graham, J. S., Pearce, G., Fox, E. A., Dreher, T. W., Ahern, K. G., Pearson, G. D. & Ryan, C. A. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 7277–7281.
11. Hill, R. E., Shaw, P. H., Boyd, P. A., Baumann, H. & Hastie, N. D. (1984) *Nature (London)* **311,** 175–177.
12. Carrell, R. & Travis, J. (1985) *Trends Biochem. Sci.* **10,** 20–24.
13. Hill, R. E. & Hastie, N. E. (1987) *Nature (London)* **326,** 96–99.
14. Laskowski, M., Jr., Kato, I., Ardelt, W., Cook, J., Denton, A., Empie, W., Kohr, W. J., Park, S. J., Parks, K., Schatzley, R. L., Schoenberger, O. L., Tashiro, M., Vichot, G., Whatley, H. E., Wieczorek, A. & Wieczorek, M. (1987) *Biochemistry* **26,** 202–221.
15. Lincoln, J. E., Cordes, S., Read, E. & Fischer, R. L. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 2793–2797.
16. Fischer, R. L. & Goldberg, R. B. (1982) *Cell* **29,** 651–660.
17. DellaPenna, D., Alexander, D. C. & Bennett, A. B. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 6420–6424.
18. Alwine, J. C., Kemp, D. J., Parker, B. A., Reisner, J., Stark, G. R. & Wahl, G. M. (1979) *Methods Enzymol.* **68,** 220-242.
19. Henikoff, S. (1984) *Gene* **28,** 351–359.
20. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
21. Cleveland, T. E., Thornburg, R. W. & Ryan, C. A. (1987) *Plant Mol. Biol.* **8,** 199–207.
22. Perlman, D. & Halvorson, H. O. (1983) *J. Mol. Biol.* **167,** 391–409.
23. Von Heijne, G. (1983) *Eur. J. Biochem.* **133,** 17–21.
24. Abeles, F. B. (1973) in *Ethylene in Plant Biology* (Academic, New York).
25. Huber, D. J. (1983) *J. Am. Soc. Hort. Sci.* **108,** 405–409.
26. Huber, D. J. (1983) *Hort. Rev.* **5,** 169–219.
27. Doolittle, R. F. (1986) *Of Urfs and Orfs: A Primer on How to Analyze Derived Amino Acid Sequences* (University Science Books, Mill Valley, CA), pp. 10–14.
28. Richardson, M. & Cossins, L. (1974) *FEBS Lett.* **45,** 11–13.
29. Laskowski, M. & Sealock, R. W. (1971) in *The Enzymes*, ed. Boyer, P. (Academic, New York).
30. Houmard, J. & Drapeau, G. R. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 3506–3509.
31. Kato, I., Kohr, W. J. & Laskowski, M., Jr. (1977) *FEBS Symp.* **47,** 197–206.