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A Putative Vacuolar Processing Protease Is Regulated by Ethylene and also during Fruit Ripening in *Citrus* Fruit[1]

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A putative citrus vacuolar processing thiolprotease cDNA (Citvac) was isolated from a cDNA library of *Citrus* fruits (*Citrus sinensis* L. Osbeck var Washington navel). The cDNA is 58 and 57% identical with vacuolar processing seed proteases from castor bean and soybean, respectively. The Citvac sequence shows a typical signal peptide for entering into the endoplasmic reticulum and two glycosylation signals. Using an in vitro transcription-translation system, we show that the Citvac precursor is able to enter a microsomal fraction and to undergo proteolytic processing and glycosylation. Transcript levels for the Citvac are developmentally regulated in the flavedo (outer colored part of the fruit peel) and increase during fruit ripening and in the flower during opening. Exogenous treatment with ethylene induces Citvac mRNA expression in both fruits and leaves. Citvac is encoded by one or two genes in the *Citrus* genome. The possible role of the Citvac gene product during fruit ripening and other ethylene-mediated processes is discussed.

The orange is a nonclimateric fruit, i.e. a sharp increase in respiration and ethylene production does not accompany the ripening process (Rasmussen, 1975). However, the peel of the orange is able to produce significant amounts of ethylene under certain conditions (Riov et al., 1969; Baldwin and Biggs, 1983, 1988). Furthermore, ethylene seems to be required for degreening of this part of the fruit (flavedo), which normally occurs during fruit ripening (Goldschmidt et al., 1993). These processes are associated with alterations at the biochemical and gene expression level (Alonso et al., 1992; Burns and Baldwin, 1994). Treatment of full-size green fruits with ethylene induces a number of changes at the morphological (Shimokawa et al., 1978), physiological, and molecular (Stewart and Wheaton, 1972; Alonso et al., 1992) levels similar to those observed during natural degreening of the fruit that occurs during ripening.

In this paper we report the molecular characterization of an mRNA (Citvac) that accumulates in the flavedo tissue of the fruit during ripening. This mRNA encodes a protein homologous to Cys proteinases from castor bean and soybean seeds that are involved in posttranslational maturation of vacuolar storage proteins (Hara-Nishimura et al., 1993; Shimada et al., 1994). The pattern of expression of this mRNA, its regulation by ethylene, and its possible role during fruit ripening will be discussed.

MATERIALS AND METHODS

Plant Materials and Ethylene Treatment

Fruits (seedless) from 25-year-old Washington navel orange trees (*Citrus sinensis* [L.] Osbeck), grafted on sour orange rootstocks, were picked at five different stages of ripening and characterized by $-a/b$ Hunter color as described previously (Alonso et al., 1992): (a) developing green fruits (three-fourths of the final size), $-a/b = 0.73$; (b) fully developed green fruits, $-a/b = 0.63$; (c) turning fruits A, $-a/b = 0.18$; (d) turning fruits B, $-a/b = -0.25$; and (e) fully colored, $-a/b = -0.79$. The positive values of $-a/b$ correspond to green fruits, zero values to yellow fruits, and negative values to orange and red fruits. Immature fruits (1 cm in diameter) and flowers at different developmental stages were obtained from the same trees. Samples were harvested, frozen immediately in liquid N_2, and stored at −80°C until use. For roots, plants were grown hydroponically in Hoagland solution in the greenhouse. Roots of 1 or 2 mm in diameter were rinsed with water, blotted with filter paper, and frozen immediately in liquid N_2. Leaf explants consisted of terminal segments of vegetative branches (about 15 cm) bearing five to eight mature leaves. Explants were quickly transported to the laboratory, and the lower part of the stem was placed in a vial with water during ethylene treatment. Ethylene treatments were carried out in the dark as described previously (Alonso et al., 1992).

Isolation of Tar4N1

A flavedo-enriched fruit cDNA library (E12 library) (Alonso et al., 1995) was screened differentially on duplicate plaque filters (Hybond-N, Amersham) with ^{32}P-labeled first-strand cDNA synthesized from orange flavedo poly(A)$^+$ RNA of green fruits treated for 12 h with either 10 μL L^{-1} ethylene (probe +) or 0 μL L^{-1} ethylene (probe −). Filters were prehybridized using standard procedures. One set of filters was hybridized with ^{32}P-labeled probe +, and

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Abbreviation: Citvac, citrus vacuolar processing thiolprotease.

the second one was hybridized with ^{32}P-labeled probe −. Hybridization was carried out overnight at 65°C in a solution of the same composition as the prehybridization solution but supplemented with ^{32}P-labeled probe. Hybond-N filters (Amersham) were washed twice for 20 min each with 2× SSC and 0.1% SDS solution at room temperature and twice for 25 min each with 0.2× SSC and 0.1% SDS at 55°C. Filters were exposed to Kodak X-Omat SX film at −80°C.

RNA Extraction and Northern Blot Analysis

RNA was extracted according to the method of Jones et al. (1985). Samples of denatured total RNA (20 μg) were run on 1.1% (w/v) agarose-formaldehyde gels. After tRNA was checked for integrity and equal loading of the rRNA bands and cross-linked to the Hybond membrane by UV irradiation, 1.2×10^5 J were supplied by UV Stratalinker 800 (Stratagene). The size of hybridized mRNA was estimated using RNA markers from Gibco-BRL.

Northern blots were prehybridized and hybridized following standard procedures (Sambrook et al., 1989). DNA probes were random-prime labeled with [α-^{32}P]dCTP. The membranes were then washed several times with 0.2× SSC and 0.1% SDS at 65°C and exposed to Kodak X-Omat SX films with an intensifying screen at −80°C.

DNA Sequence Analysis

Double-stranded plasmid (tar4N1) was sequenced with Sequenase, version 2.0 (United States Biochemical), according to the instructions of the manufacturer. DNA sequence alignments were carried out using the Genetics Computer Group (University of Wisconsin, Madison, WI) package. Search for homologies between vacuolar processing proteases and typical Cys proteases of the papain family was conducted using the Multiple Alignment Construction and Analysis Workbench program (Schuler, 1994).

Southern Blot Analysis

Genomic DNA for filter hybridization was prepared as previously described (Dellaporta et al., 1983). Ten micrograms of genomic DNA was digested with *Eco*RI, *Kpn*I, or *Hin*dIII and electrophoresed on a 0.7% agarose gel, transferred overnight in 10× SSC onto Hybond-N$^+$ (Amersham), and UV cross-linked. The filter was then prehybridized and hybridized at low (55°C) or high (65°C) stringency in 7% SDS, 0.33 M phosphate buffer, pH 7.5, and 1 mM EDTA. The probe used to examine the copy number was a 280-bp *Sac*I fragment corresponding to the 5′ end of the cDNA. The filters were washed under low-stringency conditions, twice in 2× SSC, 0.1% SDS at room temperature and twice in 1× SSC, 0.1% SDS at 65°C, or under high-stringency conditions, twice in 2× SSC, 0.1% SDS at room temperature and twice in 0.1× SSC, 0.1% SDS at 65°C. Filters were wrapped in Lab Wrap (Fisher) and exposed to Kodak X-Omat AR film.

In Vitro Transcription and Translation

The transcription plasmid was linearized at the appropriate restriction site 3′ to the coding region. Linearized templates were repurified by extraction with phenol:chloroform:isoamyl alcohol (24:24:1) before in vitro transcription. The templates were transcribed in vitro using T7 polymerase. A cap analog, 7mG(5′)ppp(5′)G (Pharmacia), was included in the transcription reactions. The transcripts were translated in a rabbit reticulocyte lysate (Amersham) containing Trans-label (ICN), which is a mixture of [^{35}S]Met and [^{35}S]Cys. For experiments of import to microsomes, the translation reaction was carried out in the presence of 2 μL of dog pancreas microsomes (Promega) following the manufacturer's instructions. Proteinase K was added to the translation mixture at a final concentration of 10 mg/mL for 1 h in an ice bath at the end of the translation/import incubation. When indicated Triton X-100 was added to the reaction mixture at 1%. Deglycosylation of in vitro translated and processed protein was carried out enzymatically by endoglycosidase H treatment as described previously (DellaPenna and Bennett, 1988). The results of the translation or processing experiments were analyzed by SDS-PAGE and fluorography.

RESULTS

Sequence Analysis

The Citvac cDNA was isolated by differential screening of a cDNA library, prepared from poly(A)$^+$ mRNAs isolated from flavedo of ethylene-treated fruits, with ^{32}P-labeled cDNAs derived from untreated and ethylene-treated flavedo mRNAs. This screening identified several cDNA clones that hybridized more intensely to the cDNA probe derived from ethylene-treated flavedo than with the cDNA from untreated fruit. Citvac cDNA was selected for further characterization.

The complete nucleotide sequence of Citvac and its deduced amino acid sequence are shown in Figure 1. The deduced polypeptide is 58 and 57% identical (74.4 and 74.2% similar) with vacuolar processing Cys proteinases from soybean (Shimada et al., 1994) and from castor bean, respectively (Hara-Nishimura et al., 1993). The protein encoded by Citvac is also homologous (55% similarity, 38% identity) to a putative Cys proteinase from the human parasite *Schistosoma mansoni* (Klinkert et al., 1989). These are the only related sequences found in the SwissProt, EMBL, and DDBS data bases. A program was used for a search of alignments based on the systematic characterization of the physicochemical properties seen at each position in a sequence (Schuler, 1994), and the regions homologous to the Cys and His in the active reaction site of typical Cys proteinases papain (Cohen et al., 1986) and cathepsin (Band and Butler, 1987) were predicted and are shown highlighted in Figure 1. These regions are also highly conserved among the different sequences. As shown in the sequence alignment in Figure 2, homology among these sequences is high except in the region spanning from D^{316} to H^{389}. In the region from G^{58} to A^{106}, identity is about 90% between them.

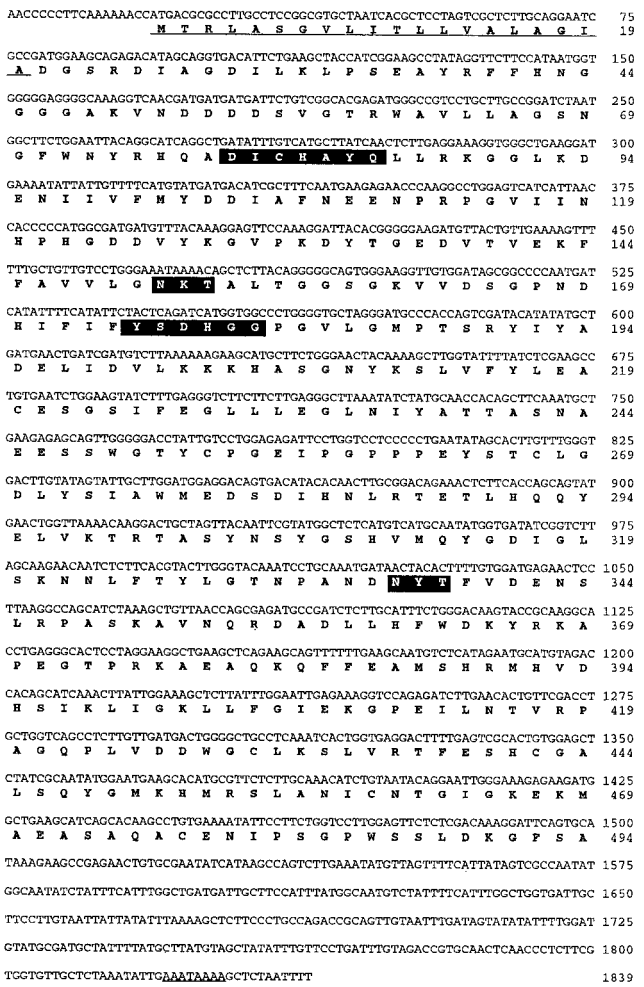

Figure 1. Nucleotide sequence and predicted protein sequence of Citvac. The putative signal peptide-coding sequence and the putative polyadenylation site (AAATAAAA) are underlined. Residues around C and H homologous to the corresponding residues in the active site of papain and cathepsins and putative glycosylation sites are highlighted.

The hydropathy plot predicts a hydrophobic N terminus with characteristics of a putative signal peptide for translocation into the ER. Transcription-translation experiments confirmed that this protein could be transported into microsomes and glycosylated in a heterologous system.

When in vitro transcripts of Citvac cDNA were used in a rabbit reticulocyte translation system, a specific polypeptide band of 53 kD was observed in SDS-polyacrylamide gels (Fig. 3) The estimated size of the band agreed well with that predicted from the cDNA (54,367 D). When the assay was conducted in the presence of a microsomal fraction, the protein became resistant to added proteases, and the protein showed slower mobility and increased in size slightly (55 kD). The deduced protein exhibits two potential glycosylation sites at positions 151 (NKT) and 336 (NYT) in the precursor molecule. To test whether the increase in size was due to glycosylation, this latter fraction was treated with endoglycosidase H. When this treatment was conducted, the molecular mass of the translation product was reduced to 51 kD. This suggests that the Citvac precursor protein is processed upon entering the microsomal fraction and is readily glycosylated, rendering the 55-kD band.

Expression of the Citvac Gene during Fruit Ripening and Flower Opening

The accumulation pattern of Citvac transcripts was determined by northern blot hybridization. The results shown in Figure 4A indicate that the Citvac mRNA is approximately 1.9 kb in size and that its levels were low in the flavedo of green fruits but accumulated concomitantly with color change occurring during ripening, reaching maximum levels in fully colored fruit.

In citrus, flower opening seems to be an ethylene-mediated process (Zacarias et al., 1991). The timing of expression of Citvac transcript during flower development was examined by northern blot analysis. As is shown in Figure 4B, the levels of Citvac transcript increased during flower development and showed highest levels in flowers at anthesis. Petals from flowers at this stage also showed high levels of expression.

Tissue Distribution of Citvac

The tissue distribution of Citvac expression was analyzed by RNA blot analysis of total RNA samples prepared from different parts of the orange tree, including leaf, stem, root, flowers at anthesis, and immature green fruits (Fig. 5). The results showed that the Citvac mRNA was found at the highest level in the flower at anthesis. It was also present in the leaf, albeit at much lower levels. Stems, roots, and developing fruits (green) have extremely low levels of this mRNA. These results indicate that high levels of Citvac expression are restricted to particular organs and only at certain stages of development.

Accumulation of Citvac in the Flavedo of Fruits and in Leaves in Response to Ethylene Treatment

Mature green fruits with very low levels of Citvac mRNA were incubated in the presence or absence of ethylene in an open-flow system for different periods. As shown in Figure 6, Citvac mRNA began to accumulate 2 h after ethylene treatment and reached very high levels after 24 h. The accumulation of Citvac mRNA was observed before any change in color induced by ethylene in the flavedo (graph in Fig. 6A) was detected. Fully developed leaves that already contained observable levels of Citvac transcript were also shown to dramatically accumulate this mRNA in response to ethylene treatment (Fig. 6B).

Gene Analysis

To estimate the number of Citvac-related genes in the *Citrus* genome, a Southern blot analysis was performed using genomic DNA from *C. sinensis* (L.) Osbeck. The DNA gel blot of *Hin*dIII-, *Pst*I-, and *Eco*RI-digested DNA samples was hybridized at high stringency with a ^{32}P-labeled probe

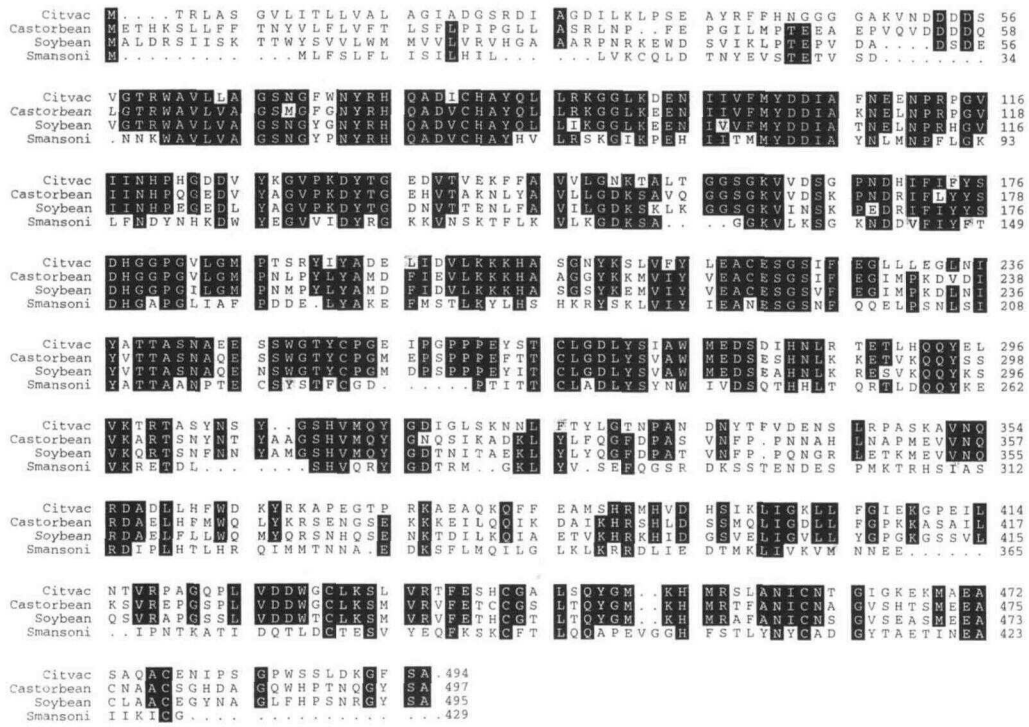

Figure 2. Sequence alignment of the predicted Citvac polypeptide with the soybean and castor bean vacuolar processing proteases, and the *S. mansoni* thiolprotease. The proteins were aligned using the PILEUP routine of the Wisconsin Genetics Computer Group software package and displayed using PRETTYBOX. Black backgrounds indicate identical amino acids, and shaded backgrounds indicate conservative substitutions.

(a 280-bp *Sac*I restriction fragment of the tar4N1 cDNA clone). As shown in Figure 7, two bands were observed in the *Pst*I and *Eco*RI digestions and one was found in the *Hin*dIII digestion, which indicate that Citvac belongs to a small gene family. When the DNA gel blot was washed at low stringency, the same bands were detected (data not shown).

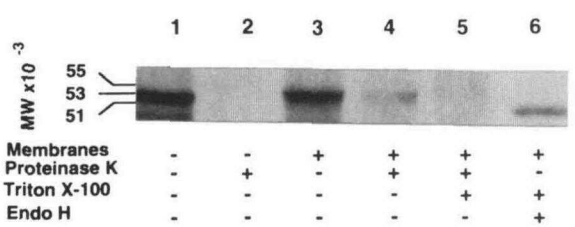

Figure 3. Translation of in vitro transcribed Citvac in a rabbit reticulocyte lysate cell-free system and effect of canine microsomal membranes and endoglycosidase H treatment on membrane-associated Citvac-processing intermediates. Translation was conducted in the absence (lanes 1 and 2) or presence (lanes 3–6) of 2 μL of dog microsomes as described in "Materials and Methods." After translation was completed, equal amounts of the reaction mixture were subjected to the following treatments: lanes 1 and 3, untreated translation products; lanes 2 and 4, treated with 5 μg of proteinase K; lane 5, treated with 5 μg of proteinase K in the presence of 1% Triton X-100; lane 6, digestion of reaction mixture in lane 3 with 10 milliunits of endoglycosidase H (Endo H) for 2 h at 37°C. Proteins were analyzed by SDS-PAGE and fluorography.

DISCUSSION

In this paper evidence is presented that a putative protease mRNA (Citvac) is induced in the flavedo tissue during fruit ripening and in the flower at the anthesis stage. Exogenous treatment of the mature green fruits with ethylene promotes degreening of the peel and also the induction of this mRNA (even before degreening is actually detected). These results suggest that ethylene is involved in both flower opening (Zacarias et al., 1991) and flavedo fruit and degreening (Goldschmidt et al., 1993) because in both cases the induction of Citvac mRNA is observed. Furthermore, it indicates that, although the orange is a nonclimacteric fruit and a sharp increase in respiration or autocatalytic ethylene production is not observed, some aspects of fruit ripening such as flavedo degreening may be regulated by ethylene (Goldschmidt et al., 1993).

Based on the homology of the putative Citvac product with the Cys proteinase from *S. mansoni* and the vacuolar-processing protease from castor bean and soybean, different roles for the putative protease can be proposed. It is interesting to note that the amino acid sequence of the Citvac differs from that of Cys proteases, which are assumed to be the main proteases involved in general protein hydrolysis (Band and Buttler, 1987) except in sequences around the catalytic domain. Furthermore, in citrus and in a number of fleshy fruits the total protein content does not decline during ripening, nor is an increase in general proteolytic activity detected (Goldschmidt, 1986). However, an

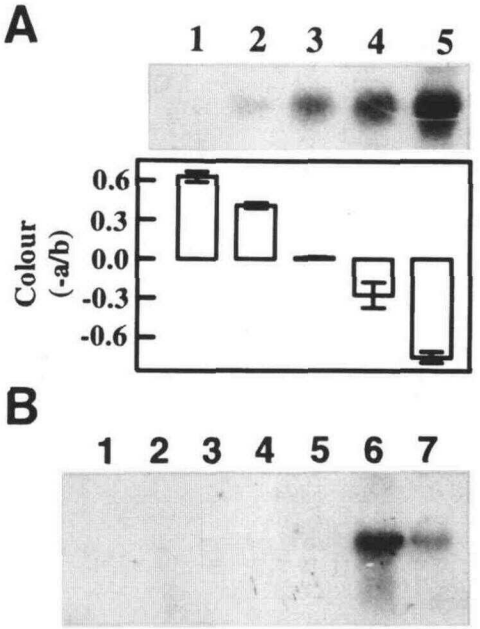

Figure 4. Expression of Citvac in different developmental processes of the citrus plant. A, Accumulation of Citvac mRNA in corresponding fruits at different developmental and ripening stages. RNA was obtained from: 6.5-cm-diameter immature fruit (lane 1), full-size green fruit (lane 2), turning fruit at stages A and B (lanes 3 and 4), and fully colored fruit (lane 5). B, Regulation of Citvac accumulation during flower development. RNA was extracted from flower buds at developmental stages corresponding to one-fifth, one-fourth, one-third, and one-half the final size and full-size fully elongated closed flower (lanes 1–5). Lane 6, Flower at anthesis; lane 7, separated petals.

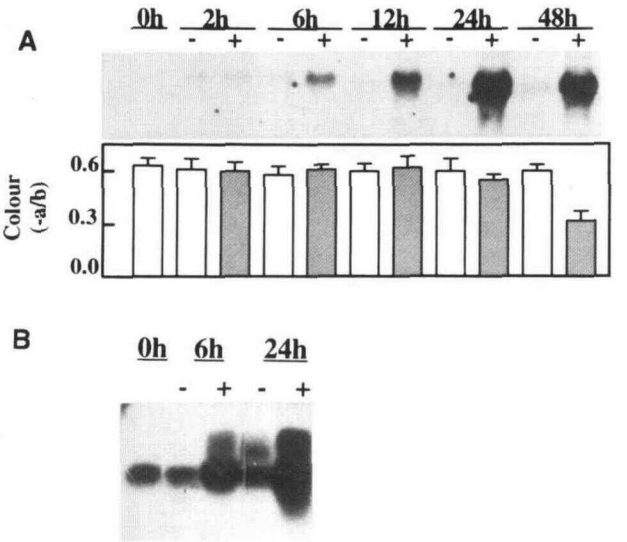

Figure 6. RNA blot analysis of RNA isolated from flavedo of full-size green fruits (A) or leaves (B) exposed to air or to 10 μL/L ethylene for the indicated durations. Full-size green fruits and leaves were harvested and incubated in an open-flow system either in a hydrocarbon-free or with 10 μL/L ethylene as described by Alonso et al. (1992). Twenty micrograms of total RNA isolated from the different samples was separated electrophoretically, blotted to nylon membranes, and hybridized to ^{32}P-labeled cDNA clones. Bar graph, Effect of ethylene treatment in flavedo degreening as represented by the Hunter color (see "Materials and Methods").

almost complete loss of the PSI and PSII light-harvesting complex proteins is observed in citrus and tomato and other fruits, which is similar to that observed during leaf senescence. In *Citrus* flavedo (and also in other fruits) a shift in the developmental program occurs during ripening in which a specific set of proteins are no longer produced and are degraded, while a new set of proteins and structures are synthesized de novo (Thompson and Whatley, 1980; Alonso et al., 1992). It is very unlikely that the protease encoded by Citvac is involved in the protein turnover occurring during the differentiation of chloroplasts to chromoplasts, since the Citvac protease is probably targeted to the vacuole and since the integrity of the plastid membrane remains preserved (Spurr and Harris, 1968) .

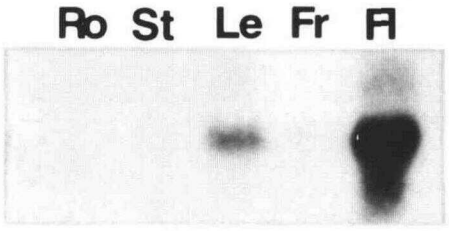

Figure 5. Accumulation of Citvac mRNA in different tissues and organs of *Citrus*. Twenty micrograms of total RNA was isolated from root (Ro), stem (St), leaf (Le), green fruit (Fr), and flower at anthesis stage (Fl), separated electrophoretically, blotted to nylon membranes, and hybridized to the ^{32}P-labeled Citvac cDNA clone.

Based on its homology to the soybean and castor bean vacuolar processing proteases, the function of the Citvac protease is more likely to process vacuolar-targeted proteins. So far, these proteases have been described mainly in maturing seeds, where they convert proproteins to their final form in the storage vacuole (Harley and Lord, 1985; Hara-Nishimura and Nishimura, 1987; Scott et al., 1992). Recent studies have shown that low levels of vacuolar processing activity can be detected in nonstorage organs, such as roots and mature leaves (Hiraiwa et al., 1993). The low level of vacuolar processing activity may be an indication of the low level of protein traffic to the vacuole in most tissues compared to that observed in seeds. However, it is known that in response to pathogen attack and ethylene plants activate the transcription of a number of genes whose protein products accumulate preferentially in the vacuole or in the extracellular space (Mauch and Staehelin, 1989). Proteins targeted to these compartments are synthesized as precursors with a signal peptide that is removed by a signal peptidase when entering into the ER. Many vacuolar proteins contain terminal extensions that are processed by vacuolar processing proteases. Ethylene treatment of *Citrus* fruits induces a number of cDNAs that encode proteins with amino-terminal sequences with characteristics typical of a signal peptide for targeting to the secretory pathway (A. Granell, unpublished results). So far, no vacuolar processing protease has been isolated from organs different from seeds. The amino acid sequence de-

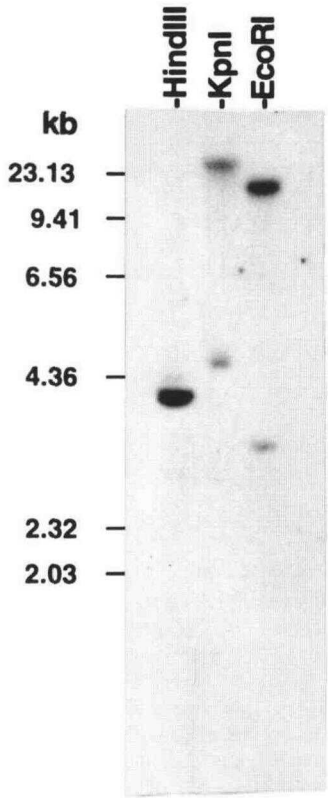

Figure 7. Representation of Citvac gene in the *Citrus* genome. Genomic DNA was isolated from citrus leaves (Dellaporta et al., 1983); 10 μg were digested with *Hin*dIII, *Kpn*I, or *Eco*RI restriction enzymes and hybridized with ^{32}P-labeled Citvac plasmid DNA, followed by autoradiography. Sizes of λ-DNA standards are expressed in kb.

duced for Citvac contains a hydrophobic N-terminal signal peptide typical of proteins targeted to the ER, and the Citvac precursor protein is able to enter into microsomes in an in vitro assay and in doing so is possibly proteolytically processed and glycosylated.

We propose that the Citvac protease is involved in processing of proteins targeted to vacuoles that accumulate during ethylene-regulated processes (namely flower opening and flavedo degreening in *Citrus*). Plants may have adapted a gene encoding a vacuolar processing protease that is regulated by ethylene when an increase in protein traffic to the vacuole exists. Since no major products are found to accumulate in the flavedo of the *Citrus* fruit during maturation or after ethylene treatment (Alonso et al., 1992), it is likely that this protease acts on a range of vacuolar-targeted proteins. The detection of only one or at most two genes of Citvac in the *Citrus* genome gives support to this idea. In this respect it has been reported that the castor bean processing enzyme is capable of converting several proprotein precursors into their respective mature forms (Hara-Nishimura et al., 1991). To define the activity, substrate specificity of this Citvac protease will help to clarify its role during ripening and other ethylene-mediated processes in *Citrus*.

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LITERATURE CITED

Alonso JM, Chamarro J, Granell A (1995) Evidence for the involvement of ethylene in the expression of specific RNAs during maturation of the orange, a non climacteric fruit. Plant Mol Biol (in press)

Alonso JM, García-Martinez JL, Chamarro J (1992) Two dimensional gel electrophoresis patterns of total, in vivo labeled and in vitro translated polypeptides from orange flavedo during maturation and following ethylene treatment. Physiol Plant **85:** 147–156

Baldwin EA, Biggs RH (1983) Ethylene biosynthesis of citrus peel explants as influenced by cycloheximide. Proc FL State Hortic Soc **96:** 183–185

Baldwin EA, Biggs RH (1988) Cell-wall lysing enzymes and products of cell-wall digestion elicit ethylene in citrus. Physiol Plant **73:** 58–64

Band JJ, Butler DE (1987) Intracellular proteinases. Annu Rev Biochem **56:** 333–364

Burns JK, Baldwin EA (1994) Glycosidase activities in grape fruit flavedo, albedo and juice vesicles during maturation and senescence. Physiol Plant **90:** 37–44

Cohen LW, Coghlan UM, Dihel LC (1986) Cloning and sequencing of papain-encoding cDNA. Gene **48:** 219–227

DellaPenna D, Bennett AB (1988) In vitro synthesis and processing of tomato fruit polygalacturonase. Plant Physiol **86:** 1057–1063

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA miniprep: version II. Plant Mol Biol Rep **1:** 19–21

Goldschmidt EE (1986) Maturation, ripening, senescence, and their control: a comparison between fruit and leaves. *In* SP Monselise, ed, Handbook of Fruit Set and Development. CRC Press, Boca Raton, FL, pp 483–491

Goldschmidt EE, Huberman M, Goren R (1993) Probing the role of endogenous ethylene in the degreening of citrus fruits with ethylene antagonists. Plant Growth Regul **12:** 325–329

Hara-Nishimura I, Inoue K, Nishimura M (1991) A unique vacuolar processing enzyme responsible for conversion of several proprotein precursors into the mature forms. FEBS Lett **294:** 89–93

Hara-Nishimura I, Nishimura M (1987) Proglobulin processing enzyme in vacuoles isolated from developing pumpkin cotyledons. Plant Physiol **85:** 440–445

Hara-Nishimura I, Takeuchi Y, Nishimura M (1993) Molecular characterization of a vacuolar processing enzyme related to a putative cysteine proteinase of *Schitosoma mansoni*. Plant Cell **5:** 1651–1659

Harley SM, Lord JM (1985) In vitro endoproteolytic cleavage of castor bean lectin precursors. Plant Sci **41:** 111–116

Hiraiwa N, Takeuchi Y, Nishimura M, Hara-Nishimura I (1993) A vacuolar processing enzyme in maturing and germinating seeds: its distribution and associated changes during development. Plant Cell Physiol **34:** 1197–1204

Jones JDG, Dunsmuir P, Bedbrook J (1985) High levels of expression of introduced chimeric genes in regenerated transformed plants. EMBO J **4:** 2411–2414

Klinkert M-Q, Felleisen R, Link G, Ruppel A, Beck, E (1989) Primary structure of Sm31/32 diagnostic proteins of *Schistosoma mansoni* and their identification of proteases. Mol Biochem Parasitol **33:** 113–122

Mauch F, Staehelin LA (1989) Functional implications of the subcellular localization of ethylene-induced chitinase and β-1,3-glucanase in bean leaves. Plant Cell **1:** 447–457

Rasmussen GK (1975) Cellulase activity, endogenous abscisic acid, and ethylene in four citrus cultivars during maturation. Plant Physiol **56:** 765–767

Riov J, Monselise SP, Kahan RS (1969) Ethylene-controlled induction of phenylalanine ammonialyase in citrus fruit peel. Plant Physiol **44:** 631–635

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Vols 1–3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schuler G (1994) MACAW: Multiple Alignment Construction and Analysis Workbench Version 2.0.2. National Center of Biotechnology Information, Bethesda, MD

Scott MP, Jung R, Müntz K, Nielsen NC (1992) A protease responsible for post-translational cleavage of a conserved Asn-Gly linkage in glycinin, the major seed storage protein of soybean. Proc Natl Acad Sci USA **89:** 658–662

Shimada T, Hiraiwa N, Nishimura M, Hara-Nishimura I (1994) Vacuolar processing enzyme of soybean that converts proproteins to the corresponding mature forms. Plant Cell Physiol **35:** 713–718

Shimokawa K, Sakanoshita A, Horiba K (1978) Ethylene-induced changes of chloroplast structure in Satsuma mandarin (*Citrus unshiu* Mark). Plant Cell Physiol **19:** 229–236

Spurr AR, Harris WM (1968) Ultrastructure of chloroplasts and chromoplasts in *Capsicum anum*. I. Thylakoid membrane changes during fruit ripening. Am J Bot **55:** 1210–1224

Stewart I, Wheaton TA (1972) Carotenoid in citrus: their accumulation induced by ethylene. J Agric Food Chem **20:** 448–449

Thompson WW, Whatley JM (1980) Development of nongreen plastids. Annu Rev Plant Physiol **31:** 375–394

Zacarias L, Tudela D, Primo-Millo E (1991) Role of the ethylene in the opening and senescence of citrus flowers. Sci Hortic **46:** 55–60