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Characterization and localization of a wound-inducible type I serine-carboxypeptidase from leaves of tomato plants (*Lycopersicon esculentum* Mill.)

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Abstract. During the course of characterization of the wound-response related proteins from tomato (*Lycopersicon esculentum* Mill.) leaves, a serine carboxypeptidase (EC 3.4.16.1) was identified. An increase in peptidase activity in response to wounding, and the isolation of a protein with carboxypeptidase (CP) activity from tomato leaves had been reported previously, but the mRNA coding for the enzyme was not identified. We now report the isolation of a tomato leaf type I serine-CP cDNA whose corresponding mRNA is induced by wounding, systemin and methyl jasmonate. The protein sequence deduced from the cDNA exhibits homology to tomato CP, and barley and rice type I CPs. Southern blot results indicated that the CP gene is probably a member of a small gene family. Tomato CP mRNA was detected within 3 h after wounding, or treatment with systemin or methyl jasmonate. Employing Western blot analysis, CP protein was shown to increase 12 h after the treatments. Using the tomato *def1* mutant, we have demonstrated that a functional octadecanoid pathway is necessary for CP transcription in response to wounding. Carboxypeptidase protein was immunolocalized as protein aggregates within the central vacuoles of palisade mesophyll cells as well as in vascular parenchyma where it had previously been found. Double labeling using antibodies specific for CP and inhibitor II indicated that the two proteins are co-localized in the vacuolar aggregates. Tomato CP is a member of the "late wound-inducible genes" whose mRNAs increase 4–12 h following wounding, in contrast to several "early wound-inducible genes", whose mRNAs appear within 30 min. The data support a role for the enzyme in protein turnover that occurs systemically in leaf cells in response to wounding.

Key words: *Lycopersicon* (wound proteins) – Methyl jasmonate – Plant defense proteins – Systemin (induced) – Serine-carboxypeptidase – Wound-inducible late genes

Introduction

Among the different strategies that tomato plants have developed to defend against polyphagous insects is the local and systemic accumulation of defense genes, including proteinases and proteinase inhibitors (Green and Ryan 1972; Karban and Baldwin 1997). The systemic activation of defense genes in response to insect attacks or to mechanical wounding has been shown in tomato plants to be mediated by the 18-amino-acid polypeptide hormone called systemin (Pearce et al. 1991).

Systemin is derived from a larger 200-amino-acid precursor, prosystemin. Tomato plants constitutively overexpressing a prosystemin transgene exhibited an abnormal phenotype that behaved as if it was in a permanent wounded state (McGurl et al. 1994). The transgenic plants overexpressed not only the prosystemin gene, but also an array of wound-related genes that resulted in a distinct protein profile when their leaf protein extracts were separated by SDS-PAGE (Bergey et al. 1996).

We report herein that among the proteins induced in leaves of plants overexpressing the prosystemin transgene and in leaves of wild-type wounded plants is an 18-kDa polypeptide with high amino acid sequence identity to the β chain of barley carboxypeptidase (CPS; Doan and Fincher 1988) and rice CP (Washio and Ishikawa 1994), a two-chain exopeptidase that plays a role in cereal seed germination. We report that the tomato CP mRNA is wound-inducible and is identical to a previously reported CP protein isolated from wounded tomato leaves (Walker-Simmons and Ryan 1980), and exhibits homology to a stress related carboxypeptidase isolated from tomato leaves (Mehta et al. 1996). The

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Abbreviations: CP = carboxypeptidase; PCR = polymerase chain reaction

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tomato leaf CP gene transcripts are also shown to increase in response to systemin and methyl jasmonate, and this pattern is similar to that shown for protease inhibitor genes. Like proteinase inhibitor proteins (Shumway et al. 1976), the CP protein accumulates in vacuoles of tomato leaf mesophyll cells and vascular parenchyma (Mehta et al. 1996) in response to wounding.

Materials and methods

Plant materials and treatments

Wild-type tomato (*Lycopersicon esculentum* Mill. cv. Castlemart) plants, transgenic tomato plants (McGurl et al. 1994), and *def1* mutant tomato plants (Howe et al. 1996) were employed. Plants were grown in a growth chamber with 17-h days of 300 μmol m^{-2} s^{-1} of light at 28 °C and 7-h nights at 18 °C. Two-week-old tomato plants were used in all of the experiments. Wounding was achieved by crushing the lower leaf perpendicular to the midvein using a hemostat. Systemin was supplied to excised plants through their cut stems at a concentration of 28 nM as described in Howe et al. (1996). Plants were exposed to methyl jasmonate vapors as previously described (Bergey and Ryan 1999). Plants, including the untreated control plants, were maintained in constant light (300 μmol m^{-2} s^{-1}) during the entire duration of the time course of all experiments. All the experiments were repeated at least twice.

Protein purification and N-terminal sequence

The general method used to study differential protein expression in transgenic tomato plants overexpressing prosystemin is as follows: frozen leaves were macerated with buffer [50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 100 mM KCl, 0.1% β-mercaptoethanol, 1% polyvinylpyrrolidone] in a blender. After passing through four layers of cheesecloth and centrifuging (7,000 *g*, 4 °C, 25 min), two ammonium sulfate precipitations were performed. The first precipitation was at 58% saturation, pH 7.0 (stirring 30 min, 4 °C), and the second, at 50% saturation, pH 5.0 (stirring overnight, 4 °C). After addition of appropriate amounts of ammonium sulfate, the precipitates were collected after centrifugation (7,000 *g*, 4 °C, 25 min) and dissolved in 50 mM Tris-HCl (pH 7.8), 5 mM EDTA and 0.1% β-mercaptoethanol. The solution was dialyzed against the same buffer. After dialysis, the ammonium sulfate precipitated fractions were separated on a DEAE-cellulose (Sigma) column using a salt gradient from 0 to 600 mM NaCl. The eluted fractions were analyzed by gradient (10–15%) SDS-PAGE. Comparisons were made between fractions from transgenic and untreated control plants, and fractions containing proteins that were overexpressed in extracts of the transgenic plants were further blotted to polyvinylidenedifluoride (PVDF) membranes. Overexpressed proteins were sequenced at their N-termini using the Edman degradation procedure at the sequencing laboratory at Washington State University.

Isolation and sequencing of cDNA clones

The degenerate primer, 5′GGA/GGTICCITGT/CACIGAT/CG3′ (I = deoxyinosine), was based on the N-terminal protein sequence obtained and designed for use in combination with the vector-based primer T7 (5′TAATACGACTCACTATAGGG3′; UniZap XR; Stratagene) at the 3′ end of the cDNA library inserts. The library was prepared using poly(A)$^+$mRNA extracted from transgenic tomato plants that constitutively overexpressed the prosystemin transgene (McGurl et al. 1994). The same library was used as template to generate cDNA fragments using standard polymerase chain reaction (PCR) methods. An amplified cDNA fragment containing the N-terminal protein sequence obtained was used to screen the library. The screening procedure was performed according to the manufacturer's instructions (Stratagene). Probes were radiolabeled using the random priming method according to the manufacturer's instructions (Pharmacia). After the secondary screening, individual positive clones were excised and sequenced using the Big-Dye terminator sequencing reaction and the Perkin-Elmer ABI-PRISM 377 sequencer. The sequence was determined at the sequencing laboratory of Washington State University.

Southern blots

Genomic DNA from tomato leaves was extracted using DNAzol reagent according to the manufacturer's instructions (Life Technologies). Five micrograms of DNA was digested using the restriction enzymes *Nde*I and *Dra*I and following the manufacturer's instructions (Promega). Probes were prepared as described for cDNA library screening. Digested DNA was separated in 0.8% agarose gels with 1 × TBE buffer. DNA fragments were transferred to nylon membranes using 10 × SSC for 18 h. After transfer, the membranes were washed with 2 × SSC, cross-linked with UV light and dried in the oven for 30 min. Prewet membranes were hybridized in 10 mL hybridization solution [1% SDS, 10% dextran sulphate (MW 500,000), 5.3% NaCl, 5 × Denhardt's and 0.05 mg/mL of sheared salmon sperm DNA], using a rotatory hybridization incubator (Robbins Scientific) adjusted for 65 °C, for 18 h. Membranes were washed twice with 2 × SSC for 30 min and 1 × SSC for 15 min. All the washes were performed at 65 °C. Membranes were exposed to Kodak X-Omat film.

Northern blots

Total RNA was extracted from tomato leaves using the TRIzol reagent following the manufacturer's instructions (Life Technologies). Fifteen micrograms of total RNA was fractionated in a 1.5% agarose gel containing 0.66 M formaldehyde and 1 × Mops buffer. The RNA was blotted to nylon membranes using the salt-transfer method (10 × SSPE) for 18 h. Membranes were hybridized in 10 mL of a solution containing 5 × SSPE, 5 × Denhardt's, 1% SDS and 10% dextran sulphate (MW 500,000). Unless stated otherwise the molecular biology procedures and solutions used here were as described in Sambrook et al. (1989).

Western blots

Protein extracts were obtained through maceration of the tomato leaves in liquid nitrogen followed by centrifugation of the macerate at 12,000 *g*, 4 °C, for 20 min. Equal amounts of the supernatant were separated by SDS-PAGE (12% polyacrylamide). Proteins were then electrotransferred to PVDF membranes. After treating the membranes with Na-periodate (1%, 30 min), they were treated for 30 min using a solution of 5% dry milk in TTBS [20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 0.05% Tween 20]. Membranes were incubated with the CP antibody (1:5,000 dilution) in TTBS containing 2% dry milk, at 4 °C overnight with gentle agitation. They were rinsed three times with TTBS and incubated with the secondary antibody (BioRad goat anti-rabbit alkaline phosphatase conjugate, 1:3,000 dilution) for 4 h at room temperature. Western blots were washed three times in TBS and developed using BCIP/NBT color development solution according to the manufacturer's instructions (BioRad). Carboxypeptidase antibody was obtained previously by injecting diisopropyl fluorophosphate (DFP)-treated CP into rabbits (Walker-Simmons and Ryan 1980; Walker-Simmons and Ryan, unpublished data).

Immunolocalization

Small pieces (1 mm × 4 mm) of unwounded leaves from wounded tomato plants were fixed overnight in 1.25% glutaraldehyde, 2% paraformaldehyde and 50 mM Pipes buffer (pH 7.2). After washing with 50 mM Pipes buffer, the leaf material was dehydrated using an ethanol step gradient (30, 50, 60, 70, 80, 95 and 100%, 10 min each). Dehydrated tissue was embedded in LR White resin for 12 h (1:3, 1:2, 1:1, 3:1, 0:1 ratio resin:ethanol). Embedded samples were transferred to gelatin capsules and polymerized overnight in an oven (65 °C). Thin (0.5 μm) and ultrathin (0.1 μm) sections were obtained using glass knives and were mounted on glass slides and Formvar-coated nickel grids. Mounted samples were incubated in TBST [10 mM Tris-HCl (pH 7.2), 500 mM NaCl, 0.1% Tween 20 and 1% BSA] for 1 h. After washing the samples with TBST (three times, 20 min), they were incubated for 4 h at room temperature with the appropriate serum or combination of sera: rabbit preimmune, rabbit anti-tomato CP, goat preimmune or goat anti-tomato inhibitor II, all of them used at 1:50 dilution in TBST. The samples were washed again and incubated with goat anti-rabbit 20-nm conjugated gold particles or rabbit anti-goat 5-nm conjugated gold particles, at a 1:50 dilution in TBST for 1 h at room temperature. Double labeling was performed by incubating ultrathin sections with two sera at the same time. After washing, thin sections were silver–enhanced and post-stained with 1% aqueous safranin. Ultrathin sections were post-stained with a freshly prepared 1:1 solution of 1% $KMnO_4$ and 2% uranyl acetate.

Results

Protein purification and N-terminal sequence analysis

An 18-kDa polypeptide was identified by SDS-PAGE in crude extracts from leaves of transgenic tomato plants constitutively overexpressing the prosystemin transgene (Bergey et al. 1996) and purified. N-terminal sequence analysis of the protein resulted in the identification of 11 amino acids, SVEVPCTDDRV. This sequence was used to design a degenerate 33-nucleotide primer (see *Materials and methods*) for use in PCR to obtain a longer fragment of the tomato leaf cDNA library to use in Northern blotting experiments.

Isolation and characterization of cDNA

A 680-bp cDNA fragment was amplified by PCR from a cDNA library prepared from leaves of the transgenic plants constitutively expressing the prosystemin transgene, and was used to screen the cDNA library. Of several cDNAs isolated from the library, the largest clone, 1731 bp, was sequenced through both strands using nested primers. The clone contains an open reading frame that codes for a 498-amino-acid protein exhibiting both a start and a stop codon (Fig. 1). According to a search of the available databases and based on the sequence homology, the deduced protein belongs to the serine-CP family. The tomato cDNA clone isolated codes for both the α and β chains that comprise the native enzyme (Doan and Fincher 1988). The deduced protein has an N-terminal hydrophobic region typical of a signal peptide, residues 1–27 (double underline in Fig. 1), flanked by charged amino acids. Five potential glycosylation sites were identified in the

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taccaaaacATGGCAAAAAAAAAACTTTGTTTTACACTATAATCTACTGTTAATTTCTTTGT 60
        M  A  K  K  N  F  V  L  H  Y  N  L  L  L  I  S  L  L  -

TGGCATTTTTGTTTTTGTTAACAGAAGGGGCACCTCAGAGTGCACTGGTAACTCAACTAC 120
  A  F  L  F  L  L  T  E  G  A  P  Q  S  A  L  V  T  Q  L  P  -
                             ↑
CTGGCTTCAATGGCACTTTTAACTCAAAACATTATGCTGGGTATGTTAATATAGATGAAA 180
  G  F  N  G  T  F  N  S  K  H  Y  A  G  Y  V  N  I  D  E  S  -

GTCATGGGAAAAATTTGTATTACTACTTTGTTGAATCTGAAAGAAATCCATCAAAGGATC 240
  H  G  K  N  L  Y  Y  Y  F  V  E  S  E  R  N  P  S  K  D  P  -

CAGTTGTTCTTTGGCTTAATGGTGGACCTGGTTGTTCAAGCTTTGATGGATTTGTCTATG 300
  V  V  L  W  L  N  G  G  P  G  C  S  S  F  D  G  F  V  Y  E  -

AGCATGGGCCTTTTAATTTTGATTTTGGAAAAGCCAAGTGGTAGCCTGCCTTCTCTGCACA 360
  H  G  P  F  N  F  D  F  G  K  P  S  G  S  L  P  S  L  H  N  -

ACAATCCATACAGTTGGTCTAAGGTTTCCAATATAATATATCTGGACTCTCCTGTTGGTG 420
  N  P  Y  S  W  S  K  V  S  N  I  I  Y  L  D  S  P  V  G  V  -

TTGGATTATCTTACTCGGGAAATAAATCTGACTATAATACAGGAGACTTAAAGACTGCAT 480
  G  L  S  Y  S  G  N  K  S  D  Y  N  T  G  D  L  K  T  A  S  -

CTGATAGCCACTCATTTCTCCTCAAGTGGTTTGAGATCTACCCGGAATTCCTCAAGAACC 540
  D  S  H  S  F  L  L  K  W  F  E  I  Y  P  E  F  L  K  N  P  -

CATTTTACATATCTGGAGAGTCTTATGCTGGAAATATACGTNCCAACTCTGGCTTCTGAAG 600
  F  Y  I  S  G  E  S  Y  A  G  I  Y  V  P  T  L  A  S  E  V  -

TAATAAAAGGTATTGATGCTGGAGTAAGGCCAGCTATCAACTTTATGGGTTACATGGTGG 660
  I  K  G  I  D  A  G  V  R  P  A  I  N  F  M  G  Y  M  V  G  -

GAAATGGCGTGGCAGATGATATAATAGACGGTAATGCTATTGTTCCATTTCAACATGGTA 720
  N  G  V  A  D  D  I  I  D  G  N  A  I  V  P  F  Q  H  G  M  -

TGGGCCTCATTTCAGATGACTTATATGAAGAAGCTGTTGTTGCATGCCATGGAAATTTCT 780
  G  L  I  S  D  D  L  Y  E  E  A  V  V  A  C  H  G  N  F  Y  -

ACGAACCAGTGGATAGTAATTGTTCAGAAAAGCTTAATAAAATTGATCAGGTAGTTTATG 840
  E  P  V  D  S  N  C  S  E  K  L  N  K  I  D  Q  V  V  Y  D  -

ATCTAAACGTTTATGACATTCTAGAACCATGTTACCACAGCAAAAAACCTAGTGTAATTA 900
  L  N  V  Y  D  I  L  E  P  C  Y  H  S  K  K  P  S  V  I  T  -

CCACTGGTAACTCAAGATTGCCTATGAGCTTTCGTAAACTAGGTGAGACAGAAAGGCCTC 960
  T  G  N  S  R  L  P  M  S  F  R  K  L  G  E  T  E  R  P  L  -

TTCCTGTCAGAAAAAGAATGTTTGGCCGTGCATGGCCTTATAAAGCCCCAGTAAGAGCGG 1020
  P  V  R  K  R  M  F  G  R  A  W  P  Y  K  A  P  V  R  A  G  -

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GTGTTGCTACACTGTGGCTCAACAATGCAGATGTTCGTAAAGCTATTCATGCTGAACCAG 1140
  V  A  T  L  W  L  N  N  A  D  V  R  K  A  I  H  A  E  P  A  -

CAACTGTGATAGGTCCCTGGGAGCTATGCACAGACAAAATAGATCTGGATCATGATTCTG 1200
  T  V  I  G  P  W  E  L  C  T  D  K  I  D  L  D  H  D  S  G  -

GAAGCATGATTCCATATCACAAAAATCTCACAGCTCGGGGCTATCGGGCAATCATATTCA 1260
  S  M  I  P  Y  H  K  N  L  T  A  R  G  Y  R  A  I  I  F  S  -

GTGGAGATCACGATATGTGTGTTCCATTCACTGGTTCAGCAGTATGGACAAAATCTCTTG 1320
  G  D  H  D  M  C  V  P  F  T  G  S  A  V  W  T  K  S  L  G  -

GATATCCGATCGTGGATGAATGGAGGCCTTGGTATGTGAATGACCAAGTTGCAGGTTTCA 1380
  Y  P  I  V  D  E  W  R  P  W  Y  V  N  D  Q  V  A  G  F  I  -

TACAAGGTTATGCCAACAACCTCATTTTTATGACTATTAAGGGAGCTGGACATACCGTGC 1440
  Q  G  Y  A  N  N  L  I  F  M  T  I  K  G  A  G  H  T  V  P  -

CAGAATACAAACCACGAGAGGCGTTGGCGTTCTACTCTCGCTGGTTAGAGGGCAAGAAGA 1500
  E  Y  K  P  R  E  A  L  A  F  Y  S  R  W  L  E  G  K  K  I  -

tatgataatatgaagattgaattggaagagtggctagagggcaacaataatataaatgaa 1560
taatgaagatgaattagaagacttaagtccaattatttcagagatttttctatgataata 1620
ttggatttttatcatcaatttctgtctgcaataagaaattagtaattgtatgttatataa 1680
tgtacctttgactgttaaggcatgcatgctcatattaattatttcactcc           1731
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Fig. 1. Tomato wound-inducible serine-CP cDNA and protein sequence. The putative signal sequenced is *double underlined*. The *arrow* indicates the likely beginning of the mature protein. Potential N-glycosylation sites are *underlined*. The *dotted underline* indicates the putative linker peptide. The eleven amino acids sequenced are *boxed*

sequence (underlined in Fig. 1). The 11 amino acids that were used to design the degenerate primer are at positions 349–359 (boxed amino acids in Fig. 1). The native enzyme apparently results from the excision of an internal peptide during enzyme maturation (dotted underline in Fig. 1). The tomato CP exhibits homology to the N-terminus of a tomato CP protein previously isolated from tomato leaves (Mehta et al. 1996). The protein has extensive identity with barley and rice CPs, with 73% and 71% amino acid similarity and 65% and 64% identity, respectively (Fig. 2). In barley, three disulfide bonds are present which are likely present in

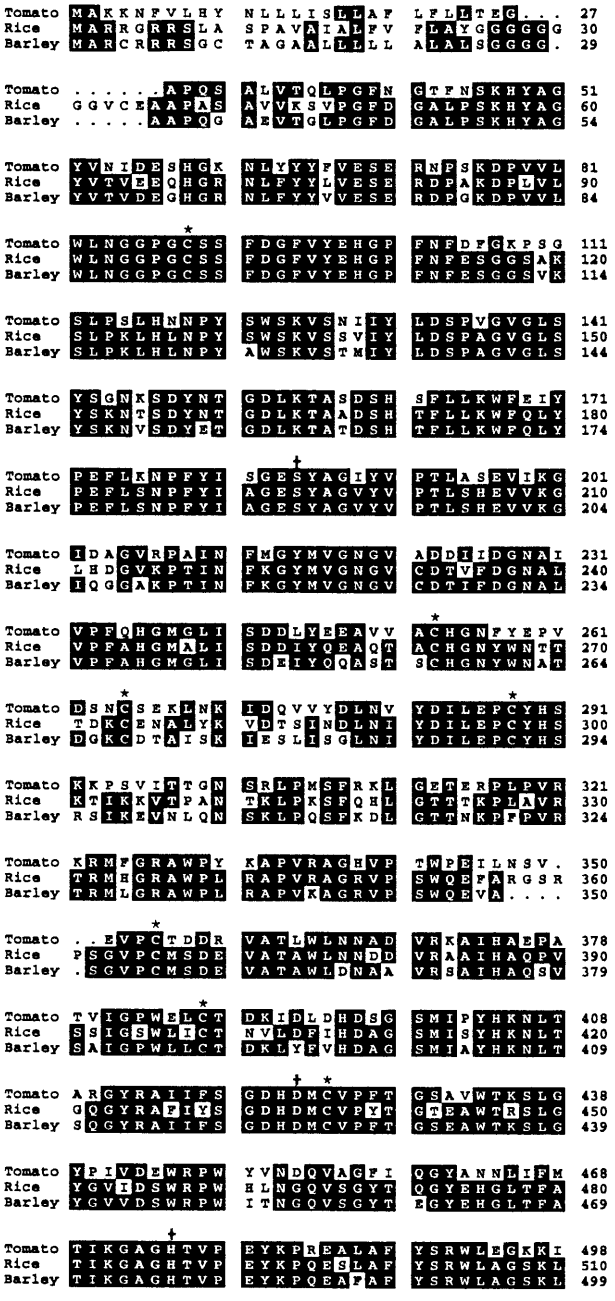

Fig. 2. Alignment of tomato (gb.ac#AF242849), rice (gb.ac#584892) and barley (gb.ac#2815493) serine-CP predicted proteins. *Black boxes* indicate identical amino acids. *Asterisks* indicate conserved cysteines. *Plus signs* indicate active-site residues

the tomato homologue, which has conserved cysteines (asterisks in Fig. 2). The tomato CP active-site residues, deduced from the barley (Dal Degan et al. 1994) and rice (Washio and Ishikawa 1994) enzymes, are serine 185, aspartate 422 and histidine 475 (plus signs in Fig. 2).

Southern blot analysis of CP

Southern blot analysis of tomato genomic DNA revealed that the CP cDNA hybridizes with five fragments

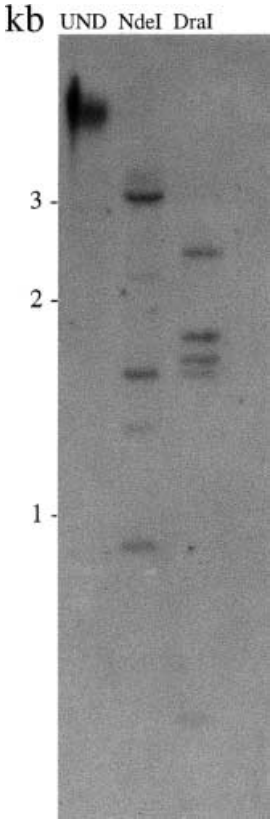

Fig. 3. Southern blot analysis of tomato genomic DNA. Five micrograms of genomic DNA was digested with the enzymes indicated. *UND*, undigested genomic DNA. *Numbers on the left*, molecular weight in kilobases

of different molecular weights, using the restriction enzymes *Nde*I and *Dra*I (Fig. 3). Undigested DNA shows only one high-molecular-weight hybridization band.

Northern blot analysis of CP

Employing the 1,731-bp fragment to evaluate the inducibility of the CP gene in response to wounding (Fig. 4A) and systemin (Fig. 4B), CP mRNA was detected within 3 h in upper unwounded leaves of plants that were mechanically wounded on the lower leaf with a hemostat. The mRNA continually increased through the 12 h of assay. Supplying systemin to young tomato plants induced CP mRNA within 3 h after initiating the treatment, with kinetics similar to wounding. Intact, untreated plants exhibited a low constitutive level of CP mRNA, and when excised and supplied with water they exhibited slightly elevated mRNA levels (Fig. 4C). Exposure of tomato plants to methyl jasmonate vapors also caused an increase in CP mRNA by 3 h and the levels remained elevated until the end of the experiment (Fig. 5A). Evaluation of CP mRNA in a tomato mutant (*def1*) that is deficient in a wound signaling pathway component, showed a weak increase in signal for CP mRNA in response to wounding (Fig. 5B,C). *Def1* plants treated with methyl jasmonate showed induction

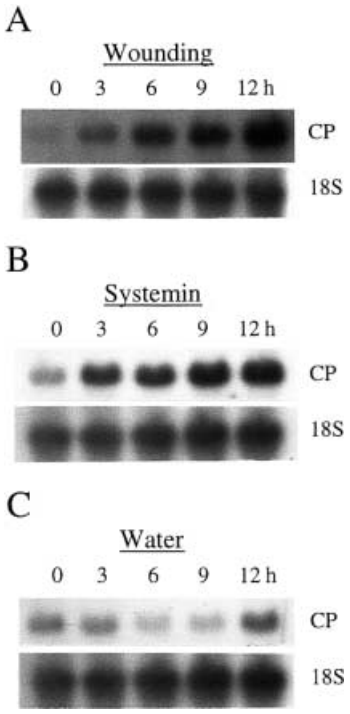

Fig. 4A–C. Northern blot analysis of a time course of tomato wound-inducible CP in tomato leaves. **A** Unwounded leaves of wounded plants. **B** Excised plants supplied with systemin peptide. **C** Control plants supplied with water only. *CP*, carboxypeptidase probe; *18S*, ribosomal RNA probe used as loading control

similar to wild-type plants when treated similarly (Fig. 5D).

Western blot analysis of CP

The CP antibody prepared against CP purified from tomato leaves cross-reacted in Western blotting experiments with a 37-kDa protein (Walker-Simmons and Ryan 1980; Mehta et al. 1996) and with two other proteins of approximately 15 kDa and 18 kDa that are present in extracts of leaves of wounded tomato plants. Time courses of CP accumulation in leaves of wounded plants and in leaves of plants supplied with systemin are shown in Fig. 6A,B. Plants supplied with water alone showed a low level of CP protein accumulation, but only after 24 h (Fig. 6C). Leaves of methyl jasmonate-treated plants also showed induction and accumulation of CP protein (Fig. 7A). Untreated plants as well as *def1* plants showed either very low levels or no CP protein (Fig. 7B and C, respectively), but, as expected, *def1* plants exposed to methyl jasmonate vapors exhibited CP protein induction, clearly shown after 24 h (Fig. 7D).

Immunolocalization of CP

Using thin sections from upper unwounded leaves of plants wounded 24 h earlier on the lower leaves, light microscopy revealed an increase in tomato CP protein,

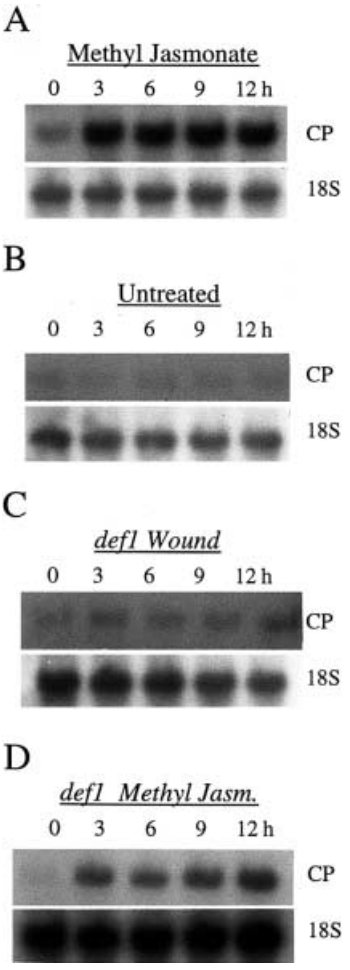

Fig. 5A–D. Northern blot analysis of a time course of tomato wound-inducible CP in tomato leaves. **A** Tomato plants exposed to methyl jasmonate vapors. **B** Untreated control plants. **C** Unwounded leaves of wounded *def1* mutants. **D** *def1* mutants exposed to methyl jasmonate vapors. *CP*, carboxypeptidase probe; *18S*, ribosomal RNA probe used as loading control

localized in the vacuoles of palisade mesophyll cells (Fig. 8B and inset in Fig. 8B). Ultra-thin sections revealed that the label was concentrated in membraneless vacuolar aggregates (Fig. 8C–E), previously associated with proteinase inhibitor accumulation (Shumway et al. 1976). Leaves of untreated tomato plants exhibited a low level of CP protein in vacuoles (Fig. 8A). Ultra-thin sections double-labeled for CP and Inh II (see *Materials and methods*) revealed that both proteins were within the same vacuolar aggregates (Fig. 8F). Preimmune serum from neither goat nor rabbit exhibited any cross-reactive proteins in any organelles of the tomato leaves. The inset in Fig. 8F shows an ultra-thin section of a vacuolar aggregate from a tomato leaf treated with a combination of goat and rabbit preimmune sera.

Discussion

Transgenic tomato plants that constitutively express the prosystemin cDNA behave as if they are constantly in a

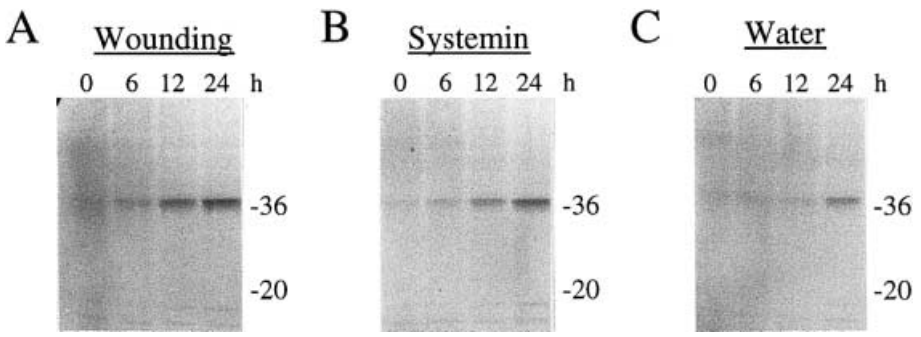

Fig. 6A–C. Western blot analysis of a time course of wound-inducible CP in tomato leaves. **A** Unwounded leaves of wounded plants. **B** Excised plants supplied with systemin peptide. **C** Control plants supplied with water only. *Numbers on the right*, molecular weight in kilodaltons

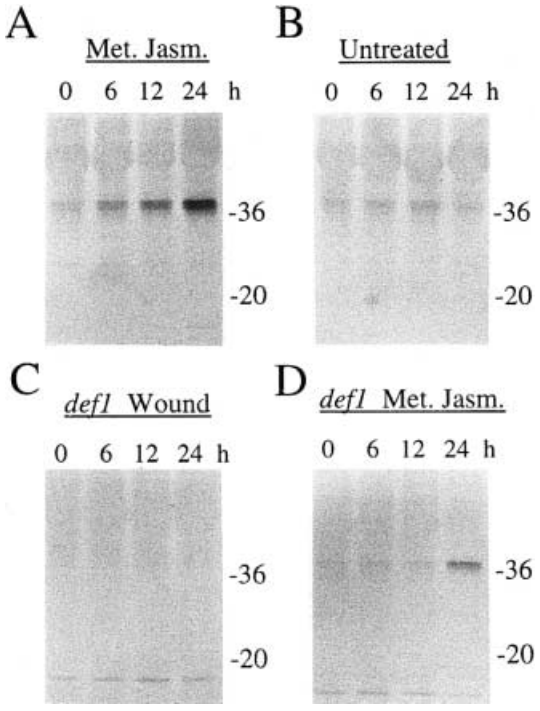

Fig. 7A–D. Western blot analysis of a time course of wound-inducible CP in tomato leaves. **A** Tomato plants exposed to methyl jasmonate vapors. **B** Untreated control plants. **C** Unwounded leaves of wounded *def1* mutans. ***D*** *def1* mutants exposed to methyl jasmonate vapors. *Numbers on the right*, molecular weight in kilodaltons

wounded state, and constitutively synthesize wound-inducible defense related proteins. Some of these proteins accumulate in leaves and are readily identified by SDS-PAGE (Bergey et al. 1996). Several wound-inducible proteinases have been identified in this way, including an aspartic proteinase, a sulfhydryl proteinase, a leucine aminopeptidase and, as reported here, a CP. The CP was identified based on a partial protein sequence obtained from an 18-kDa polypeptide that accumulates in the transgenic plants. From the partial sequence, we were able to synthesize a degenerate primer that allowed us to isolate a cDNA clone in which the open reading frame exhibited homology to a type I serine-CP.

The nucleotide sequence shown in Fig. 1 codes for a protein of 498 amino acids with a methionine at the N-terminus and an isoleucine at the C-terminus. The deduced N-terminal 27 amino acids possess the characteristics of a signal sequence (double underline in Fig. 1) with a double charge (-K-K-) at positions 3 and 4, followed by a hydrophobic region (22 residues), terminating with a -G-A-, a typical site for a signal peptidase specificity. The processed N-terminus coincides with a 13-amino-acid N-terminal sequence of the native tomato CP determined by Mehta et al. (1996) (arrow in Fig. 1). This sequence, as discussed by Mehta et al. (1996), is very similar to the barley CP-I N-terminus (Sorensen et al. 1986). The deduced protein sequence contains five potential N-glycosylation sites (underlined in Fig. 1), four in the proposed α-chain and one in the β-chain. Barley and rice CPs are known to be glycoproteins, with three potential glycosylation sites (Sorensen et al. 1986; Washio and Ishikawa 1994). Figure 2 shows an alignment of the tomato wound-inducible CP with the barley and rice enzymes. The tomato protein sequence is 73% similar (65% identity) to barley CP, and 71% similar (64% identity) to the rice enzyme. The barley enzyme contains three disulfide bonds that are responsible for linking the α- and β-chains in the mature enzyme (Doan and Fincher 1988). The alignment indicates a 100% conservation of seven cysteines (asterisks in Fig. 2) indicating the importance of disulfide bonds in each of the three enzymes.

Southern blot analysis of the wound-inducible CP gene in tomato revealed that the gene is likely a member of a small family of genes (Fig. 3). Our probe showed hybridization with five fragments of different molecular weights. Mehta et al. (1996) had reported the presence of several isoenzymes of tomato CP, based on protein purifications. While the nature of the different isoforms is not known, it is possible that some may be the product of different members of a small gene family. The presence of CPs isoforms has been reported previously in germinating barley using chromatographic techniques (Mikola 1983), wheat (Mikola 1986) and in tomato fruit (Mehta and Mattoo 1996), but it is not known if they are degradation products or multiple gene products in these tissues. To our knowledge, the tomato CP gene is first of a type I serine CP gene reported in a dicotyledonous species.

The time-course analyses of mRNA levels following wounding and systemin treatment showed induction of CP mRNA (Fig. 4A,B). The CP mRNA was present at low levels in untreated plants, and an increase in CP mRNA was not detected. However, with each treatment, CP transcription gradually increased through the 12 h of

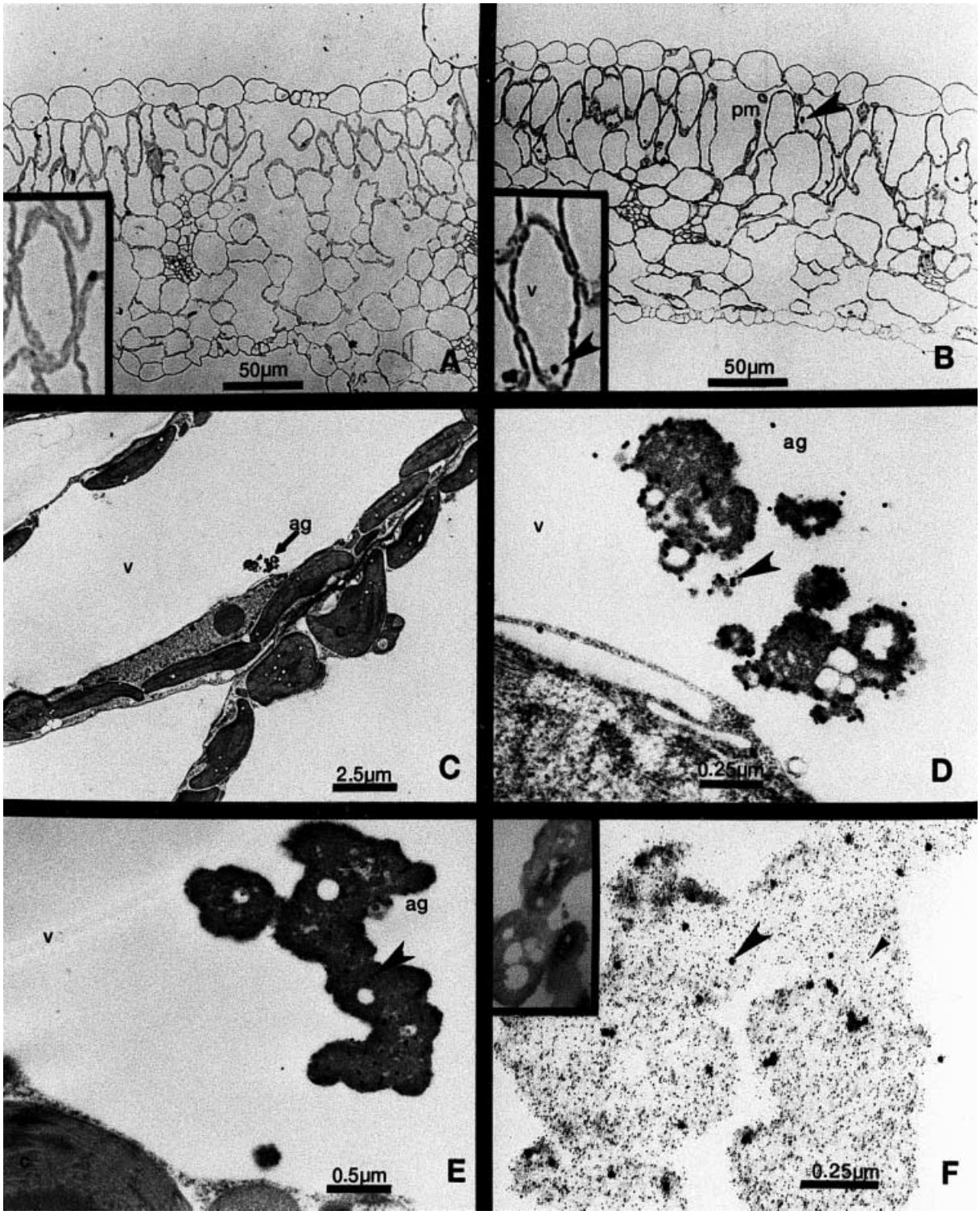

Fig. 8A–F. Immunolocalization of carboxypeptidase protein in tomato leaves. **A, B** Silver-enhanced immunogold labeling of thin sections of control (**A**) and unwounded leaf from wounded plants (**B**). *Insets*, magnifications of a selected mature palisade cell. **C** Immunogold labeling of an ultra-thin section of an unwounded leaf from a wounded plant. **D** Magnification of the protein aggregate shown in **C**. **E** Ultra-thin section of unwounded leaf from a wounded plant showing immunogold labeling of protein aggregate in the vacuole. **F** Double immunogold labeling with anti CP serum (20 nm) and anti-tomato inhibitor II serum (5 nm). *Inset*, treatment with rabbit and goat preimmune serum. *v*, vacuole; *pm*, palisade mesophyll; *ag*, protein aggregate; *c*, chloroplast. *Large arrowheads*, cross-reaction with CP antibody; *small arrowhead* (**F**), cross-reaction with Inh.II antibody

the experiments. By 18 h, the mRNA had returned to zero-time levels (data not shown). Plants supplied with water exhibited low levels of CP mRNA, due either to cutting of the stem (Fig. 4C) or handling of the plants during experimentation (Fig. 5B). Methyl jasmonate-treated plants showed a strong induction of CP mRNA at 3 h and the levels remained high through the 12 h of the experiment (Fig. 5A). All of these experiments support a role for the octadecanoid pathway in the systemic induction of CP. To verify the mediation of the octadecanoid pathway in signaling CP transcription, we assayed CP mRNA induction in a tomato mutant (*def1*) (Howe et al. 1996) that has an impairment in the octadecanoid signaling pathway. A time course analysis of mRNA in this mutant revealed that no CP mRNA was induced in response to wounding (Fig. 5C). Treatment of the *def1* mutants with methyl jasmonate, an end product of the octadecanoid pathway downstream from the mutation (Howe et al. 1996), induced CP mRNA with similar kinetics to those of the wild type (Fig. 5D).

Analysis of CP protein synthesis using Western blotting with CP antiserum confirmed the mRNA data. A CP cross-reactive protein of 37 kDa corresponding to the protein previously identified by Walker-Simmons and Ryan (1980) and Mehta et al. (1996) increased in upper unwounded leaves of plants wounded on lower leaves within 6 h of wounding (Fig. 6A), and the accumulation continued over the 24 h of the experiments. A weakly cross-reactive 18-kDa protein (Walker-Simmons and Ryan 1980) also increased in response to wounding, similar to the 37-kDa protein. A 15-kDa protein cross-reacted with the antibody, but was present at the same levels throughout the experiment. In immunoblotting experiments, no proteins larger than 37 kDa were found. This is in contrast to Mehta et al. (1996) who reported a 69-kDa form of tomato leaf CP. Since the native, unglycosylated protein has a molecular weight of about 50 kDa, and it is processed into two smaller forms, one of which is likely glycosylated, a 69-kDa form might be generated by the incomplete processing of the glycosylated precursor in the plants utilized in the study of Mehta et al. (1996). A similar pattern of CP cross-reactive proteins accumulated when plants were supplied with systemin (Fig. 6B). Plants supplied only with water showed an increase in 37-kDa and 18-kDa proteins, but only after 24 h. This correlates with the low production of CP mRNA in response to cutting the stems of the plants (Fig. 6C). Methyl jasmonate vapors induced CP protein in leaves of both wild-type and *def1* plants (Fig. 7A,D) and no detectable levels of CP protein were found in wounded *def1* mutant plants (Fig. 7C). The 15-kDa protein was again present and remained unchanged throughout the experiments.

Expression of the CP cDNA in *Escherichia coli* produced a protein that was recognized by the anti-CP serum used in this study (data not shown), confirming identity with the previously isolated enzyme and making it likely that the CP isolated previously is the same or a closely related enzyme to the wound-inducible CP protein and mRNA reported here.

Carboxypeptidase activity was shown previously to be associated with tomato vacuoles isolated from leaves of wounded plants (Walker-Simmons and Ryan 1977) and it was immunolocalized in vacuoles of vascular parenchyma tissue (Mehta et al. 1996). In this latter study, no CP was found in mesophyll cell vacuoles in response to wounding. Immunocytochemical localization of a tomato fruit CP also showed the enzyme associated with electron-dense aggregates in the vacuole (Mehta and Mattoo 1996). Immunolabeling data reported here (Fig. 8A,B) show that a protein with reactivity with anti-CP serum accumulates in the central vacuole of leaf palisade mesophyll cells in response to wounding (inset in Fig. 8B), and could also be seen sometimes in cells of the vascular parenchyma (data not shown) as reported by Mehta et al. (1996). Electron micrographs of mature palisade cells incubated with CP antibody (Fig. 8C–E) exhibited immunogold labeling in vacuolar aggregates. Double label with CP and Inhibitor II antibodies confirmed that both proteins are found in the same type of vacuolar aggregate (Fig. 8F). The targeting of CP to the vacuole is consistent with the presence of the signal peptide in the cDNA. The timing of mRNA synthesis, 3–12 h following wounding or treatment of excised plants with systemin, and the presence in vacuoles, indicate that CP protein, like the defensive proteinase inhibitor proteins, is a late product of so-called "late wound-inducible genes". This is in contrast to "early wound-inducible genes" that are synthesized in the vascular bundle cells within 0.5–2 h following wounding, and are associated with signal transduction components (Ryan 2000).

Barley CP enzymes have been shown to be involved in the mobilization of storage proteins during seed germination (Mikola 1983), and the enzyme in tomato leaves may be related to protein turnover in response to wounding, resulting from an increased demand for free amino acids for protein synthesis (Mehta et al. 1996). In a study of the relationship between protein turnover and the accumulation of the proteinase inhibitors in wounded tomato leaves, Gustafson and Ryan (1976) observed a decrease in total soluble leaf protein following wounding. The decrease in leaf proteins was accompanied by an increase in protein turnover and a selective accumulation of proteins such as proteinase inhibitors that are rich in disulfide bonds. The leaf CP enzyme is not inhibited by crude extracts of tomato leaves, nor is the enzyme able to hydrolyze the tomato inhibitors I and II (Walker-Simmons and Ryan 1980). The accumulation of CP protein with a primary role in amino acid mobilization from vacuolar proteins, excluding proteinase inhibitors, would meet the needs of an increasing cellular rate of protein turnover while accumulating the defensive proteins. It is unlikely that CP would be involved in processing (trimming) prosystemin because of its location and late timing of synthesis. Taken together, the data presented here support a role for CP in the wound-response in protein turnover rather than in processing.

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References

Bergey DR, Ryan CA (1999) Wound- and systemin-inducible calmodulin gene expression in tomato leaves. Plant Mol Biol 40: 815–823

Bergey DR, Howe G, Ryan CA (1996) Polypeptide signaling for plant defensive genes exhibits analogies to defense signaling in animals. Proc Natl Acad Sci USA 93: 12053–12058

Dal Degan F, Rocher A, Cameron-Mills V, von Wettstein D (1994) The expression of serine carboxypeptidases during maturation and germination of the barley grain. Proc Natl Acad Sci USA 91: 8209–8213

Doan NP, Fincher GB (1988) The α- and β-chains of carboxypeptidase I from germinated barley originate from a single precursor polypeptide. J Biol Chem 263: 11106–11110

Green TR, Ryan CA (1972) Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. Science 175: 776–777

Gustafson G, Ryan CA (1976) Specificity of protein turnover in tomato leaves. Accumulation of proteinase inhibitors, induced with the wound hormone, PIIF. J Biol Chem 251: 7004–7010

Howe GA, Lightner J, Browse J, Ryan CA (1996). An octadecanoid pathway mutant (JL5) of tomato is compromised in signaling for defense against insect attack. Plant Cell 8: 2067–2077

Karban R, Baldwin IT (1997) Mechanisms of induced responses. In: Karban R, Baldwin IT (eds) Induced responses to herbivory. The University of Chicago Press, Chicago, pp 47–103

McGurl B, Orozco-Cardenas ML, Pearce G, Ryan CA (1994) Overexpression of a CaMV-prosystemin gene in transgenic tomato plants generates a systemic signal that induces proteinase inhibitor synthesis. Proc Natl Acad Sci USA 91: 9799–9802

Mehta RA, Mattoo AK (1996) Isolation and identification of ripening-related tomato fruit carboxypeptidase. Plant Physiol 110: 875–882

Mehta RA, Warmbardt RD, Mattoo AK (1996) Tomato (*Lycopersicon esculentum* cv. Pik Red) leaf carboxypeptidase: identification, N-terminal sequence, stress-regulation, and specific localization in the paraveinal mesophyll vacuoles. Plant Cell Physiol 37: 806–815

Mikola L (1983) Germinating barley grains contain five acid carboxypeptidases with complementary substrate specificities. Biochim Biophys Acta 747: 241–252

Mikola L (1986) Acid carboxypeptidases in grains and leaves of Wheat, *Triticum aestivum* L. Plant Physiol 81: 823–829

Pearce G, Strydom D, Johnson S, Ryan CA (1991) A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. Science 253: 895–898

Ryan CA (2000) The systemin signaling pathway: differential activation of plant defense genes. Biochim Biophys Acta 1477: 112–121

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Shumway LK, Yang VV, Ryan CA (1976) Evidence for the presence of proteinase inhibitor I in vacuolar protein bodies of plant cells. Planta 129: 161–165

Sorensen SB, Breddam K, Fischer RL (1986) Primary structure of carboxypeptidase I from malted barley. Carlsberg Res Commun 51: 475–485

Walker-Simmons M, Ryan CA (1977) Immunological identification of proteinase inhibitors I and II in isolated tomato leaf vacuoles. Plant Physiol 60: 61–63

Walker-Simmons M, Ryan CA (1980) Isolation and properties of carboxypeptidase from leaves of wounded tomato plants. Phytochemistry 19: 43–47

Washio K, Ishikawa K (1994) Cloning and sequencing of the gene for type I carboxypeptidase in rice. Biochim Biophys Acta 1199: 311–314