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

Daniel S. Moura, Daniel R. Bergey*, Clarence A. Ryan
Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340, USA
Received: 28 April 2000 / Accepted: 5 June 2000

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 systematically 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 proystemin 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 (Berger 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

*Present address: Department of Plant Sciences, Montana State University, Bozeman, MT 59717-3140, USA
Abbreviations: CP = carboxypeptidase; PCR = polymerase chain reaction
Correspondence to: C. A. Ryan;
Fax: +1-509-3357643
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 (Berger 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 systemin 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/GGTTCCTTG/CA/CIGAT/GC3′ (I = deoxyinosine), was based on the N-terminal protein sequence obtained and designed for use in combination with the vector-based primer T7 (5′TATACGACTCATAAGGG3′; 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 proprotease transgene (McGurl et al. 1994). The same library was used as a 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 *NaeI* and *DraI* and following the manufacturer’s instructions (Promega). Probes were prepared as described for cDNA library screening. Digested DNA was separated in 0.8% agarose gel with 1 x TBE buffer. DNA fragments were transferred to nylon membranes using 10 x SSC for 18 h. After transfer, the membranes were washed with 2 x 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 x Denhardt’s and 0.05 mg/ml of sheared salmon sperm DNA], using a rotary hybridization incubator (Robbins Scientific) adjusted for 65 °C, for 18 h. Membranes were washed twice with 2 x SSC for 30 min and 1 x 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 x Mops buffer. The RNA was blotted to nylon membranes using the salt-transfer method (10 x SSPE) for 18 h. Membranes were hybridized in 10 ml of a solution containing 5 x SSPE, 5 x 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 BCP/ NBT color development solution according to the manufacturer’s instructions (BioRad). Carboxypeptidase antibody was obtained previously by injecting disopropyl fluorophosphate (DFP)-treated CP into rabbits (Walker-Simmons and Ryan 1980; Walker-Simmons and Ryan, unpublished data).