Characterization of the wound-induced metallocarboxypeptidase inhibitor from potato

cDNA sequence, induction of gene expression, subcellular immunolocalization and potential roles of the C-terminal propeptide

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Abstract A partial cDNA clone for the potato wound-inducible metallocarboxypeptidase inhibitor (PCI) was isolated from a cDNA library constructed from mRNA of abscisic acid (ABA)-treated potato leaves. The full 5′ region of the cDNA was obtained through a RACE-PCR protocol. PCI mRNA encodes a precursor polypeptide which comprises a 29 residue N-terminal signal peptide, a 27 residue N-terminal pro-region, the 39 residue mature PCI protein, and a 7 residue C-terminal extension. Northern blot analysis demonstrates that the PCI gene is transcriptionally activated by wounding, and wound signaling can be induced by ABA and jasmonic acid. Subcellular localization of the protein was investigated by immunocytochemistry and electron microscopy, showing that PCI accumulates within the vacuole. A partial PCI precursor form, comprising the mature protein and the C-terminal extension, has been expressed in *Escherichia coli* and characterized. Its inability to inhibit carboxypeptidases, and stability to carboxypeptidase digestion, suggest that the C-terminal pro-domain may have, besides a probable vacuolar sorting function, a role in modulation of the inhibitory activity of PCI.

Key words: Metallocarboxypeptidase inhibitor; Precursor protein; Vacuolar sorting; Wound-inducible expression; *(Solanum tuberosum)*

1. Introduction

Small proteic protease inhibitors of diverse specificities are widespread in higher plants. Many of these inhibitors are active against proteases from the digestive tracts of insects, and their expression in plants is activated by mechanical wounding or insect injury, and they are thus thought to be part of a defense system of plants against insect attack [1,2]. Among those wound-inducible inhibitors only two inhibitors specific for metallocarboxypeptidases, the potato carboxypeptidase inhibitor (PCI) [3] and its close homologue from tomato [4], have been reported. Both are small proteins, with a molecular mass of about 4 kDa, stabilized by three disulfide bridges and showing a high stability to denaturation or digestion by endoproteases. Accumulation of a carboxypeptidase inhibitor in leaves of wounded potato plants had been previously reported [5]. Also, the cDNA sequence of a tomato metallocarboxypeptidase inhibitor and studies on its fruit-specific and wound-induced expression have been reported [6].

The inhibitor derived from potato has been by far the best characterized at the protein level [7]. Its tertiary structure, both in solution [8] and in a crystal complex with carboxypeptidase A [9], has been determined. From previous chemical modification studies [10], and based on the structure of this complex, it was shown that the C-terminal tail of the inhibitor is directly involved in the inhibition mechanism, by interacting with carboxypeptidases in a substrate-like manner. We have previously developed a heterologous expression system, based on a synthetic gene constructed from the sequence of the mature protein [11]. Site-directed mutagenesis studies have served to further characterize the interaction between carboxypeptidases and PCI [12]. PCI shares a peculiar disulfide-stabilized loop scaffold, known as a T-knot [13], with other plant protease inhibitors and with animal peptide growth factors. In particular, we have recently shown that PCI displays structural homology with mammalian epidermal growth factor (EGF), which makes this molecule an EGF antagonist with potential significance as an antitumor agent [14].

In studies on the disulfide folding pathway of PCI [15] we found that in vitro folding of PCI was highly inefficient. Some protein precursor domains have been shown to play a role in the folding of the mature protein [16]. However, this issue has been in some cases controversial, as found for the pancreatic trypsin inhibitor [17,18]. The fact that the reported cDNA sequence of the tomato inhibitor showed that this protein is first synthesized as a precursor prompted us to carry out the cloning of a cDNA for PCI, to study the structure of its precursor and to characterize the putative roles of the precursor domains in folding, subcellular sorting and activity.

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Abbreviations: ABA, abscisic acid; MCPI, metallocarboxypeptidase inhibitor; PCI, potato carboxypeptidase inhibitor; CPA, carboxypeptidase A; CPB, carboxypeptidase B; JA, jasmonic acid; proPCI-Ct, carboxy-terminal proPCI precursor; Ct, carboxy-terminal; MALDI-TOF, matrix-assisted laser desorption ionization time of flight

The nucleotide sequence data reported here have been submitted to the GenBank Nucleotide Sequence Database and are available under accession number AF060551.

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2. Materials and methods

2.1. Materials

A *Solanum tuberosum* cDNA library constructed in λgt11 (Amersham) using RNA from abscisic acid (ABA)-induced potato leaves was screened for isolation of the PCI clone. cDNA clone pCGN2552 of the tomato metallocarboxypeptidase inhibitor (MCPI) [6], originally used as a probe, was kindly provided by Calgene Inc., Davis, CA. Preparation of rabbit antibodies against the mature PCI has been previously reported [11]. Bovine carboxypeptidase A (CPA) was from Boehringer Mannheim. Porcine carboxypeptidase B (CPB) was prepared as described [19]. Porcine elastase and the synthetic carboxypeptidase substrate benzoyl-glycyl-L-phenylalanine were from Sigma. Restriction enzymes, T4 DNA ligase and DNA polymerases were from Boehringer Mannheim and Ecogen. Synthetic oligonucleotides were obtained from MWG-Biotech (Ebersberg, Germany).

2.2. Plant material

Solanum tuberosum plants var. Désirée or Berolina were grown in the greenhouse at 22°C and 16 h light/8 h dark photoperiodic conditions. Plants at the five leaf stage were used for wounding or hormone treatment studies.

2.3. Nucleic acid isolation and analysis

An ABA-induced potato leaf cDNA library in λgt11 was screened by plaque hybridization using standard techniques [20]. The cDNA encoding the tomato MCPI, ^{32}P-labeled by random priming using the Prime-a-Gene kit (Promega), was used as a probe. Inserts from the isolated positive phage clones were excised by *Bam*HI digestion and subcloned in the plasmid vector pUCBM21 for sequencing. Total RNA from potato leaves was isolated according to the procedure of Logemann et al. [21]. Poly(A)$^+$ RNA was purified by oligo(dT) affinity separation (Promega). Isolation of genomic DNA from potato was performed according to Dellaporta et al. [22]. For Northern blot analysis, total RNA isolated from potato leaves was fractionated on a denaturing agarose/formaldehyde gel, blotted onto a Nylon membrane (Hybond, Amersham) and fixed by UV cross-linking. The ^{32}P-labeled cDNA clone pCPCI81, encoding the PCI precursor, was used as a probe. Hybridization was carried out according to Amasino [23].

2.4. 5′ RACE

The 5′ region of the transcript for the PCI precursor was isolated by 5′ RACE PCR [24]. For this, we designed the following three gene-specific synthetic primers and two adaptor primers:

GSP-RT: 5′-AACACTTGAGACAATAATATTGA, complementary to nucleotides 325–347, according to the numbering of the complete cDNA in Fig. 1.

GSP1: 5′-TATTGAAATTACAGGCCTATGGCCA, complementary to nucleotides 306–330.

GSP2: 5′-TCGCCTAGGTCGTGTGTTTTACAAGGTTTGTTAC, complementary to nucleotides 204–228, with the addition of a *Bam*HI site at the 5′ terminus.

T_{17}Adp: 5′-CCGGAATTCACTGCAGGGTACCCAATACGACTCACTATAGGGCT$_{17}$ [24]

Adp: 5′-CTGCAGGGTACCCAATACGACTCAC (part of the sequence of T_{17}Adp).

Poly(A)$^+$ RNA purified from ABA-induced potato leaves was reverse-transcribed using GSP-RT as primer, and poly(dA) was added to the 3′ end of the synthesized first strand cDNA using terminal deoxynucleotidyl transferase. A first round of PCR was performed using this cDNA as template and GSP1 and T_{17}Adp as primers. A second 'nested' PCR was carried out using the primers GSP2 and Adp. PCR products were cloned on a plasmid vector using the pGEM-T Vector System (Promega) and their nucleotide sequences were determined.

2.5. PCR amplification of genomic sequences

PCR amplification of the genomic sequence coding for PCI precursor was carried out by a 'nested' PCR strategy. First amplification was performed using potato genomic DNA as template and the synthetic primers Nt (5′- ACTTACTATCCTTTTCACCATTCT, position 61–84 of the cDNA sequence) and PolyA (5′-AACACACATAACATGATGATTTT, complementary to nucleotides 517–539). The product was subsequently used as template for a second PCR with primers Nt and GSP-RT. PCR products were purified and directly sequenced using GSP-RT as sequencing primer.

2.6. Wounding, ABA, and jasmonic acid (JA) treatments

Plants were wounded by application of a dialysis clamp to the third leaf. After 20 h, a second clamp was applied to the fifth leaf and 4 h later the directly wounded leaves were harvested as wound samples. The fourth leaf located between the injured leaves was also harvested to assay for systemic induction. ABA and JA treatments were performed by incubation of the leaves through the petiole in 100 μM ABA and 10 μM MeJA solutions in water.

2.7. Immunocytochemistry and electron microscopy

Small pieces of apex meristem of potato plant were fixed by vacuum infiltration with 2.5% glutaraldehyde and 2.5% paraformaldehyde in 20 mM phosphate buffer pH 7.2 for 2 h at room temperature. The tissue was washed in 0.2 M NH_4Cl and in three changes of 20 mM phosphate buffer. Samples were dehydrated through an ethanol series and embedded in LR White resin at −20°C. Immunocytochemistry was performed as described [25]. Ultrathin sections were incubated in PBLS buffer (20 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.1% Tween 20) containing 1% BSA (blocking solution) for 30 min at room temperature. The grids were drained and incubated with the PCI antiserum (dilution 1:1000) in blocking solution for 2 h at room temperature. Controls were incubated with non-immune serum. The grids were then washed in PBLS and incubated with protein A-colloidal gold (15 nm diameter) diluted 1:50 in blocking solution for antibody detection. Sections were extensively washed with PBLS and then with double-distilled water. Finally they were stained with uranyl acetate and lead citrate and examined under an electron microscope (Phillips EM301).

2.8. Expression of the carboxy-terminal proPCI precursor (proPCI-Ct)

An expression vector for proPCI-Ct was constructed from the pIMAM3 vector [11], which derives from the pINIII-ompA3 secretion vector [26], by adding a sequence encoding the seven residues of the carboxy-terminal precursor sequence. Mutagenesis was performed by PCR using a vector-derived primer, and the specific synthetic oligonucleotide 5′-CCCGAATTCTTACTACAGACCAATTGCCATTGCACCACCAACGTACGGGCCGCA. *Escherichia coli* strain MC1061 [27] carrying the pINIII-OmpA3-proPCI-Ct plasmid was used to produce the recombinant protein, in the conditions reported for the mature PCI expression system [11]. Purity of the isolated proPCI-Ct was assessed by MALDI-TOF mass spectrometry and N-terminal sequencing.

2.9. Protein analysis

MALDI-TOF mass spectrometry was performed on a Bruker- BIFLEX spectrometer. N-terminal sequencing was performed by automatic Edman degradation on a Beckman-LF3000 protein sequencer. Circular dichroism (CD) spectra were measured on a Jasco J-715 dichrograph (Japan).

2.10. Protease digestions

Carboxypeptidase: About 2 μg of protein was digested with bovine CPA or porcine CPB, at a 1/1.5 (w/w) ratio, in 10 μl of 50 mM Tris-HCl (pH 7.5) at room temperature for 20 h. The extent of digestion was monitored at different times by MALDI-TOF mass spectrometry. Equal aliquots of fresh enzyme were subsequently added every 24 h, and the reaction was allowed to proceed for a total of 96 h. The final reaction mixture was acidified by addition of trifluoroacetic acid to denature the carboxypeptidase, centrifuged at 13 000×*g* for 5 min, and the supernatant assayed for inhibitory activity.

Elastase: About 2 μg of protein was dissolved in 0.1 M Tris-HCl (pH 8.8) and a 4/1 (w/w) ratio of bovine elastase (Sigma) was added. The digestion was continued for 20 h at room temperature. Progress of the reaction was monitored by MALDI-TOF mass spectrometry.

2.11. Carboxypeptidase inhibition assay

Inhibition of carboxypeptidase activity was assayed using bovine CPA and *N*-benzoyl-glycyl-L-phenylalanine as a substrate. Assays were performed spectrophotometrically by monitoring substrate hydrolysis at 254 nm, as previously reported [19].

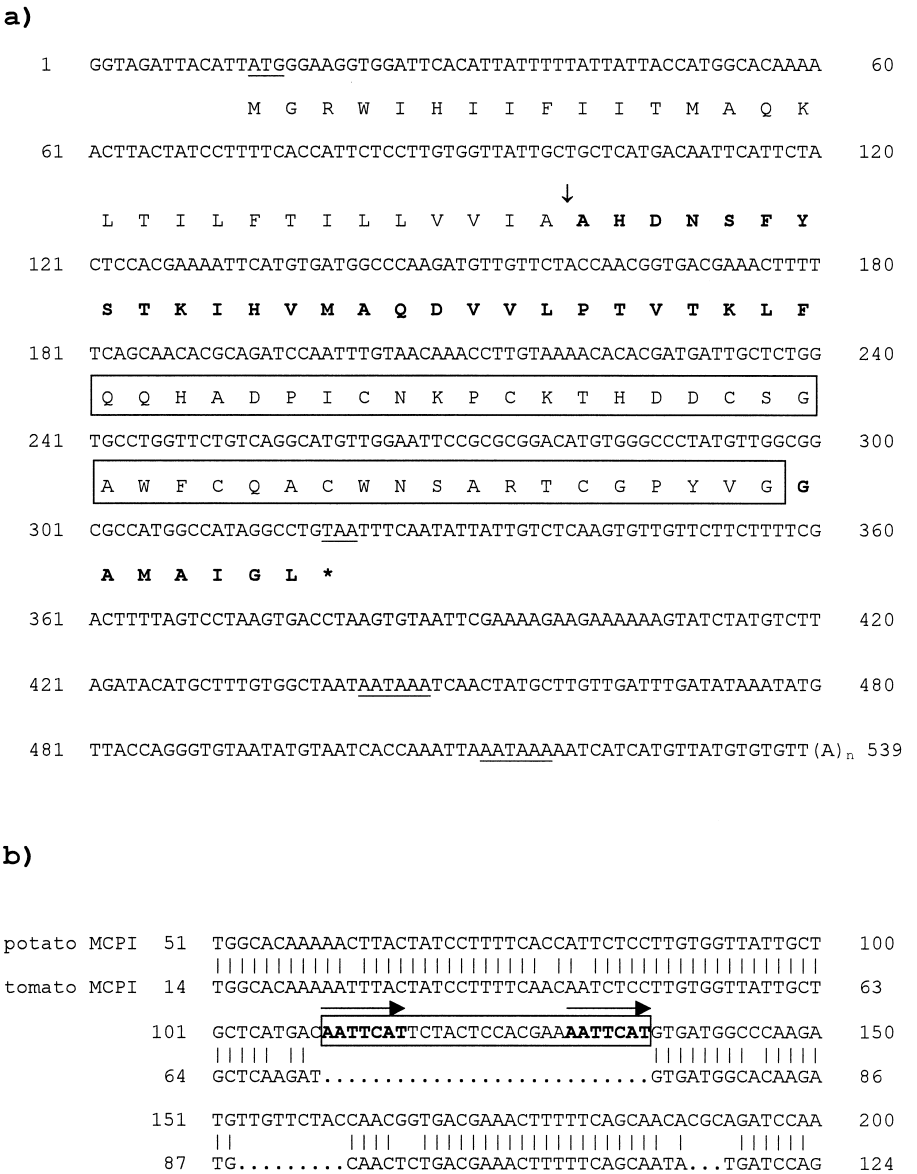

Fig. 1. a: Nucleotide and deduced amino acid sequence of the PCI precursor cDNA. The sequence of the pCPCI81 clone started at nucleotide 53. The sequence at the 5′ end was obtained by 5′ RACE. Initiation (ATG) and termination (TAA) codons and two potential polyadenylation signals are underlined. The predicted cleavage site between a putative signal peptide and the pro-protein is indicated by an arrow. The boxed amino acid sequence corresponds to the mature PCI protein. N- and C-terminal pro-sequences are printed in bold. b: Partial alignment between potato and tomato cDNAs encoding the carboxypeptidase inhibitor precursor. The box indicates the 27 nt insertion found in the potato cDNA, flanked by 7 nt direct repeats, marked in bold.

3. Results

3.1. Cloning and sequence analysis of the cDNA of PCI precursor

An ABA-induced potato leaf cDNA library was screened by plaque hybridization using ^{32}P-labeled tomato MCPI cDNA as a probe [6]. Five positive phage clones were isolated out of a total of 2.5×10^5 phage plaques screened. All purified clones contained a recombinant cDNA insert of a similar size of around 500 bp. cDNA inserts were excised with *Bam*HI, subcloned in the pUCBM21 plasmid, and the sequences of both strands determined. The sequence of one of these clones, designed pCPCI81, is shown in Fig. 1 (sequence from nucleotide 53 to the end). Identical sequences were obtained for the other isolated clones. Although analysis of the sequence showed that these clones encoded the mature PCI protein, no in-frame initiation methionine codons were found for any of the five positive cDNAs isolated.

In order to determine the nucleotide sequence of the 5′ region of the transcript encoding the PCI precursor, we carried out an amplification by a 5′ RACE PCR strategy [24] using ABA-induced potato leaf poly(A)$^+$ RNA as a template. The primers used for cDNA first strand synthesis and subsequent specific amplification are detailed in Section 2. An additional 52 bp sequence to the 5′ end of cDNA was obtained by this method, which contained an in-frame ATG codon at position 14–16, which conforms to the Kozak consensus for a translational initiation site [28].

The full cDNA sequence of the PCI precursor, shown in Fig. 1a, is 539 bp in length, with a 309 bp open reading frame,

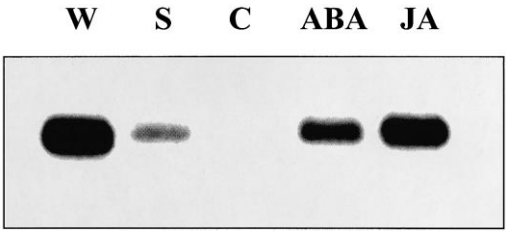

Fig. 2. Transcriptional activation of PCI gene expression. Total RNA was isolated from: directly wounded potato leaves (W), upper unwounded leaves (systemically induced) (S), control leaves (C), ABA-treated and JA-treated leaves. RNA samples (30 μg each) were analyzed by Northern blot hybridization with a PCI cDNA probe.

a 13 bp 5′ untranslated region and a 3′ untranslated sequence of 217 bp. Two putative polyadenylation sites (AATAAA) were found (underlined in Fig. 1a) at positions 443 and 513. The size of this cDNA is consistent with the observed transcript size detected by Northern blot analysis (see below). The open reading frame encodes a protein of 102 amino acid residues. A hydrophobic region at the amino-terminus conforms to a consensus signal peptide, with a predicted cleavage site after the alanine 29 according to von Heijne's rules [29] (Fig. 1). Removal of this signal sequence would result in a proprotein sequence of 73 aa, with a molecular mass of 7958 Da. This protein sequence comprises the mature protein region [30] as well as a 27 amino acid N-terminal pro-domain and a 7 amino acid C-terminal extension, the latter having a hydrophobic character. The predicted mature protein corresponds to the major isoinhibitor form IIa of CPI [30].

The cDNA for the PCI precursor presents an overall 84.9% homology to the tomato carboxypeptidase inhibitor cDNA [6]. Major differences are found located in the region encoding the N-terminal pro-domain. Particularly striking is the presence in this region of a 27 bp insertion (positions 110–137) in the potato cDNA (Fig. 1b), which is flanked by two 7 bp direct repeats. This insertion was found to be present in all

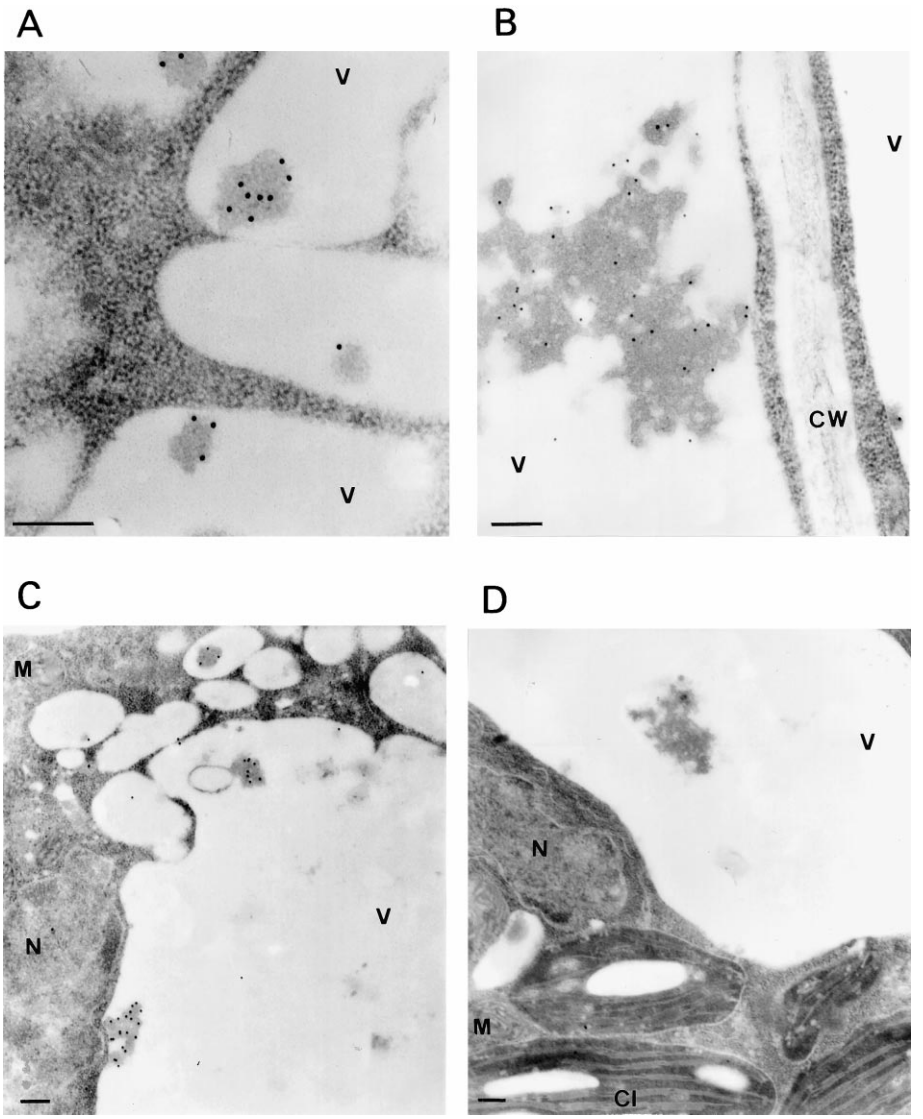

Fig. 3. Subcellular localization of PCI in apical meristems of potato plants. A: Immunolocalization of PCI in small vacuoles of young leaves using anti-PCI antibody diluted 1:1000 followed by labeling with 15 nm protein A-gold. B: A higher magnification of a labeled electron-dense structure within a large vacuole. C: Immunolocalization of PCI in small and large vacuoles of sub-epidermal parenchyma cells. D: Control experiment. Sub-epidermal cells incubated with non-immune serum. CW, cell wall; M, mitochondria; Cl, chloroplast; V, vacuole. Bar: 0.2 μm.

five positive clones isolated from the cDNA library. In order to discard an artifactual origin of this insertion, we amplified this region by a 'nested' PCR strategy using potato genomic DNA as template, and the three internal primers detailed in Section 2. A 300 bp fragment, corresponding to the PCI gene sequence between nucleotides 85 and 324 in Fig. 1, was isolated and sequenced, showing that this insertion is also present in the genomic DNA.

3.2. Wound induction of PCI expression

Analysis of expression of the PCI transcript by RNA blot is shown in Fig. 2. Plants were wounded or treated with the phytohormones ABA and JA, which have been shown to be involved in the signaling pathway of other wound-induced defense proteins [31–33], as described in Section 2. Total RNA was isolated from control and induced leaves, fractionated by gel electrophoresis and hybridized with the ^{32}P-labeled PCI cDNA probe. As seen in Fig. 2, no hybridization signal was detected in control leaves, while a clear hybridization band of about 580 bp, corresponding to the PCI transcript, was detected upon wounding or ABA or JA treatments. Wounding induced high levels of accumulation of the PCI mRNA in directly wounded as well as in systemically induced leaves, thus evidencing a systemic induction of the potato PCI gene. Treatment with ABA or MeJA resulted in an increase in the levels of transcript of about the same order of magnitude as wounding.

3.3. Subcellular immunolocalization of PCI

The subcellular localization of PCI in potato plants was determined by electron microscopic immunocytochemistry using a polyclonal antiserum directed against PCI [11]. Although the PCI gene was highly induced by wounding, we observed by immunoblot that the protein was relatively abundant in the meristematic tissues of both non-induced and induced potato plants (results not shown). Therefore, ultrathin sections of apical meristem of potato plants containing young leaves were treated with the antiserum against PCI and the antibody binding was visualized with 15 nm diameter colloidal gold linked to protein A. Representative photomicrographs of young leaf sections are shown in Fig. 3. Electron-dense protein aggregates stained with the antibody were seen in small and large vacuoles of parenchyma cells (Fig. 3A–C). No cross-reactive material was detected in the cell wall or in any subcellular fraction other than vacuole, which clearly demonstrates that PCI is a vacuolar protein. This result is in agreement with a previous report in which the localization of PCI had been inferred by immunological assays in isolated protoplasts and vacuoles from leaves of wound-induced plants [34]. No specific labeling was detected in parallel experiments using non-immune serum instead of the primary antibody (Fig. 3D). A large number of vacuoles from different types of specialized cells were analyzed (e.g. pre-vascular cells, epidermal cells, etc.) and, from all, PCI label was only observed in vacuoles of parenchyma cells which were in close association with epidermal cells.

3.4. Expression and structural characterization of the proPCI-Ct

The C-terminal tail of mature PCI is directly involved in the mechanism of inhibition of metallocarboxypeptidases [9] and is responsible for most of the stability of the carboxypeptidase-PCI complex. [12]. The presence of the seven amino acid C-terminal extension in the precursor of PCI could strongly affect its inhibitory capability, serving as a modulator of this function. In order to study this potential role, we constructed an expression vector to produce a recombinant protein, proPCI-Ct, comprising the mature part of PCI plus the seven residues forming the C-terminal extension. Site-directed mutagenesis was performed on the pIMAM3 vector, carrying a synthetic gene for mature PCI fused to the ompA signal sequence [11], to add a sequence coding for the seven residue C-terminal extension. This construction was transformed in *E. coli* strain MC1061 for expression. As observed previously for the mature PCI [11], the recombinant proPCI-Ct protein was completely released to the culture medium.

Purification of proPCI-Ct from the culture medium was accomplished by concentration of the supernatant using a reverse-phase cartridge, followed by reversed-phase HPLC. Approximately 0.3 mg of purified protein per liter of medium, giving a homogeneous HPLC peak, was obtained. The identity of proPCI-Ct was established by MALDI-TOF mass spectrometry and N-terminal sequencing. The observed mass for proPCI-Ct was 4906 Da, which coincides with that expected from the amino acid sequence, and the observed N-terminal sequence shows that proPCI-Ct is correctly processed and pure by these two criteria.

The CD spectrum of proPCI-Ct at low pH presents a minimum of ellipticity around 210 nm and a characteristic maximum at 228 nm (data not shown). This maximum is present in the CD spectrum of mature PCI (Venhudova and Avilés, unpublished results), but not in the spectra of species with mispaired disulfide bridges ([15] and Pavía and Avilés, unpublished results), and seems to be related to the three-dimensional conformation of the native protein.

3.5. Stability towards protease digestion and inhibitory activity assays of proPCI-Ct

Assays of the inhibition on metallocarboxypeptidase A (CPA) showed that proPCI-Ct is not able to inhibit CPA, even at concentrations 20 times greater than those at which CPA is totally inhibited by mature PCI. As another way to check that proPCI-Ct was correctly folded to the native, mature PCI-like structure, we tried to convert it to the active form by digestion with elastase. We had previously observed (Venhudova and Avilés, unpublished results) that this non-specific protease, due to the compact structure of PCI, can only cleave the last Gly residue of the mature protein, leaving an active inhibitor [35]. On the other hand, elastase would degrade extensively disulfide-scrambled forms of PCI ([15], and Pavía and Avilés, unpublished results). When proPCI-Ct was digested with elastase under the same conditions, a single cut digestion product was observed by MALDI-TOF mass spectrometry, even after long digestion times, which corresponded to mature PCI plus the first two residues (Gly and Ala) of the Ct pro-domain, PCI+2. This digestion product was likewise not active as an inhibitor of CPA.

We assayed next the digestion of proPCI-Ct by the same metallocarboxypeptidase A (Fig. 4a). Digestion progressed very slowly, even at very high protease/proPCI-Ct ratio, and surprisingly, the final digestion product was mature PCI plus one additional Gly residue from the Ct extension, PCI+1. Digestion did not progress further even after addition of fresh protease and long digestion times. PCI+1 showed no inhibi-

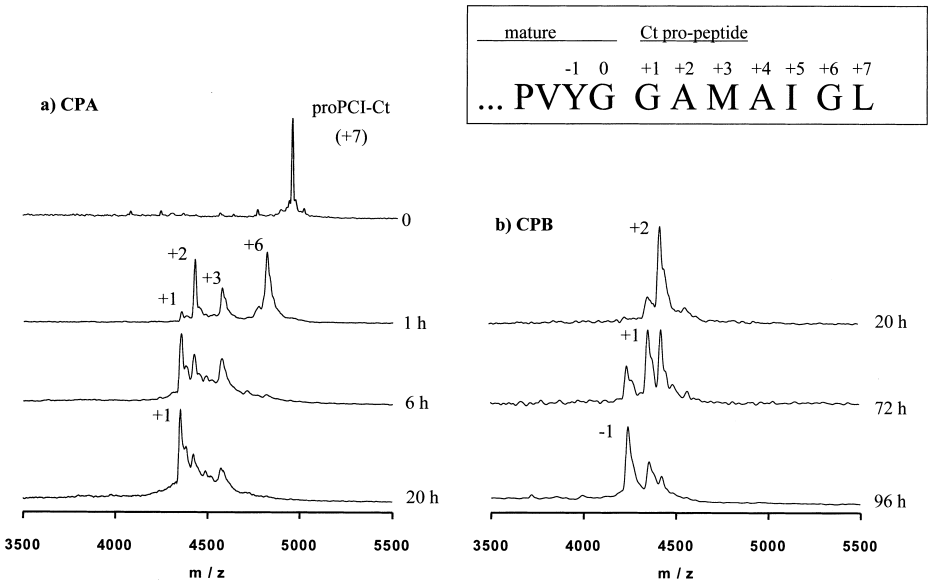

Fig. 4. Analysis of carboxypeptidase digestion of proPCI-Ct by MALDI-TOF mass spectrometry. Time course of the digestion of proPCI-Ct with bovine CPA (a) and porcine CPB (b). The different species observed in the mass spectra of the digestion mixtures are marked according to the number of extra residues at the C-terminus with respect to mature PCI, as indicated in the sequence shown above.

tory activity of CPA. Under the same digestion conditions, the C-terminal Gly of mature PCI is readily cleaved, leading to a fully active inhibitor, PCI−1 [35]. We also observed that digestion of proPCI-Ct by metallocarboxypeptidase B (a protease of different specificity) progresses extremely slowly (Fig. 4b), but gives rise to PCI−1 as the final digestion product. As expected, inhibitory activity was observed for this final digestion product, which corroborates that proPCI-Ct is properly folded.

4. Discussion

We report here the cDNA sequence encoding the precursor of the potato carboxypeptidase inhibitor. The deduced protein sequence includes the 39 residue mature sequence of the isoinhibitor form IIa isolated from potato, which is the predominant form in all tissues studied [3]. Isoinhibitors IIb and III could also derive from different processing of this same precursor protein [30]. Taken together, forms II and III represent 95% of the total inhibitor found in potato leaf [36]. The cDNA for the isoinhibitor I, the minor form in leaf, which differs in two amino acids from form II, was not found in our screening of the cDNA library.

Although the high degree of overall identity between the cDNA sequence of the PCI precursor and the cDNA sequence reported for the homologous inhibitor from tomato [6] is not surprising, considering the phylogenetic closeness of these two species, differences of particular relevance were observed between both cDNA sequences. For example, we found in the 5′ region of the PCI cDNA two in-frame ATG codons, which conform to the Kozak consensus for translational initiation sites, whereas only the downstream one was reported for the corresponding tomato cDNA clone. Initiation of translation at either the first methionine codon or that analogous to the one found in the tomato cDNA would result in a putative 29 or 17 residue long signal peptide, both sharing the same predicted cleavage site according to von Heijne's rules [29]. The predicted signal peptide length for the tomato protein, considering the equivalent cleavage site, would also be 17 residues. Although this discrepancy could be due to a sequence determination problem, it cannot be excluded that there is a true difference in the translation initiation site of both cDNAs.

A second noteworthy difference found in the cDNA of PCI, and also in the corresponding genomic sequence, is the presence of the 27 bp insertion flanked by two 7 bp direct repeats in the middle of the pro-region, as shown in Fig. 1b. Small insertions flanked by short direct repeats are often left behind after integration-excision events of transposable DNA elements. [37] Therefore, this insertion might correspond to the fingerprint of a transposable element that inserted in the proprotein region.

Aside from the presence of the above mentioned insertion, a remarkable fact in the sequence of both precursor forms is the presence of a relatively long N-terminal pro-domain (15 residues for the tomato and 27 for the potato inhibitor), which does not share significant homology to any sequence of the databases. No specific functional role was suggested for this N-terminal domain of the tomato inhibitor. It is known that for some proteins produced as precursors these pro-regions are needed for a proper folding. In fact, these regions have been referred to as intramolecular chaperones, because they perform a function similar to the molecular chaperones in assisting folding [16]. Bearing this in mind, and taking into account our previous studies about the folding in vitro of mature PCI [15], which demonstrated that folding of the mature protein is very inefficient, a hypothetical role of this precursor region in the folding of the protein seems plausible. To check this hypothesis, folding experiments on the whole precursor protein will be the object of further studies. It has to be noted, however, that this N-terminal pro-region appears to be quite tolerant to modifications, since is in this region where most of the divergences between the tomato and potato cDNAs are observed.

Results obtained by immunocytochemistry show that PCI was localized in vacuoles. In plants, vacuolar transport can be

mediated by targeting signals contained in an amino-terminal propeptide, a carboxy-terminal propeptide, or a mature portion of the protein [38]. This suggest that N-terminal and/or C-terminal propeptides of PCI could be involved on the vacuolar sorting. The N-terminal region of PCI precursor, however, does not share the common motif (NPIRL/P) present in amino-terminal extensions of some vacuolar proteins [39]. The implication of the C-terminal propeptide in vacuolar sorting seems very likely, in view of its hydrophobic character and sequence similarities to other reported vacuolar signal sequences [40]. In fact, this potential role had already been suggested for the C-terminal extension of the tomato inhibitor [6].

On the other hand, it was also suggested that this C-terminal extension may have a function in keeping the inhibitor inactive until it reaches its proper subcellular compartment [6]. This hypothesis seems plausible, because of the relevance of the C-terminal region of the mature protein in determining its inhibitory activity. It is also possible that digestion by carboxypeptidases (derived from the plant itself or from insects) results in an efficient degradation of the C-terminal extension, to lead to the active inhibitor. To study these possibilities, here we have obtained a recombinant protein comprising the mature part of PCI with the addition of the C-terminal pro-domain, proPCI-Ct. This protein is inactive as metallocarboxypeptidase inhibitor. The possibility that this lack of activity was the consequence of misfolding of the recombinant protein was ruled out by the CD spectrum, stability towards digestion with elastase, and by the fact that digestion with CPB yielded the active form of PCI. Assays of the digestion of proPCI-Ct with carboxypeptidases of different specificities, CPA and CPB, showed that the C-terminal extension is quite resistant to carboxypeptidase digestion (Fig. 4). While CPA alone cannot cleave the whole C-terminal pro-peptide and leaves a protein precursor that is inactive as inhibitor, digestion with CPB can proceed to the mature inhibitor, though very high concentrations of carboxypeptidase and long digestion times are required. These results make plausible that the presence of the C-terminal extension, besides its role in vacuolar sorting, may function to modulate the activity of the inhibitor, until it is cleaved, probably by a specific protease in the vacuolar compartment. Expression of this protease could be a further regulatory step of the wound response.

Inhibitors specific to carboxypeptidases had been previously shown to accumulate in response to wounding in potato and tomato leaves. In the case of potato, accumulation of the CPI protein upon wounding had been reported in leaves [5]. In contrast, no protein was detectable in wounded leaves of tomato plants [6,41] though accumulation of the inhibitor mRNA was observed in response to wounding [6]. In the case of PCI, accumulation of the protein was shown to parallel the wound-induced expression of serine-proteinase inhibitors I and II [5]. The wound signaling pathway for these two inhibitors is at present very well characterized [31,42,43]. In the present work we demonstrate that expression of PCI can be induced by the phytohormones ABA and JA, in the absence of mechanical wounding, in a similar way as reported for serine-protease inhibitors I and II [31,32]. In addition, we demonstrate a systemic induction of PCI, as denoted by the accumulation of PCI mRNA in unwounded leaves of a wound-induced plant. All these results support that PCI expression is triggered by wounding through a signaling pathway common to other wound-induced defense proteins such as the serine-protease inhibitors I and II.

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