Characterization of *Cucurbita maxima* Phloem Serpin-1 (CmPS-1): a Developmentally Regulated Elastase Inhibitor*

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Running title: *Cucurbita maxima* Phloem Serpin, CmPS-1

Abbreviations: CmPS-1, Cucurbita maxima phloem serpin-1; PI, proteinase inhibitor; PP1, phloem protein 1; PP2, phloem protein 2; serpin, serine proteinase inhibitor

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# On sabbatical leave from: Plant Sciences Department, University of Rhode Island, Kingston,
*This work was supported by Department of Energy Biosciences Grant DE-FG03-94ER20134 (to W. J. L.).
SUMMARY

We report on the molecular, biochemical and functional characterization of *Cucurbita maxima* phloem serpin-1 (CmPS-1), a novel 42 kDa serine proteinase inhibitor that is developmentally regulated and has anti-elastase properties. CmPS-1 was purified to near homogeneity from *C. maxima* (pumpkin) phloem exudate and, based on microsequence analysis, the cDNA encoding CmPS-1 was cloned. The association rate constant (kₐ) of phloem-purified and recombinant His₆-tagged CmPS-1 for elastase was $3 \pm 1.6 \times 10^5$ and $2.7 \pm 0.4 \times 10^5$ M⁻¹ s⁻¹, respectively. The fraction of complex-forming CmPS-1, $X_{inh}$, was estimated at 79%. CmPS-1 displayed no detectable inhibitory properties against chymotrypsin, trypsin, or thrombin. The elastase cleavage sites within the reactive center loop of CmPS-1 were determined to be Val₃₄⁷-Gly₃₄₈ and Val₃₅⁰-Ser₃₅₁ with a 3:2 molar ratio. *In vivo* feeding assays conducted with the sucking-piercing aphid, *Myzus persicae*, established a close correlation between the developmentally regulated increase in CmPS-1 within the phloem sap and the reduced ability of these insects to survive and reproduce on *C. maxima*. However, *in vitro* feeding experiments, using purified phloem CmPS-1, failed to demonstrate a direct effect on aphid survival. Likely roles of this novel phloem serpin in defense against insects/pathogens are discussed.
INTRODUCTION

The phloem long-distance translocation system of plants appears to function both as a nutrient delivery system and as an information superhighway (1–3). A central role for the phloem in the translocation of nutrients has long been recognized. The presence of plant hormones in the phloem sap (4, 5) implicated this long-distance transport pathway in the delivery of signaling molecules. Recent studies provided new insights into the nature of the information molecules being transported from mature leaves, via the phloem, to distant plant organs. Irrefutable evidence has been obtained for the translocation of certain proteins (6–9). In addition, it has also been demonstrated that specific RNA molecules are present in the phloem sap (10, 11) and some move to distant tissues, where they appear to influence post-transcriptional events (9, 12).

Given the importance of this nutrient/information delivery system to the functioning of the plant, it was axiomatic that plants had to evolve mechanisms to protect the operational integrity of the phloem. Maintenance of structural integrity required the development of systems able to rapidly respond to physical damage, imposed either by environmental forces or herbivory; sealing of disrupted sieve tubes involves deposition of material at the level of the sieve plate pore (1). In the enucleate sieve tube system of angiosperms, maintenance of membrane integrity has also been transferred to the neighboring companion cells (CC) and likely involves the delivery of essential constituents, via plasmodesmata (6, 13, 14).

To ensure the integrity of the signaling components, the plant needs also to protect against protein and RNA degradation occurring within the phloem sap. This capacity appears to have been achieved through the development of a control system that regulates the plasmodesmal-
mediated exchange of macromolecules between CC and the sieve tube system (9, 12, 13). The absence of proteinase activity (15) within the phloem sap is consistent with this model. A range of small molecular weight proteinase inhibitors (PIs; 3–10 kDa) has also been isolated from the phloem sap of several species (16–18). The smallest of these (3 kDa) are members of the serine PI family, whereas the 7–10 kDa PIs belong to the potato PI 1 family. Although nothing is known concerning the mechanism(s) by which these PIs enter the sieve tube system, and their role remains conjectural, they likely serve to protect the proteins within the phloem sap against the action of endogenous proteinases.

An important challenge to the integrity of the phloem system is also posed by piercing-sucking insects, whose primary nutrition is gained through uncontrolled access to the phloem sap. In this regard, it is of interest to note that numerous plant PIs have been shown to modify plant-arthropod interactions, via their role as digestibility reducers, toxins, or modifiers of feeding behavior (19, 20). In addition, in vitro assays have established that phloem PIs (3–10 kDa) are able to inhibit a wide range of proteinases (18, 21, 22), including a number of such enzymes extracted from the midgut of lepidopteran (chewing) larvae (23, 24).

In the present study, we report on the molecular, biochemical and functional characterization of Cucurbita maxima phloem serpin-1 (CmPS-1), a 42 kDa serine PI present in the C. maxima (pumpkin) phloem translocation stream. Biochemical studies revealed that CmPS-1 represents a novel plant serpin having anti-elastase properties. A close correlation was established between the developmentally regulated increase in CmPS-1, within the phloem sap, and the reduced ability of the sucking-piercing aphids, Myzus persicae, to survive and reproduce on C. maxima.
However, *in vitro* feeding experiments, using highly purified phloem-derived CmPS-1, failed to demonstrate a direct effect on aphid survival.

**EXPERIMENTAL PROCEDURES**

**Plant Material --** *C. maxima* Duch. cv. Big Max (pumpkin) plants were grown in an insect-free greenhouse under natural daylight conditions (14 h photoperiod). Light intensities at midday ranged from 1200 to 1500 μmol m⁻² s⁻¹ and day/night temperatures were 26 °C ± 3 °C/22 °C ± 2 °C, respectively.

**Protein Microsequencing, PCR, and cDNA Cloning of CmPS --** Phloem exudate (sap) from cut stems of 4-week-old pumpkin plants was collected, dialyzed and stored as previously described (13). Phloem sap proteins were fractionated by SDS-PAGE and those at the 40 kDa size-range were excised from gels, electro-eluted and concentrated by partial lyophilization. This protein concentrate was digested, overnight, with lysyl endopeptidase (EC 3.4.21.50; Wako, Richmond, CA) and the resultant products separated by HPLC on a 25 cm-long C18 column. Peptides were eluted and a major peak was sequenced (model 477A, Applied Biosystems, Foster City, CA).

For cloning of *CmPS-I*, stem poly(A)+ RNA, isolated from 4-week-old pumpkin plants, was used to synthesize first-strand cDNA by reverse transcription (FastTrack 2.0 kit; Invitrogen, Carlsbad, CA); this cDNA was then used as template for PCR. The following degenerate primers were employed; forward, 5’-TICCITAYWSICARGGICNGA-3’ (I = deoxyinosine, N = A + C + T + G, Y = C + T, S = C + G, W = A + T) and reverse, 5’-TCIGTICCYTCYTCRTTNAC-
YTC-3’. The resultant 342 bp DNA fragment was labeled with $^{32}$P-nucleotides by random priming (high prime DNA labeling kit, Boehringer Mannheim, Indianapolis, IN) and then used to screen a pumpkin stem cDNA library. This library was constructed in Uni-ZAP XR (Stratagene, La Jolla, CA) using the above-described poly(A)$^+$ RNA to synthesize double-stranded cDNA (ZAP synthesis kit, Stratagene). Plaques ($10^5$) were screened and five positive clones were excised into phagemids. Each putative CmPS-1 clone was then sequenced in both directions. Editing, analysis, and sequence alignments were performed using the SeqEd1.03, SeqVu1.0.1, DNASIS-Mac v2.0, DNA Strider1.2 programs and GAP, FASTA and BLAST search engines from the Genetics Computer Group (Wisconsin sequence analysis package).

**Purification of CmPS-1 from C. maxima Phloem Exudates** -- All procedures were carried out at 4 °C and chromatography was performed using FPLC. Purification was followed by Western analysis with polyclonal antibodies (R306, raised against serpin Z-type proteins from barley seed (25) and kindly provided by Dr. Jøern Hejgaard, Dept. of Biochemistry and Nutrition, Technical University of Denmark, Lyngby, Denmark). Phloem exudate collected as described above was dialyzed overnight against buffer A (50 mM Tris, pH 7.5, 1 mM EDTA, and 50 mM 2-mercaptoethanol). After clarification by centrifugation ($17,000 \times g$ for 30 min) the exudate was applied to HiTrap Q Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated with buffer A. CmPS-1 did not bind to the column and, thus, was collected from the flow-through fractions. These fractions were dialyzed against buffer B (25 mM HEPES, pH 7.0, 1 mM EDTA, and 14 mM 2-mercaptoethanol), clarified (as above), and loaded onto HiTrap SP
Sepharose equilibrated with buffer B. CmPS-1 was present in the flow-through fractions. A second cation exchange chromatography fractionation was next performed in the presence of a lower pH buffer to fractionate CmPS-1. The flow-through fractions were dialyzed against buffer C (30 mM MES, pH 5.5, 1 mM EDTA), and subjected to HiTrap SP equilibrated with buffer C. CmPS-1 was eluted at 120 mM NaCl using a linear gradient. The fractions containing CmPS-1 were pooled, supplemented to 1.7 M ammonium sulfate, and subsequently loaded onto a HiTrap Phenyl Sepharose column. CmPS-1 was eluted using a reverse gradient. Fractions of the highest purity, as judged by SDS-PAGE, were pooled and dialyzed against 25 mM HEPES, pH 7.5, and 100 mM NaCl. Dialyzed proteins were concentrated by ultrafiltration using Centricon (Millipore, Bedford, MA) and stored at 4 °C. Protein concentration was measured by the Protein Assay of BioRad (Richmond, CA) using BSA as standard.

**Expression and Purification of Recombinant CmPS-1** -- The expression vector used to produce recombinant, His6-tagged CmPS-1, was derived from pET-15b and the *Escherichia coli* host was BL21(DE3)pLysS (26). To construct the expression vector, pET-15b/CmPS-1, the CmPS-1 ORF was amplified by PCR using 5’ primer, 5’-GCGGATCCATGGA-CATCAAAGAAGCAATCAG-3’ and 3’ primer, 5’-GCGGATCCATCCACAATCCACAAGAGGGTTTAACACCTG3’. BamHI sites (underlined) were included to facilitate the cloning procedures. A PCR-amplified fragment was digested with BamHI and ligated into pET-15b previously digested with BamHI and dephosphorylated. After transformation, the orientation of the insert was verified by restriction enzyme analysis and integrity of the cloning by sequencing.
To express His6-tagged CmPS-1, transformed BL21(DE3)pLysS harboring pET-15b/CmPS-1 was grown overnight at 37 °C in Luria broth from a single colony, which was subsequently used as the primary culture. The secondary culture was initiated with 1/500 dilution of primary culture in 750 ml of M9TB media (26) at 37 °C and continued until it reached 0.8 OD600. The culture was then cooled in a 16 °C water bath and expression induced, overnight, with 1 mM IPTG. Cells were then harvested and resuspended in 50 ml of B-PER solution (Pierce, Rockford, IL) supplemented with 0.5 M NaCl and 20 μg/ml of DNase I. Cell lysis (15 min at room temperature) was followed by centrifugation (27,000 × g for 30 min) and the supernatant was batch-incubated with Ni-agarose (His•Bind resin, Novagen, Madison, WI) charged previously with 50 mM NiSO4 and equilibrated with 50 mM Tris, pH 7.8, 0.5 M NaCl, and 5 mM imidazole. The resin was extensively washed with equilibration buffer, followed by a second wash with 30 mM imidazole in equilibration buffer. Bound proteins were eluted with 400 mM imidazole and dialyzed against 50 mM Tris, pH 7.5, and 1 mM EDTA. Proteins were further purified by loading onto HiTrap Q Sepharose. His6-tagged CmPS-1 was present in the flow-through fractions, which were pooled, dialyzed against 50 mM Tris, pH 7.5 and 100 mM NaCl, and stored at 4 °C.

Inhibition Assay -- To measure proteinase inhibitory activity, 10 µl (0.5 pmoles) stock solution of each serine proteinase substrate was incubated at 24 °C for 0.25-15 min with 10 µl of an equimolar amount of CmPS-1 in 50 mM Tris, pH 8.0, 100 mM NaCl, and 0.01 % Tween 20. After incubation, 80 µl of 0.5 mM chromogenic substrate solution was added, and residual
proteinase activities monitored by detecting the time-dependent change in absorbance at 405 nm. Proteinases and substrates (Sigma, St. Louis, MO) used in these assays were as follows: porcine pancreatic elastase and \( N\)-succinyl-Ala-Ala-Pro-Leu-\( p \)-nitroanilide; porcine pancreatic trypsin and \( N\)-\( p \)-tosyl-Gly-Pro-Arg-\( p \)-nitroanilide; bovine pancreatic \( \alpha \)-chymotrypsin and \( N\)-succinyl-Ala-Ala-Pro-Phe-\( p \)-nitroanilide; human plasma thrombin and \( N\)-\( p \)-tosyl-Gly-Pro-Arg-\( p \)-nitroanilide.

**Association Rate Constants** -- Association rate constants were determined according to the scheme described by Dahl et al. (25). Active site titration was carried out as described by Jiang and Kanost (27). Trypsin was titrated first by using \( p \)-nitrophenyl-\( p' \)-guanidinobenzoate (Sigma) (28) and this was then used to titrate alpha-1-antitrypsin (Calbiochem, La Jolla, CA). This alpha-1-antitrypsin was subsequently used as a secondary standard to titrate elastase and chymotrypsin. Titrated elastase was then used to titrate CmPS-1 and His6-tagged CmPS-1.

**Complex Formation and Cleavage Site Determination** -- Serpin-proteinase complexes were formed by incubating 50 pmoles of CmPS-1 with 50 pmoles of proteinases at 24 °C for 5 min in 20 mM Tris, pH 8.4, and 100 mM NaCl. The reactions were stopped by the addition of hot SDS-PAGE sample buffer, and the mixture was then subjected to Tricine-SDS-PAGE using a 10-20 % gradient gel (Novex, San Diego, CA). After blotting onto PVDF membrane, the amino terminal residues of the cleaved 4 kDa peptide were determined by sequencing analysis. Alternatively, the 4 kDa peptide fragment was first extracted from the gel, using a combination of 50 % acetonitrile plus 5 % formic acid, followed by 70 % isoprophenol plus 5 % formic acid.
The molecular weight of the pooled extracts was then determined by matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectrometry on a Biflex III (Bruker, Billerica, MA). Theoretical values for the molecular weight of the peptide were calculated using the PeptideMass program (http://expasy.ch/tools/peptide-mass.html).

**Western Analysis** -- After fractionation by SDS-PAGE, proteins were electro-transferred onto nitrocellulose membranes and probed with rabbit anti-serpin polyclonal antibodies (R306 [25]). For visualization, either chemiluminescence (Renaissance, NEN Life Science Products, Boston, MA) or color development (NBT/BCIP) was employed. CmPS-1 levels present in the phloem sap were determined by quantitative Western analysis, in which purified recombinant CmPS-1 was used as the standard.

**In Vivo and In Vitro Aphid Feeding Assays** -- The aphid species, *Myzus persicae*, was used in feeding studies. As a cucurbit-adapted control, *Aphis gossypii* was raised on *Cucurbita pepo* L. (squash) and employed in parallel feeding experiments. Aphid isolates were collected locally and maintained in cages within a greenhouse (16 h light: 8 h dark at 27 °C); *M. persicae* was reared on *Rhaphanus sativus* L. (radish). The *in vivo* influence of CmPS-1 and plant age on aphid survival were investigated by placing *M. persicae* and *A. gossypii* on newly expanded leaves of 10, 14, 21, 27, 35, and 42-day-old pumpkin plants. Aphids were confined to the feeding surface (second- through fifth-order veins) using clip cages, mounted on the abaxial leaf surface, with 50 individuals in each cage and two cages per plant. After 3 days, aphid survival and fecundity (nymphs produced per adult) were recorded, and then phloem sap was collected from the petiole of the treated leaf. Each experiment was performed on five plants and
experiments were repeated in triplicate. The level of CmPS-1 was analyzed by SDS-PAGE and Western analysis.

For *in vitro* aphid feeding experiments, 10 neonate *M. persicae* (aphids less than 12 h old) were placed in a sterile 1.5 ml Eppendorf tube which was then covered with a thin layer of Parafilm® (stretched 4 times in length). A 20 µl drop of feeding solution was then placed onto the Parafilm® surface and immediately covered by an additional layer of Parafilm®, forming a feeding sachet. Feeding solution was composed of 20% sucrose supplemented with each protein being tested. Aphid survival was recorded at 24 h intervals for three days. Enzymatic activity of each treated protein, maintained under feeding conditions within control sachets, was tested to confirm protein stability under these experimental conditions. Each feeding experiment was repeated at least 10 times.

**RESULTS**

*Cloning of Developmentally-Regulated 40 kDa Phloem Protein* -- SDS-PAGE analysis performed on phloem exudates, collected from different aged *C. maxima* plants, revealed the presence of a developmentally-regulated 40 kDa protein; the level of other phloem proteins appeared more-or-less invariant with plant age (Fig. 1A). For further study, this protein was subjected to internal microsequencing after lysyl endopeptidase digestion. A protein database search, using the resultant twenty amino acid peptide sequence, VLALPYSQGPDRFSMYFF, identified homologous regions within the serpin superfamily of serine PIs (25 and references therein). Degenerate PCR primers, based on the internal peptide sequence LPYSQGP and a sequence within plant serpins, EVNEEGTE (29–31), were used to amplify a 342 bp fragment.
This PCR product was cloned and sequenced and confirmed to contain the expected internal peptide sequence, PRRFSMYFF.

A pumpkin stem cDNA library was then used to obtain a full-length (1363 bp) clone, 
*CmPS-1*, encoding a 389 amino acid polypeptide with a predicted molecular weight of 42.8 kDa. In the deduced *CmPS-1* ORF, amino acid residues 221-240 precisely matched the microsequenced peptide fragment (Fig. 2, dashed line). During the cDNA library screening, a second gene (1470 bp), *CmPS-2*, was cloned and the encoded polypeptide (389 amino acid residues) found to have 72% identity to CmPS-1. However, the internal microsequence obtained from the 40 kDa phloem sap protein failed to match any region within the predicted CmPS-2 ORF. In addition, immunological analyses revealed that CmPS-2 was undetectable in phloem exudate (data not shown). Collectively, these results suggested that CmPS-2 is not a phloem sap protein. Characterization of this protein will be presented elsewhere.

The deduced CmPS-1 peptide sequence exhibited greatest identity with plant serpins; BSZ7 (50%) and BSZ4 (47%), isolated and characterized from barley seeds (29, 31), and WSZ1 (50%), isolated from wheat seeds (31), and a putative serpin, AtSLP (52%; *Arabidopsis thaliana* database, accession number AAD23667). Alignments with mammalian and insect members of the serpin superfamily, including human alpha-1-antichymotrypsin (HsACT), alpha-1-antitrypsin (HsA1AT), and *Manduca sexta* alaserpin (MsALA) showed overall amino acid sequence identities in the 25-30% range (50% similarity; Fig. 2). CmPS-1 contains the carboxy-terminal active site loop which represents the signature for the serpin family of serine
PIs; the P1-P1' bond is located within this consensus loop (Fig. 2, horizontal line).

Confirmation of the relatedness between CmPS-1 and the serpins present in barley and wheat seeds was obtained through Western analysis. Polyclonal antibodies raised against a barley serpin (25) cross-reacted with CmPS-1 present in phloem sap collected from *C. maxima* plants (Fig. 1B) and confirmed the developmental up-regulation of CmPS-1. An additional, but much weaker, immunoreactive-band was also detected at the 70 kDa region; the level of this protein remained constant over the developmental period examined (data not shown).

**Purification of CmPS-1 from *C. maxima* Phloem Exudate --** Biochemical studies of native proteins obtained from the phloem sap of plants are rare, largely due to the difficulty in collecting sufficient amounts of sap to permit biochemical purification. In this regard, cucurbits represent an excellent system as they allow the efficient collection of large amounts of exudate. To purify CmPS-1 from phloem sap, dialyzed exudate was subjected to a series of chromatographic steps (Fig. 3A). Anion followed by cation exchange chromatography (at pH 7.0) was used to separate CmPS-1 from phloem protein 1 (PP1; 96 kDa) and phloem protein 2 (PP2; 24 kDa), the two major constituents of the *C. maxima* phloem sap. As a result of Q Sepharose chromatography, a number of minor proteins were retained in the anion exchange column (data not shown), whereas most proteins, including CmPS-1, PP1 and PP2, were present in the flow-through fraction (Fig. 3B, compare lanes 1 and 2). The SP Sepharose cation exchange chromatographic step, carried out at pH 7.0, removed PP1, PP2 and a number of additional proteins from the CmPS-1 fraction (Fig. 3B, lane 3). A second cation exchange
fractionation, carried out at pH 5.5, removed the remaining high molecular weight proteins; CmPS-1 was eluted using a salt gradient (Fig. 3B, lane 4). Finally, Phenyl Sepharose with reverse gradient was used to purify CmPS-1 to near homogeneity (Fig. 3B, lane 5). Western analysis confirmed the identity of the protein preparation (Fig. 3C) and the purity of CmPS-1 was confirmed by mass spectrometry (data not shown). Special attention was taken to separate CmPS-1 from low molecular weight proteins because the phloem sap contains a number of small (3-10 kDa) PIs (16-18), which would have complicated any further biochemical analyses.

**Purification of Recombinant CmPS-1** -- An amino-terminal His6-tagged recombinant form of CmPS-1 was overexpressed in and purified from *E. coli* (Fig. 4). To obtain soluble cytosolic CmPS-1, low-temperature induction was employed during expression. As a first purification step, soluble *E. coli* extracts were loaded onto Ni agarose columns (Fig. 4A, lanes 1 to 5). Based on Western analyses (data not shown), the majority of the His6-tagged CmPS-1 was retained on the column. Elution of CmPS-1 was achieved using 400 mM imidazole in equilibration buffer and, following dialysis the eluate was subjected to anion exchange chromatography that allowed purified CmPS-1 to be collected from the flow-through fraction (Fig. 4A, lane 6); only two minor contaminants were still present. Western analysis showed that these contaminants were not due to proteolytic degradation of CmPS-1 (Fig. 4B).

**CmPS-1 Displayed Antielastase Activity** -- The P1 residue in the reaction center loop generally serves to determine the inhibitory specificity of serpins (27, 33). As CmPS-1 has a
valine in the putative P1 position, it was expected that it would exhibit inhibitory specificity against the elastase class of serine proteinases. Trypsin, chymotrypsin, and thrombin were employed as controls for our serine proteinase inhibition assays. As predicted, CmPS-1 displayed specific inhibition against elastase, but was inactive against trypsin, chymotrypsin, or thrombin (Fig. 5A). The fraction of complex-forming CmPS-1, X_{inh}, was estimated as 79% (Table I). Values for X_{inh} could not be obtained for trypsin, chymotrypsin, and thrombin, as the percent inhibition was negligible (< 5%). The calculated association rate constant, k_a, for elastase was $3.5 \pm 1.6 \times 10^5$ M$^{-1}$ s$^{-1}$ (Table I), a value close to that reported for M. sexta serpin-1F (27; Table I). Purified recombinant CmPS-1 exhibited similar inhibitory activity against elastase, having a k_a of $2.7 \pm 0.4 \times 10^5$ M$^{-1}$ s$^{-1}$ (Fig. 5B, Table I).

The specificity of inhibition between CmPS-1 and elastase was supported by the proteinase-serpin complex-formation assay (25). CmPS-1 formed an inhibitory complex (C) with porcine pancreatic elastase (Fig. 6A). By mixing CmPS-1 and elastase at a molar ratio of 1:1, a fraction of the CmPS-1 was carboxy-terminally cleaved (I*), and the released carboxy-terminal peptide (P) was detected at 4 kDa. CmPS-1 fragments (18-22 kDa) produced by substrate cleavage were also detected. In addition, CmPS-1 formed a complex with human neutrophil elastase (data not shown). However, CmPS-1 was unable to interact with trypsin to form an equivalent complex; rather, CmPS-1 was degraded by trypsin, resulting in the production of many lower molecular weight bands (Fig. 6B). Neither chymotrypsin nor thrombin formed an inhibitory complex with CmPS-1 (data not shown).
Cleavage Site Determination – The porcine pancreatic elastase cleavage site on CmPS-1 was first investigated using amino-terminal sequencing of the released carboxy-terminal peptide (approx. 4 kDa). Two amino acid peaks were obtained in each Edman degradation cycle and the molar ratio of the major to the minor peak was approx. 3:2. The amino-terminal sequences for these major and minor peaks were GIVSLP and SLPINR, respectively. These results indicated that CmPS-1 cleavage occurred at both the Val347-Gly348 and Val350-Ser351 peptide bonds; these cleavage site determinations were further confirmed by using mass spectrometry. The carboxy-terminal 4 kDa peptides were extracted from the acrylamide gel and subjected to MALDI-TOF analysis. Two peaks at 4742.68 and 4473.41 Da were obtained and these were close to being identical to the predicted values of 4742.61 and 4473.26 Da for CmPS-1 carboxy-terminal peptides starting at Gly348 and Ser351, respectively. The collective results from these analyses are presented in Fig. 7. The Val350-Ser351 site matches the predicted P1-P1’ site based on the amino acid sequence alignment. An alternate cleavage site exists at Val347-Gly348 and likely represents an inhibitory cleavage site.

In Vivo and In Vitro Effects of CmPS-1 on Aphid Survival – A potential role for serpins in plants is as a feeding deterrent/inhibitor of piercing-sucking and/or chewing insects. The piercing-sucking aphid, M. persicae, was used in experiments to ascertain whether the developmentally-regulated increase in CmPS-1, within the
phloem of pumpkin plants (Fig. 1), had any deleterious effect on this insect pest. For these experiments, aphids were placed on newly expanded leaves of various-aged pumpkin plants (Fig. 8) and observed over a 3 day period. The survival rate of *M. persicae* declined from 48% on 14-day-old plants to a plateau of approx. 18% on 28- to 42-day-old plants; a parallel and significant decline in fecundity was also observed (Fig. 8). These results established a strong negative correlation between the level of CmPS-1 in the phloem sap (Fig. 1) and aphid survival. Analysis of phloem sap, collected from control and aphid-infested leaves, established that *CmPS-1* expression was not altered in these aphid-feeding treatments (data not shown). Next, *in vitro* feeding experiments were conducted to ascertain, directly, the influence of CmPS-1 on the survival of *M. persicae*. For these experiments, CmPS-1 was employed at 200 µg ml⁻¹ (4.6 µM), which reflected the level present within the phloem sap of 42-day-old pumpkin plants. As alpha-1-antitrypsin (A1AT) has anti-elastase, -trypsin, and -chymotrypsin activities (34) it was included, along with BSA, as a control for these feeding studies. Interestingly, in these experiments, neither phloem-purified CmPS-1 nor A1AT had any significant effect on the survival of *M. persicae* (Table II).

**DISCUSSION**

In the present study, we establish that the phloem sap of *C. maxima* contains a novel 42 kDa PI, CmPS-1, belonging to the serpin superfamily. Sequence analysis and biochemical assays, conducted both with phloem-purified and recombinant CmPS-1, confirmed that this protein is
an active serpin capable of specifically inhibiting elastase. Confinement of CmPS-1 mRNA to companion cells (B. Xoconostle-Cázares and W.J. Lucas, unpublished results), in conjunction with the presence of CmPS-1 in the phloem sap, strongly implicates a role for CmPS-1 in the operation of the enucleate sieve tube system. Expression of CmPS-1 is developmentally regulated, as CmPS-1 within the phloem sap of 7- to 10-day-old C. maxima seedlings was either absent, or on the threshold of detection by Western analysis. Over the ensuing 4 weeks of seedling/plant development, the level of CmPS-1 increased by three orders of magnitude (10 nM to 5 µM). As chymotrypsin/trypsin PI activity was detectable within the phloem sap of all plants tested, general proteolytic protection of the proteins within the functional sieve tubes (both mobile and immobile) may well be afforded by the small molecular weight PIs (16–18). Thus, CmPS-1 may well function to protect the phloem against proteolytic activities associated with an endogenous elastase-like proteinase.

The present study implicated CmPS-1 in the protection of the pumpkin phloem sap against the intrusive feeding activities of aphids. Members of the serpin superfamily of serine PIs have been identified in animals, insects, and plants. In animals, serpins play pivotal roles in many physiological processes (35, 36), with perhaps the most well characterized being anti-thrombin III, whose function is central to the control of blood coagulation. Little is known concerning the function of the plant serpins, although a role in the protection of seeds against insects and pathogens has been proposed (25, 29-31, 37). Given that anti-elastase activity has been shown to impart resistance to bacteria (38), it is possible that CmPS-1 confers protection against bacteria, or phytoplasma that invade the tissues of the phloem (39, 40).
A central role for PIs in the defense response of plants to chewing insects is well supported by a wide range of experimental evidence (24, 41-44). The applicability of this paradigm to piercing-sucking insects has remained unresolved (24, 45). These insects appear to lack endo-proteinase activity within their digestive tract (46) and, therefore, have not been considered likely targets for PIs. However, as our in vivo aphid infestation experiments established that increasing insect mortality and CmPS-1 levels were highly correlated (Fig. 1 and Fig. 8), it would appear that CmPS-1 may well play a role in the defense of the phloem against piercing-sucking insects. As phloem-purified CmPS-1 had no detectable effect on aphid survival (Table II), it might well be that this serpin requires additional phloem proteins to form an active complex. Future experiments will be performed to test this hypothesis. Additionally, CmPS-1 may act upon homopteran insects other than aphids. As an alternative mode of action, the possibility cannot be discounted that CmPS-1 functions, in concert with other phloem and/or insect proteins, in the sealing (possibly by occlusion) of the stylet, once the insect has probed into a sieve element. Such a mode of action would be consistent with the general observation that insect feeding is impaired on resistant plant lines (47, 48).

Acknowledgements – We thank Dr. Jøern Hejgaard, Dept. of Biochemistry and Nutrition, Technical University of Denmark, Lyngby, Denmark for providing barley serpin polyclonal antibody R306. Thanks are also due to the members of the Ullman laboratory for assistance in aphid feeding experiments.
REFERENCES

1. Zimmermann, H. M., and Milburn, J. A. (1975) Encyclopedia of Plant Physiology, New Series: Transport in Plants I. Phloem Transport (Springer, New York), Vol. 1, 535pp

2. Schaller, A., and Ryan, C. A. (1996) Bioessays 18, 27-33

3. Jorgensen, R. A., Atkinson, R. G., Forster, R. L. S., and Lucas, W. J. (1998). Science 279, 1486-1487

4. Borkovec, V., Didehvar, F., and Baker, D. A. (1994) Plant Growth Regul. 15, 137-141

5. Kamboj, J. S., Blake, P. S., and Baker, D. A. (1998) Plant Growth Regul. 25, 123-126

6. Fisher, D. B., Wu, Y., and Ku, M. S. B. (1992) Plant Physiol. 100, 1433-1441

7. Tiedemann R., and Carstens-Behrens, U. (1994). J. Plant Physiol. 143, 189-194

8. Golecki, B., Schultz, A., and Thompson, G. A. (1999) Plant Cell 11, 127-140

9. Xoconostle-Cázares, B., Xiang, Y., Ruiz-Medrano, R., Wang, H.-L., Monzer, J., Yoo, B.-C., McFarland, K. C., Franceschi, V. R., and Lucas, W. J. (1999). Science 283, 94-98

10. Kühn, C., Franceschi, V. R., Schulz, A., Lemoine, R., and Frommer, W. B. (1997). Science 275, 1298-1300

11. Sasaki, T., Chino, M., Hayashi, H., and Fujiwara, T. (1998) Plant Cell Physiol. 39, 895-897

12. Ruiz-Medrano, R., Xoconostle-Cázares, B. and Lucas, W. J. (1999) Development 126, 4405-4419

13. Balachandran, S., Xiang, Y., Schobert, C., Thompson, G.A., and Lucas, W. J. (1997) Proc. Natl. Acad. Sci. USA 94, 14150-14155
14. Ishiwatari, Y., Fujiwara, T., McFarland, K. C., Nemoto, K., Hayashi, H., Chino, M., and Lucas, W. J. (1998) *Planta* **205**, 12-22

15. Chino, M., Hayashi, H., Nakumura, S., Oshima, T., Turner, H., Sabnis, D., Borkovec, V., Baker, D., Girousse, G., Bonnemain, J.-L., and Delrot, S. (1991) in *Recent Advances in Phloem Transport and Assimilate Compartmentation*, eds. Bonnemain, J.-L., Delrot, S., Dainty, J. & Lucas, W. J. (Ouest Editions, Nantes), pp. 64-73

16. Murray, C., and Christeller, J. T. (1995) *Biol. Chem. Hoppe-Seyler* **376**, 281-287

17. Habu, Y., Fukushima, H., Sakata, Y., Abe, H., and Funada, R. (1996) *Plant Mol. Biol.* **32**, 1209-1213

18. Christeller, J. T., Farley, P. C., Ramsay, R. J., Sullivan, P. A., and Laing, W. A. (1998) *Eur. J. Biochem.* **254**, 160-167

19. Broadway, R. M., Duffey, S. S., Pearce, G., and Ryan, C. A. (1986) *Entomol. Exp. Appl.* **41**, 33-38

20. Duffey, S. S., and Stout, M. J. (1996) *Arch. Insect Biochem. Physiol.* **32**, 3-37

21. Wieczorek, M., Otlewski, J., Cook, J., Parks, K., Leluk, J., Wilimowska-Pelc, A., Polanowski, A., Wilusz, T., and Laskowski, M. (1985). *Biochem. Phys. Res. Commun.* **126**, 646-652

22. MacGibbon, D. B., and Mann, J. D. (1986) *J. Sci. Food Agric.* **37**, 515-522

23. Christeller, J. T., Laing, W. A., Markwick, N. P., and Burgess, E. P. J. (1992) *Insect Biochem. Molec. Biol.* **22**, 735-746

24. Jongsma, M. A., and Bolter, C. (1997) *J. Insect Physiol.* **43**, 885-895

25. Dahl, S. W., Rasmussen, S. K., and Hejgaard, J. (1996) *J. Biol. Chem.* **271**, 25083-25088
26 Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) *Meth. Enzymol.* **185**, 60-69

27 Jiang, H., and Kanost, M. R. (1997) *J. Biol. Chem.* **272**, 1082-1087

28 Chase, T., and Shaw, E. (1967) *Biochem. Biophys. Res. Commun.* **29**, 508-514

29 Brandt, A., Svendsen, I., and Hejgaard, J. (1990) *Eur. J. Biochem.* **194**, 499-505

30 Rasmussen, S. K. (1993) *Biochim. Biophys. Acta* **1172**, 151-154

31 Rasmussen, S. K., Dahl, S. W., Nørgard, A., and Hejgaard, J. (1996) *Plant Mol. Biol.* **30**, 673-677

32 Kanost, M. R., Prasad, S. V., and Wells, M. A. (1989) *J. Biol. Chem.* **264**, 965-972

33 Bode, W., Meyer, E., and Powers, J. C. (1989) *Biochemistry* **28**, 1951-1963

34 Beatty, K., Bieth, J., and Travis, J. (1980) *J. Biol. Chem.* **255**, 3931-3934

35 Stein, P. E., and Carrell, R. W. (1995) *Nature Structural Biol.* **2**, 96-113

36 Carrell, R. W., and Stein, P. E. (1996) *Biol. Chem. Hoppe-Seyler* **377**, 1-17

37 Dahl, S. W., Rasmussen, S. K., Petersen, L.C., and Hejgaard, J. (1996) *FEBS Lett.* **394**, 165-168

38 Simpson, A. J., Maxwell, A. I., Govan, J. R. W., Haslett, C., and Sallenave, J. M. (1999) *FEBS Lett.* **452**, 309-313

39 Lherminier, J., Bonfiglioli, R. G., Daire, X., Symons, R. H., and Boudon-Padieu, E. (1999) *Mol. Cell Probe* **13**, 41-47

40 Khadnair, A. H., and Evans, I. R. (2000) *Microbiol. Res.* **155**, 53-57

41 Ryan, C. A. (1990) *Annu. Rev. Phytopathol.* **28**, 425-429
42 Ryals, J. A., Neuenschwander, U. H., Willits, M. G., Molina, A., Steiner, H. Y., and Hunt, M. D. (1996) *Plant Cell* **8**, 1809-1819

43 Koiwa, H., Bressan, R. A., and Hasegawa, P. M. (1997) *Trends Plant Sci.* **2**, 379-384

44 Ryan, C. A., and Pearce, G. (1998) *Annu. Rev. Cell Devel. Biol.* **14**, 1-17

45 Rahbé, Y., and Febvay, G. (1993) *Entomol. Exp. Appl.* **67**, 149-160

46 Rahbé, Y., Sauvion, N., Febvay, G., Peumans, W. J., and Gatehouse, A. M. R. (1995) *Entomol. Exp. Appl.* **76**, 143-155

47 Klinger, J., Powell, G., Thompson, G. A., and Isaacs, R. (1998) *Entomol. Exp. Appl.* **86**, 79-88

48 Caillaud C. M., and Niemeyer, H. M. (1996) *Experientia* **52**, 927-931
FIGURE LEGENDS

FIG. 1. Developmentally-regulated 40 kDa protein within the phloem sap of Cucurbita maxima represents a novel plant serpin, CmPS-1. A: SDS-PAGE analysis of phloem sap collected from the petioles of newly-expanded leaves of 10-, 14-, 21-, 27-, 35- and 42-day-old Cucurbita maxima (pumpkin) plants. Proteins stained with Coomassie Brilliant Blue. B: Immunodetection of CmPS-1 by the anti-serpin antibody, R306 (25). Detection used alkaline phosphatase-linked secondary antibody and 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium as substrates. Based on quantitative Western analysis, the level of CmPS-1 increased from less than 5 µg ml⁻¹ (< 11 nM) in 10-day-old plants to approx. 200 µg ml⁻¹ (4.6 µM) in 42-day-old plants. Arrowheads indicate position of CmPS-1.

FIG. 2. Sequence alignment of the deduced CmPS-1 polypeptide and homology with other members of the serpin superfamily. Amino acids of CmPS-1 matching the microsequenced peptide are indicated by a dashed line; amino acid residues conserved among serpins are depicted by shaded areas. Reactive center loop sequences containing the P₁-P₁´ peptide bond cleavage sites are depicted with a horizontal bar. CmPS-1 (accession number AF284038); BSZ7, barley serpin Z7 (31; accession number CAA64599); BSZ4, barley serpin Z4 (25; accession number CAA66232); WSZ1, wheat serpin Z1 (31; accession number S65782); AtSLP, Arabidopsis thaliana serpin-like protein (accession number AAD23667); MsALA, Manduca sexta alaerpin (32; accession number P14754); HsACT, human α-1-antichymotrypsin (accession number
HsA1AT, human alpha-1-antitrypsin (accession number P01009). Note that, currently, there are 10 putative serpin genes deposited in the *A. thaliana* database.

**FIG. 3. Purification of CmPS-1 from *Cucurbita maxima* phloem exudate.** A: Schematic representation of the chromatographic steps used to purify CmPS-1. B: SDS-PAGE analysis of the phloem proteins present after each purification step. *Lane 1*, dialyzed phloem sap (30 µg); *lane 2*, flow-through of Q Sepharose (30 µg); *lane 3*, flow-through of SP Sepharose (pH 7.0, 20 µg); *lane 4*, fractionation by SP Sepharose (pH 5.5, 20 µg); *lane 5*, fractionation by Phenyl Sepharose in a reverse gradient (2 µg). C: Western analysis of purified CmPS-1 using R306. Protein staining and immunodetection were carried out as described in FIG. 1.

**FIG. 4. Purification of recombinant His$_6$-tagged CmPS-1.** A: SDS-PAGE analysis of the proteins present after each purification step. *Lanes 1 -- 5*, nickel-agarose affinity chromatography; *lane 6*, HiTrap Q Sepharose fraction. Loading was as follows: *lane 1*, E. coli lysate; *lane 2*, flow-through; *lane 3*, equilibration-wash fraction; *lane 4*, 30 mM imidazole wash fraction; *lane 5*, eluate obtained with 400 mM imidazole; *lane 6*, flow-through fraction of HiTrap Q Sepharose; B: Western analysis of purified His$_6$-tagged CmPS-1 using R306. Protein staining and immunodetection were carried out as described in FIG. 1.

**FIG. 5. Inhibition of elastase by phloem-purified and recombinant CmPS-1.** CmPS-1 (A) and recombinant His$_6$-tagged CmPS-1 (B) was pre-incubated with elastase (♦), or trypsin (♦),
for the indicated time and residual proteinase activity then measured. Equivalent experiments were performed with chymotrypsin and thrombin and the level of inhibition was found to be < 5% (data not shown). Inhibition is defined as the ratio of the decreased proteinase activity in the presence of the added serpin to that measured in the absence of serpin. Values are averages from three independent experiments. Fitted curves for elastase inhibition were derived based on (25).

FIG. 6. Formation of SDS-stable CmPS-1-serine proteinase complexes. Active-site titrated CmPS-1 (50 pmoles) was mixed with an equimolar amount of titrated elastase (A) or titrated trypsin (B) and incubated at 24 °C for 5 min before the addition of hot SDS-PAGE sample buffer. Reaction products were immediately analyzed by tricine-SDS-PAGE. A: Lane 1, CmPS-1; lane 2, elastase; lane 3, CmPS-1 plus elastase. B: Lane 1, CmPS-1; lane 2, trypsin; lane 3, CmPS-1 plus trypsin. The positions of intact CmPS-1-proteinase complexes (C), intact CmPS-1 inhibitor (I), cleaved CmPS-1 inhibitor (I*), intact elastase (Es), intact trypsin (Tr) and released carboxy-terminal peptides (P) are indicated to the right.

FIG. 7. Reactive center loop sequences and cleavage sites for CmPS-1 and related serpins. Experimentally determined cleavage sites of serpins, by serine proteinases (Es, elastase; Tr, trypsin; Ch, chymotrypsin), indicated by arrowheads. CmPS-1 P1 and P1’ residues were predicted on the basis of homology within the reactive center loop. Cleavage sites of BSZx and WSZ1 were reported by Dahl et al. (25) and for Manduca sexta serpin-1F (MsS-1F) by Jiang and Kanost (27).
FIG. 8. **Influence of plant age on the survival and fecundity of phloem-feeding insects.** The sucking-piercing aphid species, *Myzus* persicae, was used to demonstrate that a negative correlation exists between aphid survival/fecundity and the amount of CmPS-1 present in *C. maxima* phloem sap. (Plants used for these experiments were common to those employed for the studies presented in FIG. 1). All experiments were performed on true leaves and, hence, earlier time points were precluded. Values represent mean ± SEM. Control infestation experiments, performed with *Aphis gossypii* (raised on *C. pepo*), resulted in 50% aphid survival and 1.5 offspring/adult over the 28 – 42 day growth period.
### Table I

**Kinetic parameters for CmPS-1 interaction with porcine pancreatic elastase**

Association rate constant ($k_a$) and the fraction of complex-forming CmPS-1 ($X_{inh}$) were determined as described in (25). Values represent mean ± SEM (n=3). Data for *Manduca sexta* serpin-1F (MsS-1F) and human alpha-1-antitrypsin (HsA1AT) are from Jiang and Kanost (27) and Beatty et al. (34), respectively.

| Serpin                  | $k_a$ (M$^{-1}$ s$^{-1}$) | $X_{inh}$ (%) |
|-------------------------|---------------------------|---------------|
| CmPS-1                  | $3.5 \pm 1.6 \times 10^5$ | $79.3 \pm 3.6$ |
| His6-tagged CmPS-1      | $2.7 \pm 0.4 \times 10^5$ | $79.7 \pm 3.0$ |
| MsS-1F                  | $3.1 \pm 0.1 \times 10^5$ | --            |
| HsA1AT                  | $1.0 \pm 0.2 \times 10^5$ | --            |
**Table II**

*Effect of CmPS-1 on Myzus persicae survival during in vitro feeding assays*

*In vitro* aphid feeding assays were performed as described in Experimental Procedures. In control experiments, aphids were fed on distilled water, 20% sucrose, or 20% sucrose solution supplemented with either protein preparation buffer (2.5 mM Hepes, pH 7.5, and 10 mM NaCl) or BSA (200 µg ml⁻¹). Concentration of CmPS-1 and human alpha-1-antitrypsin (HsA1AT) was 200 µg ml⁻¹ delivered in a 20% sucrose solution. Ten neonate *M. persicae* were used per treatment and survival data were collected after 24, 48, and 72 h feeding periods, and represent mean ± SEM for ten replicate experiments.

| Period | Water (µ) | 20% Sucrose (µ) | Buffer (µ) | BSA (µ) | α1AT (µ) | CmPS-1 (µ) |
|--------|-----------|-----------------|------------|---------|---------|------------|
| Day 1  | 2.2 ± 1.7 | 6.9 ± 2.1       | 6.4 ± 1.3  | 6.5 ± 2.1 | 6.5 ± 0.7 | 7.9 ± 1.4  |
| Day 2  | 0.4       | 6.6 ± 2.3       | 6.3 ± 2.0  | 6.6 ± 1.3 | 7.5 ± 1.3 | 6.9 ± 1.4  |
| Day 3  | 0         | 7.2 ± 2.8       | 6.5 ± 1.3  | 6.3 ± 1.3 | 6.6 ± 1.9 | 5.8 ± 2.3  |
Fig. 1
Yoo et al. (2000)
Fig. 2
Yoo et al. (2000)

Cucurbita maxima, CmPS-1
Hordeum vulgare, BSZ7
Hordeum vulgare, BSZ4
Triticum aestivum, WSS1
Arabidopsis thaliana, AtSLP
Manduca sexta, MsALA
Homo sapiens, HsACT
Homo sapiens, HsA1AT
Fig. 3
Yoo et al. (2000)

(A) Schematic representation of the purification process:

- Phloem Exudate
- Q Sepharose
- SP Sepharose (pH 7.0)
- SP Sepharose (pH 5.5)
- Phenyl Sepharose

(B) Gel electrophoresis image with markers (M) and samples 1 to 5.

(C) Western blot analysis result.
Fig. 5
Yoo et al. (2000)
Fig. 6

Yoo et al. (2000)
Fig. 8
Yoo et al. (2000)
Characterization of Cucurbita maxima Phloem Serpin-1 (CmPS-1): a Developmentally Regulated Elastase Inhibitor
Byung-Chun Yoo, Koh Aoki, Yu Xiang, Leslie R. Campbell, Richard J. Hull, Beatriz Xoconostle-Cazares, Jan Monzer, Jung-Youn Lee, Diane E. Ullman and William J. Lucas
J. Biol. Chem. published online August 25, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M006060200

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