Wound-Inducible Proteinase Inhibitors in Pepper. Differential Regulation upon Wounding, Systemin, and Methyl Jasmonate[1]

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Seven small (approximately 6,000 D) wound-inducible proteinase inhibitor proteins were isolated from leaves of pepper (*Capsicum annuum*) plants that are members of the potato inhibitor II family. N-terminal sequences obtained indicated that the pepper leaf proteinase inhibitors (PLPIs) exhibit homology to two GenBank accessions that code for preproteins containing three isoinhibitors domains each that, when post-translationally processed, can account for the mixture of isoinhibitors that are reported herein from pepper leaves. A constitutive level of PLPI proteins was found in pepper leaves, and these levels increased up to 2.6-fold upon wounding of the lower leaves. Exposing intact plants to methyl jasmonate vapors induced the accumulation of PLPIs. Supplying excised young pepper plants with water through the cut stems induced PLPI proteins to levels higher than those found in intact plants, but with high variability. Supplying the excised plants with systemin did not result in an increase of PLPI levels that were statistically higher than levels found in excised plants. Gel-blot analyses of PLPI induction revealed the presence of two mRNA bands, having slightly different mobilities in agarose gels. Only the low M_r mRNA is present in untreated control plants, and it appears to be responsible for the constitutive levels of PLPI found in leaves. Both mRNA species are wound- and methyl jasmonate-inducible. Only the low-M_r species is weakly induced by systemin, indicating a differential expression of the two PLPI species.

Wound-inducible proteinase inhibitor proteins in plant leaves, when ingested by polyphagous insects, interact with proteases of the insect midgut and lead to an arrest of growth and development, and occasionally to death (Ryan, 1990). Proteinase inhibitors have been identified in seeds or leaves of nearly all agriculturally important crop plants, where they play important roles in defense against herbivores and pathogens, while also serving as storage proteins (Plunkett et al., 1982; Brown and Ryan, 1984; Cleveland et al., 1987; Thornburg et al., 1987; Bradshaw et al., 1989; Ryan, 1990; Pearce et al., 1993; Rohrmeier and Lehle, 1993; Saarikoski et al., 1996; Zhao et al., 1996; Ferrasson et al., 1997; Karban and Baldwin, 1997; Koiwa et al., 1997).

Tomato plants have been used as a model to study systemic defense gene expression in plants in response to insect and pathogen attacks. An 18-amino acid polypeptide called systemin (Pearce et al., 1991) has been shown to be a systemic wound signal in leaves that initiates a cascade of events that leads to the expression of several defense-related genes (Ryan, 2000). Systemin is processed from a 200-amino acid precursor named prosystemin. A prosystemin cDNA was cloned from tomato (McGurl et al., 1992) and other closely related species of the *Solanaceae* family (Constabel et al., 1998), among which pepper (*Capsicum annuum*) showed the most divergent sequence, i.e. 73% identity with the tomato prosystemin (Constabel et al., 1998). Pepper systemin, when supplied to pepper plants, induced an Inh II mRNA in leaves. The pepper Inh II exhibited a higher M_r than Inh II mRNA found in tomato leaves (Constabel et al., 1998), suggesting that the gene duplication-elongation events may have increased the size of the gene, similar to that found for the Inh II gene in tobacco leaves (Atkinson et al., 1993).

Constabel and Ryan (1998) had previously evaluated the wound-inducibility of polyphenol oxidase (PPO) enzyme in pepper, which indicated that PPO was weakly inducible by wounding or methyl jasmonate (MeJ), a product of the octadecanoid pathway and a potent elicitor of wound-related proteins in tomato. Proteins involved in wound-healing processes such as shikimate dehydrogenase and peroxidase were also found to be wound-inducible in pepper leaves (Diaz and Merino, 1997).

The identification of a pepper prosystemin in pepper leaves, the inducibility of PPO by wounding and MeJ, and the identification of a systemin-inducible Inh II mRNA in gel-blot analyses indicated that the systemin signaling pathway is most probably present in this species. However, the physical and biochemical properties of proteinase inhibitors that are induced in pepper leaves have heretofore not been investigated. Therefore, a study to investigate the defense response of pepper leaves was initiated.

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Seven wound-inducible proteinase inhibitors present in the leaves of pepper plants were identified, isolated, and characterized.

The data here reported provide the initial experiments to isolate and characterize wound-inducible pepper leaf proteinase inhibitor (PLPI) proteins, and to investigate expression of the PLPI genes in leaves in response to wounding, systemin, and MeJ.

RESULTS

PLPI Purification and Characterization

Table I shows the protocol for the purification of PLPIs. The procedure was basically as described by Pearce et al. (1993) for the purification of tobacco proteinase inhibitors. The inhibitors were initially purified by affinity chromatography (see "Materials and Methods"), and separated into isoinhibitors using a C-18 reversed phase (RP)-HPLC column with a gradient of 0% to 40% (v/v) acetonitrile in 0.1% (v/v) TFA. The protein profile revealed seven major protein peaks (Fig. 1) that were identified according to their retention times (near integer in minutes), i.e. 34, 35, 40, 41, 43, 45, and 46. Using a combination of strong cation exchange-HPLC and C-18 RP-HPLC, each isoinhibitor was shown to be as a pure protein by SDS/urea-PAGE and silver staining (Fig. 2). The quantities recovered for each isoinhibitor are shown in Table II. N-terminal amino acid sequences of each PLPI are presented in Table III, together with their molecular masses, their inhibitory activities against trypsin and chymotrypsin, and their K_i for chymotrypsin and trypsin. Titration curves of PLPIs for the inhibition of trypsin and chymotrypsin by each isoinhibitor are shown in Figure 3. PLPIs 34, 35, 45, and 46 inhibited trypsin and chymotrypsin, whereas isoinhibitors 40, 41, and 43 inhibited chymotrypsin, but not trypsin. The K_i for chymotrypsin inhibition among the isoinhibitors ranged from 1.0×10^{-09} to 8.0×10^{-11}M, and for trypsin the range was 1×10^{-8} to 6×10^{-9} M (Table III).

A GenBank search using the partial amino acid sequences of the seven inhibitors revealed homology with two GenBank accessions reported from pepper plants. The partial N-terminal sequences for PLPIs 34, 35, and 40 (20 residues) exhibited 100% identity to regions of GenBank accession nos. AF221097 (R. Shin, G.J. Lee, C.J. Park, T.Y. Kim, J.S. You, Y.W. Nam, and K.H. Paek, unpublished data) and AF039398 (S.H. Kim, D.S. Choi, and K.W. Lee, unpublished data; Fig. 4, A and B). PLPIs 45 and 46 (20 residues) exhibited 95% identity to a single region of the GenBank accession no. AF221097 (R. Shin, G.J. Lee, C.J. Park, T.Y. Kim, J.S. You, Y.W. Nam, and K.H. Paek, unpublished data; Fig. 4A). The partial sequences of PLPIs 41 (22 residues) and 43 (20 residues) were 100% identical to a single region of the GenBank accession no. AF039398 (S.H. Kim, D.S. Choi, and K.W. Lee, unpublished data; Fig. 4B). Accession nos. AF221097 and AF039398 code for Ser proteinase inhibitors of the potato Inh II family, and each are composed of multidomain structures (Fig. 4C).

All seven of the pepper leaf isoinhibitors had mass numbers between 5,586 and 5,952 D determined by matrix assisted laser desorption ionization-mass spectrometry (Table III). Mass spectroscopy also confirmed that each of the PLPIs was homogeneous.

Induction of PLPIs by Wounding, Systemin, and MeJ

Antibodies generated against the total mixture of PLPIs obtained by the affinity chromatography were used in radial immunodiffusion assays to evaluate the wound-inducibility of the isoinhibitors. A low level of PLPI protein was detected in control plants, estimated to range from 25 to 35 μg mL^{-1} of leaf juice (Fig. 5). Wounded plants accumulated PLPI protein, which were detectable as early as 4 h after wounding, and the levels continued to increase through 48 h (Fig. 5), reaching about 70 μg mL^{-1}. Supplying water or buffer to excised plants had a moderate effect over intact plants incubated under the same conditions, an effect that is likely due to a wound signal that is released from the cut site on the stems. Intact plants subjected to MeJ vapors accumulated levels of PLPI protein equal to the highest levels found in response to wounding. Supplying pepper plants with pepper systemin polypeptide through the cut stems resulted in PLPI levels that were statistically indistinguishable from MeJ and water/buffer controls (Table IV). SA, a suppressor of the wound response in tomato (Doares et al., 1995), also inhibited the wound induction of PLPIs in pepper plants, and appeared to slightly suppress the levels of inhibitors compared with the excised plants supplied with water/buffer (Table IV).

Table I. *Pepper proteinase inhibitor proteins table of purification*

Purification Steps	Volume	Protein	Inhibitors	Activity	Specific Activity
	mL	*mg*		*CUI*[a]	*CUI*[a]*/mg protein*
Crude extract	3,300	2,425	54.7	440,000	182
Ammonium sulfate precipitation.	1,620	2,284	53.3	438,000	192
Heat treatment + dialysis	2,110	527	38.7	298,000	566
Chymotrypsin affinity column	90	36	36.0	259,000	7,194
HPLC of most abundant inhibitors	3.3	9.94	9.94		

[a] CUI, Chymotrypsin units inhibited.

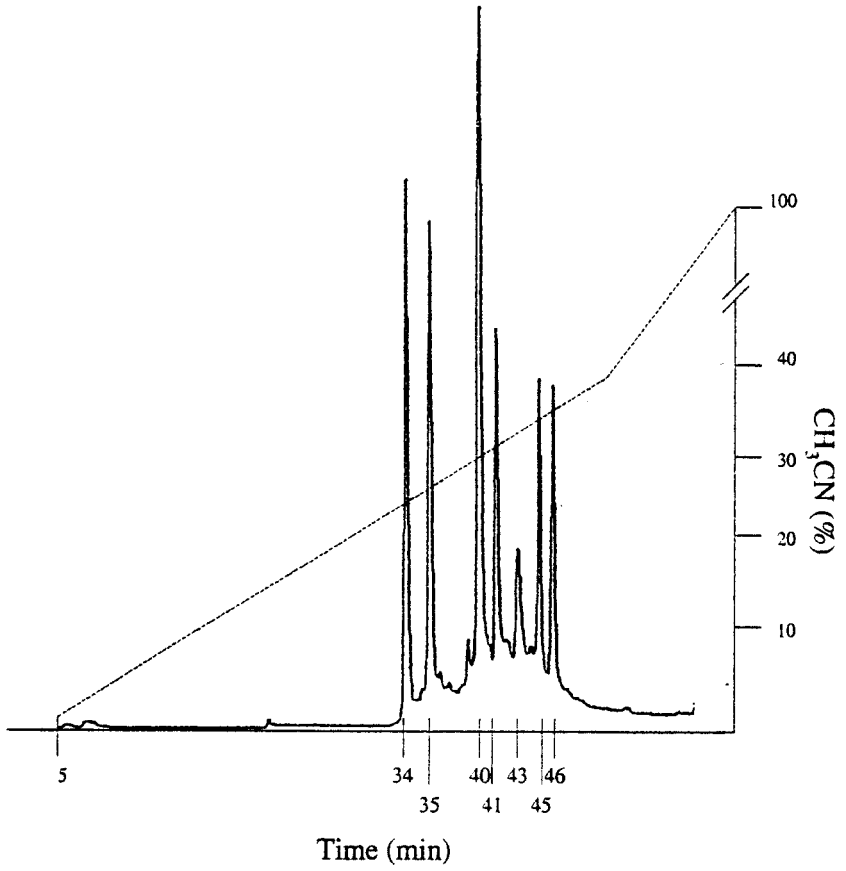

Figure 1. PLPIs separation on C-18 RP-HPLC. One-fourth of the inhibitors eluted from the chymotrypsin affinity column was loaded into the C-18 column and eluted with a gradient of 0% to 40% (v/v) acetonitrile in 0.1% (v/v) TFA for 50 min. The seven major peaks were eluted at approximately 34, 35, 40, 41, 43, 45, and 46 min and were purified to homogeneity using a combination of C-18 RP-HPLC and SCX-HPLC.

Eight varieties of pepper were evaluated for the presence and inducibility of PLPIs (Fig. 6). All varieties tested showed constitutive level of PLPI protein and all responded to wounding. The inducibility ranged from 1.8-fold (Serrano Chili) to 4.1-fold (New Mexico no. 6) over the control levels.

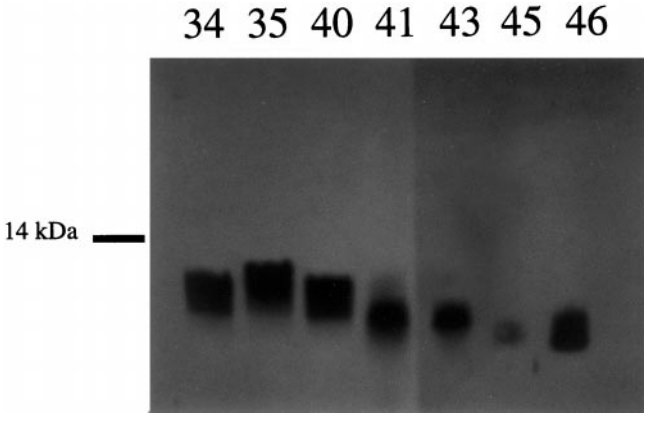

Figure 2. SDS/urea-PAGE of the seven purified PLPIs. Fifteen micrograms of each PLPI was loaded on the gel. The gel was silver stained and part of the gel containing the lanes with PLPIs 43, 45, and 46 was developed longer.

Analyses of PLPI Gene Expression

Gel-blot analyses were performed using a 634-bp cDNA fragment obtained by reverse transcriptase (RT)-PCR (amino acid residues 21–204 plus 81 bp of 3′-untranslated region) deduced from the GenBank sequence AF221097. Two distinct but closely migrating mRNA bands were detected, here called fast (F-band) and slow (S-band) bands (Fig. 7). The F-band mRNA was found in untreated plants in cotyledons, lower and apical leaves, and increased in response to wounding. By quantifying the bands in gel-blot analyses, increases of 90%, 290%, and 160% were found over the control levels in cotyledons, and

Table II. *Quantities obtained for each PLPI after using C-18 RP-HPLC and SCX-HPLC purification*

PLPI Retention Time on C-18 RP-HPLC	Inhibitor Protein	Percent
min	*mg*	
34	2.27	23
35	1.87	19
40	2.87	30
41	0.96	9
43	0.64	6
45	0.48	5
46	0.85	8

Table III. *Characteristics of the isolated PLPIs*

Inhibitor Retention Time	N-Terminal Sequence[a]	Mass	Inhibitor of Chymotrypsin	Inhibitor of Trypsin	K_i Chymotrypsin	K_i Trypsin
min		*D*			*M*	
34	RICTNCCAGRKGCNYYSADG	5,646	Yes	Yes	8×10^{-11}	5×10^{-9}
35	.....	5,671	Yes	Yes	3×10^{-10}	4×10^{-9}
40	.....	5,681	Yes	No	1×10^{-10}	–
41	AKEP.....	5,952	Yes	No	6×10^{-10}	–
43	EP.....	5,755	Yes	No	7×10^{-10}	–
45	.L.....	5,716	Yes	Yes	1×10^{-9}	1×10^{-8}
46	.L.....	5,586	Yes	Yes	3×10^{-10}	6×10^{-9}

[a] A dot represents an amino acid identical to PLPI 34.

lower and apical leaves, respectively (Fig. 7, A and B). The S-band, which is not found in tissues of untreated plants, was induced to accumulate in cotyledons and lower leaves in response to wounding (Fig. 7B). In cotyledons, the S-band was induced as early as 2 h after wounding and its level stayed high throughout the experiment. The S-band increased within 2 h in lower leaves and peaked at 4 to 6 h after wounding (Fig. 7B). The F-band species was only weakly induced by systemin (Fig. 8A), but F-band and S-band in cotyledons and leaves increased in response to MeJ (Fig. 8C). Water controls showed a slight increase in PLPI mRNA (Fig. 8B).

DISCUSSION

Seven isoinhibitors of the potato inhibitor II family of proteinase inhibitors were isolated from leaves of MeJ-treated pepper plants. Using a purification method similar to the one described by Pearce et al. (1993) for tobacco proteinase Inh II family members (tobacco trypsin inhibitor [TTI];Table I), nearly 10 mg of inhibitors was isolated from approximately 600 pepper plants. The method described here resulted in a 40-fold purification of PLPIs (based on specific activity), with a recovery of 59% of the initial activity (Table I). MeJ treatment proved to be an effective method to enrich the yields of PLPIs, as it increased the levels of the inhibitors in the plants by a factor of about 2. Following chymotrypsin affinity purification, the inhibitors were further separated using C-18 RP-HPLC and SCX-HPLC. Among the seven inhibitors that were purified (Fig. 1; Table II), PLPIs 34, 35, and 40 were the most abundant, and together represented 20% of the affinity-purified proteins that were added to the SCX-HPLC column. However, the combined purified inhibitors (Table II) represented only 33% of the total amount of inhibitors eluted from the affinity column. Thus, not all inhibitory activity was recovered. The affinity column used was specific for chymotrypsin, and strictly trypsin inhibitors may have been present that were not purified by this method.

Figure 2 shows the seven PLPI proteins that separated in SDS-urea/PAGE and were silver stained. All PLPIs showed slightly different mobilities. PLPIs 34, 35, 40, and 41 stained quickly with the silver reagent and PLPIs 43, 45, and 46 developed slowly. N-terminal sequences, masses, inhibitory activities against trypsin and chymotrypsin, and the inhibition constant for chymotrypsin and trypsin (Table III) are characteristic of a family of small isoinhibitors, with similarities to the small isoinhibitors (TTIs) isolated from tobacco leaves (Pearce et al., 1993).

Trypsin and chymotrypsin titration curves for all PLPIs are shown in Figure 3. PLPIs 34, 35, 45, and 46 were potent inhibitors of both enzymes. PLPIs 40, 41, and 43 inhibited only chymotrypsin. These differences reflect the amino acid in or near the active sites of the inhibitors. It is not known whether the inhibitors 34, 35, 45, and 46 have separate reactive sites for chymotrypsin and trypsin (double headed), or if a single site can interact with both enzymes. Examples of inhibitors having a single reactive site, but inhibiting chymotrypsin and trypsin has been reported in

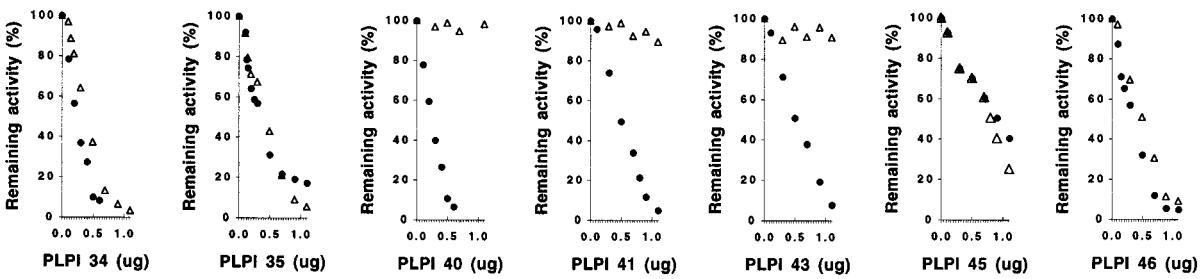

Figure 3. Titration of chymotrypsin and trypsin by each purified PLPI. Trypsin and chymotrypsin activities were measured spectrophotometrically in the presence of increasing amounts of the inhibitors. ●, Chymotrypsin activity. △, Trypsin activity.

A

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          1                                                    50
AF221097  MAVHKEVSFL AFLLVLEILL LHVDAKACSE ENAENRICTN CCAGRKGCNY
PLPI  34  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~RICTN CCAGRKGCNY
PLPI  35  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~RICTN CCAGRKGCNY
PLPI  40  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~RICTN CCAGRKGCNY

          51                                                   100
AF221097  YSADGTFICE GESDPNNPKA CPRYCDTRIA YSKCPRSEGN AENRICTNCC
PLPI  34  YSADG~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
PLPI  35  YSADG~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
PLPI  40  YSADG~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          101                                                  150
AF221097  AGRKGCNYYS VIGTFICEGE SDPNNPKACP RNCDTRIAYS KCPRSEGNAE

          151                                                  200
AF221097  NRLCTNCCAG RKVCNYYSAD GTFICEGESD PNNPKACPRN CDPNIAYSLC
PLPI  45  ~RLCTNCCAG RKGCNYYSAD G~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
PLPI  46  ~RLCTNCCAG RKGCNYYSAD G~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          201
AF221097  LYEK
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B

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          1                                                    50
AF039398  MAVPKEVSFL ASLLVLGILL LHVDAKACSQ RNAKEPICTN CCAGRKGCNY
PLPI  41  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~AKEPICTN CCAGRKGCNY
PLPI  43  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~EPICTN CCAGRKGCNY

          51                                                   100
AF039398  YSADGTFICE GESDPNNPKP CTLNCDPRIF YSKCPRSEGN AENRICTNCC
PLPI  41  YSADG~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
PLPI  43  YSADG~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
PLPI  34  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~RICTNCC
PLPI  35  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~RICTNCC
PLPI  40  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~RICTNCC

          101                                                  150
AF039398  AGRKGCNYYS ADGTFICEGE SDPNNPKPCT LNCDPRIFYS KCPRSEASAE
PLPI  34  AGRKGCNYYS ADG~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
PLPI  35  AGRKGCNYYS ADG~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
PLPI  40  AGRKGCNYYS ADG~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          151                                                  200
AF039398  QPICTNCCAG LKGCNYYNAD GTFICEGESD PNHPKACPKN CDPNIAYSLC

          201
AF039398  LYEK
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C

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Tobacco JQ2153
                                              KACTLNCDPRIAYGVCPRSEEKKND    Domain 1
          RICTNCCAGTKGCKYFSDDGTFVCEGESDPRNPKACTLNCDPRIAYGVCPRSEEKKND    Domain 2
          RICTNCCAGTKGCKYFSDDGTFVCEGESDPRNPKACPRNCDPRIAYGICPLAEEKKND    Domain 3
          RICTNCCAGKKGCKYFSDDGTFVCEGESDPKNPKACPRNCDGRIAYGICPLSEEKKND    Domain 4
          RICTNCCAGKKGCKYFSDDGTFVCEGESDPKNPKACPRNCDGRIAYGICPLSEEKKND    Domain 5
          RICTNCCAGKKGCKYFSDDGTFVCEGESDPRNPKACPRNCDGRIAYGICPLSEEKKND    Domain 6
          RICTNCCAGKKGCKYFSDDGTFICEGES                                  Domain 7
Tomato P05119
                                              KACTRECG.NLGPGICPRSEG.SPLN   Domain 1
          PICINCCSGYKGCNYYNSFGKFICEGESDPKRPNACTFNCDPNIAYSRCPRSQGKSLIYP   Domain 2
          TGCTTCCTGYKGCYYFGKDGKFVCEGESD..EPKA.....................NMYPVM   Domain 3
Pepper AF221097
                                              KACSE.............E.NAEN     Domain 1
          RICTNCCAGRKGCNYYSADGTFICEGESDPNNPKACPRYCDTRIAYSKCPRSEGNAEN     Domain 2
          RICTNCCAGRKGCNYYSVIGTFICEGESDPNNPKACPRNCDTRIAYSKCPRSEGNAEN     Domain 3
          RLCTNCCAGRKVCNYYSADGTFICEGESDPNNPKACPRNCDPNIAYSLCLYEK          Domain 4
Pepper AF039398
                                              KACSQ.............R...NAKE   Domain 1
          PICTNCCAGRKGCNYYSADGTFICEGESDPNNPKPCTLNCDPRIFYSKCPRSEGNAEN     Domain 2
          RICTNCCAGRKGCNYYSADGTFICEGESDPNNPKPCTLNCDPRIFYSKCPRSEASAEQ     Domain 3
          PICTNCCAGLKGCNYYNADGTFICEGESDPNHPKACPKNCDPNIAYSLCLYEK          Domain 4
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Figure 4. Sequence alignment of the PLPIs. A, Alignment of PLPIs 34, 35, 40, 45, and 46 with the GenBank accession no. AF221097. B, Alignment of PLPIs 34, 35, 40, 41, and 43 with the GenBank accession number AF039398. C, Alignment of deduced proteins from tobacco stigma inhibitors II precursor (GenBank accession no. JQ2153), tomato inhibitor II (GenBank accession no. P05119), and pepper (GenBank accession nos. AF221097 and AF039398) signal sequences were omitted for clarity. Reactive sites are indicated by amino acids in bold and conserved cysteines are indicated by bars between sequences.

potato (Pearce et al., 1982) and in pepper seeds (Antcheva et al., 1996).

Partial amino acid sequences were determined for each isoinhibitor and were used to search available databases for homologous. The inhibitors showed high identity with regions of the deduced protein from two unpublished pepper cDNAs, AF221097 and

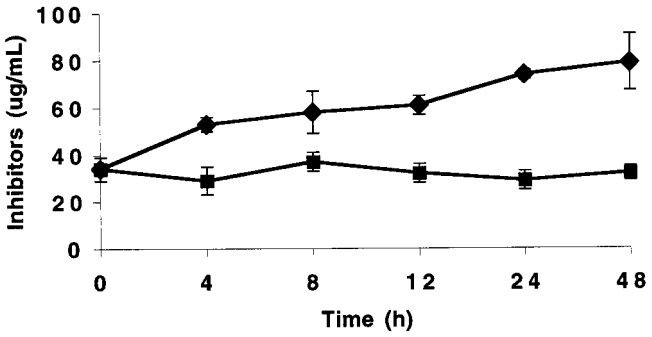

Figure 5. Wound-inducibility of PLPIs in pepper leaves. Pepper plants were wounded with a hemostat across the midvein and in three different locations along the borders of the lower leaf. Tissues were collected at times indicated and leaf juice was extracted for PLPI evaluation. PLPIs levels were evaluated using immunodiffusion assay. Six plants were used for each time point evaluated. ♦, Wounded plants. ■, Control plants.

AF039398, which share 88% identity. Both of these cDNAs contain three gene-duplicated/elongated copies of small (approximately 6,000 D) inhibitors that could be processed to small isoinhibitors as found in leaves. cDNAs coding for three isoinhibitors of the Inh II family have been reported from tomato roots (Taylor et al., 1993) and tobacco leaves (Balandin et al., 1995). Isoinhibitors amino acid sequences 34, 35, 40, 45, and 46 begin with residues R(I/L) CTN and align with accessions AF221097 and AF039398 (Fig. 4, A and B). PLPIs 41 and 43 sequences begin with a motif (AK) EPICTN and align with the accession AF039398 (Fig. 4B). Both accessions likely code for the isoinhibitors isolated in this study. AF221097 exhibits three reactive sites with Arg at the P_1 site and Tyr or Asn at the P_1'. This is usually a specific trypsin inhibitory site. This site can accommodate trypsin and chymotrypsin as seen with isoinhibitors 34, 35, 45, and 46, or there is a second reactive site heretofore not known. On the other hand, two of the three reactive sites of AF039398 have

Table IV. *Effects of MeJ, systemin, and salicylic acid (SA) on the induction of PLPIs*

Treatments[a]	Inhibitor Level[b]
	μg/mL
MeJ	84 ± 8
Systemin	60 ± 8
Water/Buffer	49 ± 16
Untreated	34 ± 5
SA	33 ± 4
SA + Wounding	30 ± 4

[a] Control plants for systemin and SA treatment were supplied with water or buffer, control plants for MeJ were left undisturbed, untreated. Pepper leaves were harvested 24 h after the experiments started and PLPI levels were evaluated by radial immunodiffusion assay. [b] Average of six plants for each treatment. Treatments with the same letter do not differ statistically at the significance level of 0.05 (Duncan, 1955).

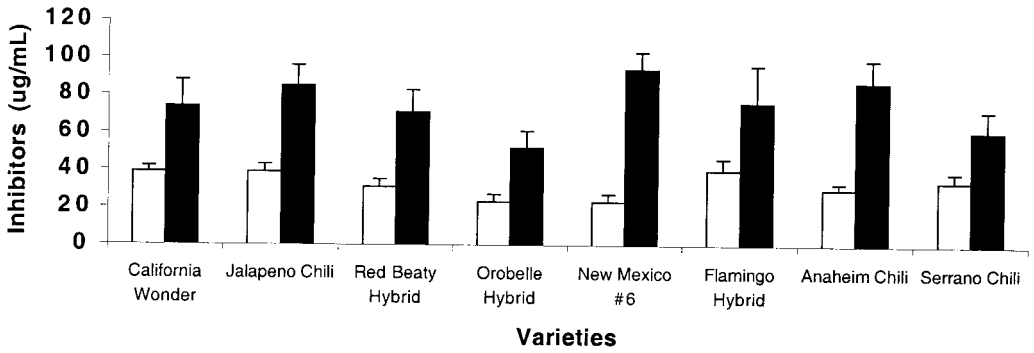

Figure 6. Analyses of PLPI protein levels in eight different varieties of pepper. Each variety was wounded with a hemostat across the midvein and in three different locations along the lower leaf. PLPI protein levels of the wounded plants (black bars) and untreated plants (white bars) were evaluated by radial immunodiffusion. The results are the average of 12 plants.

Leu at the P_1 site and Asn at the P_1' site, which are typical of a chymotrypsin inhibitor. The chymotrypsin inhibitors having no trypsin inhibitory activity are likely derived from this cDNA. Figure 4C shows a comparison of the isoinhibitor domains deduced from the two pepper cDNAs, the domains of Inh II preprotein from ornamental tobacco stigma (Atkinson et al., 1993) and those of wound-inducible Inh II from tomato leaves (Graham et al., 1985). Tobacco preprotein codes for seven domains with six reactive sites (amino acids in bold). Following translation, the N terminus of tobacco domain one and the C terminus of domain seven become covalently bound to form a large circular protein. This circular protein is then cleaved to produce six small proteinase inhibitor proteins. Tomato leaf inhibitor II preprotein contains three domains that are not circularized or processed and the translated inhibitor protein contains two reactive sites (Fig. 4C). Each of the two deduced pepper preproteins contains one short domain and three isoinhibitors domains (domains 2, 3, and 4). The short first domain is missing a reactive site, whereas the second, third, and fourth are nearly complete repeats. The linker amino acid sequences EEKKND found in the tobacco preprotein (Nielsen et al., 1996) that are thought to be processing sites are not found in pepper or tomato. Thus, the genes for tomato, tobacco, and pepper are variations of a basic ancestral domain that was gene duplicated-elongated to produce the various preproteins. Tobacco and pep-

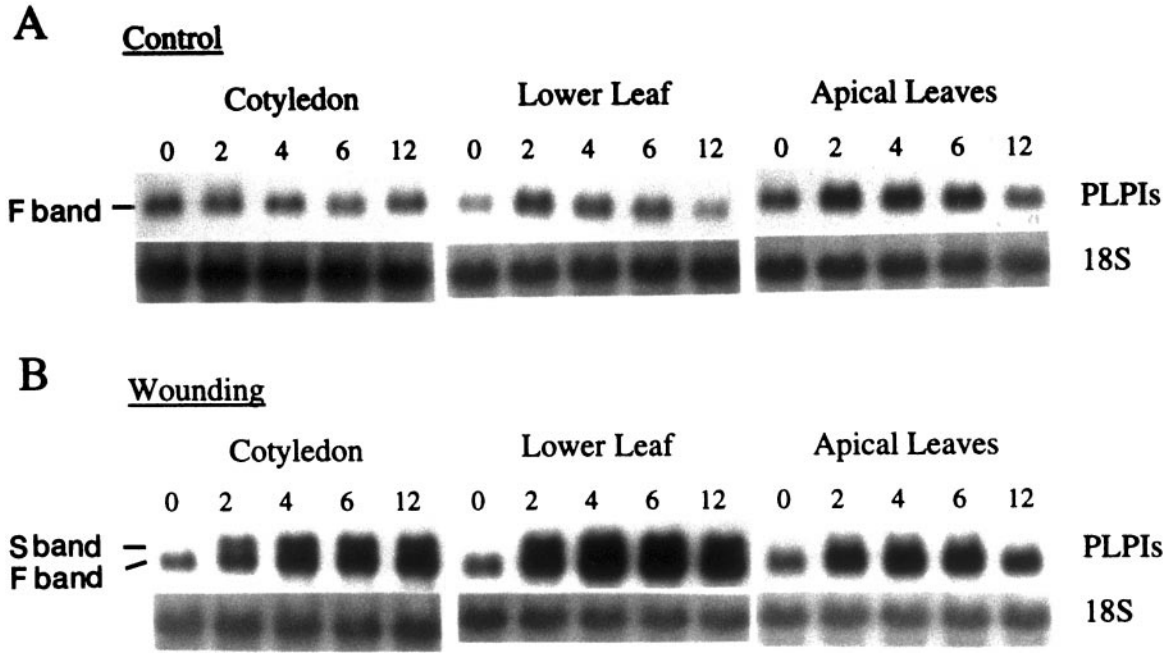

Figure 7. Gel-blot analyses of PLPI mRNA induction in control plants and upon wounding. Cotyledons and leaves were harvested at the times indicated and were immediately frozen in liquid nitrogen for RNA extraction. Fifteen micrograms of total RNA was loaded and separated on a 1.5% (w/v) agarose gel. The RNA was salt transferred to nylon membranes that were used for hybridization with DNA probes. 18S rRNA was used as loading control.

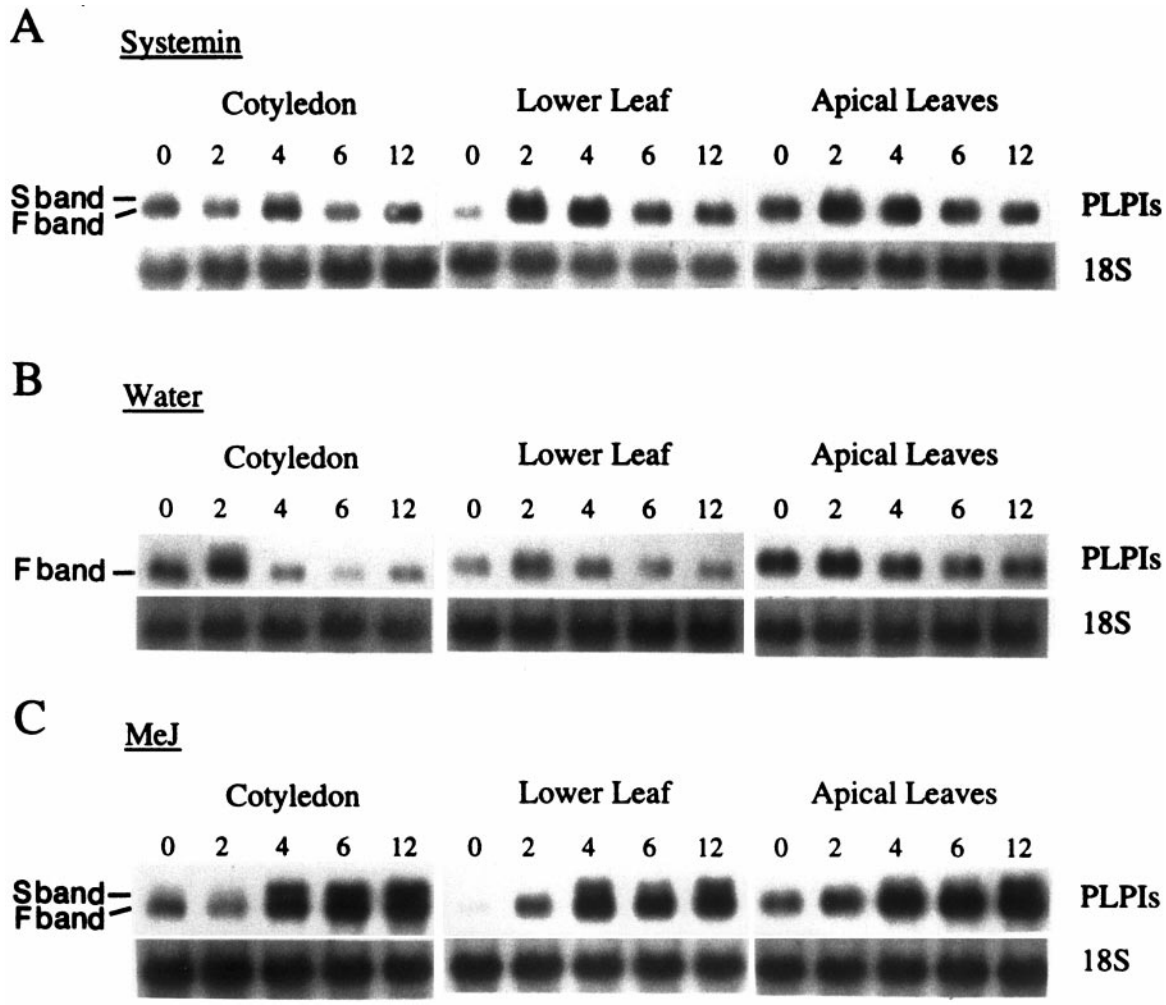

Figure 8. Gel-blot analyses of PLPI mRNA induction in systemin (A), water (B), and MeJ-treated plants (C). Cotyledons and leaves were harvested at the times indicated and were immediately frozen in liquid nitrogen for RNA extraction. Fifteen micrograms of total RNA was loaded and separated on a 1.5% (w/v) agarose gel. The RNA was salt transferred to nylon membranes that were used for hybridization with DNA probes. 18S rRNA was used as loading control.

per are processed to the small proteinase inhibitors, but the tomato Inh II preprotein, unlike tobacco and pepper, is not processed.

No inhibitors of the potato inhibitor I family were detected at any steps during the purification reported here. Inhibitor I has been found in other species of the *Solanaceae* family and in members of the *Graminaea* family as well. A possibility exists that inhibitor I type proteins exist in pepper, but have a different regulation so that the treatment with MeJ could have down-regulated its gene. In tobacco, inhibitor I protein was found to be related with senescence and did not accumulate upon wounding (Kuo et al., 1984), and in potato, inhibitor I is regulated by wounding and development (Ryan et al., 1968; Green and Ryan, 1972). In barley, inhibitor I is found in seeds, but not in leaves (Svendsen et al., 1980).

Using antibodies raised in rabbits against PLPIs, untreated pepper plants showed constitutive low levels of PLPIs in their leaves (Fig. 5). We did not study the developmental regulation of PLPIs in pepper plants, but in the young plants employed in this study the inhibitors were always present. The 2- to 3-fold variability found in wound inducibility among the eight varieties of pepper plants (Fig. 6) indicates that the wound response might be useful to genetic selection in enhancing the defense response of pepper plants to herbivores and pathogens.

PLPI genes exhibited differential induction by wounding, systemin, and MeJ. A cDNA probe with over 90% identity to AF221097 and AF039398 accessions (see "Materials and Methods") was used to evaluate PLPI gene expression. Leaves and cotyledons of control untreated pepper plants (Fig. 7A) showed hybridization only to a single mRNA species, called F-band. This constitutive level of the F-band mRNA is consistent with the low level of PLPI protein found in pepper plants. In the lower and apical leaves, the F-band exhibited a 70% and 40% increase over the zero time levels at 2 h of

incubation time. However, all plants were handled during the experiments, and these transient increases are probably due to a touch response. In lower and apical leaves the F-band had returned to zero time levels within 12 h after beginning the experiments. The wounded plants (Fig. 7B) showed an induction in all leaf types of two bands, F-band and S-band. The induction of both bands began 2 h after wounding and peaked at 4 to 6 h. This pattern of induction is similar to the one reported for wound-inducible inhibitors in tomato plants (Ryan, 2000). When pepper systemin was supplied through the cut stems of the plants, all leaves showed a weak induction of the F-band (Fig. 8A). The peak of expression of the F-band was detected within 2 to 4 h after starting the experiment. The weak induction of PLPI mRNA by systemin may explain why PLPI protein induction was not statistically different from MeJ and water/buffer controls. Increase in inhibitory activity against trypsin in leaves of pepper plants due to cutting of the stem for the systemin treatment was reported earlier to preclude evaluation of systemin effect (Constabel et al., 1998). Treatment of plants with MeJ vapors induced F-band and S-band mRNAs (Fig. 8C). The induction of the S-band mRNA is just detectable at the 4-h time point, compared with 2 h in response to wounding (Fig. 7B). The delay in response to MeJ may be a reflection of the access of the MeJ vapors to target cells.

The isolation of PLPIs from pepper leaves is the initial step in studying the defense-related genes that are induced in pepper in response to herbivore and pathogen attacks. PLPI induction by MeJ and inhibition by SA indicate that the response is regulated through the octadecanoid signaling pathway as in tomato plants, but that the Inh II isoinhibitors in pepper leaves are processed from a precursor that is larger than that found in tomato leaves, producing an array of small proteinase inhibitors with varying specificities. This indicates that the proteinase inhibitor genes may have evolved in each species to defend against the specific herbivores and pathogens that they encountered in their unique ecological niches.

MATERIALS AND METHODS

Plant Materials

Unless indicated, 19- to 20-d-old plants of bell pepper (*Capsicum annuum* var. California Wonder) were used in all experiments. The plants had two expanding leaves and a small apical leaf. Seeds for all varieties tested were obtained in local markets. Plants were grown in peat pots in a growth chamber with 17-h days of 300 mE m^{-2} s^{-1} of light at 28°C and 7-h nights at 18°C.

Purification of Inhibitors and Antibody Production

The entire aerial part of the plants, excluding roots, were used for protein extraction. To induce maximal proteinase inhibitor induction and accumulation, plants were sprayed with a MeJ solution (125 μL of MeJ in 0.1% [w/v] Triton X-100) twice at 24-h intervals, and were then harvested 24 h after the second application. The tissues were frozen in liquid nitrogen and ground in a fine powder using a mortar and pestle. An approximate 4 L of ground tissue (600 plants) was extracted with 3 L of buffer (0.01 M Na citrate, 1 M NaCl, and 14 g of Na hydrosulfite, pH 4.3). To concentrate the protein an ammonium sulfate precipitation was performed. To the crude extract was slowly added ammonium sulfate to 80% saturation and the extract was stirred for 4 h at 4°C. After centrifugation at 10,000*g* for 10 min, the precipitate was recovered and redissolved in 1.3 L of water. The solution was heated to 70°C by immersing the flask in a boiling water bath, cooled in an ice bath, and dialyzed against 150 mM KCl, 0.01 M Tris/HCl, pH 8.0. The extract containing the inhibitors was loaded into a chymotrypsin affinity column (3.5 × 11 cm) and after washing, the inhibitors were eluted with 8 M urea, pH 3.0. The mixture of inhibitors that eluted at the void volume of the affinity column was dialyzed, lyophilized, and dissolved in 0.1% (v/v) TFA. Chymotrypsin affinity chromatography was performed as described by Cuatrecasas and Anfinson (1971) using chymotrypsin immobilized in Sepharose CL-4B resin (Pharmacia, Piscataway, NJ). HPLC separation was performed using C-18 and SCX columns as described in Pearce et al. (1993). Bicinchonic acid protein assay reagent (Pierce, Rockford, IL) was used for protein quantification. Bovine serum albumin (Sigma, St. Louis) and tobacco isoinhibitors (Pearce et al., 1993) were used as standards. Inhibitory activities against trypsin and chymotrypsin were assayed using a spectrophotometer (model 2000, Hitachi, San Jose, CA) with the substrates p-tosyl-L-Arg methyl ester (Sigma) for trypsin and *N*-benzoyl-L-Tyr ethyl ester (Sigma) for chymotrypsin, according to Hummel (1959). Inhibition constants (K_i) were estimated as described in Cha (1975) using the formula $I_{50} = 0.5E_t + K_I + K_iSK_m^{-1}$, where I_{50} is the total inhibitor concentration at which the enzyme reaction velocity is 50% of the uninhibited reaction, E_t is the total enzyme concentration, and S is the substrate concentration. SDS/urea-PAGE was performed as described in Swank and Munkres (1971). N-terminal sequence of the PLPIs was determined by Edman degradation at the Washington State University Sequencing Laboratory. Mass determination of the purified isoinhibitors was performed by matrix assisted laser desorption ionization-mass spectrometry (Mass Spectroscopy Laboratory at Washington State University). Antibodies against the pepper inhibitors were obtained as described in Pearce et al. (1993). A mixture of purified PLPIs (5 mg) was cross-linked to rabbit serum albumin (1 mg) and was injected into rabbits. The antibodies were used in radial immunodiffusion assays (Ryan, 1967; Trautman et al., 1971) to quantify PLPIs, using HPLC-purified PLPIs as standards.

Treatments

Wounds were inflicted with a hemostat across the midvein and in three different locations along the border of the

pepper leaf. MeJ treatment was performed as described in Bergey and Ryan (1999) by exposing plants to MeJ vapors in closed Plexiglas boxes. Pepper systemin, 2.5 pmol/plant dissolved in water (Constabel et al., 1998), SA, 5 mM dissolved in buffer (10 mM NaP, pH 6.5), buffer alone (10 mM NaP, pH 6.5), and water were supplied through the cut stems as described in Howe et al. (1996). Proteinase inhibitor proteins concentration were assayed in leaf juice using radial immunodiffusion assay (Ryan, 1967; Trautman et al., 1971) 24 h after treatments. All leaves, including cotyledons, were used to quantify inhibitor protein accumulation. Untreated plants were left undisturbed (in separate boxes when MeJ was used) along with the treated plants during the entire experiment.

Isolation of cDNA and Gel Blots

Specific primers (5′-tctacatgttgatgccaagg-3′ and 5′-gtgacactgttcacgctttt-3′) were designed based on the sequence of PLPIs deposited in the GenBank (accession no. AF221097). The primers were used for RT-PCR with Superscript (Gibco, Gaithersburg, MD) RT with total RNA isolated from pepper plants that were exposed to MeJ vapors for 12 h. The fragments amplified by RT-PCR were cloned using the pGEM-T easy vector system (Promega, Madison, WI). The nucleotide sequence of the cloned fragment was analyzed by the Sequencing Laboratory at Washington State University. Cotyledons, lower leaves (first true leaf), and upper leaves (second and apical leaves) were collected individually after 0, 2, 4, 6, and 12 h of exposure to MeJ and were frozen in liquid nitrogen immediately after collection. RNA extractions were performed using TRIzol reagent (Life Technologies, Gaithersburg, MD) following manufacturer instructions. Fifteen micrograms of total RNA was loaded in agarose gels for separation, and gel-blot analyses were performed as described in Moura et al. (2001). Gel blots were quantified using Phosphoimager and Molecular Analyst Software (Bio-Rad, Hercules, CA). Gel blots were hybridized with 18S rRNA for loading control. All experiments were repeated at least twice.

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