Modification of luciferase to be a substrate for plant aspartic proteinase

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The possibility of using firefly luciferase as a substrate for an aspartic proteinase was explored. Several amino acid modifications to the C-terminus of the luciferase were created on the basis of the known substrate of the *Arabidopsis thaliana* aspartic proteinase, pro-(barley lectin). One luciferase with the sequence Arg-Asp-Gly-Val-Phe-Ala-Ala instead of the native Arg-Glu-Ile-Leu-Ile-Lys-Ala at position –15 to –9 relative to the C-terminus of native luciferase was found to possess 17% of the original luciferase activity. When this modified luciferase was incubated with the aspartic proteinase, a specific loss in activity occurred that was not observed with the original luciferase. However, both enzymes seemed very sensitive to the acidic conditions required for aspartic proteinase activity. The other versions of luciferase with different numbers of pro-(barley lectin) amino acids were not active luciferases. This provided

information on the structural requirements of the C-terminal portion of the protein for luciferase activity. The luciferase proteins were also monitored during the digestion by using Western blots and some were shown to be substrates for the aspartic proteinase. Contrary to what had been expected, the modified luciferase that incorporated the pro-(barley lectin) sequences was not simply cleaved at the engineered site but at additional positions in the protein. The *Arabidopsis* aspartic proteinase cleaved two other standard protein substrates at many sites, suggesting that this proteinase could have a role in the degradation of proteins in addition to processing propeptides in plants.

Key words: C-terminal propeptide, pro-(barley lectin), protein degradation, protein processing, protein structure.

INTRODUCTION

The aspartic proteinases (aspartic endopeptidases; EC 3.4.23.*n*) are one of the major classes of proteolytic enzymes found in a wide variety of organisms, including viruses of plants and animals, bacteria, fungi, and animal and plant cells [1–4]. The aspartic proteinases are characterized by having two aspartic residues in their active site, being active in the acidic pH range (pH 3–5) and being almost universally susceptible to a pentapeptide from *Streptomyces*, pepstatin A. Very often an immobilized form of this inhibitor has been used as a means of simple and rapid purification of these enzymes from extracts. These enzymes are generally found in the acidic lysosomal/vacuolar compartment of cells or secreted into the medium, which is consistent with the acidic pH optimum for activity [1,3]. They are believed to be involved in specific protein processing or general protein degradation in all systems analysed.

The plant aspartic proteinases have been isolated from numerous sources, including *Arabidopsis*, barley, cardoon, rice and tomato (reviewed in [3]). These enzymes have been implicated in the processing of seed storage protein precursors such as pro-(barley lectin) and 2 S albumin. Processing of pro-(barley lectin) by the *Arabidopsis* and barley aspartic proteinases occurs *in vitro* at the C-terminus, removing 13 of the 15 precursor amino acid residues and clipping between Phe and Val residues [5,6]. Aspartic proteinases from *Brassica napus* and castor bean have been implicated in the processing or degradation of 2 S albumin peptides, a seed storage protein from *Arabidopsis* [7,8]. The *Arabidopsis*, barley and castor bean aspartic proteinases have been localized to the protein storage vacuoles, which is consistent

with a role of these enzymes in the processing of seed storage proteins [6,8,9]. Other plant aspartic proteinases seem to have a degradative role. Secreted enzymes from tobacco and tomato have been implicated in the degradation of proteins such as some pathogenesis-related proteins [10,11]. In addition, one of the proteins in the digestive fluid of carnivorous plants is an aspartic proteinase [12], implying a role in general protein degradation similar to those enzymes in the mammalian digestive tract. However, the identification of a role in protein degradation or processing might depend on the particular substrate or assay employed *in vitro*.

General assays for aspartic proteinases have often used standard protein substrates that are commercially available, such as BSA and haemoglobin. The appearance of trichloroacetic acid-soluble UV-absorbing peptides or the appearance of new primary amines is then used as an indicator of proteolytic activity [5,13]. These assays are single endpoint assays requiring many time points and significant sample preparation to obtain one enzyme reaction rate point. One major class of synthetic substrates that can be assayed continuously, saving time and sample preparation, has been developed specifically for aspartic proteinases. These are peptides containing a Phe-nitroPhe bond cleaved by this class of enzymes, which prefer to cut between hydrophobic amino acids [14]. In these substrates, a shift in the UV absorbance at 300 nm indicates cleavage of the bond between the Phe and nitroPhe residues, allowing the assay to be run continuously. Numerous versions of these peptides have been analysed, with variations at all the other amino acid positions to probe the specificity of the aspartic proteinase under study [14,15]. How-

Abbreviation used: DTT, dithiothreitol.

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ever, a continuous assay with a protein substrate for aspartic proteinases has not yet been developed. We wished to create such a system and explored the use of firefly luciferase as a potential substrate for the aspartic proteinases.

The luciferase from *Photinus pyralis* is a 62 kDa protein that produces a flash of light on the oxidation of luciferin in the presence of ATP, oxygen and Mg^{2+} [16]. It has been used as a sensitive means for quantifying ATP levels and as a marker for gene expression in a wide variety of systems [17–19]. In plants it has been used to follow gene expression *in vivo* under the control of light-regulated promoters and to select for *Arabidopsis* mutants with altered circadian rhythms [20,21]. The crystal structure of the enzyme is known: it has two domains, an N-terminal region and a C-terminal region [22]. The N-terminal region including residues 4–436 is composed of two β -sheet domains, which are flanked on each side by α -helices. The C-terminal region, comprising residues 440–550, forms a small separate α -helix and β -sheet domain; residues 534–542 form the final α -helix with a random amino acid region composed of eight residues completing the protein. The active site is not known but has been predicted to contain amino acids in the N-terminal region because these are conserved in three families of related enzymes, acyl-CoA ligases, peptide synthetases and firefly luciferase [22]. Owing to the known cleavage by the *Arabidopsis* aspartic proteinase in the C-terminus of the barley lectin precursor, we explored whether this region of luciferase was flexible enough to accept sequence alterations.

Recent work has focused on the importance of the last 12 C-terminal amino acids for luciferase activity. Sala-Newby and Campbell [23] reported that the removal of three to seven residues from the C-terminus results in no detectable loss of activity of the luciferase enzyme. However, the stepwise removal of the next five to nine residues (a total of 8–12 residues removed) results in an enzyme lacking 50–99% of its activity. Because the protein lacking the C-terminal 12 residues binds to ATP, it was proposed that this region is not part of the active site of the enzyme [23]. Interestingly, replacement of this region with amino acids specifying a protein kinase phosphorylation site restores a significant amount of activity. Waud et al. [24] incorporated a site for the serine proteinase thrombin into the C-terminal region. This modified luciferase has significant activity, which is lost when incubated with thrombin. This proteinase had little or no effect on the activity of the unmodified luciferase protein. (No protein gels or blots were shown in this work to correlate changes in activity with luciferase peptide changes.) These studies suggest flexibility of the sequence in the C-terminal region and the possibility that this site would be suitable for the incorporation of a protein-processing signal for the aspartic proteinase.

In the present study we investigated the possibility that luciferase could be modified to become a substrate for our plant aspartic proteinase. We generated several modified luciferase enzymes with pro-(barley lectin) amino acids at the C-terminus and these were tested as substrates for the aspartic proteinase. We found the activity of luciferase was more sensitive to the kinds of amino acid that were placed at its C-terminus than had been reported previously. The native and modified enzymes were highly sensitive to acidic environments and permanently lost a significant portion of activity within minutes of exposure to low pH. Results demonstrated that luciferase, when modified with specific pro-(barley lectin) amino acids, was a substrate for the *Arabidopsis* aspartic proteinase but was not simply processed by the proteinase at the predicted site but digested at other sites as well. This is evidence that this aspartic proteinase not only processes precursor proteins but can cleave at many other sites in proteins *in vitro*.

EXPERIMENTAL

Mutagenesis of the luciferase gene

The cloning vector pSP-luc+ (Promega, Madison, WI, U.S.A.) was chosen as the template for mutagenesis; PCR was used to generate the mutations in the luciferase gene. All reactions were done in the PTC-200 DNA engine (MJ Research, Watertown, MA, U.S.A.) in a volume of 25 μ l. The reactions contained 0.5 ng of DNA template, each primer at 1 μ M (Integrated DNA Technologies, Coralville, IA, U.S.A.), 0.2 mM dNTPs (Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.), 1 $\times$ Mg^{2+} -free *Taq* buffer (Promega), 1.5 mM $MgCl_2$ (Promega) and 1 unit of *Taq* polymerase (Promega). The reaction conditions for the PCR programme cycled 30 times with each step involving incubation at 95 °C for 1 min and annealing at 42 °C or 50 °C for 2 min (depending on the primer sequence), followed by elongation at 72 °C for 1 or 2 min (depending on the length of the PCR product). The sequences of the primers used to create the luciferase mutations are given in Table 1.

To create the 12-residue deletion from the C-terminus of luciferase (del12), we used a non-mutagenic upstream primer at the *Ban*II restriction site of the luciferase gene (position 1069 in pSP-luc+; *Ban*II primer) and a mutagenic downstream primer that included the *Xba*I site in the vector (del12 primer). After PCR, the product was restricted with *Ban*II and *Xba*I, purified with a low-melting-point agarose gel, and ligated back into a *Ban*II- and *Xba*I-restricted pSP-luc+ vector. The ligation products were transformed into *Escherichia coli* and plasmid DNA from individual colonies was initially checked for the incorporation of a new restriction enzyme site, in this first case *Dra*I. The region that had been amplified was sequenced to confirm the incorporation of only the desired mutation by using the Ampli-Taq ready reaction mix (Applied Biosystems, Perkin-Elmer, Foster City, CA, U.S.A.), analysed on an Applied Biosystems Incorporated Prism Genetic Analyzer Model 310 (Perkin-Elmer). To replace the final amino acids with Asp-Gly, the del12 plasmid was used as a template for PCR with the *Ban*II primer (Table 1) and a different downstream primer, +DG (Table 1), which was then used as a template to add amino acids Val-Phe by using the *Ban*II primer and another downstream primer, +VF (Table 1). This plasmid was then used to create a luciferase with Ala-Glu-Ala-Ile-Ala-Ala added on using the *Ban*II primer and another downstream primer, +AEAIAA (Table 1); the resulting plasmid was used as a template to add the last amino acids of the pro-(barley lectin) C-terminus, Gly-Ser-Thr-Leu-Val-Ala-Glu [the native C-terminus of pro-(barley lectin) would be Asn-Ser-Thr-Leu-Val-Ala-Glu but the mutation of Asn $\rightarrow$ Gly does not alter the protein processing or targeting of the protein in tobacco cells and eliminated the complication of Asn-linked glycan modification in eukaryotes [25]] by using the *Ban*II primer and a different downstream primer, +GSTLVAE (Table 1). In each case the PCR product was restricted, purified and religated back into the pSP-luc+, and the region was sequenced as above.

To create a more conservative substitution of the pro-(barley lectin) amino acids such that only the presumed cleavage site was incorporated into the C-terminal sequence of the luciferase at position –12 (numbering from the C-terminus), two PCR products were formed and a three-way ligation with the pSP-luc+ vector was performed. The first PCR product was formed by using the upstream *Ban*II primer and the downstream mutagenic primer, consdown (Table 1); the second product was formed by using the downstream T7 promoter primer in the vector, T7 prom, and an upstream mutagenic primer, consup (Table 1). The first PCR product was restricted with *Ban*II and

Table 1 Oligonucleotides used to incorporate pro-(barley lectin) amino acid residues into the C-terminal region of firefly luciferase

Oligonucleotide name	Sequence (5' → 3')
BanII	GAATATGGGCTCACTGAGACT
del12	TATCGAATTCTCTAGATTAAATCTCTGATTTTCTTGC
+ DG	ATCGAATTCTCTAGAGCTAGCGCTCTGATTTTCTTG
+ VF	GATGATATCGAATTCTCTAGATTAAAGACGCCGTCTGATTTTCTTG
+ AEAIAA	GATGATGAATTCTCTAGACCTAGGCGGCGAATCTGGCCTCGGCAAAGACGCCGTCTGATTTTCTTG
+ GSTLVAE	GATATCGAATTCTCTAGACCTATTCTGCGACAAGAGTGGAGCCGGCGGATGGCCTCGGCAAAG
Consdown	GATCTTTCCGCCCTTCTTGGCCGCAAAGACGCCGTCTGATTTTCTTGCGTCT
Consup	GACGCAAGAAAAATCAGAGACGGCGTCTTTCGGCCAAGAAGGGCGGAAAGATC
T7 prom	TAATACGACTCACTATA

EaeI (the mutagenic primers create a unique *EaeI* restriction site); the second product was restricted with *XbaI* and *EaeI*. They were then purified on a low-melting-point agarose gel and ligated with the pSP-luc+ vector restricted with *BanII* and *XbaI*. The ligation products were transformed into bacteria; plasmid DNA was isolated and sequenced as above.

Expression, immunoprecipitation and assay of the mutant luciferase proteins *in vitro*

All translations of the modified luciferase genes *in vitro* were done with the TNT-coupled Sp6 transcription/translation reticulocyte lysate system in accordance with the manufacturer's instructions (Promega). Each time the transcription/translation was done, a positive control reaction with the luciferase DNA provided with the kit was generated; a portion of this positive control and the mutagenized luciferases were added to the luciferase assay reagent (Promega) and exposed to type 665 Polaroid film (Cambridge, MA, U.S.A.) for 8 min at room temperature to determine activity qualitatively.

In one case, the mutagenized luciferase protein synthesized *in vitro* was purified by immunoprecipitation. A portion of the protein/lysate mixture (120 µl) was mixed with 5 µl of anti-luciferase antibody (Promega) and 50 µl of immobilized Protein A linked to agarose (Sigma Chemical Company, St. Louis, MO, U.S.A.). The mixture was rocked gently at room temperature for approx. 1 h and then centrifuged at 11 000 g at room temperature for 30 s to 1 min. The pellet was then washed three times with 50 µl of PBS [137 mM NaCl/0.3 mM KCl/5 mM Na₂HPO₄/1.8 mM KH₂PO₄ (pH 7.4)]; the luciferase protein was then salted off by the addition of 50 µl of 4 M MgCl₂ and incubation at 25 °C for at least 30 min. The sample was then spun for 30 s to pellet the Protein A; this procedure was repeated twice more. The supernatants were combined and dialysed against a luciferase storage buffer [1 mM EDTA/1 mM dithiothreitol (DTT)/25 mM Tris/acetate/0.2 M (NH₄)₂SO₄ (pH 7.8)] with the use of a Sli-dalyzer dialysis cassette (Pierce, Rockford, IL, U.S.A.) at 25 °C for 4 h.

The quantitative luminescence assay of the luciferase involved adding 5 µl of each sample (translated protein) to a well on a Packard Instrument Company (Meriden, CT, U.S.A.) HTRF black 96-well Optiplat containing 20 µl of a luciferase stabilization solution (1.25 mM MgCl₂/1.25 mM CaCl₂). Then 25 µl of the LucLite substrate [reconstituted in accordance with the manufacturer's instructions (Packard Instrument Company)] was added and the signal was left to enhance for 10 min. A count delay of 3 min was used to decrease the background signal by allowing auto-fluorescence of the plate to dissipate. The two photodiodes in the TopCount (Packard Instrument Company)

were normalized by using the provided normalization plate; the instrument was then set to count each well for 5 s in luminescence mode.

The amount of luciferase protein in each of the translation mixes was quantified with a slot-blot to calculate specific activity. Dilutions of a standard luciferase protein (Promega) ranging from 1.3 µg to 130 ng and appropriately diluted translated luciferases were loaded on a nitrocellulose membrane (Micron Separations, Westborough, MA, U.S.A.) with the Minifold I slot-blot apparatus, in accordance with the manufacturer's instructions (Schleicher and Schuell, Keene, NH, U.S.A.). The blot was rinsed with luciferase dialysis buffer (see above), then blocked for 30 min with 2.5% (w/v) BSA (Sigma) in TBS/KCl buffer (25 mM Tris base/14 mM NaCl/0.2 mM KCl), followed by incubation in a 1:10000-diluted anti-luciferase antibody (Promega) in TBS/KCl with 2.5% (w/v) BSA for 1 h. The primary antibody was washed off with three 10 min washes with the TBS/KCl buffer containing 0.1% (v/v) Tween 20 (Sigma). The blot was then treated with a secondary goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma), diluted 1:10000 in TBS/KCl containing 2.5% (w/v) BSA for 1 h. Washes were performed as above and then the blot was incubated with 5-bromo-4-chloroindol-3-yl phosphate/Nitro Blue Tetrazolium (Sigma Fast alkaline phosphatase substrate tablets; Sigma) in the dark until colour development was complete. Developed blots were scanned and analysed with the Image Master VDS Software (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). The absorbance of each of the slots was determined. A curve was generated with the luciferase standard protein and used for determination of the level of luciferase protein in each translation. Specific activities were calculated by dividing the luminescence activity of the luciferase protein in c.p.s. from the luminometer by the nanogram amount of luciferase protein determined from the slot-blot.

Incubation of luciferase proteins with the *Arabidopsis* aspartic proteinase, and Western blots for luciferase

The *Arabidopsis thaliana* aspartic proteinase was isolated from the seeds of *A. thaliana* by using pepstatin A/agarose chromatography in a modification of the protocol described previously [5]. This modification permitted a more rapid preparation of the enzyme with mini pepstatin A-agarose columns in 1 ml syringes and buffer amounts scaled down accordingly. The activity of the aspartic proteinase was determined with haemoglobin in a fluorescamine assay, detecting new primary amines at pH 3.5 as described previously [5]; one unit corresponded to the enzyme activity hydrolysing the equivalent of 1 µg of haemoglobin/h. For experiments to monitor the peptides produced by the aspartic

proteinase from the digestion of BSA or haemoglobin, incubations at 37 °C with the enzyme were done in 0.1 M sodium acetate buffer, pH 3.5, with 4.3 units of the aspartic proteinase and 82 µg of the substrate protein (previously treated at 30 °C for 30 min in 0.1 M sodium acetate, pH 3.5) in a total volume of 90 µl. Aliquots (9 µl) were removed at various time points and 4 × SDS loading buffer [40 mM Tris/HCl (pH 8.0)/4 mM EDTA/10% (w/v) SDS/0.04% Bromophenol Blue/10% (v/v) glycerol] was added. The proteins were separated on a 20% (w/v) PhastSystem polyacrylamide gel (Amersham Pharmacia Biotech) with SDS buffer strips, then stained with Coomassie Blue R (0.1%) in methanol/acetic acid/water (3:1:6, by vol.).

To monitor the changes in luminescence activity of a luciferase protein after incubation with the aspartic proteinase, we performed the following reaction. A mixture containing 100 mM sodium formate buffer, pH 4.0, was used with a ratio of luciferase protein to aspartic proteinase maintained at approx. 100 ng luciferase to 1 unit of proteinase; the reaction was incubated at 37 °C for various durations. The final reaction volumes were varied on the basis of the number of time points desired. Aliquots (5 µl) were removed at various time points and the reaction was stopped by the addition of 2 µl of 1 M Tris/HCl, pH 8.0. The luminescence activity of the treated protein was assayed, after all the time points of a particular sample had been removed, with the Packard TopCount as above. The reactions were done in duplicate with both control and experimental conditions. For negative control reactions, the mixtures contained either boiled aspartic proteinase (10 min at 95 °C), pepstatin A (10 µM) or ethanol (0.1%, v/v), the solvent for pepstatin A.

All luciferase proteins were incubated with the aspartic proteinase as described above and were tested for evidence of degradation by using Western blots. At designated time points, the reactions were stopped by pipetting a 5 µl aliquot directly into 2.0 µl of 4 × SDS loading buffer and boiled immediately. Samples were then separated on precast SDS/7.5% (w/v) polyacrylamide gels with the Bio-Rad electrophoretic system, in accordance with the manufacturer's instructions (Bio-Rad, Hercules, CA, U.S.A.). The gels were transferred to nitrocellulose membranes (Micron Separations) and treated to detect luciferase proteins as described above for the slot-blots. Protein amounts were quantified as for the slot-blots by using the Image Master VDS software. As a control for the detection of the luciferase antibody, the pSP-luc+ luciferase was also incubated with the proteinase clostripain (Sigma). The clostripain was dissolved in an activation buffer containing 10 mM MOPS, 2.5 mM DTT and 1.0 mM CaCl₂ pH 7.4, at a concentration of 0.1 unit/µl and preincubated for 2 h at 25 °C. Then 1 unit of clostripain was combined with 84 ng of the original pSP-luc+ luciferase in 25 µl of 2.5 mM DTT. This mixture was incubated at 37 °C and 2 µl aliquots were removed at various times; SDS sample buffer was added and the proteins were separated on a 20% (w/v) PhastSystem gel with SDS buffer strips as described by the manufacturer (Amersham Pharmacia Biotech). The gel was blotted by using the semi-dry blotting technique with the PhastSystem (Amersham Pharmacia Biotech); the luciferase proteins were detected as above.

RESULTS

To test the ability of luciferase to act as a substrate for the *A. thaliana* aspartic proteinase, we constructed a total of six different versions of luciferase, each containing a different mutation in the C-terminal region. These mutations were based on the C-terminal propeptide sequence of barley lectin, which is a substrate for the aspartic proteinase *in vitro* [5]. Each of these luciferase con-

structions was then expressed with a coupled transcription/translation system and the activity of the luciferase was measured with luciferin (see the Experimental section). The luciferases lacking the 12 C-terminal residues, where the last two residues of this deletion were replaced with pro-(barley lectin) residues or one where two more amino acids had been added from the pro-(barley lectin) sequence, were not active (del12, DG and VF in Table 2). This is consistent with published results [23]. However, restoration of a total of 8 or 15 residues to that deleted luciferase did not restore activity (6AA and 7AE in Table 2). A more conservative substitution replacing only the five residues at the -12 position (relative to the C-terminus) with residues at the proposed cleavage site on the pro-(barley lectin) resulted in a luciferase that retained 17% of the original enzyme activity (AB1, Table 2). This engineered luciferase was then tested as a substrate for the aspartic proteinase.

The aspartic proteinase requires acidic conditions for optimal activity and is generally assayed *in vitro* in a 0.1 M sodium acetate or sodium formate buffer at pH 3.5 [5]. However, luciferase has a pH optimum between 8 and 8.5 [26]. For these experiments, incubation was done in pH 4.0 sodium formate buffer to minimize the loss of activity of the luciferases but to retain approx. 50% activity of the aspartic proteinase (A. Mutlu and S. Gal, unpublished work). Although the low pH of the buffer caused a significant loss of activity of the AB1 luciferase, the loss was even greater in the reactions that contained the aspartic proteinase (Figure 1A). In addition, increasing the activity of aspartic proteinase added to the reaction caused a more rapid loss of luciferase activity (results not shown). Control reactions containing boiled aspartic proteinase showed slightly more luciferase activity than buffer alone, possibly owing to slight buffering of the low pH of the reaction (Figure 1A). The control reaction containing pepstatin A, the specific aspartic proteinase inhibitor [5], retained most but not all of the luciferase activity lost when incubated with the aspartic proteinase (results not shown). This incomplete recovery of luciferase activity could be attributed to the fact that the solvent (ethanol) alone seemed to have a very detrimental effect on the luciferase activity (results not shown).

As a comparison, the highly active original luciferase (pSP-luc+) was tested as a substrate for the aspartic proteinase. Although this enzyme also lost a significant amount of activity owing to the low pH buffer, it lost somewhat less activity in the presence of active aspartic proteinase, possibly owing to the buffering effect mentioned above (Figure 1B). When boiled aspartic proteinase was added to the reaction, the luciferase activity was similar to that observed in the reaction with active enzyme (Figure 1B). This suggested that the original luciferase was not as good a substrate for the aspartic proteinase as the modified luciferase, AB1, containing the pro-(barley lectin) residues.

To monitor the changes in the AB1 luciferase protein after exposure to the aspartic proteinase, we performed Western blots of samples taken throughout the incubation by using polyclonal anti-luciferase antibodies. If the aspartic proteinase clipped the luciferase at the engineered pro-(barley lectin) C-terminal processing site, we would expect the protein to be appropriately decreased in molecular mass. Surprisingly, in no case did we see evidence for simple processing of the luciferase protein during incubation with the aspartic proteinase. Because the amount of AB1 protein disappeared over time with the addition of the aspartic proteinase, this modified luciferase did seem to be a good substrate for the aspartic proteinase (Figure 2A). [The complete loss of the luciferase protein on these blots is probably a limitation of the antibody detection (see below).] If processing

Table 2 Specific activities of luciferases modified with pro-(barley lectin) amino acid residues

Original pSP-luc+ luciferase residues are shown in capital letters, whereas those from pro-(barley lectin) are shown in lower-case letters. The activities of luciferase (counts per second; c.p.s.) were obtained on the Packard TopCount instrument by using black Optiplates. The quantities of luciferase protein were determined by comparison with a standard amount of luciferase protein with the use of luciferase antibodies and a slot-blot (see the Experimental section).

Construct name	C-terminal sequence (−15 → −1 relative to C-terminus)	Specific activity (c.p.s./ng of luciferase protein)	Proportion of original activity (%)
pSP	REILIKAKKGGKIAV	1070	100
del12	REI	0.5	0.05
DG	Rdg	1.2	0.11
VF	Rdgvf	1.1	0.10
6AA	Rdgvfaeiaaa	1.7	0.16
7AE	Rdgvfaeiaaagstlvae	0.9	0.09
AB1	RdgvfaAKKGGKIAV	184	17

of luciferase occurred only within the C-terminal tail at the *in vitro* cleavage site on pro-(barley lectin) [5], a luciferase protein the size of the VF construction (10 residues shorter than pSP-luc+) would have accumulated. This did not occur (Figure 2A). The control reactions, in which either boiled aspartic proteinase or pepstatin A were added to the reaction, showed no loss of the luciferase protein (Figure 2A, and results not shown).

The loss of luciferase was specific for the AB1 enzyme because the original luciferase (pSP-luc+) was not appreciably removed by the aspartic proteinase (Figure 2B). Results from two different experiments with the original luciferase showed some variation in the amount of this protein lost in the presence of the aspartic proteinase, but it was always significantly lower than that observed with the AB1 luciferase protein. The small amount of pSP-luc+ luciferase degradation that was observed was inhibited by pepstatin A and was not due to the presence of the low-pH buffer alone (Figure 2B, and results not shown). It did seem that the aspartic proteinase selectively preferred to cleave the AB1 luciferase, a protein that contains amino acids of a substrate for the aspartic proteinase, namely from the C-terminus of pro-(barley lectin). The acidic buffer alone was not responsible for the change in intensity of the luciferase proteins, because incubation with boiled aspartic proteinase showed no change in the antigenic protein (Figures 2A and B).

The method that we used for monitoring the cleavage of the modified luciferases depended on the detection of the luciferase by the antibody. It could be that this technique would not detect certain processed forms of the luciferase, because cleavage might destroy antigenic sites of recognition (despite this being a polyclonal antibody). It is clear that this antibody would recognize the del12, DG and VF forms of the luciferase (because these proteins were seen on Western blots; see below) but if processing were to proceed only slightly more, it is not clear whether the antigen would still be detected. To test this, we digested the luciferase protein with another proteinase, clostripain, known to cleave proteins at Arg residues [27]. We predicted from the luciferase sequence that this digestion would produce 21 different peptides ranging in size from 0.4 to 8.7 kDa. After incubation of the original luciferase with clostripain, samples were removed at various times and the luciferase proteins were detected with Western blots, as in the other experiments (Figure 3). We found that some initial cleavage products were detected at early times, but continued incubation of luciferase with clostripain resulted in the loss of all antigenic peptides. This suggested that the luciferase antibody did not interact with all parts of the luciferase protein. (This was also confirmed by the technical support team at Promega.) Thus the loss of detection of

luciferase in the incubations with the aspartic proteinase might not show some of the smaller products of luciferase cleavage. Therefore, although the previous experiments suggest the luciferase proteins were degraded by the aspartic proteinase, with this antibody and detection scheme we are unable to confirm that.

It is possible that the pro-(barley lectin)-processing site on AB1 was first cleaved by the aspartic proteinase but that further cleavage of this product was so rapid that this intermediate could not be detected. To rule out the possibility that other proteinases found in the reticulocyte lysate mixture in which the luciferase was translated were responsible for this degradation, we purified the AB1 protein from the lysate by using the polyclonal luciferase antibody before incubating it with the aspartic proteinase. This purified AB1 protein proved to be an excellent substrate for the aspartic proteinase: all of the antigenic protein was completely lost from the Western blot in only 2 min (Figure 2A). This supported the hypothesis that the aspartic proteinase alone was responsible for the loss of detectable AB1 and not simply the initial processing of the modified luciferase protein. This immunopurified AB1 luciferase was not active, presumably owing to the incubation in the salting-off buffer, 4 M MgCl₂, which is acidic. The amount of this purified luciferase was not sufficient to detect the bands by simply staining the gel, so we could not monitor the appearance of luciferase peptides directly. It is also formally possible that the initial cleavage by the aspartic proteinase produced a form of luciferase that was unstable in the acidic buffer. This is unlikely because the processed protein would have a structure similar to the VF or DG luciferases, which were relatively stable under acidic conditions (see below).

The various other versions of luciferase containing parts of the pro-(barley lectin) propeptide sequence were also tested as substrates for the aspartic proteinase. Because these versions of luciferase yielded inactive protein (Table 2), changes were monitored by using Western blots only (Figure 4). We observed a general trend that the longer the region of the pro-(barley lectin) amino acids that had been incorporated, the more complete the degradation by the aspartic proteinase. The amount of the del12 luciferase lost after 10 min with the aspartic proteinase was similar to the amount of original luciferase (pSP-luc+) after a similar time (compare Figures 4 and 2B). The modified luciferases containing 2, 4 or 10 pro-(barley lectin) residues (DG, VF and 6AA respectively) were somewhat more degraded by the aspartic proteinase than the original luciferase under the same conditions (compare Figures 4 and 2B). However, the luciferase containing the full pro-(barley lectin) C-terminus, 7AE, was degraded the most quickly and efficiently (Figure 4).

Figure 1 Loss of luciferase activity when incubated with the aspartic proteinase

Approximately 865 ng of AB1 (**A**) or 660 ng of pSP-luc+ luciferase (**B**) was incubated diluted in water alone (□), in 100 mM sodium formate buffer, pH 4 (○), with 2.4 units of the *A. thaliana* aspartic proteinase (AtAP) (■) or with 2.4 units of boiled aspartic proteinase (●). Reactions were stopped at the specified times by buffering aliquots up to pH 8 with the addition of 1 M Tris/HCl. Luminescent activity was recorded immediately in the Packard TopCount Instrument. The error bars show the variation between duplicate reactions.

These results indicated that replacement of pro-(barley lectin) residues on luciferase improved the ability of the protein to be cleaved by the aspartic proteinase in approximately a direct relationship to the number of pro-(barley lectin) residues that had been added. In all cases, as was observed with AB1, the aspartic proteinase did not produce an intermediate by processing of the protein at the engineered cleavage site only, but cleaved the modified luciferases at other internal sites.

From these experiments, it seemed that the aspartic proteinase was able to cleave the modified luciferase proteins at (a) site(s) beyond the engineered cleavage site to create smaller fragments of the protein. We wished to show whether the amino acid bond specificity of the aspartic proteinase was also broad with other

Figure 2 Western blots of luciferase proteins during incubation with the aspartic proteinase

Approximately 865 ng of AB1 was incubated with 2.7 units of the aspartic proteinase (**A**) or 165 ng pSP-luc+ luciferase was incubated with 2 units of the aspartic proteinase (**B**) for various durations (m, minutes). The proteins were then separated on 7.5% (**A**) or 15% (**B**) SDS gels, transferred to nitrocellulose membranes and probed with anti-luciferase antibodies (see the Experimental section). The VF form of luciferase (VF) was included as a size indicator; the position of the full-length luciferase is indicated by an arrow in each panel.

Figure 3 Western blots of pSP-luc+ luciferase protein during incubation with clostripain

Approximately 84 ng of pSP-luc+ luciferase was incubated for various durations (m, minutes) with 1 unit of clostripain. The proteins were then separated on an SDS/20% (w/v) polyacrylamide Phast gel with the use of the PhastSystem. The proteins were transferred and the blot was probed with anti-luciferase antibodies. The position of the luciferase transcribed and translated *in vitro* is indicated by an arrow.

protein substrates. For this, we incubated the aspartic proteinase with BSA or haemoglobin for various times. The proteins were then separated by SDS/PAGE and stained with Coomassie Blue (Figures 5A and 5B). From this experiment we observed that discrete large protein fragments from both BSA and haemoglobin were produced early during digestion by the aspartic proteinase but that longer incubation times produced

Figure 4 Western blots of other luciferase proteins during incubation with the aspartic proteinase

Approximately 165 ng of VF, DG or del12 (A), or 6AA or 7AE (B) luciferase protein was incubated with 2 units of the aspartic proteinase for various durations (m, minutes). The proteins were then separated by SDS/PAGE [7.5% (w/v) gel] (Bio-Rad), then transferred to nitrocellulose and probed with anti-luciferase antibodies (see the Experimental section). The position of the luciferase transcribed and translated *in vitro* is indicated by an arrow.

Figure 5 Changes in BSA and haemoglobin peptides during incubation with the aspartic proteinase

The substrate protein BSA (A) or haemoglobin (B) (82 μ g) was incubated with 4.3 units of the aspartic proteinase for various durations (m, minutes). The proteins were separated on SDS/20% (w/v) polyacrylamide Phast gels with the PhastSystem and then stained with Coomassie Blue. The positions of molecular mass marker proteins are indicated.

many smaller peptides that did not always stain well in these gels (Figures 5A and 5B). It is unlikely that the acidic buffer alone was causing the hydrolysis of these bonds because the substrate starting material was preincubated in the pH 3.5 buffer before the addition of the aspartic proteinase. These experiments suggested that the proteinase cut at many peptide bond sites within these proteins, possibly owing partly to the acidic conditions of the proteinase assay.

DISCUSSION

This study was designed to test the suitability of luciferase as a substrate for the *A. thaliana* aspartic proteinase. Previous research showed the importance of the C-terminal amino acids for luciferase activity, and the ability for other stretches of amino acids to help restore luciferase activity [23]. We therefore chose to modify the C-terminal 12 residues to replace them with the C-terminal propeptide sequence from barley lectin, a substrate for the aspartic proteinase *in vitro* [5]. In the most conservatively substituted luciferase, we replaced only five internal residues with those from the cleavage site of pro-(barley lectin). This enzyme, AB1, retained significant activity when compared with the original luciferase.

Despite the fact that the luciferase enzymes were sensitive to acidic pH conditions, we obtained evidence that the AB1 luciferase containing the pro-(barley lectin) residues was a substrate for the aspartic proteinase. We measured luciferase activity and luciferase antigenic peptides after short incubations with the aspartic proteinase. We observed a significant loss of AB1 with the addition of the aspartic proteinase that was not observed when the original pSP-luc+ luciferase was used as a substrate. This suggested that recognition by the aspartic proteinase was dependent on the presence of pro-(barley lectin) residues. From our results, it was evident that the aspartic proteinase did not simply digest incorrectly folded, inactive luciferase but showed a selectivity for those luciferase versions that specifically contained pro-(barley lectin) residues. For instance, both the del12 and 7AE forms of luciferase were inactive, but the latter with 17 residues from the pro-(barley lectin) was a much better substrate for the aspartic proteinase. It therefore seems that we have created luciferases that are substrates for our plant aspartic proteinase.

Counter to what we expected, the aspartic proteinase was not simply processing modified luciferase at the engineered site but was cleaving at other locations in the substrate. If only processing were occurring, we would have expected to see a luciferase protein the size of VF or DG, but these proteins were not clearly detected during the incubation. All of the modified forms of luciferase were cleaved at other sites and not detected in the blot. We cannot discern the extent of the degradation of the luciferase proteins, because the antibody used was limited in its detection of luciferase peptides. To test the possibility that the aspartic proteinase was able to cleave other peptide bonds, we used the protein substrates BSA and haemoglobin. The aspartic proteinase was found to cleave at several different sites in these proteins also, producing many small peptides. The fact that the aspartic proteinase processes pro-(barley lectin) *in vitro* [5] but digests luciferase and other proteins more completely to smaller peptides might indicate that this enzyme has a dual role in the plant, in both the processing and the degradation of proteins. The aspartic proteinase from castor bean has been proposed to be involved in the degradation of a propeptide from 2 S albumin, whereas the vacuolar processing enzyme is the primary processing enzyme of this seed storage protein [8]. The plant vacuolar processing enzyme or asparaginyl endopeptidase also seems to

have a dual activity. The proteinase processes the correctly folded trimers of 11 S proglubulin but degrades unassembled monomers *in vitro* [28]. Thus a dual nature of a proteinase might be a more common phenomenon for proteases than has been discussed previously and might be dependent on the substrate and assay used.

The unique mutations in the luciferase protein suggested that luciferase activity depended not only on the number of amino acid residues placed at its C-terminus but also on the type of amino acids. Thus this region of luciferase might be important in the action of this enzyme. The crystal structure of luciferase suggests that the C-terminal region contains α -helices and β -sheets ending with an α -helix created by the residues at -19 to -9 (numbered from the C-terminus) [22]. The last eight residues are disordered in the crystal structure and point away from the body of the protein into the solvent [22]. The C-terminal α -helix includes the -12 site, which was used here to engineer the proteinase substrate. The significant lack of activity that we observed in some of our luciferase mutants might be due to the inability of the modified enzyme to form an α -helix in this region because of the kinds of residue that were used to replace the native residues.

The -15 to -9 position of luciferase contains the sequence Arg⁻¹⁵-Glu-Ile-Leu-Ile-Lys-Ala, which forms part of the α -helix at the C-terminus of the protein [22]. The 7AE version of luciferase has the sequence Arg-Asp-Gly-Val-Phe-Ala-Glu in this region (Table 2). This contains the relatively conservative substitutions Glu⁻¹⁴ → Asp, Ile⁻¹³ → Gly and Leu⁻¹² → Val but then the replacement of Phe⁻¹¹ for Ile and Glu⁻⁹ for Ala. The Phe side chain is a large hydrophobic group in comparison with that of the smaller hydrophobic Ile, whereas the acidic residue Glu replaces the small hydrophobic Ala residue. The previous work found that replacement of the last twelve or eight residues of luciferase by the decapeptide Met-Arg-Ser-Ala-Met-Ser-Gly-Leu-His-Leu resulted in an enzyme with 22–35% of the activity of the wild-type luciferase [23]. The mostly smaller non-polar amino acids such as Met and Ser in this luciferase might have facilitated a correct structure in this region. Chou and Fasman [29] predict that residues such as Glu, Met, Ala and Leu are strong α -helix-supporting residues, whereas Gly strongly destabilizes this structure. Phe, Ile and Val are predicted to be weaker α -helix-stabilizing residues, whereas Asp, Arg and Ser are indifferent to the formation of an α -helix. The presence of the α -helix breaker Gly in all of the modified luciferase forms in place of the α -helix-stabilizing residue Ile might explain the lack of activity of many of these luciferases. The partly active AB1 still contained a Gly residue in this region but followed it by six α -helix-stabilizing residues, which might have formed enough of the structure in this region to be active. The observation that this protein retained 17% of the activity of the original luciferase, more than 100-fold the 7AE luciferase, suggests that although Gly might be predicted to disrupt an α -helix, the presence of several residues that promote this structure might have a positive effect on activity. There is also a significant difference in the charges on the last eight residues between the inactive 7AE luciferase and the active AB1, which might account for some of the differences. It would be interesting to crystallize these luciferase proteins and compare their structure in this region with that of the wild-type protein to observe whether the native configuration was restored in AB1 and provide evidence that this region is important for luciferase activity.

In this study we engineered a protein substrate for the analysis *in vitro* of the aspartic proteinase from *A. thaliana*. Luciferase has been used as a means for monitoring gene expression in a number of living systems, including plants, and as a mechanism for

isolating mutants in circadian rhythms [18,20,21]. The intention was that if a suitable active luciferase were created that was inactivated by the aspartic proteinase *in vitro*, it might also be a substrate *in vivo* and could be used as a method of selecting plants lacking the aspartic proteinase. Thus, in wild-type cells with the proteinase active, no luciferase activity would be detected, whereas luciferase would be active in mutant plants lacking the proteinase. This approach is impracticable with luciferase for two main reasons.

Despite the fact that the AB1 luciferase was active, it would probably not be useful in a screen *in vivo* for aspartic proteinase mutants of *Arabidopsis* because it would not be targeted to the vacuole. Targeting of this proposed substrate protein to the plant vacuole is probably necessary for interaction with the aspartic proteinase because that is the site of accumulation of the enzyme, at least in developing seed tissues [9]. The C-terminal propeptide of barley lectin is known to act as a vacuolar targeting signal but it must be present at the extreme C-terminus of the protein to function [25,30,31]. From the published work on pro-(barley lectin), we would expect that the 7AE construct containing the full propeptide of barley lectin would target the luciferase to the vacuoles of plant cells. However, this protein was inactive when synthesized *in vitro*, so it would probably not be a feasible method of monitoring the aspartic proteinase activity in plants. The addition of just three Gly residues to the Val-Phe-Ala targeting signal abolishes targeting in a transient assay system using tobacco protoplasts [25]. The C-terminus of AB1 has nine residues after the Val-Phe-Ala sequence, making this luciferase unlikely to be targeted to the vacuole in plant cells.

Another limitation of the suitability of luciferase for monitoring aspartic proteinase activity *in vivo* was the unexpected observation of the sensitivity of both the modified and original luciferase proteins to the acidic environment. The aspartic proteinase is active at acidic pH, a common property of most aspartic proteinases [1,5,13]. Luciferase has a pH optimum in the neutral range, between 8 and 8.5 [26]. For the purposes of these studies, we incubated the luciferase at low pH (4.0) for cleavage by the aspartic proteinase and then returned the luciferase protein to pH 8.0 to perform the luciferase assay. Both the original pSP-luc+ and AB1 luciferases lost a significant amount of activity in a short period on exposure to buffer at low pH. However, these buffers did not cause the degradation of luciferase because the proteins were detected on Western blots. Because low pH had such a detrimental effect on luciferase activity, there would probably be limitations on the ability of the luciferase system to monitor aspartic proteinase activity *in vivo*. The aspartic proteinase is found in vacuoles [5], an acidic organelle with an average pH of 5 [32]. However, on the basis of our work, targeting of a luciferase to this organelle would probably result in its rapid and irreversible inactivation. This would make the detection of luciferase activity in the acidic vacuoles of plants very difficult, another obstacle to the use of this system to monitor aspartic proteinase activity *in vivo*. It is possible that this system could be useful for monitoring changes in luciferase proteins targeted to other organelles with a higher pH.

We have created a novel protein substrate for the *Arabidopsis* aspartic proteinase with the use of firefly luciferase. Continued modification of the luciferase to include other specific amino acid residues and testing of these proteins with the aspartic proteinase could be used to probe the substrate specificity of the proteinase. This information from a protein substrate could then be compared with the specificity observed with peptide substrates ([15], and S. Gal, unpublished work). This rapid assay would facilitate the analysis. In addition, other proteinases might also be able to

recognize these luciferases differentially, making this assay of wider potential use.

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