Plant Stress ©2008 Global Science Books

Tissue Disruption Activates a Plant Caspase-Like Protease with TATD Cleavage Specificity

Nina V. Chichkova[1] • Raisa A. Galiullina[1] • Michael E. Taliansky[2] • Andrey B. Vartapetian[1*]

[1] Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119991, Russia
[2] Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, United Kingdom

Corresponding author: * varta@genebee.msu.ru

ABSTRACT

Recently, several caspase-like proteases have been described in plants. In particular, we have identified a tobacco caspase-like protease (CLP) that has a caspase cleavage specificity, that becomes activated in the course of plant programmed cell death (PCD), and that is essential for implementation of the cell death programme. The enzyme cleaves a peptide bond next to the Asp (D) residue within the TATD motif in the substrate VirD2 protein. Here we found that activation of tobacco CLP does occur in the course of healthy leaf tissue disruption as well, possibly through zymogen activation or enzyme de-sequestration. CLPs with identical cleavage specificity were demonstrated to be ubiquitous in mono- and dicotyledonous plants. Purified CLPs of tobacco and rice were shown to possess similar biochemical properties. Furthermore, inhibitor analysis demonstrated that CLP is sensitive to a number of peptide aldehyde inhibitors of animal caspases, with a notable exception of DEVD-CHO. Since these inhibitors have previously been employed in suppression of PCD mediated by different stress inducers, this result suggests that their inhibitory effect could be due, at least in part, to inactivation of the CLP under study.

Keywords: caspase inhibitors, programmed cell death, tobacco mosaic virus, VirD2 protein, wounding stress
Abbreviations: CHX, cycloheximide; **CLP**, caspase-like protease; **GFP**, green fluorescent protein; **HR**, hypersensitive response; **NLS**, nuclear localization signal; **PAGE**, polyacrylamide gel electrophoresis; **PCD**, programmed cell death; **TMV**, tobacco mosaic virus; **VPE**, vacuolar processing enzyme

INTRODUCTION

Programmed cell death (PCD, or apoptosis) is a basic process for elimination of redundant and damaged cells in multicellular organisms, which operates in the course of their development and in response to various stress-inducing stimuli. PCD in plant and animal kingdoms has a number of morphological features in common (Danon *et al*. 2000; Balk and Leaver 2001; Lam *et al*. 2001; Hoeberichts and Woltering 2003), yet PCD mechanisms are far better understood in animals than in plants. In animals, a key component of the PCD machinery is a family of apoptotic proteases termed caspases (for cysteine-dependent aspartate-specific proteases). These proteolytic enzymes become activated in the course of apoptosis and introduce specific cleavages into a limited set of cellular protein targets to promote cell death (Thornberry and Lazebnik 1998; Wolf and Green 1999). Accordingly, caspase inactivation is known to suppress cell death (Ekert *et al*. 1999; Zheng *et al*. 1999). Surprisingly, direct structural homologues of animal caspases with an analogous cleavage specificity and function are absent in plants.

We (Chichkova *et al*. 2004) and others (Bozhkov *et al*. 2004; Coffeen and Wolpert 2004; Danon *et al*. 2004; Hatsugai *et al*. 2004; Rojo *et al*. 2004; Vercammen *et al*. 2004; Watanabe and Lam 2005; Bosch and Franklin-Tong 2007) have described several different plant caspase-like proteases. In particular, we have identified a caspase-like protease (CLP) in tobacco that appears to represent a functional analogue of animal caspases (Chichkova *et al*. 2004). Tobacco CLP has selectivity and specificity resembling that of human caspase-3. Plant CLP introduced a single break into *Agrobacterium tumefaciens* VirD2 protein immediately after the D (aspartate) residue in the TATD motif. Tobacco CLP, like animal caspases, is dormant in healthy cells and becomes active in the course of the tobacco mosaic virus (TMV)-induced hypersensitive response [HR, a form of plant PCD (Greenberg 1997)] in tobacco. Furthermore, inactivation of tobacco CLP with a peptide aldehyde matching its cleavage site inhibited PCD in tobacco leaves (Chichkova *et al*. 2004).

Fragmentation of the *A. tumefaciens* VirD2 protein by tobacco CLP results in the detachment of the carboxy-terminal nuclear localization signal (NLS)-containing peptide of VirD2. The NLS in the VirD2 protein is essential for nuclear uptake of foreign DNA within the plant cell during bacterial infection and plant transformation (Steck *et al*. 1990; Shurvinton *et al*. 1992). Therefore CLP-mediated inactivation of VirD2 represents a protective mechanism aimed to limit delivery and expression of foreign genes in plants. Indeed, substitution of the wild type VirD2 protein with its CLP-resistant mutant was shown recently to markedly improve *A. tumefaciens*-mediated foreign gene delivery in various mono- and dicotyledonous plant species (Reavy *et al*. 2007).

Here we provide evidence that, apart from biotic stresses (such as viral and bacterial infection) mentioned above, CLP activation occurs in the course of healthy leaf tissue disruption. We demonstrate that CLPs with the specificity and biochemical properties similar to those of the tobacco enzyme are ubiquitous in plants, and that CLP activity is sensitive to a range of peptide aldehyde inhibitors of animal caspases.

MATERIALS AND METHODS

Purification of the GFP-VirD2Ct CLP substrate protein

$(His)_6$-tagged GFP-VirD2Ct protein containing, in successive

Received: 16 July, 2008. Accepted: 1 September, 2008.

Original Research Paper

order, a His tag, a GFP moiety, and an 86 amino acid long C-terminal region of the *A. tumefaciens* VirD2 protein, and its D39A point mutant were isolated from *Escherichia coli* cells overproducing the respective protein. Recombinant proteins were purified by Ni-NTA agarose (Qiagen) chromatography as described (Chichkova *et al*. 2004) and dialyzed against B1 buffer (20 mM MES, 2 mM DTT, 0.1% Tween 20, and 5% glycerol), pH 5.5. The final concentration of the protein substrate thus obtained was 0.5 mg/ml.

Preparation of leaf extracts

Tobacco (*Nicotiana tabacum* 'Xanthi nc', genotypes *NN* and *nn*) plants were grown at 24°C with a 16 h photoperiod. Fresh leaves were used either non-infected or infected with the wild-type TMV (U-1 strain). Infected leaves were kept at 33°C for 2 days, and then transferred to 24°C for 2 days. This temperature shift induced the hypersensitive response in TMV-infected *NN* leaves, as visualized by the formation of multiple necrotic lesions. Leaves (1 g samples from at least three different leaves processed in parallel) were ground under liquid nitrogen, and the samples were suspended in 2 ml of B1 buffer, pH 5.5 containing 50 mM NaCl at 0°C for 1 h. Where indicated, CHX was added to the buffer up to 1 mg/ml. Debris was eliminated by two successive centrifugation steps, 15 min at 15,000 × *g* each. The supernatants were treated with 1 mM AEBSF, 1 mM EDTA, 10 μM E-64, and 200 μg/ml chymostatin at 27°C for 30 min. The protein content of different extracts was verified by 15% SDS-PAGE fractionation with subsequent Coomassie Blue R-250 staining and equalized, if necessary. Samples in 0.1-1.0 μl aliquots were diluted 10-fold with B1+50 mM NaCl buffer and used for detection of CLP activity.

Fresh extracts of rice (*Oryza sativa)*, wheat (*Triticum vulgare*), potato (*Solanum tuberosum*), tomato (*Lycopersicum esculentum*), coffee (*Coffea arabica*), and plantain (*Plantago major*) leaves were prepared in an analogous fashion. For barley (*Hordeum vulgare*) and oat (*Avena sativa*) extracts, an additional fractionation was required to reduce contaminating proteolytic activities. Barley extract was passed through a Sephadex G-25 column, and the flow-through was applied onto a DE53 column (1 ml). Barley CLP activity was eluted with B1 buffer containing 0.1 M NaCl and used for subsequent analyses. For oat extraction, a protein fraction of the extract that precipitated within the 50-70% interval of $(NH_4)_2SO_4$ saturation was taken for analysis.

Detection of CLP activity

CLP activity was determined by assessing fragmentation of the added GFP-VirD2Ct protein as follows. Aliquots of leaf extracts or purified CLP were incubated with the GFP-VirD2Ct protein or with its D39A mutant (1-2 μg per sample) at 27°C for 1.5 h. Where indicated, biotinylated peptide aldehydes, Bio-TATD-CHO and Bio-DEVD-CHO (Bachem, from stock solution in DMSO), were added to CLP-containing samples at concentrations up to 100 μM, and the mixtures were incubated at 27°C for 30 min prior to addition of the GFP-VirD2Ct protein. In these cases, control samples were supplied with an equivalent amount of DMSO. Aliquots of chromatography fractions were diluted 10-fold with B1 buffer, pH 5.7, and incubated with a mixture of protease inhibitors (1 mM AEBSF, 1 mM EDTA, 10 μM E-64, and 200 μg/ml chymostatin) in a total volume of 20 μl at 27°C for 30 min prior to addition of GFP-VirD2Ct. Reaction mixtures were fractionated by 15% SDS-PAGE (Laemmli 1970), and substrate fragmentation was visualized by Coomassie Blue R-250 staining. The amount of extraneous proteins present in CLP preparations was usually very low (**Fig. 2A-F**, lanes 8) and did not interfere with the analysis.

Purification of tobacco and rice CLPs

Fresh tobacco 'Xanthi nc' leaves (10 g) and rice *Oryza sativa* cv. 'Leader' (80 g) leaves were ground in liquid nitrogen, resuspended in 40 and 320 ml respectively, of B1 buffer, pH 5.7, containing 1 mM PMSF and sonicated for 15 min. The debris was eliminated by two successive 15-min centrifugation steps at 15,000 × *g*, and the supernatants were fractionated by ammonium sulfate precipitation. The protein fraction that precipitated within the 50-70% interval of $(NH_4)_2SO_4$ saturation was dissolved in a start buffer containing 25 mM Tris-HCl, 2 mM DTT, 0.1% Tween 20, 5% glycerol, pH 7.6 (for tobacco CLP) or in B1 buffer, pH 5.5 (for rice CLP) and dialyzed against the same buffer to remove traces of ammonium sulfate.

The tobacco CLP sample was then subjected to chromatofocusing using a PBE 94 Polybuffer Exchanger column (Amersham Biosciences, volume = 15 ml) equilibrated with the start buffer. The column was washed with two volumes of the same buffer, and the proteins were eluted from the column with ten volumes of a mixture of 10-fold diluted Polybuffer 94 (30%) and Polybuffer 74, pH 5.0 (70%) containing 2 mM DTT, 0.1% Tween 20, and 5% glycerol at 20 ml/h. Additional elution was performed with two volumes of Polybuffer 74, pH 4.0 (eight-fold diluted and containing the same additives). For each fraction the pH was measured and 5 μl samples were then diluted 4-fold with B1 buffer, pH 6.0. Samples were processed for CLP activity determination and peak fractions were combined.

Rice CLP was subjected to anion exchange chromatography on a DE53 column (Whatman, 5 ml) equilibrated with B1 buffer, pH 5.5. Flow-through from this column was collected, dialyzed against B1 buffer, pH 7.5, and applied onto a DE53 column equilibrated with B1 buffer, pH 7.5. Elution was performed with a 0-200 mM NaCl gradient in the same buffer, and protein fractions eluted at 100 mM NaCl and containing CLP activity were collected.

Finally, peak fractions of tobacco and rice CLP preparations were applied onto a Blue agarose column (2 ml) equilibrated with B1 buffer, pH 6.0 (for tobacco CLP) or with B1 buffer, pH 7.5 (for rice CLP). Elution was performed at 6 ml/h with a 0-1.5 M NaCl gradient in the same buffer. Peak fractions containing CLP activities (0.07-0.15 M NaCl for the tobacco enzyme and 0.8-1.5 M NaCl for the rice enzyme) were combined, concentrated using a YM-30 Centricon (Amicon), and used for characterization of the respective CLP.

Characterization of CLPs

To perform accurate comparison of activities of purified tobacco and rice CLPs, GFP-VirD2Ct substrate protein was treated with the enzyme preparations under the conditions of partial hydrolysis.

To determine the optimal pH for CLP hydrolysis, tobacco and rice CLP samples were diluted 50-fold with buffers of pH 4.0-9.0 and incubated with GFP-VirD2Ct substrate protein at 27°C for 0.5 h (tobacco CLP) or 1.5 h (rice CLP). Polybuffer 74 (8-fold diluted), 25 mM MES, 25 mM HEPES, and 25 mM Tris were used to obtain buffers covering pH intervals 4.0-5.0, 5.5-6.5, 7.0-7.5, and 8.0-9.0, respectively. All buffers contained 2 mM DTT, 0.1% Tween 20, and 5% glycerol.

The stability of CLPs at different pH was determined by 10-fold dilution of CLP samples with buffers of pH 4.0-9.0 (as indicated in the previous paragraph). Samples were incubated at +4°C for two weeks, then diluted 10-fold with B1 buffer, pH 6.0, and incubated for another 20 h. GFP-VirD2Ct was then added to the samples, and reaction mixtures were incubated for 1.5 h at 27°C.

The isoelectric points of CLPs were determined by chromatofocusing on a PBE 94 column (volume = 5 ml) using Polybuffer 74 (eight-fold diluted, pH 4.0) as an eluent, essentially as described above. Two ml fractions were collected and used for pH measurements and, diluted 4-fold with B1 buffer, pH 6.0, for CLP activity determinations.

The salt dependence of CLP hydrolysis was determined by supplementing the reaction buffer B1, pH 6.0, with 0-300 mM NaCl. Samples were incubated with GFP-VirD2Ct protein for 1.5 h at 27°C.

The sensitivity of rice CLP to various inhibitors of proteolytic enzymes was assessed using an INHIB1 Protease Inhibitor Panel (Sigma) and mercuric chloride according to recommendations of the manufacturer. Rice CLP samples were pre-incubated in B1 buffer, pH 6.0, at 27°C for 30 min with the inhibitors at the following concentrations: AEBSF, 1 mM; aprotinin, 5 μg/ml; antipain, 100 μM; leupeptin, 50 μg/ml; N-ethylmaleimide (NEM), 1 mM; E-64, 10 μM; benzamidine-HCl, 4 mM; trypsin inhibitor, 10 μg/ml; 6-aminohexanoic acid, 5 mg/ml; EDTA, 1 mM; phosphoramidon, 10 μM; bestatin, 40 μM; pepstatin A, 1 μg/ml; $HgCl_2$,

200 μM; chymostatin, 200 μg/ml. Then the GFP-VirD2Ct protein substrate was added to the samples, and incubation was continued for another 1.5 h. Reaction mixtures were fractionated by 15% SDS-PAGE.

To assess the sensitivity of rice CLP to animal caspase inhibitors, CLP samples were pre-incubated in B1 buffer, pH 5.7, with Ac-VAD-CHO (Calbiochem), Ac-YVAD-CHO, Ac-WEHD-CHO, Ac-VEID-CHO, Ac-IETD-CHO, Ac-LEHD-CHO, Ac-DEVD-CHO (Bachem, from stock solution in DMSO), as well as with Bio-DEVD-CHO and Bio-TATD-CHO at 100 μM at 27°C for 30 min. Control samples were supplied with an equivalent amount of DMSO. The inhibitor-treated samples were then incubated with the GFP-VirD2Ct substrate protein at 27°C for 1 h and analyzed by 15% SDS-PAGE.

RESULTS

CLP activity in extracts from *Nicotiana tabacum* leaves

Our previous *in planta* studies have demonstrated that the CLP activity capable of target protein fragmentation at the TATD motif was undetectable in tobacco leaves unless the HR was induced by TMV infection of the *N* gene-containing *N. tabacum* plants (Chichkova *et al*. 2004). In this experimental system, at temperatures below 27°C the *N* gene mediates an HR response in *N. tabacum* cells infected with the virus, thus limiting the virus spread through the plant. At higher temperatures, the *N* gene-mediated response is inoperative which results in a lack of PCD and in systemic spread of the virus through the plant (Kassanis 1952). A common practice therefore is to infect tobacco *NN* plants with TMV at a permissive temperature (33°C) and, after a short period required for virus multiplication, to shift the infected plants to a restrictive temperature (24°C) to induce the HR. Using this scheme, the CLP activity was undetectable in *N. tabacum nn* plants lacking a functional *N* gene, both when healthy and when TMV-infected, at either temperature. In *NN* plants, the CLP activity was observed only when the TMV-infected plants were shifted to 24°C to induce the HR (Chichkova *et al*. 2004).

Proof of the inducibility of CLP activity was based on analysis of the subcellular distribution of a fluorescent chimeric GFP-VirD2Ct reporter protein. GFP-VirD2Ct contains a C-terminal portion of *A. tumefaciens* VirD2 protein (86 amino acid residues long) that encompasses a CLP cleavage site (TATD[39]) and the NLS fused at the N-terminus to GFP. Depending on whether or not the reporter protein is cleaved by the CLP, its fluorescent moiety localizes inside the nucleus (intact GFP-VirD2Ct protein) or is spread throughout the cell (caspase-truncated GFP-VirD2Ct protein that lacks the NLS) (Chichkova *et al*. 2004).

In vitro, purified tobacco CLP cleaves recombinant GFP-VirD2Ct protein at the same TATD[39] motif giving rise

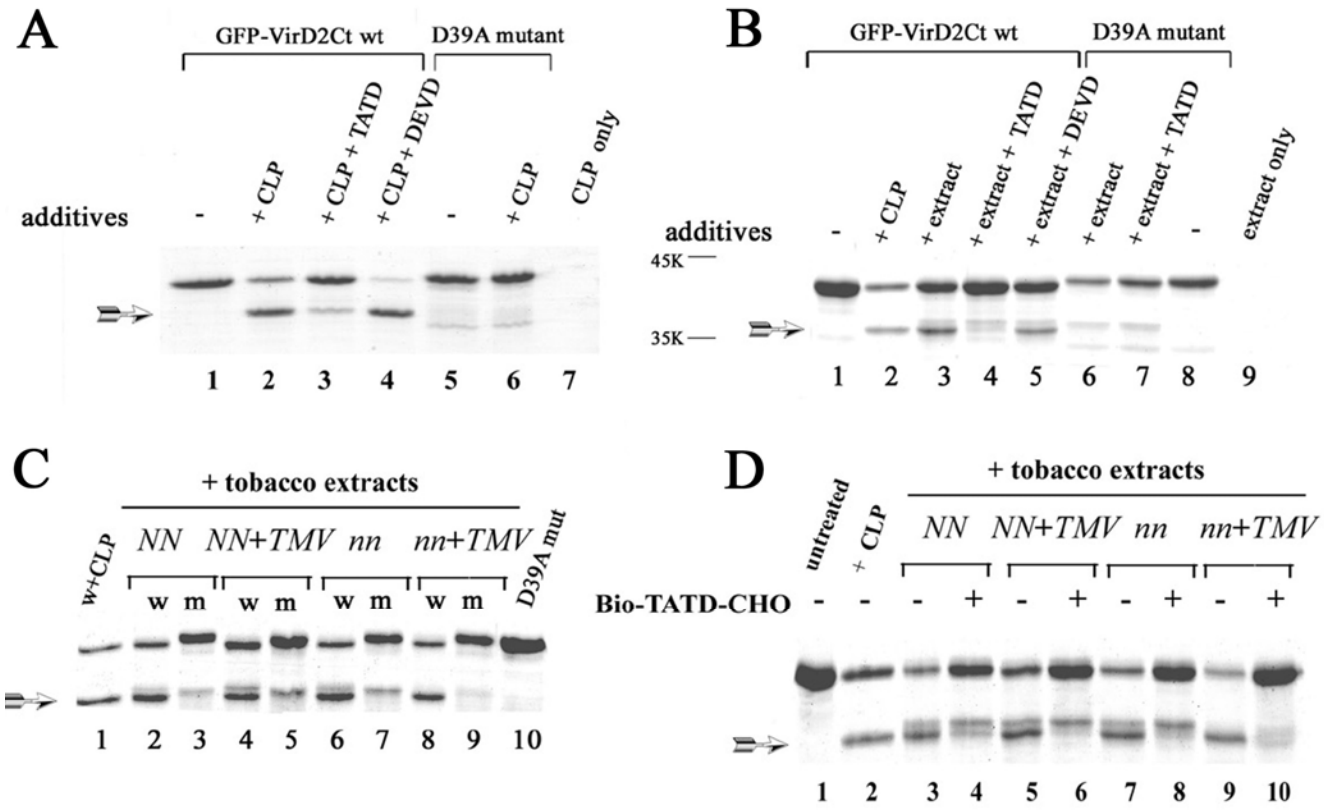

Fig. 1 Specific fragmentation of chimeric GFP-VirD2Ct substrate protein by purified tobacco CLP (A) and by CLP in tobacco leaf extracts (B-D). (**A**) Wild type (wt) GFP-VirD2Ct or its D39A mutant (calculated MW ~38 kD) were treated with CLP purified from tobacco leaves infected with TMV and incubated at 24°C to induce the HR. +TATD and +DEVD denote pre-treatment of the enzyme preparations with biotinyl-TATD-CHO and biotinyl-DEVD-CHO (100 μM), respectively. Lane 'CLP only' corresponds to the enzyme preparation without GFP-VirD2Ct added. Reaction mixtures were fractionated by electrophoresis in 15% SDS-polyacrylamide gels, and protein bands were visualized by Coomassie Blue staining. Arrows here and in **Figs. 2** and **3** point to the protein fragment generated by the CLP activity (calculated MW~33 kD). (**B**) Detection of the CLP activity in crude extracts of *N. tabacum* leaves infected with TMV and incubated at 24°C for 2 days to induce the HR. Extracts were pre-treated with AEBSF, EDTA, E-64, and chymostatin. Where indicated, extracts were also pre-treated with 100 μM biotinyl-TATD-CHO or biotinyl-DEVD-CHO. GFP-VirD2Ct and its D39A mutant were used as substrates. +CLP, substrate treatment with the purified CLP preparation. Lane 'extract only', no GFP-VirD2Ct was added to the extract. Numbers at the left correspond to positions of MW protein markers. (**C**) Evidence for the presence of CLP activity in various tobacco leaf extracts using wild-type (w) and D39A point mutant (m) GFP-VirD2Ct proteins. Extracts were obtained from *NN* tobacco leaves infected with the TMV and kept at 24°C to induce the HR (*NN + TMV*) and from either non-infected leaves (*NN* and *nn*) or TMV-infected *nn* leaves that do not exhibit HR (*nn + TMV*). w+CLP (lane 1), wild-type substrate treatment with the purified CLP preparation. (**D**) CLP activity in tobacco leaf extracts is suppressed by biotinyl-TATD-CHO. Leaf extracts prepared as in (C) were used to digest GFP-VirD2Ct protein. The extracts were either pre-treated with 100 μM biotinyl-TATD-CHO (+) or left untreated (-).

to a truncated protein. CLP was isolated by a series of chromatography steps (see Materials and methods section) from *N. tabacum NN* leaves that had been infected with TMV and incubated at 24°C to induce the HR. The truncation of the reporter protein by this enzyme could be visualized by polyacrylamide gel electrophoresis (PAGE) of the reaction mixture – **Fig. 1A**, lane 2. Suppression of the cleavage reaction by a specific biotinylated peptide aldehyde inhibitor of CLP, Bio-TATD-CHO (lane 3), and not by a control peptide Bio-DEVD-CHO (lane 4) confirms specificity of the cleavage reaction. Furthermore, lack of cleavage of the GFP-VirD2Ct(D39A) mutant protein that possesses a single amino acid substitution of the critical D^{39} residue in the TATD motif (lane 6) provides additional evidence for the cleavage specificity.

To learn whether the CLP activity can be detected with this approach in leaf extracts, we prepared crude extracts of *N. tabacum NN* leaves infected with TMV and incubated at 24°C until symptoms of the HR became visible. The extracts were supplemented with the CLP substrate protein, GFP-VirD2Ct, and possible substrate fragmentation was analyzed by gel electrophoresis. However this approach turned out to be unsuccessful due to a high level of non-specific proteolytic degradation of the target protein in crude extracts that masked putative CLP-mediated fragmentation (data not shown).

Since tobacco CLP is a highly specific protease and is not inhibited by a wide range of chemical inhibitors known to inactivate various proteolytic enzymes (Chichkova *et al*. 2004), we supplemented crude tobacco extracts with a cocktail of inhibitors prior to the addition of GFP-VirD2Ct to prevent non-specific proteolysis of the substrate. The most efficient combination of inhibitors included AEBSF (1 mM), EDTA (1 mM), E64 (1 μM), and chymostatin (200 μg/ml). Although such a pretreatment did not eliminate contaminating proteolytic activities completely, it did clarify the pattern of the substrate protein fragmentation significantly. Incubation of the treated extract with the GFP-VirD2Ct protein resulted in a specific truncation of the substrate, with the electrophoretic mobility of the cleavage product being the same as for the product generated by purified tobacco CLP (**Fig. 1B**, compare lanes 2 and 3). Pre-incubation of the extract with 100 μM Bio-TATD-CHO impaired fragmentation of the substrate (lane 4), while 100 μM Bio-DEVD-CHO produced no effect (lane 5). The specific cleavage was also abrogated when the mutant GFP-VirD2Ct(D39A) protein was used instead of the wild type one (**Fig. 1B**, lanes 6-8).

Thus, the CLP-mediated fragmentation of GFP-VirD2Ct can be monitored in tobacco leaf extracts. We then aimed to compare the level of CLP activity in leaf extracts from healthy (uninfected) and TMV-infected tobacco plants of *NN* and *nn* genotypes. TMV-infected plants were incubated at 24°C to generate necrotic lesions, a manifestation of the TMV-induced HR, in the case of the *NN* plants. Under the same conditions, PCD was not induced in *nn* plants. Surprisingly, when extracts from healthy tobacco leaves (whether of *NN* or *nn* genotype) and from TMV-infected *nn* leaves were assayed for the presence of the CLP activity, cleavage of the substrate protein occurred (**Fig. 1C**, lanes 2, 6 and 8). The efficiencies of these cleavage reactions were comparable to that exhibited by extracts from TMV-infected *NN* plants incubated at 24°C that demonstrated symptoms of the HR (**Fig. 1C**, lane 4). Cleavage specificity in all these cases was confirmed by using the CLP-resistant GFP-VirD2Ct(D39A) mutant protein (**Fig. 1C**, lanes 3, 5, 7, and 9). Furthermore, the Bio-TATD-CHO inhibitor of CLP suppressed fragmentation of the substrate protein in the case of each extract (**Fig. 1D**). Of note, CLP activity was unaltered in the presence of the translation inhibitor cycloheximide (data not shown), which indicates that formation of the active enzyme was independent of *de novo* protein synthesis.

It is therefore evident that in extracts of tobacco leaves, either healthy or apoptotic, CLP is found in an already activated state. This is in contrast to *in planta* studies showing that the CLP activity is undetectable in intact tobacco leaves unless the HR was induced by TMV infection. One of the possibilities explaining this unexpected phenomenon is that CLP activation occurs through a post-translational processing of a CLP precursor protein in the course of tissue disruption and incubation of the extract with the substrate protein. Another possibility is that cell damage causes enzyme de-sequestration.

On the one hand, such a behavior is inconvenient since the level of the CLP activity in extracts does not mirror its level in intact tissues. On the other hand, this result indicates that any virus-specific component is dispensable for CLP activity and opens the possibility to detect the presence of CLP activity in various plant species without knowing the genuine stimuli that cause activation of this enzyme *in planta*.

CLP activity is widespread in plant kingdom

To learn whether mechanical stress would cause CLP activation in evolutionary distant plants, we assayed leaf extracts of rice, wheat, potato, tomato, barley, coffee, plantain, and oat for the presence of the CLP activity. Healthy leaves were homogenized, extracts were clarified by low-speed centrifugation and pre-incubated with a set of protease inhibitors to suppress non-specific proteolysis. The GFP-VirD2Ct substrate protein or the GFP-VirD2Ct(D39A) mutant were then incubated with these extracts, and the reaction mixtures were analyzed by PAGE to assess substrate fragmentation. As shown in **Fig. 2A-H**, the presence of the CLP activity was evident in extracts of every plant species tested. Cleavage specificity of CLPs from various sources appeared to be identical to that of tobacco CLP, as judged by the inhibitory action of Bio-TATD-CHO (but not of Bio-DEVD-CHO) and by the resistance of the GFP-VirD2Ct(D39A) mutant to fragmentation.

The efficiency with which a cocktail of protease inhibitors prevented non-specific degradation of the substrate protein varied between plant species, indicating that it should be optimized for every species to obtain a most clear pattern, if necessary. In some complicated cases (e.g. barley and oat extracts), additional ion-exchange chromatography or ammonium sulfate purification steps were required (see Materials and methods section) in order to eliminate interfering proteolytic activities causing non-specific degradation of the substrate.

Thus the CLP activity was identified in extracts from various healthy plants although, as mentioned above, this does not necessary mean that the enzyme is constitutively active.

Biochemical properties of tobacco and rice CLPs

We then compared some biochemical properties of CLPs isolated from the di- and monocotyledonous plants, respectively, tobacco and rice. The enzyme purification scheme included several chromatography steps (see Materials and methods for details) and resulted in an over 1000-fold purification of these proteins. GFP-VirD2Ct was used as a CLP target in these assays.

The optimum pH for the cleavage reaction was 5.5-6.5 for tobacco CLP and 6.0-6.5 for rice CLP (**Fig. 3A**). Both the tobacco and rice CLPs displayed a remarkable stability throughout a two-week incubation over a broad pH 4.0-9.0 range (**Fig. 3B**). Both enzymes are slightly acidic proteins, with pI = 5.0 for tobacco CLP and pI = 6.4-6.5 for rice CLP (**Fig. 3C**). Both enzymes did not display any evident requirement of NaCl for their activity. Moreover, a salt concentration of 100 mM and above was clearly inhibitory (**Fig. 3D**). We then tested ability of various chemical inhibitors of proteolytic enzymes belonging to different classes to inactivate rice CLP. As was found previously for tobacco CLP (Chichkova *et al*. 2004), rice CLP turned out to be virtually insensitive to most of these treatments, with mercuric chlo-

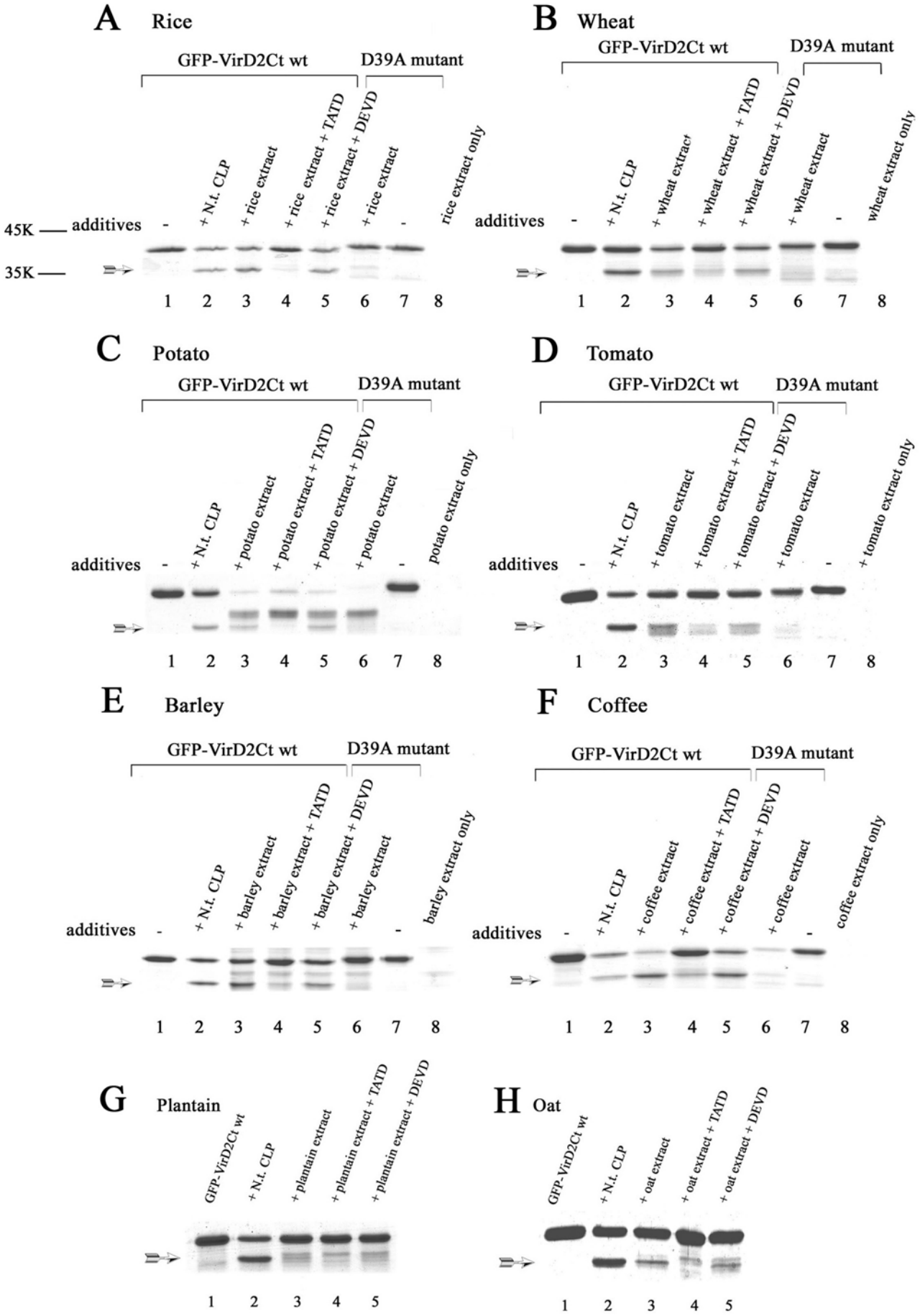

Fig. 2 CLP activity with TATD specificity is ubiquitous in plants. GFP-VirD2Ct protein (lanes 1-5) or the D39A CLP-resistant mutant (lanes 6 and 7) were treated with extracts obtained from leaves of (**A**) rice, (**B**) wheat, (**C**) potato, (**D**) tomato, (**E**) barley, (**F**) coffee, (**G**) plantain, and (**H**) oat. Extract preparation is described in detail in the Materials and methods section. Where indicated, extracts were pre-treated with 100 μM biotinyl-TATD-CHO or biotinyl-DEVD-CHO, respectively. Treatment of the same protein substrate with purified tobacco CLP (+*N.t.* CLP) is given for comparison (lane 2 in all panels). Protein bands were visualized by Coomassie Blue staining upon electrophoresis in 15% SDS- polyacrylamide gels. Numbers on the left correspond to positions of MW protein markers.

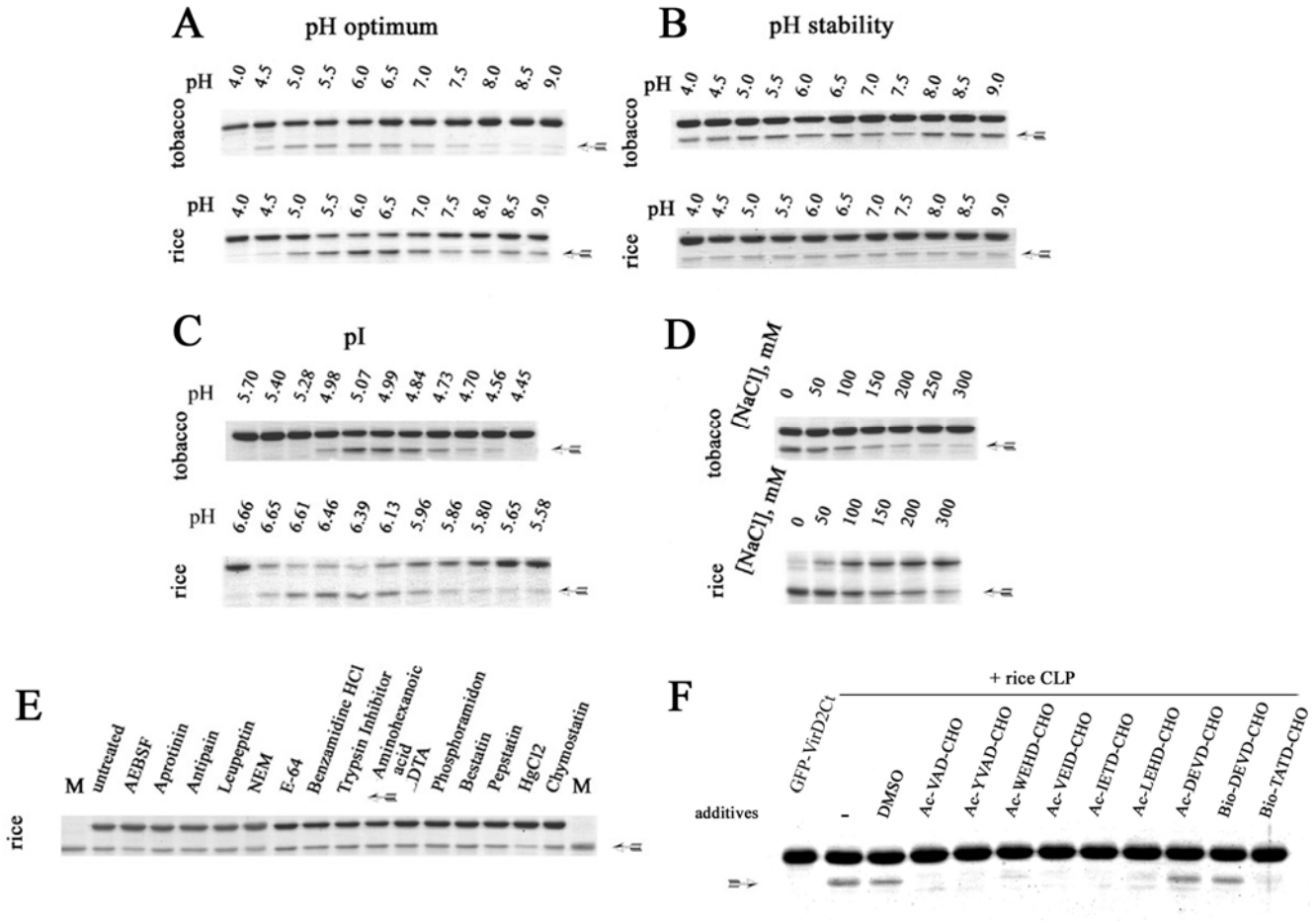

Fig. 3 Comparison of some biochemical properties of purified tobacco and rice CLPs. Recombinant GFP-VirD2Ct protein was used as a substrate to evaluate CLP activity. (**A**) Optimum pH for CLP cleavage reaction was determined by performing digestions of the GFP-VirD2Ct protein at different pH values within the pH 4.0-9.0 range. (**B**) Stability of CLPs at different pH. Enzyme preparations were incubated at indicated pH values at +4°C for two weeks. Then the samples were brought to pH 6.0 and used to digest the substrate protein. (**C**) Isoelectric points of CLPs were determined by chromatofocusing of the enzyme preparations on a Polybuffer Exchanger 94 column. For each eluted fraction, the pH value was determined, and CLP activity was assessed after bringing the pH of the sample to pH 6.0. (**D**) Salt dependence of CLP activity was determined by supplementing reaction buffer B1, pH 6.0, with the indicated amount of NaCl. (**E**) Rice CLP is insensitive to the majority of chemical inhibitors known to inactivate various proteolytic enzymes. Rice CLP preparation was pre-incubated with the indicated reagents before incubation with GFP-VirD2Ct. (**F**) Inhibition of rice CLP with peptide aldehyde inhibitors of animal caspases. Rice CLP preparation was pre-incubated with the indicated peptide aldehydes (100 μM) or with DMSO only before incubation with GFP-VirD2Ct. Fragmentation of the substrate protein was assessed by 15% SDS-PAGE and staining with Coomassie Blue. Lanes M in (E), fragmentation of GFP-VirD2Ct by purified rice CLP under the optimum conditions used here as an electrophoretic mobility marker.

ride being the most potent inhibitor (**Fig. 3E**). We therefore conclude that CLPs from distant plant species possess similar biochemical properties.

Finally, we assessed the sensitivity of rice CLP to a set of peptide aldehyde inhibitors of animal caspases. The inhibitors were employed at 100 μM concentration shown previously to be effective with the Ac-TATD-CHO inhibitor matching the CLP cleavage site. With the exception of Ac-DEVD-CHO and Bio-DEVD-CHO, all other inhibitors dramatically suppressed rice CLP activity (**Fig. 3F**). This result further confirms that the aspartate specificity is an intrinsic property of CLP.

DISCUSSION

A CLP capable of specific fragmentation of the GFP-VirD2Ct protein substrate strictly after a D residue within the TATD motif has previously been shown to be activated in tobacco leaves in the course of the HR caused by TMV infection of *N* gene-containing plants (Chichkova *et al*. 2004). Furthermore, this CLP was found to be essential for the implementation of the TMV-induced PCD in tobacco since CLP inactivation markedly suppressed PCD. The same CLP is likely to become activated in the course of the *A. tumefaciens* infection as well, since the CLP-resistant VirD2 mutant markedly increased *A. tumefaciens*-mediated gene delivery into plants (Reavy *et al*. 2007). Here we report that, apart from these biotic stresses, activation of the enzyme also occurs in the course of healthy leaf disruption. Since CLP activation in this model was found to be independent of *de novo* protein synthesis, our data indicate that the formation of an active CLP requires either activation of a zymogen, as is the case with many proteolytic enzymes including animal caspases (Salvesen and Dixit 1999), or desequestration of the already processed enzyme. For example, activation (processing) of another plant PCD-related protease, cathepsin B, takes place upon secretion into the apoplast in the absence of PCD (Gilroy *et al*. 2007).

Whatever the exact mechanism of CLP activation is, the presence of an active CLP in leaf extracts turned out to be helpful. It has allowed us to detect CLPs with identical cleavage specificities in diverse plant species, both mono- and dicotyledonous, even in the absence of knowledge of the genuine physiological stimuli that trigger CLP activation *in planta*. Purified CLPs of tobacco and rice were obtained and shown to possess similar biochemical properties. Broad distribution of CLPs likely points to a functional importance of the enzyme.

In *A. tumefaciens* VirD2 protein, CLP cleaves a peptide bond next to the TATD motif. Accordingly, a TATD-CHO peptide aldehyde matching the cleavage site was shown to inhibit CLP activity (this study and Chichkova *et al*. 2004). To assess CLP substrate specificity in greater detail, we employed various peptide aldehyde inhibitors of animal cas-

pases with the purified rice enzyme. Surprisingly, the majority of inhibitors tested could inactivate CLP, including: Ac-VAD-CHO (a general caspase inhibitor), Ac-YVAD-CHO (a caspase-1 inhibitor), Ac-WEHD-CHO (a caspase-5 inhibitor), Ac-VEID-CHO (a caspase-6 inhibitor), Ac-IETD (a caspase-8 inhibitor), and Ac-LEHD-CHO (a caspase-9 inhibitor). Ac-DEVD-CHO, a caspase-3 inhibitor, represented a notable exception. It seems possible therefore that CLP activation could account for at least some of the caspase-like proteolytic activities observed in a variety of plant PCD models using peptide substrates of animal caspases (reviewed in Bonneau *et al*. 2008). Likewise, inhibition of PCD in various plant systems with a range of peptide inhibitors of animal caspases could possibly be mediated by CLP inactivation.

A peculiarity of the CLP under study is that it appears to differentiate between the two general caspase inhibitors, Ac-VAD-CHO and z-VAD-fmk. While both of them are active against animal caspases, the latter was inefficient in CLP inhibition (Chichkova *et al*. 2004). By the same criterion, the CLP under study appears to be distinct from the two other plant proteases proposed to represent analogues of animal caspases, VPE and "saspase". Both of them were reported to be z-VAD-fmk-sensitive (Coffeen and Wolpert 2004; Rojo *et al*. 2004). The CLP is also clearly different from plant metacaspases that possess arginine/lysine cleavage specificity (Vercammen *et al*. 2004; Watanabe and Lam 2005). Identification of CLP is of importance and is currently underway.

In conclusion, we present evidence that the CLP under study becomes activated not only as a result of pathogen attack but as a consequence of tissue disruption as well. This finding suggests that CLP may be involved in various pathways initiated by both biotic (such as viral or bacterial infections) and abiotic (for example, wounding) stresses. Our study demonstrates ubiquity of CLP in plants and provides an approach for isolation of an active enzyme from various plant sources for further studies. Finally, a CLP inhibition pattern should be instructive for interpreting data on plant PCD suppression using animal caspase inhibitors.

ACKNOWLEDGEMENTS

We thank Tatyana N. Chernysheva for plant care. This work was supported in part by grants from the Russian Foundation for Basic Research (to N.V.C. and A.B.V.), by Scottish Government (M.E.T.) and by the Royal Society Joint Project Grant (to M.E.T and A.B.V.).

REFERENCES

Balk J, Leaver CJ (2001) The PET1-CMS mitochondrial mutation in sunflower is associated with premature programmed cell death and cytochrome *c* release. *The Plant Cell* **13**, 1803-1818

Bonneau L, Ge Y, Drury G, Gallois P (2008) What happened to plant caspases? *Journal of Experimental Botany* **59**, 491-499

Bosch M, Franklin-Tong VE (2007) Temporal and spatial activation of caspase-like enzymes induced by self-incompatibility in *Papaver* pollen. *Proceedings of the National Academy of Sciences USA* **104**, 18327-18332

Bozhkov PV, Filonova LH, Suarez MF, Helmersson A, Smertenko AP, Zhivotovsky B, von Arnold S (2004) VEIDase is a principal caspase-like activity involved in plant programmed cell death and essential for embryonic pattern formation. *Cell Death and Differentiation* **11**, 175-182

Chichkova NV, Kim SH, Titova ES, Kalkum M, Morozov VS, Rubtsov YP, Kalinina NO, Taliansky ME, Vartapetian AB (2004) A plant caspase-like protease activated during the hypersensitive response. *The Plant Cell* **16**, 157-171

Coffeen WC, Wolpert TJ (2004) Purification and characterization of serine proteases that exhibit caspase-like activity and are associated with programmed cell death in *Avena sativa*. *The Plant Cell* **16**, 857-873

Danon A, Delorme V, Mailhac N, Gallois P (2000) Plant programmed cell death: a common way to die. *Plant Physiology and Biochemistry* **38**, 647-655

Danon A, Rotari VI, Gordon A, Mailhac N, Gallois P (2004) Ultraviolet-C overexposure induces programmed cell death in Arabidopsis, which is mediated by caspase-like activities and which can be suppressed by caspase inhibitors, p35 and Defender against Apoptotic Death. *The Journal of Biological Chemistry* **279**, 779-787

Ekert PG, Silke J, Vaux DL (1999) Caspase inhibitors. *Cell Death and Differentiation* **6**, 1081-1086

Gilroy EM, Hein I, van der Hoorn R, Boevink PC, Venter E, McLellan H, Kaffarnik F, Hrubikova K, Shaw J, Holeva M, López EC, Borras-Hidalgo O, Pritchard L, Loake GJ, Lacomme C, Birch PR (2007) Involvement of cathepsin B in the plant disease resistance hypersensitive response. *The Plant Journal* **52**, 1-13

Greenberg JT (1997) Programmed cell death in plant-pathogen interactions. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**, 525-545

Hatsugai N, Kuroyanagi M, Yamada K, Meshi T, Tsuda S, Kondo M, Nishimura M, Hara-Nishimura I (2004) A plant vacuolar protease, VPE, mediates virus-induced hypersensitive cell death. *Science* **305**, 855-858

Hoeberichts FA, Woltering EJ (2003) Multiple mediators of plant programmed cell death: interplay of conserved cell death mechanisms and plant-specific regulators. *Bioessays* **25**, 47-57

Kassanis B (1952) Some effects of high temperature on susceptibility of plants to infection with viruses. *Annals of Applied Biology* **26**, 358-369

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685

Lam E, Kato N, Lawton M (2001) Programmed cell death, mitochondria and the plant hypersensitive response. *Nature* **411**, 848-853

Reavy B, Bagirova S, Chichkova NV, Fedoseeva SV, Kim SH, Vartapetian AB, Taliansky ME (2007) Caspase-resistant VirD2 protein provides enhanced gene delivery and expression in plants. *Plant Cell Reports* **26**, 1215-1219

Rojo E, Martín R, Carter C, Zouhar J, Pan S, Plotnikova J, Jin H, Paneque M, Sánchez-Serrano JJ, Baker B, Ausubel FM, Raikhel NV (2004) VPEgamma exhibits a caspase-like activity that contributes to defense against pathogens. *Current Biology* **14**, 1897-1906

Salvesen GS, Dixit VM (1999) Caspase activation: the induced-proximity model. *Proceedings of the National Academy of Sciences USA* **96**, 10964-10967

Shurvinton CE, Hodges L, Ream W (1992) A nuclear localization signal and the C-terminal omega sequence in the *Agrobacterium tumefaciens* VirD2 endonuclease are important for tumor formation. *Proceedings of the National Academy of Sciences USA* **89**, 11837-11841

Steck TR, Lin TS, Kado CI (1990) VirD2 gene product from the nopaline plasmid pTiC58 has at least two activities required for virulence. *Nucleic Acids Research* **18**, 6953-6958

Thornberry NA, Lazebnik Y (1998) Caspases: enemies within. *Science* **281**, 1312-1316

Vercammen D, van de Cotte B, De Jaeger G, Eeckhout D, Casteels P, Vandepoele K, Vandenberghe I, Van Beeumen J, Inzé D, Van Breusegem F (2004) Type II metacaspases Atmc4 and Atmc9 of Arabidopsis thaliana cleave substrates after arginine and lysine. *The Journal of Biological Chemistry* **279**, 45329-45336

Watanabe N, Lam E (2005) Two Arabidopsis metacaspases AtMCP1b and AtMCP2b are arginine/lysine-specific cysteine proteases and activate apoptosis-like cell death in yeast. *The Journal of Biological Chemistry* **280**, 14691-14699

Wolf BB, Green DR (1999) Suicidal tendencies: apoptotic cell death by caspase family proteinases. *The Journal of Biological Chemistry* **274**, 20049-20052

Zheng TS, Hunot S, Kuida K, Flavell RA (1999) Caspase knockouts: matters of life and death. *Cell Death and Differentiation* **6**, 1043-1053