Do Plant Caspases Exist?

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Programmed cell death (PCD) is a functional concept that refers to cell death that is part of the normal life of a multicellular organism; it involves controlled disassembly of the cell. In animal systems PCD is synonymous with apoptosis, a cell death process characterized by a distinct set of morphological and biochemical features, mediated by a class of specific Cys proteases called cysteinyl aspartate-specific proteinases (caspases). Although to date no functional homologs of animal caspases have been identified in plants, a vast amount of indirect evidence suggesting the existence in plants of true caspase-like activity and its functional involvement in plant cell death has accumulated.

PCD AND APOPTOSIS

Apoptosis in animal cells is characterized by specific features such as cell shrinkage, blebbing of the plasma membrane, condensation and fragmentation of the nucleus, and internucleosomal cleavage of DNA. The final stage of apoptosis is the fragmentation of the cell into cellular debris-containing vesicles called "apototic bodies" that are being phagocytosed by other cells (for review, see Hengartner, 2000). Inappropriate apoptosis has been implicated in many human diseases, including a number of birth defects, ischemic vascular diseases (e.g. heart attack and stroke), neurodegenerative diseases (e.g. Alzheimer's and Parkinson's diseases), autoimmune diseases (e.g. rheumatoid arthritis), AIDS, and diabetes mellitus type I.

There are numerous examples of cell death during plant development that conform to the general definition of PCD such as cell death during xylogenesis, aerenchyma formation, plant reproductive processes, leaf and petal senescence, and endosperm development. Furthermore, cell death in response to pathogen attack, and in response to a variety of abiotic factors such as ozone and UV radiation also fall within the definition of PCD. A number of morphological similarities were found between animal cells undergoing apoptosis and dying plant cells, including compaction and shrinkage of the cytoplasm and nucleus, DNA and nuclear fragmentation, and the formation of DNA-containing (apoptotic-like) bodies (Wang et al., 1996; De Jong et al., 2000). Although such typical apoptotic hallmarks have not been established in all the cases of plant PCD, the observations do suggest the existence of an apoptotic machinery in plant cells. In mammalian cells, these typical hallmarks of apoptosis are ascribed to caspase-mediated processing of specific target molecules such as the activation of a caspase-activated DNase (CAD) by caspase-mediated cleavage of the CAD inhibitory subunit (ICAD; Nagata, 2000). It is tempting to speculate that comparable caspase-mediated proteolytic events cause the apoptotic phenotype observed in dying plant cells.

CYSTEINYL ASP-SPECIFIC PROTEINASES (CASPASES)

Caspases belong to a class of specific Cys proteases that show a high degree of specificity with an absolute requirement for cleavage adjacent an Asp residue and a recognition sequence of at least four amino acids N-terminal to this cleavage site. Determination of the tertiary structure of human caspases by crystallography has revealed a unique topology designated as the "caspase-hemoglobinase fold." Caspases are synthesized as inactive pro-enzymes and are activated by directed proteolysis that removes the N-terminal peptide and cleaves the proteolytic domain at specific recognition sites (Fig. 1). Cleavage of a caspase molecule yields a large (α) subunit and a small (β) subunit that form the enzymatically active heterodimer. Each active heterodimer consists of six β-strands that form a twisted β-sheet structure with five α-helices. Recognition of the substrate occurs in a cleft formed by the loop regions of the α- and β-subunits. In situ, the active caspase molecule exists as a $(\alpha/\beta)_2$-tetramer (Nicholson, 1999; Grütter, 2000).

In general, apoptotic cell death involves a sequence of caspase activation events in which initiator caspases activate downstream executioner caspases that process a variety of target proteins eventually leading to the apoptotic phenotype. Initiator caspases may be activated by autoprocessing when clustering occurs e.g. at the cytosolic part of (activated) cell death receptors. Caspase-8 is the key initiator caspase in the death-receptor pathway. Upon ligand binding, receptors such as CD95 (Apo-1/Fas) aggregate and

[1] This work was supported by the European Union (grant no. FAIR CT95–0225) and by the Dutch Ministry of Agriculture, Nature Management, and Fisheries.

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www.plantphysiol.org/cgi/doi/10.1104/pp.006338.

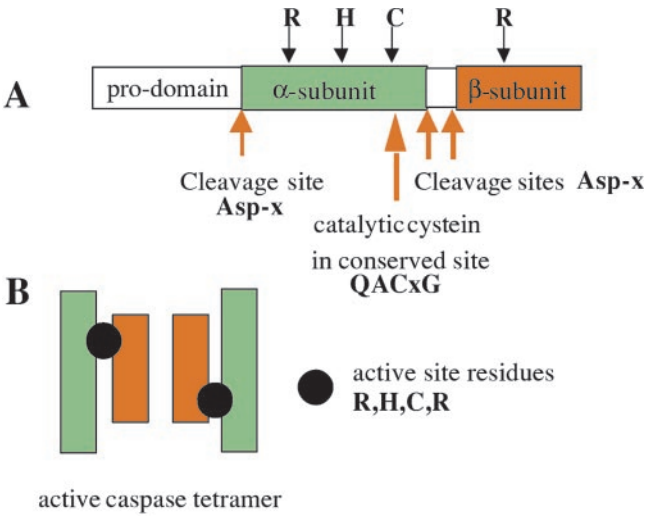

Figure 1. Schematic representation of structural features of mammalian caspases. A, Caspases are synthesized as inactive pro-enzymes with an N-terminal prodomain and a large and small subunit of 17 to 21 and 10 to 13 kD, respectively. In most procaspases, the subunits are separated by a linker peptide. B, Proteolytic activation yields an active caspase tetramer bearing two active sites (Nicholson, 1999; Grütter, 2000).

form membrane-bound complexes that then recruit, through adaptor proteins, several procaspase-8 molecules. Under these conditions of induced proximity, the low intrinsic protease activity of procaspase-8 molecules is sufficient to cleave and activate each other. Autoprocessing and activation of initiator caspase-9 is mediated through association with specific activators such as Apaf1 and cytochrome *c*. Together, these proteins form a large protein complex (the apoptosome) that may contain additional proteins as well. Initiator caspases may directly process and activate executioner caspases 3, 6, and 7 (for review, see Hengartner, 2000).

Caspases can selectively be inhibited by small peptides, mimicking the substrate recognition site, carrying electrophiles such as aldehydes, nitriles, or ketones at their C terminus that react with the active site Cys. In addition, macromolecular proteins such as the cowpox serpin crmA, members of the inhibitor of apoptosis protein (IAP) family, and the broad spectrum caspase inhibitor p35 from baculovirus are able to specifically block caspase activity (Ekert et al., 1999). To date, 14 different human caspases have been identified, which have been subdivided into different groups based on their substrate preferences and extent of sequence and structural similarities. Given their pivotal role in regulation of apoptosis, caspases are considered important therapeutic targets.

PROTEOLYTIC ACTIVITY AND PLANT CELL DEATH

In plants, different types of proteolytic enzymes are known to be associated with developmental and pathogen- and stress-induced PCD. This proteolytic activity is generally assumed to function in the random autolysis of intracellular proteins rather than being regulatory in an ordered breakdown process. However, analogous to the established participation of proteases, specifically caspases, in the regulation of animal PCD, specific plant proteases are expected to regulate plant PCD likewise. There are several reports that link protease activity to the regulation of plant PCD. Proteasome inhibitors can prevent tracheary element differentiation in zinnia cell cultures when added at the time of culture initiation, and the appearance of a secreted protease is coordinated with secondary cell wall synthesis and cell death during tracheary element differentiation. Protease activity and the cell death process are inhibited by soybean (*Glycine max*) trypsin inhibitor, whereas exogenous application of another Ser protease prematurely triggers cell death (Groover and Jones, 1999). Inhibitor studies also implicate Ser proteases in signaling during elicitin-induced hypersensitive response (HR) cell death (Beers et al., 2000). In soybean cells, PCD-activating oxidative stress induces a set of Cys proteases. Inhibition of the induced Cys protease activity by ectopic expression of the Cys protease inhibitor, cystatin, blocks PCD triggered either by an avirulent pathogen or directly by reactive oxygen species (Solomon et al., 1999). These data suggest that the interplay between proteases and endogenous protease inhibitors may play a regulatory role in plant cell death.

THE USE OF SYNTHETIC CASPASE INHIBITORS AND SUBSTRATES IN PLANT PCD RESEARCH

There has been a tremendous effort to develop specific caspase inhibitors for pharmacological use. Such inhibitors mimic the caspase substrate recognition site. An increasing number of reports show that inhibitors to various mammalian caspases markedly suppress plant cell death as well. PCD in tobacco (*Nicotiana tabacum*) leaves induced by the bean pathogen *Pseudomonas syringae* pv *phaseolicola* can effectively be blocked by treatment with the human caspase-1 (Ac-YVAD-CMK) and caspase-3 (Ac-DEVD-CHO) inhibitors (Table I). In this system, some other Cys and Ser protease inhibitors (PMSF and TPCK) also partially suppressed cell death, whereas leupeptin, TLCK, and E64 had no effect (Lam and Del Pozo, 2000). Menadione-induced PCD in tobacco protoplasts, showing typical apoptotic features such as chromatin condensation and nuclear and DNA fragmentation, can be blocked by low concentrations of caspase-3 inhibitor (Ac-DEVD-CHO) and high concentration of PMSF (Sun et al., 1999b).

We studied chemical-induced PCD in tomato (*Lycopersicon esculentum*) suspension cells. Treatment of the cells with low concentrations of e.g. camptothecin or fumonisin-B1 resulted in cell death exhibiting typ-

Table I. *Selection of protease inhibitors used in plant PCD research*

Short Name	Chemical Name	Target
Ac-DEVD-CHO (-CMK, -FMK)	*N*-acetyl-Asp-Glu-Val-L-aspartic acid-aldehyde (-Chloromethylketone, -fluoromethylketone)	Caspase-3
Ac-YVAD-CHO (-CMK, -FMK)	*N*-acetyl-Tyr-Val-Ala-aspartic acid-aldehyde (-Chloromethylketone, -fluoromethylketone)	Caspase-1
Z-asp-CH2-DCB	Benzyloxycarbonyl-Asp-2,6-dichlorobenzoyloxymethylketone	Caspases
TPCK	*N*-tosyl-L-phenylalanine chloromethylketone	Cys proteases
E64	*N*[*N*-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine	Cys proteases
IA	Iodoacetamide	Cys proteases
NEM	*N*-ethylmaleimide	Cys proteases
PMSF	Phenylmethylsulphonylfluoride	Cys/Ser proteases
Leupeptin	*N*-acetyl-Leu-Leu-Arg-aldehyde	Cys/Ser proteases
Cystatin	Natural protein	Cys/Ser proteases
AEBSF	4-(2-Aminoethyl)benzenesulfonylfluoride	Cys/Ser proteases
TLCK	*N*-tosyl-Lys-chloromethylketon	Ser proteases

ical apoptotic features such as nuclear and DNA fragmentation. Cell death was effectively inhibited by the human caspase-1 (Ac-YVAD-CHO and Ac-YVAD-CMK) and caspase-3 (Ac-DEVD-CHO) inhibitors as well as by the broad-range caspase inhibitor (Z-asp-CH_2-DCB; De Jong et al., 2000, 2002). To be effective, the caspase inhibitors must be added within the 1st h after addition of cell death-inducing chemicals (E.J. Woltering, A.J. De Jong, and E.T. Yakimova, unpublished data). These inhibitors proved to be effective at very low concentrations, with 50% inhibition of chemical-induced cell death at concentrations close to 1 nM (Fig. 2). A number of other Cys and Ser protease inhibitors [4-(2-aminoethyl)benzenesulfonylfluoride, IA, TLCK, and TPCK] were also able to block cell death; however, this generally required 10 to 100 times higher concentrations. Small caspase-unrelated peptides with similar reactive groups that served as negative controls (e.g. methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone, leupeptin) did not affect cell death in this system (De Jong et al., 2000).

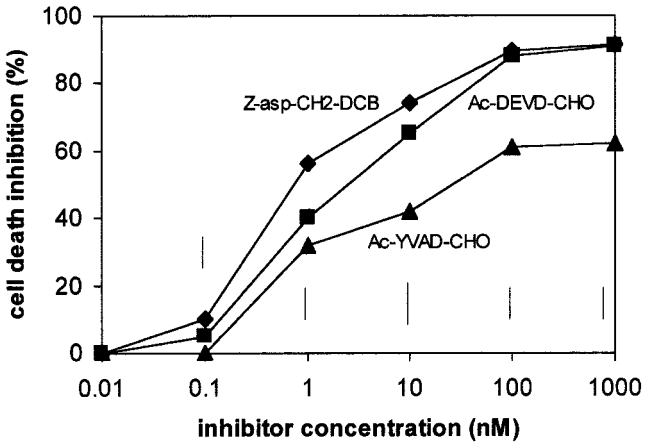

Figure 2. Effect of human caspase inhibitors on cell death in camptothecin-treated tomato suspension cells. Cells were simultaneously treated with 5 μM of the anticancer drug camptothecin and different concentrations of caspase inhibitor. Cell death was assessed after 24 h. Vertical bars represent LSD (LSD 5%). For details on experimental procedures see De Jong et al. (2000, 2002).

Using synthetic fluorogenic substrates to caspase-1 (Ac-YVAD-aminomethylcoumarin [AMC]), caspase-like activity has been demonstrated in extracts from tobacco mosaic virus-infected tobacco leaves, and this caspase-like activity could be inhibited with caspase-1 (Ac-YVAD-CMK) and caspase-3 (Ac-DEVD-CHO; Ac-DEVD-FMK) inhibitors but not by caspase-unrelated protease inhibitors. No activity was detected using caspase-3 fluorogenic substrate (Ac-DEVD-AMC; Lam and Del Pozo, 2000). Using fluorogenic substrate to caspase-1 (Ac-YVAD-AMC) and caspase-3 (Ac-DEVD-AMC), it was similarly shown that cytosolic extracts from barley (*Hordeum vulgare*) embryonic suspension cells exhibit both caspase-1 and -3 activity. Caspase-3-like activity was enhanced at low pH and could only be blocked by caspase-3 inhibitor (Ac-DEVD-FMK), whereas caspase-1-like activity was not sensitive to low pH and could be blocked by both caspase-1 (Ac-YVAD-FMK) and -3 (Ac-DEVD-FMK) inhibitors. Caspase-like activity was not affected by the addition of high concentrations of PMSF, leupeptin, E64, or a cocktail of several other Ser, Cys, and metalloprotease inhibitors. Authors concluded that at least two different caspase-like proteases (CLPs) are present in plants (Korthout et al., 2000). Cytosolic extracts from carrot (*Daucus carota*) suspension cells were, upon addition of cytochrome *c*, able to induce apoptotic-like changes, including chromatin condensation, formation of apoptotic bodies, and DNA fragmentation, in purified mouse liver. This could be blocked by caspase-1 (Ac-YVAD-CHO) and caspase-3 (Ac-DEVD-CHO) inhibitors (Zhao et al., 1999). These experiments suggest the existence of functional caspase activity in plant extracts.

Proteolytic activity in plant cells undergoing PCD has also been studied using poly(ADP-Rib) polymerase (PARP) as a substrate. In both mammals and plants, two different types of PARP exist, and both

types are presumably involved in DNA repair. The Arabidopsis PARP-1 shows high homology to human PARP-1 including a conserved caspase-3 recognition site (DSVD-N). In mammalian cells, PARP cleavage by caspase 3 initially yields a 89-kD "signature" fragment (Doucet-Chabeaud et al., 2001). Exogenous (bovine) PARP is endoproteolytically cleaved by extracts from fungus-infected cowpea (*Vigna unguiculata*) plants that were developing a HR but not by extracts from noninfected leaves. This cleavage activity was inhibited by caspase-3 inhibitor (Ac-DEVD-CHO) but not by caspase-1 inhibitor (Ac-YVAD-CHO; D'Silva et al., 1998). Interestingly, a polypeptide (GDEVDG-IDEV) mimicking the PARP caspase-3 cleavage site (DEVD-G) partially inhibited PARP cleavage, whereas a modified peptide in which the essential Asp was replaced by Ala (GDEVAGIDEV) did not affect PARP cleavage. This cleavage activity was also inhibited by other Cys protease inhibitors (E-64, IA, and NEM). Inhibitors to other types of proteases (Ser-, metallo-, Asp proteases, and calpain) were without effect in this system. In these experiments, PARP cleavage eventually yielded four different fragments of 77, 52, 47, and 45 kD (D'Silva et al., 1998). Cleavage of endogenous (plant) PARP occurs during menadione-induced PCD in tobacco protoplasts, and this was inhibited by caspase-1 (Ac-YVAD-CHO) and caspase-3 (Ac-DEVD-CHO) inhibitors. PARP cleavage initially yielded a 84-kD fragment (Sun et al., 1999a). Also in heat shock-induced PCD in tobacco suspension cells, endogenous PARP was cleaved, yielding a 89-kD fragment (Tian et al., 2000).

The applied caspase-specific inhibitors are considered to be more specific than general Cys protease inhibitors such as IA, E-64, and NEM. However, they may also nonspecifically inhibit other (caspase-unrelated) proteases if applied in sufficiently high concentrations. General Cys protease inhibitors conversely will likely also target CLPs to a certain extent. The caspase inhibitors often exert their effect in the low-nanomolar range in systems where even high concentrations of general Cys protease inhibitors may be without effect. Although this could partly be an effect of different rates of uptake in plant cells, the observations do suggest that plant proteases exist that specifically recognize peptide sequences that mimic caspase substrate recognition sites. Moreover, these CLPs seem involved in plant cell death. This view is strengthened by the observations that caspase inhibitor-sensitive plant proteases exist that can cleave the endogenous caspase-3 substrate, PARP, presumably at a caspase recognition site.

MACROMOLECULAR CASPASE INHIBITORS BLOCK PLANT CELL DEATH

Caspase activity may be controlled by a variety of viral or cellular macromolecular inhibitors thought to interact directly with the protease. In mammals, the endogenous IAP protein family has been postulated to play its regulating role by inhibiting caspase activity. IAP proteins, conserved between numerous organisms, are distinguished both by their ability to suppress apoptosis and by the presence of at least one baculoviral IAP repeat required for their anti-death activity (Ekert et al., 1999). IAPs interact with caspases in such a way that substrate access is blocked but without directly docking into the substrate pockets on the enzyme surface (Henning et al., 2002). It has been reported that *Agrobacterium tumefaciens*-induced PCD in maize (*Zea mays*) cells with typical features of apoptosis, such as cytochrome *c* release and DNA fragmentation, can be suppressed by ectopic expression of the baculovirus IAP (Hansen, 2000). Likewise, heterologous expression of baculovirus Op-IAP in tobacco conferred resistance to several necrotrophic fungal pathogens through suppression of cell death (Dickman et al., 2001). The baculovirus broad-range caspase inhibitor p35 contains an extremely large and flexible reactive site loop with caspase recognition site (DQMD-G), and after appropriate cleavage, it forms a complex with the caspase (mechanism-based inactivation). p35 can inhibit *Caenorhabditis elegans* CED-3 and mammalian caspases 1, 3, 6, 7, 8, and 10 with K_i values of less than 10 nM (Ekert et al., 1999; Snipas et al., 2001; Henning et al., 2002). *A. tumefaciens*-induced PCD in maize can be suppressed by ectopic expression of p35 (Hansen, 2000). Likewise, tobacco plants expressing p35 were partially inhibited in HR cell death. Different mutant versions of the p35 protein, impaired in caspase inhibition, when expressed in tobacco were ineffective (Lam and Del Pozo, 2000). p35 shows a high degree of specificity toward caspases and shows little or no cross reactivity with other proteases including those exhibiting a "caspase-hemoglobinase fold" such as legumains and gingipains (Snipas et al., 2001; discussed below). It is predicted from structural studies that only proteases with caspase active site geometry will be inhibited by p35 (Henning et al., 2002). The functionality of IAPs and p35 as inhibitors of plant cell death is a strong indication that cell death-associated CLPs exist that recognize and process (in the case of p35) these inhibitors.

CASPASE TERTIARY STRUCTURE

Iterative homology searches have recently revealed two new groups of caspase-related Cys proteases designated paracaspases (in e.g. humans and *C. elegans*) and metacaspases (in e.g. fungi and plants; Uren et al., 2000; Koonin and Aravind, 2002). The plant metacaspases fall into two types. Type I metacaspases contain a predicted caspase-like proteolytic domain and a prodomain with a Pro-rich motif. In plants, the type I prodomain in addition contains a zinc finger motif similar to those of the plant HR

protein LSD-1. Type II metacaspases (only in plants) contain no prodomain. Modeling of the three-dimensional protein structure of para- and metacaspases indicates significant tertiary structure homology to animal caspases (the so-called caspase-hemoglobinase fold). Mutational studies in *Trypanosoma brucei* first suggested that metacaspases function as Cys proteinases (Scallies et al., 2002). It was recently shown that the only metacaspase present in Brewer's yeast (*Saccharomyces cerevisiae*) displays caspase-like proteolytic activity that is activated when yeast is stimulated by H_2O_2 to undergo apoptosis (Madeo et al., 2002). The actual function(s) and substrate specificity of the metacaspases from plants have not yet been investigated, and until now, no functions that relate them to cell death have been defined.

Another subgroup of proteases exhibiting tertiary homology to caspases are the legumains, Cys endopeptidases present in e.g. humans and plants. Legumains have a strict specificity for an Asn (and not Asp) residue immediately N-terminal to the substrate's cleavage site and possess a protein fold similar to animal caspases (Chen et al., 1998). Clostripain and gingipain, Cys endoproteases from *Clostridium histolyticum* and *Porphyromonas gingivalis*, respectively, are also thought to exhibit a similar topology. Although caspases, para- and metacaspases, legumains, clostripains, and gingipains show significant tertiary structure homology and are thought to belong to the same clan of evolutionary-related endoproteases, both their substrate specificity and biological functions may markedly differ from each other, and it remains to be seen whether plant metacaspases exist that possess real caspase activity.

DO PLANT CASPASES EXIST?

"Do plant caspases exist" is a question that cannot be answered easily and highly relies on the way we define them. From a physiological perspective, any plant cell death-related protease could be named "plant caspase," but this would not be in line with the definition used in animal research. Caspase nomenclature is based on the existence of Cys proteases with an absolute requirement for Asp at the P1 position and neither a specific function in cell metabolic processes (apoptosis) nor the presence of a specific tertiary structure (caspase-hemoglobinase fold) is required for classifying a protein as a caspase. Therefore, the question whether plant caspases exist relies on the existence in plants of Cys endoproteases that cleave adjacent an Asp residue. The available evidence strongly suggests that true caspase-like proteolytic activity is present in plants and, moreover, that this activity does play a pivotal role in plant PCD suggesting an evolutionary relationship. This view is based on the following observations: (a) the existence of cell death-related plant proteases that recognize and process synthetic peptide inhibitors and fluorogenic substrates that mimic the caspase substrate recognition sites; (b) the existence of plant proteases that recognize and process the natural caspase substrate PARP apparently at a caspase recognition site; and (c) the functionality of macromolecular caspase inhibitors (IAPs and in particular p35) in plants and the observation that modification of the caspase recognition site in p35 abolishes its effect. It remains to be seen whether these plant CLPs exhibit the characteristic caspase topology as well. Until proven otherwise, the plant metacaspases are considered prime candidates because of their structural and evolutionary relationship to the caspases and the existence, in type 1 metacaspases, of an LSD1-like zinc-finger supposed to regulate PCD. Although molecular modeling studies suggest that the metacaspase substrate recognition site may not specifically target Asp (Uren et al., 2000), the caspase-like activity of the yeast metacaspase (Madeo et al., 2002) suggests that some of the plant metacaspases may also function as true caspases.

Apart from specific caspase inhibitors, cell death in some systems can effectively be blocked by chemical or endogenously expressed broad-range Cys or Ser protease inhibitors. Regulation of plant cell death therefore apparently involves, but does not solely depend on, activation of Cys proteases, some of which are expected to be similar to caspases in having specificity for aspartic residues, whereas others may not have a defined specificity. One could easily envision a proteolytic cascade or network consisting of different classes of regulatory proteases involved in plant cell death (Fig. 3). Given the postulated pivotal role played by plant Cys proteases that specifically target Asp residues (CLPs) and their activation very early in the cell death process, we envision the CLPs upstream of the additional regulatory proteases (plant cell death proteases [PCDPs]). The role of CLPs may be the activation of PCDPs or transacting factors involved in their expression, inactivation of endogenous proteinase inhibitory proteins (PIs) or the direct processing of proteins involved in ordered breakdown of the cells.

The hypothetical targets of CLPs could be among the plant proteins having a CLP substrate recognition site. By convention, the side chains of each residue of the substrate are numbered sequentially (P1, P2,. . . P*n*) N-terminal, or (P1′, P2′,. . . P*n*′) C-terminal from the scissile bond, and complementary pockets on the surface of the proteases are given the "S" designation. In human caspase-1, the topology of the active site is such that the carboxylate of the P1 Asp fits into a highly restrictive pocket and is oriented by hydrogen-bond interactions with other residues. The S2- and S3-binding sites are more tolerant, whereas the S4 site is the primary determinant for substrate specificity among caspases (Grütter, 2000). In most plant systems, inhibitors specific to different human

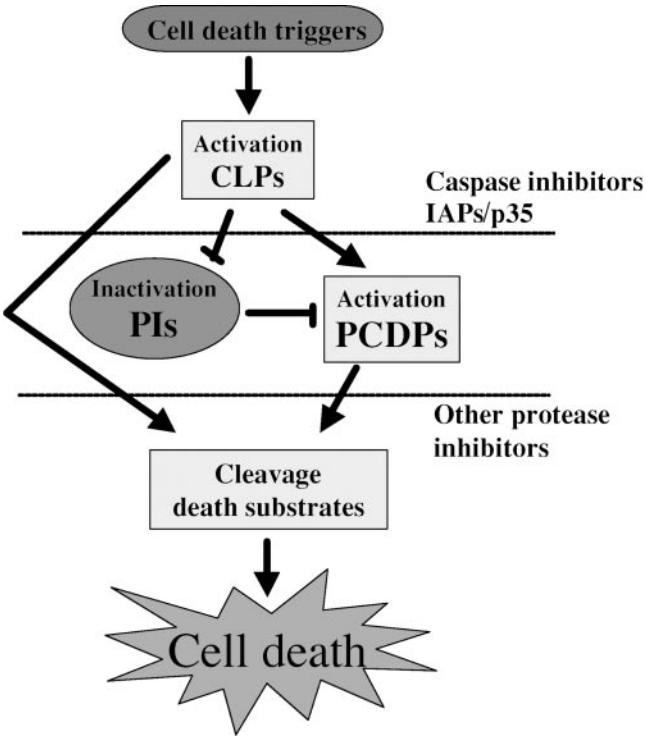

Figure 3. Schematic representation of the possible involvement of caspase-like proteases (CLPs), plant cell death proteases (PCDPs), and endogenous proteinase inhibitors (PIs) in plant (apoptotic) cell death. For explanation see text.

caspases all exert a significant effect on cell death. Because of the chemical structure of the inhibitors, recognition of the Asp is expected to immediately inactivate the protease even if the inhibitor has a poor match with the putative S2-S4 sites. Without a sensible means to quantify the extent of the inhibition compared with that in (purified) mammalian caspases, these experiments do, apart from the requirement of Asp in P1 position, not tell us much about additional residues involved in substrate recognition. A similar reasoning applies for the cleavage of caspase peptide substrates.

More useful information about the putative substrate recognition sites of CLPs can be derived from the apparent recognition of the caspase cleavage sites in human PARP (DEVD-G), plant PARP (DSVD-N), and baculovirus p35 caspase inhibitory protein (DQMD-G). On the basis of these "natural" substrates, one could speculate that the substrate recognition site for plant CLPs could have a general structure DxxD. Many plant proteins are potentially candidates, and this criterion alone is largely insufficient to recognize possible CLP targets in the protein databases. Information about the expected tertiary structure of such a putative CLP substrate would greatly help to estimate accessibility of the putative CLP recognition sequence and to identify possible plant cell death substrates. Apart from further functional characterization of plant metacaspases and other proteases and protease inhibitors involved in cell death, the identification of endogenous CLP protein targets is necessary to elucidate the proposed regulatory role for specific Cys proteases in plant PCD.

Received March 25, 2002; returned for revision April 15, 2002; accepted September 1, 2002.

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