Caspases and programmed cell death in the hypersensitive response of plants to pathogens

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The hypersensitive response (HR) is induced by certain plant pathogens and involves programmed cell death (PCD) to restrict the spread of pathogens from the infection site [1]. Concurrent with the induction of cell death, the host activates a defense response [2]. The cell death associated with the HR in several plant–pathogen systems has morphological similarities to animal apoptosis [3,4], which suggests that cell death mechanisms in plants and animals may share common components that lead to similar cellular events. Caspases are conserved cysteine proteases that regulate animal PCD [5]; caspase activity or an involvement of caspases in cell death has yet to be reported in plants. In this work, we investigated the participation of caspases in HR cell death. Caspase-specific peptide inhibitors, Ac-YVAD-CMK [6] and Ac-DEVD-CHO [7], could abolish bacteria-induced plant PCD but did not significantly affect the induction of other aspects of HR, such as the expression of defense genes. This result confirmed our previous model that cell death can be uncoupled from defense gene activation during HR [8]. Caspase-like proteolytic activity was detected in tobacco tissues that were developing HR following infection with tobacco mosaic virus (TMV). Our results provide evidence for the presence of caspase-like plant protease(s) that participate in HR cell death.

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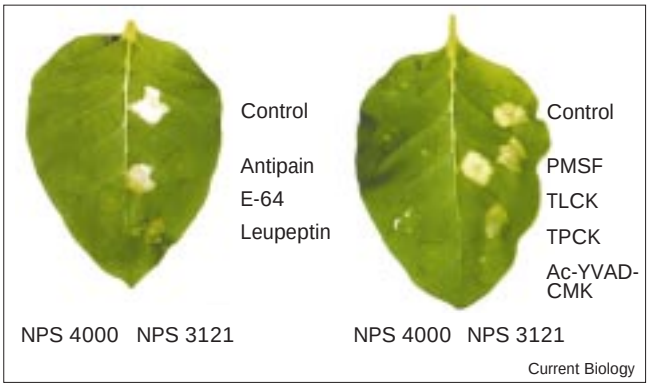
Results and discussion

Synthetic caspase inhibitors abolish HR cell death

We examined the effects of specific peptide inhibitors of caspases [6,7] to determine whether a caspase-like activity is involved in cell death in tobacco caused by *Pseudomonas syringae* pv. *phaseolicola*, a bean pathogen that induces HR in this species [9]. We co-infiltrated tobacco leaves with an HR-inducing *P.s. phaseolicola* strain (NPS 3121) and either one of two caspase inhibitors — the chloromethylketone Ac-YVAD-CMK (which is irreversible and more specific for caspase-1) or the aldehyde Ac-DEVD-CHO (which is reversible and more specific for caspase-3). The YVAD and DEVD sequences (in single-letter amino acid code) of these caspase inhibitors are based on the sequences cleaved by caspase-1 in prointerleukin-1β and by caspase-3 in poly(ADP)-ribose polymerase [6,7]. The addition of these caspase inhibitors abolished HR cell death. At 42 hours after co-infiltration, symptoms of HR cell death were not evident, although cell death could be observed in control samples after 16–18 hours (Figure 1, and data not shown for Ac-DEVD-CHO). As a negative control, we performed the same infiltrations with an isogenic *P.s. phaseolicola* strain that does not induce the HR (*Hrp*$^-$; NPS 4000) [9].

To test the specificity of action of these caspase inhibitors and to rule out the possibility that the highly reactive chloromethylketone group was inhibiting other proteases during the HR, several protease inhibitors with different target preferences and containing different chemical groups were tested. Each compound was co-infiltrated with NPS 3121 or with NPS 4000 (Figure 1). At 20 hours after infiltration, tissues that had been co-infiltrated with TLCK (N^{α}-tosyl-lys chloromethylketone), leupeptin and E-64 (trans-epoxysuccinyl-L-leucylamido-[4-guanidino]-butane)

Figure 1



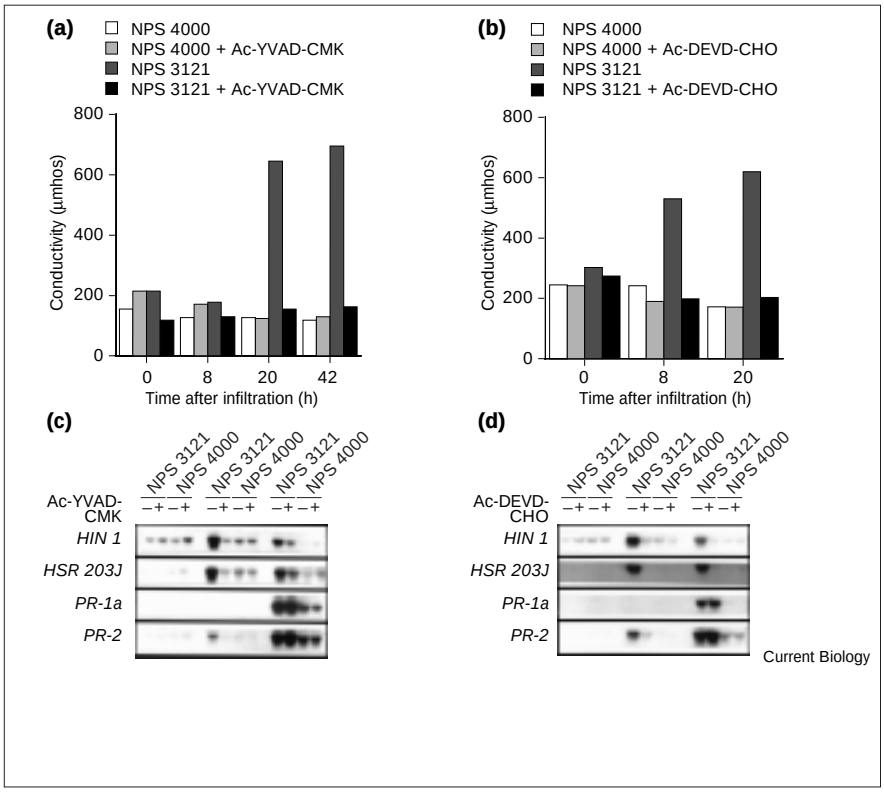
Synthetic caspase-specific inhibitors abolish HR cell death induced by bacteria. Tobacco leaves (Samsun *NN*) were hand-infiltrated with *P.s.* pv. *phaseolicola* strains NPS 4000 or NPS 3121 in the presence of protease inhibitors. Antipain, E-64, leupeptin, PMSF, Ac-YVAD-CMK and Ac-DEVD-CHO were used at 0.5 mM each (data not shown for Ac-DEVD-CHO). TLCK and TPCK were used at 1 mM each. The picture was taken 42 h after bacteria infiltration. Bacteria were prepared as described [8], resuspended in milliQ water and diluted to 10^9 colony-forming units (cfu) in milliQ water before use. Protease inhibitors were added to bacteria immediately before infiltration. Plants infected with bacteria were kept at 23°C under continuous illumination.

did not show any difference from control inoculated samples. PMSF (phenylmethylsulfonyl fluoride) and TPCK (N^{α}-tosyl-phe chloromethylketone) showed slower HR cell death 20 hours after infiltration, whereas after 42 hours, cell death in these samples was almost indistinguishable from the control. Antipain, a broad-range serine/cysteine protease inhibitor, was the only exception and it inhibited the formation of HR lesions even 42 hours after inoculation. The fact that both chloromethylketones TPCK and TLCK did not inhibit HR cell death even when used at a higher concentration than that of Ac-YVAD-CMK rules out the possibility of non-specific HR cell death suppression by the chloromethylketone group.

One of the morphological differences between plant and animal cells undergoing PCD is that dying plant cells are not engulfed by neighboring cells, because of the presence of their cell wall. Therefore, at late stages of cell death, contents from dying cells leak into the intercellular space. This allows ion leakage to be used to compare the relative amount of cell death in plant tissues. We measured the progress of cell death by means of conductivity at different times after infiltration with NPS 3121 alone and in the presence of caspase inhibitors. The same set of samples was infiltrated with NPS 4000 as a negative control. Ac-YVAD-CMK quantitatively inhibited HR cell death (Figure 2a) and similar results were obtained using Ac-DEVD-CHO (Figure 2b). We also examined the effects of caspase inhibitors on the expression of plant genes that are activated during the HR (Figure 2c,d). *HSR203J* and *HIN1* are tobacco genes whose expression is specifically induced upon incompatible plant–bacteria interactions [10,11]. Both genes are used as early markers for HR cell death, as they accumulate 3–6 hours after inoculation with bacteria and several hours before the appearance of HR lesions. In the presence of Ac-YVAD-CMK or Ac-DEVD-CHO, *HIN1* and *HSR203J* transcripts did not accumulate to significant levels compared with samples inoculated with NPS 3121 alone. Considering that *HSR203J* and *HIN1* transcripts accumulate soon after bacteria inoculation, the step regulated by putative plant caspases during HR cell death is likely to be early and upstream of the activation of these molecular markers.

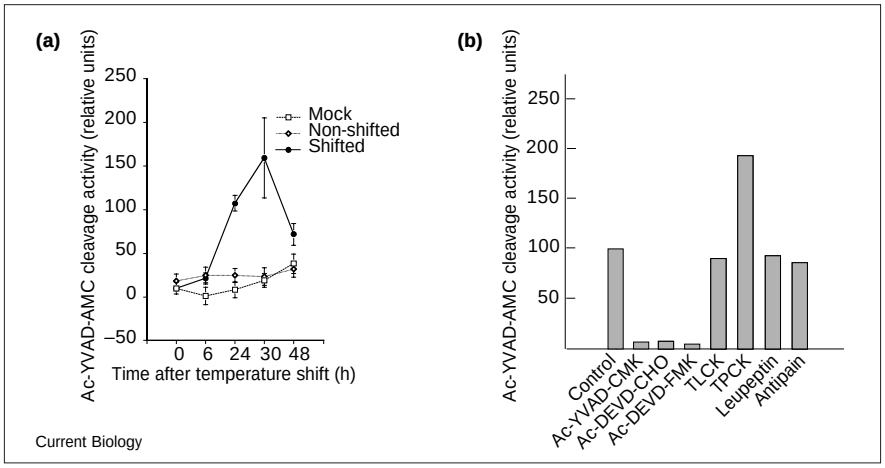
Pathogenesis-related (*PR*) genes are used as molecular markers for the local activation of host defense mechanisms during the HR [12]. Under our assay conditions in which cell death is completely suppressed by caspase inhibitors, *PR-1a* and *PR-2* expression was not affected 20 hours after infiltration, although there was a slight delay in *PR-2* expression 8 hours after infiltration (Figure 2c,d). No significant induction of *PR-1a* or *PR-2* was observed upon infiltration of NPS 4000 with or without the inhibitors. The fact that the induction of *PR* genes was not suppressed by

Figure 2



Synthetic caspase inhibitors block HR cell death induced by bacteria but do not affect activation of host defense mechanisms. (a) Ac-YVAD-CMK and (b) Ac-DEVD-CHO inhibited cell death induced by bacteria. Cell death was determined by ion leakage from leaf discs obtained from tobacco leaves infiltrated with NPS 3121 or NPS 4000 in the presence and in the absence of Ac-YVAD-CMK or Ac-DEVD-CHO respectively at 0, 8, 20 and 42 h after infiltration. Caspase inhibitors were dissolved in DMSO and the same volume of DMSO was added to control samples. A representative experiment out of four independent trials is shown, all of which showed similar results. Cell death was assayed by measuring ion leakage from leaf discs; three leaf discs (11 mm diameter) per sample were collected at different time points after infiltration and floated abaxial side up on 3 ml distilled water for about 6 h at room temperature. The conductivity (in μmhos) of the bathing solution was then measured with a conductivity meter (model 604; VWR Scientific). (c) Ac-YVAD-CMK and (d) Ac-DEVD-CHO suppressed HR cell death gene expression but did not affect accumulation of *PR* gene transcripts. Samples were infiltrated as described in Figure 1. Total RNA per sample (30 μg) was loaded and the RNA blot was probed for the expression of the genes indicated on the left.

Figure 3



Ac-YVAD-AMC cleavage activity can be detected in extracts prepared from tobacco plants undergoing HR cell death. (a) Activation of caspase-like activity during TMV-induced HR. Three tobacco plants (Samsun *NN*) were mock infected or TMV infected (six plants) and kept at 30°C for 2–3 weeks. Mock-infected plants and half of the TMV-infected plants were shifted to 23°C, and the rest of the TMV-infected plants were kept at 30°C (non-shifted). Samples were collected at 0, 6, 24, 30 and 48 h after temperature shift and caspase activity was assayed as described below using 75 μM Ac-YVAD-AMC as substrate (Calbiochem). YVAD proteolytic activity was detected in TMV-infected, shifted plants but not in mock-infected or non-shifted tobacco plants. Bars represent SEM of three plants per treatment. (b) Caspase-like activity was specifically inhibited by caspase inhibitors but not affected by other protease inhibitors. Protease inhibitors were added to plant extracts to a final concentration of 0.5 mM and caspase assay was performed using Ac-YVAD-AMC (75 μM final concentration) as a substrate. Plant caspase-like assay: leaf tissues were frozen in liquid nitrogen immediately and kept at –80°C until processing. Frozen samples were ground to a fine powder with a mortar and pestle and 400 μl caspase extraction buffer (50 mM Hepes, pH 7.5, 20% glycerol, 1 mM EDTA, 1 mM DTT, 1% BSA, 1 mM PMSF) was added and the samples were quickly homogenized using the pestle. Samples were transferred to a 2 ml microfuge tube and incubated with shaking on ice for 15 min, centrifuged (12,000 rpm, 10 min, 4°C) and the supernatants collected. Samples (50 μl) were mixed with 50 μl caspase assay buffer (caspase extraction buffer with 150 μM YVAD-AMC). Half of the mixture (50 μl) was immediately transferred to a tube containing 25 μl stop solution (1% sodium acetate in 175 mM acetic acid) and this sample was designated as (t_0). The remaining sample was incubated at 30°C for 2 h before the stop solution was added (t_2). Samples were diluted in 2 ml water and their relative fluorescence (F) was measured with a TKO 100 fluorimeter (Hoeffer Scientific). The relative caspase activity at the different time points was calculated as $F(t_2) – F(t_0)$.

caspase inhibitors is consistent with the suggestion that host defense mechanisms and HR cell death may be regulated by different pathways coordinately activated through the same signal [8]. This observation also rules out the possibilities that caspase inhibitors suppress gene expression non-specifically in our system or that they affect the signalling events between pathogen and host cells. Our results contradict those of Levine *et al.* [13], who found that Ac-YVAD-CMK did not suppress cell death induced in cell suspensions with hydrogen peroxide. This discrepancy may be due to the different systems used (intact plants versus cell suspensions) and the abiotic induction of cell death with 8 mM hydrogen peroxide in the other study [13].

Detection of caspase enzymatic activity in plant tissues developing HR

For the biochemical detection of the caspase-like activity in tobacco during the HR, we used the temperature-sensitive *N*–TMV tobacco plant–pathogen system [14] which allows a more synchronized population of cells undergoing PCD to be obtained. In the tobacco–NPS 3121 system, upon induction of the HR by bacteria infiltration, cell death is not synchronous and also not temperature-sensitive, unlike the situation with TMV. At 30°C, TMV can systemically infect *N* tobacco plants as induction of cell death and defense gene expression are completely suppressed [3,14]. Upon shifting the infected plant to 23°C, large regions of HR cell death appear all over the plant within 18–24 hours. Using a synthetic fluorogenic substrate for animal caspase-1 (Ac-YVAD-AMC) [6], we found that extracts from TMV-infected tobacco plants undergoing HR cell death showed YVAD-specific proteolytic activity (Figure 3a). No activation of YVAD-specific protease activity could be detected in mock-inoculated plants, or in TMV-infected tobacco plants kept at 30°C and therefore unable to trigger HR cell death (Figure 3a, non-shifted control). The increase of this caspase-like activity could be detected rapidly upon HR activation and preceded the onset of visible lesions and increases in ion leakage (about 12 hours after induction by temperature shift). These induction kinetics are consistent with a possible role for this caspase-like activity in mediating PCD execution during the HR.

The induced YVAD proteolytic activity could be inhibited by Ac-YVAD-CMK, Ac-DEVD-CHO, Ac-DEVD-FMK, but not by PMSF, TPCK, TLCK, leupeptin or antipain, even when used at the higher concentration (Figure 3b). This inhibitor specificity is similar to that shown by known caspases. Furthermore, these results are compatible with the inhibitor specificity for bacteria-induced HR cell death (Figure 1), suggesting that the caspase-like activity could

be involved in cell death activation during the HR. On the other hand, the inability of antipain (a relatively non-specific serine/cysteine protease inhibitor) to inhibit the caspase-like activity in extracts from TMV-infected plants suggests the involvement of other antipain-sensitive proteases in HR. Interestingly, no DEVD-AMC proteolytic activity could be observed in extracts from TMV-infected tobacco (data not shown). This observation is unexpected because DEVD is a good inhibitor of the caspase-like activity in extracts from TMV-infected plants (Figure 3b) and because DEVD is a more promiscuous substrate for known caspases than YVAD [15]. Our results suggest a model in which the plant caspase-like protease can bind DEVD as a competitive inhibitor, but is unable to cleave this substrate after the first aspartate residue.

Interesting parallels between components of host–pathogen interactions and cell death in animal and plant systems have been revealed in recent studies [14,16–21]. These include sequence similarities between the *Drosophila* Toll receptor, which activates defense responses mediated by the transcription factor Cactus, and several resistance genes from different plant species [14]. In addition, homologies between *Arabidopsis* NIM1/NPR1 and the Cactus/IκB class of transcription regulators further strengthen this comparison [16,17]. NIM1/NPR1 appears to function downstream of disease resistance proteins such as RPP5 [16,17] and may mediate the activation of systemic acquired resistance in plants. The requirement of reactive oxygen species for HR cell death in plants may also be analogous to their role in neutrophil cell death [19]. Indeed, inhibitors of mammalian NADPH oxidase can prevent the oxidative burst that typically occurs after the induction of the HR [20]. Recently, a putative homolog of the human Bax-inhibitor-1 (BI-1) has been reported in *Arabidopsis* [21], and the Ced-4 pro-apoptotic protein from *C. elegans* [22] and its human homolog Apaf-1 [23] share conserved structural and functional domains with plant resistance gene products [18]. Ced-4 and Apaf-1 interact with anti-apoptotic proteins of the Ced-9/Bcl-2 class, in addition to mediating caspase activation [22,23]. These homologies suggest that cell death mechanisms may be conserved in plant and animal kingdoms. Although PCD has been described in several different species of unicellular eukaryotes (reviewed in [24]), regulators of cell death have not been identified outside of animal systems. Our present work shows that the involvement of similar proteases in controlling PCD may be conserved between animals and higher plants. This observation suggests that the pathway for cell death control may predate the divergence of plants and animals.

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References

1. Greenberg JT: Programmed cell death: a way of life for plants. *Proc Natl Acad Sci USA* 1996, 93:12094-12097.
2. Hammond-Kossack KE, Jones DG: Resistance gene-dependent plant defense responses. *Plant Cell* 1996, 8:1773-1791.
3. Mittler R, Lam E: Sacrifice in the face of foes: pathogen-induced programmed cell death in plants. *Trends Microbiol* 1996, 4:10-15.
4. Ryerson DE, Heath MC: Cleavage of nuclear DNA into oligonucleosomal fragments during cell death induced by fungal infection or by abiotic treatments. *Plant Cell* 1996, 8:393-402.
5. Whyte M: ICE/CED-3 proteases in apoptosis. *Trends Cell Biol* 1996, 6:245-248.
6. Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, *et al.*: A novel heterodimeric cysteine protease is required for interleukin-1ß processing in monocytes. *Nature* 1992, 356:768-774.
7. Nicholson DW, All A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, *et al.*: Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 1995, 376:37-43.
8. Mittler R, Shulaev V, Seskar M, Lam E: Inhibition of programmed cell death in tobacco plants during pathogen-induced hypersensitive response at low oxygen pressure. *Plant Cell* 1996, 8:1991-2001.
9. Lindgren PB, Peet RC, Panopoulos NJ: Gene cluster of ***Pseudomonas syringae*** pv. '***phaseolicola***' controls pathogenicity of bean plants and hypersensitivity on nonhost plants. *J Bacteriol* 1986, 168:512-522.
10. Pontier D, Godiard L, Marco Y, Roby D: ***hsr203J***, a tobacco gene whose activation is rapid, highly localized and specific for incompatible plant pathogen interactions. *Plant J* 1994, 5:507-521.
11. Gopalan S, Wei W, He SY: ***hrp*** gene-dependent induction of ***hin1*** a plant gene activated rapidly by both harpins and the ***avrPto*** gene-mediated signal. *Plant J* 1996, 10:591-600.
12. Linthorst JM: Pathogenesis related proteins of plants. *Crit Rev Plant Sci* 1991, 10:123-150.
13. Levine A, Pennell RI, Alvarez ME, Palmer R, Lamb C: Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. *Curr Biol* 1996, 6:427-437.
14. Whitman S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C, Baker B: The product of the tobacco mosaic virus resistance gene ***N***: similarity to Toll and the Interleukin-1 receptor. *Cell* 1994, 78:1101-1115.
15. Talanian RV, Quinian C, Trautz S, Hackett MC, Mankovich JA, Banach D, *et al.*: Substrate specificities of caspase (interleukin-1β converting enzyme) family proteases. *J Biol Chem* 1997, 272:9677-9682.
16. Cao H, Glazebrook J, Clark JD, Volko S, Dong X: The ***Arabidopsis NPR1*** gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* 1997, 88:57-63.
17. Ryals J, Weyman K, Lawton K, Friedrich L, Ellis D, Steiner H-Y, *et al.*: The ***Arabidopsis*** NIM1 protein shows homology to the mammalian transcription factor IκB. *Plant Cell* 1997, 9:425-439.
18. Van der Biezen EA, Jones JGN: The NB-ARC domain: a novel signalling motif shared by plant resistance gene products and regulators of cell death in animals. *Curr Biol* 1998, 7:226-227.
19. Levine A, Tenhaken R, Dixon R, Lamb C: H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* 1994, 79:583-589.
20. Keller T, Damude HG, Werner D, Doerner P, Dixon RA, Lamb C: A plant homolog of the neutrophil NADPH oxidase $gp91^{phox}$ subunit encodes a plasma membrane protein with Ca^{2+} binding motifs. *Plant Cell* 1998, 10:255-266.
21. Xu Q, Reed JC: Bax inhibitor-1, a mammalian apoptosis suppressor identified by functional screening in yeast. *Mol Cell* 1998, 1:337-346.
22. Chinnaiyan AM, Chaudhary D, O'Rourke K, Koonin EV, Dixit VM: Role of CED-4 in the activation of CED-3. *Nature* 1997, 388:728-729.
23. Zou H, Henzel WJ, Liu X, Lutschg A, Wang X: Apaf-1, a human protein homologous to ***C. elegans*** CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 1997, 90:404-413.
24. Ameisen JC: The origin of programmed cell death. *Science* 1996, 272:1278-1279.