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# Cleavage of Nuclear DNA into Oligonucleosomal Fragments during Cell Death Induced by Fungal Infection or by Abiotic Treatments

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**It is often claimed that programmed cell death (pcd) exists in plants and that a form of pcd known as the hypersensitive response is triggered as a defense mechanism by microbial pathogens. However, in contrast to animals, no feature in plants universally identifies or defines pcd. We have looked for a hallmark of pcd in animal cells, namely, DNA cleavage, in plant cells killed by infection with incompatible fungi or by abiotic means. We found that cell death triggered in intact leaves of two resistant cowpea cultivars by the cowpea rust fungus is accompanied by the cleavage of nuclear DNA into oligonucleosomal fragments (DNA laddering). Terminal deoxynucleotidyl transferase–mediated dUTP nick end in situ labeling of leaf sections showed that fungus-induced DNA cleavage occurred only in haustorium-containing cells and was detectable early in the degeneration process. Such cytologically detectable DNA cleavage was also observed in vascular tissue of infected and uninfected plants, but no DNA laddering was detected in the latter. DNA laddering was triggered by ≥100 mM KCN, regardless of cowpea cultivar, but not by physical cell disruption or by concentrations of $H_2O_2$, $NaN_3$, $CuSO_4$, or $ZnCl_2$ that killed cowpea cells at a rate similar to that of ladder-inducing KCN concentrations. These and other results suggest that the hypersensitive response to microbial pathogens may involve a pcd with some of the characteristics of animal apoptosis and that DNA cleavage is a potential indicator of pcd in plants.**

## INTRODUCTION

Programmed cell death (pcd) is found in many animal tissues and occurs as part of normal development as well as in pathological processes associated with some diseases (Kerr et al., 1972; Steller, 1995). Both the presence and the inhibition of pcd may be crucial in mammalian tissue, given the suggestion that pcd occurs by default unless suppressed by signals from other cells (Raff, 1992). Therefore, it is of considerable importance to know whether pcd plays a similarly vital role in the development and survival of cells of higher plants.

Endogenously triggered cell death occurs during the development of a number of plant tissues (Chasan, 1994; Smart, 1994; Mittler and Lam, 1995a). Moreover, it is often claimed that the cell death commonly triggered by pathogens in resistant plants is also a form of pcd (Greenberg et al., 1994; Mittler and Lam, 1995b; Mittler et al., 1995; Tenhaken et al., 1995), controlled by genes that, when mutated, cause cells to die spontaneously or in response to stress (Dietrich et al., 1994; Greenberg et al., 1994). However, as yet, there is no feature that universally defines pcd in plants. This contrasts with the striking cellular events that characterize the most commonly studied form of pcd, known as apoptosis, in animal cells. These events include condensation of the nucleus and chromatin, fragmentation of nuclear DNA at internucleosomal regions, nuclear and membrane blebbing, and the fragmentation of the cell into apoptotic bodies (Earnshaw, 1995). DNA cleavage, in particular, appears to be a hallmark of animal apoptosis, and the responsible endonuclease may be activated indirectly by proteases that act as apoptosis regulators (Nicholson et al., 1995).

Rapid, pathogen-induced cell death in resistant plants is usually called the hypersensitive response (Stakman, 1915) and can be induced by viruses, bacteria, fungi, and nematodes. However, only for pathogens that do not immediately kill cells in susceptible tissue is this response clearly separable from adverse cellular changes induced by successful pathogenesis. For this reason, we have studied the cell death induced in resistant cowpea plants by the biotrophic cowpea rust fungus. This fungus invades living cells in susceptible plants, and although infected plants are debilitated and invaded leaves may senesce prematurely, invaded cells usually stay alive for days or weeks. We report here that cell death in resistant plants is accompanied by DNA cleavage into oligonucleosomal fragments that form a "ladder" on agarose gels. Similar cleavage is caused in uninfected cowpea leaves by KCN but not by other abiotic treatments, including $H_2O_2$, which has been suggested to induce a cell death identical to the hypersensitive response (Tenhaken et al., 1995). We also demonstrate, using

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a terminal deoxynucleotidyl transferase (TdT)–mediated dUTP nick end-labeling procedure (the TUNEL procedure) that adds fluorescently labeled nucleotides to DNA strand breaks in situ, that DNA cleavage occurs only in those cells invaded by the fungus and occurs at an early stage in the degeneration of the invaded cell.

## RESULTS

### DNA Laddering in Fungus-Infected Plants

DNA was extracted from cowpea leaves 24 hr after inoculation with urediospores of the fungus. By this time, each fungus had penetrated the leaf via the stomata, formed a single infection hypha in the substomatal cavity, and produced an intracellular haustorium in an adjacent mesophyll cell. DNA from the infected resistant cowpea cultivars Dixie Cream and Calico Crowder showed a ladder of DNA fragments that differed by just under 200 bp when run on an agarose gel, as shown in lanes 3 and 4 of Figure 1. This laddering was virtually identical to an authentic, apoptosis-related DNA ladder shown in lane 6, which was derived from apoptotic B6 mouse thymocytes. Figure 1 also illustrates that no laddering was observed with DNA from fungal spores, uninoculated plants, or plants of the inoculated, susceptible cultivar California Blackeye.

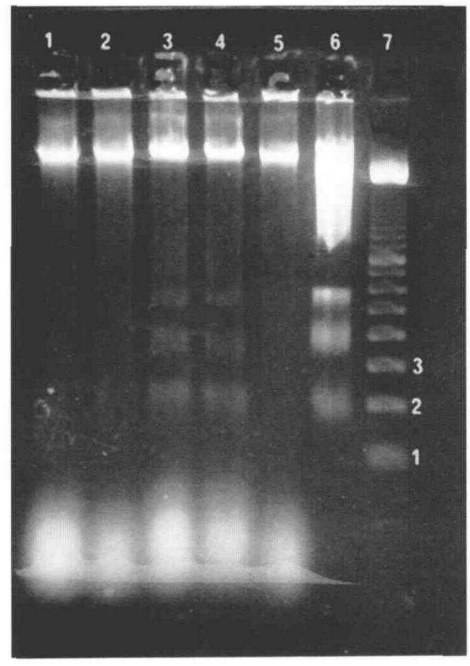


**Figure 1.** DNA Laddering Induced by the Fungus in Resistant Plants Extracted 24 Hr after Inoculation.

Shown is a 2% agarose gel of DNA extracted from infected or uninfected leaves or fungal urediospores. For DNA from plant tissue, each lane received DNA from an equivalent area of the leaf. Lane 1 contains the uninoculated susceptible cultivar California Blackeye; lane 2, fungus-infected California Blackeye; lane 3, fungus-infected resistant cultivar Dixie Cream; lane 4, fungus-infected resistant cultivar Calico Crowder; lane 5, fungal urediospores; lane 6, apoptosis-induced ladder from ionomycin-treated B6 mouse thymocytes; lane 7, 100-bp restriction endonuclease ladder control. The 100-, 200-, and 300-bp bands are labeled 1, 2, and 3, respectively. DNA laddering that appears identical to that produced by mouse thymocytes can be seen only in samples from infected resistant cowpea cultivars.

### Cytologically Detectable DNA Cleavage in Fungus-Invaded Cells and Vascular Tissue

As illustrated in Figures 2A through 2C for the cowpea cultivar Dixie Cream, sections of infected leaves tested for DNA cleavage by the TUNEL procedure revealed that at 24 hr after inoculation, ~15% (Dixie Cream) and ~37% (Calico Crowder) of plant mesophyll cells containing fungal haustoria had nuclei (identified by 4′,6-diamidino-2-phenylindole [DAPI] staining) that fluoresced a bright yellow, indicative of DNA cleavage. In the remaining invaded cells, the plant nucleus was not detectable; this may have been because the DNA had been degraded, but possibly because the deposition of phenolics in the cell wall and cytoplasm made it more difficult for DAPI and TdT to access the nuclear material. These phenomena may also account for the difference in frequency of stained nuclei between cultivars, because cells die after invasion in cultivar Dixie Cream more rapidly than they do after invasion in cultivar Calico Crowder (Heath, 1989). Thus, the results of the TUNEL assay suggest that DNA cleavage had occurred in all of the detectable nuclei in invaded cells of both resistant cultivars. No more than one DAPI-positive or TUNEL-positive body was ever seen in each invaded plant cell, indicating that the nucleus did not fragment during cell death. No brightly fluorescent nuclei were seen in haustorium-containing cells in the susceptible cultivar California Blackeye after the TUNEL procedure.

Thin sections that were not treated with β-glucanase to aid penetration of TdT and thicker sections treated with glucanase before the TUNEL procedure gave similar results, except that patches of epidermal cells had brightly fluorescent nuclei in the glucanase-treated tissue. This latter staining was considered to be an artifact related to the shearing and distortion of the epidermis that often occurred during sectioning of enzyme-treated tissue.

Nuclei of uninvaded mesophyll cells in all situations generally did not show yellow fluorescence following the TUNEL procedure, as illustrated in Figures 2B and 2C. However, brightly fluorescent, TUNEL-positive, and DAPI-positive (i.e., DNA) material was sporadically seen in vascular tissue in all sections regardless of cultivar, infection, or glucanase treatment. This phenomenon was also observed in chemically treated tissue and is illustrated in Figures 3M and 3N. The

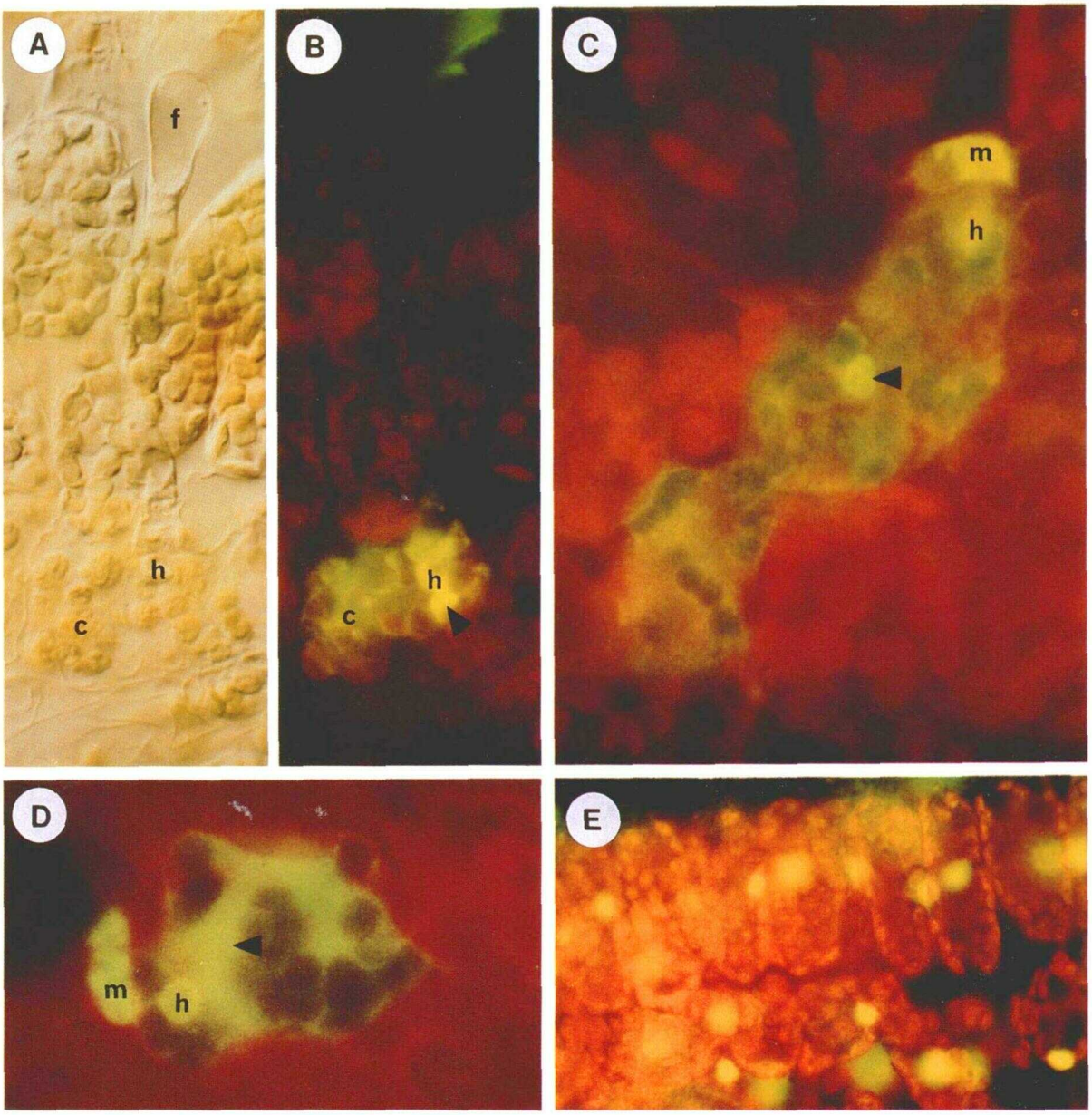


**Figure 2.** Cytological Detection of DNA Cleavage by the TUNEL Procedure Applied to Fungus-Infected Tissue.

**(A)** A thin leaf section of cowpea cultivar Dixie Cream 24 hr after inoculation with the cowpea rust fungus. The fungal infection hypha (f) has formed a haustorium (h) in a mesophyll cell (c) that has disorganized chloroplasts. DIC optics were used. Magnification, ×717.

**(B)** The same section as shown in **(A)** viewed under blue light epifluorescence irradiation. Uninvaded mesophyll cells contain nonfluorescent nuclei (indicating no DNA cleavage) and chloroplasts that fluoresce red. The haustorium (h) and the invaded cell (c) exhibit autofluorescence; the nucleus (arrowhead) of this cell fluoresces more brightly than the plant cytoplasm, indicating DNA cleavage. Magnification, ×717.

**(C)** Another autofluorescent plant cell in cultivar Dixie Cream containing an autofluorescent haustorium (h) formed by the extracellular haustorial mother cell (m). DNA cleavage in the plant cell nucleus (arrowhead) is indicated by brighter fluorescence compared with that of the plant cytoplasm. Magnification, ×1293.

**(D)** Negative control section not treated with TdT. The haustorium (h), the haustorial mother cell (m), and the plant cytoplasm are all autofluorescent; the plant nucleus (arrowhead) is not more brightly fluorescent than the surrounding cytoplasm. Magnification, ×1271.

**(E)** A section of a leaf of Dixie Cream treated before the TUNEL procedure with cellulysin containing DNase activity as a positive control. DNA cleavage in all nuclei of every cell is indicated by bright fluorescence. Magnification, ×462.

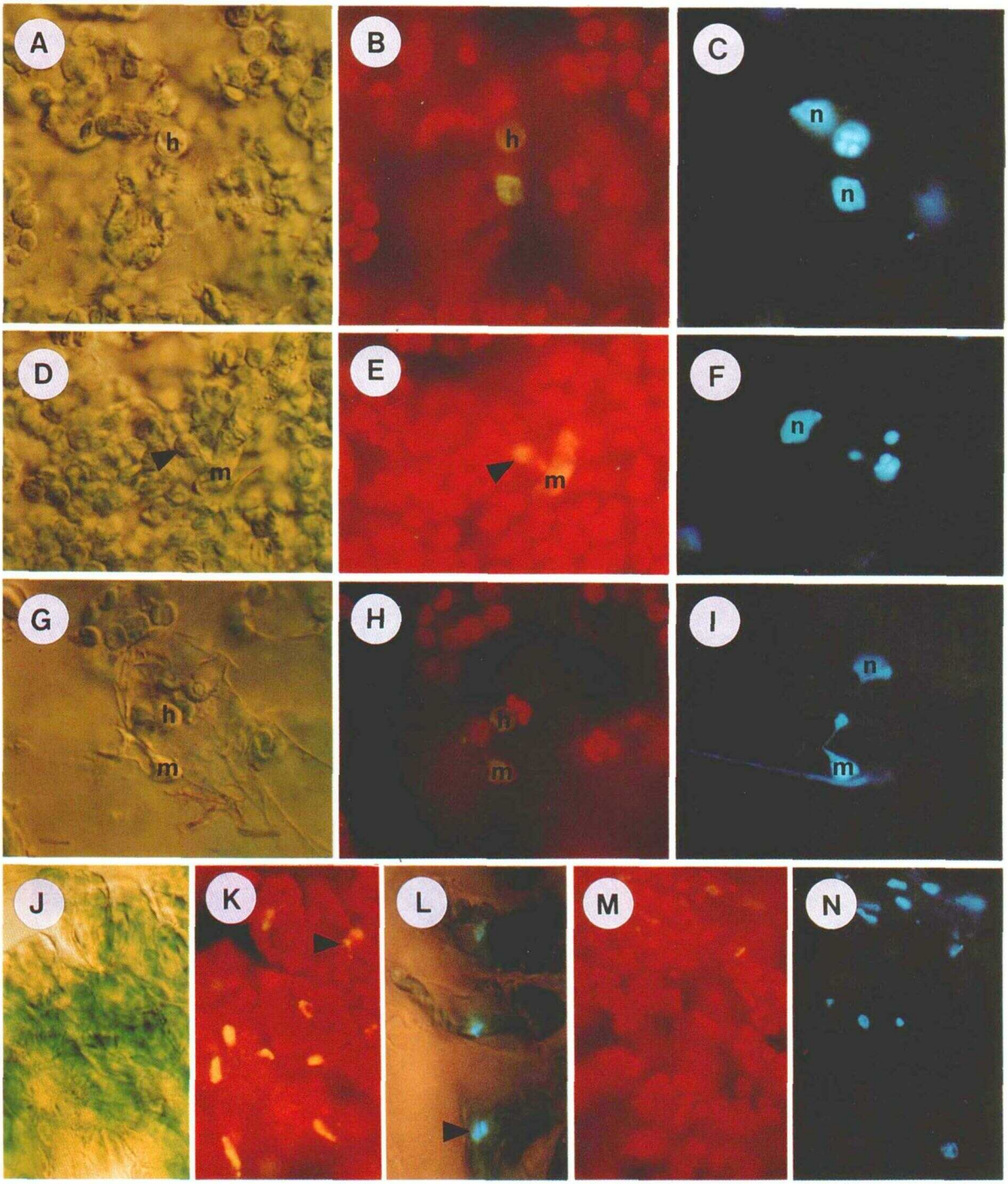


**Figure 3.** Sections of Fungus-Infected or Chemically Treated Leaves Viewed under DIC Optics or Using Blue Light to Detect DNA Cleavage and UV Irradiation to Detect Total DNA.

Cells were permeabilized by using glucanase prior to the TUNEL treatment. **(A)** to **(I)** and **(L)** are all ×712. **(J)**, **(K)**, **(M)**, and **(N)** are ×450. **(A)** to **(C)** DIC, blue light, and UV irradiation images of a haustorium (h) in a mesophyll cell of resistant cultivar Dixie Cream 15 hr after inoculation. Note that the invaded cell does not differ in appearance from surrounding uninvaded cells in **(A)** but that its chloroplasts do not fluoresce a bright red in **(B)**. The nucleus (n) in the invaded cell (the lower one visualized in **[C]**) is TUNEL positive in **(B)**, indicative of DNA cleavage. Four haustorial nuclei can be seen in **(C)**, but these show no signs of DNA cleavage in **(B)**; the haustorium (h) is autofluorescent.

cleaved DNA sometimes was seen inside xylem elements but usually appeared in other, unidentifiable cells of the vascular tissue.

Haustorium-containing cells in control sections that had not been treated with the TdT exhibited a dull orange–yellow autofluorescence of cell wall and cytoplasm, as was also seen in sections in which TdT had been included in the TUNEL procedure. However, in the control sections, plant nuclei did not exhibit the much brighter fluorescence typical of sections exposed to TdT (compare Figures 2B and 2C with Figure 2D), indicating that the fluorescence in the experimental sections was due to DNA cleavage, not autofluorescence. By contrast, fungal haustoria and haustorial mother cells in control sections as well as those subjected to the TUNEL procedure showed the same bright yellow autofluorescence (compare Figures 2B and 2C with Figure 2D). Autofluorescence has been shown to be indicative of an advanced stage of death of both fungal and plant cells and is usually attributed to the presence of phenolic substances in the latter (Heath, 1984). Previous ultrastructural studies have shown that in the resistant cultivars Dixie Cream and Calico Crowder, plant cell death precedes the death of the fungal haustorium and haustorial mother cell (Heath, 1972).

As a positive control, uninfected tissue was treated with 2% cellulysin (a hydrolase mixture found to contain high levels of DNase activity) to induce DNA cleavage prior to the TUNEL procedure; as illustrated in Figure 2E, all plant nuclei indicated a high degree of DNA cleavage by their bright fluorescence.

### Timing of DNA Cleavage Relative to Cellular Disorganization

To determine at what stage of fungus-induced plant cell death DNA cleavage is first detectable, additional tissue was harvested 15 hr after inoculation (~3 hr after the haustorium had formed), at which time many haustorium-invaded cells showed no, or a very faint, yellow autofluorescence. From previous ultrastructural studies (Heath, 1972; M.C. Heath, unpublished data), these cells were expected to show relatively few signs of disorganization of organelles or other cytoplasmic structures. Approximately 60% of nuclei in haustorium-containing cells in both resistant cultivars were slightly to highly fluorescent after the TUNEL procedure, indicating varying degrees of DNA cleavage; the remaining nuclei were not fluorescent. Invaded plant cells generally showed a reduction in the degree of red chlorophyll fluorescence compared with surrounding mesophyll cells, suggesting that the chloroplasts were damaged even though they were green and looked otherwise normal under differential interference contrast optics. Figures 3A to 3C are examples of this phenomenon for an invaded cell with a TUNEL-positive nucleus. As illustrated in Figures 3D to 3F, nuclei in haustorium-containing cells that had normal chlorophyll fluorescence showed no signs of DNA cleavage after the TUNEL procedure.

In control sections not treated with TdT, 8% (resistant cultivar Dixie Cream) and 10% (resistant cultivar Calico Crowder) of nuclei in invaded cells showed very faint autofluorescence that could have accounted for only a very few of the fluorescent nuclei seen after the TUNEL procedure; the remaining nuclei were not autofluorescent, as illustrated in Figures 3G to 3I. In the control and experimental sections, haustoria and haustorial mother cells were moderately autofluorescent, which was typical of all fungal cytoplasm and appeared to be an effect of the fixation procedure. No increase in fluorescence of fungal nuclei was seen following the TUNEL procedure; for example, the haustorial nuclei shown in Figure 3C following DAPI staining for total DNA cannot be detected in Figure 3B, in which the leaf section is examined for TUNEL-induced fluorescence.

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**Figure 3.** (continued).

**(D)** to **(F)** DIC, blue light, and UV irradiation images of a haustorium (arrowhead) in a mesophyll cell that has normal-appearing chloroplasts under DIC **(D)** and blue light **(E)**. The plant nucleus (n in **[F]**) shows no sign of DNA cleavage because it is not TUNEL positive in **(E)**. Five fungal nuclei can be seen in **(F)**, four in the haustorial mother cell (m) and one in the process of migrating into the haustorium; none of these show any signs of DNA cleavage in **(E)**.

**(G)** to **(I)** DIC, blue light, and UV irradiation images of a haustorial mother cell (m) and haustorium (h) in a control section not treated with TdT. The DNA of the plant nucleus (n in **[I]**) does not fluoresce in **(H)**. One of the fungal nuclei seen in **(I)** is in the process of migrating into the haustorium.

**(J)** Section of a leaf of California Blackeye fixed 6 hr after injection with 100 mM KCN and viewed under DIC optics. The tissue is still green, but the cells have collapsed and their contents are disorganized (compare with uninvaded cells in **[A]**).

**(K)** The same cells as shown in **(J)** viewed under blue light after TUNEL staining. All cells exhibit nuclear DNA cleavage. Some cells have a single, brightly fluorescent region of cleaved DNA, whereas in others (arrowhead), the cleaved DNA appears fragmented.

**(L)** Different area of the same tissue as in **(J)** viewed under combined UV and visible irradiation to show total DNA. A single DNA region is visible in two cells and two adjacent regions (arrowhead) in the third.

**(M)** and **(N)** Azide-treated tissue showing yellow, fluorescent cleaved DNA material associated with the vascular tissue (top right in **[M]**) and the same area of tissue viewed under UV irradiation to show total DNA **(N)**. Note that the four nuclei away from the vascular tissue in **(N)** do not stain for cleaved DNA in **(M)**.

### DNA Laddering in Plant Cells Killed by Chemical Means

KCN, $H_2O_2$, $NaN_3$, $CuSO_4$, and $ZnCl_2$ all killed cowpea mesophyll tissue, regardless of cultivar, when injected into intact leaves. The percentage of dead cells (detected by the absence of staining with fluorescein diacetate) in tissue harvested at 6 hr after treatment was similar in each cultivar and increased with chemical concentration, as shown in Table 1. $H_2O_2$, $NaN_3$, $ZnCl_2$, and $CuSO_4$ did not induce DNA laddering regardless of concentration (Table 1) or time of DNA extraction after application. As an example, lanes 7 to 11 in Figure 4 illustrate the lack of laddering in cowpea California Blackeye leaves treated with 0.29 M $H_2O_2$ and harvested at 1, 2, 6, 12, and 24 hr after injection, respectively. Some random cleavage of the DNA into small fragments can be detected in the 1-hr sample but was not consistently observed at other times or concentrations.

As shown in Table 1, KCN at concentrations ≥100 mM induced DNA laddering, and it did so in cultivars California Blackeye (fungus susceptible) and Dixie Cream (fungus resistant). Surprisingly, 50 mM KCN did not cause laddering, even though, as shown in Table 1, it killed 89% of the plant cells by 6 hr after injection. With 100 mM KCN, faint laddering was detectable in DNA extractions from either cultivar California Blackeye or Dixie Cream made at 4 and 6 hr after treatment, as shown for California Blackeye in lanes 3 and 4 of Figure 4. The periodicity of the DNA bands closely resembled that seen in fungus-infected tissue. Figure 4 also shows that no laddering was observed in the 1-hr extraction (lane 2) and that the DNA appeared to be degraded into random-sized, small fragments in the 12- and 24-hr samples (lanes 5 and 6). Observations similar to these were made from tissue treated with 200 mM KCN, except that DNA degradation occurred at a faster rate and laddering began to fade sooner, at ~4 hr after treatment.

### Cytologically Detectable DNA Cleavage in KCN- and $NaN_3$-Treated Leaves

California Blackeye leaves fixed at 6 hr after treatment with 100 mM KCN or $NaN_3$ were sectioned and subjected to the TUNEL procedure. Under differential interference contrast (DIC) microscopy, mesophyll cells did not look normal (compare Figure 3J with the uninfected cells in Figure 3A) but were collapsed, with individual chloroplasts often difficult to distinguish. Under blue light, Figure 3K illustrates that the nucleus of each cell was brightly fluorescent, indicating DNA cleavage. Occasionally, the cleaved DNA appeared as a large, TUNEL-positive body associated with one or more smaller ones, as can be seen in Figure 3K, but it was impossible to determine whether these DNA clumps were enclosed within a single nucleus or whether the nucleus had fragmented. Figure 3L shows two large, adjacent DNA bodies revealed by DAPI (for total DNA) staining that similarly could have been part of a single nucleus. Figures 3K and 3L illustrate the maximum amount of potential nuclear fragmentation observed in KCN-treated tissue.

Similar irregularity in the shape of nuclear DNA was not seen in azide-treated mesophyll cells, which had only one DNA body per cell, as can be seen in Figure 3N. Away from the vascular tissue, this DNA showed no signs of cleavage after the TUNEL procedure, as seen by comparing Figures 3M and 3N. As discussed earlier, the TUNEL staining of the vascular tissue seen in Figure 3M was identical to that seen in all tissues, regardless of infection or chemical treatment.

### Lack of DNA Laddering in Cells Killed by Freezing

To demonstrtate the effect of physical cell disruption on DNA breakdown, leaf tissue of California Blackeye was frozen for 1 hr at −20°C, thawed, and allowed to stand at room tempera-

**Table 1.** Effect of Different Concentrations of Chemicals on Cell Death and DNA Laddering in Cowpea Leaves

| $H_2O_2$ | | | $CuSO_4$ | | | $ZnCl_2$ | | | KCN | | | $NaN_3$ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C[a] | %D[b] | L[c] | C | %D | L | C | %D | L | C | %D | L | C | %D | L |
| 0 | 2A | – | 0 | 2A | – | 0 | 2A | – | 0 | 2A | – | 0 | 1A | – |
| 0.29 mM | 1A | – | 1 μM | 1A | – | 1 μM | 1A | – | 100 μM | 1A | – | 1 μM | 1A | – |
| 2.9 mM | 2A | – | 10 μM | 7B | – | 10 μM | 1A | – | 1 mM | 1A | – | 10 μM | 6A | – |
| 29 mM | 10B | – | 100 μM | 68C | – | 100 μM | 3A | – | 10 mM | 16B | – | 100 μM | 15B | – |
| 0.29 M | 86C | – | 1 mM | 100C | – | 1 mM | 13B | – | 25 mM | 73C | – | 1 mM | 83C | – |
| 2.9 M | 99C | – | 10 mM | 100C | – | 10 mM | 75C | – | 50 mM | 89D | – | 10 mM | 95D | – |
| | | | 100 mM | 100C | – | 100 mM | 100D | – | 100 mM | 100D | + | 100 mM | 100D | – |
| | | | | | | | | | 200 mM | 100D | + | | | |

[a] Concentration of chemical injected into each leaf.

[b] Percentage of dead cells as detected by lack of fluorescein diacetate fluorescence at 6 hr after injection. Values for $H_2O_2$ are means from four plants of cultivar California Blackeye; values for the remaining treatments are means from four observations from cultivar California Blackeye and four from cultivar Dixie Cream. Means in the same column followed by the same letter are not significantly different (Fisher's least significant difference, P = 0.05).

[c] Presence (+) or absence (–) of laddering detected in isolated DNA.

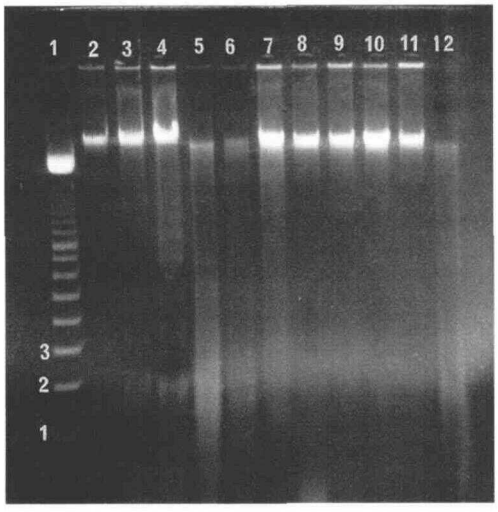


**Figure 4.** DNA Laddering Induced by Chemical or Physical Means in Uninoculated Cowpea Leaves.

Shown is a 2% agarose gel of DNA extracted from treated leaves. Each lane received DNA from an equivalent amount of leaf tissue. Lane 1 contains a 100-bp restriction endonuclease ladder control; the 100-, 200-, and 300-bp bands are labeled 1, 2, and 3, respectively. Lanes 2 to 12 are from cultivar California Blackeye. Lanes 2 to 6 contain samples from tissue treated with 100 mM KCN and extracted at 1, 4, 6, 12, and 24 hr after injection, respectively. Lanes 7 to 11 contain samples from tissue treated with 0.29 M $H_2O_2$ and extracted at 1, 2, 6, 12, and 24 hr after injection. Lane 12 contains frozen tissue sampled 5 min after thawing. Faint DNA laddering is detectable only in the 4- and 6-hr samples from KCN-treated tissue.

ture for 5 min, 1 hr, or 2 hr prior to DNA extraction. On an agarose gel, this DNA produced a continuous smear indicative of randomly shattered DNA, as shown for the 5-min sample in lane 12 of Figure 4.

## DISCUSSION

Despite observations that higher plants can control the death of specific cells during development and that plant cells often die rapidly when resistant plants are invaded by pathogens, there has been little previous evidence that these forms of cell death have any features in common. Indeed, developmental pcd does not seem to be associated with features such as browning, phytoalexin accumulation, and activation of defense genes (Greenberg et al., 1994; Tenhaken et al., 1995) that typify the hypersensitive response elicited by microbial pathogens and their products. Therefore, if the hypersensitive response is a form of pcd (rather than being caused by some directly toxic activity of the microbe), it must be a special form that has evolved primarily as a microbial defense mechanism (Heath and Skalamera, 1996). Nevertheless, our data, coupled with those of Wang et al. (1996) and Mittler and Lam (1995a, 1995b), suggest that there may be some homology between the hypersensitive response, animal apoptosis, and more indisputable forms of pcd in plants, although no two of these forms of cell death appear to be identical.

We have shown that DNA laddering, a hallmark of apoptosis in animal cells, is detectable in DNA from rust-infected resistant cowpea leaves and that cytological evidence of DNA cleavage can be found in fungus-infected cells. No DNA cleavage was detected in rust-infected susceptible leaves, in which the fungus obtains its nutrients from living plant cells that do not die despite fungal invasion. Because <50% of mesophyll cells in the resistant cultivars were invaded by the fungus, it might seem surprising that DNA laddering was so easily detected; however, these results are consistent with reports in animal cells that oligonucleosomal ladders on agarose gels can be seen even when only ~2% of the cells show morphological signs of apoptosis (Collins et al., 1992). Cytological signs of DNA cleavage were not observed until the chloroplasts of the invaded cell lost their red autofluorescence, indicating that DNA cleavage does not significantly precede other signs of cell damage. However, DNA cleavage was common before the invaded cell became autofluorescent, suggesting that as in apoptotic animal cells (Kressel and Groscurth, 1994), DNA cleavage occurs within a few hours of the onset of cell degeneration. Both DNA laddering and cytologically detectable DNA cleavage rapidly occur in tomato cells treated with fungal toxins that, like the fungus that produces them, kill tissue susceptible but not resistant to the fungus (Wang et al., 1996). These and our results suggest that plant genotype-specific cell death caused by fungi in either resistant or susceptible plants may involve similar endonuclease activity. By contrast, however, Mittler and Lam (1995b) recently reported that DNA fragmentation and increased nuclease activity occurred only during later stages of the hypersensitive response of tobacco to tobacco mosaic virus and were not accompanied by DNA laddering. It is possible, therefore, that different nucleases may be activated during cell death caused by viruses and fungi.

Cleaved DNA has been cytologically detected in vascular tissue (Mittler and Lam, 1995a; Mittler et al., 1995; Wang et al., 1996) and in onion root cap cells (Wang et al., 1996), both tissues that exhibit pcd. Cleavage associated with the vascular tissue of uninfected cowpea leaves was observed in our study, but no DNA laddering was detected in DNA extracted from such tissue. Given the sensitivity with which laddering was detected in infected plants, its absence in uninfected tissue suggests (1) that oligonucleosomal fragments are not formed during pcd in vascular tissue, (2) that they occur transiently during the differentiation of xylem elements (and, possibly, phloem sieve tubes in which the nucleus disintegrates during differentiation) and cannot be detected once differentiation is complete, or (3) that the DNA cleavage is an artifact induced by specimen preparation. A search for DNA laddering during vascular differentiation is needed to show whether this phenomenon is a universal indicator of pcd in plants.

In support of a relationship between DNA laddering and

specific forms of cell death, we have shown that physically disrupted or chemically killed cells do not necessarily exhibit DNA laddering. Several tested chemicals that caused cell death in cowpea leaves did not cause laddering, regardless of chemical concentration, time of DNA extraction, or speed at which cells were killed. Significantly, $CuSO_4$ and $H_2O_2$ were among these ineffective chemicals despite the fact that $CuSO_4$ within the range of concentrations tested in the present study has been suggested to generate active oxygen species, such as $H_2O_2$, in plants (Luna et al., 1994) and that $H_2O_2$ has been suggested to orchestrate the hypersensitive response of plants to pathogens (Tenhaken et al., 1995). Taken at face value, our results with those of Wang et al. (1996) do not support the hypothesis that $H_2O_2$ causes the same type of cell death as a rust fungus or some fungal toxins. In accord with this conclusion, $CuCl_2$ has been shown to induce a cell death in cowpea that is ultrastructurally different from that caused by infection with an incompatible fungus (Meyer and Heath, 1988a, 1988b).

Of the substances tested, only KCN at high concentrations induced rapid, cytologically detectable DNA cleavage and DNA laddering. This laddering appeared transiently during the process of DNA degradation, and the faintness of the ladders, given that all nuclei in the tissue showed cytological signs of DNA cleavage, suggests that only a small part of the DNA was broken into oligonucleosomal fragments. Nevertheless, the lack of similar ladders in DNA from tissue killed equally rapidly by other chemicals indicates that laddering is associated with only certain types of cell death and is related to the mode of action of the toxic chemical. Whether death induced by KCN has any functional similarity with that induced by the fungus remains to be determined. However, it is significant that no laddering and little cytologically detectable DNA cleavage was triggered by the concentration of $NaN_3$ that killed cowpea cells at a rate similar to the laddering-inducing concentration of KCN. Both KCN and $NaN_3$ block electron transport at the location of cytochrome $a_3$, but their differing effects in the present study suggest that cyanide's effect on the nucleus is unrelated to its effect on respiration and ATP depletion. $NaN_3$ has been shown not to cause apoptotic cell death or early DNA fragmentation in animal cells (Kressel and Groscurth, 1994).

Later stages of apoptosis in animal cells, such as membrane blebbing and the formation of apoptotic bodies, were not observed in response to any treatments in the present work, although there may have been some fragmentation of nuclei in KCN-killed tissue. Nuclear fragmentation was not seen in fungus-infected cells, nor has it been seen in ultrastructural studies of rust-infected, resistant plants or in dying, fungus-infected cells observed in living tissue by light microscopy (Chen and Heath, 1991; M.C. Heath, unpublished results). Its apparent occurrence in other forms of plant cell death (Wang et al., 1996) emphasizes the possibility that even when there are similarities between various forms of death with respect to DNA cleavage, there may be differences in subsequent or accompanying cellular events.

## METHODS

### Plant Material

Seeds of cowpea (*Vigna unguiculata*) cultivars California Blackeye, Dixie Cream, and Calico Crowder were planted in Pro-Mix (Premier Brands Inc., Red Hill, PA) in growth chambers maintained at 21 to 23°C, with a 16-hr photoperiod at about 250 $\mu E\ m^{-2}\ sec^{-1}$ provided by cool-white fluorescent and tungsten lamps. California Blackeye is susceptible, whereas Dixie Cream and Calico Crowder are resistant, to race 1 of the cowpea rust fungus (Heath, 1989). Plants used for experiments were 9 to 11 days old; all experiments were conducted with intact plants.

### Production of Inocula

Urediospores of the cowpea rust fungus (*Uromyces vignae*) were produced on the susceptible cultivar California Blackeye (Heath, 1989). Spores were harvested with a cyclone collector and stored at −20°C.

### Inoculation Procedure

Self-inhibitors of germination were removed from urediospores by washing in 0.1% Tween 20 (v/v). Washed spores were rinsed with distilled water and dried on filter paper (Whatman No. 1) in a Buchner funnel. A soft brush dipped in sterile water was used to apply spores to both surfaces of both primary leaves of cowpea plants. Plants were then sprayed with a fine mist of sterile water and incubated in a dark, humid chamber for 24 hr.

### Injection Procedure

Aqueous solutions of $H_2O_2$ (0.29, 2.9, and 29 mM and 0.29 and 2.9 M; Sigma), KCN (100 μM and 1, 10, 25, 50, 100, and 200 mM; J.T. Baker, Inc., Phillipsburg, NJ), $NaN_3$ (1, 10, and 100 μM and 1, 10, and 100 mM; Sigma), $CuSO_4$ (1, 10, and 100 μM and 1, 10, and 100 mM; J.T. Baker, Inc.), and $ZnCl_2$ (1, 10, and 100 μM, and 1, 10, and 100 mM; Fisher Scientific Co., Fair Lawn, NJ) were injected into the intercellular spaces of leaves of California Blackeye or Dixie Cream, using a 3-mL syringe (Becton Dickinson and Co., Rutherford, NJ) with a 30-gauge needle (Becton Dickinson and Co.). The needle was inserted adjacent to the primary leaf vein, and ~10 $cm^2$ of leaf area was flooded with the injected solution. Water was injected as a control.

### Cell Viability Test

Pieces of leaf tissue of ~1 $cm^2$ were cut from the plant at 6 hr after injection with water or chemical solutions and vacuum infiltrated with 0.005% (w/v) fluorescein diacetate (Sigma) diluted from a 5 mg $mL^{-1}$ stock in acetone (0.1% [v/v] final acetone concentration). After incubation in the fluorescein diacetate solution for 15 min, tissue was mounted in water and viewed with a Reichert–Jung Polyvar microscope equipped with blue light epifluorescence (Heath, 1984). The living cells showing the bright yellow–green fluorescence of fluorescein over a constant leaf area were counted, and the percentage of dead cells

was determined (Bowen, 1984). Data from California Blackeye and Dixie Cream were combined because there was no significant difference in the response of the two cultivars.

### DNA Extraction and Analysis

Chemically injected and fungus-infected areas (6.7 $cm^2$) were cut from leaves with a cork borer and placed in 1.5-mL Eppendorf centrifuge tubes. Infected tissue was harvested at 24 hr after inoculation, whereas chemically injected tissue was harvested at 1, 2, 4, 6, 12, 24, and 36 hr after injection. Tubes were immediately placed in liquid nitrogen until frozen. A cold metal spatula was used to grind the leaf tissue into a fine powder. One milliliter of extraction buffer (100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% [w/v] hexadecyl triethyl ammonium bromide [CTAB; ICN Biochemicals, Irvine, CA]), 0.2% [v/v] β-mercaptoethanol) was added to each sample, the tubes were stirred briefly on a vortex mixer, and the mixture was incubated for 45 min at 60°C. Subsequent extraction procedures followed standard protocols for isolating plasmid DNA, including RNase digestion, except that all centrifugations were at 10,000*g* (Sambrook et al., 1982). To observe DNA fragmentation, samples were run on a 2% (w/v) agarose gel stained with 0.626 μg $mL^{-1}$ ([w/v] final concentration) ethidium bromide.

For fungal spores, ~1 mL of spores was frozen in liquid nitrogen and ground with a fine glass powder for 5 min in 5 mL of extraction buffer. DNA was extracted from 1 mL of the slurry as described for leaf tissue. All extractions were repeated at least twice with a different set of spores or cowpea tissue.

### Cytological Staining

Small pieces (1 $cm^2$) of leaf tissue were dropped into 4% paraformaldehyde in PBS, pH 7.4, immediately after cutting and were then vacuum infiltrated with the fixative. Leaf sections were cut, without further treatment, with a Hooker plant microtome (Lab-Line Instruments, Inc., Melrose Park, IL). Sections were stained for DNA with 5 μg $mL^{-1}$ 4′,6-diamidino-2-phenylindole in 0.02 M sodium phosphate buffer, pH 7.2, and were observed under UV epifluorescence irradiation (Heath, 1984) or differential interference contrast (DIC) optics.

To detect nuclear DNA cleavage, leaf sections were treated with the terminal deoxynucleotidyl transferase (TdT)–mediated dUTP nick end-labeling (TUNEL) procedure, using the fluorescein–dUTP-based in situ death detection kit (Boehringer Mannheim). Before a permeabilization step with 0.1% Triton X-100 in 0.1% sodium citrate, some sections were treated for 30 min at room temperature with 2% β-glucanase (InterSpex Products, Inc., Foster City, CA) in 0.05 M acetate buffer, pH 4.5, containing 0.1 M NaCl; this treatment facilitated entry of the TdT through the cell wall in thicker sections that did not cut through each cell. This hydrolase solution was found to have negligible DNase activity by using the EnzChek DNase Solution Assay Kit (Molecular Probes, Inc., Eugene, OR). This assay kit was also used to demonstrate high levels of DNase activity in cellulysin (Calbiochem, La Jolla, CA) used as a positive control. Negative controls were sections treated in a similar manner, except that TdT was omitted. Sections were viewed microscopically, using DIC optics or blue light epifluorescence irradiation (Heath, 1984).

Each experiment was repeated at least twice, and in each experiment, >25 haustorium-containing cells were observed for each plant/fungus/time combination.

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