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Apoptosis: A Functional Paradigm for Programmed Plant Cell Death Induced by a Host-Selective Phytotoxin and Invoked during Development

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The host-selective AAL toxins secreted by *Alternaria alternata* f sp *lycopersici* are primary chemical determinants in the Alternaria stem canker disease of tomato. The AAL toxins are members of a new class of sphinganine analog mycotoxins that cause cell death in both animals and plants. Here, we report detection of stereotypic hallmarks of apoptosis during cell death induced by these toxins in tomato. DNA ladders were observed during cell death in toxin-treated tomato protoplasts and leaflets. The intensity of the DNA ladders was enhanced by Ca^{2+} and inhibited by Zn^{2+}. The progressive delineation of fragmented DNA into distinct bodies, coincident with the appearance of DNA ladders, also was observed during death of toxin-treated tomato protoplasts. In situ analysis of cells dying during development in both onion root caps and tomato leaf tracheary elements revealed DNA fragmentation localized to the dying cells as well as the additional formation of apoptotic-like bodies in sloughing root cap cells. We conclude that the fundamental elements of apoptosis, as characterized in animals, are conserved in plants. The apoptotic process may be expressed during some developmental transitions and is the functional process by which symptomatic lesions are formed in the Alternaria stem canker disease of tomato. Sphinganine analog mycotoxins may be used to characterize further signaling pathways leading to apoptosis in plants.

INTRODUCTION

An unresolved question in plant biology is whether conserved signal transduction pathways lead to cell death by specifically ordered metabolic changes during normal development, environmental stress, or pathogen attack. The concept of a functionally conserved, gene-directed program for the maintenance of cellular homeostasis that is dependent on localized cell death is established in animal systems, including humans, nematodes, and insects (Tomei and Cope, 1991, 1994; Gerschenson and Totello, 1992; Hengartner and Horvitz, 1994). This orderly process of programmed cell death (pcd), known as apoptosis, depends on the active participation of the dying cell and is regulated by a number of well-characterized genes (Schwartzman and Cidlowski, 1993; Williams and Smith, 1993). Functionally, apoptosis is a distinct form of pcd that differs from degenerative death or necrosis in its nature and biological significance (Martin, 1993; Vaux et al., 1994; Kerr et al., 1995; Steller, 1995). Although apoptosis is a process in which the cell directs its own death, necrosis is essentially the opposite. It is the outcome of severe injurious changes in the environment of affected cells and is not an active gene-dependent form of cell death (Kerr et al., 1995).

Apoptosis, invoked in morphological development and in circumstances in which cells are unnecessary or would otherwise prove harmful to the organism (Tomei and Cope, 1991), has been legitimized scientifically by both genetic and molecular studies of the process (Alison and Sarraf, 1992; Williams and Smith, 1993; Whyte and Evan, 1995). Although apoptosis may have evolved for morphogenetic and developmental reasons, it appears to represent both a positive effector against disease and a target of active suppression by many successful pathogens (Savill, 1994; Vaux et al., 1994). Apoptosis also can be invoked as a suicide program after the appearance or disappearance of many different signals, including pathogens (Carson and Ribeiro, 1993; Martin, 1993; Barr and Tomei, 1994; Tomei and Cope, 1994) and toxins (Corcoran et al., 1994). Conversely, in disease situations in which immortalization of a cell occurs, the expression of various proto-oncogenes and virus-encoded genes that actively suppress apoptosis appears to be widespread (Raff, 1992; Smith et al., 1993; Cartier et al., 1994). In each of these cases, it is clear that multiple, distinct signaling pathways are involved in the induction of apoptosis, but eventually there is convergence in the seminal events that give rise to the phenotype of apoptosis.

There is a series of morphological and biochemical hallmarks of apoptosis in animals (Tomei and Cope, 1991, 1994). Animal

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cells undergoing apoptosis exhibit shrinkage, loss of cell-to-cell contact in organized tissues, and orderly fragmentation of nuclear DNA at internucleosomal sites. The fragmented DNA may then be organized into sharply defined membrane-bound bodies referred to as apoptotic bodies. In animal cells, these apoptotic bodies are taken up by adjacent cells and degraded within minutes to hours (Bursch et al., 1990). Internucleosomal cleavage of DNA is catalyzed by an endogenous Ca^{2+}-dependent endonuclease that generates 180-bp fragments and multiples of these units, which are resolved by gel electrophoresis as a laddered pattern of DNA that corresponds to the nucleosomal fragments (Collins et al., 1992; Cohen et al., 1994). Fragmentation of DNA during apoptosis also can be detected in situ by reagents that react with the exposed 3′ hydroxyls on the nucleosomal units (Gavrieli et al., 1992; Gold et al., 1994; Li et al., 1995). The assay procedure involves end labeling the DNA fragments by terminal deoxynucleotidyl transferase (TdT) with UTP conjugated to a detectable marker (Gorczyca et al., 1993).

Phenotypically analogous death-enforced changes in homeostasis occur in plants both during development (Woodson et al., 1992; O'Neill et al., 1993; Zhang and O'Neill, 1993; Chasan, 1994; Smart, 1994) and in response to pathogens (Keen, 1990; Lamb, 1994). Recently, there has been considerable speculation that hypersensitive resistance–associated cell death in several incompatible plant–microbe interactions may follow a death pattern similar to apoptosis in animals (Bachmair et al., 1990; Greenberg and Ausubel, 1993; Dietrich et al., 1994; Greenberg et al., 1994; Lamb, 1994; Vaux et al., 1994; Mittler et al., 1995). Because much is known about both the process and modulating genes in animals, the search for functional homologs of these genes in plants may prove useful if aspects of the process have been conserved in plants as they appear to be in animals. The stereotypic hallmarks of apoptosis, including DNA ladders and apoptotic bodies (Wyllie, 1995), and homologs of genes shown to regulate apoptosis in animals have not been reported in plants. However, recent reports, based on in situ detection of DNA fragmentation in dying cells, presumably resulting from specific endonuclease cleavage of nuclear DNA at internucleosomal sites during xylem differentiation and disease resistance expression, have suggested that an initial event consistent with pcd in animals can occur in plants (Mittler et al., 1995).

The metabolic and genetic bases of the functionally significant localized death of plant cells associated with necrotrophic disease (susceptibility), the hypersensitive reaction (HR) (resistance), reproduction, tissue differentiation, and natural senescence have not been characterized (see Ryerson and Heath, 1996). We have been studying the process of cell death in plants undergoing a susceptible response to pathogens to identify gene products that may modulate the process. This is part of a long-term effort to detect potential targets for genetic engineering of novel resistance genes by modifying the expression of host genes that either facilitate or fail to block susceptibility.

One of the systems we have been using to look for genetic and cellular markers of disease-dependent cell death is the Alternaria stem canker disease (Grogan et al., 1975; Gilchrist et al., 1992, 1995; Moussatos et al., 1993a, 1993b, 1994) of tomato caused by *Alternaria alternata* f sp *lycopersici*. Key features of this system include (1) expression by the pathogen of the host-selective toxins (AAL toxins) that are primary determinants of the disease (Siler and Gilchrist, 1983; Clouse and Gilchrist, 1986) and (2) expression by the host of the *Asc* (Alternaria stem canker) gene that determines susceptibility (*asc/asc*) and resistance (*Asc/Asc*) to the pathogen and to the AAL toxins (Clouse and Gilchrist, 1986). Cell death in tomato leaflets, caused by either the pathogen or purified AAL toxin, appears as localized spots of dead cells (Gilchrist and Grogan, 1976). Protoplasts of *Asc* isolines also react to the AAL toxins in a genotype-specific, toxin concentration–dependent manner, indicating that the alleles of the *Asc* gene are functionally active in protoplasts (Moussatos et al., 1993a). Toxin-induced cell death in protoplasts and leaflets requires between 36 to 48 hr, during which toxin-treated cells appear to maintain integrity of the plasma membrane until symptoms of death are observed (Gilchrist, 1983).

AAL toxins are a family of monoesters of tricarballylic acid and polyhydric alcohols (Bottini et al., 1981; Caldas et al., 1994, 1995) structurally related to sphinganine, a precursor of the chemical backbone of all sphingolipids. The AAL toxins and the fumonisins, a group of chemically related congeners produced by *Fusarium moniliforme* (Nelson et al., 1993), are potent and widespread mycotoxins with important health implications (Sydenham et al., 1991; Hopmans and Murphy, 1993; Merrill et al., 1993). The most abundant and active toxin congeners are AAL toxin TA and fumonisin B_1 (FB_1). The spectrum of biological effects induced by these sphinganine analog mycotoxins (SAMs) includes cell death in plants (Gilchrist and Grogan, 1976; Clouse and Gilchrist, 1986; Gilchrist et al., 1992, 1995; Abbas et al., 1994) and maladies in animals ranging from cancer (Gelderblom et al., 1992; Syndenham et al., 1990) to renal, neural, and hepatic degenerative diseases (Ross et al., 1990; Norred et al., 1992; Thiel et al., 1992; Norred, 1993).

Functionally, these toxins are potent inhibitors of ceramide synthase in plants (Abbas et al., 1994; Gilchrist et al., 1995) and animals (Wang et al., 1990, 1992; Norred et al., 1992; Merrill et al., 1993; Schroeder et al., 1994), which suggests that changes in cellular levels of sphingoid bases may be linked to cell death. As second messengers, sphingolipids and sphingoid bases now are known to regulate cell behavior at many levels, including cell communication, growth factor receptors, growth, differentiation, and transformation (Ghosh et al., 1994; Jarvis et al., 1994a, 1994b; Natarajan et al., 1994; Bose et al., 1995; Chao, 1995; Jones and Murray, 1995; Khan et al., 1995; Merrill et al., 1995; Obeid and Hannun, 1995; Ohata et al.,1995). Recently, we observed that fumonisins and AAL toxins induce cellular hallmarks of apoptosis in African green monkey kidney (CV-1) cells (Wang et al., 1996; Winter et al., 1996), most likely through interdiction of lipid-based signaling pathways.

Given the relationship between ceramides and apoptosis in animals, SAMs and ceramide metabolism, and our observation that SAMs induce apoptosis in CV-1 cells, we reasoned that *asc/asc* tomato cells might be a useful system to ascertain whether stereotypical hallmarks of the apoptotic process occur during toxin-induced cell death.

Evidence for the existence in plants of a pcd process that is available for use during developmental transitions and that could be triggered during disease would likely be dependent on genes of interest to both plant biologists and pathologists. Protoplasts and intact tomato leaflet tissue were analyzed for characteristics of apoptosis following treatment with host-selective SAMs. Simultaneously, we histologically examined tomato leaf sections during formation of tracheary elements and onion root cap cells undergoing sloughing during root elongation for evidence of developmentally regulated apoptotic characteristics. We report here (1) the presence of DNA cleavage at internucleosomal linker sites during toxin-induced cell death, using the TdT end-labeling (TUNEL) method; (2) Ca^{2+}-dependent DNA ladders of nuclear DNA from toxin-treated protoplasts and leaflets; and (3) the formation of distinct bodies, resembling apoptotic bodies, containing fragmented DNA in both SAM-treated protoplasts and sloughing root cap cells.

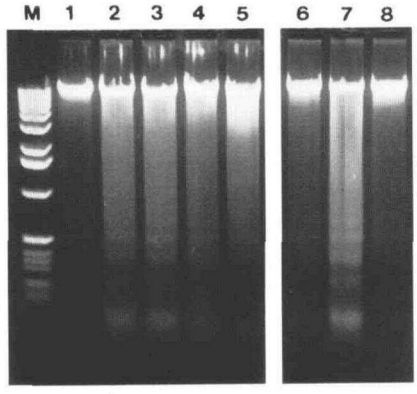

Figure 1. Agarose Gel Analysis of Tomato Protoplasts for DNA Fragmentation.

Lane 1 contains protoplasts that were incubated in TM-2 medium containing 3.3 mM $CaCl_2$; lane 2, medium plus 20 μM AAL toxin; lane 3, medium plus 20 μM FB_1; lane 4, medium plus 20 μM FB_1 and 50 μM cycloheximide; lane 5, medium plus 5 mM Fe_2SO_4; lane 6, medium containing 1 mM $CaCl_2$ plus 20 μM FB_1; lane 7, medium containing 10 mM $CaCl_2$ plus 20 μM FB_1; lane 8, medium containing 10 mM Ca^{2+} and 10 mM $ZnSO_4$ plus 20 μM FB_1; and lane M, DNA markers of 1-kb fragments. DNA was isolated 48 hr after treatment, separated on a 1.5% agarose gel by electrophoresis, stained with ethidium bromide, and photographed under UV illumination.

RESULTS

Detection of DNA Ladders after Toxin Treatment of *asc/asc* Protoplasts

Protoplasts were preincubated in TM-2 medium (Shahin, 1985) for 6 hr and subjected to gentle centrifugation in 0.6 M sucrose to remove cells that had died during protoplast isolation; the healthy protoplasts were then treated with 20 μM TA for 48 hr. This toxin concentration was chosen because it led to the death of ~50% of the total number of treated cells within 48 hr in both leaflets and protoplasts. This level of cell death permitted detection of both increases and decreases in death due to various treatments imposed in these studies.

As shown in Figure 1, agarose gel analysis of DNA isolated from TA-treated cells revealed DNA ladders consisting of fragments increasing in size by multiples of 180 bp when stained with ethidium bromide (Figure 1, lane 2). Healthy control cells, maintained in culture medium alone, did not show DNA fragmentation under these conditions (Figure 1, lane 1). The addition of 20 μM FB_1 to the protoplasts induced DNA ladders equivalent to those induced by TA (Figure 1, lane 2). Additional treatments that induced DNA ladders during cell death, including arachidonic acid, a fungal elicitor of the HR from *Phytophthora infestans* (Bostock et al., 1981), and KCN (see Ryerson and Heath, 1996), are listed in Table 1.

Another frequent observation regarding the induction of DNA ladders in animals is that apoptosis is inhibited by cycloheximide, suggesting that the process requires gene expression, at least in some situations, to go to completion (Schwartzman and Cidlowski, 1993). In tomato protoplasts, the addition of 50 μM cycloheximide reduced substantially the degree of DNA fragmentation induced by 20 μM FB_1 (Figure 1, lane 4) or TA (data not shown). The reduction of Ca^{2+} (as $CaCl_2$) from 3.3 to 1 mM in the TM-2 medium (Figure 1, lane 6) led to a reduction in DNA fragmentation, whereas an increase to 10 mM Ca^{2+} (Figure 1, lane 7) increased the intensity of the DNA ladders induced by 20 μM FB_1. The activating effect of Ca^{2+} on DNA fragmentation was completely negated by the addition of 10 mM $ZnSO_4$ (Figure 1, lane 8). The concentration- dependent activation by Ca^{2+} and inhibition by Zn^{2+} of toxin-induced DNA fragmentation of the plant DNA are consistent with the widely recognized effect of these divalent cations on the endogenous endonuclease responsible for cleavage of DNA during apoptosis in animal systems (Barry and Eastman, 1992; Lohmann and Beyersmann, 1993; Peitsch et al., 1993). Whereas cell death in the tomato protoplast system generated DNA ladders from many effectors (Table 1), we found that 5 mM Fe_2SO_4 killed 90 to 100% of the protoplasts within 12 hr but did not induce DNA fragmentation (Figure 1, lane 5; Schwartzman and Cidlowski, 1993).

Table 1. Compounds or Treatments Tested in Tomato Protoplasts or Leaflets for Induction of DNA Ladders[a]

Compounds	Concentration[b] or Duration	% Cell Death[c]	DNA Fragmentation[d] Protoplasts	Leaflets
AAL toxin TA	5 μM	30	+ + +	+ + +
FB_1	5 μM	33	+ + +	+ + +
KCN[e]	1 μM	75	+ + + +	NT[f]
KCN	300 μM	100	+ + + +	+
Arachidonic acid	100 μM	30	+ + +	NT
Heat shock	4 hr at 42°C	55	+ + +	NT
Heat shock	5 min at 65°C	10	NT	ø[f]
Staurosporine	500 nM	20	+ +	NT
C-2 ceramide	20 μM	30	+ +	NT
Fe_2SO_4	5 mM	95	ø	ø

[a] All tissues were incubated for 48 hr at 23°C before DNA isolation and vital staining.

[b] Each concentration listed is the lowest concentration tested that induced ladders or the highest concentration tested that did not induce ladders.

[c] Percentage of cell death is based on fluorescein diacetate vital staining.

[d] Relative intensity of DNA ladders compared with Figure 1 is shown: +, lane 4 to + + + +, lane 7.

[e] KCN-induced protoplast DNA fragmentation was at concentrations as low as 1 μM. DNA ladders could be resolved within 4 hr at 100 μM KCN.

[f] NT, not tested; ø, tested but not detected.

Detection of DNA Ladders by DNA Gel Blot Analysis after Treatment of Leaflets with SAMs

Detached leaflets were treated with TA by the standard leaflet bioassay (Gilchrist and Grogan, 1976) in which toxin is taken up for 36 hr through the petiole of the leaflet from filter paper impregnated with 20 μM TA or FB_1 in H_2O. At this toxin concentration, typical lesions covered ~50 to 60% of the total leaflet, indicating that DNA from the dead areas would be combined with a nearly equal amount of DNA from apparently healthy areas outside the lesions when the entire leaflet was extracted. Preliminary experiments indicated that gel blot analysis was more sensitive than ethidium bromide staining in detecting DNA ladders. Hence, to maximize visualization of ladders from treated leaflets containing both lesion and healthy regions, DNA gel blot analysis was performed on DNA that was isolated by the nonphenol method (Tai and Tanksley, 1991) and then separated on 1.5% agarose gel. The separated DNA was transferred to a nylon membrane and hybridized with ^{32}P-labeled tomato total genomic DNA. As shown under these conditions in Figure 2, both toxins (Figure 2, lanes 1 and 2) induced DNA fragmentation in the treated leaflets; this fragmentation appeared as a series of bands corresponding to increasing multiples of 180- to 200-bp fragments. No fragmentation was observed in the water control tissue (Figure 2, lane C). In addition, identical results were obtained with DNA isolated from leaflets taken directly from plants infected with the *A. a. lycopersici* that induced typical symptoms of the Alternaria stem canker disease (Gilchrist and Grogan, 1976).

In Situ Detection of Nuclear Fragmentation by the TUNEL Method in Protoplasts

Apoptosis is further revealed in animals by the orderly compartmentation of DNA into distinct membrane-bound apoptotic bodies that persist for varying periods of time. The bodies containing fragmented DNA eventually move to the periphery of the affected cells, which then become distorted in shape around these bodies, a process called blebbing (Tomei and Cope, 1991). In situ detection of nuclear DNA fragmentation and visualization of apoptotic bodies involves using a TUNEL of the free 3′ –OH groups on the nucleosomal fragments with a detectable marker (Gorczyca et al., 1993). The TUNEL method was used to determine whether fragmentation was restricted to nuclear DNA of TA-treated protoplasts and whether the fragmented DNA was then compartmentalized into several distinct bodies, as occurs in animal systems. In these experiments, the *asc/asc* protoplasts were incubated inside plastic rings directly on microscope slides for 24 hr in TM-2 medium containing 20 μM TA and compared with cells incubated in TM-2 medium alone after application of the TUNEL method and counterstaining the cells with Hoechst 33342 for total DNA.

As shown in Figure 3, light microscopy revealed the location and shape of the TA-treated cells compared with the control

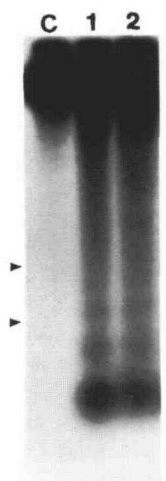

Figure 2. Toxin-Induced DNA Fragmentation in Tomato Leaflets.

Excised tomato leaflets were treated for 36 hr with H_2O (lane C), 20 μM AAL toxin (lane 1), or 20 μM FB_1 (lane 2). DNA was isolated and separated as described in Methods. Gel blot analysis was performed by hybridizing the DNA with a ^{32}P-labeled tomato genomic DNA. The arrowheads indicate 500- and 1000-bp DNA markers.

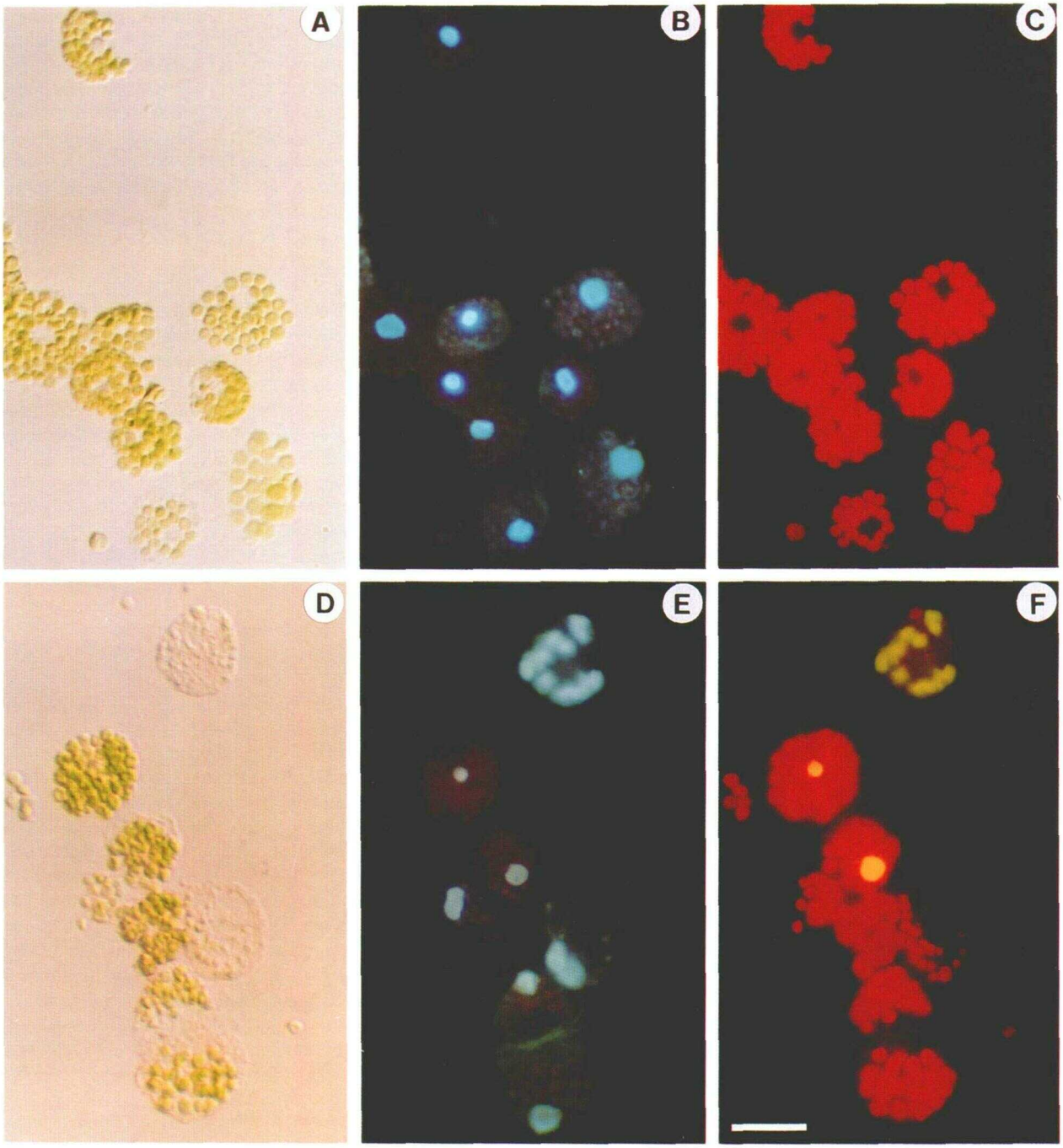

Figure 3. In Situ Detection of Apoptosis in Tomato Protoplasts.

(A) to **(C)** Protoplasts incubated for 24 hr in TM-2 medium (control).
(D) to **(F)** Protoplasts incubated for 24 hr in TM-2 medium containing 20 μM AAL toxin.
Nomarski optics were used for **(A)** and **(D)**. In **(B)** and **(E)**, Hoechst 33342 was used to stain the DNA. In **(C)** and **(F)**, the TUNEL assay was used to detect DNA fragmentation. Bar in **(F)** = 20 μm.

protoplasts (Figures 3A and 3D) and permitted distinction of cells that were becoming chlorotic, darkened, and slightly distorted in shape after a 24-hr exposure to the toxin. DNA staining revealed single nuclei within the individual cells that appeared to be centrally located and of similar size and shape in control cells (Figure 3B). In contrast, cells treated with TA exhibited nuclei with more variable size and shape, frequently with multiple distinct bodies containing DNA that often were associated with the periphery of the cell (Figure 3E).

Application of the TUNEL method to end-labeled fragmented DNA with dUTP-digoxigenin followed by treatment with anti-digoxigenin antibody and fluorescein isothiocyanate (FITC)–labeled anti–goat antibody revealed fragmented nuclear DNA in toxin-treated cells (Figures 3C and 3F). Together, these techniques permitted viewing the exact same cells for general appearance with light microscopy (Nomarski), localization of nuclear DNA (Hoechst 33342), and detection of fragmented nuclear DNA (TUNEL), using different wavelengths of UV light. The TUNEL method revealed that ~50% of the cells were positive for excess 3′ –OH groups and that some of these cells contained multiple TUNEL-positive, distinctly separated bodies. The size, shape, and location of the Hoechst-stained DNA in each of the TUNEL-positive cells corresponded exactly with the TUNEL-positive bodies (compare Figures 3F and 3E) in the TA-treated cells. No FITC-derived fluorescence was observed when the TdT, the dUTP substrate, or the respective antibodies were omitted from the reaction. Vital staining of dying protoplasts with fluorescein diacetate indicated that these distinct bodies, even when separated from the cell, were bounded by an intact membrane, although we did not conduct electron microscopy studies to confirm this point. These results are strikingly consistent with the characteristics of apoptosis in animals cells, in which similar techniques have been used as in situ strategies to visualize nuclei undergoing fragmentation during apoptosis and the subsequent formation of apoptotic bodies (Gorczyca et al., 1993).

In Situ Detection of Nuclear Fragmentation in TA-Treated Tomato Leaflets

Tomato leaflets (*asc/asc*) were allowed to take up 20 μM TA for 24 hr in the standard detached leaflet bioassay procedure. Localized dead areas and surrounding areas at the lesion margin without symptoms were fixed in 10% buffered formalin, cut into small pieces, imbedded in HistoPrep (Fisher), and frozen in liquid nitrogen before sectioning with a freezing microtome at a thickness of 10 μm. Areas of dead cells were readily detected by their brown color under normal light as well strong autofluorescence in the dead areas under UV light. In addition, there was a complete loss of cell-to-cell contact, which is seen in Figures 4B and 4D. Strong autofluorescence in the lesion areas quenched FITC fluorescence in contrast to protoplasts. This problem was circumvented by using anti-digoxigenin antibody conjugated to alkaline phosphatase with leaflet tissue, although the TUNEL-positive color stain could block the Hoechst stain. As shown in Figure 4, the areas containing dead cells consistently exhibited a positive TUNEL reaction that was limited to the area of the lesion (designated as dc in Figure 4B). The TUNEL-positive area did not extend beyond the margin of the lesion, nor did TUNEL-positive cells appear in the water-treated controls (Figure 4C). The same section (Figure 4A) counterstained with Hoechst 33342 revealed that DNA was present in all cells, but intact nuclei were confined to cells in areas that did not express a positive alkaline phosphatase reaction.

Dependence on end labeling by TdT in the lesion areas was confirmed by the results of negative control reactions conducted in the absence of the transferase (Figure 4D) that showed no color development in the area of the dead cells. In addition, the cells in the lesion area appeared to have lost cell-to-cell contact and exhibited distorted margins but retained the fragmented DNA within the cell margins, even though the DNA was no longer confined to a single nuclear body, as can be seen in DNA counterstained cells outside the lesion area (compare Figures 4A and 4B). All of these results are consistent with the morphological characteristics of apoptosis in organized animal tissues (Gorczyca et al., 1993).

Quantification of TA-Induced Cell Death in *asc/asc* Protoplasts

The positive association between detection of DNA ladders and TUNEL-positive DNA in protoplasts treated with 20 μM TA or FB_1 suggested that most protoplasts may have died in a manner consistent with the induction of an apoptotic process. We examined this relationship further in additional experiments in which the protoplasts were isolated in the same manner as those in Figures 3 but were cultured at 24°C for 48 hr in 250 μL of TM-2 medium with or without either toxin in 24-well plates with a concentration of 10^5 protoplasts per mL. Viable cells, detected by staining with fluorescein diacetate, and total cells were viewed on an inverted microscope. For in situ application of the TUNEL assay, protoplasts from the same isolations as shown in Figure 3 were cultured directly on slides, and the TUNEL reaction was run with alkaline phosphatase conjugated to the anti-digoxigenin antibody. Use of alkaline phosphatase was necessary because FITC fluorescence faded too quickly to permit accurate counting.

Approximately 50% of the cells died in the TA and FB_1 treatments; of these, 85 and 95%, respectively, were TUNEL positive (Figures 5A and 5B). The addition of 10 mM Ca^{2+} to the TA treatment resulted in 100% of the dead cells reacting TUNEL positive, in contrast to the addition of 10 mM Zn^{2+}, which did not protect against cell death but reduced the fragmentation detectable by the TUNEL reaction to <10% of the total dead cells. Cycloheximide treatment resulted in a reduction of both TA-associated death and TUNEL-positive cells to the respective control values, suggesting that inhibition of gene

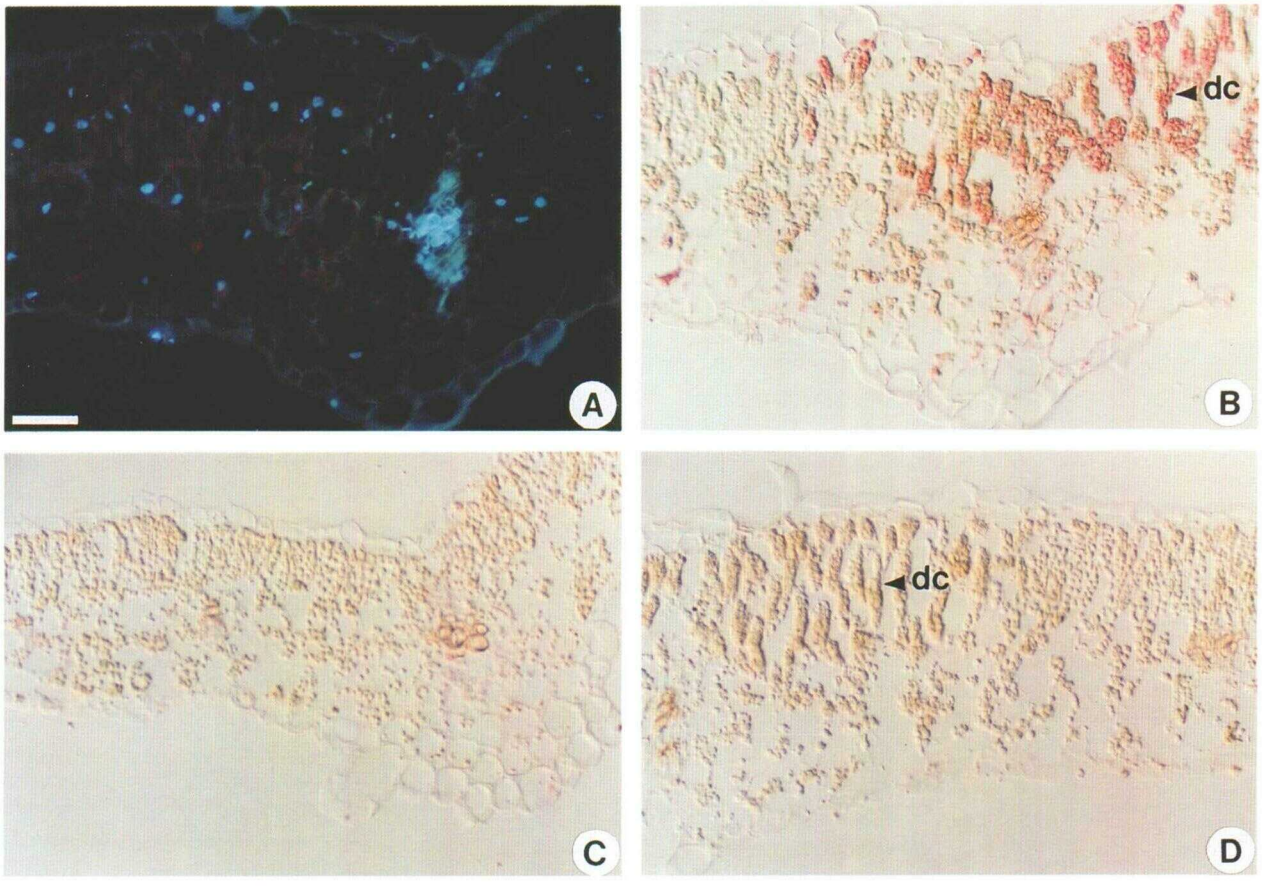

Figure 4. In Situ Detection of Apoptosis in Tomato Leaflets.

Tomato leaflets in **(A)**, **(B)**, and **(D)** were treated for 24 hr with AAL toxin (20 μM). Tomato leaflets in **(C)** were treated for 24 hr with H_2O.
(A) Toxin-treated tomato leaf stained for DNA with Hoechst 33342.
(B) The same field of tomato leaf section as shown in **(A)** stained by using the TUNEL method as described in Methods.
(C) Section from a water-treated (control) tomato leaflet stained by using the TUNEL method.
(D) AAL toxin–treated tomato leaf tissue stained as shown in **(B)**, except for the absence of TdT.
Note that the detection of fragmented DNA in dead cell (dc) areas was TdT dependent. Bar in **(A)** = 50 μm.

expression reduced the absolute amount of cell death resulting from toxin treatment. These data also suggest that the effect of 10 mM Ca^{2+} added to the TM-2 medium was not to increase the number of dead cells but to increase the activity of the endonuclease in those cells that had been triggered to die.

Progression of Nuclear Changes during TA-Induced Cell Death in *asc/asc* Protoplasts

In the course of viewing thousands of protoplasts harvested at different times after treatment with TA that were undergoing cell death, we observed that the changes in nuclear DNA followed a consistent pattern once the nuclear material first reacted positively in the TUNEL procedure. As shown in Figure 6, this progression of changes began with an intact nucleus (Figures 6A and 6F) that maintained its shape and size but reacted TUNEL positive with the DNA localized in a single nuclear body (Figures 6B and 6G). This was followed by an expansion or spreading of the nuclear material, which then began to show delineation into several nuclear bodies (Figures 6C and 6H). These bodies maintained a generally round shape but sometimes took on a more elongated form as the process proceeded (Figures 6D and 6I). Following migration of these bodies to the periphery of the cell, the final stage appeared to be one in which the cell began to lose shape (blebbing) and then disintegrate, at which point the distinct bodies containing the fragmented DNA were released into the medium (Figures

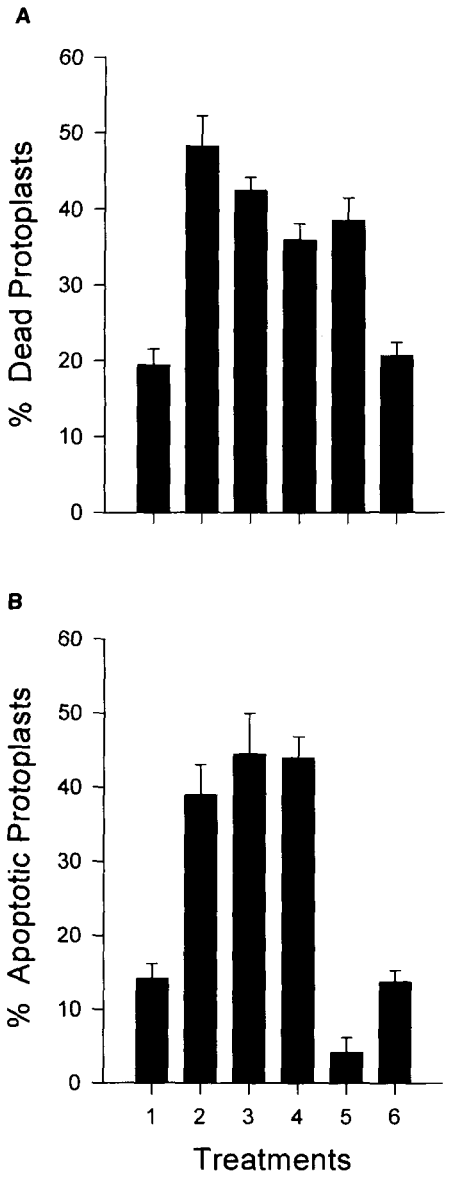

Figure 5. Quantification of AAL Toxin–Induced pcd in Tomato Protoplasts.

(A) Protoplasts were isolated and cultured in 250 μL of treatment solution in a 24-well plate at a concentration of 10^5 protoplasts per mL. Plates were incubated at 24°C for 48 hr. Viable cells were counted in three wells with 10 views per well and each view containing ~100 cells.
(B) Protoplasts were cultured directly on slides and fixed, as described in Methods. After in situ TdT-digoxigenin end labeling, the slides were stained with anti-digoxigenin antibody followed by alkaline phosphatase–labeled anti-goat antibody. Apoptotic protoplasts were counted in two slides with 10 views per slide and each view containing ~200 cells.
Means and SEM from three experiments are indicated above the respective bars: (1) indicates the control; (2), treatment with 20 μM TA; (3), 20 μM FB_1; (4), 20 μM TA plus 10 mM Ca^{2+}; (5), 20 μM TA plus 10 mM Ca^{2+} and 10 mM Zn^{2+}; and (6), 20 μM TA and 15 μM cycloheximide.

6E and 6J). After the bodies moved outside the cell, many appeared to remain intact, retained their shape, and remained closely associated with each other in the high osmotic protoplast medium.

We were unable to estimate how long it took for a cell to traverse these changes once fragmentation begins, because the death process in these protoplasts is not fully synchronized and all determinations are made on destructively sampled cells. However, we were able to detect stages equivalent to those illustrated here from 6 to 36 hr under standard assay conditions. In animal cells undergoing apoptosis, analogous events can occur in minutes to a few hours. In some cases, discrete stages are not readily or consistently detectable because the events proceed so quickly. Condensation and budding to form apoptotic bodies can be completed within minutes (Matter, 1979) and may remain visible for only a few hours (Bursch et al., 1990), prompting Kerr et al. (1991) to comment that apoptotic bodies are remarkably inconspicuous histologically. We suspect that the tomato protoplast system is biased toward detection of stages, because we are able to sample periodically during the 24- to 48-hr death window. This likely improved our chances of seeing progressive stages, in contrast to viewing similar events in organized tissue undergoing either toxin-induced or developmentally triggered death by this process.

Effect of AAL Toxin on Tomato Protoplasts and Leaflet Tissue in Resistant *Asc* Isolines

Both protoplasts and leaflets of the *Asc/Asc* isoline were treated with TA at 5 to 20 μM under the same conditions as the *asc/asc* isoline. There was no apparent toxin-induced death observed in either situation, nor were there any DNA ladders or TUNEL-positive reactions observed, and the tissues did not differ from untreated controls. However, both protoplasts and leaflets produced DNA ladders in the *Asc/Asc* isoline when treated with other inducers of cell death (Table 1). These other effectors with potential to induce apoptosis based on animal studies included sphinganine metabolites (C-2 ceramide), a lipid-based elicitor of cell death (arachidonic acid), the protein kinase inhibitor staurosporine, and heat shock. Concentrations used were based on previous reports in the literature on animals. Among the chemicals tested, many induced DNA ladders and cell death in both genotypes, but only the SAMs were genotype specific in toxicity and DNA fragmentation. However, Fe_2SO_4 killed cells but did not induce the formation of DNA ladders and therefore was used as a negative control for nonapoptotic cell death.

Detection of TUNEL-Positive Cells and Apoptotic-like Bodies in Sloughing Root Cap Cells

Detection of morphological characteristics of apoptosis in tomato cells challenged by a number of negative external stimuli has confirmed the existence of several functional elements of the apoptotic process in plant cells under stress (see

also Ryerson and Heath, 1996). Although an orderly elimination of cells under stress may be sufficient reason for this process to have been conserved in plants, it seemed likely that such a complex process with the potential for multiple signal inputs and parallel pathways leading to expression of genetic determinants of cell death might be invoked during normal development in plants as it is in animals.

Because apoptosis in animals is recognized as a rapid process once invoked, to observe the various steps in this process in plants likely would be facilitated by examining tissues in which the process is ongoing continuously. In this context, we reasoned that root cap cells may be synchronized in sufficient numbers to enable observation of where and when, in relation to their meristematic origin, a cell death program may be invoked. The root cap consists of living parenchyma cells derived continuously from the apical meristem (Esau, 1977). As new cells are produced in the interior, those on the periphery of the root are shed in an orderly manner reminiscent of Kerr's original basis for derivation of the term apoptosis to describe pcd as a natural process (Kerr et al., 1972). It has been estimated that in oat roots, the time from origin to shedding is a period of 5 to 6 days (Harkes, 1973), thus suggesting that stages, if they exist, might be detectable.

Fresh onion root tips, fixed and sectioned cyrostatically, were treated by using the TUNEL method and counterstained with Hoechst 33342. As shown in Figure 7, the DNA counterstain revealed nuclear DNA in cells throughout the root sections, from the apical meristem, where cells were undergoing division, to the outer surface, where cap cells were sloughing from the root (Figure 7A). The TUNEL method detected fragmented DNA only in cells near the surface of the root cap (Figure 7B) in longitudinal sections, which was confirmed in cross-sections (data not shown). The area of TUNEL-positive cells was restricted to the outermost one to three cells of the cap, and no DNA fragmentation in the interior of the root, including cells that were undergoing active DNA replication during cell division, was detected. When viewed under higher magnification, the appearance of nuclear DNA (Figure 7C) changed from a compact circular form to elongated TUNEL-positive structures that eventually took on the form of numerous well-defined circular bodies (Figure 7D). The formation of these bodies appeared to be the final step reached before a cell was shed from the root cap (Figure 7D). The same view fields of the root tip that reacted with the TUNEL method (Figures 7B and 7D) were counterstained for DNA with Hoechst 33342 (Figures 7A and 7C). The weaker DNA-staining signal in the outer layer of cap cells was due to the significant loss of DNA as well as the fact that the fragmented DNA was no longer in a single compact nucleus in these cells in contrast to the strong TUNEL labeling in the same cells due to the high sensitivity of this assay (see cautionary statement in Methods). The ease with which these findings can be observed was revealed by the

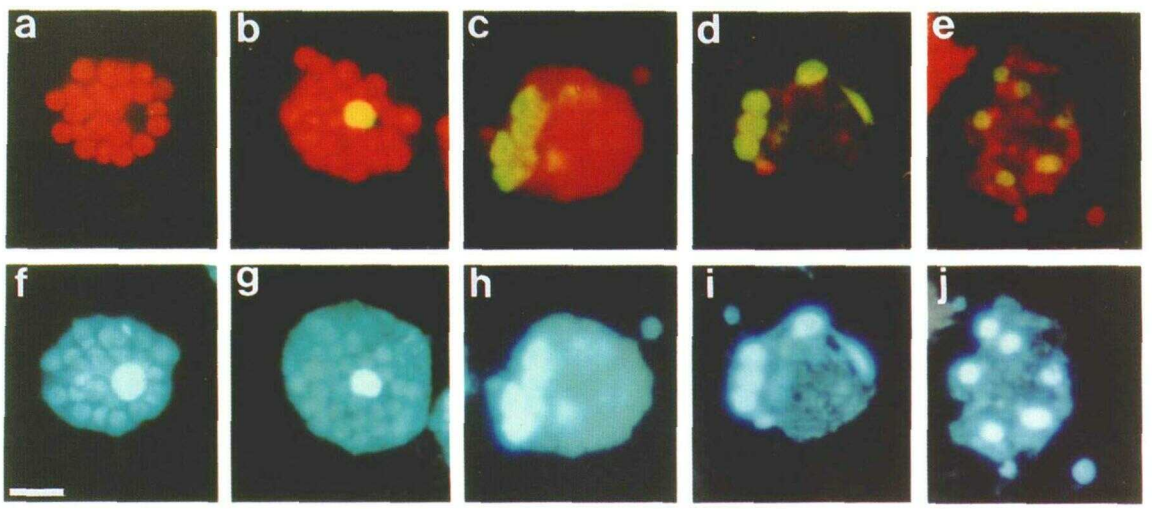

Figure 6. Temporal Progression of Nuclear Changes Associated with AAL Toxin–Induced Cell Death in *asc/asc* Tomato Protoplasts.

Tomato protoplasts were treated with 20 μM TA for 24 hr. **(A)** to **(E)** show protoplasts labeled by using the TUNEL method. Fragmented DNA and apoptotic bodies were viewed at 510 to 560 nm as yellow to greenish fluorescence. The red fluorescence represents autofluorescence of chlorophyll. **(F)** to **(J)** show the same view field as in the upper row, except protoplasts were stained with the DNA counterstain Hoechst 33342.
(A) and **(F)** Normal, apparently healthy protoplast.
(B) and **(G)** Early stages of apoptosis, with nuclear DNA fragmentation and nuclear budding shown as light green fluorescence.
(C) and **(H)** Same stages as shown in **(B)** and **(G)**.
(D) and **(I)** Late stages of apoptosis with DNA fragments and distinct bodies shown as green fluorescence following the TUNEL assay.
(E) and **(J)** Same stages as shown in **(D)** and **(I)**.
Bar in **(F)** = 10 μm.

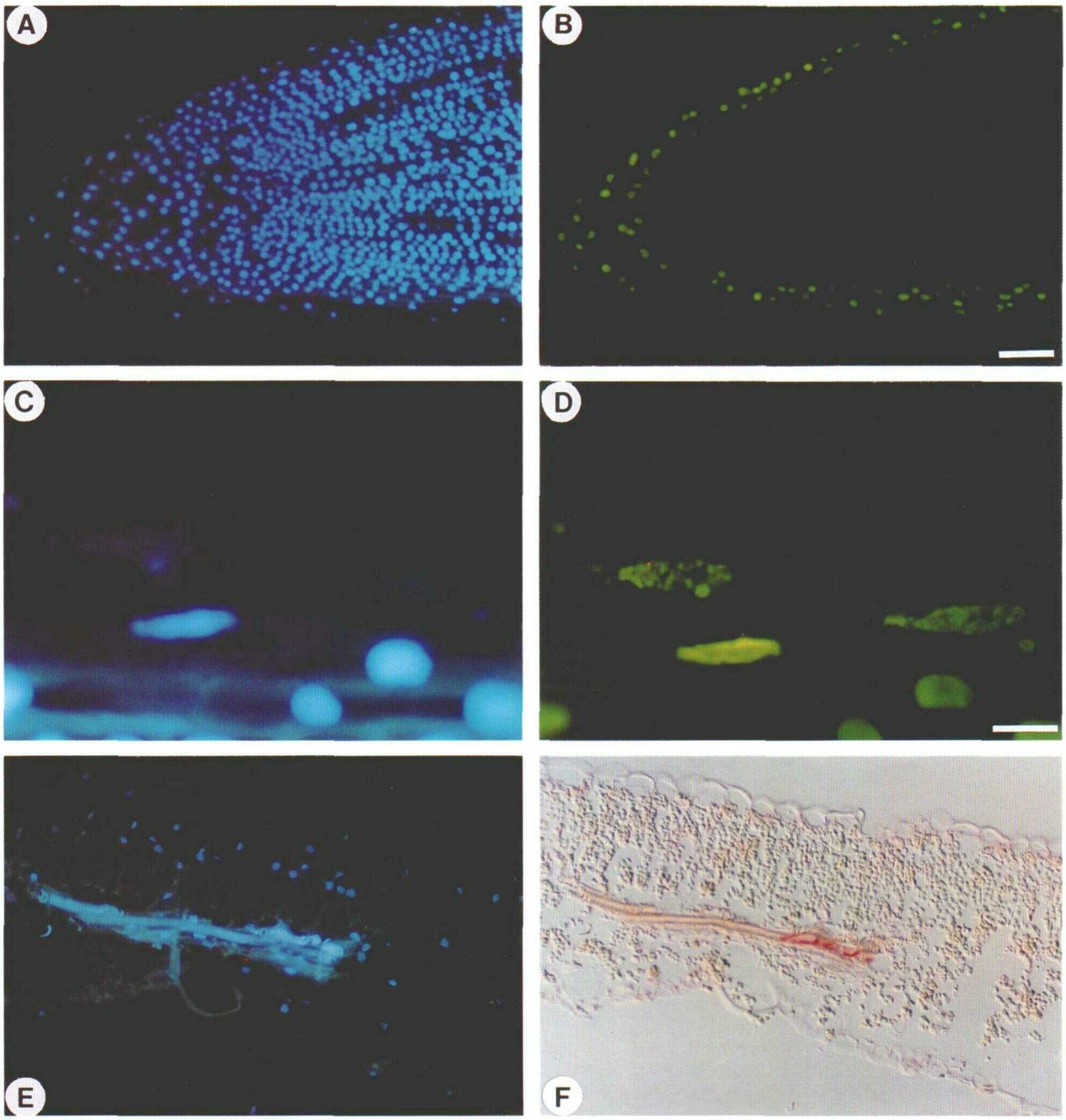

Figure 7. Apoptosis during Plant Development.

(A) Longitudinal section of onion root tip stained for DNA with Hoechst 33342.
(B) Same section as shown in **(A)** but labeled by using the TUNEL assay. Shown are root cap cells undergoing apoptosis.
(C) Root cap cells stained for DNA with Hoechst 33342.
(D) The same field of root cap cells as shown in **(C)** stained by using the TUNEL method. Shown is the formation of apoptotic bodies.
(E) Cross-section of young expanding tomato leaflet stained for DNA with Hoechst 33342.
(F) The same field of leaf section as shown in **(E)** stained by using the TUNEL method. Shown are developing tracheids undergoing DNA fragmentation.
Bar in **(B)** = 50 μm for **(A)**, **(B)**, **(E)**, and **(F)**; bar in **(D)** = 2 μm for **(C)** and **(D)**.

fact that sections of tomato root caps readily gave the same results as those seen with onion (data not shown).

We also examined cryosections of young expanding tomato leaflets stained by using the TUNEL method (Figure 7F) and counterstained with Hoechst 33342 (Figure 7E). Developing tracheary elements consistently were positive for the TUNEL reaction only at the point at which the elements were first forming (Figure 7). All other areas of the leaflet were free of TUNEL-positive DNA, even though Hoescht 33342 counterstain confirmed that DNA was present in all cells surrounding the tracheary elements. The same field and leaf section shown in Figure 7E was stained for DNA. End labeling of DNA in sections showing tracheid development is consistent with the conclusion of Mittler et al. (1995), who provided histological evidence that fragmented DNA is present during development of tracheary elements.

DISCUSSION

The ability of a pathogen, via secreted signals, to interdict and disrupt evolutionarily conserved signal transduction pathways dedicated to plant development would fulfill the functional requisites of pathogenicity factors and expose a fundamental genetic vulnerability of plants to pathogens. Our results indicate that a process leading from Ca^{2+}-activated endonuclease cleavage of DNA to the formation of apoptotic bodies can be induced in diseased tissues by host-selective toxins or arachidonic acid, an elicitor of the HR derived from *P. infestans*, and can appear during normal plant development. Coupled with the appearance of DNA ladders and TUNEL-positive cells in regions of leaves undergoing HR in an incompatible interaction between *Uromyces vignae* (an obligate parasite) and cowpea (see Ryerson and Heath, 1996), these data suggest that diverse pathogens may interdict conserved developmental pathways in plants, leading to cell death. Molecular dissection of the signaling pathways involved could identify genes common to plant development and plant disease. Elucidation of the role of plant genes in host-determined susceptibility could provide the basis for molecular strategies to reduce disease severity and may be especially important in situations in which no effective genetic resistance currently exists. For example, if the expression of a plant gene(s) linked to cell death predisposes tissue to infection or if a plant gene induced during infection by a specific pathogen contributes to enhanced disease severity, the reduced expression by antisense technology, under control of a pathogen-sensitive promoter, has the potential to reduce or limit the disease.

Plant gene expression has been studied widely in plants undergoing resistant reactions to microbial pathogens (Lamb, 1994) but has been addressed sparingly as a necessary component of susceptibility in plant disease. Instead, studies of genes that may play a determinative role in plant susceptibility have focused on expression of microbial genes or the activity of microbial gene products in pathogenesis or increased virulence (Collmer, 1986). The results reported here, wherein a host-selective toxin and a fungal elicitor of HR induced equivalent characteristics of cell death, suggest that similar plant genes regulate susceptibility and resistance to pathogens, at least in some cases. DNA ladders, detected in tomato protoplasts treated with arachidonic acid, an elicitor of the HR phenotype in potato and other plants (Bostock et al., 1981), are consistent with the results of Ryerson and Heath (1996).

The association of lipid peroxidation and active oxygen species in plant disease is well documented (Keppler et al., 1989; Levine et al., 1994), as is the critical role of lipid-based second messengers in regulating cell stability in animals (Khan et al., 1995). Hydroperoxides of arachidonate, known to induce all the major morphological and chromatin changes of apoptosis in human T cells (Sandstrom et al., 1995), are formed within minutes in plant cells exposed to arachidonic acid (Ricker and Bostock, 1994). Our initial results indicate that oxidative stress involving lipid peroxidation may trigger signal transduction pathways leading to the apoptotic phenotype in plants, as has been reported for animals.

In contrast to coevolution of pathogenicity factors and susceptibility or resistance, our results lead us to speculate that the genes conditioning sensitivity to host-selective toxins and elicitors may predate the pathogenic interaction. In the present situation, the plant would function as a sensitive assay for an acquired ability on the part of the pathogen to secrete biologically active functional homologs of endogenous signals of apoptosis. This molecular interaction, facilitating either susceptibility or resistance, could occur as the result of these signaling molecules arising for developmental or ecological reasons unique to the pathogen as well as the plant and not by directed coevolution. In this context, a "necrotizing" pathogen capable of secreting signals to which the plant is sensitive could lead to the fortuitous acquisition of a food base somewhat sooner than would be acquired following normal senescence but without any selection pressure to do so (Gilchrist et al., 1995). Conversely, pathogen-regulated suppression of plant cell death in biotrophic interactions, in which the nutrient base is a living plant cell, could be the positive selection pressure on the pathogen rather than a failure on the part of the host to react with an HR response. Although we know of no analogies in plants, there are at least a dozen mammalian viruses that have been shown to encode activities that specifically modulate apoptosis during infection (Shen and Shenk, 1995). It may be useful to look directly for evidence of active suppression of apoptosis in plants during infection by obligate parasites.

Clearly, there are several options afforded the cell following perception of an endogenous or exogenous signal potentially capable of disrupting homeostasis. The final outcome may be proliferation, quiescence, death, or differentiation, depending on the cell type, the physiological status of the cell, the cell-cycle stage, or the blend of other signals being processed by the cell at the moment a different stimulus is perceived (Williams et al., 1992; Williams and Smith, 1993). In animal

medicine, both the signals that regulate gene expression and the impact of altered gene expression on apoptosis (Wyllie, 1995) are the subject of intense interest in basic research in human diseases ranging from cancer (Anderson et al., 1994; Fisher, 1994) to autoimmune diseases (Critchfield and Lenardo, 1995; Thompson, 1995).

Interestingly, the term apoptosis was derived from the Greek by J.F.R. Kerr in 1972 to reflect the process by which leaves fall from trees to emphasize the natural process of a programmed form of cell death he was studying in liver tissue (Kerr et al., 1972). Analogous to animals, the confirmation and manipulation of a gene expression–dependent apoptotic process in plants will require linkage to specific genes, sets of interacting genes, or gene products that respond to the signals transduced by the range of stimuli that elicit this form of pcd. For now the principal criteria for apoptosis in plants are those used by animal biologists, namely, rapid changes in chromatin structure, in situ labeling of fragmenting DNA, DNA ladders, and the formation of apoptotic bodies.

We are cognizant of the limitations of relying solely on a single criterion such as DNA fragmentation to ascribe cell death to an apoptotic process (Collins et al., 1992); hence, we have examined additional facets and metabolic connections between the events observed in plants and those reported in animals. Cloning of plant genes functionally homologous to those shown to affect apoptosis in animals will be necessary to resolve further the similarities and the differences in cross-kingdom conservation of this process (Takayama et al., 1994). Unfortunately, homology at the DNA level is often <20% between different animal species, and dilution of expressed messages in populations of differentially responding plant cells could be another difficulty (Hengartner and Horvitz, 1994; Shen and Shenk, 1995). For induced genes, the number of cells in an organized tissue undergoing apoptosis may be small compared with the total cell mass, the process may be asynchronous, and the number of cells induced to die apoptotically compared with those that actually complete the stereotypical stages of the process before metabolic collapse may represent only a fraction of the total.

A corollary issue relates to the basis by which particular cell groups (spots) are targeted for death in leaf spot diseases, HR-induced spots, and lesion mimic spots. The fact that only certain areas die, even in situations in which cells outside the responding area are exposed simultaneously to the stimulus (elicitor, toxin, infiltrated pathogen, stress, or inappropriate developmental signals), suggests that either temporal or permanent diversity exists in elements controlling the response of individual cells to different stimuli in organized tissue networks.

The fate of the apoptotic-like bodies containing fragmented DNA is unknown. The role of apoptotic bodies in animal tissues is thought to facilitate phagocytosis of the debris from a dying cell as an efficient means to protect the surrounding tissues from potentially harmful cell contents. In the absence of phagocytic cells, the contents of a plant cell, packaged in apoptotic bodies similar to the role of vacuoles but containing additional constituents, could represent a prophylactic function during release of cellular contents from a dying cell into tissue. What is clear is that the mechanism for organizing this material into stable bodies, equivalent to apoptotic bodies in animal cells, is conserved in plants, as evidenced by the formation of such bodies during the final phases of sloughing of root cap cells during onion root growth.

Whether the full program for apoptosis or only phases of the program occur in plant cells that are programmed to die is not known. Also, the types and number of pathways that may converge to give the stereotypic hallmarks are unknown. If the situation in plants were like apoptosis in animals, the induction of pcd in one or a few cells in a given region or tissue may trigger the rapid death of surrounding cells; the latter cells may not show the apoptotic phenotype but die nonetheless (Collins et al., 1992). The most salient conclusion we draw from these studies and those of Ryerson and Heath (1996) is that the functional properties of apoptosis occur in plants, and the role of pcd in plants appears to encompass that proposed in animals, both in health and sickness.

METHODS

Plant Materials

Tomato (*Lycopersicon esculentum*) plants used for leaflet assays were grown from seed of Alternaria stem canker (*Asc*) isolines (Clouse and Gilchrist, 1986). Plants were grown in soil under greenhouse conditions for 4 weeks, transferred to growth chambers (25°C/21°C day/night, 16-hr photoperiod) at the five-leaf stage, and used in experiments at the seven-leaf stage. Plants were illuminated with a combination of cool-white fluorescent and incandescent lamps giving an intensity of 112 W m^{-2} irradiance at the leaf canopy. Excised tomato leaflets were put on a filter paper saturated with treatment solutions in a glass Petri dish and placed under continuous light for 36 to 48 hr at 25°C, as described previously (Moussatos et al., 1993b).

Fumonisin, AAL Toxin, and Other Chemical Treatments

Fumonisin B_1 (FB_1) was purchased from Sigma (>95% pure). AAL (*Alternaria alternata* f sp *lycopersici*) toxin TA was produced and purified as described previously (Caldas et al., 1994). The preparation used was >98% pure based on analysis by HPLC and electrospray mass spectrometry. C-2 ceramide was obtained from Calbiochem (La Jolla, CA), and staurosporine and arachidonic acid, prepared as an emulsion in water (Bostock et al., 1981), were from Sigma.

Protoplast Preparation

Tomato leaf protoplasts were isolated from *Asc* isoline plants grown under aseptic conditions in Murashige and Skoog medium (Moussatos et al., 1993a). After 3 to 4 weeks of growth, the plants were transferred to a dark chamber at 24°C for 2 days, followed by 6 to 8 hr of dark

at 4°C. After the cold treatment, leaflets were harvested, and protoplasts were isolated as described earlier (Moussatos et al., 1993a). After isolation, protoplasts were freed from cellular debris by flotation on a 0.6 M sucrose solution, using low-speed centrifugation (100*g* for 5 min). Viable protoplasts were collected at the interface and washed three times by centrifugation in 10% mannitol solution before they were resuspended in culture medium. The fractionated protoplasts were preincubated for 12 hr in TM-2 medium containing 3.3 mM Ca^{2+} with no added hormones (Shahin, 1985) at a density of 2×10^5 protoplasts per mL. After preincubation, protoplasts were centrifuged on a sucrose cushion as given above, and viable protoplasts were washed and resuspended in the same incubation medium before exposure to the treatment as indicated. After treatment, protoplasts were centrifuged at 100*g* for 5 min. The pelleted protoplasts were placed immediately in liquid N_2 and stored at −80°C for DNA analysis. For in situ detection of DNA fragmentation, protoplasts were incubated directly on Super Frost Plus microscope slides (Fisher Scientific) inside an Incu-Ring (Polyscientific, Inc., Warrington, PA) at 24°C under continuous light.

Isolation of Nuclear DNA for Fragmentation Analysis

Protoplast incubations were performed at 24°C in TM-2 medium in the presence of the respective toxins or other amendments at the concentrations indicated. Protoplast DNA was isolated first in 100 mM Tris, 50 mM EDTA, 1% SDS, pH 7.5, as the extraction buffer, followed by extraction in phenol, chloroform, and a final ethanol precipitation. After RNase digestion at 100 μg/mL for 1 hr at 37°C, identical amounts of DNA were separated on a 1.5% agarose gel by electrophoresis. Standard leaflet bioassays were performed on *Asc/Asc* and *asc/asc* leaflets, as described earlier (Gilchrist and Grogan, 1976), at 20 μM AAL toxin to induce ~50% cell death on a leaf area basis. Toxin-treated tomato leaf DNA was isolated using the nonphenol method described by Tai and Tanksley (1991), followed by 100 μg/mL Rnase A digestion for 1 hr at 37°C. Agarose gel electrophoresis was performed immediately after DNA isolation. Finally, DNA was transferred onto a Nytran Plus membrane (Scheicher & Schuell) and hybridized with a ^{32}P-labeled tomato genomic DNA probe.

Histochemical Detection of Nuclear DNA Fragmentation and Apoptotic Bodies

For in situ detection of DNA fragmentation, equal volumes of 10% buffered formalin (Fisher Scientific) were added directly to the protoplasts on slides. After drying at room temperature, the fixed protoplasts were washed with TBS buffer (150 mM NaCl, 100 mM Tris-HCl, pH 7.4), digested with proteinase K (10 μg/mL) for 10 min at room temperature, washed with distilled water, briefly rinsed with terminal deoxynucleotidyl transferase (TdT) buffer (25 mM Tris-HCl, 200 mM sodium cacodylate, 5 mM cobalt chloride), and then incubated with the labeling mix (20 units of TdT; Boehringer Mannheim) and 1 nM digoxigenin-11-dUTP (Boehringer Mannheim) in 100 μL of TdT buffer for 1 hr at 37°C. The reaction was terminated by rinsing the section in 300 mM NaCl, 30 mM sodium citrate for 30 min at room temperature and then blocked with TBS buffer containing 2% BSA, 0.3% Triton X-100 for 10 min. The slides were washed three times in TBS buffer and then incubated for 1 hr at room temperature with anti-digoxigenin antibody (Boehringer Mannheim) at a dilution of 1:25 in TBS containing 1% BSA and 0.05% Triton X-100.

After BSA blocking and washing as given above, the fixed protoplasts were incubated with second antibody (anti-goat conjugated with fluorescein isothiocyanate [FITC]; Pierce, Rockford, IL) and diluted 1:25 in TBS with BSA for 2 hr before counterstaining the DNA with Hoechst 33342 (Sigma) at 5 μg/mL for 10 min at room temperature. The slides were viewed with a Nikon SA fluorescence microscope (Nikon, Foster City, CA).

For quantitation of toxin-induced programmed cell death (pcd) in tomato protoplasts, the TdT end-labeling (TUNEL) assay was performed. The anti-digoxigenin antibody was conjugated with alkaline phosphatase (1:1000), and Immuno Pure Fast Red was used as the substrate (Pierce) to label the cells with fragmented DNA. The viability of the protoplasts was determined using fluorescein diacetate stain (Sigma) at 0.01 mg/mL on a Nikon Diaphot TMD inverted microscope. The number of protoplasts that reacted with the respective stain treatments was determined by counting cells within individual fields before and after the counterstain treatment. Each determination was done with at least 3000 protoplasts in each of three independent experiments for each treatment.

Preparation of Whole Tissue and Root Tip Sections

For in situ detection of TUNEL-positive cell death in plant tissues, tomato leaflets, tomato root tips, and onion root tips were fixed in 10% buffered formalin for 20 min under vacuum and then transferred to 0.6 M sucrose and exposed to a vacuum for an additional 20 min. The tissues were then cut into small pieces and embedded in Histo Prep (Fisher Scientific), frozen in liquid N_2, and stored at −80°C before sectioning on a Cyrocut 1800 microtome (Reichet-Jung, Leica, Foster City, CA) at a thickness of 10 μm. After a 5-min postfixation in buffered formalin, the sections were digested with proteinase K (10 μg/mL) for 10 min at room temperature and then subjected to the TUNEL method as described above, except that antibody conjugated with alkaline phosphatase was used rather than FITC because of strong autofluorescence in the tissue sections.

Cautionary Notes When Applying the TUNEL Procedure to Plant Tissues

We found that the TUNEL assay possessed high labeling capacity (Gorczyca et al., 1993) that could easily generate end-labeling artifacts due to inappropriate reagent concentrations or tissue handling. Heavy background and nonspecific staining were seen frequently when high TdT and nucleotide concentrations were used. Substantial reduction of nucleotide and TdT enzyme concentration below that prescribed for staining animal cells enabled strong staining of positive controls in the absence of background interference. As an example of the sensitivity of the assay, it is possible to end-label uncut λ DNA if the reaction is run with excess reagents. We routinely set reaction conditions with uncut λ DNA as a negative control. Excess proteinase K treatment also was found to cause nonspecific staining in tissue sections.

We found that certain approaches to preparation, for sectioning, of plant as well as animal tissue (Grasl-Kraupp et al., 1995) can lead to generation of artifacts and the inability to produce positive control treatments. For example, embedding in either plastic or paraffin, followed by standard fixation, did, in our hands, cause sufficient nicking of nuclear DNA to give a positive TUNEL reaction in the fixed sections. Early in our studies, we noted that approaches similar to those used by Mittler

et al. (1995) caused both treated and control sections to be heavily labeled. Hence, unless DNA counterstaining is used to ensure that healthy DNA is not nicked during processing of the sections, a TUNEL-positive artifact can occur. Clearly, this technique, although useful and reliable, must be conducted under controlled conditions, and appropriate control treatments must be conducted at all times. All experiments reported herein were repeated at least five times, with all results in agreement across the experiments.

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