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Review

Mycotoxins reveal connections between plants and animals in apoptosis and ceramide signaling

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Abstract

Plants undergo programmed cell death during development and disease in contexts that are functionally analogous to apoptosis in animals. Recent studies involving plant cell death induced by mycotoxins, pathogens and lethal mutations along with the cell-autonomous death during development now point to several conserved connections to apoptosis in animals. Morphological markers indicative of apoptosis recently reported in plants include TUNEL positive cells, DNA ladders, Ca^{2+}-activated nucleosomal DNA cleavage, and formation of apoptotic-like bodies that occur in some but not all situations involving ordered cell death. In parallel studies with animal and plant cells treated with sphinganine analog mycotoxins our results indicate that the induction and inhibition of death may be mediated by ceramide-linked signaling systems. The presence and significance of ceramide-linked second messenger systems is well documented in animals but is virtually unknown in plants. Further research will discern the manner in which the important function of programmed cell death is conserved as well as diverged between the two kingdoms.

Keywords: fumonisins; AAL toxins; *Fusarium moniliforme*; *Alternaria alternata* f. sp. *lycopersici*; host-selective toxins; disease resistance

Abbreviations: SAMs, sphinganine analog mycotoxins; FB_1, fumonisin B_1; AAL-TA, AAL toxin TA; HR, hypersensitive response; ROIs, reactive oxygen intermediates

Introduction

Understanding the genetic basis and functional steps of conserved signal transduction pathways that regulate cell homeostasis is a basic research goal in contemporary biology. The practical benefit to be accrued from these studies may include insight into how to design strategies to modify the action of genes or signal responses that disrupt the homeostatic state of complex organisms during development and disease. 'Apoptosis' was coined by J.F.R. Kerr and colleagues in 1972 to describe a process where programmed cell death is an integral requirement for life of the organism (Kerr *et al*, 1972). This term is now used widely to define a series of genetically-ordered physiological and morphological changes that occur during the death of animal cells; the novelty of which is that this death process requires the active participation of the dying cell (Tomei and Cope, 1994). The initially surprising idea that intentional cell death could be an essential control mechanism for multicellular integrity has taken nearly two decades to gain general acceptance. However, a literal explosion of discoveries in laboratories worldwide in the past few years has placed the structure and function of genes, signal molecules and signal transduction pathways regulating apoptosis in the forefront of biological and pharmaceutical research (Danheiser, 1995; Jacobson *et al*, 1997; Vaux and Strasser, 1996; Wyllie, 1995).

Apoptosis is now accepted among animal biologists as a fundamental genetically controlled process that directs cells to commit suicide following transduction of a wide range of inter-, intra- and extracellular signals through multiple converging pathways (Evans, 1993). The functional elements of apoptosis, both genes and signaling events, are conserved from nematodes to insects to humans albeit with subtle differences in the actual mechanisms. The service to the organism is to remove cells that (1) have completed an essential but transient role, (2) are in surplus, (3) develop improperly and (4) have the potential to be deleterious to the organism (Jacobson, 1997). Although frequently co-opted during pathogenesis (Barr and Tomei, 1994; Savill, 1994; Thompson, 1995), the evolutionary significance of apoptosis appears to reside in the timely elimination of specific cell populations as an essential prerequisite for normal development of multicellular organisms (Ameisen, 1996; Vaux *et al*, 1994).

Apoptosis has achieved wide interest among biologists because the functional bases of the genetic connections are rooted in normal development (Camp and Martin, 1996) yet may be co-opted in disease (Savill, 1994). In what may seem at first to be a paradox, apoptosis plays a key role in both degenerative and proliferative diseases. Elucidation of controlling genes and signaling pathways has provided evidence for an evolutionarily conserved link between maladies ranging from Alzheimer's disease (Vito *et al*, 1996) to cancer (Thompson, 1995). It is perhaps intuitive that any process that is highly conserved to maintain order can result in harsh consequences for an organism when circumstances beyond the control of the organism disrupt that order. For example, excessive cell death can lead to impaired development and degenerative diseases, (Savill,

1994) whereas insufficient gene-directed cell death can lead to cancer (Fisher, 1994), permit viral infections (Shen and Shenk, 1995), and contribute to autoimmune diseases (Mountz *et al*, 1996). The genes and signal molecules involved in apoptosis (Wyllie, 1995; Allison and Sarraf, 1992) have become widely sought targets for therapeutic manipulation in both degenerative and proliferative diseases of animals and humans (Thompson, 1995), including diseases of the immune system (Critchfield and Lenardo, 1995). At the level of scientific interactions, the discovery of the existence and role of programmed cell death in healthy and diseased tissue has led to new collaborations among cell biologists, geneticists and pathologists.

Although apoptosis in animals was initially likened to senescence of plant leaves (Kerr *et al*, 1972), evidence of genetic controls and signaling molecules in plants, analogous to those now widely studied in animals, has emerged only recently. This is in spite of the fact that programmed cell death regimes are recognized to occur in numerous situations in plants ranging from the development of vascular elements and floral organs to flower petals undergoing senescence. Like in animals, localized cell death in plants appears to play opposite roles in plant disease where cell death is a disease symptom in both susceptible (Wang *et al*, 1996b) and resistant plant genotypes. With respect to resistance, a classical symptom of pathogen limitation is localized cell death at the site of infection. The term applied to this type of resistance in plants is the hypersensitive response (HR). However, it is not clear whether the role of cell death in the HR is a consequence or a cause of resistance (Morel and Dangl, 1997). There also are many examples of spontaneous cell death in plants where localized lesions occur in the absence of a pathogen that appear to mimic the HR in both appearance and in the biochemical changes that are coincident with death (Jones and Dangl, 1996; Morel and Dangl, 1997). These genetic lesions are controlled by single genetic loci and have been designated as lesion mimic mutations. The following discussion will highlight a few instances in which programmed cell death appears to be associated with specific developmental events, genetic mutations or in association with plant-microbe interactions.

Programmed cell death in plants: occurrence in development, disease and genetic lesions

Programmed cell death regimes in plants are recognized to occur at specific points during development including senescence (Woodson *et al*, 1992; Smart, 1994), pollination (O'Neill *et al*, 1993; Zhang and O'Neil, 1993); tracheary element development (Chasan, 1994; Demura and Fukuda, 1994; Fukuda, 1994) and in the formation of aleurone cells in barley and wheat (Wang *et al*, 1996; Kuo *et al*, 1996). Analogous to apoptosis in animals, cell death may be triggered in response to pathogens (see reviews by Dangl, 1995; Keen, 1990; Lamb, 1994; Mittler *et al*, 1995; Jones and Dangl, 1996; Morel and Dangl, 1997). Recently, morphological evidence of apoptosis including TUNEL positive cells (Gorczyca *et al*, 1993), DNA ladders and apoptotic bodies has been reported in at least three situations: (a) plant development (Wang *et al*, 1996b), (b) disease associated death in susceptibility (Wang *et al*, 1996b) and (c) in response to arachidonic acid, an elicitor of HR (Wang *et al*, 1996b). Both TUNEL positive cells and DNA ladders were reported by Ryerson and Heath (1996) in cowpea leaf cells exhibiting the HR type of resistance to the cowpea rust fungus, *Uromyces vignae*, but not in infected cells from a susceptible cowpea genotype that was successfully invaded by the fungal pathogen.

In addition, appearance of TUNEL staining has been reported in cells that die to form the aleurone layer of barley and wheat grains (Wang *et al*, 1996), a gibberellin induced process that is blocked by okadaic acid (Kuo *et al*, 1996), and in microspore cells during diploid parthenogenesis of Norway spruce (Havel and Durzan, 1996). This suggests that DNA fragmentation, consistent with an apoptotic process, occurs in these situations where death clearly is a programmed and necessary part of normal development of the plant. Several studies by Lam and coworkers have detected the presence of TUNEL positive cells in developing tracheary elements, in cells expressing HR in response to infection by tobacco mosaic virus and following the transgenic expression of a bacterial proton pump (Mittler and Lam, 1995; Mittler *et al*, 1995; reviewed by Mittler and Lam, 1996). In regard to the latter situation, the activation of proton pump ATPase has also been shown to be part of early defense responses of plants to pathogens (Atkinson and Baker, 1989).

While there are a number of recent examples in plants where many of the stereotypical hallmarks of apoptosis have been observed, it also is clear that they have not been detected in all cases where programmed death appears to occur. Several explanations are plausible. First is that the death of specific cells, while programmed, occurs by mechanisms that do not invoke an apoptotic template and therefore the markers will not be present. Second is the fact that programmed cell death in plant tissues, like animals, is initiated in a few cells and the wave of death that may spread into surrounding cells is asynchronous. This results in immense difficulty in detecting common controlling events and a serious dilution of key signal molecules or other markers when extracts are made from a mix of responding and non responding cells. Notice also is taken of the fact that all programmed cell death in animals does not proceed by a strictly apoptotic process nor do all the typical hallmarks appear in all cases where apoptosis is involved (Schwartz *et al*, 1993; Columbano, 1995).

Among the disease-related circumstances in which cell death has been suggested to play a role is the previously mentioned HR that is characteristic of several incompatible plant-microbe interactions. In this case, the moribund cells may eventually be compressed by surrounding healthy cells while losing their form and contents in the process. While this is not mechanistically analogous to phagocytosis, the functional outcome is similar. In other cases where death appears to be programmed, the fate of the cellular contents is unclear but compartmentation of potentially toxic cellular contents into smaller bodies, prior to the loss of membrane integrity, could serve a protective

role. Included among the resistance markers expressed in cells undergoing a HR cell death are the expression of pathogenesis related proteins, phytoalexins, and generation of reactive oxygen intermediates (ROIs). In several studies of inducible host responses to infection by incompatible strains of bacterial and fungal pathogens exhibiting the HR phenotype, the affected host cells show dramatic changes in calcium influx that precede an oxidative burst. Both hydrogen peroxide and superoxide anion have been suggested as factors important in the execution of infected cells by the HR (Baker and Orlandi, 1995; Jabs *et al*, 1996; Levine *et al*, 1994). The sum of extensive research in this area is that, although these markers have been shown to be toxic to certain pathogens *in vitro* and their induction is correlated with resistance *in vivo*, the exact mechanistic role that any or all of them play in resistance is not known. At this point it appears that isolation and characterization of genes conditioning altered phenotypes in the HR or genes that regulate the generation of spontaneous lesions in the absence of pathogens (lesion mimic mutations) are needed to further identify the pathways involved and to dissect the role of plant cell death in resistance.

Plants express lesion mimic mutations at defined genetic loci in many species of commercial plants including, tomato, maize and barley (Wolter *et al*, 1993) as well as the model genetic plant, *Arabidopsis thaliana* (Dietrich *et al*, 1994; Greenberg *et al*, 1994). Similarities between the phenotypes of these and other genetically regulated cell death examples in plants and the apoptotic process in animals has been noted by several authors (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). In the case of several of these genes there appears to be a negative relationship between the expression of the lesion mimic phenotype and susceptibility to several different pathogens (Morel and Dangl, 1997). In each of these cases resistance, to at least some pathogens, seems to be enhanced in plants capable of expressing genetic lesions that resemble a wild-type resistance response. The Arabidopsis lesion mimic mutant *lsd1*, in fact, shows both enhanced expression of disease resistance markers and increased resistance to bacterial and oomycete pathogens when spontaneous lesions are absent (Dietrich *et al*, 1994). This suggests that the *lsd1* mutation may condition a constitutively heightened responsiveness to exogenous stress including pathogens.

A second mutation in Arabidopsis, *acd2*, developed spontaneous lesions in the absence of pathogens and expressed an HR phenotype in otherwise healthy areas of the plant when distal tissues were inoculated with either virulent (cause disease) or avirulent (trigger HR) bacterial pathogens (Greenberg *et al*, 1994). In contrast, wild-type plants lacking these recessive mutations express the HR phenotype only when inoculated with avirulent strains of the pathogen. Barley plants expressing the recessive *mlo* alleles that condition resistance to the obligate fungal pathogen that causes powdery mildew, *Erysiphe graminus* f. sp. *hordei*, sporadically show spontaneous formation of lesions in the absence of the pathogen when subjected to environmental stress such as low temperature (Wolter *et al*, 1993). While a number of defense-related gene products, including pathogenesis related proteins and phytoalexins, have been detected in these mutant backgrounds when lesions are expressed, the mechanistic relationship between lesion mimic genes and host resistance is unclear.

With respect to lesion mimic mutants and oxidative bursts, the recent map-based cloning of the *LSD1* gene adds an exciting dimension to the death process initiated by lesion mimic genes (Dietrich *et al*, 1997). In this case it appears that reactive oxygen intermediates do play a role in mediating the response associated with the alleles of the lesion mimic mutation. The predicted LSD1 protein indicates the presence of three zinc finger domains that is indicative of a DNA binding function. In addition, this study suggests that *LSD1* is linked to a superoxide-dependent pathway and negatively regulates a plant cell death pathway at the level of transcription. In the plants carrying the *lsd1* mutation, the presence of superoxide is a necessary and sufficient signal for cell death (Dietrich *et al*, 1997; Morel and Dangl, 1997). The mechanism by which the gene functions could involve either the repression of a prodeath or the activation of an antideath pathway. For additional discussion of lesion mimic genes and the HR in relation to programmed cell death and disease the reader is referred to recent reviews by Dangl *et al*, (1996), Jones and Dangl (1996) and Morel and Dangl (1997).

The sum of the studies in plants is that the appearance of the morphological features of apoptosis, in contexts analogous to its appearance in animals, indicates that at least the terminal steps in the process that condition these stereotypical hallmarks are conserved in plants. Triggers of cell death like the sphinganine analog mycotoxins and arachidonic acid that induce apoptosis in animal cells lead to expression of the same apoptotic markers in sensitive plant cells. (Wang *et al*, 1996a,b) These observations suggest that at least portions of common signaling pathways and regulatory genes are conserved in both animals and plants. However, the extent to which other signaling pathways and functional homologs of the controlling genetic elements of programmed cell death regimes are conserved in plants is unresolved.

As indicated earlier, no genes regulating programmed cell death in plants with functional homologies to apoptotic genes in animals have been isolated and shown to function in analogous pathways in the reciprocal hosts from the two kingdoms. However, *dad1* (*d*efender against *a*poptotic *d*eath) from *C. elegans* and humans has highly conserved homologs in plants (Sugimoto *et al*, 1995). The human *dad1* gene functions in *C. elegans*. It has now been reported that the plant homolog from rice is functionally active in animal cells and can rescue mutant hamster cells lacking *dad1* (Tanaka *et al*, 1997). With respect to other conserved domains in cell death-associated plant genes, the recent cloning of the N gene from tobacco encoding HR resistance against tobacco mosaic virus provides another interesting observation (Whitham *et al*, 1994). Sequence analysis of the N gene showed that it encoded a 131.4 kDa protein with an amino-terminal domain similar to that of the cytoplasmic domain of the Drosophila Toll protein and the

interleukin-1 receptor in mammals (Belvin and Anderson, 1996). Although no functional connection between this domain and an apoptotic signaling pathway has been reported, the link between interleukins, the interleukin converting enzyme (ICE) and integral role of ICE and ICE-like proteases in apoptosis suggests that the presence of this domain in N gene-expressing plants may link to a death program.

Ceramide signaling and apoptosis in animals and plants

Signal transduction involving ceramide and sphingoid bases has emerged as an important lipid-based second messenger system in development, cell proliferation and degenerative diseases in animals through regulating a diversity of responses from gene expression to kinase cascades (Chao, 1995; Merrill *et al*, 1996, 1997; Obeid and Hannun, 1995; Pena *et al*, 1997). The linkage of ceramide signaling to apoptosis, cancer, and degenerative disease in animals is now driving a rapidly emerging novel area in signal transduction in animal biology (Hannun, 1996). The reader is directed to a recent review by Merrill *et al* (1997) which summarizes the current state of knowledge on sphingolipids and the myriad of functions that they may serve in regulating life and death of cells. Results from our laboratory and others suggest that plants also may share a common connection to apoptosis through ceramide-based signaling pathways.

Sphinganine analog mycotoxins (SAMs)

We have been studying host-selective phytotoxins as triggers of physiological cell death in disease for several years. Phytotoxins produced by the fungus *Alternaria alternata* f. sp. *lycopersici*, collectively known as AAL toxins, were described initially as host-selective determinants of the Alternaria stem canker disease of tomato (Gilchrist and Grogan, 1976). Only strains of the pathogen that produce the toxins are pathogenic on tomato and only cultivars of tomato homozygous for the recessive alleles at the Asc locus are sensitive to the toxins and susceptible to the pathogenic strains (Clouse and Gilchrist, 1986). The toxin-induced symptoms in *asc/asc* tomato leaves consist of localized interveinal death of cells that, at threshold toxic concentrations, appear as distinct dark spots. Structural characterization of AAL toxins TA and TB indicates that each of the toxins are synthesized as a pair of regioisomers of 1,2,3 propanetricarboxylic acid (tricarballylic acid) esterified to 1-amino-11,15-dimethylheptadeca-2,4,5,13,14-pentol (TA) (Figure 1) and 1-amino-11,15-dimethylheptadeca-2,4,13,14-tetraol (TB) (Bottini and Gilchrist, 1981; Bottini *et al*, 1981). The most prevalent and toxic pair of regioisomers (Figure 1) is designated AAL toxin TA (AAL-TA). Subsequently, three additional sets of regioisomers were identified in culture filtrates of the pathogen including two sets of isomers that lack detectable biological activity (Caldas *et al*, 1994).

Initially, interest in this class of toxins was limited to the role of AAL toxins in the stem canker disease of tomato. However, research on this class of toxins has broadened greatly in the past few years following reports from South

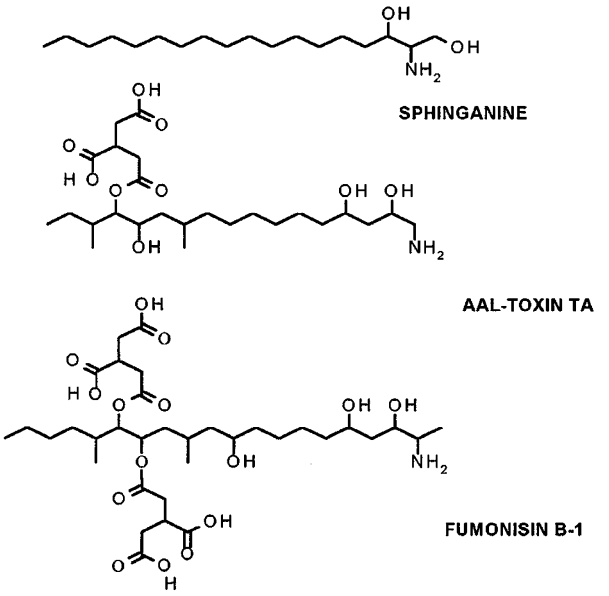

Figure 1 Relationship between sphinganine and representative sphinganine analog mycotoxins

Africa that structural analogs of the AAL toxins produced by *Fusarium moniliforme*, a common pathogen of maize, were toxic to animals. Such toxins fall into the class of fungal secreted metabolites referred to as mycotoxins. These new mycotoxins, characterized as bistricarballylic aminopolyol esters (Figure 1), were given the trivial name of fumonisins. Like the AAL toxins, the fumonisins are comprised of a family of structural congeners with differing levels of toxic activity (Bezuidenhout *et al*, 1988). Consumption of maize colonized by *F. moniliforme* was linked first to leukoencephalomalacia, a fatal disease of horses (Marasas *et al*, 1988a; Gelderblom *et al*, 1988a), and subsequently to esophageal cancer in humans (Gelderblom *et al*, 1988b; Marasas *et al*, 1988b; Sydenham *et al*, 1990). The most prevalent isoform, fumonisin B_1 (FB_1), is also the most toxic. In studies where they have been compared directly, the AAL toxins and fumonisins share similar biological properties: both are effective inhibitors of ceramide synthase in animal cells (Merrill *et al*, 1993), both inhibit cell proliferation in rat liver and dog kidney cells (Mirocha *et al*, 1992), and both induce cell death in tomato (Abbas *et al*, 1994; Gilchrist *et al*, 1992; Moussatos *et al*, 1993; Lamprecht *et al*, 1994; Wang *et al*, 1996b) and African green monkey kidney (CV-1) cells (Huang *et al*, 1995; Wang *et al*, 1996b).

Both *A. alternata* and *F. moniliforme* are ubiquitous saprophytes as well as economically important pathogens of many food crops. *F. moniliforme* causes both stalk and ear rot of maize worldwide. Fumonisin contamination has been detected in substantial amounts ($\geqslant$2.7 p.p.m.) in 74% of US corn and is found in all areas of the world where corn is produced, with levels ranging as high as 1200 p.p.m. or greater (Nelson *et al*, 1993; Sydenham *et al*, 1991).

Ingestion of fumonisin through contaminated feed, or as pure FB_1, has been shown to induce a variety of responses in the challenged animals including neuro-, renal- or hepatoxicosis and neoplasms as well as cell death (Gelderblom *et al*, 1988a,b, 1992; Marasas *et al*, 1988a; Merrill *et al*, 1996; Nelson *et al*, 1993; Tolleson *et al*, 1996). Because of the frequency and level of contamination of maize and the toxicity of fumonisin to animals, serious concern exists over the dual roles involving proliferation and apoptosis these mycotoxins may play in animal and human disease. As will be discussed in the following sections, part of this concern arises from emerging information linking their biological activity to apoptosis in animal cells.

Connection of ceramide signaling to toxicity of SAMs and apoptosis

In concurrent studies with animal and plant cells, we observed stereotypical hallmarks of apoptosis in tomato cells (Wang *et al*, 1996a) and in African green monkey kidney (CV-1) cells (Wang *et al*, 1996b) exposed to either FB_1 or AAL-TA. The induction of cell death in both animal and plant cells occurred at similar toxin concentrations (10–50 nm) and time frames (12–24 h). Morphological markers indicative of apoptosis in the tomato and the CV-1 cells included TUNEL positive cells, DNA ladders, Ca^{2+}-activated nucleosomal DNA cleavage, and formation of apoptotic-like bodies (Figure 3). In addition, these markers also were found in onion root cap cells undergoing death associated with the natural and continuous sloughing of these cells as the root tip elongates (Wang *et al*, 1996b). The fact that the SAMs bear structural relationships to sphinganine and sphingosine suggested a connection to sphingolipids. Several reports now link the affects of the fumonisins on animals and cultured animal cells to altered sphingolipid metabolism (reviewed by Merrill *et al*, 1996, 1997; Yoo *et al*, 1994).

Current information indicates that the triggering of cell death by SAMs is linked in plants to a concerted effect on sphingolipid synthesis resulting in rapid and dramatic changes in sphingoid bases and ceramide (Abbas *et al*, 1994). While the existence and role of ceramide-based signaling in plant cells is virtually unknown, our ongoing studies with animal and plant cells responding to diverse stimuli that affect apoptosis suggest that lipid-based second messengers may play a substantial, and heretofore unrecognized, role in plants (H Wang, unpublished). Sphingolipid synthesis in plants occurs by the same pathway as in animals but, by comparison, sparingly little is known about the enzymes involved, their kinetic properties, regulatory mechanisms, or intracellular location in plants (reviewed in Lynch, 1993). Even less is known about ceramides in plants although it has been reported that 17% of the lipids in the tonoplast of mung bean consists of ceramide monohexoside (Yoshida and Uimura, 1986) and that leaves of 22 plant species contain cerebrosides in both the tonoplast and plasma membrane (Imai *et al*, 1997).

Until recently, little was known about the mechanism by which SAMs induce toxigenic or carcinogenic effects. The structural relationship to sphinganine, an intermediate in the biosynthesis of the sphingosine backbone of more complex sphingolipids such as ceramides, sphingomyelin cerebrosides, gangliosides, and sulfatides, is the basis for current models linking mode of action to alterations in the synthesis of sphingoid bases. There is considerable evidence now that ingestion of SAMs leads to disruption of sphingolipid metabolism (summarized in Merrill *et al*, 1996) and it is clear that one site of metabolic disruption is the reaction catalyzed by sphingosine N-acetyltransferase (ceramide synthase) (Figure 2). FB_1 is a potent competitive inhibitor of ceramide synthase from liver and brain microsomes as well as in several mammalian cell lines (Wang *et al*, 1991; Merrill *et al*, 1993). Both FB_1 and AAL inhibit ceramide synthase in rat hepatocytes (Merrill *et al*, 1993), and in microsomal preparations from green tomato fruit (Gilchrist *et al*, 1995). An increase in sphinganine, consistent with inhibition of ceramide synthase, is commonly observed following exposure to SAMs as are increases in sphingosine-1-phosphate (Merrill *et al*, 1995, 1996). Inhibition of ceramide synthase in animal cells by both fumonisins and AAL-toxins (Wang *et al*, 1991; Merrill *et al*, 1993) suggested a molecular target for the molecules in animals leading to perturbation of biosynthesis of sphingoid bases and potential changes in production of ceramides. Physiological studies have confirmed that animals fed fumonisins at toxic concentrations show changes in levels of sphinganine and sphingosine consistent with *in vivo* inhibition of ceramide synthase (Wang *et al*, 1992). Similar observations have been reported for toxin-treated plant cells (Abbas *et al*, 1994). We also have confirmed that ceramide synthase activity in microsomal preparations from

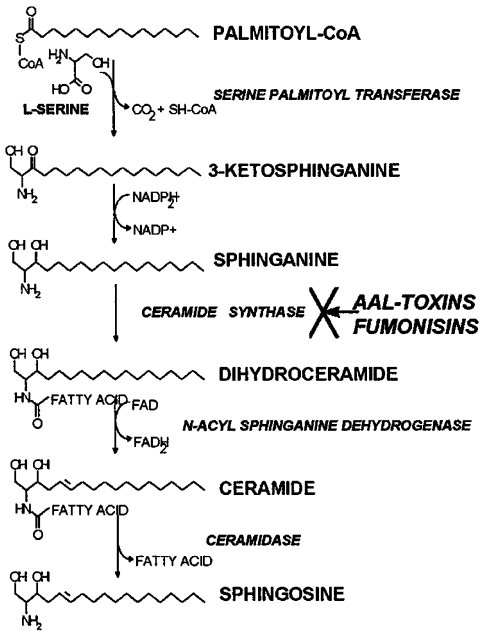

Figure 2 Sphingolipid biosynthetic pathway in plants and animals. AAL toxins and fumonisins inhibit sphingolipid synthesis at the step catalyzed by ceramide synthase

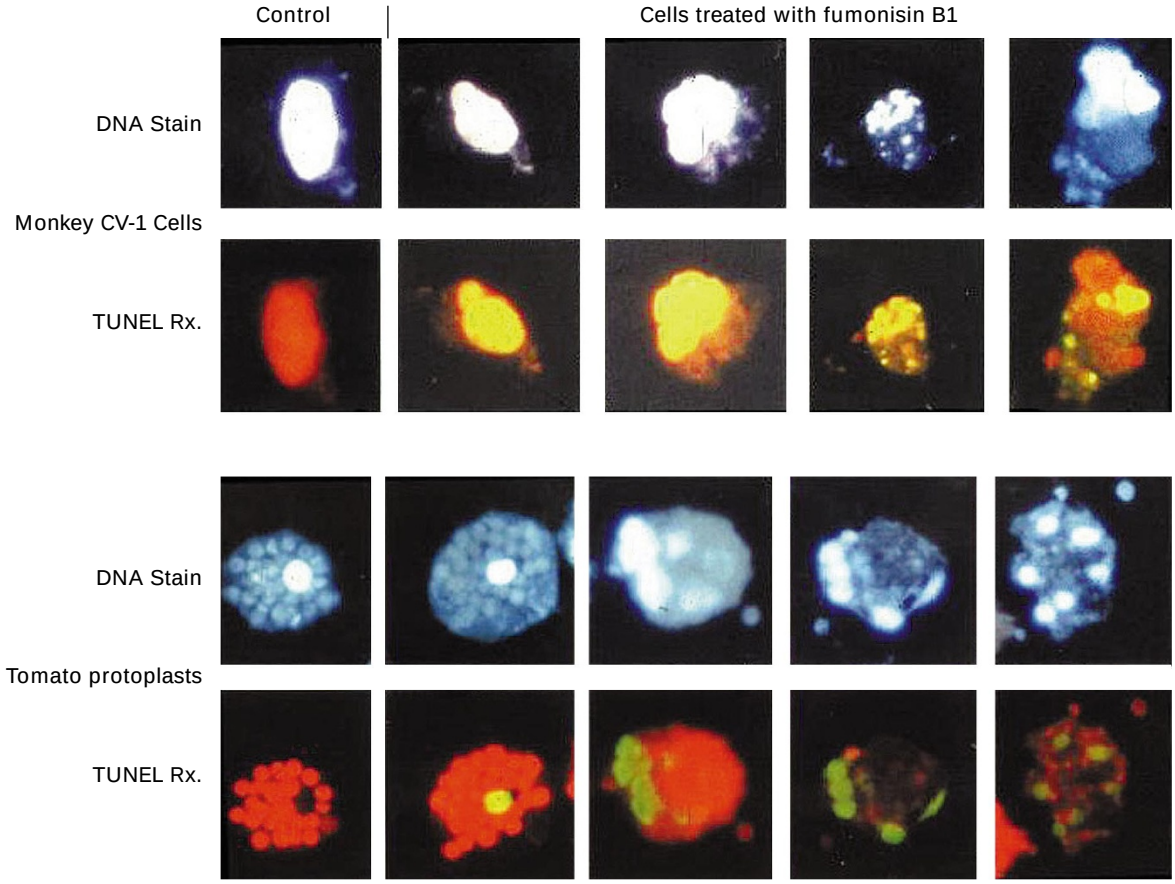

Figure 3 Comparison of the nuclear changes observed in African green monkey kidney cells (CV-1) and tomato leaf protoplasts after treatment with 50 nM fumonisin B_1. The cells were sequentially stained with Hoechst 33342 to determine the location and form of nuclear DNA and then subjected to the TUNEL reaction to detect fragmentation of the nuclear DNA. The red fluorescence in the tomato protoplasts is the autofluorescence of chlorophyll. These two plates appeared separately in Wang *et al*, 1996a and b and are presented here for direct comparison of the progression of nuclear changes, including the formation of apoptotic bodies, in the animal and plant cells respectively

green tomato fruit and leaf tissue is inhibited by purified AAL-TA and FB_1 (H Wang and D Gilchrist, unpublished). In tomato there was a significant and equivalent inhibition of the enzyme at 20 nmolar with an IC_{50} in the range of 35–40 nmolar for both AAL-TA and FB_1. In plants, fumonisin B_1 induces cell death at nm concentrations in *asc/asc* (susceptible) but not *Asc/Asc* (resistant) tomato tissues and protoplasts (Gilchrist *et al*, 1992).

FB_1 also is reported to repress expression of two isoforms of protein kinase C (PKC) (α and θ) in CV-1 cells and inhibit transcription by activator protein 1 (AP-1) tenfold within 3 h after toxin treatment. In contrast FB_1 stimulated transcription from a promoter containing a cAMP response element (Huang *et al*, 1995). Repression of PKC under conditions where sphinganine accumulates may be related to the fact that sphingoid bases are potent inhibitors of PKC. Disruption of cell cycle progression is frequently associated with cells undergoing apoptosis (King and Cidlowski, 1995; Meikrantz and Schlegel, 1995). Treatment of CV-1 cells with FB_1 resulted in G1 arrest. Moreover, CV-1 cells transformed by the simian virus 40 (SV40) large T-antigen (COS-7) were unaffected by the same levels of FB_1 or AAL-TA that kill CV-1 cells (Wang *et al*, 1996a). Since large T-antigen has pleiotropic effects on cell cycle regulation and induction of apoptosis (Fanning and Knippers, 1992), large T-antigen may overcome the antiproliferative or apoptotic properties of FB_1. Taken together, these results are consistent with an effect of the toxins on regulation of cell homeostasis through the apoptotic process that involves ceramide signaling and disruption of the cell cycle.

In spite of the elegant *in vitro* and *in vivo* studies on the effects of SAMs on ceramide synthase and demonstration of altered levels of related metabolites in animals treated with fumonisins by Merrill's group (reviewed in Merrill *et al*, 1996, 1997), it is not clear how, or whether, disruption of this step leads directly to the symptoms associated with ingestion of the SAMs. It remains possible that these toxins could act directly on ceramide signaling pathways coupled to apoptotic programs. A number of possibilities involving ceramide-linked signaling pathways are now being intensely studied in a number of systems as critical second messengers regulating cellular homeostasis (Kolesnick and Fuks, 1995; Hannun, 1996; Hannun and Obeid, 1995). For

example, sphingolipids have been implicated as playing a direct role in cell contact, growth, and differentiation as well as influencing the proliferative potential of mammalian cells by induction or suppression of apoptosis (reviewed in Hannun, 1996). FB_1 alters cell morphology, cell-cell interactions, the behavior of cell surface proteins, protein kinase activity and cell growth and viability (as reviewed by Merrill *et al*, 1996, 1997). Since fumonisin and AAL toxins resemble sphingoid bases, these toxins could alter cell homeostasis leading to either death or carcinogenesis by inducing or suppressing apoptosis through mechanisms affecting the levels of lipid-derived second messengers in addition to inhibition of ceramide synthesis.

In summary, there now are many reports in the literature indicating that ceramides and sphingosine derivatives are potent second messengers which trigger apoptosis or programmed cell death in animals and regulate other physiological processes (see review by Hannun, 1996). It appears that plants also may utilize ceramide-linked signaling systems, at least in some stress responses as noted above. In the context of connections between ceramide intermediates and the superoxide anion, a rapid Ca^{2+} influx has been noted in plant cells undergoing cell death in the HR and in lesion mimics (Hammond-Kosack and Jones, 1996). Conversely, N-acetylsphingosine (C-2 ceramide) inhibits both effects in neutrophils (Wong *et al*, 1995) and suppresses FB_1 induced cell death in tomato protoplasts (H Wang, unpublished). While the complexity of cellular responses observed and the direct connections between toxicity of these mycotoxins and ceramide-mediated responses is unresolved, the SAMs should be useful tools for deciphering the role of ceramides in development and disease.

Level of parasitism and consequences of plant cell death to pathogens

The molecular and biochemical events that occur at the site of infections in plants include a plethora of changes that are triggered by both host and pathogen genes (Morel and Dangl, 1997). The biochemical range of plant response to pathogens exhibits similarities to those associated with apoptosis in animals. With respect to the nutritional and growth requirements of the pathogen, the consequences of an apoptosis-like induction or suppression of host cell death during the early phases of infection could determine the eventual outcome (Gilchrist *et al*, 1995; Wang *et al*, 1996b) of disease for both obligate and nonobligate parasites. Facultative parasites would be able to use the contents of dead cells as a nutrient source and thus benefit from host cell death. In contrast, the early death of host cells, as in the classical HR, could serve to limit infection by obligate parasites deprived of a living host cell in which to either multiply (viruses) or from which to extract nutrients during growth of the pathogen in infected tissues (rusts). Reference was made earlier to the studies of Ryerson and Heath (1996) where several morphological hallmarks of apoptosis were observed in an incompatible interaction between a rust fungus and its host.

Conversely, active suppression of default programs to eliminate cells undergoing pathogen-induced stress (suppression of apoptosis) is a formal possibility in plant-microbe interactions involving compatible obligate parasites including viruses, fungi and plant parasitic nematodes as well as symbiotic interactions as with Rhizobia and endomychorrizal fungi. The existence and role of both exogenous and endogenous inhibitors of apoptosis (IAPs) is well documented in animals (Shen and Shenk, 1995). For example, many animal viruses encode IAPs such as p35 from baculovirus and CrmA from cowpox virus that serve to block apoptosis during viral replication (Clem and Miller, 1994; Ray and Pickup, 1996). No evidence has been reported for IAPs in plants or pathogens but the formal possibility remains since the issue seems to be unexplored.

In the case of nonobligate parasites, the general result of infection is death of tissues during colonization. Both bacterial and fungal pathogens that are not obligately parasitic secrete toxic substances that are either necessary for infection or facilitate infection by causing cell death in advance of the growth of the pathogen through the affected tissue. Physiological changes in the toxin-stimulated cells could trigger default genetic programs leading to cell death. Except for the fumonisins and AAL toxins, no studies have been reported that directly test this hypothesis.

Another established line of information suggesting that a programmed cell death mechanism like apoptosis may be invoked during infection is that death during disease of plants does require new gene expression, especially in the case of resistance (Dixon *et al*, 1994). As indicated earlier, the class of mutants known as lesion mimic mutants exhibiting phenotypes that resemble lesions caused by pathogens may share functions in common with HR-coupled cell death (Morel and Dangl, 1997). One or more lesion mimic mutants have been detected in most cultivated plants and many appear to be under the control of a large number of loci. Whether the mutations that give rise to these lesions are in pathways that determine cell death in the HR response or in lesions occurring in compatible plant-microbe interactions is not known. However, in the case of the *lsd* and *acd* mutations in Arabidopsis, there is a corresponding increase in the expression of a number of defense-related genes in plants expressing the lesion mimic phenotype and an increased resistance to some pathogens (Dietrich *et al*, 1994; Jones and Dangl, 1995; Morel and Dangl, 1997).

A role for apoptosis in plants: development and disease

Analogous to animals, the confirmation, characterization and manipulation of a gene expression-dependent cell death process in plants will require linkage to specific genes, sets of interacting genes, or gene products that respond to the signals transduced by a range of stimuli. Cloning of genes in plants functionally homologous to those shown to affect apoptosis in animals will be necessary to further resolve the similarities and the differences in the two kingdoms. This may not be simple even if major portions of the apoptotic process are conserved. Homology at the DNA level between genes that will compliment loss of function mutants in evolutionarily divergent species may be as little as 20%. In addition, the

dilution of expressed messages in small sub-populations of asynchronously responding plant cells is a potential hurdle to both cloning and characterizing the putative apoptotic genes. For expressed genes, the number of cells in an organized tissue undergoing programmed cell death at any point in time may be small compared to the total cell mass; the process may be asynchronous; and the number of cells that actually complete the stereotypical steps of apoptosis prior to metabolic collapse may represent only a fraction of the total.

Whether the full template or only portions of the animal-resolved program occur in plant cells that are programmed to die is unclear at this time. Since the final morphological characteristics of apoptosis do occur in plants and complimentary signal responses appear also to be conserved in specific situations, it is likely that many of the functional elements will be similar. Regardless of the extent of similarity, it is likely that the genes and signal molecules that define programmed cell death in plants will become important targets for both understanding and manipulating this process in development and disease.

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