Bcl-2 family members localize to tobacco chloroplasts and inhibit programmed cell death induced by chloroplast-targeted herbicides

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Abstract
In mammalian cells, apoptosis is often mediated via organelles. While apoptotic-like cell death occurs in plants, the mechanistic details are unresolved. Transgenic tobacco plants have been generated that harbour selected animal anti-apoptotic genes. Subcellular fractionation followed by western blot analysis indicated that chloroplasts serve as a location for these animal anti-apoptotic proteins in addition to the established mitochondrial location. To explore the functional significance of this observation, tobacco plants were treated with three chloroplast-directed herbicides. Wild-type plants died and exhibited features associated with apoptosis. Transgenic plants survived and did not show any apoptotic-like characteristics. Moreover, the herbicide-induced apoptotic-like cell death was light requiring. It was concluded that chloroplasts may be involved in mediating certain types of plant programmed cell death.

Key words: Apoptosis, Bcl-2, chloroplast, herbicide.

Introduction
In mammalian cells, programmed cell death (PCD) generally proceeds by one of two signalling pathways; intrinsic or extrinsic. In the latter, induction of apoptosis is mediated by extracellular receptors; binding of death ligands to these specialized death receptors (e.g. FAS, TNF) causes receptor oligimerization, recruitment of adaptor molecules, and activation of initiator caspases. Once activated, these upstream caspases activate downstream effector caspases which are involved in the execution of the cell (Earnshaw et al., 1999). The intrinsic pathway is organelar; generally mediated by the mitochondrion, whereby following apoptotic stimulation, cytochrome c (and other apoptotic factors) are released. Cytochrome c and ATP or dATP form an oligomeric complex with the cytosolic adaptor protein, Apaf-1. Together, these proteins form a large multimeric complex termed the apoptosome which recruits and activates caspase 9, which is then released and processes the downstream executioner caspases 3, 6, and/or 7 (Ferri and Kroemer, 2001). PCD is characterized by a number of hallmark features including cell shrinkage, plasma membrane blebbing, nuclear condensation, internucleosomal DNA cleavage, and externalization of plasma membrane phosphatidylserine, which lead to fragmentation and coalescing of DNA into apoptotic bodies that migrate to the periphery of the cell for eventual phagocytosis by neighbouring macrophages. Thus, during the PCD process, unwanted cells are cleanly removed without inflammation. It is now well established that inappropriate cell death (too much, too little) significantly contributes to a number of important mammalian diseases (e.g. cancer, AIDS, stroke) (Cory and Adams, 2002).

In plants, PCD plays a normal physiological role in a variety of developmental processes including xylem formation, senescence, sloughing of root cap cells, and embryogenesis, among others (reviewed in Dickman and Reed, 2003). Plant cell death in response to pathogen challenge and in response to abiotic stresses has also been documented and, at least in some cases, is clearly genetically programmed (Li and Dickman, 2004; Navarre and Wolpert, 1999; Dickman et al., 2001). A question that has arisen is whether plant PCD and mammalian PCD share similar mechanistic features. A primary argument against this idea stems from the fact that homologues of the core machinery of apoptotic regulators (Bcl-2 family, caspases) have not been identified in plants, at either the sequence or functional levels. Although the biochemical mechanisms responsible for cell suicide in
plants are largely unknown, an increasing number of reports suggest similarities to the programmed cell death that occurs in animal species. For example, PCD in plants typically requires new gene expression, and thus can be suppressed by cycloheximide and similar inhibitors of protein or RNA synthesis (Havel and Durzan, 1996). The morphological characteristics of plant cells undergoing PCD also bear some striking similarities to apoptosis in animals, although the presence of a cell wall around plant cells imposes certain differences. Akin to animal cells, PCD in plants is associated with internucleosomal DNA fragmentation (DNA ladders) and the activation of proteases (Ryerson and Heath, 1996; Stein and Hansen, 1999; Solomon et al., 1999). For instance, genes encoding cysteine proteases are induced during tracheary element development in Arabidopsis and tomato plants (Groover and Jones, 1999; Nam, 1997; Fukuda, 1996). It should be noted, however, that PCD processes in plants do not always exhibit these hallmark characteristics (Heath, 1998).

In addition to its role in developmental processes in plants, cell suicide plays an important role in the interactions of plants with pathogens, including bacteria, fungi, and viruses. One of the best studied of plant responses to pathogens is the hypersensitive response (HR). Upon exposure to certain pathogens, plant cells in the immediately affected area undergo a rapid cell suicide response that results in cell death at and near the site of infection, thereby limiting spread of pathogens (Dangl and Jones, 2001). The HR is associated with the expression of a variety of plant defence genes and the induction of programmed cell death. The HR is usually preceded by rapid and transient responses including ion fluxes, alterations in protein phosphorylation patterns, pH changes, changes in membrane potential, release of reactive oxygen species (ROS; oxidative burst), and oxidative cross-linking of plant cell wall proteins (Richberg et al., 1998; Bolwell and Wojtaszek, 1997). HR appears to be coupled to cytosine protease expression akin to animal PCD although it is unclear whether the analogy extends to caspase-like proteases. Parallels with the animal cell death machinery have been suggested by reports that (a) the HR induced by tobacco mosaic virus (TMV) in tobacco plants is associated with the generation of caspase-like protease activity; and (b) caspase-inhibitory peptides can block bacteria-induced PCD in Arabidopsis without significantly affecting the induction of HR-associated defence genes (del Pozo and Lam, 1998).

Previously, it has been shown that transgenic expression of anti-apoptotic Bcl-2 family members (Bcl-2, Bcl-XL, CED-9, Op-IAP) in tobacco plants conferred heritable disease resistance to several necrotrophic fungi, including Sclerotinia sclerotiorum and Botrytis cinerea (Dickman et al., 2001). Hallmark features of apoptosis have also been observed in plants that are sensitive to toxin-producing necrotrophic fungi, including Fusarium moniliforme (fumonisin), Alternaria alternata (AAL toxin), and Cochliobolus victoriae (victorin) (Navarre and Wolpert, 1999; Wang et al., 1996). When S. sclerotiorum was inoculated to wild-type tobacco, DNA fragmentation was observed in the form of a characteristic ‘ladder’ and by terminal deoxyribonucleotide transferase-mediated dUTP end labelling (TUNEL) of DNA 3’-OH groups, both common features of apoptotic responses. Importantly, when transgenic plants were inoculated with S. sclerotiorum, not only were the plants resistant, but there was no ladder nor were there any TUNEL positive cells. Wild-type and transgenic plant responses to selected abiotic stresses, including heat, cold, salt, drought, and oxidative stress, have recently been evaluated (Awada et al., 2003; Li and Dickman, 2004). Transgenic plants were protected from lethal levels of these stresses and sensitive wild-type tobacco during the death process exhibited features associated with mammalian apoptosis during the death process. Thus, at least in some cases, abiotic stress-induced cell death in plants can be accompanied by apoptotic-like features that are inhibited by expression of Bcl-2. These observations add to the growing body of evidence indicating transkingdom conservation of programmed cell death mechanisms.

In the following, the subcellular localization of the animal anti-apoptotic proteins in tobacco is described. It is shown that Bcl-2, Bcl-xL, and CED-9, not only localize to the mitochondrial and nuclear fractions as in mammals, but also to chloroplast membranes. The localization to chloroplasts may have functional significance. Selected chloroplast-directed herbicides killed tobacco cells in an apoptotic-like, light-requiring manner, but transgenic tobacco expressing anti-apoptotic genes survived and did not exhibit apoptotic characteristics. Taken together, these data suggest that under conditions of oxidative stress, chloroplasts can mediate plant apoptosis.

Materials and methods

Plasmid construction and plant transformation

Separate binary plasmid vectors containing human Bcl-2: Bcl-2ΔBH4; chicken Bcl-xL, and Caenorhabditis elegans CED-9 were constructed and introduced into wild-type tobacco (Nicotiana tabacum cv. Glurk NN) as described previously (Dickman et al., 2001).

Reagents

Bcl-2 and Bcl-xL antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). CED-9 antibody was kindly provided by Dr Robert Horvitz (MIT, Cambridge, MA, USA), the tobacco Rubisco large (RBSL) subunit antibody was obtained from Agrisera (Viannya, Sweden), PEP carboxylase (PEPC) antibody was a gift from Dr Ray Chollet (University of Nebraska, USA), and plant perin antibody was obtained from Dr Tom Elthon (University of Nebraska, USA). Methyl viologen (paraquat) was obtained from Sigma (St Louis, MO, USA), acifluorfen from Riedel-de Haen (Sezde, Germany), and sulfentrazone from Du Pont (Wilmington, DE, USA).

Chloroplast and mitochondria isolation

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Subcellular fractionation of transgenic tobacco lines harbouring animal anti-apoptotic genes followed by western blotting was performed to determine the cellular location of selected Bcl-2 family anti-apoptotic proteins. The purity of each preparation was confirmed by immunoblotting with the following specific markers: PEP carboxylase for cytoplasm; maize porin for mitochondria, and the tobacco RBSL for the chloroplast (Fig. 1A). These studies indicated that all three selected transgenes (Bcl-2, Bcl-xL, CED-9) localize not only to both mitochondrial and cytosolic fractions, as might be expected, but also to the chloroplast (Fig. 1B).

These anti-apoptotic proteins all contain transmembrane binding domains, thus, the organellar associations are not entirely surprising. Mitochondrial localization of Bax, a Bcl-2 family member, has been shown to occur in tobacco (Lacombe and Santa Cruz, 1999); thus there was particular interest in determining whether chloroplast localization was of functional significance. To address this question, antibodies were used whose mode of action is coupled to impairment of chloroplast function. Biperidyl herbicides such as methyl viologen (MV) or paraquat, are redox-active contact compounds that become reduced within the cell and subsequently catalyse the photoreduction of O₂ forming O₂⁻ and H₂O₂ (Asada and Takahashi, 1987; Halliwell and Gutteridge, 1989). Herbicide activity is light-dependent, and the ROS that is generated is lethal to the plant cell. The diphenyl-ether herbicide acifluorfen is an inhibitor of protoporphyrinogen oxidase (PPO). PPO is a chloroplast enzyme that oxidizes protoporphyrinogen to produce protoporphyrin IX. This product is a precursor for both chlorophyll (photosynthesis) and haem (electron transfer). When PPO is inhibited, substrate accumulation occurs and,

Subcellular localization of animal anti-apoptotic proteins

Preweighed leaf discs were ground in 0.5 M sucrose, 0.1% ascorbic acid, 0.1% cysteine-HCl, and 0.1 M TRIS-HCl, pH 7.5. Cleared preincubated in the dark for 1 h, and incubated at 25 °C under continuous light conditions (300 μmol m⁻² s⁻¹) for 48 h. Following treatment, discs were visually inspected and chlorophyll concentrations were determined (Arnon, 1949).

Analysis of apoptotic markers

To determine whether DNA laddering occurred, genomic DNA was extracted from treated plant samples and run on 2% agarose gel as described by White and Kaper (1989). DNA fragmentation in situ was detected by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labelling (TUNEL) with fluorescein isothiocyanate (FITC)-conjugated dUTP (in situ cell death detection kit, Roche Biochemicals). Following TUNEL, sections were counterstained with propidium iodide and examined using a Bio-Rad MRC1024ES confocal microscope (Hercules, CA, USA). Confocal images of the FITC- or PI-labelled signals were collected simultaneously using a dual-line excitation/emission mode (488/520 nm for FITC and 560/598 nm for PI) using the Bio-Rad LaserSharp imaging program.

Results

Subcellular fractionation of transgenic tobacco lines harbouring animal anti-apoptotic genes followed by western blotting was performed to determine the cellular location of selected Bcl-2 family anti-apoptotic proteins. The purity of each preparation was confirmed by immunoblotting with the

Chloroplast involvement in PCD

Fig. 1. Subcellular localization of Bcl-2 family proteins in tobacco. (A) Purity of mitochondria (Mit) and chloroplast (Chl) fractions from transgenic tobacco plants were confirmed by immunoblotting with selective antibodies PEP, RBSL, and porin. (B) Purified mitochondria (Mit) and chloroplasts (Chl) from transgenic tobacco plants were immunoblotted with antibodies Bcl-2, Bcl-xL, and Ced-9, respectively. Wt: wild-type tobacco. For further details see Materials and methods.
in the presence of light, protoporphyrin excites to the triplet state and interacts with molecular O2 to produce singlet oxygen which is toxic (Lermontova and Grimm, 2000). Sulfentrazone, a member of the aryetriczolinone family, also inhibits PPO.

Herbicide treatment of wild-type tobacco at concentrations used under field conditions was lethal to whole plants, detached leaves, and leaf discs (Fig. 2A). However, when transgenic tobacco plants harbouring Bcl-2, Bcl-xL, or CED-9 were treated in an identical manner, the plants survived and appeared similar to control plants, treated with buffer alone. Chlorophyll levels of the treated plants were consistent with these observations (Fig. 2B). To confirm that specific transgene expression was responsible for these phenotypes, a tobacco line harbouring Bcl-2ΔBH4 was generated. This mutant has a deletion of the BH4 domain rendering it null for anti-apoptotic activity in mammalian cells (Hanada et al., 1995). As shown in Fig. 2A, tobacco leaf discs carrying Bcl-2ΔBH4, lost cytoprotection following herbicide treatment. Thus, transgenic expression of anti-apoptotic genes inhibits herbicide-induced plant cell death. To confirm that these results are chloroplast-dependent, the same experiments were conducted in the dark by covering the plants with aluminium foil. Herbicide treatments had no effect on dark-grown plants, indicating that herbicide-induced cell death is light-dependent (data not shown).

Since anti-apoptotic gene expression prevented herbicide-induced cell death, the mechanism of this protection was explored next by evaluating whether a programmed type of apoptotic cell death was occurring. DNA was extracted from herbicide-treated leaf tissue. Figure 3A shows the presence of DNA laddering in MV, AC, and ST-treated wild-type leaves; however, DNA appears to be intact and not fragmented in treated transgenic leaves. As a control, tobacco expressing Bcl-2ΔBH4 was also herbicide treated and, as shown in Fig. 3A, DNA laddering occurred. There was no evidence for DNA fragmentation in chloroplasts, based on evaluating isolated chloroplast DNA as well as using chloroplast DNA as a probe in DNA blots from DNA laddering gels (data not shown). Moreover using fluorescence microscopy, it is evident that TUNEL positive nuclei are present in the herbicide-treated wild-type leaves (Fig. 3B). It therefore appears that these herbicides kill plants via the generation of toxic levels of ROS, in an apoptotic-like manner. Such programmed cell death is inhibited by the expression of animal anti-apoptotic genes. Moreover, when the chloroplast-directed herbicide glyphosate was administered to tobacco, cell death occurred, but there was no generation of ROS, DNA fragmentation did not occur, nor was protection afforded by the transgenes (data not shown). Thus, based on the available evidence, the localization of the anti-apoptotic proteins to the chloroplast may be relevant for protection in certain facets of plant PCD.

Discussion

Apoptosis is often mediated via organelles (Ferri and Kroemer, 2001). While the mitochondrion has received the bulk of the attention, evidence has shown that the endoplasmic reticulum, nucleus, lysosomes, and Golgi can also be involved in the triggering of the death programme (Ferri and Kroemer, 2001). In this report, evidence is presented to show that plant chloroplasts, not only associate with Bcl-2 family members, but importantly this interaction may have functional significance.

Chloroplasts are green photosynthetic plastids that are responsible for energy capture during the process of converting
light energy to chemical energy. Thus, chloroplasts are potentially the major source of toxic oxygen derivatives in plant tissue (Foyer et al., 1994). Accumulation of active oxygen species is an unavoidable consequence of photosynthesis. Under high doses of illumination, singlet oxygen is generated through the interaction of triplet-state chlorophyll with ground-state oxygen which can also produce the superoxide radical ($O_2^-$). Chloroplasts are particularly sensitive to damage by ROS because electrons that escape from the photosynthetic electron transfer system are able to react with the relatively high concentrations of $O_2$ in chloroplasts.

Chloroplasts have been previously suggested to play significant roles in the programmed death of plant cells. For example, on epidermal peels of pea leaves, CN$^-$ induces guard cell death (containing chloroplasts and mitochondria), but not epidermal cell death (containing mitochondria only) and only in the presence of light (Samuilov et al., 2002, 2003). The association of Bcl-2, Bcl-xL, and CED-9 with chloroplast membranes should not be unexpected. These proteins all have transmembrane domains at their C termini and have been previously reported to associate with numerous membrane-bound structures. Thus, the key question is whether chloroplast localization has functional importance in modulating cell death responses. The evidence that plant chloroplasts are causally involved in programmed cell death is based on the following. (i) Treatment of tobacco with herbicides whose primary site of action is the chloroplast result in a plant cell death that is typified by apoptotic-like features. It should be noted that expression of these transgenes did not protect against the herbicide glyphosate (‘Round-up’), which is also a chloroplast-targeted herbicide that does not generate ROS. In addition, cell death induced by glyphosate did not exhibit apoptotic-like features. (ii) Light is required for cell death to occur. (iii) Transgenic tobacco harbouring animal anti-apoptotic genes inhibit herbicide-mediated plant cell death and apoptotic-like characteristics are not observed. (iv) Null mutations in the transgenes no longer confer cytoprotection.

A common theme shared by these chemical stresses is the induction of toxic levels of reactive oxygen. Bcl-2 and Bcl-xL have been shown in mammalian systems to confer tolerance to oxidative stress, although the precise manner by which this occurs is not clear (Hockenbery et al., 1993). Previously, it has been shown that these anti-apoptotic genes, including CED-9 can protect yeast from lethal levels of oxidative stress induced by menadione and H$_2$O$_2$ (Chen et al., 2003). Paraquat has often been used as an inducer of photo-oxidative stress and the site of action has generally been associated with the chloroplast. When isolated chloroplasts were illuminated in the presence of paraquat, both stromal and thylakoid-bound ascorbate peroxidases were rapidly inactivated (Mano et al., 2001). The chloroplastic glutamine synthetase, a key enzyme that catalyses the rate-determining step in the photorespiratory pathway, also showed rapid decline when exposed to paraquat (Palatnik et al., 1999). Thus, the association of paraquat activating with chloroplasts is well documented. However, paraquat also functions in mammalian systems presumably generating toxicity via mitochondrial electron transport (Kelner et al., 1993).
programmed cell death events are manifested in plants. Oxidative stress in chloroplasts is a means by which certain clearly established. It is suggested that regulation of mitochondrial involvement in apoptosis have been secondary cell wall synthesis. Thus, chloroplast-directed PCD is mediated via the nucleus.

Light is known to be required for the development of a number of plant diseases and chloroplasts are a well established site of action for certain diseases. For example, in the compatible interaction between tobacco mosaic virus and its host, chlorosis is observed as the virus replicates and spreads. In the incompatible hypersensitive response, a programmed cell death occurs that involves morphological changes in the chloroplast prior to chromatin cleavage and death (Mitter et al., 1997). Chloroplasts appear to be the key mediators of cell death in maize lesion mimic lsl1 mutants (Gray et al., 2002) and a tobacco salicylic acid binding protein was found to be a chloroplast carbonic anhydrase that was shown to harbour antioxidant activity in plants and yeast. Further, when this gene was silenced, the HR was suppressed (Slaymaker et al., 2002).

The role of mitochondria and PCD has been extensively discussed from an evolutionary perspective (Blackstone and Green, 1999). Considerable evidence indicates a bacterial origin for mitochondria and chloroplasts, which resemble and appear to be descended from photosynthetic bacteria. Mitochondria and chloroplasts have several common features including (i) they arise by growth and division not by de novo synthesis, (ii) they are not inherited in a Mendelian manner, (iii) have different DNA organization, (iv) are generally devoid of introns, (v) are involved in energy metabolism, and (vi) are semi-autonomous, containing the genetic machinery required to synthesize some of their own proteins. All of these designations are consistent with the endosymbiotic theory. Thus it is postulated that these bacteria evolved into the modern day mitochondria (and chloroplasts), which provided not only critical antioxidants, but also a source of ROS as a by-product of oxidative phosphorylation. It has been hypothesized that the endosymbiotic origins of mitochondria and the evolution or aerobic metabolism in eukaryotes was a result of active cell death that is illustrated by apoptosis in metazoans (Kroemer, 1997). While mitochondria (and chloroplasts) are not involved in all forms of cell death (e.g. apoptosis can occur in the absence of mitochondria), roles for mitochondrial involvement in apoptosis have been clearly established. It is suggested that regulation of oxidative stress in chloroplasts is a means by which certain programmed cell death events are manifested in plants.

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