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Mannose Induces an Endonuclease Responsible for DNA Laddering in Plant Cells

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The effect of D-mannose (Man) on plant cells was studied in two different systems: Arabidopsis roots and maize (*Zea mays*) suspension-cultured cells. In both systems, exposure to D-Man was associated with a subset of features characteristic of apoptosis, as assessed by oligonucleosomal fragmentation and microscopy analysis. Furthermore, D-Man induced the release of cytochrome *c* from mitochondria. The specificity of D-Man was evaluated by comparing the effects of diastereomers such as L-Man, D-glucose, and D-galactose. Of these treatments, only D-Man caused a reduction in final fresh weight with concomitant oligonucleosomal fragmentation. Man-induced DNA laddering coincided with the activation of a DNase in maize cytosolic extracts and with the appearance of single 35-kD band detected using an in-gel DNase assay. The DNase activity was further confirmed by using covalently closed circular plasmid DNA as a substrate. It appears that D-Man, a safe and readily accessible compound, offers remarkable features for the study of apoptosis in plant cells.

Apoptosis, a morphologically defined form of programmed cell death, plays an essential role in animal development and tissue homeostasis. The apoptotic process can be divided into three phases: an induction phase, the nature of which depends on the specific death-inducing signals; an effector phase, during which the cell becomes committed to die; and a degradation phase, during which the biochemical and morphological features of apoptosis can be observed (Martins and Earnshaw, 1997). In this cascade of events, the core components of the pathway include regulatory proteins that are conserved across animal phyla and a family of Cys proteases known as caspases because they cleave selected substrates at Asp residues (Jacobson, 1997; McCall and Steller, 1997). In addition, the release of Cyt *c* to the cytosol, a protein that is normally sequestered in the mitochondrial intermembrane space, is often an early and committing step in apoptosis (Reed, 1997; Manon et al., 1997). The orchestrated disassembly of cells culminates in a series of distinct morphological changes, including cell and nuclear shrinkage, membrane blebbing, and disintegration into discrete packets called apoptotic bodies (Kerr et al., 1987). The biochemical hallmark of apoptosis is the cleavage of DNA at internucleosomal sites, which generates oligonucleosomal fragments (Wyllie, 1980; Liv et al., 1997). The endonuclease responsible for this cleavage was only recently characterized (Enari et al., 1998; Sakahira et al., 1998).

There are many examples of cell death in plants occurring as part of development, pathogen interaction, or abiotic stress, and they all share common apoptotic mechanisms. Much of the evidence for the concept of apoptosis in plants derives from the observation of oligonucleosomal fragmentation in cells entering the cell death phase. This form of DNA fragmentation can be detected using in situ cytological methods, but can also be detected by the formation of DNA ladders, multimers of 170 to 200 bp, on agarose gels. DNA laddering has been observed in plant tissues responding to fungal infection or phytotoxin exposure (Ryerson and Heath, 1996; Wang et al., 1996a), in senescing carpels (Orzáez and Granell, 1997), in hormone-treated aleurone cells (Wang et al., 1996b), and in cells or tissues responding to abiotic treatments (Ryerson and Heath, 1996; Katsuhara, 1997). In some of these examples, other features of apoptosis are also present, including nuclear shrinkage (Katsuhara and Kawasaki, 1996; Orzáez and Granell, 1997) and the formation of apoptotic bodies (Wang et al., 1996a). Other instances of plant cell death result in an apoptosis-like morphology—cell and nucleus shrinkage and/or membrane blebbing and apoptotic body formation—in the absence of internucleosomal fragmentation. In these cases DNA is either degraded into approximately 50-kb fragments (Levine et al., 1996), which is considered a possible precursor to oligonucleosomal fragmentation, or is degraded into more or less random sizes (McCabe et al., 1997).

Characterization of the endonucleases responsible for DNA degradation will have an important impact on the understanding of plant cell death, and will ultimately help to resolve questions regarding the relationship between plant cell death and apoptosis. At least four different endonucleases are induced in the hypersensitive response of tobacco to virus infection (Mittler and Lam, 1995, 1997). These enzymes are correlated with the degradation of DNA into approximately 50-kb fragments during the hypersensitive response (Mittler and Lam, 1997). However, there is as yet no information regarding the endonuclease activities responsible for oligonucleosomal fragmentation in plant cell death.

We have developed a system for studying apoptosis that is suitable for both Arabidopsis roots and a maize (*Zea*

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mays) cell culture. This system is based on the toxicity of Man to plants, a phenomenon first described over 40 years ago. Man, a hexose sugar, strongly inhibits root growth and respiration in wheat and tomato (Stenlid, 1954; Morgan and Street, 1959). The sugar is readily taken up by roots and converted to Man-6-P by the action of hexokinase. However, Man-6-P is not further utilized due to a deficiency of Man-6-P isomerase, which is necessary for its conversion to Fru-6-P (Goldsworthy and Street, 1965). The high accumulation of Man-6-P inhibits phospho-Glc isomerase, thus blocking glycolysis (Goldsworthy and Street, 1965). The irreversible formation of Man-6-P also inhibits respiration by depleting cells of the orthophosphate required for ATP production (Goldsworthy and Street, 1965; Loughman, 1966). In addition to these metabolic effects, Man and other hexoses repress the transcription of genes required in photosynthesis and the glyoxylate cycle (Jang and Sheen, 1994, 1997; Graham et al., 1997). The toxic action of Man makes it a useful selection agent for the generation of transgenic plants in which the *Escherichia coli* Man-6-P-isomerase gene is used as a resistance marker (Joersbo et al., 1998).

In this report, we show that Man toxicity in Arabidopsis roots and maize cells is associated with a subset of features characteristic of apoptosis, including internucleosomal fragmentation. Using a maize cell suspension, we characterized the dose response, time dependence, and specificity of Man-induced internucleosomal fragmentation. The endonuclease responsible for internucleosomal fragmentation in Man-treated cells was detected in vitro, and initial characterization of this activity is presented.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals and reagents were supplied by Sigma.

Plant Culture and Treatments

Sterilized seeds of Arabidopsis ecotype Columbia were germinated on vertically oriented agar plates in a culture room at 20°C with a 13-h/11-h light/dark cycle. After 1 week, the young plants were transferred to new plates containing either 1% (w/v) D-Glc or 1% (w/v) D-Man. To monitor new root growth, the plates were re-oriented at a right angle to the original growth vector and further incubated in the growth room. After the indicated time period, the plants were analyzed as described below.

The maize (*Zea mays*) suspension cell line issued from an elite genotype related to B73 was cultured in 250-mL flasks in 50 mL of N6 liquid medium (Chu et al., 1975) supplemented with 2 mg/L 2,4-D and 3% (w/v) Suc (2N63S medium). Cultures were grown at 28°C on a rotary shaker at 125 rpm in the dark. Three days after subculture, the medium was replaced with 25 mL of fresh medium containing the indicated hexose compound. Cells were harvested at different times over a strainer with vacuum applied to remove excess liquid, and then washed extensively with 2N63S medium.

Microscopy Analysis

Roots were simultaneously stained with 10 μg/mL fluorescein diacetate (FDA) and 0.5 μg/mL propidium iodide (PI) in water and immediately observed by epifluorescence microscopy using a fluorescence microscope (Orthoplan, Leitz, Midland, Ontario). The microscope was fitted with a blue filter set (excitation at 450–490 nm and emission above 515 nm) for FDA fluorescence and a green filter set (excitation at 530–560 nm and emission above 580 nm) for PI fluorescence. Nuclei were stained using 4,6-diamidinophenylindole (DAPI) at 1 μg/mL in 0.1% (v/v) Triton X-100 for 10 min, and observed using a UV filter set (excitation at 340–380 nm and emission above 430 nm).

Isolation and Analysis of Plant DNA

Maize cells were frozen in liquid nitrogen, ground to a fine powder, and extracted with a solution of 50% (v/v) phenol, 2% (w/v) *p*-aminosalicylic acid, and 0.5%(w/v) 1,5 napthalenedisulfonic acid, and were then extracted with an equal volume of chloroform. The aqueous phase was ethanol precipitated, and the resulting nucleic acid pellet was resuspended with water. RNA was precipitated with the addition of LiCl to a final concentration of 3 M. After centrifugation, DNA within the supernatant was ethanol precipitated and resuspended in water. Arabidopsis tissue was frozen in liquid nitrogen and ground with a Teflon pestle in a microfuge tube. DNA was isolated using a nucleic acid extraction kit (IsoQuick, Orca Research, Bothell, WA) according to the directions provide by the manufacturer. DNA concentrations were assayed using a fluorometer (TKO, Hoefer, San Francisco). For analysis of DNA laddering, equal amounts of DNA (see specific values in each figure legend) were treated with DNase-free RNase A (Boehringer Mannheim) at 25 μg/mL for 1 h at 37°C. DNA was subjected to electrophoresis on 2% (w/v) agarose gels, stained with 0.5 μg/mL ethidium bromide for 45 min, destained in 1 mM $MgSO_4$ for 1 h, and observed on a UV light box. For M_r determination, a 100-bp ladder (GIBCO-BRL) was used for the standards.

Preparation of Cytosolic Extracts

All steps were carried out at 4°C or on ice. Maize cells (1 g fresh weight) were homogenized in 1 mL of buffer A (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM EDTA, 0.25 M Suc, 1 mM EDTA, 1 mM AEBSF, 2 μg/mL pepstatin A, 10 μg/mL leupeptin, and 10 μg/mL aprotinin) using three 10-s pulses of a polytron at a speed setting of 7. The homogenate was pushed through one layer of Miracloth (Calbiochem) using a syringe. Cellular debris and nuclei were spun down using a microfuge at 1,000*g* for 10 min. The supernatant was spun at 10,000*g* for 10 min, giving a pellet fraction enriched in mitochondria. This pellet was resuspended in buffer A. The supernatant was spun at 100,000*g* for 30 min. The supernatant fraction recovered from this final centrifugation step contained cytosolic protein.

Western Analysis for Cyt *c*

The Bradford method (Bio-Rad) was used according to the manufacturer's instructions for protein determination. Equal amounts of protein (10 μg/lane), along with M_r standards (Mark12, Novex, Frankfurt), were subjected to SDS-PAGE using precast 10% (w/v) gels (Novex). Gels were electroblotted onto PVDF membranes (Immobilon, Millipore) and stained with Ponceau S to visualize the markers. For Cyt *c* detection, the blots were incubated in blocking solution consisting of 4% (w/v) nonfat dry milk in TBST (10 mM Tris-HCl, pH 8, 150 mM NaCl, and 0.05% [v/v] Tween 20) for 30 min. The mouse monoclonal antibody clone 7H8.2C12 (PharMingen, San Diego) was added at a concentration of 2 μg/mL and incubated for 1 h. After three 10-min washes in TBST, the membrane was incubated with 0.27 μg/mL peroxidase-conjugated goat-anti-mouse IgG (Pierce) in blocking solution for 1 h. The blot was further washed as indicated above, and the immunolabeled proteins were detected using a chemiluminescent substrate (SuperSignal, Pierce).

Nuclei Preparation

All steps were carried out at 4°C or on ice. Maize suspension cells (approximately 35 g) were homogenized with 50 mL of buffer B (10 mM Tris-HCl, pH 8.0, 40 mM KCl, 10 mM NaCl, 2.5 mM EGTA, 0.15 mM spermine, 0.15 mM spermidine, and 40% [w/v] Suc) using a mortar and pestle. The homogenate was filtered through three layers of Miracloth and further washed with 40 mL of buffer B. The filtrates were pooled and centrifuged at 150*g* for 6 min. The supernatant was centrifuged twice at 150*g* for 30 min and the resulting pellets, P1 and P2, were each resuspended in 200 μL of buffer B. Using a microfuge, contaminating cells and large vesicles were spun down by successive centrifugation at 30*g* for 1 min and 90*g* for 30 s. The supernatant derived from P1 was pipetted to a new tube. To remove contaminating small vesicles, the supernatant derived from P2 was further centrifuged at 210*g* for 5 min. The pellet was resuspended with buffer B and pooled with the nuclei derived from P1. The concentration of nuclei was approximately 4.3×10^7/mL, as determined using a hemocytometer. The nuclei were aliquoted and stored at −80°C.

In Vitro Assay of Endonuclease Activity

The cytosolic fractions were diluted to a final protein concentration of 0.5 to 1.0 mg/mL with buffer A. Nuclei (3 μL) were added to 50 μL of extract or buffer A and incubated for 2 h at 28°C. After incubation, an equal volume of 2× nuclear extraction buffer (50 μL of 200 mM Tris-HCl, pH 8.0, 100 mM EDTA, 250 mM NaCl, 1% [w/v] sarkosyl, 1 mg/mL proteinase K, and 0.2 mg/mL RNaseA) was added to the reaction and incubated at 55°C for 1 h. Nuclear debris were spun down in a microfuge for 10 min, and the DNA in the supernatant was precipitated with a 0.8 volume of isopropanol for 10 min at −20°C. The DNA precipitate was washed with 70% (w/v) ethanol, dried, and resuspended in 20 μL of water. The DNA was treated with RNase A and run on agarose gels as described above.

For the nicking and linearization assay, the cytosolic fractions were diluted to 1 mg/mL in 10 μL to which 1 μL (3.6 μg) of supercoiled plasmid Bluescript KS+ (Stratagene) was added. The reactions were incubated at room temperature and 2.5-μL aliquots were removed at 15-min intervals and flash-frozen in liquid nitrogen. The aliquots were analyzed by electrophoresis on 1% (w/v) agarose gels. For controls, equivalent amounts of non-digested plasmids and plasmids linearized with *Hin*dIII were loaded.

For the in-gel nuclease assay, samples (10 μg protein/lane) were heated in sample buffer at 73°C for 10 min and run on a 12% (w/v) polyacrylamide gel containing 50 μg/mL heat-denatured salmon-sperm DNA with no SDS. SDS was included in the running buffer and sample buffer at the final concentrations of 0.1% (w/v) and 2% (w/v), respectively. These conditions did not alter the migration of standards (Novex) normally observed. Following electrophoresis, the gel was washed twice with 25% (v/v) isopropanol in 10 mM Tris-HCl, pH 7.5, and once in Tris buffer without isopropanol, both at room temperature and 30 min per wash. The gel was then incubated in 10 mM Tris-HCl, pH 7.5, for 11 h at room temperature. To visualize the DNA, the gel was stained with 2 μg/mL ethidium bromide for 30 min and viewed with a UV-light box. Nuclease activity was identified as bands devoid of DNA.

RESULTS

D-Man Induces Cell Death and DNA Laddering in Arabidopsis

Arabidopsis seeds were germinated and grown on plates oriented vertically for 1 week and then transferred to new plates containing no additions, 1% (w/v) D-Man, or 1% (w/v) D-Glc (these concentrations are equal to 56 mM). To illustrate the effect of Man on root growth, the plates were oriented at a 90° angle relative to the original growth vector, providing a reference point for new growth. As shown in Figure 1, root growth was completely inhibited in plants exposed to D-Man, confirming previous observations made in wheat and tomato (Stenlid, 1954; Morgan and Street, 1959). Plants exposed to D-Glc, an epimer of D-Man with respect to the second carbon, did not show inhibited root growth. Like the untreated controls, these roots grew several millimeters toward the new gravity vector in the days following transfer (Fig. 1).

The viability of roots was examined using the fluorescent probes FDA and PI. FDA detects living cells, whereas PI detects dead cells. Arabidopsis roots treated with D-Man showed a dramatic loss of viability, as indicated by the intense red fluorescence contributed by PI (Fig. 2), whereas roots treated with D-Glc and the untreated controls showed intense FDA fluorescence (Fig. 2). Loss of viability was accompanied by changes in nuclear morphology, as exam-

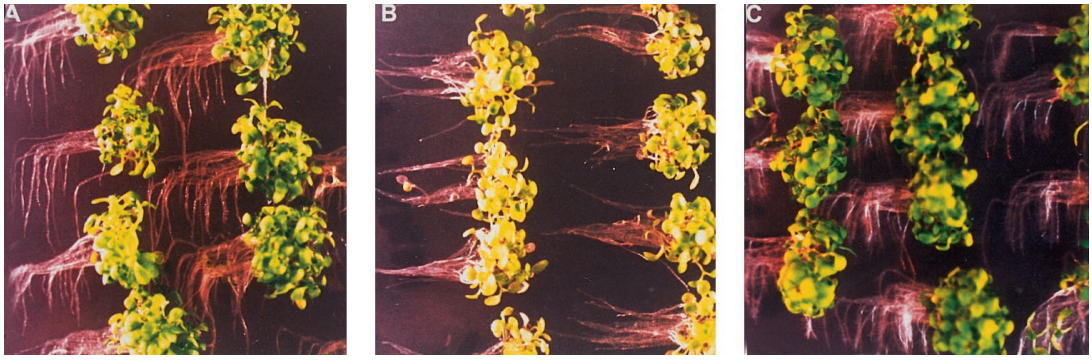

Figure 1. Effect of D-Man on Arabidopsis plants. Plants were grown on agar medium with Glc (A), D-Man (B), or no hexose (C).

ined using DAPI. Nuclei in the D-Man-treated roots were shrunken and often misshapen. Furthermore, the high intensity of the fluorescence was possibly due to chromatin condensation in the nuclei. By comparison, the nuclei of D-Glc-treated and untreated controls were larger and rounded (Fig. 2). Also, differential interference contrast imaging showed that D-Man treated roots were slightly brown compared with the controls. Higher magnification revealed some morphological differences: roots from plants grown on medium with no hexose or with Glc exhibited organized rows of cells with dense cytoplasm, small vacuoles, and a prominent nucleus in the center of

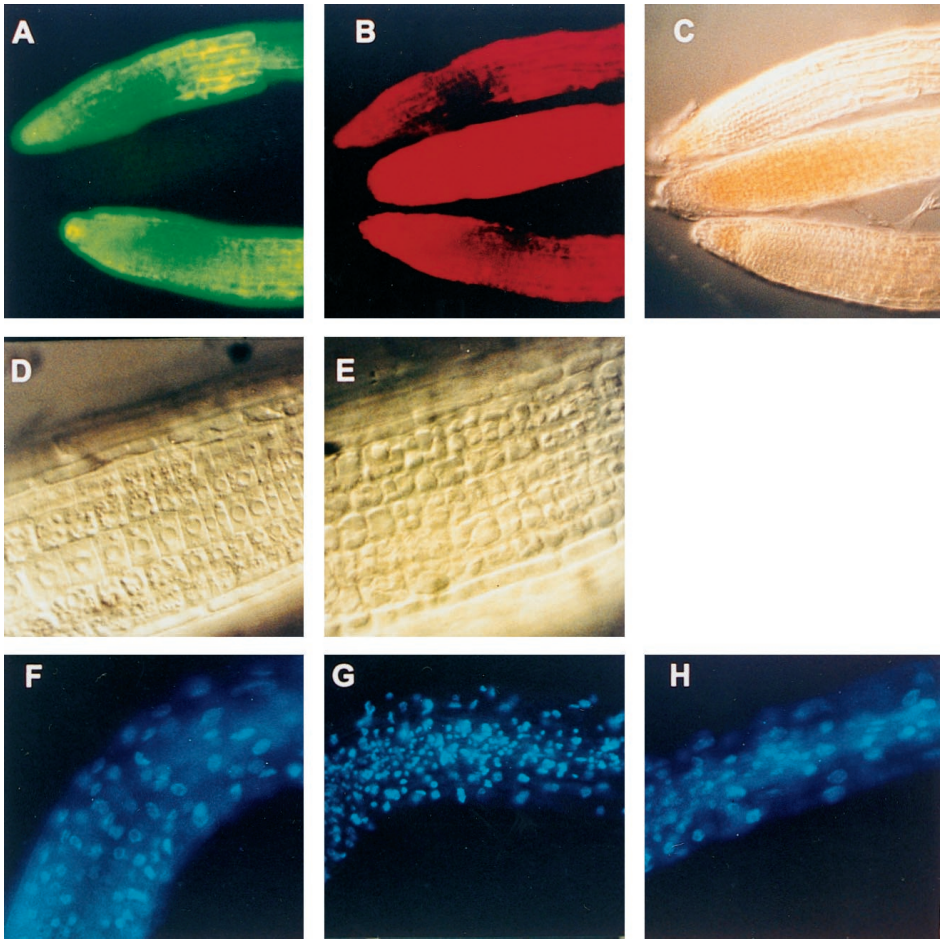

Figure 2. Effect of D-Man on Arabidopsis roots from plants grown on medium with no hexose (top), with Man (middle), and with Glc (bottom). Roots were stained in situ with FDA (A) and PI (B). C, Corresponding differential interference contrast image. D and E, Higher magnification of roots from plants grown on medium with (E) or without (D) Man. F to H, In situ staining of roots with DAPI from plants grown with Glc (F), with Man (G), or with no addition of hexose (H).

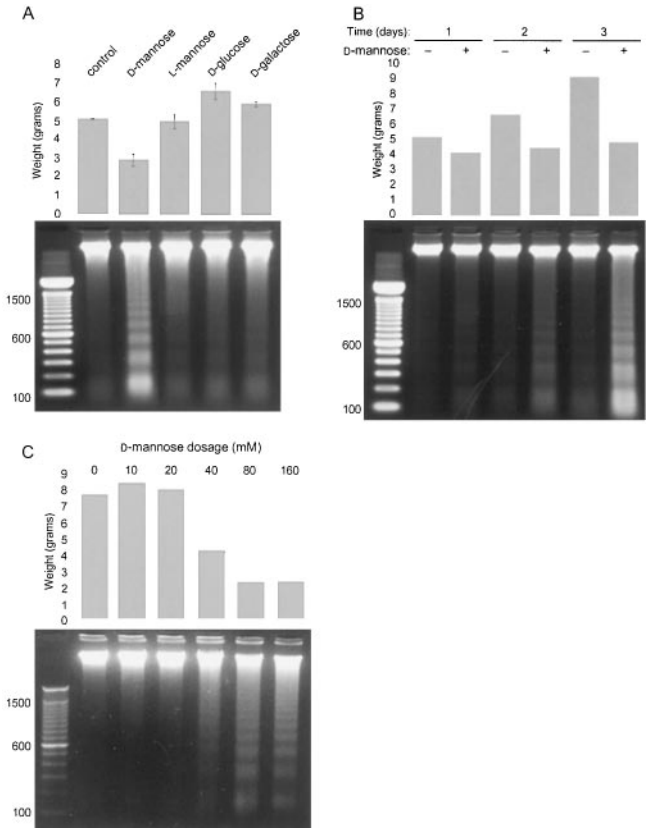

Figure 3. Effect of D-Man on maize cell culture growth and induction of oligonucleosomal fragmentation. Maize cells were treated with the indicated hexoses for 3 d. The histogram shows the mean (± SD) fresh weight for three replicates per treatment. The agarose gel shows DNA extracted from maize cells. Numbers to the left of the figures indicate DNA size in bp. The M_r marker corresponds to a 100-bp ladder. A, Specificity of D-Man. Lane 1, M_r marker; lane 2, untreated control; lane 3, D-Man treated; lane 4, L-Man treated; lane 5, D-Glc treated; lane 6, D-Gal treated. B, Time course of D-Man effects. Maize cells were grown for 1 to 3 d in the absence (−) or presence (+) of 56 mM D-Man. Lane 1, M_r marker; lanes 2, 4, and 6, without D-Man for 1, 2, and 3 d, respectively; lanes 3, 5, and 7, plus D-Man, for 1, 2, and 3 d, respectively. C, Dose dependence of D-Man. Lane 1, M_r marker; lanes 2 through 7, with 0, 10, 20, 40, 80, and 160 mM D-Man, respectively.

each cell. Roots from plants grown on medium containing Man exhibited some cellular disruption.

DNA was prepared from roots and green tissues and assayed for oligonuncleosomal fragmentation by agarose gel electrophoresis. DNA laddering was observed in D-Man-treated plants, but not in the D-Glc treated plants or untreated control plants (data not shown). The sizes of DNA bands were multiples of 175 bp, which is consistent with fragmentation at internucleosomal sites. The DNA ladder pattern was most obvious in roots, whereas it was only slightly detectable in green tissues. These data show that the toxicity of D-Man in roots is associated with features characteristic of apoptosis. All cells in the root responded to Man, as shown by PI and DAPI. This is consistent with the metabolic explanation of Man action, but also presents another facet of Man-induced cell death as a model system: it induces apoptosis in a large population of cells, making biochemical approaches much easier.

Man Induces DNA Fragmentation in Maize Cells

The specificity of Man toxicity was evaluated by comparing the effects of the diasteromers D-Man, L-Man, D-Glc, and D-Gal. Of these treatments, only D-Man caused a reduction in final fresh weight (56% that of the untreated controls) and induced internucleosomal fragmentation (Fig. 3A). In the time-course study shown in Figure 3B, DNA laddering was evident after 24 h of exposure to D-Man and increased over a 3-d period, with concomitant inhibition of culture growth. No increase in DNA fragmentation was observed in untreated control cells over the equivalent time period. The dose of D-Man required to inhibit growth and induce oligonucleosomal fragmentation is shown in Figure 3C. No effect was observed in cultures treated with 0, 10, or 20 mM D-Man. Treatment with 40 mM D-Man resulted in growth inhibition and DNA laddering, and the severity of these effects was increased by treatment with 80 mM D-Man. However, no further fragmentation was noted using 160 mM D-Man, indicating that 80 mM is near the saturating dose for this process (Fig. 3C).

D-Man-Induced Cell Death Is Associated with Cyt *c* Release

Cyt *c* release from mitochondria into the cytosol is an early event of apoptosis in animal cells. It is required for the activation of the caspase protease cascade, as well as downstream events such as DNA laddering (Liu et al., 1996; Kluck et al., 1997a, 1997b; Yang et al., 1997). Thus, Cyt *c* release constitutes an additional biochemical marker for apoptosis. To track Cyt *c* distribution within maize cells, protein extracts were separated into particulate and soluble fractions containing mitochondrial and cytosolic proteins, respectively, and analyzed by western blot with an anti-Cyt *c* monoclonal antibody (Jemmerson et al., 1991) (Fig. 4). Virtually all of the Cyt *c* was detected in the particulate fraction in untreated cultures, which is consistent with localization within mitochondria. In contrast, in the D-Man-treated culture, Cyt *c* was distributed in both the particulate and soluble fractions, indicating release from mitochondria into the cytosol. This effect was specific to D-Man, since no Cyt *c* release was observed in cultures treated with either D-Glc or L-Man. Detection of the band was elimi-

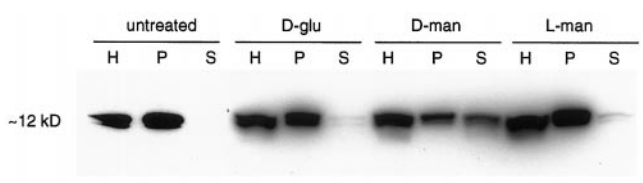

Figure 4. Detection of Cyt *c*. Maize cells treated with the indicated hexose were fractionated into pellet (P) and soluble (S) fractions containing particulate and cytosolic fractions, respectively. H, Homogenate. Cyt *c* in each fraction was determined by western analysis.

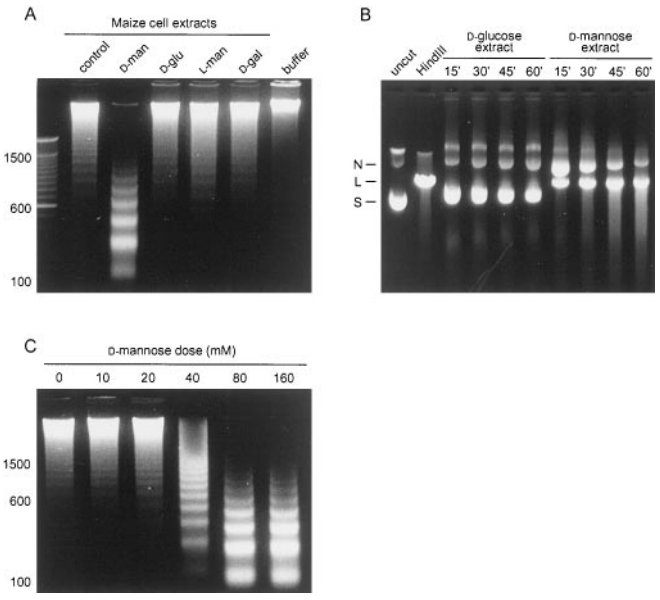

Figure 5. Detection of endonuclease activity in the cytosol of maize cells treated with D-Man. Endonuclease activity was assayed in cytosolic extracts prepared from maize cells following treatment with the indicated hexose. A, Assay performed using purified maize nuclei as substrate. Lane 1, M_r marker; lane 2, extract, untreated control; lane 3, extract, D-Man; lane 4, extract, D-Glc; lane 5, extract, L-Man; lane 6, extract, D-Gal; lane 7, buffer control. B, Assay showing degradation of covalently closed circular plasmid using cytosolic extracts of cells treated with D-Glc or D-Man. Uncut and *Hin*dIII-cut plasmid provide migration standards for supercoiled (S), nicked (N), and linearized plasmid (L). C, Assay showing degradation of purified nuclei exposed to extracts of maize cells treated with 0, 10, 20, 40, 80, and 160 mM D-Man, respectively.

nated by competition with Cyt *c* from horse heart (data not shown).

In a time-course study, maize cells were cultured in the absence or presence of Man for 48, 62, 72, and 88 h. Cultures grown in the presence of Man displayed DNA laddering that increased over the course of infection, whereas no DNA laddering was detected in cultures grown in the absence of Man over the same time period (data not shown). The proportion of Cyt *c* localized to the cytoplasm increased significantly with time, reaching a maximum at 88 h (data not shown). This increase was correlated with the degree of DNA laddering observed over this period.

In Vitro Detection and Characterization of the Maize Apoptotic Nuclease

To characterize the endonuclease responsible for the DNA laddering triggered by D-Man, maize cytosolic extracts were incubated in vitro with purified maize nuclei, and the DNA was analyzed by agarose gel electrophoresis. As shown in Figure 5A, the cytosol of D-Man-treated cells exhibited an activity capable of degrading nDNA into a DNA ladder. This activity was not found in extracts of untreated cells or in cells treated with other hexoses. The nuclease activity was further confirmed using covalently closed circular plasmid DNA as a substrate (Fig. 5B). Over time, the extract of D-Man-treated cells progressively degraded the supercoiled plasmid first into a nicked, relaxed circle, then into a linearized form, and finally into a smear. The extract of D-Glc-treated cells did not exhibit such activity.

The concentration of D-Man required for the induction of the endonuclease correlated with the observed dose dependence for DNA ladder formation in vivo (Fig. 5C). No endonuclease activity was observed in cells treated with 0, 10, or 20 mM D-Man, as measured using purified nuclei. Endonuclease activity was detected in cells treated with 40 mM D-Man, and was increased in cells treated with 80 mM D-Man. However, no further increase in endonuclease activity was found in cells treated with 160 mM D-Man.

Several treatments were capable of inhibiting the endonuclease activity in vitro, as measured using either purified nuclei or plasmid DNA as the substrate (data not shown). Aurintricarboxylic acid, an inhibitor of DNA laddering in apoptotic cells (Hallick et al., 1977; Batistatou and Green, 1993), strongly inhibited the D-Man-induced activity. Unfortunately, because aurintricarboxylic acid was extremely deleterious to maize cells (data not shown), it was not possible to test its effect on DNA laddering in vivo. The Man-induced endonuclease was also inhibited in assays containing either 25 mM EDTA or EGTA, suggesting that the enzyme has a requirement for divalent cations.

M_r Determination of the D-Man-Induced Endonuclease

To determine the M_r of the endonuclease, an in-gel nuclease activity assay was used. In this method, high-M_r salmon-sperm DNA is incorporated into a standard SDS-polyacrylamide gel. Following electrophoresis of cell ex-

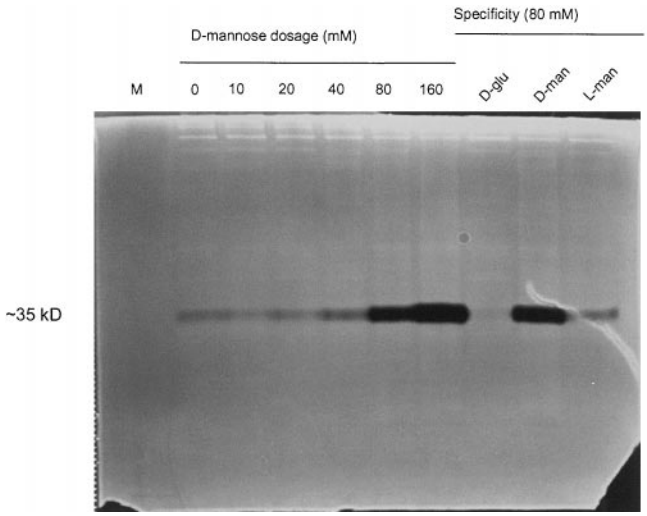

Figure 6. M_r determination of the endonuclease induced by D-Man. Maize cells were treated for 3 d with the indicated concentrations of D-Man, D-Glc, or L-Man, and cytosolic extracts were subjected to an in-gel nuclease assay. The gel was then stained with Coomassie Blue. Lane 1, Molecular mass markers (kD); lane 2, untreated controls; lanes 3, 4, 5, 6, and 7, 10, 20, 40, 80, and 160 mM of D-Man, respectively; lanes 8, 9, and 10, 80 mM of D-Glc, D-Man, and L-Man, respectively.

tracts, proteins are renatured and the gel is stained with ethidium bromide. Bands of DNase activity are then visible as areas devoid of DNA. This method allowed the identification of an approximately 35-kD DNase that correlated perfectly with D-Man dosage and specificity (Fig. 6). The intensity of the DNase band increased with dosage from 40 to 160 mM D-Man, and was barely detectable in cultures treated with 0 to 20 mM D-Man. Furthermore, only basal levels of the nuclease activity was detected in extracts of cells treated with 80 mM of either D-Glc or L-Man (Fig. 6). The hexose specificity and the dose response required for the appearance of the 35-kD nuclease indicate that this band represents the nuclease responsible for DNA laddering in maize cells exposed to Man.

DISCUSSION

Clear parallels exist between programmed cell death in plants and apoptosis in animals (Pennell and Lamb, 1997), and several examples of plant cell death accompanied by apoptosis-like morphology and/or DNA laddering have been reported (Katsuhara and Kawasaki, 1996; Levine et al., 1996; Ryerson and Heath, 1996; Wang et al., 1996a; Wang et al., 1996b; McCabe et al., 1997; Orzáez and Granell, 1997; O'Brien et al., 1998). However, further research is still required to determine the extent to which these parallels indicate similar mechanisms at the molecular level. We show here that Man toxicity is a potentially useful system for probing the events leading to plant cell death and/or subsequent processing of dead cells. The remarkable features of this system include: (a) applicability to both Arabidopsis plants and maize cell suspension cultures, making it amenable to research approaches based on genetics, cell biology, and biochemistry; and (b) phenomenology of apoptosis, including nuclear shrinkage, Cyt *c* release from mitochondria, and DNA laddering. Among the hexoses tested, these effects were specific to D-Man, a safe and readily available compound. Another useful feature is that Man is toxic to certain mammalian tumor cell lines that, like plants, lack sufficient Man-6-P-isomerase activity for its utilization (Hernández and de la Fuente, 1989). Thus, comparative studies between Man toxicity in plant and mammalian cells are possible.

We show that Man-induced DNA laddering coincides with the induction or activation of a DNA endonuclease. Three lines of evidence indicate that this endonuclease is responsible for the observed DNA laddering. First, the activity generated DNA ladders in vitro when purified maize nuclei were used as a substrate. Second, among the hexoses tested, induction of the endonuclease was specific for D-Man. Third, the level of activity in maize extracts correlated with the dosage required for Man-induced DNA laddering. These latter characteristics also apply to the appearance of a single 35-kD DNase detected using an in-gel nuclease assay. Endonucleases of a similar size range are induced in tobacco by virus infection and wounding (Mittler and Lam, 1995, 1997). However, these may be fundamentally different in that the latter are not associated with DNA laddering activity (Mittler and Lam, 1995, 1997).

The enzyme responsible for DNA laddering in mammalian apoptosis is a novel 39-kD endonuclease called CAD (caspase-activated DNase) (Enari et al., 1998; Sakahira et al., 1998). Like the Man-induced endonuclease described here, CAD is inhibited by aurintricarboxylic acid (Enari et al., 1998). CAD is constitutively expressed in the cytoplasm but is kept inactive by association with its inhibitor, ICAD. In apoptotic cells, cleavage of ICAD by the caspase-3 protease allows CAD to enter the nucleus and to degrade DNA (Enari et al., 1998; Sakahira et al., 1998). We do not know whether the induction of the endonuclease by Man is the result of regulation at the transcriptional or posttranslational levels. However, a system of regulation similar to CAD/ICAD may not exist, since this predicts that in noninduced cells endonuclease activation would be evident following separation by denaturing gel electrophoresis. This was not observed using the in-gel nuclease assay with cell extracts from maize cells exposed to Man.

This is the first report, to our knowledge, to associate DNA laddering in plants with Cyt *c* release from mitochondria. Various stimuli of apoptosis lead to the release of Cyt *c* from mitochondria in animal cells. This release can be inhibited by two mitochondrial proteins that negatively regulate apoptosis, Bcl-2 and Bcl-x_L (Liu et al., 1996; Chauhan et al., 1997; Du et al., 1997; Kharbanda et al., 1997; Kim et al., 1997; Kluck et al., 1997a; Vander Heiden et al., 1997; Yang et al., 1997). The role of Cyt *c* in triggering apoptosis has been shown by microinjection experiments (Li et al., 1997a) and by using cell-free systems in which the apoptosis pathway is reconstituted in vitro (Liu et al., 1996; Kluck et al., 1997a; Yang et al., 1997). Cyt *c* binds to Apaf-1 (Li et al., 1997b; Zou et al., 1997), the human homolog of the CED-4 protein that is genetically required for apoptosis in *Caenorhabditis elegans* (Horvitz et al., 1994). Once complexed, Apaf-1 initiates a proteolytic cascade pathway leading to caspase-3 activation and the downstream events in apoptosis (Li et al., 1997b).

The Cyt *c* release observed in cells treated with Man is consistent with the hypothesis that the molecular mechanism of apoptosis execution in plants and animals is evolutionarily conserved. By analogy with the animal pathway, Cyt *c* may interact with an as-yet-unidentified homolog of Apaf-1. It is interesting that a subclass of plant disease resistance genes that are genetically required for programmed cell death in the hypersensitive response share significant similarity with Apaf-1 and CED-4 (Graham et al., 1997). It is possible that resistance proteins themselves are able to interact with Cyt *c* in a manner similar to Apaf-1. Alternatively, there may be other members of this family that are functionally distinct from resistance proteins. However, the role of Cyt *c* release in Man-induced DNA laddering remains to be determined.

There are several possible routes by which Man could induce apoptosis. The specificity of the D- over the L-enantiomer indicates that Man toxicity is not the result of osmotic stress. Rather, the effect of Man could be the result of interference with Glc utilization and phosphate availability (Goldsworthy and Street, 1965). Analogous treatment of mammalian cells with 2-deoxy-Glc affects metabolism in a similar fashion and triggers apoptosis, probably

as a result of reduced ATP (Marton et al., 1997). A second means by which Man could induce apoptosis is by compromising the cell's ability to detoxify reactive oxygen species. In erythrocytes, Man inhibits defense against oxidants by lowering ATP required for the regeneration of reduced pyridine nucleotides and glutathione (Lachant and Zerez, 1988).

Reactive oxygen species are known to trigger apoptosis in animal cells (Jacobsen, 1996) and programmed cell death in plants (Jabs et al., 1996; Levine et al., 1996). Either model predicts that carbon starvation causes the same effects as D-Man. However, maize cell cultures transferred to medium lacking Suc or other carbon sources were inhibited in growth but did not display DNA laddering (data not shown). Therefore, it is unlikely that all of the effects of D-Man can be attributed to carbon starvation. An additional possibility is that D-Man either directly or indirectly activates a cell death pathway as a result of its effect on gene regulation. Hexoses are known to repress or activate the transcription of genes involved in photosynthesis (Jang and Sheen, 1994), glyoxylate metabolism, starch metabolism (Mita and Suzuki-Fujii, 1995), nitrogen metabolism (Cheng et al., 1992), pigmentation (Tsukaya et al., 1991), and pathogen defense (Johnson and Ryan, 1990; Herbers et al., 1996). In all cases tested, Man is a potent regulator of gene transcription (Jang and Sheen, 1994).

A free form of Man exists in trace amounts in some species at the time of breakdown of the mannan reserve (Koch, 1996). Man-containing polysaccharides are primarily found in the endosperm cell walls of seeds that exhibit coat-enhanced dormancy, such as legumes, tomato, and lettuce, and mannanases participate in their enzymatic depolymerization (Bewley, 1997). During endosperm mobilization in germinated seeds, synthesis of these enzymes occurs in the endosperm prior to germination. This step could be a prerequisite to permit radicle emergence by weakening of the surrounding tissue. Interestingly, these enzymes are also present in some vegetative tissues of plants such as alfafa, and are not known to contain appreciable levels of mannans in the cell wall (Dirk et al., 1995). Because Man could be available during specific seed and plant growth phases, one could speculate that Man provides the means to regulate cell differentiation and the cell cycle, and to adjust to developmental changes by apoptosis.

An important role for hexokinase has been proposed in sensing and signaling of the sugar status. This pathway acts by a mechanism independent of its role in hexose metabolism (Jang and Sheen, 1997). However, the mechanism by which hexokinase transmits the signal to downstream elements to initiate changes in gene expression in the pathway is not well understood. Man can be phosphorylated by hexokinase, but Man-6-P, unlike Glc-6-P, is not further metabolized as a carbon or energy source. It was also recently proposed that Man inhibits hexokinases (Pego et al., 1999). The system used in the present study thus presents the potential to study the regulation of hexokinase in plants. Efforts to dissect the sugar-sensing pathway by the isolation of sugar-insensitive mutant Arabidopsis lines (Mita and Suzuki-Fujii, 1997; Van Oosten et al., 1997) should help to establish the route by which Man induces apoptotic effects.

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