Programmed cell death induced by (β-D-galactosyl)₃ Yariv reagent in *Nicotiana tabacum* BY-2 suspension-cultured cells

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Programmed cell death (PCD) is involved in plant development and pathogen defence and can be triggered in vitro by several biotic and abiotic stimuli. In this report (β-D-galactosyl)₃ Yariv reagent, a chemical that specifically binds to arabinogalactan-proteins (AGPs), completely inhibited cell growth and induced PCD in tobacco BY-2 suspension cultured cells. Analysis of DNA from these cells, by agarose gel electrophoresis, revealed a DNA ladder consisting of multimers of 140–170 bp, similar to apoptotic animal DNA internucleosomal fragmentation. Complementary morphological studies revealed additional PCD characteristics in the Yariv-treated BY-2 cells, including cell shrinkage and cytoplasmic condensation. These studies demonstrate the usefulness of BY-2 cells as a model plant PCD system and confirm a link between AGPs and PCD.

Introduction

Unlike necrosis, programmed cell death (PCD) is an active process, which occurs during normal biological development. Despite the early recognition that PCD is required for growth of vascular plants, its study has been mostly neglected over the intervening years. However, it is known that PCD is essential for development and defence and that it is also induced in response to many environmental stresses, such as chilling, salt and drought (Greenberg 1996, Solomon et al. 1999). Although a detailed understanding of how plant cells die is still largely unknown, recent evidence suggests that, at least in some cases, plant PCD might be mechanistically similar to animal apoptosis. Different experimental approaches have provided evidence for the mechanistic similarity between plant and animal cell death at the molecular level. For example, extracts of *Xenopus* eggs or carrot cells induce apoptosis in purified animal and plant nuclei (Jiang et al. 1999, Zhao et al. 1999). Moreover, in tobacco, expression of *bax*, a death-promoting member of the Bcl-2 family of proteins, triggers cell death (Lacomme and Santa Cruz 1999) and expression of *bcl-x*$_L$, which encodes a mammalian pro-survival member of the Bcl-2 family, suppresses cell death induced by UV-B irradiation, paraquat treatment or the hypersensitive reaction to tobacco mosaic virus (TMV) infection (Mitsuhara et al. 1999). Enhanced resistance to cell death was also found in transgenic tobacco plants over-expressing the *ced-9* gene, a *Caenorhabditis elegans* homologue of *bcl-x*$_L$ (Mitsuhara et al. 1999). Finally, as in animal apoptosis, cysteine proteases are also involved in plant PCD (del Pozo and Lam 1998, Solomon et al. 1999). Our future aim is to study differential gene expression, at the protein and mRNA levels, in order to a better understand the underlying mechanisms of plant PCD and to further examine the relationship between plant PCD and animal apoptosis. Thus, we intend to set up a pathogen-free experimental plant system using BY-2 cells, in which PCD can be easily induced and manipulated. Apoptotic-like changes have already been described for tobacco BY-2 suspension-cultured cells in response to low temperature treatment (5–6°C) (Koukalová et al. 1997) and after H₂O₂ treatment (Houot et al. 2001).

Recently, it was reported, that (β-D-galactosyl)₃ Yariv

Abbreviations –AGPs, arabinogalactan-proteins; BY-2, Bright Yellow-2; 2,4-D, 2,4-dichlorophenoxyacetic acid; FDA, fluorescein diacetate; MS medium, Murashige and Skoog medium; PBS, phosphate buffered saline; PCD, programmed cell death; PI, propidium iodide.

reagent, a chemical which specifically binds arabinogalactan proteins (AGPs), induces *Arabidopsis* suspension-cultured cells to undergo PCD (Gao and Showalter 1999). According to the authors these results indicate that AGPs may be an important component of the signal transduction pathway for PCD in plants. Here, we report that (β-D-galactosyl)$_3$ Yariv reagent also induces PCD in BY-2 suspension-cultured cells in a dose and time dependent manner. To our knowledge this is only the second report of induction of PCD in plant cells by (β-D-galactosyl)$_3$ Yariv reagent. Thus, these results corroborate the implication that AGPs are involved with PCD and further establish tobacco BY-2 cells as a model plant system to study PCD.

Materials and methods

Plant material

Nicotiana tabacum Bright Yellow-2 suspension-cultured (BY-2) cells were grown under weak light (9 µmol photons m^{-2} s^{-1}) at 24°C on a rotatory shaker (110 r.p.m.). Cells were grown on Murashige and Skoog (MS) medium (Sigma, St. Louis, MO, USA) supplemented with 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D, Sigma, St. Louis, MO, USA).

Three ml of 4-day-old cultures, at a 2×10^3 cells µl^{-1} density, were subcultured to fresh medium with either 50 µM or 100 µM (β-D-galactosyl)$_3$ Yariv reagent, 100 µM (α-D-galactosyl)$_3$ Yariv reagent or in the absence of any Yariv reagent and grown for the desired length of time (24–96 h) after treatment. Yariv reagents were synthesized as described by Yariv et al. (1962). Necrosis was induced by fast cooling BY-2 cells to -70°C for 10 min followed by a heat treatment at 90°C for 3 min.

Cell staining and fluorescent and light microscopy

Fluorescein diacetate (FDA) was used as a fluorescent indicator of cell viability. FDA was used in combination with propidium iodide (PI) in order to determine the percentage of cell death. PI is a nucleic acid stain that can only penetrate cells with damaged or leaking cell membranes, so PI-positive staining will only be observed in dying or dead cells. The cells were washed twice with PBS (10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, 136.9 mM NaCl, 2.7 mM KCl, pH 7.2) and stained with 5 µg ml^{-1} PI and 2.5 µg ml^{-1} FDA in PBS, for 15 min, at room temperature. After three washes with PBS, cells were observed under a Nikon Labophot-2 fluorescence microscope. To detect cytoplasm shrinkage, cells were treated in the same way as for PI or FDA, but stained with 200 µg ml^{-1} Toluidine blue O and observed in a Nikon Labophot-2 fluorescence microscope under white-light illumination. The cells were counted in an haemacitometre 24, 48, 72 and 96 h after the beginning of each treatment.

DNA extraction and analysis

DNA fragmentation analysis was performed on BY-2 cells treated for 96 h with (β-D-galactosyl)$_3$ Yariv reagent (50 or 100 µM), untreated cells and necrotic cells. DNA was isolated from these cells with the *MasterPure^{TM}·Plant Leaf DNA Purification Kit* (Promega, Madison, WI, USA). DNA samples (20 µg) were electrophoresed on 1.6% (m/v) agarose gels at constant voltage (70 V) and stained with ethidium bromide to visualize DNA fragmentation patterns.

Results

Inhibition of cell growth in BY-2 suspension cultured cells after treatment with (β-D-galactosyl)$_3$ Yariv reagent

In order to evaluate if Yariv reagent interferes with BY-2 cell growth, cells were treated with 50 µM or 100 µM (β-D-galactosyl)$_3$ Yariv reagent and counted in a haemacitometer following 24, 48, 72 and 96 h of treatment. Toluidine blue O was used as the staining agent since dead cells stain with toluidine blue O and the living cells do not because the stain cannot cross an intact cell membrane. Cells treated with 50 µM or 100 µM (β-D-galactosyl)$_3$ Yariv did not increase in number between 24 h and 96 h of treatment (Fig. 1) i.e. the number of cells remained constant at about 400 cells µl^{-1} at 24, 48, 72 and 96 h of treatment. In contrast, the number of untreated cells increased about 2-fold between 24 h and 96 h (Fig. 1). Moreover, cells treated with (α-D-galactosyl)$_3$ Yariv reagent, a useful control reagent that does not bind AGPs (Gao and Showalter 1999), also demonstrated a 2-fold increase in cell number 96 h after treatment; specifically, 1436 cells µl^{-1} were detected in this control treatment.

In order to evaluate whether inhibition of growth of BY-2 cultured cells treated with (β-D-galactosyl)$_3$ Yariv reagent involves cell death, cells were stained with PI and FDA (Fig. 2). When the plasma membrane is intact and
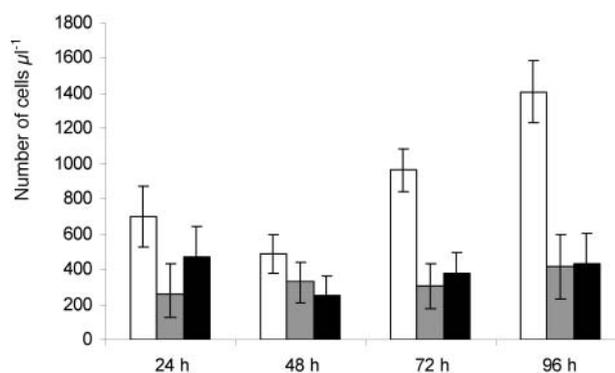

Fig. 1. Effect of (β-D-galactosyl)$_3$ Yariv reagent on the number of cells. BY-2 cells were treated for up to 96 h with 50 µM or 100 µM (β-D-galactosyl)$_3$ Yariv reagent. Untreated cells (□); 50 µM (β-D-galactosyl)$_3$ Yariv reagent treated cells (▨) and 100 µM (β-D-galactosyl)$_3$ Yariv reagent treated cells (■). Cells were counted in an haemacytometer after toluidine blue O staining.
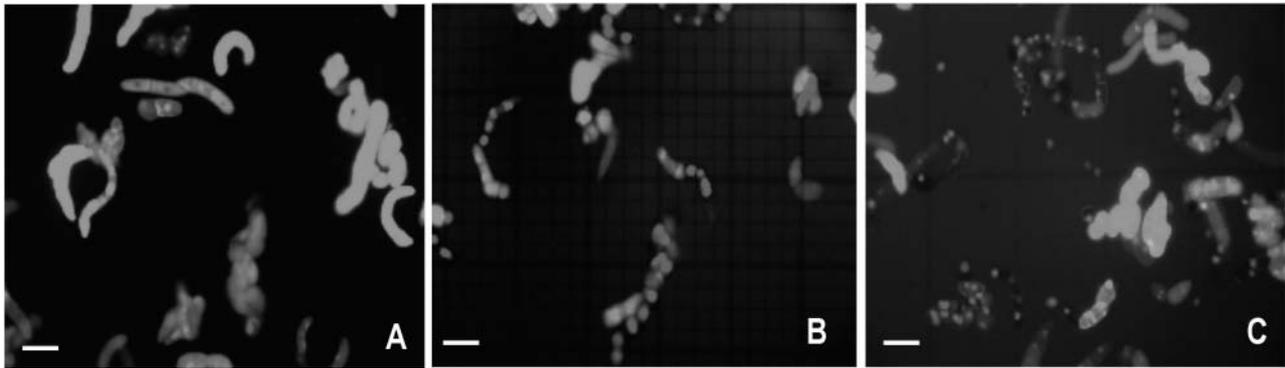
Fig. 2. Fluorescent microscopy of FDA and PI stained BY-2 cells after 96 h of (β-D-galactosyl)$_3$ Yariv reagent treatment. A, Untreated cells; B, 50 μM (β-D-galactosyl)$_3$ Yariv reagent treated cells; C, 100 μM (β-D-galactosyl)$_3$ Yariv reagent treated cells. Bar in = 0.25 mm.

metabolically active, cells exhibit FDA-positive staining (i.e. living cells appear green under fluorescent light). When cells die and consequently their plasma membrane integrity is lost, FDA will not stain these cells. Instead, PI can enter the dead cells and stain the nuclei so that these nuclei appear red under fluorescent light, thus providing a convenient method to count the number of dead cells.

Cell death increased markedly 24 h after treatment with (β-D-galactosyl)$_3$ Yariv reagent reaching 22% for 50 μM and 16% for 100 μM (β-D-galactosyl)$_3$ Yariv reagent (Fig. 3). In the presence of 50 μM (β-D-galactosyl)$_3$ Yariv reagent, the percentage of dead cells remained nearly constant at 24, 48, 72 and 96 h after treatment. In contrast, in the presence of 100 μM (β-D-galactosyl)$_3$ Yariv, the cell death percentage increased dramatically between 72 and 96 h of treatment, reaching a value of 91% at 96 h. For untreated cells, the cell death percentage remained at approximately 10% throughout the time course of the experiment.

Internucleosomal DNA fragmentation in BY-2 cells treated with (β-D-galactosyl)$_3$ Yariv reagent

Endonucleolysis is considered as the key biochemical event of programmed cell death, resulting in cleavage of nuclear DNA into oligonucleosomal-sized fragments. Such cleavage is reflected in the appearance of DNA ladders following agarose gel electrophoresis and is considered a diagnostic characteristic of PCD (Koukalová et al. 1997, Stein and Hansen 1999).

BY-2 cells were subjected to a 96-h treatment with either 50 μM or 100 μM (β-D-galactosyl)$_3$ Yariv reagent and their DNA subsequently analysed by agarose gel electrophoresis. For comparison, cells were treated by a fast cooling to $-70°C$ for 10 min and subsequently heated to 90°C for 3 min to induce necrosis and serve as a control (McCabe et al. 1997). Such necrotic cells are known to produce random DNA cleavage patterns appearing as broad molecular weight smears following agarose gel electrophoresis. Untreated cells were also used as a control here since DNA cleavage should be

minimal and result in a relatively narrow band of high molecular weight DNA following agarose gel electrophoresis.

Examination of the DNA patterns generated from cells treated with (β-D-galactosyl)$_3$ Yariv reagent (50 μM and 100 μM) indicated clear differences compared to necrotic and untreated control cells (Fig. 4). Specifically, the observed DNA laddering for the cells treated with 50 μM, and particularly 100 μM (β-D-galactosyl)$_3$ Yariv reagent consisted of multimers of 140–170 bp, and was consistent with the internucleosomal hydrolysis characteristic of animal apoptosis. In contrast, DNA from the untreated control cells exhibited a single, relatively sharp band of high molecular weight DNA, while DNA from necrotic cells appeared as a very broad molecular weight smear, indicating random cleavage of DNA.

Morphological changes in BY-2 cells treated with (β-D-galactosyl)$_3$ Yariv reagent

DNA laddering on agarose gels cannot provide information regarding PCD in individual cells. Cytoplasmic shrinkage is a characteristic event of PCD in contrast to cytoplasmic swelling that often accompanies necrotic cell death (Kerr et al. 1972, Cohen 1993).
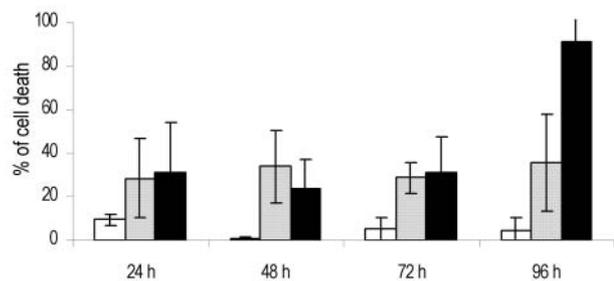
Untreated control cells showed normal morphology

Fig. 3. Percentage of cell death over time in relation to the total number of cells, evaluated by PI-positive for dead cell. Untreated cells (□); 50 μM (β-D-galactosyl)$_3$ Yariv reagent treated cells (▨) and 100 μM (β-D-galactosyl)$_3$ Yariv reagent treated cells (■).
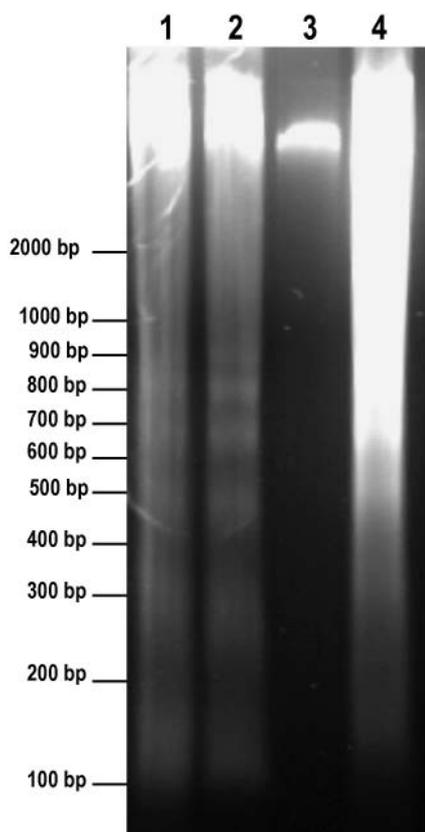
Fig. 4. Agarose gel electrophoresis of DNA from BY-2 cells treated with (β-D-galactosyl)₃ Yariv reagent for 96 h. Lane 1, cells treated with 50 µM of (β-D-galactosyl)₃ Yariv reagent; lane 2, cells treated with 100 µM (β-D-galactosyl)₃ Yariv reagent; lane 3, untreated cells; lane 4, necrotic cells. The 100 bp size ladder is indicated.

(i.e. plasma membrane appressed to the cell wall and an evident nucleus) (Fig. 5A). In contrast, cells grown in the presence of 100 µM (β-D-galactosyl)₃ Yariv reagent for 96 h showed marked cytoplasmic shrinkage with either no evident nucleus or a very condensed nucleus having a peripheral location (Fig. 5C). In necrotic cells, there is no cytoplasmic shrinkage; instead the plasma membrane and cellular contents are broken down resulting in toluidine blue O staining of residual debris (Fig. 5B). The percentage of cells showing such cytoplasmic shrinkage increased with time and with concentration of the (β-D-galactosyl)₃ Yariv reagent, similar to the percentage of cell death, which occurred more rapidly and to a greater extent than cytoplasmic shrinkage (Figs 3 and 6). The percentage of cells showing cytoplasm shrinkage at 96 h was 14% in cells treated with 50 µM of (β-D-galactosyl)₃ Yariv and 62% in cells treated with 100 µM of (β-D-galactosyl)₃ Yariv (Fig. 6). The increase in the percentage of cells showing cytoplasmic shrinkage was first detected after 48 h of treatment; in contrast, an increase in the percentage of cell death was first detected after 24 h of treatment (Figs 3 and 6).

Discussion

Unlike necrosis, which is an 'accidental' process, PCD is a genetically programmed process. When dying animal cells exhibit certain morphological characteristics such as DNA strand breaks with 3′ OH ends, chromatin condensation, fragmentation of the nucleus, cell shrinkage, membrane blebbing and disassembly into membrane-enclosed vesicles (apoptotic bodies), this form of PCD is named apoptosis. Despite the early recognition that PCD is required for growth of vascular plants, its study has been mostly neglected over the intervening years. However, experimental evidence suggests that PCD in plants, in some cases at least, might be mechanistically similar to apoptosis in animal cells (del Pozo and Lam 1998, Lacomme and Santa Cruz 1999, Mitsuhara et al. 1999, Solomon et al. 1999, Zhao et al. 1999). These observations raise the question of to what degree the PCD process is conserved between animals and plants.

Cell suspension cultures represent an ideal research tool to establish a pathogen-free experimental model system in which PCD can be induced and manipulated. BY-2 suspension cultured-cells represent a good pathogen-free experimental system to study PCD. Apoptotic like changes have already been described for BY-2 suspension cultured cells after low temperature treatment (Koukalová et al. 1997) and more recently after H_2O_2 treatment (Houot et al. 2001).

In 1999, Gao and Showalter (1999), reported that (β-D-galactosyl)₃ Yariv reagent induces PCD in *Arabidopsis thaliana* cell suspension cultures. (β-D-galactosyl)₃ Yariv phenylglycoside specifically binds arabinogalactan-proteins (AGPs), a family of highly glycosylated, hydroxyproline-rich glycoproteins, implicated in many aspects of plant growth and development (Showalter 1993, 2001, Knox 1995, Greenberg 1996).

Growth of BY-2 suspension cultured cells was completely inhibited in the presence of either 50 µM or 100 µM (β-D-galactosyl)₃ Yariv reagent (Fig. 1). However, growth inhibition was not observed in untreated, control cells (Fig. 1). Identical results were reported for *Arabidopsis* cell cultures treated with 80 µM (β-D-galactosyl)₃ Yariv reagent (Gao and Showalter 1999) and ancient and newly established lines of rose suspension cultured cells treated with (β-D-glucosyl)₃ Yariv reagent (Serpe and Nothnagel 1994, Langan and Nothnagel 1997). Growth inhibition in the ancient rose-cultured line involved suppression of cell division, whereas some type of cell death was gradually induced in the newly established cell line. Treatment of BY-2 cultured suspension cells with (α-D-galactosyl)₃ Yariv reagent, a control reagent which does not bind AGPs, does not inhibit cell growth in this system, as already reported for *Arabidopsis* cultured cells (Gao and Showalter 1999). Overall, these results indicate that (β-D-galactosyl)₃ Yariv reagent induces some kind of cell death on the tobacco cultured cells.

In BY-2 cultured cells, cell death, as evaluated by PI staining using fluorescent microscopy, increased sig-
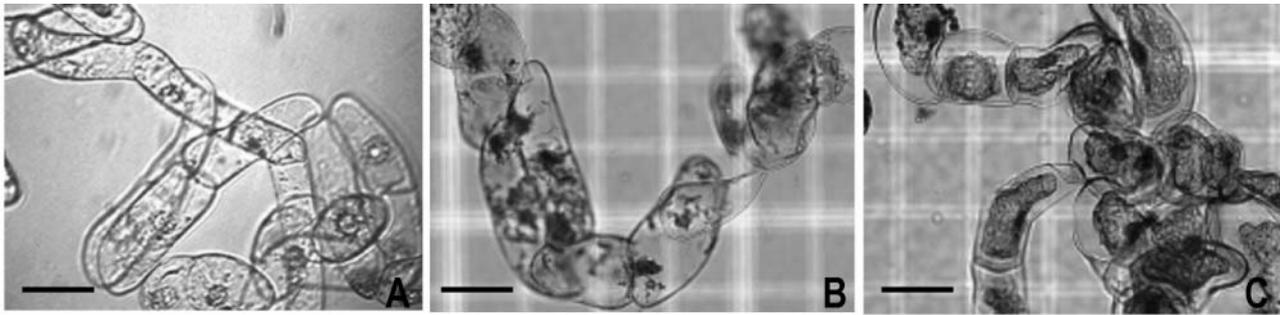
Fig. 5. Cell structure of BY-2 cells observed by light microscopy following Toluidine blue O staining. A, Untreated cells; B, necrotic, heat shocked cells (fast cooled at −70°C for 10 min followed by 3 min at 90°C); C, cells grown in the presence of 100 µM β-Yariv reagent for 96 h. Bar in = 0.25 mm.

nificantly after treatment with (β-D-galactosyl)₃ Yariv reagent reaching a value of 91%, 96 h after treatment with 100 µM (β-D-galactosyl)₃ Yariv reagent (Figs 2 and 3). These results indicate that in BY-2 cultured cells inhibition of growth after treatment with (β-D-galactosyl)₃ Yariv reagent indeed involves some kind of cell death.

Endonucleolysis is considered a hallmark biochemical event for PCD, resulting in the cleavage of nuclear DNA into oligonucleosomal-sized fragments. DNA laddering observed following agarose gel electrophoresis reflects such cleavage and is therefore considered a diagnostic indicator of PCD. Detection of DNA laddering, however, may be difficult for technical reasons (Gao and Showalter 1999), since detection depends on the simultaneous occurrence of a high percentage of cells undergoing PCD. DNA laddering consisting of multiples of 140–170 bp, characteristic of apoptotic animal cells, was observed after treatment of BY-2 cells with 100 µM (β-D-galactosyl)₃ Yariv reagent in contrast to untreated cells or necrotic cells used as controls (Fig. 4). Treatment with 50 µM (β-D-galactosyl)₃ Yariv reagent was also able to induce DNA laddering, but to a lesser extent (Fig. 4). These results indicate that DNA laddering is induced by (β-D-galactosyl)₃ Yariv reagent on BY-2 cultured cells, in a dose and time dependent manner. Overall, these results

indicate that (β-D-galactosyl)₃ Yariv reagent induces PCD in BY-2 cultured cells.

Morphological changes characteristic of animal cell apoptosis include convolutions at the cell surface with infolding and blebbing of the plasma membrane, cell shrinkage, condensation of the cytoplasm and nucleus, and formation of apoptotic bodies. Cell shrinkage and cytoplasmic condensation were observed in BY-2 cultured cells treated with either 50 µM or 100 µM (β-D-galactosyl)₃ Yariv reagent, in contrast to control cells (Figs 5 and 6). Based on these results, the cell death induced in BY-2 cultured suspension cells treated with (β-D-galactosyl)₃ Yariv reagent represents PCD.

The hallmarks of animal apoptosis observed in tobacco BY-2 suspension cultured cells treated with (β-D-galactosyl)₃ Yariv reagent, namely, internucleosomal DNA fragmentation and cytoplasmic shrinkage and condensation, clearly demonstrates that this system represents a useful pathogen-free experimental model system to study plant PCD.

Finally, the results presented here further strengthen the idea that AGPs are involved in PCD in plants as initially suggested by Gao and Showalter (1999). This link between AGPs and PCD is also observed in zinnia mesophyll cells where AGPs are reported to act as endogenous inducers of tracheary element differentiation (Motose et al. 2001). While the mechanisms by which AGPs or perturbation of AGPs by Yariv reagents induce PCD are unknown, there are several relevant pieces of information to bear in mind. First, AGPs are found at the plasma membrane, and a recent work has indicated their attachment is via glycosylphosphatidylinositol (gpi) anchors (Borner et al. 2002). Such gpi-anchored proteins are known to be important signal molecules in other systems. Second, Yariv reagent is known to signal release of intercellular Ca^{2+} (Roy et al. 1999). Third, Yariv reagent is known to elicit H_2O_2 production and subsequently PCD in tobacco cells (Chen and Showalter, unpublished results). Consequently, we hypothesize that AGP perturbation at the plasma membrane by Yariv reagent can signal a release of intracellular Ca^{2+} leading to H_2O_2 production and subsequent PCD. Testing this model and identifying the signal molecules associated
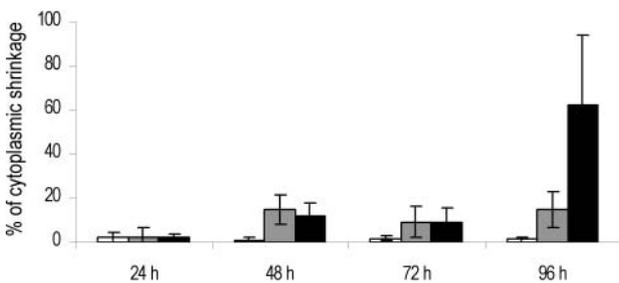

Fig. 6. Percentage of cells with cytoplasmic shrinkage over time in relation to the total number of cells, evaluated by toluidine blue O. Untreated cells (□); 50 µM (β-D-galactosyl)₃ Yariv reagent treated cells (▨) and 100 µM (β-D-galactosyl)₃ Yariv reagent treated cells (■).

with this PCD pathway remain a challenge for the future.

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