

Yariv reagent treatment induces programmed cell death in *Arabidopsis* cell cultures and implicates arabinogalactan protein involvement

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Summary

Arabinogalactan proteins (AGPs) are a family of highly glycosylated, hydroxyproline-rich glycoproteins implicated in various aspects of plant growth and development. (β -D-glucosyl) $_3$ and (β -D-galactosyl) $_3$ Yariv phenylglycosides, commonly known as Yariv reagents, specifically bind AGPs in a non-covalent manner. Here (β -D-galactosyl) $_3$ Yariv reagent was added to *Arabidopsis thaliana* cell suspension cultures and determined to induce programmed cell death (PCD) by three criteria: (i) DNA fragmentation as detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) of DNA 3'-OH groups; (ii) internucleosomal DNA fragmentation as visualized by genomic Southern blotting; and (iii) structural changes characteristic of PCD including cytoplasmic shrinkage and condensation, chromatin condensation and nuclear membrane blebbing. These findings implicate AGP involvement in PCD in plants, presumably by perturbation of AGPs located at the plasma membrane-cell wall interface.

Introduction

Arabinogalactan proteins (AGPs) constitute a family of proteoglycans (Fincher *et al.*, 1983; Jermyn and Yeow, 1975) that belong to the hydroxyproline-rich glycoprotein (HRGP) superfamily, which also includes extensins, proline-rich proteins (PRPs) and Solanaceous lectins (Showalter, 1993). These proteoglycans are widely distributed in the plant kingdom and reside in various plant organs including leaves, stems, roots, flowers and seeds (Basile and Basile, 1987; Clarke *et al.*, 1979; Jermyn and Yeow, 1975). At the subcellular level, AGPs occur on the plasma membrane (Komalavilas *et al.*, 1991; Pennell *et al.*, 1989), in the cell wall (Basile and Basile, 1987; Serpe and Nothnagel, 1994), in the extracellular space (Samson *et al.*, 1984), in multi-

vesicular bodies (Herman and Lamb, 1992), and in the medium of cultured cells (Komalavilas *et al.*, 1991).

The widespread distribution of AGPs in plants, particularly at the cell surface, has long implied that AGPs play important roles in plant development and cell-cell interactions. Indeed, evidence indicates that some AGPs are involved in cell-cell communication and cell-matrix interactions during plant development, cell proliferation and somatic embryogenesis (reviewed by Nothnagel, 1997). Tissue-specific and spatio-temporal expression of some AGP epitopes as determined by monoclonal antibodies (Dolan *et al.*, 1995; Knox *et al.*, 1989; Pennell and Roberts, 1990; Schindler *et al.*, 1995) indicate that AGPs act as cell surface markers. The ability of (β -D-glucosyl) $_3$ or (β -D-galactosyl) $_3$ Yariv reagent to bind specifically with AGPs has been used to perturb AGPs and alter cell elongation and cell division (Serpe and Nothnagel, 1994). Growth of rose cell suspension cultures was completely inhibited by the addition of (β -D-glucosyl) $_3$ Yariv reagent due to the reversible suppression of cell division, while rose cell growth was not inhibited by other 'control' Yariv reagents, such as (α -D-galactosyl) $_3$ or (β -D-mannosyl) $_3$ Yariv reagent, which do not bind AGPs (Serpe and Nothnagel, 1994). Similarly, perturbation of AGPs by (β -D-glucosyl) $_3$ Yariv reagent inhibited lily pollen tube growth (Jauh and Lord, 1996; Roy *et al.*, 1998) and *Arabidopsis* root growth (Willats and Knox, 1996), blocked elongation of suspension-cultured carrot cells (Willats and Knox, 1996), and induced cell death in young rose cell cultures, as well as *Nicotiana* and *Arabidopsis* cell cultures (Langan and Nothnagel, 1997). However, it was not clear whether these cell cultures died as a result of necrosis or programmed cell death (PCD).

In plants, PCD is a normal developmental process (Barlow, 1982; Jones and Dangl, 1996; Pennell and Lamb, 1997) involved in anther, megagametophyte and vascular tissue development as well as in senescence, pollination and sex determination (Chasan, 1994; DeLong *et al.*, 1993; Dietrich *et al.*, 1994; Groover and Jones, 1999; Groover *et al.*, 1997; Orzáez and Granell, 1997; Wang *et al.*, 1996b). Plants also employ PCD as a precisely controlled response to different biotic and abiotic stimuli (Baillieul *et al.*, 1995; Dietrich *et al.*, 1994; Greenberg and Ausubel, 1993; Greenberg *et al.*, 1994; Greenberg, 1996; McCabe *et al.*, 1997; Orzáez and

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Granell, 1997; Ryerson and Heath, 1996). Several studies have elucidated similarities between cell death associated with the hypersensitive response (HR) in plants and a particular type of PCD in animals known as apoptosis (Greenberg *et al.*, 1994; Wang *et al.*, 1996a). Apoptosis occurs in animals during normal development to remove unwanted cells and in response to infection (Greenberg, 1996). Apoptosis is an ordered process leading to the breakdown of the dying cell. These dying cells display certain morphological changes such as plasma membrane and nuclear membrane blebbing, cytoplasmic condensation and shrinkage, chromatin condensation and internucleosomal DNA fragmentation caused by endogenous Ca^{2+} -dependent endonucleases, as well as the appearance of 'apoptotic bodies' (i.e. small membrane bound structures containing the fragmented DNA) (Dangl *et al.*, 1996). Such fragmented DNA can be detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) of DNA 3'-OH ends (Gavrieli *et al.*, 1992; Mittler and Lam, 1995) which is one of the most useful diagnostic assays for apoptosis (Gavrieli *et al.*, 1992; Vaux and Strasser, 1996).

In animal cells, the extracellular matrix (ECM) plays an essential role in integrin-mediated signal transduction pathways which affects important cellular events such as motility, cell division, differentiation and PCD (Howe *et al.*, 1998). For example, basement membrane ECM can suppress apoptosis of mammary epithelial cells in tissue culture and *in vivo* (Boudreau *et al.*, 1995, 1996). Moreover, alteration of the ECM induces an increase in Fas mRNA, which encodes a human cell surface antigen, and therefore significantly enhances Fas-induced apoptosis in epithelial cells (Fine *et al.*, 1998). Evidently, disruption of cell-ECM interactions causes apoptosis in some animal cell types (Cardone *et al.*, 1997; Fine *et al.*, 1998; Singhal *et al.*, 1998). Such a disruption of plasma membrane-cell wall interactions may have the same effect in plants and may involve AGPs at the cell surface. Some epitopes of AGPs have been found in the secondary cell wall of maturing xylem elements in maize coleoptiles and in the plasma membrane and cell wall of developing metaxylem elements in *Arabidopsis* roots (Dolan *et al.*, 1995; Schindler *et al.*, 1995). Pre-xylem cells are committed to undergo PCD in xylem differentiation; therefore, it has been suggested that AGPs can identify such cells that are predetermined for PCD.

Here, we report that $(\beta\text{-D-galactosyl})_3$ Yariv reagent, a chemical which specifically binds AGPs, inhibits growth of *Arabidopsis* suspension-cultured cells by inducing these cells to undergo PCD in a time- and dose-dependent manner. These results, for the first time, strongly implicate that AGPs are involved in PCD in

plants, and indicate that AGPs may be an important component of the signal transduction pathway for this process.

Results and Discussion

Perturbation of AGPs induces DNA fragmentation in Arabidopsis suspension-cultured cells as detected by TUNEL

In preliminary experiments, growth of *Arabidopsis* suspension-cultured cells was inhibited in the presence of $30\ \mu\text{M}$ $(\beta\text{-D-galactosyl})_3$ Yariv reagent and was completely blocked by treatment with $80\ \mu\text{M}$ $(\beta\text{-D-galactosyl})_3$ Yariv reagent (data not shown). No growth inhibition, however, was observed in untreated (control) cells or in cells treated with $(\alpha\text{-D-galactosyl})_3$ Yariv reagent, which does not bind AGPs and hence serves as an important additional control. Similar growth inhibition was observed in ancient and newly established lines of rose suspension-cultured cells (Langan and Nothnagel, 1997; Serpe and Nothnagel, 1994) in response to $(\beta\text{-D-glucosyl})_3$ Yariv reagent treatment. It was noted that growth inhibition in the ancient line involved suppression of cell division, whereas in the newly established cell line, some type of cell death gradually occurred. Intriguingly, in animal cell cultures, several cell types, such as epithelial cells (Frisch and Francis, 1994) and endothelial cells (Meredith *et al.*, 1993), were induced to undergo apoptosis when the cell-ECM interactions were disrupted. Therefore, we predicted that AGPs, which are important components of plasma membranes and cell walls in plant cells, when perturbed may also cause PCD. Consistent with this hypothesis, we found that cells grown in $(\beta\text{-D-galactosyl})_3$ Yariv reagent displayed an increasing percentage of TUNEL-positive nuclei in a time- and dose-dependent manner (Figure 1 and Table 1). The TUNEL reaction is a sensitive assay to detect the increased 3'-OH ends caused by DNA degradation in individual cells and is widely used as a diagnostic test for PCD, although it does not detect DNA degradation occurring exclusively at internucleosomal regions (Orzáez and Granell, 1997).

As shown in Figure 1 and Table 1, most of the untreated *Arabidopsis* suspension-cultured cells and cells treated with different concentrations of $(\alpha\text{-D-galactosyl})_3$ Yariv reagent for different periods of time were TUNEL-negative, but propidium iodide (PI)-or 4',6-diamidino-2-phenylindole (DAPI)-positive, indicating that DNA degradation did not occur to any appreciable extent in these cells. Notably, with longer treatment times (i.e. 48 and 72 h), nearly all of the $(\alpha\text{-D-galactosyl})_3$ Yariv reagent-treated cells showed a relatively lower percentage of TUNEL-positive nuclei, indicating that low cell density may account for the higher percentage of TUNEL-positive nuclei at the early stage of the cultures. In other words, the higher cell density

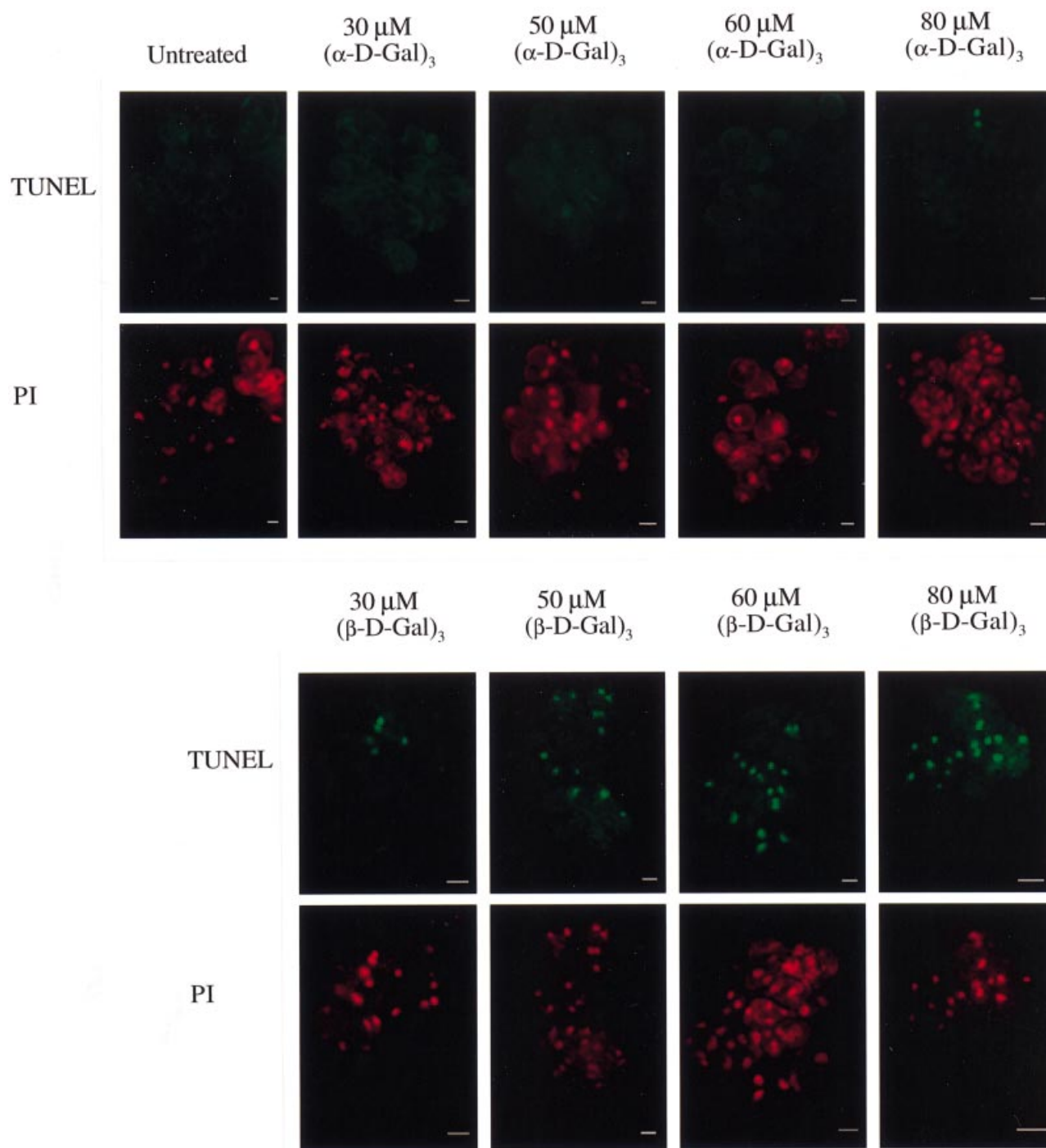


Figure 1. DNA fragmentation and nuclear staining of *Arabidopsis* suspension-cultured cells treated with Yarov reagent.

Cells grown for 72 h in the absence (i.e. untreated cells) or presence of various concentrations of (α -D-galactosyl) $_3$ Yarov reagent, which do not bind AGPs, or (β -D-galactosyl) $_3$ Yarov reagent, which bind AGPs, were double-stained by TUNEL and PI to detect fragmented DNA and nuclei, respectively. (α -D-Gal) $_3$ (α -D-galactosyl) $_3$ Yarov reagent; (β -D-Gal) $_3$ (β -D-galactosyl) $_3$ Yarov reagent. Bars = 10 μ m.

resulted in fewer TUNEL-positive cells. Carrot cells cultured at low density are known to activate a PCD pathway, which can be prevented by the addition of a cell-free, cell-conditioned growth medium (McCabe *et al.*, 1997).

Similarly, animal cells grown in medium absent of growth factors initiated PCD (Barres *et al.*, 1992), which can be suppressed by signal molecules released by other cells (Raff, 1992). Thus, 'social control' of cell death, as

Table 1. Average percentage of apoptotic (TUNEL positive) cells in *Arabidopsis* suspension-cultured cells treated with Yariv reagent^a

Percentage (%) of apoptotic cells (mean±SD) treated with (α-D-galactosyl) ₃ and (β-D-galactosyl) ₃ Yariv reagents									
Time (h)	Untreated	30 μM (α-D-Gal) ₃	30 μM (β-D-Gal) ₃	50 μM (α-D-Gal) ₃	50 μM (β-D-Gal) ₃	60 μM (α-D-Gal) ₃	60 μM (β-D-Gal) ₃	80 μM (α-D-Gal) ₃	80 μM (β-D-Gal) ₃
24		4.53±2.93	16.51±2.53	5.98±1.74	30.38±3.19	4.59±1.75	31.24±6.11	7.30±1.34	47.51±5.45
48		2.04±0.92	19.80±2.95	2.29±1.69	34.66±5.28	2.82±1.10	38.57±4.63	3.47±1.14	80.14±6.73
72	0.87±0.47	1.99±1.04	30.33±5.82	2.44±1.35	45.43±5.12	1.33±1.33	75.65±5.29	4.52±1.19	100.00±0.00

^aThe percentage of dying cells was calculated as the number of TUNEL-positive cells divided by the number of DAPI-positive cells.

Table 2. Average percentage of dying and dead cells over time as a function of Yariv reagent treatment evaluated by the PI staining^a

Percentage (%) of dying/dead cells (mean±SD) treated with (α-D-galactosyl) ₃ and (β-D-galactosyl) ₃ Yariv reagents									
Time (h)	Untreated	30 μM (α-D-Gal) ₃	30 μM (β-D-Gal) ₃	50 μM (α-D-Gal) ₃	50 μM (β-D-Gal) ₃	60 μM (α-D-Gal) ₃	60 μM (β-D-Gal) ₃	80 μM (α-D-Gal) ₃	80 μM (β-D-Gal) ₃
24	8.21±4.34	6.03±5.60	15.63±11.40	11.55±11.13	27.71±16.33	7.69±5.40	30.12±22.34	5.78±5.42	42.84±24.33
48	5.74±5.61	3.36±2.56	29.49±10.99	3.62±3.27	44.44±12.57	3.67±2.37	49.99±15.06	5.45±4.48	64.67±14.75
72	1.27±1.23	0.55±0.54	37.00±14.17	1.17±0.89	52.60±8.65	1.31±1.13	68.88±16.01	2.44±2.39	75.41±7.88

^aThe percentage of dying/dead cells was calculated as the number of PI-positive cells divided by the total number of cells.

observed in animal cells and carrot cells, may also operate here in *Arabidopsis* cell cultures.

In contrast, cells grown with increasing concentrations of (β-D-galactosyl)₃ Yariv reagent showed an increasing percentage of TUNEL-positive nuclei. Moreover, at later time points of these treatments, an increasing percentage of TUNEL-positive nuclei was also observed. Remarkably, in cells treated with 80 μM (β-D-galactosyl)₃ Yariv reagent for 72 h, all nuclei detected by PI or DAPI staining were also identified by TUNEL. This observation coupled with subsequent experimental evidence presented below indicates that perturbation of AGPs by (β-D-galactosyl)₃ Yariv reagent under such conditions was so substantial that all cells were induced to undergo PCD. It should be noted that cells which were already dead and lacked DNA (i.e. empty cells) were not stained with DAPI or PI, nor labeled by TUNEL.

Yariv reagents strongly self-associate in aqueous solutions to form complexes of 10–50 molecules (Woods *et al.*, 1978) with an apparent molecular weight range of 10–50 kDa, most of which should be small enough to enter cell walls (Carpita *et al.*, 1979) but not plasma membranes (Serpe and Nothnagel, 1994). When cells were subcultured into fresh medium containing (β-D-galactosyl)₃ Yariv reagent, cell wall AGPs and AGPs existing at the plasma membrane–cell wall interface were selectively and non-covalently bound by this Yariv reagent. Although such Yariv-AGP complexes may remain bound in these locations or be secreted as small red pellets, binding of (β-D-galactosyl)₃ Yariv reagent to AGPs leads to aggregation of

AGPs and can disrupt their normal interactions with other cell surface components so as to trigger the PCD pathway.

Vital staining was also applied in time course analyses to evaluate the effect of (β-D-galactosyl)₃ Yariv reagent on cell death directly (i.e. without fixation) relative to the TUNEL data in which chemically fixed cells were examined. Live cells with intact cell membranes can exclude specific dyes such as propidium iodide (PI) used in this experiment, whereas dead cells (and chemically fixed cells) with compromised cell membranes are unable to exclude vital dyes and thus exhibit positive staining (Loo and Rillema, 1998). Consistent with results obtained by TUNEL detection (Figure 1 and Table 1), PI staining of cells treated with (β-D-galactosyl)₃ Yariv reagent showed an increasing percentage of positively stained cells in a time- and dose-dependent manner (Table 2). These values, however, should be viewed as underestimates of cell death given that cells which already died and lost their nucleic acid will not stain with PI. Such dead cells, as discussed below, appeared empty under the light microscope.

The cell-density dependent 'social control' observed for (α-D-galactosyl)₃ Yariv reagent in Figure 1 and Table 1, was also substantiated with vital staining (Table 2). Such social control was also apparent in untreated cells with increasing culture time (i.e. increasing cell density) (Table 2). These observations, when coupled with (β-D-galactosyl)₃ Yariv reagent's ability to induce cell death, indicate that AGPs may represent an important component for plant cell growth and survival.

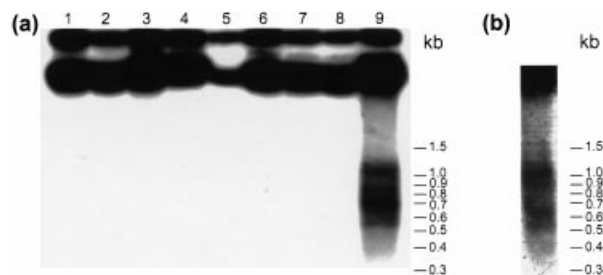


Figure 2. Dose effects of Yariv reagents on *Arabidopsis* suspension-cultured cells treated for 72 h as determined by genomic Southern blotting using *Arabidopsis* total genomic DNA as a probe.

(a) Lanes include untreated cells (lane 1) and cells treated with (α -D-galactosyl) $_3$ Yariv reagent at concentrations of 30 μ M (lane 2), 50 μ M (lane 4), 60 μ M (lane 6), and 80 μ M (lane 8) and with (β -D-galactosyl) $_3$ Yariv reagent at concentrations of 30 μ M (lane 3), 50 μ M (lane 5), 60 μ M (lane 7), and 80 μ M (lane 9). The 100 bp size ladder is indicated.

(b) Overexposure of lane 7 from panel (a) demonstrating DNA laddering.

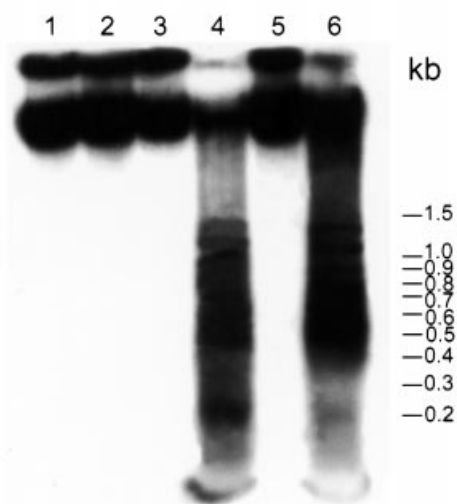


Figure 3. Effects of Yariv reagents on *Arabidopsis* suspension-cultured cells at different time points as determined by genomic Southern blotting using *Arabidopsis* total genomic DNA as a probe.

Cells were treated with 80 μ M (α -D-galactosyl) $_3$ Yariv reagent for 24 h (lane 1), 48 h (lane 3) and 72 h (lane 5), and with 80 μ M (β -D-galactosyl) $_3$ Yariv reagent for 24 h (lane 2), 48 h (lane 4) and 72 h (lane 6). The 100 bp size ladder is indicated.

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

Internucleosomal cleavage of nuclear DNA into nucleosomal fragments which are multiples of 180 bp is usually identified by agarose gel electrophoresis as DNA ladders in apoptotic animal cells (Boudreau *et al.*, 1995; Singhal *et al.*, 1998). Similarly, DNA laddering is also detected in cowpea undergoing the hypersensitive response triggered by cowpea rust fungus (Ryerson and Heath, 1996), in tomato protoplasts and leaflets treated with host-selective AAL toxins (Wang *et al.*, 1996a), and in senescent carpels (Orzáez and Granell, 1997). Detection of DNA laddering, however, may be difficult for technical reasons (Groover

et al., 1997; McCabe *et al.*, 1997). Detection depends on having a substantial population of cells undergoing PCD synchronously. Fortunately, in cells treated with 80 μ M (β -D-galactosyl) $_3$ Yariv reagent for 72 h, all nuclei detected by DAPI or PI staining were TUNEL-positive (Table 1) and resided in dying cells (i.e. the 75% PI-positive cell population) as detected by vital staining (Table 2). This observation indicated that it may be possible to detect DNA laddering in such a substantial population of dying cells. Indeed, Southern analysis of DNA isolated from *Arabidopsis* suspension-cultured cells treated with different concentrations of (α -D-galactosyl) $_3$ or (β -D-galactosyl) $_3$ Yariv reagent for 72 h showed that DNA laddering occurred only in cells treated with high concentrations of (β -D-galactosyl) $_3$ Yariv reagent (Figure 2). Here, DNA laddering, consisting of multiples of approximately 180 bp, was observed from cells treated with 80 μ M (β -D-galactosyl) $_3$ Yariv reagent (Figure 2a); treatment with 60 μ M (β -D-galactosyl) $_3$ Yariv reagent was also capable of producing less intensely labeled ladders as revealed by overexposure of the blot (Figure 2b). No fragmentation was seen in DNAs from cells grown in low concentrations of (β -D-galactosyl) $_3$ Yariv reagent. DNAs from untreated cells and cells grown in different concentrations of (α -D-galactosyl) $_3$ Yariv reagent did not show any degradation. This evidence strongly supports the idea that perturbation of AGPs with (β -D-galactosyl) $_3$ Yariv reagent causes PCD in *Arabidopsis* suspension-cultured cells in a dose-dependent fashion.

Southern analysis of DNA isolated from *Arabidopsis* suspension-cultured cells treated with 80 μ M (α -D-galactosyl) $_3$ or (β -D-galactosyl) $_3$ Yariv reagent for various times showed that DNA laddering occurred in a time-dependent fashion only in (β -D-galactosyl) $_3$ Yariv treated cells (Figure 3). This DNA laddering was observed at 48 h and was more pronounced at 72 h. Interestingly, cells treated with 80 μ M (β -D-galactosyl) $_3$ Yariv reagent for 48 h and 60 μ M (β -D-galactosyl) $_3$ Yariv reagent for 72 h resulted in 80% and 75% of the nuclei detected by DAPI or PI staining being TUNEL-positive, respectively (Table 1). These two treatments reflect threshold detection levels for DNA laddering in this system (Figures 2 and 3). In any event, such time-dependent induction of DNA laddering is consistent with a PCD response as opposed to an immediate necrotic response (Bielawska *et al.*, 1997; Dangl *et al.*, 1996; Matylevitch *et al.*, 1998; McCabe *et al.*, 1997; Mignotte and Vayssiére, 1998; Orzáez and Granell, 1997; Pennell and Lamb, 1997; Ryerson and Heath, 1996; Wang *et al.*, 1996a; Wyllie, 1980).

Structural changes in cells treated with (β -D-galactosyl) $_3$ Yariv reagent

As shown in Table 1 and Figure 1, all cells grown in 80 μ M (β -D-galactosyl) $_3$ Yariv reagent for 72 h that were stained

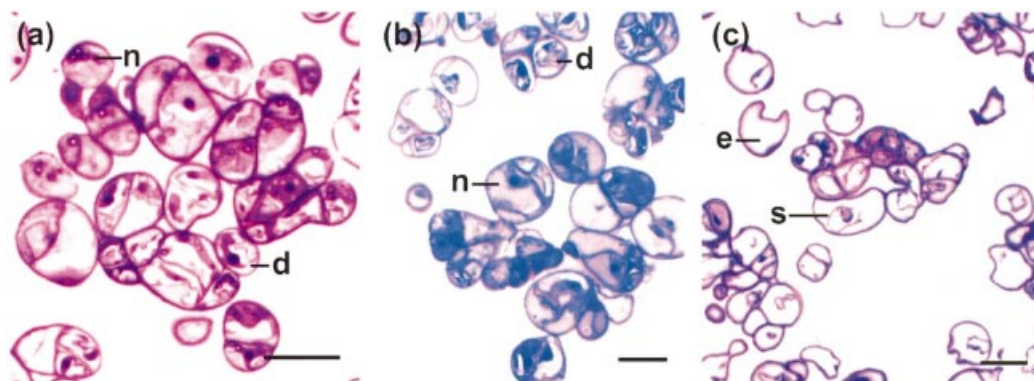


Figure 4. Structural changes in *Arabidopsis* suspension-cultured cells treated with Yariv reagents for 72 h and observed by light microscopy with Toluidine blue O staining.

(a) Untreated cells. (b) Cells grown in the presence of $80\ \mu\text{M}$ (α -D-galactosyl) $_3$ Yariv reagent. (c) Cells grown in the presence of $80\ \mu\text{M}$ (β -D-galactosyl) $_3$ Yariv reagent. Most of the cells in (a) and (b) showed normal morphology, with the plasma membrane appressed to the cell wall and an evident nucleus, whereas some cells showed a limited degree of cytoplasmic detachment. In contrast, most of the cells in (c) showed strong cytoplasmic shrinkage with no nucleus clearly seen, whereas some cells were empty. n, normal cell; d, cell showing cytoplasmic detachment; s, cell showing strong cytoplasmic shrinkage; e, empty cell. Bars = $50\ \mu\text{m}$.

Table 3. Effects of $80\ \mu\text{M}$ (α -D-galactosyl) $_3$ and (β -D-galactosyl) $_3$ Yariv reagents on *Arabidopsis* suspension-cultured cells treated for 72 h as determined by light microscopy^a

Treatment	Total cells	Normal cells	Cells with cytoplasmic detachment	Cells with cytoplasmic shrinkage	Empty cells
Untreated	533	244 (45.8%)	253 (47.5%)	0 (0.0%)	36 (6.7%)
(α -D-Gal) $_3$	425	234 (55.1%)	160 (37.6%)	0 (0.0%)	31 (7.3%)
(β -D-Gal) $_3$	423	0 (0.0%)	0 (0.0%)	315 (74.5%)	108 (25.5%)

^aThese data were obtained by scoring multiple fields of cells on semithin sections stained with toluidine blue O. Numbers in parentheses are percentages of cells with indicated morphology.

with DAPI or PI were also TUNEL-positive. Given the robust nature of this response, it was convenient to determine whether cells induced by this treatment display structural features characteristic of PCD. At the light microscope level, untreated cells and cells treated with $80\ \mu\text{M}$ (α -D-galactosyl) $_3$ Yariv reagent showed normal morphology (i.e. plasma membrane appressed to the cell wall and evident nucleus) or some degree of detachment of the plasma membrane from the cell wall (Figure 4, Table 3). Limited detachment may result from osmotic changes occurring in fixation and dehydration. Moreover, a small number of these cells were empty (Figure 4, Table 3). In stark contrast, 75% of the cells grown in $80\ \mu\text{M}$ (β -D-galactosyl) $_3$ Yariv reagent showed strong cytoplasmic shrinkage with no evident nucleus, while the remaining cells were empty (Figure 4, Table 3). Dying cells release their contents into intercellular spaces or culture media with cell walls left behind. Such empty cells are not engulfed by neighboring cells because of the presence of cell walls (del Pozo and Lam, 1998).

At the transmission electron microscope level, most cells treated with $80\ \mu\text{M}$ (β -D-galactosyl) $_3$ Yariv reagent for 72 h were characterized by cytoplasmic shrinkage and

condensation (Figure 5a,d), whereas the remaining cells were empty (Table 4). These observations were consistent with light microscopic analysis. Further examination of the shrunken cells revealed 5 out of 13 nuclei with chromatin condensation (Figure 5b,e, Table 4) and 6 out of 13 nuclei with nuclear membrane blebbing (Figure 5b,e, and f, Table 4). Although apoptotic bodies and plasma membrane blebbing were not observed, cytoplasmic shrinkage and condensation, chromatin condensation, and nuclear membrane blebbing were observed and represent structural hallmarks of animal cell apoptosis (Dangl *et al.*, 1996; Earnshaw, 1995; Groover *et al.*, 1997; Hockenbery *et al.*, 1993; McCabe *et al.*, 1997; Mignotte and Vayssiere, 1998; Pennell and Lamb, 1997). Cell condensation and shrinkage were also found in carrot suspension-cultured cells grown at low density or under moderate heat shock; both of these conditions were shown to activate a PCD pathway (McCabe *et al.*, 1997). In addition, organelles such as mitochondria still remained intact (Figure 5f) and membrane-bound vesicles with unknown contents were observed (Figure 5c,f).

Untreated cells (Figure 6a,b) and cells treated with $80\ \mu\text{M}$ (α -D-galactosyl) $_3$ Yariv reagent for 72 h (Figure 6c,d)

Figure 5. Ultrastructure of *Arabidopsis* suspension-cultured cells treated with 80 μM (β -D-galactosyl)₃ Yariv reagent for 72 h. Cells showed strong cytoplasmic shrinkage and condensation (a and d), chromatin condensation (b and e), nuclear membrane blebbing (b, e, and f), and the appearance of membrane-bound vesicles (c and f). Notably, organelles such as mitochondria remained intact (f). cc, chromatin condensation; cw, cell wall; m, mitochondrion; mv, membrane-bound vesicle; n, nucleus; nm, nuclear membrane; pm, plasma membrane; s, strong cytoplasmic shrinkage. Bars in (a) and (d) = 2 μm . Bars in (b), (c), and (e) = 1 μm . Bar in (f) = 0.5 μm .

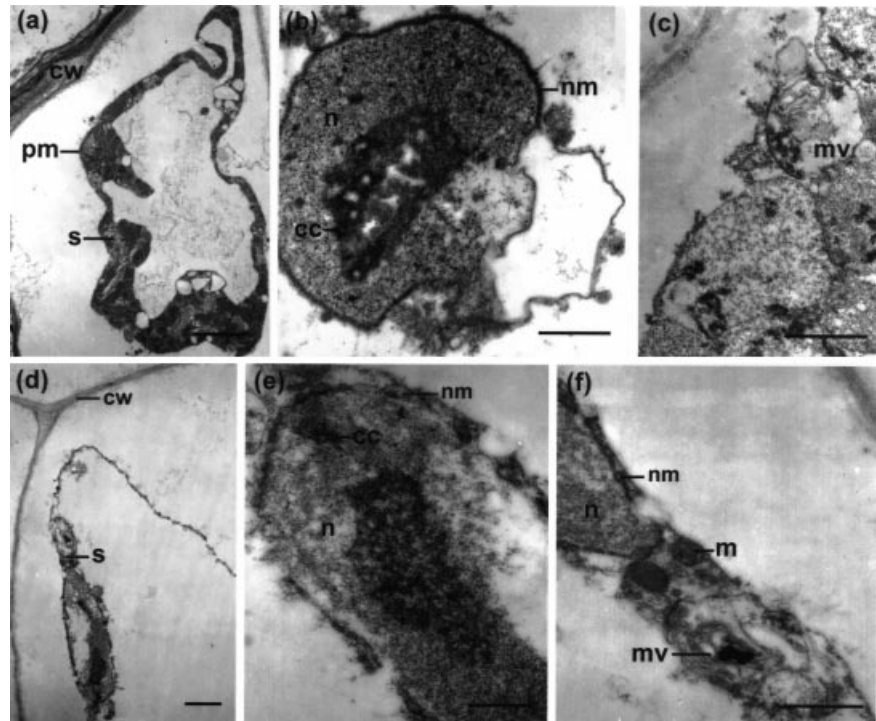


Table 4. Effects of 80 μM (α -D-galactosyl)₃ and (β -D-galactosyl)₃ Yariv reagents on *Arabidopsis* suspension-cultured cells treated for 72 h as determined by transmission electron microscopy^a

Treatment	Total cells	Normal cells	Cells with cytoplasmic detachment	Cells with cytoplasmic shrinkage	Empty cells	Nuclear characterization ^b		
						Nuclei observed	Chromatin condensation	Nuclear membrane blebbing
Untreated	26	10 (38.5%)	15 (57.7%)	0 (0.0%)	1 (3.8%)	23	0 (0.0%)	0 (0.0%)
(α -D-Gal) ₃	16	7 (43.8%)	8 (50.0%)	0 (0.0%)	1 (6.2%)	14	0 (0.0%)	0 (0.0%)
(β -D-Gal) ₃	36	0 (0.0%)	0 (0.0%)	26 (72.2%)	10 (27.8%)	13	5 (38.5%)	6 (46.2%)

^aThese data were obtained from ultra-thin sections. Cells observed were photographed and scored. Numbers in parentheses are percentages of cells (or nuclei) with indicated morphology.

^bChromatin condensation and nuclear membrane blebbing were scored in the nuclei observed in each treatment group.

appeared normal or showed a limited degree of detachment of the plasma membrane from the cell wall (Table 4). These observations were generally consistent with light microscopic analysis. Moreover, only one empty cell was observed in each of these cases, and there was no evidence of cells showing cytoplasmic shrinkage and condensation, chromatin condensation or nuclear membrane blebbing (Table 4).

In this paper, we show that perturbation of AGPs by Yariv reagent induces PCD by several criteria in *Arabidopsis* suspension-cultured cells and suggest that AGPs may be involved in other plant PCD responses. This

suggestion is based not only on this work, but also on other studies which have elucidated correlations between cells that are predetermined for, or have undergone, PCD and the occurrence of specific AGP epitopes at their cell surface (i.e. plasma membrane and/or cell wall). Xylem and pre-xylem cells, as examined in maize coleoptiles, *Arabidopsis* roots and tomato stems and petioles, afford compelling examples of AGPs serving as such tissue-specific markers of cells fated to die (Dolan *et al.*, 1995; Gao *et al.*, 1999; Schindler *et al.*, 1995). It now becomes important to examine such systems with causality in mind as well as to attempt the isolation of an endogenous

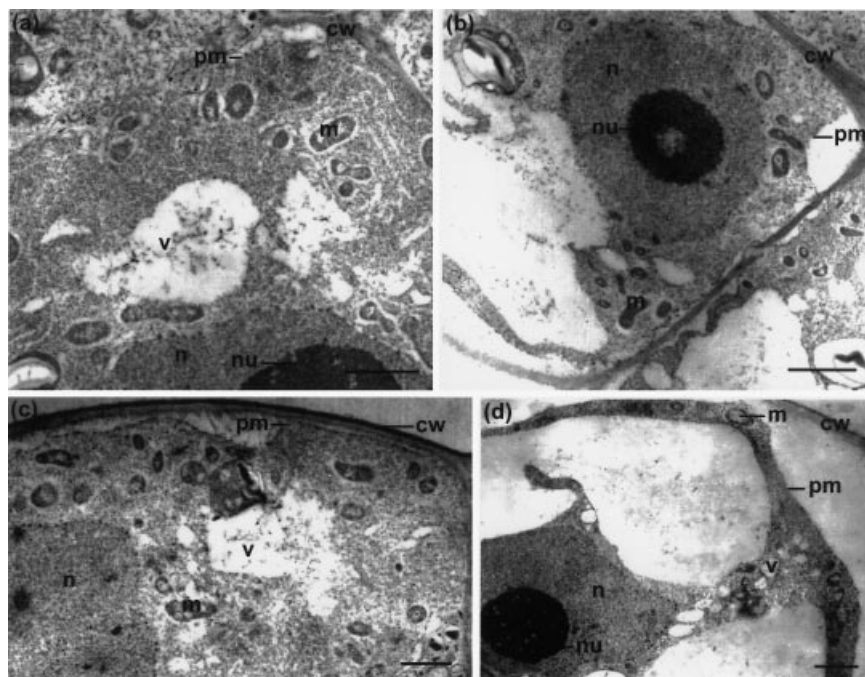


Figure 6. Ultrastructure of untreated *Arabidopsis* suspension-cultured cells and cells treated with $80\ \mu\text{M}$ (α -D-galactosyl) $_3$ Yariv reagent for 72 h.

Untreated cells (a and b) and cells treated with $80\ \mu\text{M}$ (α -D-galactosyl) $_3$ Yariv reagent (c and d) showed intact plasma membrane along with the cell wall, normal organelles such as mitochondria, vacuoles, and a nucleus with a nucleolus (a and c). Some cells (b and d) showed a limited degree of cytoplasmic detachment. cw, cell wall; m, mitochondrion; n, nucleus; nu, nucleolus; pm, plasma membrane; v, vacuole. Bars in (a–d) = $2\ \mu\text{m}$.

molecular counterpart to Yariv reagent. Furthermore, we suggest a mechanism by which Yariv reagent treatment, and hence AGP perturbations, can activate the PCD pathway in suspension-cultured cells as well as in other plant developmental events such as xylem differentiation. We postulate that Yariv reagent, through its ability to bind and aggregate AGPs, can disrupt plasma membrane–cell wall connections involving AGPs and/or can alter the normal distribution of plasma membrane AGPs by causing their aggregation so as to activate an intracellular PCD signal transduction pathway. Similarly, in animal cells, a disruption of ECM–cell interactions triggers apoptosis (Boudreau *et al.*, 1995; Boudreau *et al.*, 1996; Fine *et al.*, 1998; Meredith *et al.*, 1993; Singhal *et al.*, 1998). It might be equally informative, as well as consistent with the social control displayed here and elsewhere, to consider that AGPs serve as important signal molecules promoting cell growth and survival. In this context, it is interesting to note that Yariv reagent treatment of plant cell cultures, roots and germinating pollen leads to various forms of growth inhibition (Jauh and Lord, 1996; Langan and Nothnagel, 1997; Roy *et al.*, 1998; Willats and Knox, 1996) and, if examined further, may also reveal evidence of PCD.

Experimental procedures

Cell cultures and treatments

Arabidopsis suspension-cultured cells were grown under weak light (i.e. $9\ \mu\text{mol photons m}^{-2}\text{sec}^{-1}$) at 24°C on a rotary shaker (110 rpm). Cells were subcultured in Gamborg's B-5 medium

(Sigma, St. Louis, MO, USA) with different concentrations of (α -D-galactosyl) $_3$ or (β -D-galactosyl) $_3$ Yariv reagent (i.e. 30, 50, 60 and $80\ \mu\text{M}$) or in the absence of any Yariv reagent (i.e. untreated) as follows. Four ml of 7-day-old cultures were transferred into 20 ml of freshly prepared medium in a 50 ml flask containing the appropriate amount of Yariv reagent and subcultured for 1.5–72 h. Yariv reagents were synthesized as described by Yariv *et al.* (1962).

TUNEL assay, fluorescent microscopy and confocal laser scanning microscopy

To detect DNA fragmentation and determine the percentage of cells undergoing such fragmentation (i.e. TUNEL positive) in response to different treatments (i.e. untreated, (α -D-galactosyl) $_3$ and (β -D-galactosyl) $_3$ Yariv reagent) and treatment times (i.e. 24, 48, and 72 h), cells were fixed with 4% paraformaldehyde in PBS (10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 136.9 mM NaCl, 2.7 mM KCl, pH 7.2) and labeled by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) of DNA 3'-OH ends with an apoptosis detection kit as described by the manufacturer (Promega Co., Madison, WI, USA) with some modifications. These modifications include: (i) staining nuclei after the TUNEL reaction with 4',6-diamidino-2-phenylindole (DAPI) at a concentration of $1\ \mu\text{g ml}^{-1}$ in PBS rather than with propidium iodide (PI) solution for 10 min at room temperature; and (ii) mounting slides with 50% glycerol (in PBS) prior to observation under a Nikon Labophot-2 fluorescence microscope to determine the percentage of apoptotic cells. Many dispersed cell populations were sampled and scored randomly under the fluorescent microscope for both TUNEL-reactivity and DAPI staining. Approximately 1200 cells were scored in each treatment group by observing 23–27 different microscopic fields of view on multiple slides. To obtain images with much lower background, cells treated as above were also labeled with the apoptosis detection kit following procedures provided by the manufacturer (Promega), including use of PI, and observed under a confocal laser scanning microscope (Sarastro

2000 with a Nikon Optiphot-2 microscope using Molecular Dynamics software). Here, PI was used since the confocal microscope was not equipped to detect DAPI staining.

To determine the number of cells dying as a function of Yariv reagent treatment time, treated cells were washed twice with PBS and stained with $5\ \mu\text{g ml}^{-1}$ PI prepared in PBS at 25°C for 15 min. Following three washes in PBS to remove the staining solution, cells were observed with a Nikon Labophot-2 fluorescence microscope under green-light epifluorescence. PI is a nucleic acid stain and can only permeate cells with damaged or leaking cell membranes. Dying or dead cells with nucleic acid showed strong red fluorescence. Approximately 1000 cells were scored in each treatment group by observing 21–24 different microscopic fields of view on multiple slides. Furthermore, because cell cultures contained many small clumps of cells, these viability data should only be regarded as semi-quantitative estimates.

DNA extraction and analysis

To examine DNA fragmentation as a function of Yariv reagent dosage, DNA was isolated from *Arabidopsis* suspension-cultured cells treated with (β -D-galactosyl) $_3$ and (α -D-galactosyl) $_3$ Yariv reagent (30, 50, 60 and $80\ \mu\text{M}$) and untreated cells after 72 h by the method of Doyle and Doyle (1990). For each sample, $20\ \mu\text{g}$ of DNA was loaded per lane and electrophoresed in a 1.6% agarose gel at constant voltage (70 V). DNA was blotted onto a nylon (Zeta-Probe) membrane (Bio-Rad, Hercules, CA, USA) and hybridized to a ^{32}P -labeled *Arabidopsis* total genomic DNA probe at 54°C overnight. The *Arabidopsis* genomic DNA probe was prepared with a random primer labeling kit following the instructions of the manufacturer (Promega Co., Madison, WI, USA). Similarly, to examine DNA fragmentation as a function of Yariv reagent treatment time, DNA was isolated from *Arabidopsis* suspension-cultured cells treated with $80\ \mu\text{M}$ (β -D-galactosyl) $_3$ and (α -D-galactosyl) $_3$ Yariv reagent for various times (0 [untreated], 1.5, 3, 6, 9, 12, 24, 48, and 72 h) and analyzed by Southern blotting. Treatments from 0 to 12 h are not shown in Figure 3 since they did not exhibit any DNA degradation.

Light and electron microscopy

Cells treated with $80\ \mu\text{M}$ (β -D-galactosyl) $_3$ and (α -D-galactosyl) $_3$ Yariv reagent and untreated cells after 72 h were collected and washed in PBS. Cells were fixed in 2% paraformaldehyde and 1% glutaraldehyde (in 50 mM citrate-phosphate buffer, pH 7.4) for 2 h at 4°C and heated in a 1.25 KW microwave oven for 12 sec. Cells were washed with 50 mM citrate-phosphate buffer and post-fixed in 1% osmium tetroxide (in 50 mM citrate-phosphate buffer) for 1 h at room temperature. After cells were washed in 50 mM citrate-phosphate buffer for 5×10 min, they were dehydrated in an ethanol series and infiltrated with ethanol/L.R. White (Sigma, St. Louis, MO, USA) mixtures (3:1, 1:1, and 1:3 ethanol:L.R. White, each for 30 min) and L.R. White (2×1 h, 1×2 days). Cells were embedded in L.R. White resin in gelatin capsules and polymerized at 60°C for 24 h. Ultra-thin sections were stained with uranyl acetate for 45 min at room temperature and observed with a Zeiss EM 109 electron microscope. Semi-thin sections ($5\ \mu\text{m}$ thick) were also collected during the process of trimming blocks for ultra-thin sectioning and stained with 0.1% Toluidine blue O in 0.5% borax buffer for 30 sec on the hot plate before rinsing in distilled water. Slides were mounted with Fisher permount mounting medium (Fisher Scientific, Pittsburgh, PA, USA) and observed with a Nikon Labophot-2 fluorescence microscope under white-light illumination.

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