Plant Physiol. (1996) 112: 433–444

Correlation of Rapid Cell Death with Metabolic Changes in Fungus-Infected, Cultured Parsley Cells

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To study in detail the hypersensitive reaction, one of the major defense responses of plants against microbial infection, we used a model system of reduced complexity with cultured parsley (*Petroselinum crispum*) cells infected with the phytopathogenic fungus *Phytophthora infestans*. Experimental conditions were established to maintain maximal viability of the cultured cells during co-cultivation with fungal germlings, and a large proportion of the infected parsley cells responded to fungal infection with rapid cell death, thereby exhibiting major features of the hypersensitive reaction in whole-plant-pathogen interactions. Rapid cell death clearly correlated with termination of further growth and development of the fungal pathogen. Thus, the system fulfilled important prerequisites for investigating cell-death-related metabolic changes in individual infected cells. Using cytochemical methods, we monitored the increase of mitochondrial activity in single infected cells and the intracellular accumulation of reactive oxygen species prior to the occurrence of rapid cell death. We obtained strong correlative evidence for the involvement of these intracellularly accumulating reactive oxygen species in membrane damage and in the resulting abrupt collapse of the cell.

Plants defend themselves against pathogens in a largely cell autonomous manner, greatly distinct from the vertebrate immune system. Each plant cell is capable of defense against pathogens by both preformed and induced mechanisms. One of the most effective induced responses is the hypersensitive reaction (Keen, 1990; Goodman and Novacky, 1994). At the infection site immediately after invasion by a pathogen, the affected plant cell undergoes highly dynamic metabolic and structural changes and suddenly dies. This process of induced cell death is extremely rapid and is considered to be a major means of halting growth of the pathogen.

In animals, programmed cell death, or apoptosis, long has been known as a ubiquitous, active, and cell-autonomous control mechanism that is important in developmental and pathological processes (Ellis et al., 1991). Genetic data strongly suggest that cell death in animals is brought about by a differentiation program requiring the activation of specific sets of genes (Yuan and Horvits, 1990), at least one of which may be involved in regulating the generation of reactive oxygen species (Hockenbery et al., 1993). In plants, the molecular mechanisms leading to the abrupt hypersensitive death of cells attacked by a pathogen are largely unknown. However, the discovery of mutants exhibiting aberrant regulation of cell death (disease lesion mimic mutants) in several plant species, for instance in Arabidopsis (Dietrich et al., 1994; Greenberg et al., 1994), and recent results indicating the requirement of protein synthesis for hypersensitive cell death (He et al., 1994) also point to a genetic control of cell death in plants.

Our histological and cytological investigations aim at contributing to a mechanistic understanding of hypersensitive cell death in plants. We recently examined the defense response of living epidermal cells of potato leaves infected by the fungal pathogen *Phytophthora infestans*, the causal agent of late blight (Freytag et al., 1994). By analyzing in vivo numerous infection processes of single cells within an intact leaf, we obtained an extensive survey of various stages of the hypersensitive reaction, including the actual incidence of cell death.

Such a detailed study of individual steps of the defense response in whole-plant tissue has many limitations, since with few asynchronously occurring infection sites, only single or very few cells react that are spatially separated by the bulk of nonaffected tissue. Therefore, we established a system of reduced complexity that consists of immobilized fungal germlings (*P. infestans*) infecting cultured parsley (*Petroselinum crispum*) cells (Gross et al., 1993). This model system closely reflected the nonhost relationship between parsley plants and *Phytophthora megasperma* f. sp. *glycinea* (Jahnen and Hahlbrock, 1988; Schmelzer et al., 1989) and exhibited major features of the early plant defense reactions that we observed in the potato cells. It proved to be very useful in investigating the participation of the cytoskeleton in the translocation of cytoplasm and nucleus as well as the simultaneous expression of defense-related genes (Gross et al., 1993; Schmelzer et al., 1995).

Here we report that under modified experimental conditions infected cultured parsley cells also displayed rapid cell death as a major defense response. Thus, the model system now enabled the examination of physiological changes in relation to cell death. Two earlier observations are of particular interest in this connection. First, infected tissue showed an increased respiration rate (Uritani and Asahi, 1980; Hutcheson and Buchanan, 1983), and second,

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Abbreviations: DCF, 2′,7′-dichlorofluorescein; DCFH, 2′,7′-dichlorofluorescin; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; FDA, fluorescein diacetate; RH 123, rhodamine 123.

the generation of reactive oxygen species has frequently been found in plant-pathogen interactions (Doke, 1983; Sutherland, 1991; Mehdy, 1994) as well as elicitor-treated cultured cells (Apostol et al., 1989a), including parsley (Nürnberger et al., 1994). In the present study we measured the increase of the mitochondrial membrane potential and the accumulation of reactive oxygen species in individual fungus-infected cells. We demonstrate that the activation of energy metabolism and the accumulation of intracellular peroxides is correlated with induced rapid cell death, and that this rapid cell death terminates growth of the fungal pathogen.

MATERIALS AND METHODS

Cultivation of Plant Cells and Production of Fungal Cysts

Cell-suspension cultures of parsley (*Petroselinum crispum*) were grown in modified B5 medium as described by Kombrink and Hahlbrock (1986) and were used for experiments 5 to 7 d after subculturing.

Mycelium of *Phytophthora infestans* (race 1) was grown on vegetable juice agar. Small pieces of mycelium were transferred to rye agar and grown for 15 to 20 d at 15°C in high humidity to induce sporangia formation (Cuypers and Hahlbrock, 1988). Liberation of zoospores was accomplished by adding 10 mL of sterile water per plate and incubating at 4°C overnight. Synchronized encystment was achieved by vigorous shaking of the zoospore suspension (60 s on a whirl mixer).

Infection of Suspension-Cultured Cells

Infection experiments were performed by co-cultivation of germinated cysts and cultured parsley cells on microscopic slides as described by Gross et al. (1993). Briefly, microscopic slides were boiled in 0.1 N HCl for at least 1 h prior to use, rinsed two times in sterile water, and coated with an aqueous solution of 0.3% (w/v) gelatin and 0.01% chrome(III)-potassium sulfate dodecahydrate. Within a 50- × 20-mm oval well made of rubber cement, 100 to 500 μL of a cyst suspension (equivalent to 5×10^4 cysts) was evenly distributed on a microscopic slide with a small Drigalski spatula. Cysts germinated and adhered to the coated surface by incubation in a dark, humid chamber for 2 h at 17°C. The supernatant fluid was replaced by 400 μL of a parsley suspension culture (50% packed cell volume in Suc-free culture medium). Infection of cultured cells by fungal structures occurred during 2 h of incubation at 26°C in the dark and was stopped by dipping the microscopic slides in cell culture medium. The slides were again placed in the humid chamber, and the infected cells were covered with 400 μL of conditioned culture medium (cell culture supernatant enriched with 2 g/L Suc and 2 mg/L kinetin). To avoid evaporation, the specimens were covered with Biofolie 25 (Heraeus, Hanau, Germany).

Treatment with salicylic acid was at a final concentration of 20 μM for 24 h (Kauss et al., 1992). Before onset of co-cultivation, the cells were washed twice and tested for viability (FDA staining), and at the salicylic acid concentration that was used, no difference in viability was found between pretreated and control cells. *n*-Propyl gallate was used at a concentration (50 μM) that was effective in experiments of C. Douglas and M. Ellard (personal communication) with cultured parsley cells without affecting their viability; this concentration had no effect on fungal growth. When fungal cysts germinated in the presence or absence of 50 μM *n*-propyl gallate, the germ tubes reached a similar length. Application of scavengers of reactive oxygen species during co-cultivation were at the following final concentrations: superoxide dismutase, 100 μg/mL (Schwacke and Hager, 1992); catalase, 100 μg/mL (Schwacke and Hager, 1992); sodium ascorbate, 25 mM; mannitol, 50 mM.

Light and Electron Microscopy

Light microscopy was carried out with a photomicroscope (Axiophot, Zeiss) equipped with differential interference contrast (Nomarski), epifluorescence, and a dark-field condenser. The defense response of living infected cells was recorded by video microscopy as described earlier (Gross et al., 1993; Freytag et al., 1994). Cells stained with fluorescent dyes were examined under epifluorescent light using appropriate filter sets. Infected cells were fixed, embedded, and sectioned for transmission electron microscopy as described by Gross et al. (1993). Series of ultrathin sections of infected microcalli were used for ultrastructural analysis.

Cytochemical Staining Procedures

Staining with the vital dye FDA was used to distinguish living from dead cells (Widholm, 1972). A stock solution (5 mg/mL) was prepared in acetone and diluted with conditioned medium to a final concentration of 10 μg/mL. Cells were incubated in this staining solution for 15 min. Subsequently, the staining solution was replaced by conditioned medium. Cells were examined under epifluorescence light using an appropriate filter set (excitation filter, 450–490 nm; dichroic mirror, 510 nm; barrier filter, 520 nm). Cells that showed characteristic features of death, such as collapse of the protoplast and Brownian motion of organelles, remained unstained.

The vital dye neutral red was used to test for membrane integrity. Accumulation of the dye within the vacuole was observed under either bright light or UV epifluorescence (excitation filter, 365 nm; dichroic mirror, 395 nm; barrier filter, 420 nm). A stock solution of 5 mg/mL in water was diluted for staining to a final concentration of 0.05% in conditioned medium.

For staining of callose, cells were incubated in an alkaline solution of aniline blue (0.1% in culture medium, pH 8) for 5 min. Callose was detected under UV epifluorescence (excitation filter, 365 nm; dichroic mirror, 395 nm; barrier filter, 420 nm).

Nuclei were stained with the fluorescent dyes bisbenzimide trihydrochloride (no. 33,342 or 33,258; Hoechst, Frankfurt am Main, Germany) or 4,6 diamidino-2-phenylindole dihydrochloride. The stock solution contained 2 mg/mL 4,6 diamidino-2-phenyl-indole-dihydrochloride in distilled water and was diluted to a concentration of 2

μg/mL for staining. Nuclei were visible under UV epifluorescent light (excitation filter, 365 nm; dichroic mirror, 395 nm; barrier filter, 420 nm).

Staining of Active Mitochondria

Mitochondria were stained with the positively charged fluorescent dye RH 123 (Sigma) as described by Wu (1987), Chen (1988), and Vannini et al. (1988). A final RH 123 concentration of 10 μg/mL (stock solution: 1 mg/mL in distilled water was used, and cells were loaded with the dye for 10 min. Excess dye was removed by rinsing the microscope slides twice for 10 min in conditioned medium. Staining resulted in intense fluorescence of small rod-like organelles of mitochondrial size moving within cytoplasmic strands. RH 123 fluorescence was never associated with other cellular compartments. Addition of the protonophore 2,4-dinitrophenol (100 μM) or the Cyt *c* oxidase inhibitor NaN_3 (10 mM) caused a rapid decrease of the mitochondrial fluorescence. Only a weak, gradually decreasing fluorescence of the cytoplasm remained. A decrease in staining intensity was also observed when cells were kept under anaerobic conditions. Dead cells never showed any RH 123 fluorescence.

Cells were examined by epifluorescence microscopy (excitation filter, 450–490 nm; dichroic mirror, 510 nm; barrier filter, 520 nm). The relative intensity of the yellow-green fluorescence of the dyes was estimated by comparing infected with adjacent, noninfected cells. Micrographs were taken on Ektachrome 800/1600 film (Kodak).

Alternatively, 3,3'-dihexyloxacarbocyanide iodide (Sigma) was used at a final concentration of 0.1 to 1 μM in conditioned medium (stock solution: 2, 5 mg/ml in ethanol) according to the method of Matzke and Matzke (1986). In contrast to RH 123, 3,3'-dihexyloxacarbocyanide iodide stained in addition to mitochondria a region surrounding the nucleus and, in collapsed cells, the cytoplasm and small particles of an unknown nature.

Visualization of Intracellular Peroxides

A stock solution of DCFH-DA (2 mg/mL acetone; Molecular Probes, Eugene, OR) was diluted 1:500 with culture medium immediately before use. Cultured cells were loaded with DCFH by incubation for 10 min and then washed briefly and covered with conditioned medium. Oxidation by intracellular peroxides produced the fluorescing dye DCF (Bass et al., 1983; Cathcart et al., 1983).

To avoid photooxidation under blue epifluorescent light (excitation filter, 450–490 nm; dichroic mirror, 510 nm; barrier filter, 520 nm), pictures were taken using light-intensifying video microscopy. A charge-coupled device video camera combined with a light-intensifying head was mounted to the microscope and connected to a frame grabber board within a PC/AT computer. Epifluorescent light was allowed for 2 s using a shutter, and the microscopic image was recorded with the light-intensifying video camera; individual frames were converted into digital information and stored in computer memory. Images were improved by background subtraction and digital contrast enhancement using appropriate image-processing software (BioScan Optimas, Edmonds, WA). Pictures were displayed on a high-resolution monitor and photographs were taken on Agfa Pan 400 film (Agfa, Leverkusen, Germany).

Time-Course Experiments and Statistical Evaluation

Prior to infection experiments the viability of suspension-cultured cells was verified by staining with FDA. Usually more than 95% of the cells were viable, as indicated by their rapid accumulation of the dye. Occasionally a very small number of dead microcalli were found, but these were never infected by the fungus. Therefore, we concluded that no dead, infected cells were present at the beginning of infection.

For time-course experiments at least three microscope slides with infected cells were prepared for each time. With the addition of cultured cells co-cultivation was started. The first assessment was not made until 1.5 h after the onset of co-cultivation to allow elongation of the fungal germ tubes, formation of appressoria, and adhesion of cells. A minimum of 100 infected microcalli from the three microscope slides were randomly selected for inspection at each time. When cells had been stained with FDA, DCFH-DA, RH 123, or neutral red, the staining intensity of infected cells was estimated relative to adjacent, noninfected cells. Of the total number of inspected, infected cells at each time, the percentage of infected cells displaying highly intensive staining was determined. In addition, infected cells were examined for cytoplasmic streaming, translocation of cytoplasm and nucleus toward the penetration site, cell death, and development and growth of the fungal pathogen (formation of an appressorium, penetration of the cell wall, formation of secondary hyphae). Experiments were usually performed three times.

Linear regression analysis determined whether the differences of the kinetics were significant due to the various cell ages and treatments. The linear regression model also included a linear trend over time and an indicator variable for cell age or treatment. The null hypothesis, asserting that there was no change over time (slope = 0) and that there were no differences between the kinetics of age dependency or of treatment, was tested with F tests. All experiments were considered to be independent and representative and hence were analyzed jointly. Nonlinear kinetics were analyzed in two steps, the linear part by linear regression and the nonlinear part (plateau) by a two-sided nonparametric Wilcoxon-Sum-Rank test or median test. P values of less than 0.05 were regarded as significant. Linear regression coefficients were compared using a two-sided Student's t test. Fit of the regression models was evaluated by the R^2, which was generally high. All calculations were performed using SAS 6.09 software (SAS Institute, Cary, NC).

RESULTS

Types of Parsley Cell-Fungus Interaction

Previous co-cultivation of parsley cells with germlings of *P. infestans* was performed in the cell culture supernatant (Gross et al., 1993). These conditions were now modified to

ensure maximal viability of the plant cells. The cell culture supernatant was complemented with 2 mg/L kinetin and 2g/L Suc. Furthermore, for prolonged observation in vivo on microscope slides, the infected microcalli were covered with Biofolie 25 rather than with coverslips. Since Biofolie 25 is biocompatible (Hoffmann et al., 1994) and permeable for gases such as O_2 and CO_2 but impermeable for water, the evaporation of water was prevented but the diffusion of O_2 was not. Thus, the viability of the plant cells, as indicated by their constant cytoplasmic streaming, was maintained for at least 24 h.

Using these modified conditions, we examined numerous infection events in vivo by video microscopy. We observed in one type of parsley cell-fungus interaction that fungal penetration induced rapid death of the major proportion (up to 60% at 8 h after onset of co-cultivation) of infected cells (Fig. 1, A–D). This event showed major features of the hypersensitive cell death observed with fungus-infected cells in intact potato leaf tissue (Freytag et al., 1994). Subsequent to translocation of cytoplasm and nucleus to the penetration site, and again similar to previous findings with potato and parsley cells (Gross et al., 1993; Schmelzer et al., 1995), the cytoplasm appeared to be more and more granular, cytoplasmic streaming ceased, and finally the protoplast collapsed and shrank. Prior to this collapse no change in appearance or in size of the nucleus was noticed by light microscopy. The shrunken cellular content autofluoresced bluish under UV epifluorescent light (Fig. 1B) and the cell was no longer stained by the vital dye FDA (Fig. 1D). The parsley cell-suspension culture consisted of small microcalli as well as single cells. It is noteworthy that such single, infected cells also showed induced rapid cell death with the same appearance and at a rate similar to cells within microcalli. Rapid cell death was usually associated with arrest of further growth and development of the fungal germling (Fig. 1C; no formation of secondary hyphae). We named this type of parsley cell-fungus interaction "prevented infection."

In a second type of interaction with the fungus, the cultured parsley cell rapidly formed a local callose-containing barrier underneath the fungal appressorium, as described earlier for the previous conditions (Gross et al., 1993) as well as for epidermal cells of potato leaves (Freytag et al., 1994). By applying the modified conditions, we observed that this local barrier prevented fungal invasion and that no further growth and development of fungal structures occurred (Fig. 1, E and F). When the local barrier was complete the nucleus migrated away from its position close to the attempted penetration site and the cell survived. Using the term introduced by Gees and Hohl (1988), we called this type of cultured parsley cell-fungus interaction "abortive penetration."

A third type of interaction was characterized by the development of secondary intracellular or extracellular hyphae of the fungus and delayed death of the infected, cultured parsley cell (Fig. 1, G and H). As revealed by FDA staining, the infected cell often was still living at 24 h of co-cultivation (Fig. 1H). In most cases the infected cell did not collapse but gradually ceased cytoplasmic streaming and finally showed Brownian motion of intracellular particles. In many cases extracellular secondary hyphae infected other adjacent parsley cells. We named this type of cultured parsley cell-fungus interaction "successful infection."

Fine-Structural Comparison of Infected and Noninfected Cells

To detect possible morphological changes in infected, cultured parsley cells, we used electron microscopy. Clear differences in the appearance of membranes and in cytoplasmic morphology were observed between infected and adjacent, noninfected cells (Fig. 2). At 5 h of co-cultivation in the infected cell, all membranes (plasmalemma, tonoplast, ER, and mitochondrial membranes) were stained diffusely and thus appeared to be degenerated. In addition to this blurred image of the entire membrane system, the mitochondria in particular showed substantial morphological changes. The mitochondrion in the infected cell (Fig. 2A) was considerably swollen and tubuli were disintegrated in contrast to mitochondria in noninfected cells (Fig. 2B).

Influence of Cell Age on Infection-Induced Death

To examine the influence of the age of cultured parsley cells on their defense response, particularly on infection-induced cell death and on fungal development, cells were taken at three different times (5, 6, and 7 d) after subculturing for infection experiments with *P. infestans*. The proportion of dead cells increased rapidly during the first 6 h after onset of co-cultivation (Fig. 3A) with a clear-cut dependence on cell age: the older the cells, the smaller the increase of dead, infected cells. During the subsequent period of co-cultivation the death rate of the cells increased only slowly and slightly to similar values of 51 to 54% at 24 h (Fig. 3B, left half of the pie charts).

In an additional experiment at 24 h of co-cultivation, the parsley cell-fungus interaction was characterized with respect to both cell death and fungal growth (formation of secondary hyphae). At this late time the correlation between cell age and the ability to defend fungal infection was even clearer (Fig. 3B). Regardless of whether the infected cells were dead or still alive, the formation of secondary fungal hyphae was more reduced with the younger cells, and dead cells always had fewer secondary hyphae than the cells that were still alive.

Mitochondrial Activity in Infected Cells

We then investigated whether the changes that occurred in the infected cells correlated with an increase in energy metabolism. The fluorescent dye RH 123 specifically stains mitochondria in living cells (Chen, 1988). According to previous studies (Johnson et al., 1981; Emaus et al., 1986) the staining is based on the potential-dependent partitioning of the positively charged lipophilic dye molecules specifically across mitochondrial membranes; the higher the membrane potential of mitochondria, the more of the dye is accumulated. In animal cells a correlation between the mitochondrial accumulation of RH 123 and energy-

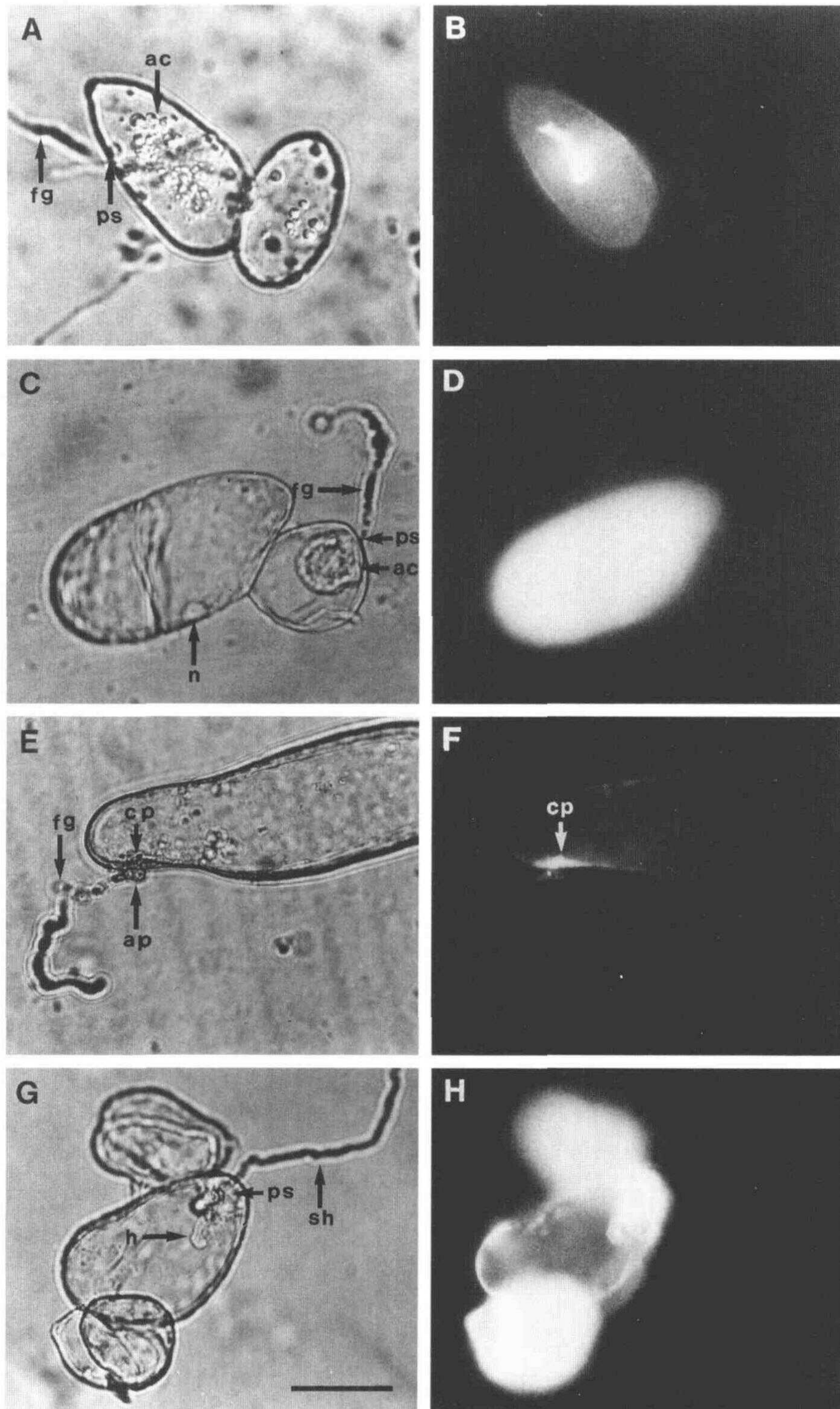

Figure 1. Types of parsley cell-fungus interactions. During co-cultivation of cultured cells of parsley and germlings of *P. infestans,* infected cells were inspected at various times by bright-light and epifluorescence microscopy. Micrographs indicate infected cells that underwent rapid cell death under bright-light (A and C) and epifluorescence optics (B and D). The dead cell shows autofluorescence under UV epifluorescent light at 6 h of co-cultivation (B) and the noninfected adjacent cell FDA staining at 24 h of co-cultivation (D). Also shown is apposition of a callose plug at 5 h of co-cultivation (E and F), bright-light micrograph (E), epifluorescence micrograph after staining with aniline blue (F), and a successfully parasitized cell at 24 h of co-cultivation under bright light (G), and epifluorescence light after FDA staining (H). ac, Aggregated cytoplasm; ap, appressorium; cp, callose plug; fg, fungal germ tube; h, haustorium; n, nucleus; ps, penetration site; sh, secondary hyphae. Bar = 40 μm.

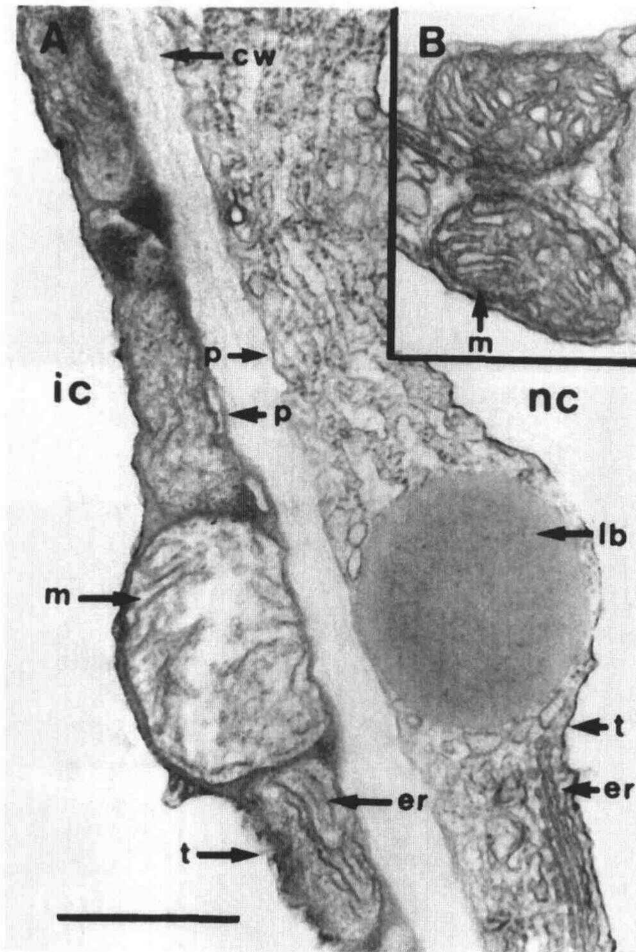

Figure 2. Electron microscopical analysis of the fine structure of infected and noninfected cells at 5 h of co-cultivation. A, Electron micrograph of part of an osmium tetroxide-stained ultrathin section of an infected (ic) and directly adjacent, noninfected (nc) cultured parsley cell. B, Mitochondria of the noninfected cell are shown. cw, Cell wall; er, ER; lb, lipid body; m, mitochondrion; p, plasmalemma; t, tonoplast. Bar = 1 μm.

consuming processes, such as motility or contraction, has been demonstrated (Johnson et al., 1981).

RH 123 proved to be suitable to cytochemically monitor the mitochondrial activity in infected microcalli of parsley. After a short preincubation period, the majority of infected cells displayed a massive accumulation of RH 123 specifically in mitochondria, whereas noninfected cells showed only a low background fluorescence (Fig. 4B). Cytoplasmic aggregation and hence accumulation and high motility of mitochondria at the penetration site resulted in an intense, localized fluorescent blur morphologically not resolved because of the long exposure time needed for epifluorescence photography.

When the proportion of living, infected cells stained by RH 123 was determined during the first 6 h after onset of co-cultivation, the values were highest (70–80%) at 1.5 h, the first time of the assessment, and decreased steadily to 40 to 50% by 6 h. In contrast to the death rate, the accumulation of RH 123 in infected cells was not dependent on the cell age. Also irrespective of cell age, we observed translocation of the nucleus to the penetration site during the first 5 h after onset of infection in 70% of the infected cells. It appeared that energy-consuming processes such as cytoplasmic rearrangements were activated in a high proportion of infected cells immediately upon fungal penetration, irrespective of the age and the rate of rapid death of the cells, and that these processes were associated with an elevation of the mitochondrial membrane potential.

Double-staining experiments with RH 123 and the vital dye neutral red revealed that the accumulation rate of neutral red decreased during the first 6 h after onset of co-cultivation simultaneously with the RH 123 fluorescence. This finding indicated that increasing membrane degeneration resulted in the gradual loss of mitochondrial functionality.

Production of Reactive Oxygen Species

When cultured cells were incubated wih salicylic acid prior to infection, rapid cell death was promoted, resulting in an increased rate at 24 h after onset of co-cultivation (20% above the control without salicylic acid). Conse-

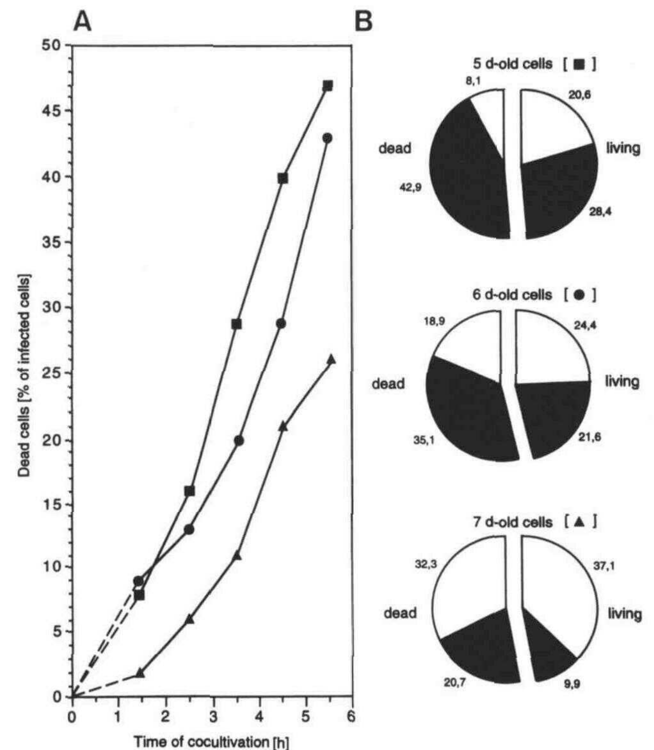

Figure 3. Rate of rapid cell death and development of the pathogen. Infected cells of different ages (■, 5-d-old cells; ●, 6-d-old cells; ▲, 7-d-old cells) were assayed for viability using FDA staining. At various times of co-cultivation the number of dead cells was determined (A). At 24 h after onset of co-cultivation, determination of the number of dead (B, left half of the pie charts) and living cells (B, right half of the pie charts) by FDA staining was combined with assessment of pathogen development. White area indicates formation of secondary hyphae (successful infection); black area indicates lack of fungal development (prevented infection).

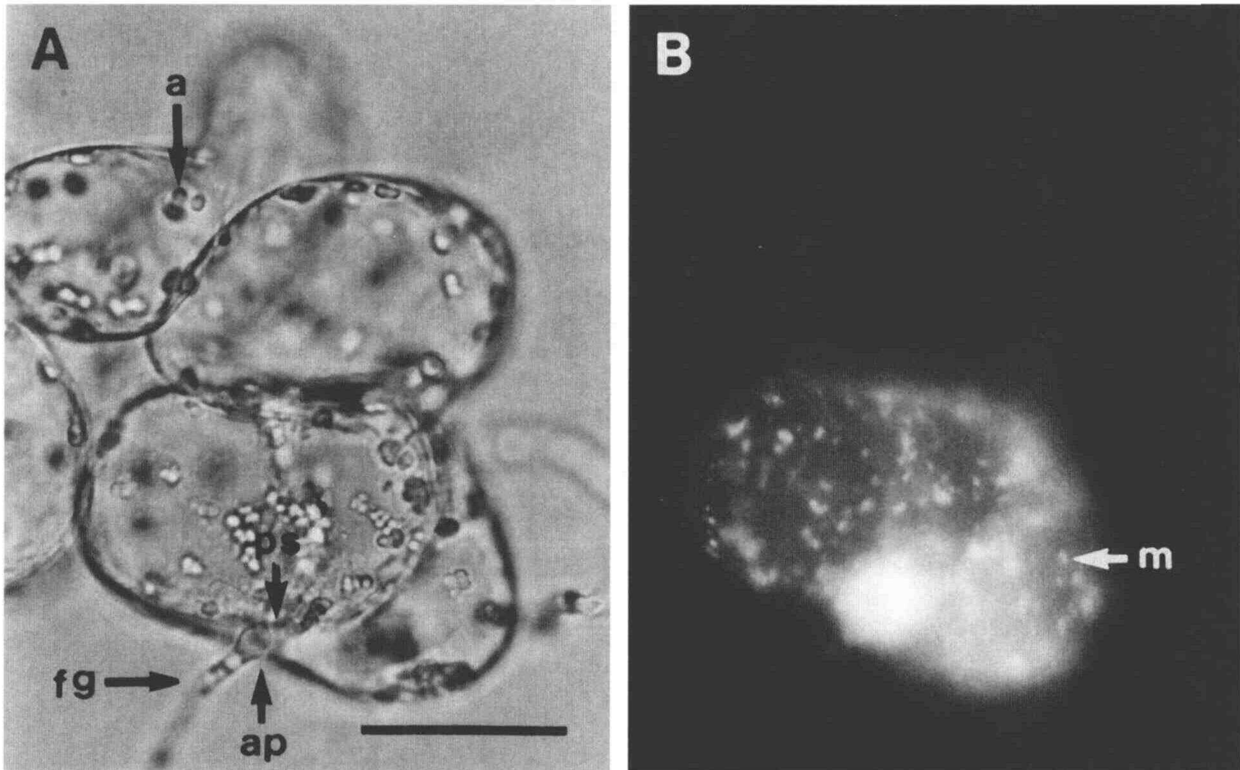

Figure 4. Staining of active mitochondria in infected cells. At 2 h after onset of co-cultivation parsley cells were stained with RH 123. Micrographs were taken under bright light (A) and epifluorescence light (B). a, Amyloplast; ap, appressorium; fg, fungal germ tube; p, penetration site; m, mitochondrion. Bar = 40 μm.

quently, formation of secondary hyphae of the fungus was reduced. The opposite effect was observed in the presence of *n*-propyl gallate. At 24 h after onset of co-cultivation the proportion of rapidly dying infected cells was drastically reduced (40% less than the control without *n*-propyl gallate) and formation of secondary fungal hyphae was enhanced 2-fold. Since salicylic acid has been suggested as an inhibitor of catalase (Chen et al., 1993; Sanchez-Casas and Klessig, 1994), whereas *n*-propyl gallate is a scavenger of reactive oxygen species and inhibitory to lipoxygenases (Peterman and Siedow, 1983), we investigated whether the level of intracellular reactive oxygen species is correlated with the rate of rapid cell death.

We monitored directly the accumulation of intracellular peroxides by using the membrane-permeable, nonfluorescing compound DCFH-DA. This compound is trapped in living cells in a manner similar to FDA, by cleavage by intracellular esterases. Intracellular peroxides then oxidize the trapped DCFH to the highly fluorescing DCF (Bass et al., 1983; Cathcart et al., 1983). The same conversion occurs by photooxidation (Burow and Valet, 1987). By using short light flashes and light-intensifying video microscopy for recording the fluorescence, we were able to avoid photooxidation. Under such experimental conditions a large proportion of infected cells showed a bright fluorescence after staining with DCFH-DA as compared with weakly fluorescing noninfected cells (Fig. 5B). By contrast, when the cells were subjected to prolonged irradiation with the excitation light (blue light), noninfected cells always fluoresced with similar intensity as infected cells (Fig. 5C). With this control experiment we could demonstrate that all cells contained the nonfluorescing precursor DCFH, but in the absence of light only the infected cells possessed sufficiently high levels of peroxides to convert the nonfluorescing to the fluorescing compound DCF.

An additional set of experiments was performed to monitor the accumulation of peroxides within infected parsley cells in relation to cell age and in the presence of salicylic acid and *n*-propyl gallate. The proportion of infected cells with intense DCF fluorescence was remarkably dependent on cell age (Fig. 6A). At the first point of observation, the vast majority of the 5-d-old infected cells, only one-half of the 6-d-old infected cells, and less than 40% of the 7-d-old infected cells showed accumulation of intracellular peroxides. These values decreased during the first 6 h after onset of infection. As shown in Figure 3A, the rate of rapid death of infected cells increased during the same period with a similar dependence on cell age. When 5-d-old cells that exhibited a high capacity to accumulate intracellular peroxides were treated with *n*-propyl gallate during the infection, the percentage of infected cells displaying intense DCF fluorescence was drastically reduced, and, concomitantly, the rate of rapid cell death was reduced appreciably (Fig. 6B). The opposite results were obtained when 7-d-old cells with their low capacity of peroxide accumulation were treated with salicylic acid prior to infection. The rate of peroxide accumulation by the cells was increased concomitantly with the rate of rapid cell death (Fig. 6C). In

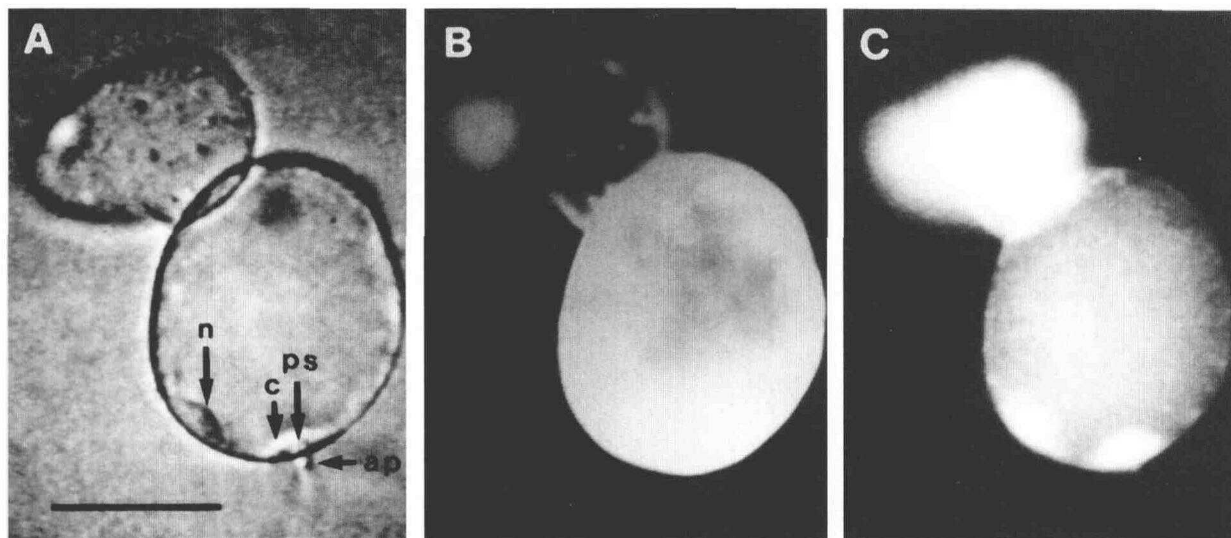

Figure 5. Monitoring of intracellular reactive oxygen species in infected cells. After 1.5 h of co-cultivation infected cells were stained with DCFH-DA, washed, and covered with dye-free medium. Pictures were taken using a light-intensifying charge-coupled device video camera under bright light (A) or epifluorescence light (B and C). B, Picture of the microcallus using a short pulse of blue light; C, same microcallus after a 2-min irradiation with blue light. ap, Appressorium; c, cytoplasm; ps, penetration site; n, nucleus. Bar = 40 μm.

summary, these data demonstrate a clear correlation between the accumulation of intracellular peroxides and the occurrence of rapid cell death.

We also examined whether extracellularly generated reactive oxygen species are involved in the process of infection-induced rapid cell death by applying scavengers of reactive oxygen species during co-cultivation of the cultured parsley cells and the fungal germlings. The substances used (superoxide dismutase and catalase, either alone or in combination, sodium ascorbate, and mannitol) had no effect on infection-induced rapid cell death and formation of secondary fungal hyphae.

Double-staining experiments using DCFH-DA and the vital dye neutral red enabled us to examine simultaneously the accumulation of intracellular peroxides and the loss of membrane integrity. Four types of staining pattern with the two dyes were found: (a) Infected cells displaying both intense DCF fluorescence and strong neutral red accumulation; such cells were obviously vital and peroxide producing. (b) Infected cells showing intense DCF fluorescence only after treatment with blue light but nevertheless accumulating neutral red; these cells were still vital but did not produce peroxides. (c) Infected cells with weak fluorescence that could not be enhanced by blue light treatment and weak neutral red staining; these cells were considered to be "leaky" and possibly dying. (d) Infected cells stained neither with DCF nor with neutral red; these cells were obviously dead.

During the first 6 h after onset of co-cultivation, the two major, most drastically changing populations were the vital, peroxide-producing cells and the dead cells. The vital, peroxide-producing cells decreased strongly and the dead cells increased to a similar extent (Fig. 7). This inverse correlation indicated the rapid transition of living, peroxide-producing cells with intact membranes to collapsed, dead cells. Of the two minor populations of double-stained infected cells, the low proportion of leaky cells remained constant (15% of infected cells) and the proportion of nonperoxide-producing but still living cells increased slightly (from 14 to 25% of infected cells). The latter cells were considered to be those that stayed alive and might be successfully infected by the fungus.

DISCUSSION

In a previous paper (Gross et al., 1993) we reported that the defense response of fungus-infected, cultured parsley cells resembled closely the hypersensitive reaction of tissue cells in whole-plant-fungus interactions, with one major exception: the almost complete lack of rapid cell death. Now, we have included this reaction by slightly changing the conditions of co-cultivation of the cultured cells and fungal germlings. Obviously, supplementation of the cell culture supernatant with low concentrations of Suc and/or kinetin stimulated the cells to perform infection-induced rapid cell death without altering growth, development, or virulence of the fungal pathogen. It is known that exogenously applied kinetin promotes or facilitates rapid cell death in infected plant (Chen and Heath, 1991) and callus tissue (Miller et al., 1984) but the mechanism for this effect is obscure. One important clue, however, is the observation that low concentrations of kinetin activated respiration of suspended soybean callus cells (Moore and Miller, 1972), suspension-cultured maize cells, and epidermal cells of barley and onion (Liu et al., 1987).

Under the improved conditions of co-cultivation the viability of the cells was maintained over a long period. This was demonstrated by the detection of sustained expression of defense-related genes using in situ RNA hybridization. At a time as late as 15 h after onset of co-cultivation,

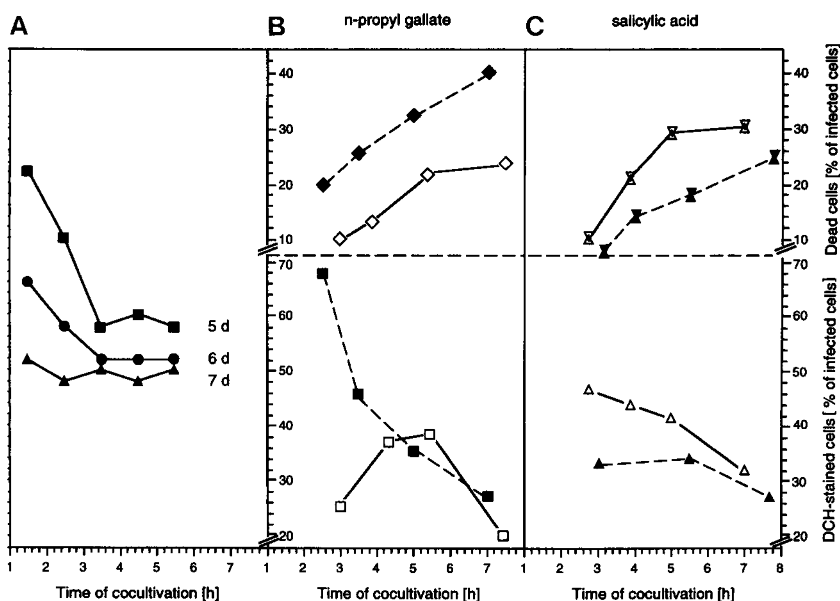

Figure 6. Accumulation of intracellular peroxides and occurrence of rapid cell death. Cultured cells of different ages were co-cultivated with fungal germlings and assayed at various times for accumulation of reactive oxygen species using DCFH-DA staining (A) and, after pretreatment with *n*-propyl gallate (B) or salicylic acid (C), for occurrence of induced rapid cell death by staining with FDA (B and C). At the times indicated the number of cells of different ages (■, 5-d-old cells; ●, 6-d-old cells; ▲, 7-d-old cells) displaying bright DCF staining was determined (A). Prior to co-cultivation with fungal germlings, 5-d-old cells were treated with *n*-propyl gallate (B) and 7-d-old cells were treated with salicylic acid (C). At various times the number of infected cells stained with DCF and the number of infected, dead cells was determined. Cells accumulating reactive oxygen species: ■, *n*-propyl gallate-treated 5-d-old cells; ■, untreated 5-d-old control cells; △, salicylic acid-treated 7-d-old cells; ▲, untreated 7-d-old control cells. Dead cells: ◇, n-propyl gallate-treated 5-d-old cells; ◆, untreated 5-d-old control cells; ⧖, salicylic acid-treated 7-d-old cells; ⧗, untreated 7-d-old control cells.

defense-related genes such as Phe ammonia-lyase and chitinase were still expressed in infected microcalli (B. Naton, Y. Ponath, and E. Schmelzer, unpublished results). All defense-related genes examined so far, including Phe ammonia-lyase, an anionic peroxidase, chitinase (B. Naton, Y. Ponath, and E. Schmelzer, unpublished results), and pathogenesis-related protein 1 (Gross et al., 1993), were activated simultaneously in the infected cell and in all other cells of an affected microcallus, whether or not induced rapid cell death occurred. Hence, expression of these genes cannot be involved in the triggering of hypersensitive cell death. A similar conclusion had been drawn by Jakobek and Lindgren (1993) from their studies of expression of defense-related genes in bean.

Defense reactions such as the synthesis of cell-wall material, translocation of cytoplasm and nucleus, and expression of defense-related genes, all of which precede rapid cell death (Freytag et al., 1994), are certainly energy-demanding processes. Thus, it is conceivable that the infected cells need to be supplied with sufficient energy to fulfill all the requirements to complete the hypersensitive reaction, including rapid cell death. The increase in mitochondrial activity that we have observed in the cultured parsley cells immediately after infection with *P. infestans* may serve to provide a sufficient level of ATP and thus may be an important contribution toward reaching such a state. In substantiating and extending earlier findings obtained by Kombrink and Hahlbrock (1986), Norman et al. (1994) reported an immediate increase and a long-term enhancement in the rate of respiratory CO_2 evolution in elicitor-treated cultured parsley cells. The enhanced rate of CO_2 evolution corresponded to the activation of phosphofructokinase and Glc-6-P dehydrogenase, the key enzymes of glycolysis and the oxidative pentose-P pathway. These data indicate a role for the activation of primary metabolism in the defense response supplying the increased need for certain metabolites, such as ATP, NADPH, and ribulose 5-P, as well as other substrates necessary for the biosynthesis of defensive compounds.

However, since in our experiments, in contrast to the rate of rapid cell death, the increase in mitochondrial activity was not dependent on the age of the cultured cells, the mere activation of energy metabolism does not appear to be the only criterion for reaching a state of competence to perform rapid cell death. In addition, other as-yet unknown factors may determine the responsiveness of the cells. Previous observations demonstrated that cultured cells at the early exponential growth stage were much more responsive to external stimuli, such as elicitor or UV light, than cells at later stages (Heller et al., 1979; Kombrink and Hahlbrock, 1985; Apostol et al., 1989b). For maximal responsiveness and the capability to perform rapid cell death, the cultured cells need to be in a very distinct physiological state that may be favored by an early growth stage and the presence of Suc and/or kinetin. These observations may be related to the variation in resistance levels

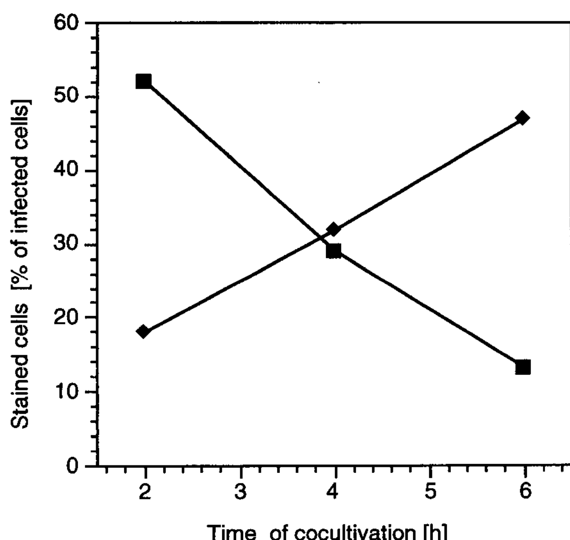

Figure 7. Double staining with DCFH-DA and neutral red. At various times of co-cultivation cells were stained simultaneously with DCFH-DA and neutral red. The resulting staining patterns of infected cells were grouped into four categories and the respective numbers of cells were determined. ■, Cells with intense DCF fluorescence as well as neutral red staining (viable and peroxide producing cells); ◆, cells with no staining at all (dead cells).

that have been observed for several plants; often, young plant parts and tissues display higher resistance levels as compared with old ones (Bell, 1981).

Our data strongly suggest a key role for the intracellular accumulation of reactive oxygen species in the process of induced rapid cell death. We do not think that extracellularly generated reactive oxygen species are of major significance for the occurrence of rapid cell death, at least in our in vitro model system, since addition of catalase, superoxide dismutase (either alone or in combination), and the scavengers of reactive oxygen species, sodium ascorbate and mannitol, applied before or during co-cultivation, had absolutely no effect on the infection-induced rapid cell death of cultured parsley cells. Extracellular reactive oxygen species may be involved in limiting pathogen growth (Merzlyak et al., 1990; Peng and Kuc, 1992), strengthening of the cell wall (Bradley et al., 1992; Brisson et al., 1994), and triggering activation of defense-related genes (Chen et al., 1993; Levine et al., 1994; Mehdy, 1994). In contrast, intracellular reactive oxygen species had never been monitored previously in individual infected plant cells (due to difficulties in measurement) and hence could not be examined as possible mediators of rapid cell death.

We have now cytochemically detected the accumulation of reactive oxygen species in infected parsley cells and, moreover, have found this process to be related to rapid cell death. We argue first that the rate of accumulation of reactive oxygen species showed the same dependency on cell age as did rapid cell death. Second, when the rate of rapid cell death was either promoted by application of salicylic acid or reduced by application of *n*-propyl gallate, the rate of accumulation of reactive oxygen species changed in the same way. Third, the double-staining experiments with DCFH-DA and neutral red, together with the electron microscope pictures, imply a close correlation between the intracellular accumulation of reactive oxygen species and the loss of membrane integrity. Reactive oxygen species, mainly highly aggressive oxygen radicals, are thought to cause lipid peroxidation, which may represent a self-propagating process and a source of new radical species (Sutherland, 1991; Tzeng and De Vay, 1993). Unsaturated fatty acids may become more and more oxidized and be removed from the lipid bilayer. H_2O_2 has been proposed as being the trigger of the activation of lipoxygenases (Kulkarni et al., 1990). Thus, enhanced degradation of fatty acids that have been released by lipases may occur via the lipoxygenase pathway. This may cause increasing membrane deterioration, finally resulting in the collapse of the protoplast. The origin of reactive oxygen species in plant cells is still a matter of debate (Sutherland, 1991). Oxygen radicals may be generated by the major electron transport processes, mitochondrial respiration, photosynthesis, and microsomal activity (Richter et al., 1977; Rich and Bonner, 1978; Michalski and Kaniuga, 1981). Studies by Doke (1985) and Doke and Chai (1985) suggest the existence of a membrane-located superoxide-generating NADPH oxidase that may be involved in the oxidative burst in association with the hypersensitive reaction. In animals, reactive oxygen species are thought to play a pivotal role in programmed cell death (Arends and Wyllie, 1991). In this case, genetic evidence implies that repression of an antioxidant pathway is important for the occurrence of cell death (Hockenbery et al., 1993).

The concept that rapid cell death halts growth of the pathogen is clearly supported by our results on the inverse correlation between the rates of cell death and formation of secondary fungal hyphae. The actual mechanism for this limitation of pathogen growth remains unclear. In some cases a major reason for the termination of pathogen growth may be the collapse of the intracellular fungal structure immediately following the collapse of the host cell, as we observed recently in the potato-*P. infestans* interaction (Freytag et al., 1994). However, disruption of the fungus was not seen in our present study, although further development of the fungus was efficiently prevented. Several different factors may be involved, among them being reactive oxygen species and possibly other toxic substances produced during the hypersensitive reaction and liberated with the collapse of the host cell.

In cultured parsley cells, the observed defense reactions, except the expression of defense-related genes, were largely confined to individual infected cells within a microcallus and occurred in the same manner in single cells. Thus, we consider the hypersensitive reaction, including rapid cell death, to be a cell-autonomous process that does not depend on the presence of adjacent cells. The identification and functional characterization of genes that are causally related to rapid cell death will greatly help to answer the question of which specific genetic program determines hypersensitive cell death in plants.

ACKNOWLEDGMENTS

We thank Christiane Julius for carrying out electron microscopy and Ila Rouhara for skillful technical assistance. We are grateful to Thorsten Nürnberger and Paul Rushton for critically reading the manuscript.

Received January 3, 1996; accepted June 8, 1996.
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