Hormonally Regulated Programmed Cell Death in Barley Aleurone Cells

Paul C. Bethke, Jennifer E. Lonsdale, Angelika Fath, and Russell L. Jones
Department of Plant and Microbial Biology, University of California, Berkeley, California 94720-3102

Cell death was studied in barley (cv Himalaya) aleurone cells treated with abscisic acid and gibberellin. Aleurone protoplasts incubated in abscisic acid remained viable in culture for at least 3 weeks, but exposure to gibberellin initiated a series of events that resulted in death. Between 4 and 8 days after incubation in gibberellin, >70% of all protoplasts died. Death, which occurred after cells became highly vacuolated, was manifest by an abrupt loss of plasma membrane integrity followed by rapid shrinkage of the cell corpse. Hydrolysis of DNA began before death and occurred as protoplasts ceased production of α-amylase. DNA degradation did not result in the accumulation of discrete low molecular weight fragments. DNA degradation and cell death were prevented by LY83583, an inhibitor of gibberellin signaling in barley aleurone. We conclude that cell death in aleurone cells is hormonally regulated and is the final step of a developmental program that promotes successful seedling establishment.

INTRODUCTION

The barley endosperm is a triploid nutritive tissue that arises after fertilization of the central cell by one of two sperm cells released into the embryo sac (Cass and Jensen, 1970). The endosperm differentiates into the aleurone and starchy endosperm 8 to 10 days after pollination (Bosnes et al., 1992). Although both aleurone and starchy endosperm act as storage tissues, the time of death for starchy endosperm and aleurone cells is markedly different. Starchy endosperm cells die as the grain ripens, which is generally 5 to 6 weeks after pollination (Brown and Morris, 1890; Brenchley, 1909). Dead cells remain filled with undigested starch and protein reserves; in the case of wheat, remnants of organelles, including nuclei, are also found (Brenchley, 1909). Aleurone cells remain alive through grain maturation.

Exposure of mature grain to water triggers a series of events that result in the secretion of hydrolytic enzymes by the aleurone and scutellum to the starchy endosperm (Fincher, 1989). These enzymes degrade the contents of the dead starchy endosperm, making nutrients available that can be absorbed by the scutellum and used for the heterotrophic growth of the embryo (Jones and Jacobsen, 1991). The protein and lipid reserves of the aleurone are used in the synthesis of secreted hydrolases. Stored mineral nutrients are released from the aleurone and provide additional nourishment for the embryo. Living aleurone cells are unnecessary after hydrolase secretion and die a few days after germination.

1 To whom correspondence should be addressed. E-mail pcbethke@nature.berkeley.edu; fax 510-642-4995.
most often referred to as programmed cell death (PCD) (Ellis et al., 1991; Greenberg, 1996). In animal cells, various classes of PCD are recognized, the most commonly studied of which is apoptosis (Kerr and Harman, 1991). Apoptotic cell death in animals is characterized by well-defined changes in cell structure. These changes culminate in the fragmentation of the cell into apoptotic bodies (Ellis et al., 1991). Blebbing of the plasma membrane, lobing of the nucleus, and cleavage of nuclear DNA into discrete fragments separated in length by ~180 bp are also hallmarks of apoptosis (Kerr and Harman, 1991; Borner et al., 1995).

Although PCD is ubiquitous in plants (Greenberg, 1996; Jones and Dangl, 1996; Pennell and Lamb, 1997), there is uncertainty about the extent to which apoptosis occurs in plants. Cellular changes that mimic those found during apoptosis in animals have been shown to occur in several types of plant cells undergoing PCD (Mittler and Lam, 1995; Levine et al., 1996; H. Wang et al., 1996; M. Wang et al., 1996; Pennell and Lamb, 1997). These changes include cytoplasmic fragmentation and DNA breakdown into 180-bp ladders. However, other researchers have argued that PCD in plants is not apoptotic because many of the hallmarks of apoptosis have not been observed (Fukuda, 1996; J. Jones and Dangl, 1996). Indeed, the presence of the plant cell wall prevents the phagocytosis of apoptotic bodies that occurs during apoptosis in animal cells. There is a growing consensus that PCD in plants only rarely follows the apoptotic route described for PCD in animals.

As a uniform, nondividing population of hormonally regulated cells, the cereal aleurone is a good experimental system for studies of plant cell death. Bush et al. (1986) conducted a detailed study of barley aleurone protoplast vacuolization and viability in response to incubation in the presence and absence of GA and Ca$^{2+}$. The conclusion was that aleurone protoplasts die during culture, regardless of the presence of GA and/or Ca$^{2+}$, but that GA synchronized and accelerated the process of cell death. More recently, Kuo et al. (1996) examined GA responses in wheat aleurone layers and showed that, as in barley, GA treatment accelerated cell death. GA-induced cell death in wheat was prevented by the protein phosphatase inhibitor okadaic acid, leading Kuo et al. (1996) to propose that protein phosphatases were key links in GA signaling via a change in cytosolic Ca$^{2+}$.

In this study, we show that PCD in the aleurone cell is tightly regulated by GA and ABA. GA promotes and ABA inhibits PCD in the barley aleurone. We also report on the cellular changes that accompany PCD in aleurone protoplasts. We provide a detailed analysis of aleurone cell death by using video time-lapse photomicrography and show that the integrity of the plasma membrane is lost abruptly as aleurone cells die. Finally, we extracted DNA from aleurone cells at various times after incubation in GA or ABA and determined the amount of DNA present and the degree to which it had been degraded to low molecular weight fragments. Our data are inconsistent with the hypothesis that barley aleurone cells die by apoptosis. We suggest that PCD in barley aleurone cells more likely results from autolysis followed by plasma membrane rupture.

**RESULTS**

**GA and ABA Regulate PCD in Barley Aleurone Cells**

Freshly isolated aleurone layers (Chrispeels and Varner, 1967) and protoplasts (Bush et al., 1986) of cv Himalaya barley grain synthesize $\alpha$-amylase in the absence of added GA because small amounts of endogenous GAs are present in the endosperm of the mature grain (Green et al., 1997). To eliminate responses to endogenous GAs, we isolated protoplasts in the presence of ABA by including 5 $\mu$M ABA in all of the solutions used in protoplast preparation. As shown in Figure 1A, when protoplasts made in this manner were treated with 5 $\mu$M ABA, $\alpha$-amylase did not accumulate in the culture medium for up to 8 days of incubation. The low rates of $\alpha$-amylase activity in the culture medium of freshly isolated protoplasts (Figures 1A and 2, 0 days) result from trace amounts of fungal $\alpha$-amylase in the Onozuka cellulase that is used in protoplast isolation (Bush et al., 1986).

Protoplasts prepared in the presence of 5 $\mu$M ABA respond normally to GA. When protoplasts prepared in the presence of 5 $\mu$M ABA were treated with 5 $\mu$M ABA plus 25 $\mu$M GA, large amounts of $\alpha$-amylase were synthesized and secreted (Figure 1A). Secretion of $\alpha$-amylase began ~1 day after the addition of GA and ended 4 to 5 days later, whether GA was added to freshly isolated protoplasts or to protoplasts up to 6 days old (Figure 1A). Because preparing protoplasts in the presence of ABA minimized their response to endogenous GA but did not prevent them from responding to exogenous GA, we used protoplasts prepared in ABA for all of the experiments described in this article.

GA treatment results in the death of barley aleurone protoplasts. When freshly isolated protoplasts were incubated in GA, the number of living protoplasts remained constant for at least 4 days and then declined precipitously (Figure 2). The number of living protoplasts was reduced by 40% between days 4 and 6 of culture in GA, and there was a further reduction of 20 to 30% in the number of living protoplasts during the following 2 days (Figure 2). A decrease in the number of living protoplasts is comparable with the number of protoplasts that died because barley aleurone protoplasts do not divide in culture. For these same protoplasts, the activity of $\alpha$-amylase in the incubation medium increased until 6 days after GA treatment (Figure 2).

We tested the hypothesis that cell death in barley aleurone depends on incubation in GA and not on the duration of protoplast incubation. Protoplasts were incubated in a culture medium containing 5 $\mu$M ABA, and GA (25 $\mu$M) was added 0, 2, 4, 6, or 7 days later. The experiment was terminated 8 days after the start of incubation, regardless of when GA was added, and the number of living protoplasts...
Taken together, the data shown in Figures 1 and 2 indicate that GA induces death in barley aleurone cells.

Like many responses of cereal aleurone cells to GA, the effects of GA on cell death can be antagonized by ABA. Figure 3 shows that ABA-treated protoplasts remained alive much longer than did GA-treated protoplasts. Whereas only 20 to 30% of GA-treated protoplasts survived for 8 days in culture (Figures 1 and 2), nearly all ABA-treated protoplasts survived for 22 days (Figure 3). From this experiment, we conclude that ABA inhibits PCD in barley aleurone and that protoplast preparation and incubation do not lead a priori to rapid cell death. Note that the increase in the number of ABA-treated protoplasts during the first 15 days of culture (Figure 3) did not result from cell division. Instead, it resulted from the continued activity of cell wall-degrading enzymes on incompletely protoplasted cells. In all protoplast preparations, there are cells at the end of the enzyme treatment that have not had their walls removed fully by cell wall-digesting enzymes. These cells are easily recognized because they remain attached to neighboring cells or are not spherical. Because it is very difficult to distinguish live cells from dead cells when they are surrounded by a cell wall, we did not include these cells in our counts of live protoplasts. The continued activity of residual wall-degrading enzymes, however, converts some partially digested cells into protoplasts. Hence, even though the number of live cells does not increase, the number of protoplasts does. The extent to which this occurs varies but is comparable from flask to flask within an experiment.

**Figure 1.** GA Treatment of ABA-Pretreated Barley Aleurone Protoplasts Results in α-Amylase Secretion and Cell Death.

(A) α-Amylase activity in the incubation media of barley aleurone protoplasts. Each symbol represents a flask of protoplasts to which GA was added as specified. Each flask was repeatedly sampled 0 to 8 days after protoplast preparation. Arrowheads indicate the time of GA addition to the flask of protoplasts represented by the corresponding symbol. Open square, ABA alone, no added GA; open circle, GA added at day 0; filled square, GA added at day 2; open triangle, GA added at day 4; filled circle, GA added at day 6. The data represent one experiment with five flasks of protoplasts and are representative of three replicates. U, units.

(B) Number of protoplasts per milliliter of incubation medium 8 days (d) after protoplast preparation. GA (25 μM) was added to ABA-pretreated cells as indicated in the figure. Before GA treatment, protoplasts were incubated without GA as necessary so that all cells were 8 days old at the end of the GA incubation period. The data were pooled from three replicate experiments, each of which gave a similar result. The data in (A) are from one of these replicates. Bars represent the mean ± SD of three flasks of protoplasts.

and the amount of secreted α-amylase were determined. As seen in Figure 1B, treatment of protoplasts with GA for ≥2 days resulted in a dramatic decrease in the number of protoplasts surviving for 8 days. This was not an effect of cell age, because protoplasts in all treatments were the same age. Hence, GA treatment, not aging, resulted in cell death.

**Figure 2.** GA Promotes α-Amylase Secretion and Cell Death in Barley Aleurone Protoplasts.

The number of living protoplasts per flask 0 to 8 days after treatment of freshly isolated protoplasts with GA (25 μM) is indicated by filled circles. α-Amylase activity in the incubation media of barley aleurone protoplasts treated with GA (25 μM) immediately after protoplast preparation is indicated by open circles. Each data point represents the mean ± SD of three flasks of protoplasts, and each flask was used for only one time point. The data from a replicate experiment with three flasks of protoplasts at each time point were similar. U, units.
To determine whether ABA could block or slow the cell death program in GA-treated protoplasts, we added ABA to protoplasts that had been incubated in GA for 24 hr. This corresponds to the time at which $\alpha$-amylase secretion has just begun (see Figures 1A and 2). We then measured the amount of secreted $\alpha$-amylase and the number of living cells. As can be seen in Figure 4A, whether or not ABA was added at 24 hr, protoplasts responded to GA by synthesizing and secreting $\alpha$-amylase until ~4 days after the addition of GA. The rate and amount of amylase secretion for both treatments were nearly identical. However, the rate of cell death was significantly different (Figure 4B). Protoplasts treated with ABA 24 hr after the addition of GA lived longer than did those that did not receive additional ABA. Compared with GA-treated controls, a significantly greater number of protoplasts survived 5 to 8 days in culture when ABA was added at 24 hr ($P < 0.01$). By day 8, however, even protoplasts treated with ABA at 24 hr began to die. These data suggest that the death program can be hormonally modulated and that completion of $\alpha$-amylase secretion is not sufficient to trigger rapid PCD.

GA-treated barley aleurone layers and protoplasts secrete numerous hydrolytic enzymes, including proteases and nucleases as well as $\alpha$-amylases (Jones and Jacobsen, 1991). To determine whether cell death resulted from exposure to a lethal amount of hydrolase activities, we incubated protoplasts in hydrolase-containing medium by using the procedure diagramed in Figure 5A. In the first part of this experiment, GA-treated protoplasts were incubated in B5 medium for 8 days. As shown in Figure 5B, 90% of the initial population of protoplasts died during this time. The hydrolase-containing medium from these flasks (conditioned medium [CM]) was collected and used as the incubation medium for freshly prepared protoplasts. Figure 5C shows that incubation of protoplasts in enzyme-containing conditioned medium did not accelerate the rate of death. There was no significant difference between the mean number of ABA-treated protoplasts per flask incubated for 3 days in CM or B5 medium ($P > 0.2$). Likewise, there was no significant difference between the mean number of GA-treated protoplasts per flask incubated for 3 days in either CM or B5 medium ($P > 0.05$). Regardless of hormone treatment, the number of protoplasts per flask after incubation in CM for 3 days was not significantly different from the number of protoplasts per flask for day 0 controls ($P > 0.2$). Taken together, these experiments suggest that incubation of protoplasts in enzyme-containing conditioned medium does not accelerate the rate of death.

Figure 3. ABA Slows the Rate of Cell Death for Barley Aleurone Protoplasts.

Freshly isolated barley aleurone protoplasts were incubated in ABA (5 $\mu$M) for the times indicated. Each point represents the mean $\pm$SD of three or four flasks of protoplasts, and each flask was used for only one time point. The number of living GA-treated protoplasts (Figure 2) is plotted as a dashed line for comparison.

Figure 4. ABA Delays PCD in GA-Treated Barley Aleurone Protoplasts.

Freshly isolated barley aleurone protoplasts were treated with 25 $\mu$M GA. ABA (125 $\mu$M) was added to the incubation medium of some flasks 24 hr later. (A) $\alpha$-Amylase activity in the incubation media of barley aleurone protoplasts incubated in GA for the number of days indicated with (open squares) or without (filled circles) the addition of ABA at 24 hr. U, units. (B) The number of living cells per flask after incubation in GA for the number of days indicated with (open bars) or without (filled bars) the addition of ABA at 24 hr.

Each data point in (A) or bar in (B) represents the mean $\pm$SD of four flasks of protoplasts. For each flask, determinations of cell number and $\alpha$-amylase activity were made at only one time of incubation. Note that addition of ABA at 24 hr results in there being significantly more living protoplasts per flask on days 5 to 8 ($P < 0.01$).
Coalescence and enlargement of protein storage vacuoles occurred more slowly in ABA-treated protoplasts, and rarely did a single large protein storage vacuole fill the cell (Figure 6B). Older GA-treated cells appeared as thin films of cytoplasm wrapped around a large protein storage vacuole (Figure 6A and see below), whereas older ABA-treated cells were packed with protein storage vacuoles (Figure 6B). All of our observations indicate that cell death is highly correlated with the extent of vacuolation. Cells that do not reach the highly vacuolate stage remain viable; those that vaculate, die. This change in vacuolar morphology may provide a clue to the mechanism of PCD in barley aleurone cells.

**PCD in Aleurone Protoplasts Culminates in an Abrupt Loss of Plasma Membrane Integrity**

We used video time-lapse photomicrography to characterize structural changes that occurred in highly vacuolated barley aleurone protoplasts immediately before cell death. Figure 7A shows a population of barley aleurone protoplasts 4 days after GA treatment. The same population of cells is shown 9 hr later in Figure 7B. In this experiment, eight of the original 24 cells died (Figure 7A, numbered cells). In each case, an abrupt transition was observed as healthy cells collapsed to form a compact mass of cellular debris \( \sim 20 \mu m \) in diameter (Figure 7B). Dead cells in Figure 7B are indicated by numbers that correspond to those shown in Figure 7A. The onset of this collapse was manifest as a sudden increase in refraction from the plasma membrane (Figures 7C and 7D, labeled Death). Figures 7C and 7D show two of the protoplasts in Figures 7A and 7B enlarged and at closely spaced time intervals and demonstrate that this transition was detected easily in \( \sim 1 \) min.

To determine whether a loss of plasma membrane integrity occurred as aleurone protoplasts died, we added YO-PRO-1 to the incubation medium and observed protoplast death by using time-lapse microscopy. YO-PRO-1 is a fluorescent DNA binding probe that is impermeant to living aleurone cells (data not shown). In these experiments, protoplasts were illuminated continuously with dim white light. Every 5 min, they were given a 1-sec pulse of light from the fluorescence light source on the microscope, and digital images were captured. The intensity of the white light source was adjusted to allow simultaneous detection of cell shape and YO-PRO-1 fluorescence. In the absence of bound DNA, fluorescence from YO-PRO-1 was below the level of detection. Video images of apparently healthy aleurone protoplasts with nonrefractile plasma membranes are shown at left in Figures 7E and 7F. These protoplasts were able to exclude YO-PRO-1 to the extent that no fluorescence was detectable from their nuclei. However, death resulted in a loss of plasma membrane integrity that allowed YO-PRO-1 to enter protoplasts, resulting in intense fluorescence from their nuclei (Figures 7E and 7F, at right). As in our other time-lapse experiments, dead cells had refractile plasma membranes.

---

**Figure 5.** Death of Barley Aleurone Protoplasts Does Not Result from a Lethal Accumulation of Secreted Hydrolytic Enzymes.

(A) A flow diagram of the experiment. d, days.

(B) The number of GA-treated protoplasts per flask at the beginning of the experiment (0 days) and 8 days later. The hydrolase-containing medium from 8-day flasks was collected, filtered, and used as the incubation medium in (C). Note that CM was collected from flasks in which 90% of the protoplasts had died.

(C) A comparison between the survival of protoplasts incubated in fresh B5 medium and hydrolase-containing CM. Note that incubation in CM does not result in accelerated rates of death for ABA- or GA-treated protoplasts.

The data in (B) and (C) are means $\pm$ SD of five flasks of protoplasts, except for the bar at right in (B), where $n = 10$.

---

**Barley Aleurone Protoplasts Undergo Characteristic Patterns of Vacuolation**

Freshly prepared barley aleurone protoplasts, like the cells in the mature aleurone layer, contain hundreds of small protein storage vacuoles. Within these protein storage vacuoles are storage proteins as well as numerous inclusions of phytin and protein/carbohydrate (Jacobsen et al., 1971). As shown in Figure 6A, these vacuoles coalesced and enlarged after GA treatment of protoplasts, until one large protein storage vacuole nearly filled the cell.

---

Together, these data demonstrate that GA-induced cell death does not result from nonspecific cytotoxic enzyme activities in the media.
and death was followed by collapse of the protoplast. Similar results were obtained using 4’,6-diamidino-2-phenylindole (DAPI) instead of YO-PRO-1 (data not shown). In our time-lapse recordings, fluorescence from the nucleus was apparent in all cells that died, even at the earliest times after death (<5 min). From these experiments, we conclude that death and loss of plasma membrane integrity are tightly linked in barley aleurone cells.

Lobing of Aleurone Nuclei Does Not Correlate with PCD

Cells undergoing apoptotic cell death often show highly distended or lobed nuclei. We used Syto 11 and DAPI to observe the shape of aleurone nuclei after incubation in ABA and GA (Figure 8). Syto 11 is a fluorescent DNA binding probe that readily permeates living barley aleurone protoplasts and has no effect on cell viability or α-amylase synthesis or secretion (data not shown). Protoplasts that were incubated in ABA often showed extreme lobing of the nucleus beginning 4 to 6 days after incubation (Figures 8A to 8D). These nuclei extended between and around the protein storage vacuoles but generally returned to an ellipsoidal shape with prolonged incubation. Nuclear lobing was infrequent in GA-treated protoplasts, and lobing was not observed in cells at advanced stages of vacuolation (Figures 8E, 8F, and 8H). Although lobing of nuclei is expected in cells undergoing apoptosis, lobing of the nucleus was not correlated with the death of barley aleurone cells because it was most commonly observed in ABA-treated protoplasts that were not undergoing PCD and was never observed in highly vacuolated GA-treated protoplasts.

DNA Content of Living Aleurone Cells Is Reduced before Death

DNA was isolated from living aleurone protoplasts to quantify the amount per protoplast and to determine if the DNA was cut into the discrete low molecular weight fragments separated in length by ~180 bp that are characteristic of apoptosis. As shown in Figures 9A and 10A, the DNA content of living GA-treated barley aleurone protoplasts declined before the protoplasts died. Freshly isolated barley aleurone protoplasts contained ~60 μg of extractable DNA per million protoplasts (Figure 9A). Living protoplasts incubated in GA for 5 days, when many of the cells were highly vacuolated (Figure 6A) and most had ceased α-amylase synthesis (Figure 1A), contained an average of 30 μg of extractable DNA per million living protoplasts (Figure 9A). Protoplasts incubated in ABA for 5 days, on the other hand, were not highly vacuolated (Figure 6B) and contained ~60 μg of extractable DNA per million protoplasts (Figures 9A and 10A). Similarly, protoplasts incubated in GA for 4 days and actively synthesizing α-amylase contained the same amount of DNA as did freshly isolated and ABA-treated protoplasts (Figure 9A). These data indicate that GA treatment led to DNA degradation in highly vacuolate living protoplasts and that ABA prevented both DNA degradation and cell death.
Figures 9B and 10B show that virtually all of the DNA extracted from aleurone protoplasts was high molecular weight DNA. The prominent, discrete low molecular weight fragments of DNA observed by others in dying aleurone protoplasts (M. Wang et al., 1996) were not found, regardless of hormone treatment or duration, when protoplasts were washed free of the enzymes used in protoplast preparation before DNA isolation.

To determine whether DNA degradation and cell death could be prevented by blocking a GA-signaling pathway, we treated barley aleurone protoplasts with the guanylyl cyclase inhibitor LY83583. In a previous report, we demonstrated that incubation of barley aleurone layers with LY83583 prevented a transient rise in cGMP 2 to 4 hr after GA treatment (Penson et al., 1996). LY83583 also inhibited the synthesis and secretion of α-amylase but had little effect on the transcription of the ABA-inducible Rab gene (Penson et al., 1996). These data suggest that cGMP plays an important role in GA but not ABA signaling in barley aleurone cells. As shown in Figure 10A, LY83583 at 50 to 75 μM significantly inhibited GA-induced α-amylase secretion (P < 0.05), nuclear DNA degradation (P < 0.01), and cell death (P < 0.05). These effects of LY83583 were dose dependent because protoplasts treated with 25 μM LY83583 secreted

![Figure 7](image-url)
more α-amylase, contained less DNA, and died more rapidly than did those treated with 50 to 75 μM LY83583. The DNA in barley aleurone protoplasts incubated for 5 days in GA, GA plus 25 to 75 μM LY83583, or ABA was virtually all high molecular weight DNA (Figure 10B). From this experiment, we conclude that blocking a GA signal transduction pathway containing cGMP can prevent GA-induced PCD.

DISCUSSION

In this report, we show that GA induces death of barley aleurone protoplasts (Figures 1 and 2) and that ABA antagonizes this effect of GA (Figures 3 and 4). We demonstrate that GA promotes cell death regardless of protoplast age (Figure 1) and that death does not result from a toxic accumulation of secreted hydrolases (Figure 5). Finally, we show that aleurone cell DNA is degraded before death (Figures 9 and 10) and that LY83583, an inhibitor of GA signaling, prevents GA-induced DNA degradation and cell death as effectively as ABA (Figure 10). From these data, we conclude that GA is a signal that initiates PCD in barley aleurone and that ABA is a negative regulator of this program.

Death of aleurone cells is the final step in a series of ordered biochemical and morphological changes that are initiated by imbibition and promoted by GA. At the time of death, a large protein storage vacuole occupies virtually all of the cytoplasm, and the nucleus, along with other organelles, is confined to a thin layer of cytoplasm between the tonoplast and plasma membrane (Figures 6 and 7). These data are in agreement with our previous observations of vacuolation in the cells of GA-treated aleurone layers and protoplasts (Jones and Price, 1970; Bush et al., 1986). Cells do not die before this highly vacuolated configuration is obtained. The hydrolysis of nuclear DNA begins around the time that aleurone cells reach the highly vacuolated stage (Figures 9 and 10). DNA degradation is rapid and extensive. Within 24 hr, living GA-treated protoplasts lose more than half of their DNA (cf. 4 to 5 days in Figure 9). Finally, the integrity of the plasma membrane is lost abruptly and the protoplast collapses (Figure 7). Between 4 and 8 days after the addition of GA, all but 10 to 30% of the protoplast population dies (Figures 1, 2, and 5).

Nearly all of the events described above are slowed or prevented by ABA. Secreted hydrolases are not synthesized rapidly (Figure 1), the rate of storage protein breakdown remains low (Fincher, 1989), protein storage vacuoles coalesce at a much slower rate than for GA-treated cells, and rarely does a single large vacuole fill the cell (Figure 6). Degradation of nuclear DNA is delayed by ABA (Figures 9 and 10), and the rate of cell death is considerably reduced (Figures 3, 4, and 10). The rate of cell death for ABA-treated cells is low enough that some protoplasts remain viable in

Figure 8. Lobing of Nuclei in Aleurone Protoplasts Is Not Correlated with Cell Death.

(A) to (D) Fluorescence microscopy of living ABA-treated barley aleurone protoplasts labeled with the nucleic acid probe Syto 11. (E) and (F) Fluorescence microscopy of living GA-treated barley aleurone protoplasts labeled with Syto 11. (G) and (H) Fluorescence microscopy of fixed ABA (G) and GA-treated (H) barley aleurone protoplasts stained with the nucleic acid probe DAPI. Note that brightly fluorescent, irregularly shaped nuclei (N) are common in ABA-treated cells (A) to (D) and (G) but rare in GA-treated cells (E), (F), and (H). Bars = 10 μm.
culture for >6 months (data not shown).

Our data are consistent with the hypothesis that PCD in barley aleurone is by way of autolysis followed by plasma membrane rupture. How this is accomplished is unknown. Before death, aleurone cells catabolize much of their cytoplasm, including lipid and protein reserves and nuclear DNA. An attractive hypothesis is that PCD-dependent autolysis reduces mitochondrial activity below the threshold required for cellular homeostasis. Reduced ATP might result in an inability to fuel the plasma membrane proton pump, thus disturbing the transport properties of the cell. Reduced ATP also might diminish the ability of the cell to repair or replace essential proteins, including those that protect the cell from environmental damage. This proposal is consistent with the mechanism proposed by Aubert et al. (1996) for the regulation of cell death in sucrose-starved sycamore cells.

Although an abrupt increase in refraction from the plasma membrane and a loss of plasma membrane integrity are the first markers that we can correlate with death, we cannot rule out vacuole rupture as an earlier event. In particular, video microscopy does not allow us to observe all of the small, secondary vacuoles that exist in GA-treated protoplasts as they approach death. Secondary vacuoles, like protein storage vacuoles, are protease-containing, acidic organelles (Swanson et al., 1998). Rupture of one or more of these organelles and release of vacuolar proteases could initiate events that culminate in plasma membrane rupture. Vacuole rupture leading to programmed death in plants occurs in differentiating tracheary elements (Groover et al., 1997).

Our data do not support the hypothesis that barley aleurone cells die by apoptosis (Ellis et al., 1991). In apoptotic cell death, the cytoplasm is partitioned into apoptotic bodies that are subsequently engulfed by neighboring cells. We have been unable to detect the lobing of the cytoplasm that leads to the formation of apoptotic bodies by using light microscopy. Furthermore, the thick cell walls and resistant inner wall of the aleurone cell would preclude the engulfment of apoptotic bodies by adjacent cells. Although we have observed highly distended and lobed nuclei, these nuclei are common in ABA-treated cells and much less common in GA-treated cells, and the lobed shape disappears as the number of vacuoles decreases and the nucleus migrates to the cell periphery (Figure 8 and data not shown).

In apoptotic cells, nuclear DNA is typically broken down into discrete low molecular weight fragments that produce a ladder of ~180 bp on agarose gels after electrophoresis. The latter characteristic often has been considered sufficient to classify death as apoptotic. Although we have been able to create artificially ladders of ~180 bp from protoplast DNA by using both exogenous and endogenous nucleases (A. Fath and R.L. Jones, unpublished data), DNA isolated by methods that reduce hydrolysis of DNA in vitro was almost exclusively high molecular weight DNA (Figures 9 and 10). We did not observe DNA ladders similar to those previously reported (M. Wang et al., 1996), even though the population of cells from which the DNA was extracted was undergoing PCD and even though the amount of DNA per cell was decreasing (Figures 9 and 10).

We tested the hypothesis that aleurone cell death is not PCD but results from exhaustion of nutrients. The rate and amount of α-amylase secreted by cells treated with GA and then ABA 24 hr later were nearly identical to those from cells that did not receive the subsequent ABA treatment (Figure 4A). Yet, cells treated with ABA at 24 hr did not die during

Figure 9. Nuclear DNA Is Degraded before Cell Death but Does Not Accumulate as Low Molecular Weight Fragments.

DNA was isolated from 120,000 living GA- or ABA-treated protoplasts immediately after the addition of hormone or 4 or 5 days later. (A) Amount of DNA in living protoplasts before cell death. DNA was quantified spectrophotometrically after purification from living protoplasts 0, 4, or 5 days after treatment with GA (filled bars) or ABA (open bars). Values represent the mean ± SD of three samples for GA-treated and two samples for ABA-treated protoplasts. (B) DNA is present as high molecular weight DNA. DNA (1.75 μg lane) was loaded on a 1.5% agarose gel, electrophoresed, and stained with ethidium bromide. A 500-bp DNA ladder was used for a molecular weight (MW) marker.
Because α-amylase is the predominant protein secreted by aleurone cells and because production of secretory proteins is the primary sink for aleurone reserves, it is unlikely that hydrolase secretion expends the metabolic reserves of the cell and renders it nonviable. Rather, these data are consistent with the idea that GA initiates cascades of events, including the synthesis of secretory proteins and cell death, that are temporally separated. For example, α-amylase production in barley aleurone layers and protoplasts is initiated during the first 24 hr of exposure to GA, whereas the synthesis of degradative enzymes such as nuclease I and ribonuclease does not begin until ~36 hr after GA treatment (Chrispeels and Varner, 1967; Brown and Ho, 1986). The ability of ABA to counter the effects of GA on cell death but not on α-amylase synthesis when it is added 24 hr after GA is consistent with the idea that these events are temporally separated and can be differentially regulated in the aleurone cell (Figure 4).

We also tested the hypothesis that aleurone cell death results from an increase in the activities of extracellular proteases, nucleases, or other hydrolases. Incubation of aleurone protoplasts in media containing hydrolases secreted by live aleurone cells and released from the corpses of dead aleurone cells did not accelerate the rate of protoplast death (Figure 5). This experiment argues against the hypothesis that aleurone cell death results from nonspecific, cytotoxic enzyme activities. On the contrary, these data show that living aleurone protoplasts are relatively insensitive to attack by extracellular enzymes because exposure to a battery of enzyme activities for 72 hr had no effect on aleurone protoplast survivability.

Currently, little is known about how the cell death program is executed in barley aleurone. Necessary steps include a signal to trigger the program, signal transduction elements to relay the signal to various effectors and to coordinate their activities, and effector molecules whose activities result in death. As described above, our data indicate that GA is an initial death signal. How this signal is transduced is largely unknown. Our experiments with the guanylyl cyclase inhibitor LY83583 implicate cGMP as a component of at least one part of this signal transduction pathway (Figure 10). LY83583 at >50 μM effectively blocks PCD, preventing both DNA degradation and cell death. LY83583 prevents α-amylase secretion from barley aleurone layers by preventing a transient rise in the amount of cGMP 2 to 4 hr after GA addition (Penson et al., 1996). LY83583 might inhibit PCD by blocking this same step, although a later effect of LY83583 cannot be ruled out. Previous reports have suggested that one or more protein phosphatases are involved in PCD signal transduction in wheat aleurone cells (Kuo et al., 1996).

We hypothesize that hormonal regulation of life and death in cereal endosperm plays a crucial role in successful seedling establishment. Under the influence of ABA, aleurone cells remain alive for many years, long after genetically identical starchy endosperm cells have died. When dormancy is

**Figure 10.** DNA Degradation and Cell Death Are Prevented by Blocking a GA Signaling Pathway with the Guanylyl Cyclase Inhibitor LY83583.

(A) Effect of LY83583 (LY) on α-amylase secretion, DNA content, and number of living cells for barley aleurone protoplasts incubated in 25 μM GA, 25 μM GA plus 25 to 75 μM LY83583, or 5 μM ABA for 5 days. Data represent the average ±SD of three replicates. U, units.

(B) DNA is present as high molecular weight DNA. DNA was isolated from protoplasts treated for 5 days with either 25 μM GA with or without 25, 50, or 75 μM LY83583 or 5 μM ABA. DNA (2 μg per lane) was analyzed on an 1.5% agarose gel. A 500-bp DNA ladder was used as a molecular weight (MW) marker.

In (A) and (B), the hormone treatment is indicated by (+). No addition of GA, 5 μM ABA, or LY83583 is indicated by (−).
broken and the grain is imbibed, GA produced by the embryo signals the aleurone to synthesize and secrete hydroases that convert reserves stored in dead starchy endosperm cells into nutrients available to the embryo (Bewley and Black, 1994). After hydroase secretion is complete, living aleurone cells are superfluous and die. At this time, the corpses of aleurone cells, like those of starchy endosperm cells, are made available for digestion by apoplastic enzymes. Degradation of macromolecules remaining within dead aleurone cells allows for additional nutrient release and brings to an end the endosperm’s role as a storage tissue.

**METHODS**

**Preparation of Barley Aleurone Layers and Protoplasts**

Aleurone layers and protoplasts were prepared from deembryonated barley grains (Hordeum vulgare cv Himalaya; 1991 harvest; Washington State University, Pullman, WA), as described previously (Chrispeels and Varner, 1967; Bush et al., 1986; Bethke et al., 1996). To prevent endogenous gibberellin (GA) from triggering the cell death pathway before treatment with exogenous GA, we added 5 μM abscisic acid (ABA) to the solutions used for protoplast preparation as well as to the baseline Gamborg’s B-5 (Sigma) culture medium. When protoplasts were treated with GA, 25 μM GA was added to the culture medium. In experiments that used ABA to reverse the effect of GA on cell death, we added a fourfold excess of ABA (125 μM) to GA (25 μM)-containing media. Protoplasts used for DNA quantitation were prepared using the method of Lin et al. (1996). This method incorporates an extensive wash step that uses centrifugation on a Percoll density gradient immediately after the release of protoplasts from the testa/pericarp. This wash step was required to remove nucleases found in the Onozuka cellulase (Yakult Honsha Co. Ltd., Tokyo, Japan) from contaminating the DNA extract and degrading the DNA during the isolation procedure. For DNA isolation, only living protoplasts were used. Living protoplasts were separated from dead protoplasts on a Nycodenz (Sigma) density gradient (Bethke et al., 1996).

In some experiments, protoplasts were incubated in hydroase containing conditioned medium (CM). The culture medium from GA-treated protoplasts was collected 8 days after hormone treatment. This culture medium was then filter sterilized to remove dead cells and debris, producing the CM, which was used to incubate freshly isolated protoplasts. Because of the unknown hormone composition of CM, we treated cells with either 25 μM GA and 5 μM ABA or with 125 μM ABA alone.

In experiments investigating the effect of the guanylyl cyclase inhibitor LY3583 (6-anilinoquinoline-5,8-quinoine) on α-amylase activity, cell number, and DNA degradation, LY3583 (50 mM in DMSO) was added to the culture medium to a final concentration of 25, 50, or 75 μM.

**Cell Counting**

Living protoplasts were counted using an improved Neubauer hemacytometer (Fisher Scientific, Pittsburgh, PA). Flasks of cells were gently mixed to produce a uniform suspension before removing samples for counting. Only intact, round protoplasts were counted. Dead shriveled cells and aspherical nonprotoplasted cells were not counted. With time, the continued activity of residual cell wall-digesting enzymes may convert some partially protoplasted cells into protoplasts. The extent to which this occurs varies but is comparable from flask to flask within an experiment. Because mixing the protoplasts imposes a mechanical stress on them that could have effects on the rate of cell death, each flask of cells was counted at only one time point.

**Light Microscopy**

Living protoplasts were examined using a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Plan-Neofluor 40× dry objective, 0.75 numerical aperture objective, and differential interference contrast optics. Protoplasts were photographed with Kodak Ektachrome 160T film.

**Time-Lapse Photomicrography**

Protoplasts were prepared in optically clear polystyrene tissue culture dishes (Corning Glass Works, Corning, NY) sealed with Parafilm (American National Can, Greenwich, CT). Four days after treatment with 25 μM GA, dishes were examined using a Zeiss Axiosvert 35 microscope equipped with a 32× LD Achrostopmat or 40× Achromplan objective (Carl Zeiss). Images of a field of protoplasts were captured every 30 sec to 5 min with a video camera (model CCU-84; Pulnix America Inc., Sunnyvale, CA; or model SIT-66x; Dage-MTI, Michigan City, IN). Image capture was controlled by Spectrum IP Lab, version 3.1a (Scanalytics, Inc., Fairfax, VA) software on a Macintosh 7100 Power PC (Apple Computer, Cupertino, CA).

In some time-lapse experiments, YO-PRO-1 (1 μM; Molecular Probes, Eugene, OR) was added to the incubation solution immediately before the time-lapse experiment. Protoplasts were illuminated continuously with dim white light and given a 1-sec pulse of blue light, during which digital images were captured. The intensity of fluorescence from YO-PRO-1 when bound to the DNA in the nucleus was great enough to be detected easily in these experiments. White light was supplied by the tungsten bulb of the Zeiss Axios Vert microscope, and blue light was supplied from the mercury bulb filtered through the standard fluorescent filter set (450- to 490-nm excitation; 520-nm and longer emission).

**Loading and Staining of Protoplasts with Fluorescent Probes**

To label protoplast DNA with Syto 11 (Molecular Probes), we incubated protoplasts with 5 μM Syto 11 for 3 hr. To stain DNA in fixed protoplasts with 4’,6-diamidino-2-phenylindole (DAPI), we isolated protoplasts on a Nycodenz gradient and then diluted them 1:10 in PBS, pH 7.2, containing 4% paraformaldehyde, 0.3% glutaraldehyde, 50 mM EDTA, and NaCl as needed to make the fixative isosmotic with the incubation medium. Protoplasts were fixed for 1.5 hr and then washed three times for 40 min with staining solution (0.5 × PBS, 50 mM EDTA, and 1 μg/mL DAPI). Syto 11-labeled and DAPI-stained cells were observed with a Zeiss Axiophot microscope using standard fluorescent and UV filter sets.
Amylase Assay

α-Amylase activity was measured using the starch-iodine method (Jones and Varner, 1967).

DNA Isolation and Electrophoresis

DNA from barley aleurone protoplasts was purified using a modification of the method described by M. Wang et al. (1996). Briefly, frozen protoplasts were thawed on ice in the presence of lysis buffer (750 μL of 0.1 M Tris-HCl, pH 7.5, 50 mM EDTA, 0.5 M NaCl, and 100 μL of 10% SDS) and vortexed. Without further incubation in lysis buffer, sodium acetate was added to a final concentration of 0.3 M, mixed, and incubated for 10 min on ice. After centrifugation for 1 min at ~12,000g, the supernatant was transferred to a new tube. One volume of 100% isopropanol was added, mixed by inversion, incubated on ice for 1 min, and centrifuged for 5 min at ~12,000g. The supernatant was discarded, and the pellet was dissolved in 250 μL of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and 250 μL of CTAB buffer (0.2 M Tris-HCl, pH 7.5, 50 mM EDTA, 2 M NaCl, and 2% CTAB [cetyltrimethylammonium bromide; Aldrich, Milwaukee, WI]) and incubated for 15 min at 65°C. The DNA was extracted with 1 volume of chloroform and centrifuged. The aequous phase was transferred to a new tube, and 2 volumes of 100% ethanol were added. DNA was allowed to precipitate for at least 20 min at ~20°C. The DNA was washed, dried, and resuspended in 50 μL of TE buffer supplemented with 1 mg/mL RNase A. The amount of DNA was quantified by measuring absorbance at 260 and 280 nm. The quality of the DNA was examined by loading 1.75 or 2 μg of DNA on a 1.5% agarose gel and by staining with 0.5 μg/mL (w/v, final concentration) ethidium bromide after electrophoresis.

ACKNOWLEDGMENTS

The technical assistance of Juan Zhang and the editorial assistance of Eleanor Crump are gratefully acknowledged. Dr. Steve Ruzin and the staff of the College of Natural Resources Biological Imaging Facility at the University of California, Berkeley, provided excellent technical support. This work was supported by grants from the National Science Foundation (Grant No. IBN-9818047) and the Competitive Research Grants Program of the United States Department of Agriculture (Grant No. 92-37304-7909).

Received December 22, 1998; accepted March 26, 1999.

REFERENCES


