A Gibberellin-induced Nuclease Is Localized in the Nucleus of Wheat Aleurone Cells Undergoing Programmed Cell Death*

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The aleurone layer of cereal grains undergoes a gibberellin-regulated process of programmed cell death (PCD) following germination. We have applied a combination of ultrastructural and biochemical approaches to analyze aleurone PCD in intact wheat grains. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay revealed that PCD was initiated in aleurone cells proximal to the embryo and then extended to distal cells. DNA fragmentation and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling analysis revealed PCD of aleurone cells in maize grains, although the process was delayed as compared with wheat. Aleurone cells undergoing PCD showed a rapid vacuolation with high lytic activity in the cytoplasm, whereas the nucleus, which adopted an irregular shape, appeared essentially intact and showed symptoms of degradation at the end of the process. A nuclease activity was identified localized in the nucleus of aleurone cells undergoing PCD, just prior to the appearance of DNA laddering. This nuclease was induced by gibberellic acid treatment and was not detected when gibberellar synthesis was inhibited or in gibberellin-acid-insensitive mutants. This nuclease was activated by Ca²⁺ and Mg²⁺, strongly inhibited by Zn²⁺, and showed optimum activity at neutral pH, resembling nucleases involved in apoptosis of animal cells.

Programmed cell death (PCD) is a selective and ordered elimination of unwanted cells, thus playing a critical role in the control of development (1, 2). Death in animal cells occurs by a process termed apoptosis, which is characterized by a dramatic reorganization of the nucleus (3–5). The signaling pathway of apoptosis is initiated either at the mitochondria or at death receptors, activating a cascade of caspases, which execute cell death (6).

In plants, PCD is both an important process of development (7–10) and a mechanism of defense against pathogens (11, 12).

However, plant PCD shows important differences from the process of apoptosis of animal cells at the morphological and biochemical levels (13, 14). At the morphological level, apoptotic bodies that are phagocytosed by other cells (15) are not formed during plant PCD because of the presence of the cell wall. At the biochemical level, despite the description of caspase-like activity in cells undergoing hypersensitive response (16) and a caspase 3-like protease in Chara cells (17), no evidence for the participation of a cascade of caspases in cells undergoing PCD during plant development has been reported (18). Finally, the vacuole, a multifunctional plant cell organelle (19), seems to play an important role in PCD in different plant systems such as the formation of trachyean elements (20) or the cereal aleurone layer (21–23).

Cereal grains have become one of the model systems for the study of plant PCD. At initial stages of grain development, maternal tissues such as the nucellus (24, 25) and the nuellar projection cells (26) degenerate by a process of PCD (27). During endosperm development, the cells differentiate into two tissues: the aleurone layer, formed by cells that remain alive in the mature grain, and the starchy endosperm, formed by cells specialized in the accumulation of reserve compounds, which undergo PCD during grain maturation (28–30). In germinating grains, gibberelins synthesized in the embryo diffuse to the endosperm and are perceived by the aleurone cells (31). In response to the hormone, aleurone cells synthesize and secrete hydrolytic enzymes for the mobilization of the storage compounds of the starchy endosperm (32, 33), thus producing nutrients to support the initial growth of the seedling. Gibberelins not only promote the metabolic activation of the dormant aleurone cells but also accelerate their PCD (34).

In a previous report we have shown the existence of a spatio-temporal pattern of gene expression and starch endosperm acidification in germinating wheat grains (35). It was deduced from this study that the external pH exerts a great influence on GA₃ perception and response by the aleurone cells. In our attempt to establish how different gibberellin-regulated processes are coordinated in cereal grains, we have analyzed the spatio-temporal pattern of PCD in aleurone cells of wheat grains following germination and its hormonal control. In addition, electron transmission microscopy was used to analyze the morphology of aleurone cells during PCD. Our results show a rapid vacuolation and degradative activity in the cytoplasm, whereas the nucleus remained almost intact. Aleurone PCD was characterized by DNA fragmentation, which was activated by GA₃. Using in-gel analysis of nucleolytic activity, we identified a Ca²⁺/Mg²⁺-activated endonuclease induced by GA₃, which is localized in the nucleus of aleurone cells showing internucleosomal fragmentation of DNA.

* This work was supported by Grants BMC2001-2366-C03-01 from the Ministerio de Ciencia y Tecnología (Spain) and Grant CVI-182 from Junta de Andalucía (Spain). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ The abbreviations used are: PCD, programmed cell death; PEPC, phosphoenolpyruvate carboxylase; TdT, terminal deoxynucleotidyl transferase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; GA, gibberelic acid; GA₃, gibberelic acid 3; MOPS, 4-morpholinopropanesulfonic acid; MES, 4-morpholinethanesulfonic acid.

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EXPERIMENTAL PROCEDURES

Plant Material—The experiments described here used the wheat (Triticum aestivum) cv. Chinese Spring and maize (Zea mays) cv. Arista as source of wild-type grains. The Tom Thumb dwarf (Rht-B1c/Rht-D1a) of April Bearded wheat, kindly provided by Dr. J. E. Flintham (John Innes Centre, Norwich, UK) was used as the source of GA-insensitive mutant (36). The maize dkl1 mutant, kindly provided by Dr. C. Vicent (Institute of Molecular Biology, Barcelona, Spain) and Dr. P. Becraft (Iowa State University) was used as the source of grains lacking aleurone cells. Grains or de-embryonated half grains were sterilized in 3% (v/v) 2-mercaptoethanol, 2% (w/v) SDS). After extraction with phenol/chloroform/isomyl alcohol (25:24:1), DNA was precipitated with two volumes of absolute ethanol, resuspended in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) buffer, again ethanol-precipitated, and finally resuspended in 0.25 ml of TE buffer. Contaminating RNA was removed by incubation for 3 h at 37 °C in the presence of RNase A (final concentration, 60 ng ml⁻¹). DNA was then ethanol-precipitated, resuspended in TE buffer, resolved on 2% (w/v) agarose gels, and stained with ethidium bromide.

Preparation of Nuclear and Cytoplasmic Extracts—The nuclei were isolated from aleurone layers dissected from de-embryonated half grains, which were incubated in the presence or absence of GA₃ for up to 5 days. Aleurone layers were ground with a mortar and pestle with liquid nitrogen and then resuspended in 5 ml of homogenization buffer (0.25 M sucrose, 10 mM NaCl, 10 mM MOPS-NaOH, pH 7.0, 1 mM CaCl₂). When required, incubation medium was supplemented with 5 μM GA₃. After 15 min, the nuclei-enriched pellet was harvested, washed in homogenization buffer, and resuspended in 100 μl of extraction buffer (25 mM sodium phosphate, pH 7.8, 40 mM KCl, 20% (v/v) glycerol, 1% plant protease inhibitor mixture (Sigma), 0.4 μM NH₄SO₄). After extraction on ice for 30 min and centrifugation at 13,000 × g for 20 min at 4 °C, the supernatant constituted the nuclear extract.

In-gel Nuclease Activity Assay—In-gel nuclease activity assay was performed according to the method described by Young and Gallie (29) with minor modifications. Cytoplasmic and nuclear extracts (100 μg of protein) obtained as described above were subjected to SDS-PAGE gels containing 0.3 mg ml⁻¹ salmon sperm DNA at 4 °C and 20 mA plate. After electrophoresis, the gels were washed twice for 15 min in distilled water. The gels were then incubated overnight in 100 mM MOPS-NaOH, pH 7.0, 1 mM CaCl₂, 1 mM MgCl₂ at 37 °C, unless otherwise indicated. To discriminate between real and apparent nucleolytic activity associated with some proteins, the gels were incubated in 1% (w/v) SDS for 2 h at room temperature and then washed in water for 10 min as described by
Alnemri and Litwack (37). Finally, the gels were stained with 1 μg ml⁻¹ ethidium bromide for 10 min. Nuclease activity was photographed on a UV light box.

Western Blot Analysis—The extracts were subjected to SDS/PAGE (10% acrylamide). After electrophoresis, the proteins were electroblotted onto nitrocellulose membranes (Sigma) at 0.8 mA cm⁻² for 1 h using the electrophoretic transfer kit (Amersham Biosciences). The membranes were blocked in Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 5% (v/v) powdered milk, and PEPC was immunologically labeled by overnight incubation of the membranes at 4 °C in 20 ml of Tris-buffered saline and affinity-purified polyclonal Sorghum C4 PEPC IgG (21 μg of protein). Subsequent detection was performed by a peroxidase assay (affinity-purified goat anti-rabbit IgG horseradish peroxidase conjugate from Sigma).

TUNEL Assay—Grains harvested at different days after imbibition were longitudinally sectioned after removing shoots and roots, fixed in FAE (50% (v/v) ethanol, 5% (v/v) acetic acid, 3.7% (v/v) formaldehyde), and embedded in Paraplast Plus (Sigma). In situ detection of DNA fragmentation was carried out with a modification of the TUNEL method (38, 39). The sections were obtained from the above described Paraplast Plus embedded material. Paraplast Plus was removed by treatment with xyol, and the sections were then hydrated with a decreasing ethanol series, treated with proteinase K (20 μg ml⁻¹) in phosphate-buffered saline (10 mM sodium phosphate buffer, 130 mM NaCl), and rinsed twice in phosphate-buffered saline. Endogeneous peroxidase activity was then quenched by incubation in 1% (v/v) H₂O₂ in methanol for 30 min and rinsed twice in phosphate-buffered saline. For labeling, the sections were incubated for 60 min at 37 °C in the presence of terminal deoxynucleotidyl transferase (TdT) with an in situ cell death detection kit (Roche Applied Systems), according to the manufacturer’s instructions. Controls were performed in which TdT was omitted.

Electron Microscopy—For morphology analysis, small fragments of wheat grains harvested at 1 or 5 days after imbibition were fixed in 4% (v/v) glutaraldehyde prepared in 0.1 M cacodylate buffer, pH 7.2, for 3 h at 4 °C and post-fixed in 1% (w/v) OsO₄ for 2 h at 4 °C. The samples were dehydrated in an acetone series and embedded in Epon (epoxy embedding medium). Toluidine blue-stained semi-thin sections used as controls were viewed in a Leitz (Aristoplan) light microscope. Thin sections (60–80 nm thick) were cut on a Reichert-Jung Ultratcut E ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Philips CM-10 transmission electron microscope.

RESULTS

DNA Fragmentation in Aleurone Cells of Wheat and Maize Grains Following Germination—Fig. 1A shows the evolution of DNA isolated from aleurone cells of wheat grains following germination. One may observe the appearance of DNA ladder, which was first observed in grains after 4 days of imbibition and increased progressively up to 7 days. The spatio-temporal pattern of aleurone PCD in wheat grains following germination was established with the TUNEL assay, which labels 3'-OH DNA ends, thus allowing in situ detection of DNA fragmentation. Sections of wheat grains after 1 day of imbibition, when no DNA fragmentation was observed (Fig. 1A), showed no TUNEL-stained cells (Fig. 1B). In contrast, in grains after 7 days of imbibition most cells proximal to the embryo showed an intense TUNEL staining (Fig. 1C), whereas staining of cells of the distal part of the grain was less intense (Fig. 1D). Therefore, it can be concluded that the process of PCD is initiated in aleurone cells proximal to the embryo and progressively extended to the distal part of the grain. That is, as the time after imbibition increases, more cells undergo PCD, in agreement with the progressive increase of DNA fragmentation shown in Fig. 1A. This spatio-temporal pattern of PCD resembles previously reported GA-regulated processes in wheat aleurone cells (35); we therefore tested the effect of exogenous GA₃ on aleurone PCD. GA₃ treatment of de-embryonated half grains promoted an intense TUNEL staining of most aleurone cells (Fig. 1E); in contrast, half grains incubated in the absence of GA₃ showed only background staining (Fig. 1F). No signal was observed in control sections incubated in the absence of TdT (Fig. 1G).

To test whether aleurone PCD is general in cereal grains, we
analyzed this process in maize grains following germination. Fig. 2A shows DNA laddering of maize aleurone DNA; however, this process was delayed as compared with wheat grains because laddering was detected only after 12 days of imbibition. The TUNEL assay showed background labeling of aleurone nuclei in maize grains after 6 days of imbibition (Fig. 2B), whereas labeling was intense in grains after 12 days of imbibition (Fig. 2C), therefore confirming the delay of aleurone PCD in maize shown by the DNA laddering (Fig. 2A). The TUNEL assay was also performed on maize dek1 mutant, which lacks the aleurone layer and is unable to germinate (40). As expected, no signal was observed in dek1 grains (Fig. 2D). After 12 days of imbibition, no labeling was detected in the control sections of wild-type grains, which were incubated in the absence of TdT (Fig. 2E).

**Ultrastructural Analysis of Aleurone PCD**—Most studies on aleurone PCD have been carried out on protoplasts. It is clear from these studies that aleurone death is preceded by a high vacuolation and the loss of the plasma membrane integrity (41). However, to our knowledge, no studies have been reported on the morphological changes that occur during aleurone PCD in situ. Taking advantage of the gradient of PCD in aleurone layers of wheat grains following germination (Fig. 1), we have studied the ultrastructural changes associated with the progression of cell death by transmission electron microscopy analysis. Live aleurone cells from grains after 1 day of imbibition contained regularly shaped nuclei with intact nuclear envelope (Fig. 3A, arrow). The cytoplasm showed a highly ordered structure, composed of abundant oleosomes surrounding protein storage vacuoles (Fig. 3B). In grains after 5 days of imbibition, aleurone cells undergoing PCD lost this ordered structure and showed high vacuolation (Fig. 3C), and the plasma membrane appeared partially separated from the cell wall, as observed in a magnified image (Fig. 3D, arrow). The presence of electron-dense bodies and the rest of the membranes in the vacuoles is indicative of a high hydrolytic activity (Fig. 3, C, E, and F). As cytoplasm vacuolation increased, the nucleus presented an irregular shape showing invaginations (Fig. 3, E and F). A detailed image of a nuclear envelope showed regions with the outer membrane interrupted (Fig. 3G, arrow) and with small discontinuities (Fig. 3H, arrow). The nuclear contents did not appear as degraded as the surrounding cytoplasm (Fig. 3, E and F), a result confirmed by the analysis of proteins extracted from nuclei of cells undergoing PCD, which did not show clear symptoms of degradation (results not shown). Aleurone cells close to the embryo, which have completed the process of PCD in grains after 5 days of imbibition, showed a fully degraded cytoplasm, whereas the nucleus appeared formed by the rest of electron-dense heterochromatin, and the nuclear envelope was degraded (Fig. 3I). It is remarkable that despite the high degradation, the cells did not collapse and retained, at least in part, the cell wall (Fig. 3I).

**A GA3-induced Nuclease Is Localized in the Nucleus of Aleurone Cells Undergoing PCD**—As shown above, a hallmark of wheat aleurone PCD is the fragmentation of nuclear DNA. To further characterize this process we searched for nucleases in the nucleus of cells undergoing PCD. To establish the precise kinetics of the appearance of DNA laddering, de-embryonated half grains were treated with GA3. As expected, GA3 promoted DNA fragmentation, which was observed after 4 days of treatment (Fig. 4, A and B). These cells were fractionated into nuclei and cytoplasm, and protein extracts from both fractions were subjected to in-gel nuclease activity analysis, which showed a similar pattern of nucleolytic activity in cytoplasmic fractions of treated and untreated aleurone cells, although activity was higher in GA3-treated cells (Fig. 4, C and D). No nucleolytic activity was detected in nuclear extracts from untreated half grains (Fig. 4C). However, a band showing endonuclease activ-
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...starchy endosperm; scutellum. The scutellum corresponds to 100 mm C in 100 mM NaOH, pH 7.0, supplemented with 10 mM CaCl2 in the presence of 5 mM GA3. The protein extracts (150 µg) from nuclear (N) or cytoplasmic (C) fractions were subjected to in-gel nuclease activity by overnight incubation at 37 °C in 100 mM MOPS-NaOH, pH 7.0 in the absence of added cations (C) or supplemented as indicated with CaCl2, MgCl2, or MnCl2 at 2 mM or ZnCl2 at 4 mM (final concentration). EDTA was added at 0.2 mM (final concentration). Molecular mass markers (in kDa) are indicated on the left.

...ality was detected in nuclear extracts from GA3-treated half grains (Fig. 4D), which was detected after 3 days of treatment, therefore prior to the appearance of DNA fragmentation (Fig. 4B). To rule out any possible contamination of nuclear extracts with cytoplasmic nucleases, we analyzed the presence of PEPC, a well characterized cytoplasmic enzyme, which we have previously shown to be present in wheat aleurone cells (42). The Western blot analysis of cytoplasmic extracts showed the presence of a band corresponding to the PEPC monomer in extracts from untreated cells (Fig. 4E). Although the amount of PEPC remained almost unaltered in these cells, it progressively disappeared from treated cells (Fig. 4F), probably reflecting the high hydrolytic activity in these cells, as shown by electron microscopy analysis (Fig. 3). No signal was detected in nuclear extracts, hence showing that such extracts were free of cytoplasmic contamination.

To further test whether the nuclear-localized nuclease was induced by GA, we analyzed aleurone PCD and the presence of this nuclease in wheat grains that were imibed in the presence of paclobutrazol, a GA synthesis inhibitor (43). Paclobutrazol treatment resulted in delayed germination and exerted a severe inhibition of seedling growth (results not shown). No fragmentation of DNA was observed in paclobutrazol-treated grains in contrast with the characteristic laddering observed in control grains imibed for the same time in the absence of the inhibitor (Fig. 5A). In agreement with this result, no TUNEL staining was detected in paclobutrazol-treated grains (Fig. 5B, PCB), whereas the untreated grains showed intense labeling of the aleurone cells (Fig. 5B, WT). Similarly, no DNA fragmentation even after 10 days of imbibition (Fig. 5A) nor TUNEL stain (Fig. 5B, A) was observed in the dwarf mutant Rht-B1c/Rht-D1a, which is affected in the response to GA (36). The in-gel nuclease activity analysis of nuclear extracts from these samples identified increasing nucleolytic activity in the nucleus of wild-type grains (Fig. 5C) previous to the detection of DNA fragmentation (Fig. 5, A and B). No nucleolytic activity was detected in paclobutrazol-treated grains or in the GA-insensitive mutant (Fig. 5C) in agreement with the absence of DNA fragmentation in these samples (Fig. 5, A and B). These results lend further support to our proposal that the nucleolytic activity detected in the nucleus of aleurone cells undergoing PCD is regulated by gibberellins.

To characterize the DNase activity localized in the nucleus of aleurone cells, nuclear and cytoplasmic extracts were prepared from cells that had been treated with GA3 for 4 days. The in-gel activity assay showed a clear activation of this nucleolytic activity by Ca2+ and Mg2+ (Fig. 6). These cations could be substituted by Mn2+, which exerted a higher activating effect. In contrast, Zn2+ exerted a strong inhibitory effect even in the

Fig. 5. Analysis of PCD and nuclease activity in paclobutrazol-treated and Rht wheat mutant. A, wild-type wheat grains were imibed in the absence (control) or the presence of 0.5 mM paclobutrazol for up to 5 days as indicated. The Rht-B1c/Rht-D1a wheat mutant was imibed on filter paper soaked with water up to 10 days. At the days indicated the aleurone layers were dissected, DNA was isolated, and the aliquots (10 µg) were fractionated on agarose gels. B, longitudinal sections of wild-type (WT), Rht-B1c/Rht-D1a (Rht), and paclobutrazol-treated wild-type (PCB) wheat grains after 5 days of incubation were subjected to the TUNEL assay. al, aleurone layer; p, pericarp; se, starchy endosperm; sc, scutellum. The bar corresponds to 100 µm. C, cells from the different samples were separated into cytoplasm and nuclear fractions, and protein extracts from both fractions (100 µg) were subjected to in-gel nuclease activity. The nucleus-localized nuclease activity is marked with an arrow.

Fig. 6. Effect of divalent cations on nuclease activity. De-embryonated wheat grains were incubated for 4 days on filter paper soaked with 20 mM MOPS-NaOH, pH 7.0, supplemented with 10 mM CaCl2 in the presence of 5 mM GA3. The protein extracts (150 µg) from nuclear (N) or cytoplasmic (C) fractions were subjected to in-gel nuclease activity by overnight incubation at 37 °C in 100 mM MOPS-NaOH, pH 7.0 in the absence of added cations (C) or supplemented as indicated with CaCl2, MgCl2, or MnCl2 at 2 mM or ZnCl2 at 4 mM (final concentration). EDTA was added at 0.2 mM (final concentration). Molecular mass markers (in kDa) are indicated on the left.
presence of Ca\(^{2+}\) and Mg\(^{2+}\). The assay in the presence of EDTA resulted in the complete inhibition of the nuclear DNase activity, which was restored by the addition of Ca\(^{2+}\) and Mg\(^{2+}\) (Fig. 6). These results show that the GA\(_3\)-induced nuclear DNase localized in the nucleus of aleurone cells undergoing PCD is a Ca\(^{2+}/\text{Mg}^{2+}\)-type nuclease.

**DISCUSSION**

The response of wheat aleurone cells to gibberellins may be subdivided in two stages. In the short term, a few hours after treatment, GA\(_3\) promotes the expression of genes encoding hydrolytic enzymes and the acidification of the external medium (35). In the long term, 3–4 days after treatment, aleurone cells undergo a process of autolysis or PCD (44). In this study we have used a combination of biochemical and ultrastructural approaches to analyze the hormonal regulation of PCD in wheat aleurone cells.

A characteristic feature of cells undergoing PCD is the formation of DNA laddering as a consequence of the internucleosomal fragmentation of nuclear DNA. The analysis of nuclear DNA from GA\(_3\)-treated barley aleurone protoplasts detected DNA laddering (45–47), but more recently Path et al. (48) concluded that it was due to the enzymes used for protoplast preparation. In view of these controversial results, we have studied aleurone PCD in intact germinating wheat grains rather than isolated protoplasts, and all of the DNA extractions were carried out in the presence of 50 mM EDTA and in the absence of MgCl\(_2\), conditions that, according to Path et al. (48) and our own results (Fig. 6), markedly reduced nuclear activity. The possibility that the DNA laddering reported here is due to endogenous nucleases not related to PCD could be ruled out because no DNA laddering was observed in untreated de-embryonated half grains (Fig. 4A), despite the significant nucleolytic activity detected in cytoplasmic extracts (Fig. 4C). Based on the analysis of DNA laddering and the TUNEL assay, we conclude that internucleosomal fragmentation of DNA is associated with wheat aleurone PCD, this process being activated by GA\(_3\). However, whereas gene expression and acidification were detected shortly after hormone treatment (35), DNA laddering was delayed 3–4 days after treatment (Fig. 4). These results suggest that GA\(_3\) is not only involved in the activation of these processes but also in its temporal coordination.

Most studies on aleurone PCD have been carried out with barley and wheat grains. Here we show evidence that maize aleurone cells of germinated grains also undergo PCD; therefore, it is most likely that this is a general process in cereal grains. However, the temporal pattern of PCD in maize is delayed as compared with wheat, probably reflecting differences in the germination strategy of both types of grain. In this regard it is worth mentioning the larger scutellum of the maize grain, which seems to play an important role supporting the initial stages of the seedling growth.

The ultrastructural analysis of aleurone PCD (Fig. 3) revealed an intense degradation of the cytoplasm by a progressive increase of vacuoles, which become lytic compartments (21, 23, 41). The rapid disappearance of PEPC, a cytoplasmic enzyme, from extracts of GA\(_3\)-treated half grains (Fig. 4F) probably reflects the intense hydrolytic activity occurring in the cytoplasm of aleurone cells undergoing PCD. Most likely, some of the GA\(_3\)-induced proteases identified in aleurone cells are actually involved in this process. This seems to be the case with a cathepsin B-like protease (49) and a carboxypeptidase III (50), expressed at high level in aleurone cells, but also associated with PCD in the nucellus (51) or during the differentiation of tracheid elements (52). Although the cytoplasm of aleurone cells undergoing PCD showed symptoms of intense degradation, the nucleus appeared with an irregular morphol-
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J. Biol. Chem. 2004, 279:11530-11536.
doi: 10.1074/jbc.M308082200 originally published online December 29, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M308082200

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