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Proteinase Activity during Tracheary Element Differentiation in Zinnia Mesophyll Cultures

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The zinnia (*Zinnia elegans*) mesophyll cell culture tracheary element (TE) system was used to study proteinases active during developmentally programmed cell death. Substrate-impregnated gels and single-cell assays revealed high levels of proteinase activity in differentiating TEs compared with undifferentiated cultured cells and expanding leaves. Three proteinases (145, 28, and 24 kD) were exclusive to differentiating TEs. A fourth proteinase (59 kD), although detected in extracts from all tissues examined, was most active in differentiating TEs. The 28- and 24-kD proteinases were inhibited by thiol proteinase inhibitors, leupeptin, and *N*-[*N*-(L-3-*trans*-carboxirane-2-carbonyl)-L-leucyl]-agmatine (E-64). The 145- and 59-kD proteinases were inhibited by the serine proteinase inhibitor phenylmethylsulfonyl fluoride (PMSF). Extracts from the TE cultures contained sodium dodecyl sulfate-stimulated proteolytic activity not detected in control cultures. Sodium dodecyl sulfate-stimulated proteolysis was inhibited by leupeptin or E-64, but not by PMSF. Other tissues, sucrose-starved cells and cotyledons, that contain high levels of proteolytic activity did not contain TE-specific proteinases, but did contain higher levels of E-64-sensitive activities migrating as 36- to 31-kD enzymes and as a PMSF-sensitive 66-kD proteinase.

pcd is a process by which the self-directed death of specific cells occurs as a necessary component of normal development. It is distinct from a pathological or necrotic cell death process. Several regulatory genes required for pcd in vertebrates and invertebrates have been characterized using neuronal (Rubin et al., 1994), insect intersegmental muscle (Jones et al., 1995), and *Caenorhabditis elegans* (Hengartner and Horvitz, 1994) model systems. In contrast, relatively little is known about this essential process in higher plants. Leaf and flower senescence are certainly the most visible examples of pcd in plants, but pcd is also responsible for the tissue sculpting that results in the fenestrations in the leaves of *Monstera* (Melville and Wrigley, 1968), and recent evidence indicates that toxin- and pathogen-induced cell death in plants displays some of the hallmarks of a particular type of pcd, apoptosis, during which nDNA fragmented at internucleosomal sites (Ryerson and Heath, 1996; Wang et al., 1996) is partitioned into membrane-bound apoptotic bodies (Wang et al., 1996).

In addition to the pcd events listed above, the terminal differentiation that results in primary and secondary (woody) xylem formation is of particular fundamental and economic importance. Xylem is characterized by the presence of TEs, which are formed as a result of a shift in nuclear and cytoplasmic activities toward the formation and lignification of new cell wall thickenings. The differentiating cells then undergo autolysis, resulting in degradation and release of cellular contents, establishing a free path for the transport of water and minerals. Using mesophyll cells isolated from the young leaves of *Zinnia elegans*, TE differentiation can be reproduced in culture (Kohlenbach and Schmidt, 1975; Fukuda and Komamine, 1980). With few exceptions (most notably in the degradation of interthickening wall material), the events of TE differentiation in culture proceed at a similar rate and in the same manner as those in the intact leaf (Burgess and Linstead, 1984a). In culture, isolated cells are initially homogeneous, and differentiation into TEs occurs semisynchronously in 40 to 60% of living cells by 72 h.

The *Z. elegans* system has been used to study the ultrastructural changes associated with TE differentiation and cell wall thickening (Burgess and Linstead, 1984b; Haigler and Brown, 1986; Taylor et al., 1992). Cultured *Z. elegans* TEs also provide an inducible system for synthesis of the enzymes involved in catalyzing formation of the lignified secondary wall, including xylan synthase (Suzuki et al., 1991), Phe ammonia-lyase (Fukuda and Komamine, 1982; Lin and Northcote, 1990), 4-coumarate:CoA ligase (Church and Galston, 1988), peroxidase (Fukuda and Komamine, 1982; Church and Galston, 1988), and *O*-methyltransferases (Ye et al., 1994; Ye and Varner, 1995). Several cDNAs associated with in vitro TE differentiation in *Z. elegans* have been cloned. Those that appear to encode known proteins include a lipid transfer protein, an adenylate kinase, a thiol proteinase (Ye and Varner, 1993), and *O*-methyltransferases (Ye and Varner, 1995). Some of the genes expressed in differentiating TEs in vitro are also expressed in differentiating TEs in vivo (Demura and Fukuda, 1994; Ye et al., 1994; Ye and Varner, 1995).

Proteinases implicated in the triggering and/or modulation of pcd in animal systems include the Cys proteinases calpain and members of the interleukin-1β coverting enzyme family, and the Ser proteinases of the granzyme B family (for reviews, see Yuan, 1995; Greenberg, 1996; Squier and Cohen, 1996). In plants, examples of developmentally programmed senescence or cell death events that correlated with increased Cys proteinase gene expression

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Abbreviations: E64, *N*-[*N*-(L-3-*trans*-carboxirane-2-carbonyl)-L-leucyl]-agmatine; pcd, programmed cell death; TE(s), tracheary element(s).

and/or activity include TE differentiation (Minami and Fukuda, 1995; Ye and Varner, 1996), anther tissue degeneration (Koltunow et al., 1990), pea ovary senescence (Granell et al., 1992), leaf senescence (Hensel et al., 1993; Drake et al., 1996; reviewed by Feller, 1986), and flower senescence (Valpuesta et al., 1995). The plant proteinases described in these reports probably function to mobilize amino acids and/or as autolytic enzymes. Additional roles for proteinases during pcd in plants may include promotion of cell death via the degradation of regulatory proteins that prevent pcd and the processing of inactive cell death promoters to active cell death promoters. Such regulatory pcd proteinases have not been identified in plants. We have used the *Z. elegans* mesophyll cell system to partially characterize four proteinases that may be involved in autolysis during TE differentiation, and those enzymes have been compared with proteinases that are active during Suc starvation and in cotyledons. The use of a single-cell assay for the demonstration of the high levels of proteinase activity in differentiating TEs versus undifferentiated cells in the same culture is also presented.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of *Zinnia elegans* cv Envy (Stokes Seeds, Buffalo, NY) were sown in 4-inch pots containing Sunshine Mix 1 (Sungrow Horticulture, Bellevue, WA). Plants were grown for 1 week in a growth chamber at 27°C under a 16-h photoperiod at 85 μmol photons m^{-2} s^{-1} and were watered as needed with distilled water.

Mesophyll Cell Isolation and Culture

The first true leaves were harvested from 7-d-old plants, and mesophyll cells were isolated and cultured in either an inductive TE medium or a noninductive control medium according to Roberts et al. (1992). These media were identical except that BA concentrations were 900 and 4.5 nM in inductive and noninductive cultures, respectively. In the Suc-starvation experiments cells were cultured in a complete noninductive medium for 48 h before the medium was replaced either with a fresh complete noninductive medium or a noninductive medium without Suc.

Sample Preparation

Z. elegans cells were collected from suspension cultures at the times indicated in the figure legends by centrifugation at 50*g* for 2 min. Cell pellets were stored at −80°C until extraction. For proteinase activity experiments, cells were lysed by four freeze/thaw cycles in 150 mM NaCl, 50 mM sodium phosphate, pH 7.2, and 14 mM 2-mercaptoethanol. Leupeptin (20 μM final concentration) was included either in the lysis buffer or in SDS-PAGE sample buffer (see below). Lysed cells were pelleted by centrifugation at 12,000*g* for 20 min at 4°C. The supernatant was concentrated approximately 10-fold using YM10 or YM3 concentrators (Amicon, Beverly, MA) and stored at −80°C until use. Extracts from leaves (7 d old) and cotyledons (3 weeks old) were prepared as described above for cells, except that a mortar and pestle were used at 4°C rather than freeze/thaw cycles. For cellular protein content measurements, cells were extracted as described above, except that the extraction buffer included additional proteinase inhibitors at the following final concentrations: PMSF, 2 mM; EDTA, 2 mM; *p*-chloromercuriphenylsulfonic acid, 5 mM; and iodoacetamide, 5 mM.

Total Protein Determination

Protein content of cell extracts was determined using bicinchoninic acid (Sigma) according to the supplier's instructions.

Single-Cell Proteinase Assays

Cell suspensions (1 mL at 72 h) from induced cultures were allowed to settle to the bottom of a microfuge tube. The culture medium was removed from the cell pellet and replaced with a proteinase assay medium consisting of acrylamide:*N,N'*-methylene-bisacrylamide (7.0:0.18, w/v), 1% gelatin (porcine, type A, 300 bloom, Sigma), 14 mM 2-mercaptoethanol, and 0.12% ammonium persulfate. Polymerization of polyacrylamide was catalyzed by the addition of 0.5 μL of *N,N,N',N'*-tetramethylethylenediamine. Cells were resuspended in the above medium and 50-μL aliquots were applied to microscope slides and covered with glass coverslips. Immediately after polymerization (approximately 15 min), coverslips were lifted and 25 μL of 50 mM Mes, pH 5.5, was applied to the polymerized cell suspension. Coverslips were replaced and microscope slides were placed on a moistened paper towel, wrapped in plastic, and incubated overnight in the dark at 27°C. Following incubation, coated microscope slides were fixed in destain for 30 min followed by staining with Coomassie brilliant blue. Destained slides were inspected for cells surrounded by unstained halos, representing zones of gelatin hydrolysis.

SDS-PAGE

SDS-PAGE was conducted using the buffer system of Laemmli (1970). Resolving gels were 12% (w/v) acrylamide. Urea (2 M final concentration) and leupeptin (20 μM final concentration) were added to the SDS-PAGE sample buffer for activity gels. Samples for the activity gels were not boiled prior to electrophoresis. In some experiments samples were preincubated for 15 min at room temperature with proteinase inhibitors, as indicated in the figure legends. To test for SDS-stimulated proteolysis, samples were incubated in SDS (1% final concentration) for 4 h at 27°C prior to boiling in SDS-PAGE sample buffer.

Two-Dimensional PAGE

IEF was conducted on a vertical, 8.5 × 7.0 × 0.15 cm 7% (w/v) acrylamide gel. The final concentrations of the ampholytes (Bio-Rad) were pH 4.0 to 6.0, 2%; pH 6.0 to 8.0, 2%; and pH 3.0 to 10.0, 0.2%. The cathode and anode buffers were 100 mM 3-(cyclohexylamino)-1-propane-

sulfonic acid (pH 8.0) and 10 mM Glu (pH 3.0), respectively. Gels were prefocused for 30 min at 500 V and focused at 1500 V for 2 h. For the second dimension, a strip of gel containing the focused proteins was equilibrated in SDS-PAGE running buffer, positioned across the top of a 12% (w/v) gel, and resolved as described above for SDS-PAGE.

Proteinase Activity Gels

Following resolution of proteins by SDS-PAGE, gels were incubated in 1 M potassium acetate, pH 5.5, 14 mM 2-mercaptoethanol (with or without proteinase inhibitors, as indicated in the figure legends) for 7 min, placed next to substrate-impregnated 7% (w/v) polyacrylamide gels, sandwiched between 3MM paper wetted with resolving gel incubation buffer, sealed in a plastic bag, and incubated in darkness for 16 h at 27°C. The substrates used were total *Z. elegans* protein (0.25%, w/v) or gelatin (0.5%, w/v). *Z. elegans* protein for the substrate gels was extracted in the presence of E-64 and PMSF from cells that were cultured in an inductive medium for 72 h as described above. Unreacted proteinase inhibitor was removed by dialysis against the cell extraction buffer without 2-mercaptoethanol. Substrate-impregnated gels were stained with Coomassie brilliant blue (*Z. elegans* protein) or amido black (gelatin). Amido black-stained gels (1% [w/v] in 7% acetic acid) were destained with 7% acetic acid. Hydrolysis of the substrates resulted in unstained bands in the substrate-impregnated gels, indicating the position of the proteolytic activity in resolving gels.

RESULTS

Changes in Levels of Extractable Protein

Total protein measurements of mesophyll cell extracts prepared over the course of a 120-h culture indicated that the protein levels in induced and noninduced cultures changed little for the first 48 h of culture (Fig. 1). After 48 h, the level of protein extracted from noninduced cultures increased steadily through 120 h. In contrast, after reaching its highest level by 72 h, extractable protein from the induced cultures decreased to a value that by 120 h was 2.5-fold less than that observed for the noninduced cultures that were harvested at the same time (Fig. 1).

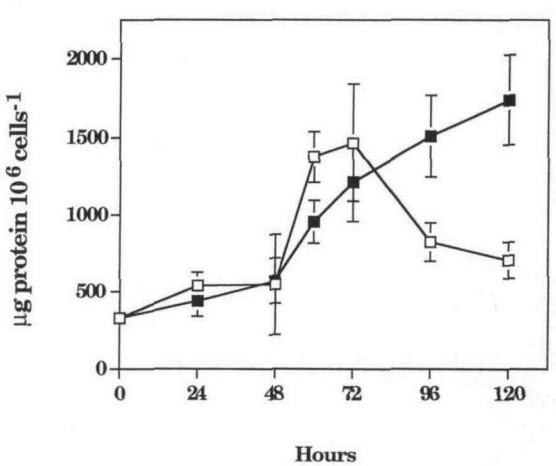

Figure 1. Change in extractable protein content of induced (□) and noninduced (■) cultured *Z. elegans* mesophyll cells. Each point represents the mean ± SD of three or four replicates.

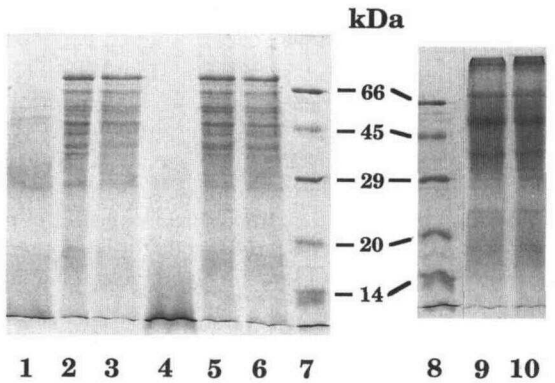

Figure 2. SDS-stimulated autolysis of extracted proteins by endogenous proteinases. Coomassie blue-stained gel following SDS-PAGE of protein (lanes 1–6, 30 μg; lanes 9 and 10, 40 μg) extracted from 72-h-induced (lanes 1–6) or noninduced (lanes 9 and 10) *Z. elegans* cells. Boiled controls (lanes 6 and 10) were preincubated with E-64 (25 μM final concentration) prior to boiling in SDS-PAGE sample buffer. Following preincubation with E-64 (lanes 3 and 5), PMSF (5 mM final concentration) (lanes 4 and 5), or no inhibitor (lanes 1, 2, and 9), samples were incubated for 4 h at 27°C with (lanes 1, 3–5, and 9) or without (lane 2) SDS (1% final concentration). Following incubation, E-64 was added to those samples not preincubated in E-64 and all samples were then boiled in SDS-PAGE sample buffer. Molecular masses of the standards (lanes 7 and 8) are shown between gels.

Increased Proteinase Activity in TEs

The decrease in extractable protein that we observed in the induced cultures occurred concomitant with the appearance of cell wall thickenings, i.e. detectable TE differentiation. If a decrease in the cellular protein content during TE differentiation is the result of increased proteolysis in differentiating TEs, extracts from the induced cultures should be capable of degrading *Z. elegans* proteins when incubated under conditions favoring proteinase activity. When extracts from the induced cultures were tested for autolytic activity at pH 5.5 (data not shown) or pH 7.2, no detectable changes in the profile of the Coomassie blue-stained proteins occurred during a 4-h, 27°C incubation unless SDS was present (Fig. 2). This SDS-stimulated autolytic activity was prevented by the inclusion of either of two thiol proteinase inhibitors, leupeptin (data not shown) or E-64. Remarkably, inclusion of the Ser proteinase inhibitor PMSF repeatably enhanced SDS-stimulated proteolysis (Fig. 2, compare lanes 1 and 4). In contrast, regardless of SDS or inhibitor treatment, the noninduced culture extracts exhibited no autolytic activity. A boiled control and a SDS-treated noninduced culture extract are presented as a representative comparison (Fig. 2, lanes 9 and 10, respectively). Subsequent to these experiments, cell extracts were never exposed to SDS in the absence of leupeptin (see "Materials and Methods"). Leupeptin is a reversible thiol proteinase inhibitor that is removed during SDS-PAGE,

thereby permitting detection of thiol proteinases in activity gels following electrophoresis (see below).

A representative 72-h *Z. elegans* mesophyll cell TE culture that was used in these studies is best described as consisting of approximately equal proportions of dead mesophyll cells (killed during the initial isolation), live undifferentiated cells, and differentiating TEs. To address the possibility that proteinases extracted from undifferentiated cells present in TE cultures could catalyze the nonspecific proteolysis illustrated by Figure 2, we developed a single-cell assay to visualize proteolysis associated with different cell types. Cells from 72-h-induced cultures were immobilized on gelatin-coated microscope slides and incubated overnight. Subsequent Coomassie blue staining of slides revealed zones of hydrolyzed gelatin (unstained halos) surrounding cells in all of the stages of differentiation (Fig. 3). Undifferentiated cells were not associated with the zones of hydrolysis. These results suggest that the proteolytic activity illustrated in Figure 2 was extracted from differentiating TEs, and not from undifferentiated cells that were present in the induced cultures.

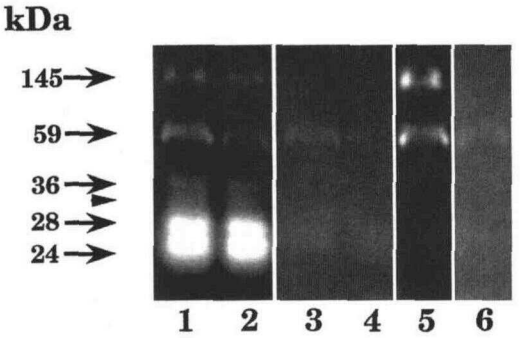

Figure 4. Digitized image of gelatin- and total *Z. elegans* protein-impregnated gels revealing proteinase activity following resolution by SDS-PAGE of proteins extracted from 72-h-induced *Z. elegans* cells. Lanes 1, 2, and 5, Gelatin-impregnated activity gel; and lanes 3, 4, and 6, total *Z. elegans* protein-impregnated activity gel. SDS-PAGE gel was loaded with 40 μg of protein in each lane. Samples and gels were incubated with proteinase inhibitors as follows: lanes 1 and 3, no inhibitor; lanes 2 and 4, 5 mM PMSF; and lanes 5 and 6, 25 μM E-64.

Partial Characterization of TE Proteinases

The SDS-stimulated protein degradation shown in Figure 2 indicated that proteinases present in TEs would still be active after SDS-PAGE. Both *Z. elegans* protein- and gelatin-impregnated activity gels were used to visualize proteinases following SDS-PAGE. Three bands of hydrolyzed *Z. elegans* protein were evident in Coomassie blue-stained activity gels, representing the activity of 59-, 28-, and 24-kD proteinases capable of nonspecific hydrolysis of *Z. elegans* proteins in the presence of SDS (Fig. 4). Proteinases capable of gelatin hydrolysis co-migrated with all three bands of activity detected in *Z. elegans* protein activity gels (Fig. 4, compare lanes 1 and 3). Two additional bands of hydrolysis were detected in gelatin activity gels, representing activities of the 36- and 145-kD proteinases. A poorly resolved activity migrating between 36 and 28 kD was detectable in some preparations (Fig. 4, arrowhead).

The 36-, 28-, and 24-kD proteinases were sensitive to E-64 (Fig. 4). The 59-kD proteinase was largely, but not completely, inhibited by 5 mM PMSF. At 10 mM PMSF, however, inhibition of proteolysis by the 145- and 59-kD proteinases in gelatin-impregnated gels was complete (see below). In addition to the apparent molecular masses and proteinase inhibitor sensitivities, we were able to determine pI values for two of the proteinases. Figure 5 shows the pI values of the two most active TE proteinases, 59 and 24 kD, to be 4.9 and 6.2, respectively. We were not able to detect the other TE proteinases by this method, perhaps due to inactivation during IEF.

When preparations from noninduced and induced cultures were compared using gelatin-impregnated activity gels, a 36-kD proteinase (also visible in Fig. 4) was active in both samples (Fig. 6A). The only other major activity detectable in the noninduced culture extracts migrated as a 66-kD enzyme. The 59-kD activity detected in the induced culture extracts was present at minimal levels in most noninduced culture extracts. Although a 66-kD proteinase was not visible in Figure 4, it was detectable in extracts from most of the induced cultures (Figs. 6A and 7A, lanes 1). Hence, it appears that there are three constitutive *Z. elegans* proteinases migrating at 36, 59, and 66 kD, with the 59-kD proteinase more active in the induced than in the noninduced cultures. The 145-, 28-, and 24-kD proteinases appear to be exclusive to differentiating TEs.

Comparison of TE Proteinases with Proteinases from Suc-Starved Cells and *Z. elegans* Cotyledons

To determine whether the TE proteinases detected in this study were unique to TE autolysis or could be detected under another cell culture condition known to induce proteolytic activities (James et al., 1993), we compared protein-

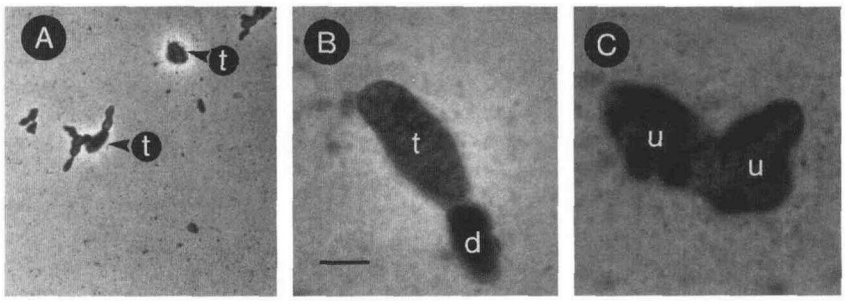

Figure 3. Single-cell proteinase assay. *Z. elegans* cells from 72-h-induced cultures were immobilized on gelatin-impregnated, polyacrylamide-coated microscope slides, incubated overnight, and stained with Coomassie blue. Unstained halos surrounding differentiating TEs result when gelatin hydrolysis is catalyzed by proteinases diffusing from cells (A and B). Gelatin hydrolysis by undifferentiated cells is not detectable (C). d, Dead cell; t, TE; and u, undifferentiated cell. Bar = 115 μm for A and 19 μm for B and C.

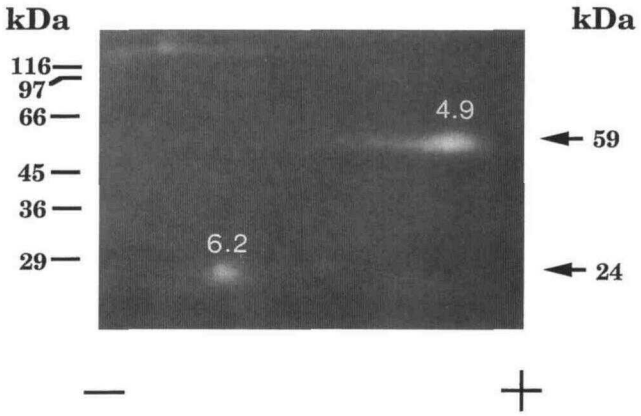

Figure 5. Gelatin-impregnated gel revealing proteinase activity following two-dimensional PAGE of proteins extracted from 72-h TE cultures. The gel slice from the first IEF dimension that was used for the second SDS-PAGE dimension contained 100 μg of protein. The pI values of the 59- and 24-kD TE proteinases (arrows) are indicated above the spots corresponding to gelatin-hydrolyzing activity. Molecular masses of second-dimension SDS-PAGE standards are indicated at the left of the figure.

ases active in Suc-starved *Z. elegans* cells with TE proteinases (Fig. 6A). The levels of activities co-migrating with the constitutive 66- and 36-kD proteinases were greater in extracts from the Suc-starved cells, relative to that detected in an equal quantity of protein from 72-h-induced extracts (Fig. 6A, compare lanes 1 and 4). In addition, a high level of poorly resolved activity migrating between 36 and 31 kD was detected in extracts from Suc-starved cells. The 66- and 36- to 31-kD activities were also detectable in extracts of mesophyll cells cultured for 120 h in complete (Suc-containing), noninductive medium, but at reduced levels, and only when 50% more protein, relative to the extract from Suc-starved cells, was assayed (Fig. 6). The profile of proteinases induced by Suc starvation, therefore, is distinct from that of the TE proteinases. Extracts from fresh leaves exhibited a minimal amount of activity of the three enzymes that co-migrated with the constitutive proteinases (66, 59, and 36 kD), as well as a unique, 30-kD enzyme (Fig. 6A, lane 5).

At equivalent levels of total protein, TE proteinases were compared with those that were active during another developmentally programmed cell death event, cotyledon senescence. Only extracts from nonsenescent (green) and advanced senescent (yellow) cotyledons are shown, since activity in these samples effectively summarizes changes occurring during advancing senescence (Fig. 7). Activity gels revealed slightly higher levels of the 66-kD proteinase in yellow cotyledons relative to TE cultures, Suc-starved cells, and green cotyledons. Proteinase activity migrating at 59 kD, readily detected in TE cultures, was barely detectable in cotyledon extracts (Fig. 7A). Proteinase activity co-migrating with the 36- to 31-kD activity, which increased during Suc starvation (Fig. 7A, lane 4), was detected in green cotyledons and appeared to decrease as the cotyledons yellowed (Fig. 7A, compare lanes 2 and 3). Neither cotyledons nor Suc-starved cells contained detectable levels of the 145-, 28-, or 24-kD proteinases (Figs. 6 and 7).

Regardless of the cell culture experiment or the tissue type we examined, results from the proteinase inhibitor treatments can be summarized as follows: proteinases 36 kD or smaller were inhibited by E-64 (Figs. 6B and 7B), and those migrating as 59-kD or larger proteins were inhibited by PMSF (Figs. 6C and 7B). Other Ser proteinase inhibitors, such as 3,4-dichloroisocoumarin and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, did not inhibit any of the proteinases detected by the activity gels (data not shown).

DISCUSSION

We have used a single-cell assay for the first unequivocal demonstration, to our knowledge, of increased proteinase activity specific to differentiating *Z. elegans* TEs rather than to undifferentiated cells present in TE cultures. The four proteinases with activities that are either increased in TE

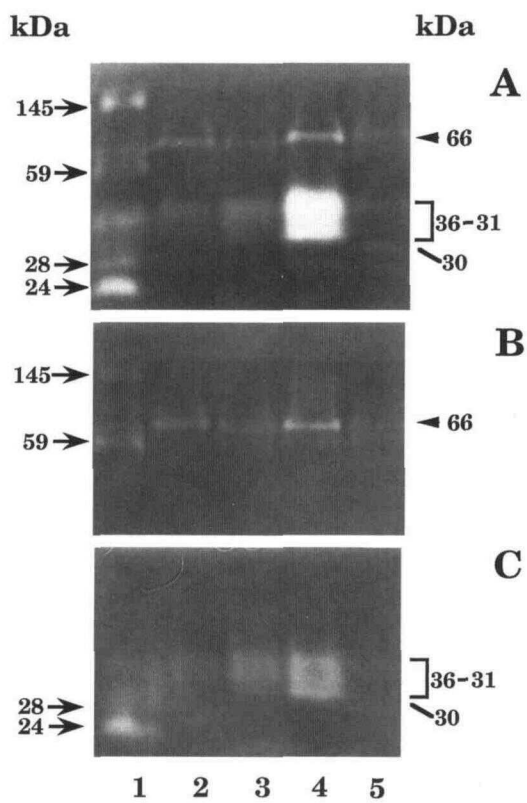

Figure 6. Gelatin-impregnated gels reveal proteinase activity following SDS-PAGE of *Z. elegans* proteins extracted from 72-h-induced (lane 1) or noninduced (lane 2) cultures; 120-h, with (lane 3) or without (lane 4) Suc cultures; and expanding leaf (lane 5). Molecular masses of TE proteinases (arrows) are indicated at the left of the figure. Molecular masses of Suc-starvation-induced proteinases (arrowheads and brackets) are indicated at the right of the figure. A 30-kD leaf proteinase (bars) is indicated at right. A, Activity gel without proteinase inhibitors. B, Activities after 25 μM E-64 treatment. C, Activities after 10 mM PMSF treatment. The amount of protein loaded in lanes 1 and 4 was 30 μg, and the amount of protein loaded in lanes 2, 3, and 5 was 45 μg.

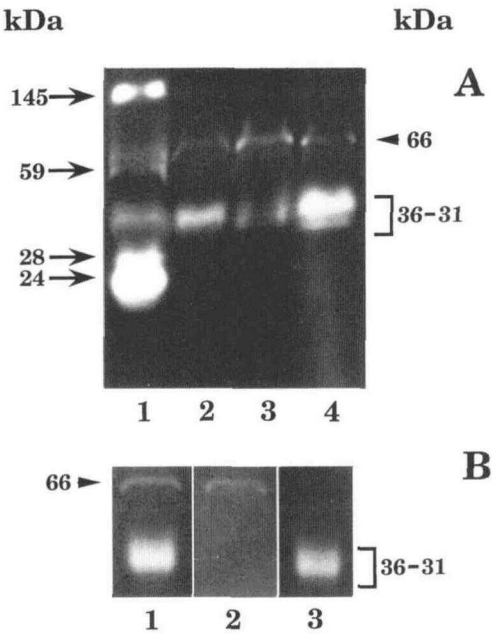

Figure 7. Gelatin-impregnated gels reveal proteinase activity following SDS-PAGE of *Z. elegans* proteins extracted from induced cultures, cotyledons, or Suc-starved cells. A, Activities in 72-h-induced cultures (lane 1), 7-d-old green cotyledons (lane 2), senescent cotyledons (lane 3), or Suc-starved cells (lane 4). B, Activities in green cotyledons without proteinase inhibitors (lane 1) and after treatment with 25 μM E-64 (lane 2) or 10 mM PMSF (lane 3). Molecular masses of TE proteinase (arrows) are indicated at the left of the figure. Molecular masses of cotyledon and Suc-starvation-induced proteinases (arrowhead and brackets) are indicated at the right of the figure. The amount of protein loaded in all lanes was 30 μg.

cultures or exclusive to TE cultures (compared with proteinases detected in extracts from undifferentiated cells, Suc-starved cells, whole leaves, and green and senescing cotyledons) have apparent molecular masses of 145, 59, 28, and 24 kD. The 145- and 59-kD proteinases were tentatively identified as Ser proteinases based on sensitivity to PMSF. The faster-migrating 28- and 24-kD proteinases were tentatively identified as thiol proteinases based on their sensitivity to leupeptin and E-64. All organs and cell types examined contained an E-64-sensitive 36-kD proteinase as well as PMSF-sensitive 66- and 59-kD proteinases.

Two limitations to the experiments described here should be noted. First, assays conducted in the presence of SDS (the in vitro autolysis experiment [Fig. 2] and the activity gels following SDS-PAGE [Figs. 4–7]) may have detected proteinases distinct from those that are active in the absence of SDS (single-cell assays [Fig. 3]). We were not able to demonstrate a consistent effect of the proteinase inhibitors E-64, leupeptin, and PMSF on activity during single-cell assays (data not shown). Effective inhibition of proteolysis may require preincubation of the extracted enzyme with the inhibitor, and this was not possible with the single-cell assays. Second, because the activity gels described here use protein samples that are not boiled prior to electrophoresis, the apparent molecular mass of a detected proteinase may differ from its true molecular mass. Nevertheless, we assumed that this method would provide a basis for a comparison of proteinases active in TE cultures with those in the noninduced controls and with proteinases in other extracts prepared from *Z. elegans*. Gelatin-SDS-PAGE analysis of unboiled samples has been used to characterize developmentally regulated changes in proteinase activity in *Dictyostelium* (North and Cotter, 1991).

The ability of the 59-, 28-, and 24-kD proteinases to degrade crude *Z. elegans* protein (Fig. 4) indicates that these enzymes may function independently as nonspecific proteinases during TE autolysis. Moreover, the activity of the 28- and 24-kD enzymes following SDS-PAGE indicates that they may be the E-64-sensitive proteinases responsible for SDS-stimulated, nonspecific protein degradation (Fig. 2, lane 1). The further stimulation of proteolysis observed when SDS and PMSF were combined (Fig. 2, lane 4) suggests that (a) PMSF-sensitive proteinases were not responsible for the observed SDS-stimulated nonspecific proteolysis, and (b) PMSF protected the SDS-stimulated Cys proteinases from degradation catalyzed by Ser proteinases. We did not determine whether SDS stimulated proteolysis through a direct effect on proteinases or endogenous proteinase inhibitors, or by rendering the substrates more accessible to proteinases. Stimulation of activity by SDS has also been reported for proteasomes (Orlowski, 1990). However, the physiological significance of proteolytic activity stimulated by SDS is not known. Although proteolytic activity of the 145-kD proteinase was detected using gelatin-impregnated gels, this enzyme did not catalyze degradation of crude *Z. elegans* protein in activity gels. The 145-kD proteinase may have a more restricted substrate specificity than the faster-migrating enzymes.

The majority of proteinase activity in plant cells has been reported to be vacuolar (reviewed by Callis, 1995). Hence, the TE proteinases described here and in other reports (Minami and Fukuda, 1995; Ye and Varner, 1996) are probably vacuolar. Their subcellular location, however, has not been determined, and the presence of an amino-terminal ER-targeting domain in the TE Cys proteinase cloned by Ye and Varner (1996) may indicate vacuolar or cell wall localization (Holwerda et al., 1990). A dehydration-inducible Cys proteinase has been localized to the cell wall of pea (Jones and Mullet, 1995). The unstained halo surrounding TEs after the single-cell assay could therefore result from the activity of extracellular proteinases. It is expected, however, that intracellular enzymes leaking from cells following autolysis would also contribute to gelatin hydrolysis.

It is possible that three classes of proteinases are represented by the enzymes described in this report. The first class consists of proteinases that are regularly involved in protein turnover, in which the activity is increased in response to specific environmental, metabolic, or developmental cues (Drake et al., 1996). Examples of this class of proteinases may include the apparently constitutive 36-, 59-, and 66-kD enzymes, in which the activity levels vary among the tissues examined. The second consists of proteinases induced in response to one or more environmental, metabolic, or developmental cues, excluding pcd. The activities that migrate between 36 and 31 kD and are active

following Suc starvation and in cotyledons may be members of this class. The third class consists of autolytic proteinases induced only during pcd. This class may be represented by the 145-, 28-, and 24-kD TE proteinases.

Although James et al. (1993) did not perform chromatographic procedures for size determination of the Ser and Cys proteinases induced by Suc starvation of maize root tips, several recent reports provide more detailed information on proteinases active in multiple tissues including those active in cotyledons. In tomato, mRNAs for two Cys proteinases, SENU2 and SENU3, that reached maximum levels in senescent leaves were also detectable in young, fully expanded leaves and germinating seeds (Drake et al., 1996). SENU3 is predicted to encode a prepropeptide of 38 kD. Wolf and Storey (1990) used azocasein and synthetic peptides as the substrates during the characterization of proteinases active in the cotyledons of jojoba seedlings. Separation by ion-exchange chromatography and detection in chromogenic peptide substrate-impregnated, nondenaturing gels confirmed the presence of four proteinases. Subsequent SDS-PAGE analysis revealed the presence of a 58-kD Ser proteinase and a 35-kD Cys proteinase, similar perhaps to the 66-kD Ser and 36- to 31-kD Cys proteinases detected in *Z. elegans* cotyledons (Fig. 7). In another study of cotyledon enzymes, gelatin-impregnated IEF gels were used to characterize imbibition-induced, light-induced cotyledon proteinases extracted from cucumber (Yamauchi et al., 1996). However, because second-dimension SDS-PAGE gels were not produced by these authors, the molecular masses of proteinases were not determined.

The 24-kD proteinase reported here may be the same as a previously described TE Cys proteinase (Ye and Varner, 1996). The mRNA encoding the proteinase cloned by Ye and Varner (1996) is present in the xylem of *Z. elegans* stems and predicts a 38-kD prepropeptide. Predicted processing to the mature form would yield a protein of 22.7 kD, closer in molecular mass to the 24-kD TE proteinase detected by activity gels reported here than the 20-kD proteinase reported by Ye and Varner (1996). It is possible that the 10-min preincubation of the TE extracts in SDS-PAGE sample buffer employed by these authors resulted in partial proteolysis of the mature proteinase. The predicted pI of 5.7 for the mature Cys proteinase described by Ye and Varner (1996) was not in agreement with the experimentally determined pI of 6.2 for the 24-kD TE proteinase (Fig. 5). These authors also detected a 60-kD PMSF-sensitive enzyme, which may be the same as the 59-kD proteinase described here. The 30-kD Cys proteinase purified by Minami and Fukuda (1995) is probably distinct from the proteinases we have described here, since it is inhibited by both PMSF and leupeptin. However, PMSF can partially inhibit Cys proteinases (Wolf and Storey, 1990), and gelatin-impregnated activity gels may not be sensitive enough to detect partial inhibition of proteinases.

We have used gelatin- and crude *Z. elegans* protein-based assays to visualize proteinases that probably participate in the autolysis of TEs. Of equal importance, however, are the upstream regulatory events that commit cells to pcd and depend, at least in part, on other proteinases, including the 26S proteasome of the ubiquitin-dependent pathway (Callis, 1995). The development of relevant model substrates and suitable assays for detecting proteinases involved in the turnover of regulatory proteins would greatly facilitate our understanding of the role of protein turnover in the early stages of pcd. Characterization of proteinases specific to pcd and of the molecular mechanisms responsible for their regulation would provide important fundamental information concerning pcd during TE differentiation. Such information may be relevant to other agronomically important cell death events, including leaf and flower senescence and local lesion formation during the hypersensitive response.

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