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Proteasome Inhibitors Prevent Tracheary Element Differentiation in Zinnia Mesophyll Cell Cultures[1]

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To determine whether proteasome activity is required for tracheary element (TE) differentiation, the proteasome inhibitors *clasto*-lactacystin β-lactone and carbobenzoxy-leucinyl-leucinyl-leucinal (LLL) were used in a zinnia (*Zinnia elegans*) mesophyll cell culture system. The addition of proteasome inhibitors at the time of culture initiation prevented differentiation otherwise detectable at 96 h. Inhibition of the proteasome at 48 h, after cellular commitment to differentiation, did not alter the final percentage of TEs compared with controls. However, proteasome inhibition at 48 h delayed the differentiation process by approximately 24 h, as indicated by examination of both morphological markers and the expression of putative autolytic proteases. These results indicate that proteasome function is required both for induction of TE differentiation and for progression of the TE program in committed cells. Treatment at 48 h with LLL but not *clasto*-lactacystin β-lactone resulted in partial uncoupling of autolysis from differentiation. Results from gel analysis of protease activity suggested that the observed incomplete autolysis was due to the ability of LLL to inhibit TE cysteine proteases.

The role of proteases in regulating PCD is currently an extremely active area of research. Principal among the proteases shown to be required for PCD in animal systems is the large and still growing family of proteases known as caspases (cysteinyl aspartate-specific proteases; for review, see Nicholson and Thornberry, 1997), which appear to be involved in disabling cell-repair processes essential for maintaining homeostasis, thereby leading to apoptosis. However, caspases are not the only proteases demonstrated to play a role in PCD. Animal apoptosis pathways can also be initiated by several other mechanistically distinct proteases, including the Ser protease granzyme B (Greenberg, 1996), members of the cathepsin family (Deiss et al., 1996), and the Cys protease calpain (Squier and Cohen, 1996).

In plants it is well documented that increases in Ser and Cys proteases are associated with two developmentally programmed suicide pathways, organ senescence (for review, see Hadfield and Bennett, 1997) and TE differentiation (for review, see Beers, 1997; Fukuda, 1997; Pennell and Lamb, 1997). Although it is generally assumed that these plant enzymes function in the autolysis of intracellular proteins rather than as components of regulatory proteolytic cascades, the apparent participation of multiple proteolytic pathways during animal PCD indicates that the ability of plant proteases to regulate PCD may be underestimated.

An additional proteolytic system, the ATP-dependent ubiquitin-proteasome pathway of proteolysis, is known to regulate numerous cellular processes via degradation of short-lived regulatory proteins in mammals, yeast, and plants (Shanklin et al., 1987; Glotzer et al., 1991; Hochstrasser et al., 1991). The importance of the ubiquitin-proteasome pathway in degrading long-lived proteins is well-established for mammals, but its function in this capacity in lower eukaryotes is uncertain (for review, see Goldberg, 1997). Ubiquitin is a 76-amino acid protein that becomes covalently ligated to cellular proteins via isopeptide bonds between the carboxy-terminal Gly of ubiquitin molecules and the ε-amino group of Lys residues of the target protein (for review, see Ciechanover and Schwartz, 1994; Varshavsky, 1997). Attachment of ubiquitin to protein targets requires the activity of multiple enzymes, including an ATP-dependent ubiquitin-activating enzyme (E1), one of a family of ubiquitin-conjugating enzymes (E2s), and, in some cases, one of a number of ubiquitin-protein ligases (E3s). Ubiquitin may be attached to protein substrates as a monomer or it may be ligated to a Lys residue of another ubiquitin molecule, forming polyubiquitin chains. Polyubiquitination of proteins is sufficient to target them for degradation by a large (26S), ATP-dependent multicatalytic protease, the proteasome. Additionally, proteasome-mediated degradation of a few proteins has been shown to occur without ubiquitination (Murakami et al., 1992; Jariel-Encontre et al., 1995).

Recent evidence indicates that the ubiquitin-proteasome pathway may regulate PCD in some systems. Levels of ubiquitin-proteasome pathway components were observed to increase during animal PCD events, including molt-induced claw muscle atrophy in lobster (Shean and Mykles, 1995) and intersegmental muscle degeneration during metamorphosis of the hawkmoth (Haas et al., 1995; Jones et al., 1995). Depending on the experimental system under investigation, proteasome activity may promote PCD (Grimm et al., 1996; Sadoul et al., 1996; Cui et al., 1997)

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Abbreviations: LAC, *clasto*-lactacystin β-lactone; LLL, carbobenzoxy-leucinyl-leucinyl-leucinal; LLM, acetyl-leucinyl-leucinyl-methional; LLnL, acetyl-leucinyl-leucinyl-norleucinal; PCD, programmed cell death; TE, tracheary element.

or prevent it (Shinohara et al., 1996; Drexler, 1997; Monney, 1998). Requirements for proteasome activity during differentiation events not involving PCD have also been documented from animal systems, including maturation of starfish oocytes (Sawada et al., 1997) and differentiation of photoreceptor cells in the *Drosophila melanogaster* eye (Li et al., 1997). In plants up-regulation of components of the ubiquitin pathway has been detected during diverse developmental PCD events, including leaf senescence (Garbarino and Belknap, 1994), anther dehiscence (Li et al., 1995), and fruit ripening (Picton et al., 1993). Thus, the ubiquitin-proteasome pathway is broadly implicated as a regulator of cell fate.

Using the zinnia (*Zinnia elegans*) mesophyll cell culture system for TE differentiation (Fukuda and Komamine, 1980a; Church, 1993; Fukuda, 1997), we investigated the role of proteolysis during cell differentiation and PCD. Mature TEs function in planta in water and solute transport and are characterized by a patterned deposition of lignified, cellulosic, secondary cell wall thickenings and the absence of a protoplast within the cell corpse. The zinnia mesophyll cell system permits the study of plant cell differentiation, death, and autolysis during the semisynchronous TE differentiation of 40% to 60% of the cultured cells. Although no genes or proteins controlling cell death or the initiation of the autolytic phase in differentiating TEs have been identified, hydrolytic enzyme activity increases dramatically late in the differentiation process.

Markers for TE autolysis include an endonuclease (Thelen and Northcote, 1989), a RNase (Ye and Droste, 1996), and proteases (Minami and Fukuda, 1995; Ye and Varner, 1996; Beers and Freeman, 1997). The ubiquitin-proteasome pathway also appears to be required for proper vascular tissue development. Transgenic tobacco expressing a mutant ubiquitin unable to form polyubiquitin chains exhibited aberrant vasculature (Bachmair et al., 1990), and in *Coleus*, regeneration of xylem vessel elements after wounding was accompanied by increased levels of ubiquitin and/or ubiquitin-protein conjugates (Stephenson et al., 1996).

Ubiquitin-protein conjugating activity is detectable in zinnia TE culture extracts (B.J. Woffenden and E.P. Beers, unpublished data). Using inhibitors of the proteasome, we asked whether the proteasome has a regulatory role during TE differentiation. Proteasome inhibitors used included LAC (Dick et al., 1997), which was derived from the microbial metabolite lactacystin (Omura et al., 1991), and the synthetic tripeptide-aldehyde inhibitors LLnL, LLM (Sasaki et al., 1990), and LLL (Tsubuki et al., 1996). LAC irreversibly inhibits the tryptic, chymotryptic, and peptidylglutamic cleavage activities of the proteasome by covalently binding to the amino-terminal Thr residue of the β-subunits (Fenteany et al., 1995). Radiographic, crystallographic, and mutagenesis studies have demonstrated that this residue provides the active-site nucleophile (Löwe et al., 1995; Seemüller et al., 1995). Specificity of LAC for the proteasome has been established to the exclusion of the Cys proteases calpain, papain, and cathepsin B, and the Ser proteases chymotrypsin and trypsin (Fenteany et al., 1995). Conversely, peptide aldehydes are reversible, competitive inhibitors that act as transition-state analogs (Löwe et al., 1995) and inhibit both calpain and the proteasome (Rock et al., 1994; Tsubuki et al., 1996).

The data presented here implicate the proteasome as a regulator in both early and late stages of TE differentiation in zinnia mesophyll cell cultures but do not support a direct role for the proteasome in TE autolysis. Rather, autolysis appears to depend at least in part on LLL-sensitive Cys proteases.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of zinnia (*Zinnia elegans* cv Envy; Grimes Seeds, Concord, OH) were sown in 4-inch pots containing Sunshine Mix 1 (Wetsel Seed Co., Harrisonburg, VA). Plants were grown at 27°C under a 16-h photoperiod at 85 μmol photons m^{-2} s^{-1} and watered as needed with distilled water.

Mesophyll Cell Isolation and Culture

The first true leaves were harvested from 7- to 9-d-old seedlings, and mesophyll cells were isolated and cultured in TE inductive medium according to the method of Roberts et al. (1992), except that cells were cultured in scintillation vials in 2.4 mL of medium. Cells were collected from suspension cultures by centrifugation at 50*g* for 2 min at the times indicated in the figure legends. Pellets were stored at −70°C until extraction (when harvesting cells for RNA extraction, this step was preceded by freezing in liquid nitrogen).

Proteasome Inhibitor Treatments

The proteasome inhibitors LLnL, LLM (Sigma), LLL (sold as MG132, Calbiochem), or LAC (Calbiochem) were added to TE cultures as follows: Because the level of solubility of LLL in aqueous solution is approximately 50 μM, LLL was added to cultures as 20 μM pulses every 6 h, either between 0 and 18 h or between 48 and 66 h, to a final concentration as indicated in the figure legends. LAC was added to a final concentration as indicated in the figure legends. DMSO solvent controls were evaluated for all treatments. Regardless of the method of inhibitor addition (i.e. pulsed multiple times or single additions), inhibitor additions begun at the time of culture initiation and at 48 h are referred to as t_0 and t_{48}, respectively.

Cell Counts

For counting, an aliquot of cells was mixed with an equal volume of 1% Evans blue (Sigma), which is excluded from live cells (Roberts and Haigler, 1989), in culture medium. Values for three populations were recorded using a hemacytometer: live, nondifferentiated cells, live TEs, and dead TEs. To score incomplete autolysis, dead TEs were counted as either autolytically cleared or retaining cellular contents.

Hoechst 33342 Staining of Cultured Cells

Paraformaldehyde-fixed (Planchais et al., 1997) cells stained with 1 μg mL^{-1} Hoechst 33342 in Galbraith buffer (20 mM Mops, pH 7.0, 45 mM $MgCl_2$, 30 mM sodium citrate, and 1% [w/v] Triton X-100; Galbraith et al., 1983) were viewed and photographed using a fluorescence microscope (model MC 63, Zeiss).

Protein Extraction

In each of two independent experiments for each inhibitor, four replicates (scintillation vials) were pooled at each harvest (72 and 96 h) and scored for TE differentiation. Extracts were prepared by four freeze-thaw cycles in 100 mM $NaPO_4$ buffer, pH 7.2, containing 20 μM leupeptin and 14 mM 2-mercaptoethanol. Lysed cells were pelleted by centrifugation at 12,000*g* for 10 min at 4°C. The supernatant was concentrated approximately 25-fold using YM10 concentrators (Millipore) and stored at −70°C for subsequent use in either activity gels or immunoblots.

Antibody Production and Purification and Immunoblot Analysis of Ubiquitin-Protein Conjugates

Antibodies to denatured, cross-linked bovine ubiquitin (Sigma) were prepared in chickens at Cocalico Biologicals (Reamstown, PA). The immunoglobulin fraction was purified from egg yolk using the caprylic acid extraction protocol of McLaren et al. (1994) and was then subjected to affinity purification (Hershko et al., 1982; Haas and Bright, 1985). After resolution by SDS-PAGE (13.5% [w/v] acrylamide) using the buffer system of Laemmli (1970), zinnia proteins were electrophoretically transferred to PVDF membranes (Immobilon-P, Millipore) using a semidry transfer apparatus (Amersham-Pharmacia Biotech) according to the manufacturer's recommendations. The transfer buffer was 48 mM Tris, 39 mM Gly, pH 8.4, 1.3 mM SDS, 20% methanol. Blocking and incubation in primary and secondary antibodies were performed with "Blotto" made according to the method of Johnson et al. (1984). Blots were incubated in antibody diluted 1:1000 in Blotto at room temperature, with rotation for 2 h (primary) or 1 h (secondary). Blots were washed between steps using 200 mM NaCl buffered with 50 mM Tris-HCl, pH 7.4. Colorimetric detection with the substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (both from Sigma) was catalyzed by alkaline phosphatase-conjugated, goat anti-chicken antibody (KPL, Gaithersburg, MD).

Protease Activity Gels

Protease activity gels were prepared essentially according to the method of Beers and Freeman (1997). Aliquots from each extract, representing an equal number of cells (1×10^5), were resolved by SDS-PAGE (12% [w/v] acrylamide). Samples were not boiled prior to electrophoresis. Hydrolysis of the gelatin substrate (0.5% [w/v]) resulted in unstained bands in the substrate-impregnated gels, indicating the position of the proteolytic activity in resolving gels. For in vitro inhibitor studies, polyacrylamide gel lanes were excised and incubated at room temperature for 15 min in 4 μM LAC, 20 μM LLL, or 0.1% DMSO as a solvent control prior to exposure to the substrate gels, as described above.

RNA Isolation

Following each of two independent experiments for LLL and one experiment for LAC, 11 replicates were pooled, an aliquot was removed and scored for TE differentiation, and the balance was frozen in liquid nitrogen and stored at −70°C until extraction. Total RNA was prepared by the method of Chirgwin et al. (1979). Immediately after the addition of 2 mL of a guanidine thiocyanate stock solution (4 M guanidine thiocyanate, 0.5% *N*-lauroylsarcosine, 25 mM sodium citrate, pH 7.0, 100 mM 2-mercaptoethanol, and 0.1% antifoam A), the sample was homogenized on ice for a total of 3 min and clarified by centrifugation. RNA was precipitated overnight at −20°C by the addition of 0.025 volume of 1 N acetic acid and 0.75 volume of ethanol. The pellet was resuspended at one-half the original volume in guanidine-HCl solution (7.5 M guanidine-HCl, 25 mM sodium citrate, pH 7.0, and 50 mM 2-mercaptoethanol), and precipitated (0.025 volume of 1 N acetic acid and 0.5 volume of ethanol) at −20°C for at least 3 h. This was repeated twice, reducing the volume of guanidine-HCl by one-half each time. The pellet was washed by suspension in absolute ethanol (−20°C), extracted twice in 100 μL of diethyl-pyrocarbonate-treated water, and precipitated (0.1 volume of 2 M potassium acetate, pH 5.0, and 2 volumes of ethanol) overnight at −20°C. The pellet was washed twice with 95% ethanol, dried, and resuspended in diethyl-pyrocarbonate-treated water.

RNA Probe Synthesis

For template preparation, 1 μg of plasmid p48h-17 in pBluescript K/S (Stratagene) was linearized with *Bgl*II, purified by electrophoresis through low-melting-point agarose (FMC, Rockland, ME), recovered, and rendered free of RNase by phenol extraction and precipitation. Biotinylated antisense p48h-17 was prepared using T7 polymerase and biotin RNA-labeling reagents (Boehringer Mannheim) according to the manufacturer's directions. The probe was checked for integrity by RNA gel electrophoresis as described below.

RNA Gel-Blot Analysis

Samples and biotinylated RNA molecular mass markers (New England Biolabs) were separated on 1.2% agarose gels containing formaldehyde as described by Sambrook et al. (1989), except that the formaldehyde gel-running buffer contained 5 mM sodium acetate. After electrophoresis, gels were photographed and washed three times for 10 min each in 2× SSC and transferred to positively charged nylon membranes (Boehringer Mannheim) by overnight capillary transfer in 2× SSC. The membranes were UV cross-linked and then dried for 2 h at 80°C.

The membranes were hydrated for 2 min in 5× SSC and prehybridized in prehybridization/hybridization buffer (NorthernMAX, Ambion, Austin, TX) in a bag at 68°C for at least 1 h. The bag was drained, refilled with fresh buffer containing biotinylated antisense probe, and hybridized overnight at 68°C. Membranes were washed twice for 5 min each time at room temperature in 2× SSC/0.1% SDS, at 0.2× SSC/0.1% SDS, and then twice for 15 min at 68°C in 0.1 × SSC/0.1% SDS. Bands were visualized by chemiluminescent detection (Phototope K6 kit, New England Biolabs) following the manufacturer's directions except that one or two additional washes were added following incubation in streptavidin.

RESULTS

Lactacystin and LLL Prevent TE Formation when Applied at Culture Initiation and Delay Differentiation when Applied after Cell Fate Determination

Preliminary experiments using the proteasome inhibitors LLnL, LLM (data not shown), LAC, and LLL revealed that only the latter two effectively inhibited TE formation when added at culture initiation (t_0). Therefore, subsequent experiments were conducted using only LAC and LLL. Figure 1 shows the effect of a range of concentrations of LAC (Fig. 1A) and LLL (Fig. 1B) on the inhibition of TE differentiation. The observed effective doses for nearly complete inhibition of TE development that were used in subsequent experiments were 4 μM LAC and 80 μM LLL.

The concentrations of LAC and LLL used in these studies are comparable to the nontoxic levels reported previously from research in animal systems (Palombella et al., 1994; Grimm et al., 1996), and several parameters indicate that these concentrations of inhibitors are nontoxic in the zinnia system. Most significantly, cell growth was not affected. The mean length of nondifferentiating cells present in cultures treated at culture initiation with either LAC or LLL increased 1.8-fold (from 48 to 88 μm) over 150 h in culture, as did the cells of control cultures. Additionally, these levels of proteasome inhibitors did not increase cell mortality above that seen in the solvent controls (data not shown), as determined from the ability of cells to exclude the nonpermeant vital stain Evans blue. Cells in inhibitor-treated cultures also continued to exhibit obvious cytoplasmic streaming and secrete characteristic protoplast fragments, as observed by Groover et al. (1997) to be normal behavior of cells in healthy zinnia mesophyll cell cultures. Finally, a washout experiment demonstrated that a high percentage of cells (near control levels for LAC) scored at 96 h could respond to signals leading to TE differentiation, even after a 24-h exposure to inhibitor (Fig. 1, A and B).

In three independent experiments, the addition of either 4 μM LAC (Fig. 1A) or 80 μM LLL (Fig. 1B) at t_0 of culture resulted in virtually complete inhibition of TE differentiation over the 96-h culture period. Although new TEs did develop in inhibitor-treated and control cultures during an additional 2 d of culture beyond 96 h, LAC-treated cultures reached only 35% of control levels and LLL-treated cultures attained only 22%.

Delaying addition by as little as 6 h after culture initiation resulted in decreased efficacy, and inhibition was no longer detected following additions at and beyond 48 h of culture (Fig. 1C). At or soon after 48 h in TE cultures, differentiation was evident as secondary cell wall thickenings became visible. A similar time course of LLL addition was not conducted because the method of application (four pulses over 18 h) required to achieve the effective dose of 80 μM in solution precluded single-time-point additions.

To determine whether the proteasome plays a role late in the differentiation process, for example, during the cell death or autolytic programs, TE cultures were treated with 4 μM LAC or 80 μM LLL at t_{48}. Table I shows that following LAC or LLL addition at t_{48} the percentage of TEs visible by 72 h in treated cultures was reduced to 13% and 70%, respectively, of the levels in control cultures. That these lower levels of TEs represented a delay and not a prohibition of differentiation was evident by 96 h, when the per-

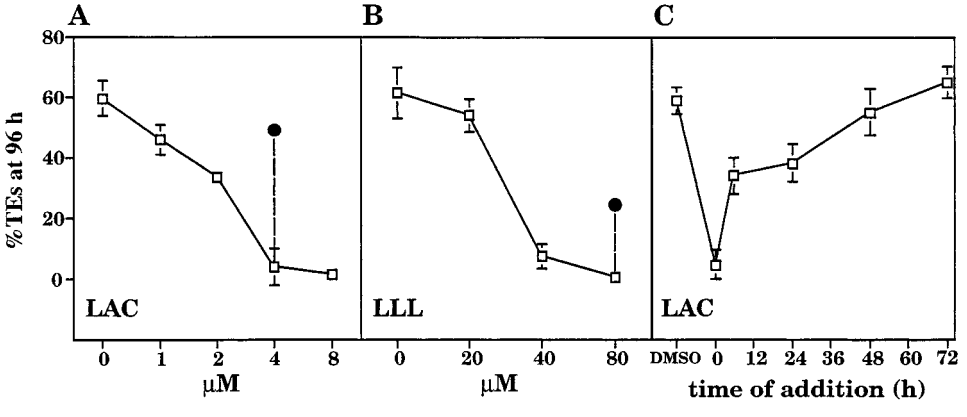

Figure 1. Effects of inhibitor dosage and time of addition on TE differentiation. Shown are the percentages of TEs in total cells (live, undifferentiated cells plus TEs; □) detectable at 96 h following treatment with the indicated concentrations of LAC (A) or LLL (B) at t_0. Also shown are the percentages of TE detectable at 96 h following removal of inhibitors at 24 h of culture (●). C, Percentages of TEs in total cells detectable at 96 h following addition of 4 μM LAC to TE cultures at the times indicated. Values are the mean percentages ± SD of a minimum of four replicates.

Table I. *Effect of t_{48} treatment with proteasome inhibitors on the percentage of TE differentiation*

Numbers within parentheses indicate the total number of replicates from a minimum of two independent experiments.

Treatment	Total TEs[a]		Live TEs[b]
	72 h	96 h	
LLL	35 ± 16 (10)	60 ± 6 (10)	4 ± 1 (7)
DMSO	50 ± 5 (10)	58 ± 7 (10)	4 ± 4 (7)
LAC	5 ± 4 (7)	59 ± 7 (11)	20 ± 8 (11)
DMSO	38 ± 14 (7)	53 ± 6 (11)	4 ± 3 (11)

[a] Mean ± SD percentages of TEs in total cells (TEs plus live, undifferentiated cells). [b] Mean percentages of live TEs in total TEs ± SD.

centage of differentiated cells in inhibitor-treated cultures was nearly identical to the control levels.

Despite the ultimate attainment of control-level numbers of TEs in t_{48} LAC-treated cultures, Table I illustrates that the LAC-induced delay in TE differentiation apparent by 72 h was still evident at 96 h of culture, as reflected by a 5-fold higher level of live TEs in treated cultures compared with controls. The observed level of live TEs in LAC-treated cultures at 96 h was intermediate between the levels observed in control cultures at 72 h (39%) and 96 h (4%). The stages of differentiation represented among live TEs in LAC-treated cultures at 96 h ranged from cells with barely detectable cell wall thickenings to those with extensive thickenings (data not shown). This observed higher level of live TEs at 96 h in LAC-treated cultures was apparently not the result of an uncoupling of secondary cell wall thickening from cell death, as was revealed by culturing cells an additional 24 h, by which time equivalent numbers of mature, dead TEs were detected in inhibitor-treated cultures and controls (data not shown). In contrast to these results with LAC, no such disparity in the number of live TEs in control versus treated cultures was evident at late times in the LLL experiments (Table I).

LLL Prevents Completion of TE Autolysis

Despite equivalent numbers of dead TEs present at 96 h in t_{48} LLL-treated and control cultures (Table I), we observed that LLL treatment induced an approximately 6-fold increase (from 15% to 85%) in the percentage of TEs that had not yet completed autolytic clearing by 96 h and retained some portion of intracellular contents. In contrast, t_{48} LAC treatment did not result in retention of protoplasmic material by dead TEs above the levels observed in control cultures (data not shown). Bright-field micrographs shown in Figure 2, A and B, depict cells harvested at 96 h from control and LLL-treated cultures. All TEs visible in Figure 2A are mature TEs of the control culture, lacking any detectable contents (representative of 85% of control TEs). All TEs exhibiting protoplasmic retention in LLL-treated cultures appeared plasmolyzed, with the collapsed protoplasm most often localized to one or two tight masses within the cell (Fig. 2B, arrowhead) but sometimes dispersed throughout the cell (Fig. 2B, arrow).

Three other cytological characteristics did not distinguish dead TEs exhibiting incomplete autolysis in LLL-treated cultures from mature TEs in control cultures. First, secondary cell wall thickenings of LLL-treated and control dead TEs appeared equivalent under bright-field microscopy (Fig. 2, compare A and B). Second, phloroglucinol staining of cells harvested at 96 h revealed no detectable differences in the extent of cell wall lignification between dead TEs in LLL-treated cultures and those in control cultures (data not shown). Finally, Calcofluor white staining of cells at 96 h did not distinguish TEs of LLL-treated cultures from those in control cultures with respect to the degree of cell wall cellulose deposition (data not shown).

It has been reported that degradation of the nucleus and other organelles occurs late during the autolysis of differentiating TEs, just before or after tonoplast disruption (Groover et al., 1997). To characterize the intracellular material retained by TEs in LLL-treated cultures, we determined whether TEs present at 96 h in LLL-treated cultures contained a nucleus (or at least dye-binding DNA) by staining with a fluorescent DNA-binding dye (Hoechst 33342). Figure 2, C and D, are bright-field and fluorescence micrographs, respectively, of paraformaldehyde-fixed cells demonstrating two important features of TEs and cells present at 96 h in LLL-treated cultures. First, live TEs exhibiting a diffuse and uniformly distributed protoplasm similar to that observed in live, undifferentiated cells contained intact nuclei (Fig. 2, C and D). Second, we did not observe TEs exhibiting both plasmolysis (i.e. incomplete autolysis) and dye-binding DNA. These results indicate that the apparent ability of LLL to stabilize intracellular contents against autolysis does not include the preservation of nDNA.

LAC and LLL Have Different Effects on the Activity and mRNA Levels of Cys Proteases Associated with Late Stages of TE Differentiation

Data presented thus far consist of a characterization of morphological markers associated with TE development as indicators of apparent inhibitor-induced disruptions in the differentiation program. To evaluate potential LAC- and LLL-induced changes in biochemical and molecular markers of TE differentiation, the activities and mRNA levels of

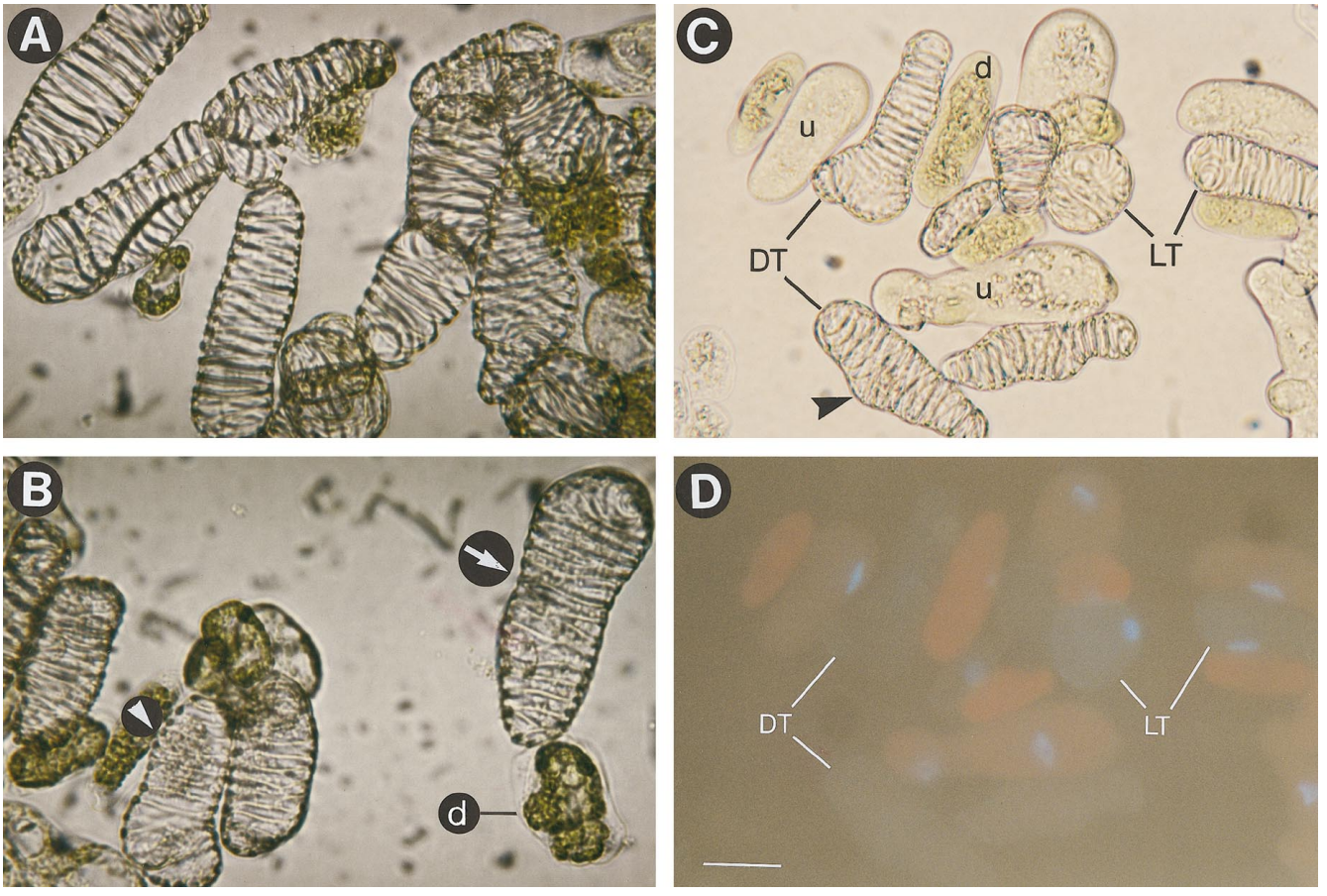

Figure 2. Characterization of LLL-induced, incomplete TE autolysis. For all panels, samples were taken from TE cultures at 96 h for staining and microscopy. C and D show the same field of view. A, Bright-field microscopy of unstained control culture TEs after autolysis. B, Bright-field microscopy of unstained TEs from the t_{48} LLL-treated culture. The two forms of protoplasmic retention by dead TEs are indicated: condensed to a single or few locations (arrowhead) or distributed throughout the cell (arrow). C, Bright-field microscopy of paraformaldehyde-fixed cells from the t_{48} LLL-treated culture. Arrowhead indicates protoplasm retained by dead TE that failed to complete autolysis. Live TEs showed diffuse protoplasm similar to that of live, undifferentiated cells. D, Fluorescence microscopy of cells from the t_{48} LLL-treated culture following DNA staining with Hoechst 33342. Blue fluorescence indicates the presence of nDNA; red fluorescence is due to chlorophyll autofluorescence. LT, live TE; DT, dead TE; u, live, undifferentiated cell; d, dead, undifferentiated cell (likely killed during isolation of mesophyll cells from leaves). Bar = 10 μm.

Cys proteases putatively involved in autolysis were examined. TE-specific proteases are well-documented markers for the late stages of TE differentiation (Minami and Fukuda, 1995; Ye and Varner, 1996; Beers and Freeman, 1997). Examining the expression and activity levels of these proteases may therefore indicate the extent to which the TE-differentiation program is affected by proteasome inhibition and provide clues as to the apparent inability of LLL-treated cells to complete autolysis.

Because the level of intracellular components decreases during TE autolysis, it was decided that a comparison of TE markers on an equal-cell-number basis would best represent relative effects of proteasome inhibitors on the progression of differentiation. Proteins were protected from degradation during and following isolation by inclusion of leupeptin in the extraction buffer (Beers and Freeman, 1997). Protease activity gels were prepared using extracts from 1.5×10^5 cells harvested at 72 and 96 h to determine the effects of t_{48} application of proteasome inhibitor on the activity of two Cys proteases (28 and 24 kD) postulated to participate in the autolysis of TEs (Beers and Freeman, 1997).

The activities of the 28- and 24-kD proteases were detectable at very low levels in extracts from LAC-treated cells harvested at 72 h compared with the control (Fig. 3A). By 96 h, however, protease activities in extracts from LAC-treated cultures had increased to levels similar to those observed in 72-h control extracts. During the same period in control cultures, activity of the 24-kD protease decreased to a barely detectable level, whereas activity of the 28-kD enzyme was undetectable by 96 h. In contrast, LLL treatment resulted in the recovery of a slightly higher level of the 24-kD protease at 72 h compared with controls (Fig. 3B). By 96 h, as was observed in extracts from LAC experiments, activity of the 24-kD protease decreased to a barely detectable level in control samples, whereas the level of

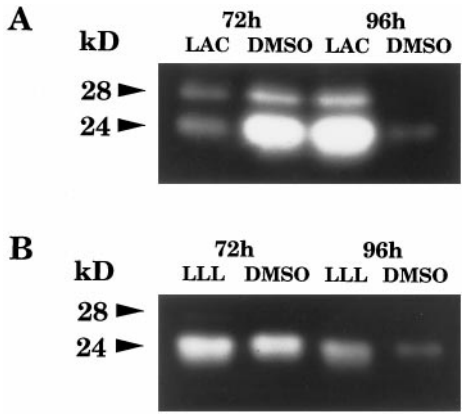

Figure 3. LAC or LLL treatment at 48 h alters the activity of TE Cys proteases. LAC (A) or LLL (B) was applied to TE cultures at t_{48}, and cells were harvested at 72 and 96 h. Shown are activity gels prepared following SDS-PAGE of total protein extracted from 1.5×10^5 zinnia cells per lane (see "Materials and Methods"). Bands represent regions of proteolytic activity in gelatin-impregnated substrate gels. Molecular masses of protein standards are indicated on the left (in kD).

activity of this enzyme remained relatively high in extracts from LLL-treated cells. Despite the apparent overall lower level of protease activity in LLL versus LAC experiments (Fig. 3, compare A and B, 72-h DMSO controls), identical results concerning the relative levels of protease activity (treated versus control) were obtained in a second independent experiment for each inhibitor. These protease activity profiles indicate that the addition of proteasome inhibitors results in altered regulation of expression and/or activity of TE-associated Cys proteases, and in the case of the LAC experiments, the results are consistent with an inhibitor-induced delay in the progression of the differentiation program of approximately 24 h.

Expression of p48h-17, a Cys protease that is upregulated in the late stages of TE differentiation (Ye and Varner, 1993, 1996), was examined following inhibitor treatment at t_{48}. During our efforts to prepare total RNA from treated and control cells for p48h-17 RNA gel-blot analysis, we discovered that equivalent numbers of cells yielded markedly different quantities of RNA. Cells harvested from LAC- and LLL-treated cultures yielded 1.7- and 3.4-fold higher levels of RNA, respectively, compared with controls at 72 h, and less than 0.5 μg of total RNA per 10^6 cells at 96 h. This low level of RNA recovery from inhibitor-treated cultures at 96 h occurred despite our efforts to denature RNases throughout RNA extraction (Chirgwin et al., 1979). Back-extraction of cell pellets yielded no additional RNA (data not shown), revealing that the observed differences in RNA yield apparently were not due to treatment-induced differences in the retention of RNA by the cells. Since it was not possible to isolate useful quantities of RNA at 96 h following LAC or LLL treatments, we have presented RNA gel-blot data (equal cell number and equal RNA comparisons) for the 72-h time point only.

Cells treated with LLL and harvested at 72 h yielded a higher level of p48h-17 mRNA compared with the control, whether analysis was conducted on an equal-cell-number or an equal-RNA basis (Fig. 4A). Similarly, but to a lesser extent, p48h-17 mRNA was more abundant in cultured cells following LAC treatment than in controls when compared on an equal-cell-number or on an equal-RNA basis (Fig. 4B).

LAC Is More Effective than LLL at Stabilizing Endogenous Ubiquitinated Proteins in Cultured Zinnia Cells

To confirm that the proteasome inhibitors used in this study were capable of exerting their effects via inhibition of the proteasome, we examined the effects of LAC and LLL on levels of endogenous ubiquitin-protein conjugates. Immunoblots of total zinnia protein probed with anti-ubiquitin antibody reveal that treatment of cells with either LAC or LLL resulted in the stabilization of conjugates extracted from 72-h cultures (Fig. 5). The apparent stabilization by the reversible inhibitor LLL was no longer evident by 96 h in culture (data not shown). In contrast, treatment of cells at t_{48} with the irreversible inhibitor LAC resulted in a high degree of ubiquitin-protein conjugate stabilization through 96 h of culture (Fig. 5B).

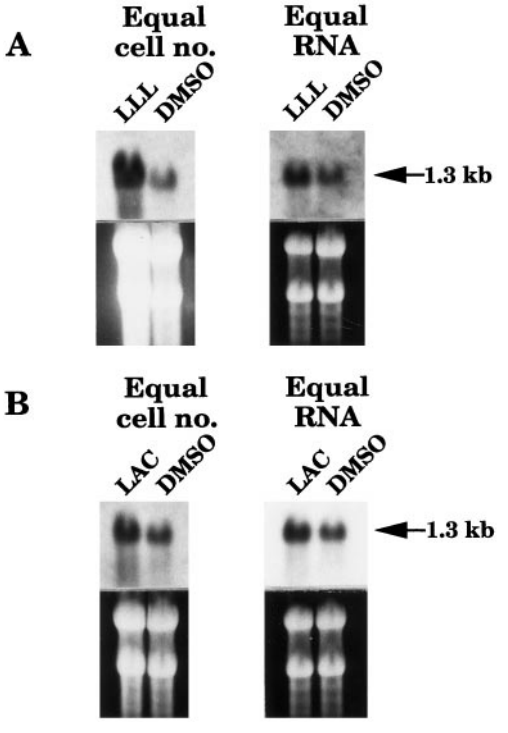

Figure 4. LLL and LAC treatments at 48 h result in elevated levels of p48h-17 expression. LLL (A) or LAC (B) was added to t_{48} TE cultures, and cells were harvested at 72 h. Shown are RNA gel blots of total RNA performed on both an equal-cell-number (10^6) and an equal-RNA (3.5 μg per lane) basis probed with biotinylated antisense p48h-17. RNA levels loaded for equal-cell-number analyses are as follows: LLL, 13.8 μg, and corresponding DMSO, 4.1 μg; LAC, 8.1 μg, and corresponding DMSO, 4.8 μg. Corresponding ethidium bromide-stained agarose gels are shown below each blot.

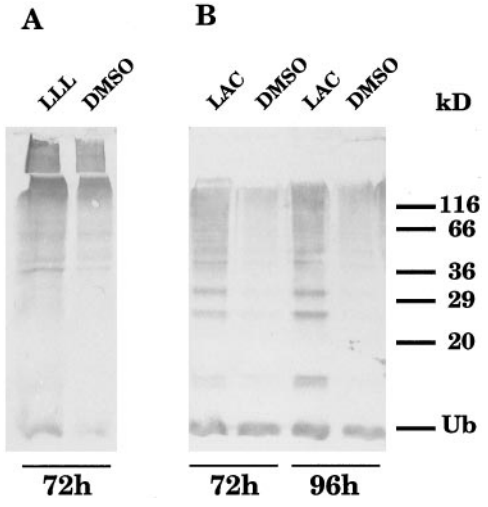

Figure 5. Endogenous ubiquitin-protein conjugates are stabilized by LLL and LAC treatments at 48 h. LLL (A) or LAC (B) was added to t_{48} TE cultures, cells were harvested at 72 h (LLL and LAC) and 96 h (LAC only), and protein was extracted for immunoblot analysis as for Figure 3. Extracts from 1.5×10^5 cells (LAC) and 2×10^5 cells (LLL) were loaded per lane. Blots were probed with anti-ubiquitin antibody. Molecular masses of protein standards and the position of free ubiquitin (Ub) are indicated on the right (in kD).

LLL Inhibits the Activity of Cys Proteases of Potential Importance to TE Autolysis

LLL has been reported to inhibit the Cys protease calpain in addition to the proteasome (Tsubuki et al., 1996). To address the possibility that LLL inhibition of Cys proteases putatively involved in TE autolysis might explain the failure of TEs in LLL-treated cultures to complete autolysis, protease activity gels were prepared in the presence of LLL and LAC. LLL treatment resulted in complete inhibition of the 28- and 24-kD TE-specific proteases (Fig. 6). As expected, LAC treatment had no effect on the activity of these proteases (Fig. 6).

DISCUSSION

We have demonstrated that, when added at the time of culture initiation, inhibitors of the proteasome can prevent TE differentiation, apparently without significantly affecting the health of cultured zinnia cells. In addition, proteasome inhibition following the appearance of cell wall thickenings resulted in an approximately 2-fold increase in the time required to complete differentiation. This delay was demonstrated by characterization of morphological markers and by evaluation of the expression of Cys proteases putatively involved in autolysis. Although LLL is capable of inhibiting both the proteasome and Cys proteases, the observation that application of the specific proteasome inhibitor LAC prevents TE differentiation indicates that proteasome inhibition by LLL is sufficient for prevention of TE differentiation. In contrast, the ability of LLL to partially uncouple autolysis from the TE-differentiation program is probably due to its inhibition of autolytic Cys proteases.

The Proteasome as Mediator of Early Signals Leading to TE Differentiation

The ubiquitin-proteasome pathway is known to regulate the cell cycle (for review, see Pagano, 1997), and although cell division can occur in up to 40% of cells undergoing TE differentiation, the remaining 60% of TEs form without intervening mitosis (Fukuda and Komamine, 1980b). Therefore, disruption of cell cycling by proteasome inhibitors is not likely to account for the nearly complete prevention of TE formation reported here. Rather, our results indicate that TE differentiation may require the proteolytic removal of an endogenous differentiation inhibitor(s), perhaps due to a role for the proteasome in transducing differentiation signals initiated by the phytohormones auxin and/or cytokinin, which are required for TE development (Church and Galston, 1988). It has been proposed that the turnover of short-lived repressor proteins is a requirement for auxin response (for review, see Abel and Theologis, 1996), and recent work by Estelle and colleagues (Ruegger et al., 1998) specifically implicates the ubiquitin-proteasome pathway by showing that two Arabidopsis genes, *TIR1* and *AXR1*, which encode proteins related to ubiquitin pathway components, are required for normal auxin signal transduction.

The Proteasome as a Regulator of the Time Course of TE Differentiation

That application of LAC after cellular commitment to TE development causes a strong delay in the overall program is corroborated by the 24-h delay in the peak of Cys protease activity noted in extracts from LAC-treated cells compared with controls (Fig. 3A). One interpretation of the apparently contrasting 72-h profile of protease activity extractable from LLL-treated cultures is that LLL does not result in a similar delay in peak activity of TE proteases. However, the higher level of 24-kD protease activity detectable at 72 h following LLL treatment compared with controls (Fig. 3B) may have resulted from up-regulation of the expression of genes encoding LLL-sensitive proteases in response to LLL-mediated inhibition of Cys proteases (Fig. 6). Increases in mRNA levels of enzymes following application of competitive inhibitors has been documented, as in the case of 3-hydroxy-3-methylglutaryl CoA reduc-

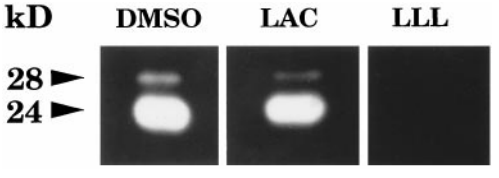

Figure 6. LLL but not LAC inhibits the activity of TE Cys proteases. Following SDS-PAGE of total zinnia protein, excised gel lanes were incubated in 4 μM LAC, 20 μM LLL, or 0.1% DMSO prior to exposure to gelatin-impregnated substrate gels. Bands represent regions of proteolytic activity in substrate gels. Molecular masses of protein standards are indicated on the left (in kD).

tase (Cohen and Griffioen, 1988). Alternatively, the higher level of protease activity observed at 72 h following LLL treatment may have resulted from inhibition by LLL of an unknown protease(s) that normally functions to degrade the 24-kD enzyme as part of a posttranslational mechanism for regulating its activity prior to autolysis. Such a posttranslational mechanism would not be expected to be affected by LAC treatment, which has no activity against Cys proteases.

Our inability to harvest useful quantities of RNA from inhibitor-treated TE cultures at 96 h may indicate that RNA was degraded prior to or during isolation from inhibitor-treated cells. RNase activity levels have been shown to increase after 48 h in normal TE cultures (Thelen and Northcote, 1989; Ye and Droste, 1996), with the highest levels detected at 84 h (Thelen and Northcote, 1989). A proteasome-inhibitor-induced delay in the overall differentiation program would be expected to include a delay in peak RNase activity relative to the controls. Thus, recovery of low levels of RNA at 96 h from proteasome inhibitor-treated cultures, despite the presence of 40% nondifferentiated cells, may represent indirect evidence of a delay in the differentiation process relative to control cultures, in which mostly empty, mature TEs contain little or no RNase to degrade RNA released from nondifferentiating cells during RNA isolation. Similarly, the recovery at 72 h of higher levels of RNA from inhibitor-treated cells compared with controls may reflect the presence of higher levels of early- stage (preautolysis) TEs in treated cultures at this time.

The apparent higher level of p48h-17 mRNA observed at 72 h in both LLL- and LAC-treated cultures is also consistent with an inhibitor-induced delay in the TE differentiation process, assuming that p48h-17 expression peaks prior to 72 h in normal TE cultures. Ye and Droste (1996) reported the highest level of p48h-17 mRNA expression at 60 h in TE cultures, although data from later times in culture were not presented. Whether p48h-17 encodes the 24-kD protease detected in this study is not known. However, when expressed in transgenic tobacco, p48h-17 yielded a 20-kD mature Cys protease, close to both its predicted size (22.7 kD; Ye and Varner, 1996) and to the 24-kD Cys protease detected here and previously (Beers and Freeman, 1997). Therefore, it seems reasonable to speculate on the significance of p48h-17 expression relative to the activity of the 24-kD Cys protease.

The inverse relationship evident between 72-h p48h-17 mRNA and 72-h protease activity levels following LAC treatment may indicate that 72-h p48h-17 mRNA levels predict the much higher 96-h, 24-kD protease activity levels. Alternatively, as discussed above, protease levels may normally be kept low, despite high transcript levels, by some as yet undescribed posttranslational proteolytic mechanism that is inhibited by LLL and not by LAC. Thus, uncoupling of this regulatory mechanism might be expected in LLL-treated cells, revealing an apparent direct correlation between p48h-17 transcript level and TE protease levels.

Ubiquitin-Protein Conjugate Stabilization by LLL and LAC

It is possible that the endogenous conjugate profiles evident in cell extracts from inhibitor-treated cultures were not the direct result of proteasome inhibition but, rather, represent the profile of a delayed culture relative to that of a normally progressing culture. However, if this were the case, we would expect the levels of conjugates extracted at 96 h from LAC-treated cells to appear more similar to those from 72-h control cells, thereby reflecting the approximately 24-h delay observed at both the morphological (Table I) and biochemical (Fig. 3) levels. Instead, conjugate stabilization in LAC-treated cultures clearly persisted through 96 h.

The Role of Cys Proteases in TE Autolysis

In contrast to LAC treatment, LLL treatment resulted in the partial uncoupling of autolysis from differentiation of what otherwise appeared to be normal TEs (Fig. 2). We have presented evidence that LLL but not LAC inhibits TE-associated Cys proteases (Fig. 6), suggesting that the apparent prohibition of TE autolysis in LLL-treated cultures is due to inhibition of the activity of the 28- and 24-kD Cys proteases or other LLL-sensitive proteases not detected by our activity gels and not caused by inhibition of the proteasome.

Hoechst 33342 staining of cells following LLL treatment demonstrated that the cellular material retained by 85% of the dead TEs in these cultures does not include nDNA, indicating that nuclear integrity is no longer maintained in TEs that exhibit incomplete autolysis. This absence of DNA is consistent with the proposal that endonucleases expressed during TE differentiation function to degrade DNA and RNA during autolysis (Thelen and Northcote, 1989) independently of Cys proteases. Although tonoplast rupture is known to be associated with autolysis of intracellular components in developing TEs (Groover et al., 1997), the results presented here represent the first demonstration to our knowledge that application of a Cys protease inhibitor prevents the complete autolysis of zinnia TEs.

Additionally, although the activity of the ubiquitin-proteasome pathway has been previously implicated as a necessary component of vascular differentiation (Bachmair et al., 1990; Stephenson et al., 1996), to our knowledge this is the first demonstration that a specific inhibitor of the proteasome can reversibly prevent TE differentiation and that proteasome function is also required for regulating the time course of TE differentiation in zinnia mesophyll cell cultures. The proteasome, however, does not appear to participate directly in TE autolysis.

As used in this study, LLL and LAC were not able to uncouple the differentiation process from cell death. If TE death is dependent on the proteasome or on LLL-sensitive proteases, perhaps the window of opportunity to uncouple death from TE differentiation is narrow, occurring at a time not tested in this study. Although it is possible that proteases do not play an important role in the regulation

of plant PCD, further investigation using inhibitors against proteases other than the proteasome and Cys proteases may lead to the identification of proteases specifically involved in the regulation of cell death during TE differentiation.

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