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The Involvement of Cysteine Proteases and Protease Inhibitor Genes in the Regulation of Programmed Cell Death in Plants

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Programmed cell death (PCD) is a process by which cells in many organisms die. The basic morphological and biochemical features of PCD are conserved between the animal and plant kingdoms. Cysteine proteases have emerged as key enzymes in the regulation of animal PCD. Here, we show that in soybean cells, PCD-activating oxidative stress induced a set of cysteine proteases. The activation of one or more of the cysteine proteases was instrumental in the PCD of soybean cells. Inhibition of the cysteine proteases by ectopic expression of cystatin, an endogenous cysteine protease inhibitor gene, inhibited induced cysteine protease activity and blocked PCD triggered either by an avirulent strain of *Pseudomonas syringae* pv *glycinea* or directly by oxidative stress. Similar expression of serine protease inhibitors was ineffective. A glutathione *S*-transferase–cystatin fusion protein was used to purify and characterize the induced proteases. Taken together, our results suggest that plant PCD can be regulated by activity poised between the cysteine proteases and the cysteine protease inhibitors. We also propose a new role for proteinase inhibitor genes as modulators of PCD in plants.

INTRODUCTION

Cell death is a basic biological process that functions in many aspects of animal and plant development and in their responses to stress (Greenberg, 1996; Wang et al., 1996; Martins and Earnshaw, 1997). The discovery that cell death is a tightly regulated (programmed) process has stirred a great deal of interest in its mechanisms. Studies of animal systems have shown that the execution of programmed cell death (PCD) or apoptosis is controlled by a multistep signaling pathway (McConkey and Orrenius, 1994; Stewart, 1994). In plants, PCD has been implicated in xylogenesis (Fukuda, 1996; Groover et al., 1997), in some forms of senescence, and in the hypersensitive response to pathogens and environmental stresses (Greenberg, 1996; Mittler and Lam, 1996; Lamb and Dixon, 1997).

Although a detailed understanding of how plant cells die is still largely unknown, recent studies have shown that the apoptotic pathways of the animal and plant kingdoms are morphologically and biochemically similar (Greenberg, 1996; Levine et al., 1996; Wang et al., 1996). Specifically, the morphological hallmarks of apoptosis include cytoplasmic shrinkage, nuclear condensation, and membrane blebbing (Earnshaw, 1995; Martins and Earnshaw, 1997); the biochemical events involve calcium influx, exposure of phosphatidylserine, and activation of specific proteases and DNA fragmentation, first to large 50-kb fragments and then to nucleosomal ladders (McConkey and Orrenius, 1994; Stewart, 1994; Wang et al., 1996; O'Brien et al., 1998). All of the above-mentioned phenomena were shown to occur in plant PCD. Also, the stimuli that activate apoptosis are similar in plant and animal cells (O'Brien et al., 1998). Although it should be noted that not all of the events were demonstrated in the same plant system, taken together these results infer a common basic cell death process in plants and animals.

Moreover, the isolation of spontaneous cell death mutants in Arabidopsis and maize, in which PCD is activated in the absence of pathogens or stress, suggests that PCD in plants is under genetic control (Dietrich et al., 1994; Greenberg et al., 1994). In at least one of these mutant plants, PCD is initiated and propagated via the generation of reactive oxygen species (ROS) (Jabs et al., 1996). Compelling evidence points to the active participation of ROS in plant (Levine et al., 1994; Chamnongpol et al., 1996; Schraudner et al., 1997) and in animal (Korsmeyer et al., 1995) PCD.

Three distinct phases that depend on the concentration of ROS characterize cellular responses to oxidative stress (Dypbukt et al., 1994; Levine et al., 1994). Low doses induce antioxidant enzymes; however, when the concentration of ROS reaches a certain threshold, a signal transduction pathway that results in PCD is activated. (Levine et al., 1996; Desikan et al., 1998). High doses result in necrosis. Earlier

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work using biochemical/pharmacological experiments, in which PCD was blocked by channel blockers or inhibitors of different signaling steps, identified a number of essential events. For example, in cultured soybean cells, synthetic protease or kinase inhibitors effectively suppressed PCD triggered by oxidative stress or by infection with avirulent pathogens (Levine et al., 1996). It is noteworthy that only a subset of the tested protease inhibitors (phenylmethylsulfonyl fluoride [PMSF], 4-[2-aminoethyl]-benzesulfonylfluoride [AEBSF] hydrocholride, and leupeptin) blocked PCD. No inhibition and in some cases even increased cell death were observed with other inhibitors (*N*-α-tosyl-L-lysine chloromethyl ketone, tosyl-L-phenylalanine chloromethyl ketone, *N*-acetyl-Leu-Leu-norleucinal, or *N*-acetyl-Leu-Leu-methioninal), suggesting activation of specific isozymes rather than general proteolysis. The compounds that were effective in reducing the degree of PCD in soybean, namely, leupeptin, PMSF, and AEBSF, all have inhibitory activity against cysteine proteases: leupeptin is an established inhibitor of cysteine proteases, whereas PMSF and AEBSF, although generally used as inhibitors of serine proteases, also can act against certain cysteine proteases, especially those in the papain family (Alonso et al., 1996; measurements in this study were generated with commercially obtained papain).

Activation of cysteine proteases constitutes the critical point in the PCD pathway of animal cells (Earnshaw, 1995; Martin and Green, 1995; Martins et al., 1997). Recently, it has been demonstrated that cysteine proteases also are induced in plant systems undergoing PCD, such as tracheary element differentiation in *Zinnia elegans* (Minami and Fukuda, 1995; Ye and Varner, 1996) and certain forms of cell aging (Drake et al., 1996). Importantly, in the Zinnia system, the terminal differentiation that ends in cell death could be blocked by the addition of a specific cysteine protease inhibitor, *trans*-(epoxysuccinyl)-L-leucylamino-(4-guanidino)butane (E-64; Minami and Fukuda, 1995). The cultured *Z. elegans* cells may, however, constitute a specific case, because autolysis of tracheary elements is mediated by an early collapse of the vacuole (Fukuda, 1996; Groover et al., 1997).

One important difference, however, exists between the animal and the plant proteases that are involved in PCD. It involves their substrate specificity. Most animal cysteine proteases associated with apoptosis regulation cleave after an aspartic acid (Cohen, 1997); thus, they have been termed caspases. Recently, such activity has been reported in tobacco mosaic virus–infected tobacco plants when a short peptide (Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin [AMC]) substrate was used (del Pozo and Lam, 1998). Our own studies using extracts from H_2O_2-treated suspension-cultured cells of soybean or from superoxide-treated Arabidopsis plants failed to detect proteolytic activity by using the peptide 4-(4-dimethylaminophenylazo)benzoic acid (DABCYL)-Tyr-Val-Ala-Asp-Ala-Pro-Val-5-([2-aminoethyl]amino)naphthalene-1-sulfonic acid (EDANS), which is a longer substrate for interleukin-1β–converting enzyme (ICE)—like animal caspases (Howard et al., 1991).

In plants and other organisms, protease activity can be regulated at different levels: by transcription/translation, by post-translational processing, and by specific protease inhibitor proteins (Bode and Huber, 1992). Plants possess a large arsenal of protease inhibitor genes that are known mostly for their function in defense against herbivores (Johnson et al., 1989). Here, we show that in soybean cells, endogenous cysteine protease inhibitors may function in modifying PCD that is activated during oxidative stress and pathogen attack. Because oxidative stress and PCD have been shown to accompany many of the environmental stresses, such as chilling (Prasad et al., 1994), ozone (Pell et al., 1997), salt (Hernandez et al., 1993), and drought (Smirnoff, 1993) stresses, our results may be important in the future engineering of plant resistance to different environmental stresses by genetically modulating the activity of endogenous protease inhibitor genes.

RESULTS

Oxidative Stress–Induced Protease Activity

Activation of cysteine proteases constitutes the critical point in the PCD pathway in animal and plant cells. As a first step to visualize changes in activity of proteases after oxidative stress, we separated cellular proteins by SDS-PAGE and used the in-gel protease activity method. The advantage of the activity gel assay is that it makes possible an analysis of individual proteases. Suspension-cultured soybean Williams 82 cells were treated with H_2O_2 at a concentration that was shown to induce PCD (Levine et al., 1994; Desikan et al., 1998), and the cells were harvested at different intervals after the stimulus was applied. Soybean cell extracts were fractionated into the cytosol and membranes by ultracentrifugation and loaded onto a gel containing 0.12% gelatin cross-linked into the acrylamide matrix. The results shown in Figure 1A reveal a number of new protease bands that appear as early as 30 min after induction of PCD by H_2O_2. Proteolytic activity could be traced to the cytosolic fraction, and no additional bands were detected after a longer incubation of up to 4 hr after stimulation (data not shown). The results also show that the activity of a major protease declined after PCD induction, indicating bidirectional changes in the activity of proteases after oxidative stress.

To determine whether the induced activity originated from the activation of preexisting proteases, we pretreated cells with cycloheximide before they were induced with H_2O_2 (Figure 1A). Because the pretreatment of cells with cycloheximide did not alter the pattern of induced proteolytic activity, we tested whether proteases could be activated directly by H_2O_2, for example, by H_2O_2-dependent oxidation of critical sulfhydryls. However, in vitro treatment of extracts prepared from the uninduced cells with H_2O_2 did not change the pattern of bands with proteolytic activity (data not shown).

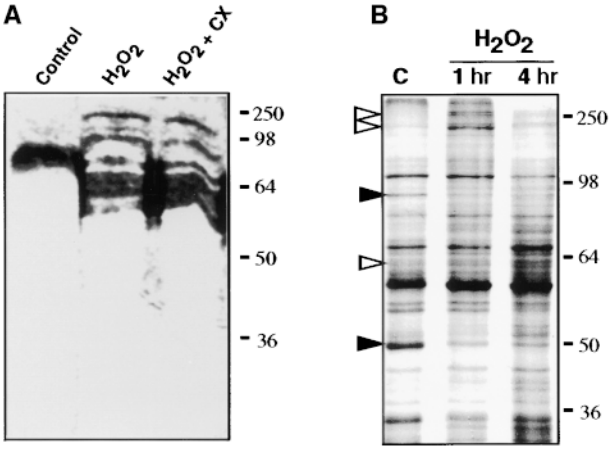

Figure 1. Changes in Protein Degradation and Synthesis Induced by H_2O_2.

(A) Activation of proteases during oxidative stress. Suspension-cultured soybean cells were treated with 5 mM H_2O_2 or water (Control). Proteins were resolved by SDS-PAGE, and the cytosolic fraction was assayed in gel for proteolytic activity with gelatin as substrate. Cycloheximide (CX) was added (+) to one set of cells to a final concentration of 40 μM 15 min before H_2O_2 was added.

(B) Analysis of protein synthesis and degradation after treatment with H_2O_2. Proteins were labeled for 12 hr and then treated with H_2O_2 as given in **(A)**. Cytosolic proteins were prepared 1 and 4 hr after the addition of H_2O_2, separated by SDS-PAGE, and analyzed by autoradiography. Filled arrowheads show the rapidly degraded polypeptides, and open arrowheads indicate the newly synthesized ones. Numbers at right indicate molecular weight markers in kilodaltons. C, control.

Therefore, additional signaling or metabolic events that occurred in the intact cells were required to activate the proteases.

An obvious consequence of protease activation is increased degradation of proteins. The type of proteins that are degraded depends on the specificity and compartmentation of the induced proteolytic activity. To check whether oxidative stress induced nonspecific degradation of a large number of proteins or cleavage of specific polypeptides, we labeled the cells for 12 hr with a mixture of ^{35}S-cysteine and ^{35}S-methionine, chased the cultures with 1 mM "cold" cysteine and methione, and immediately treated them with H_2O_2 at the PCD-inducing concentration. Cells were harvested 1 and 4 hr later, separated on an SDS–polyacrylamide gel, and observed by autoradiography (Figure 1B). Surprisingly, no overall degradation was detected. Two bands that showed relatively rapid degradation are indicated by arrowheads (Figure 1B). H_2O_2 treatment also induced a small number of proteins, which were probably labeled from the internal amino acid pool (indicated by open arrowheads in Figure 1B). Transient induction of a high molecular weight polypeptide was detected 1 hr after stimulation and disappeared 3 hr later. In brief, H_2O_2 treatment did not produce gross changes in the pattern of expressed proteins but rather induced specific changes in both the synthesis of certain new proteins and the degradation of a number of specific polypeptides.

Cell Death Induced by Oxidative Stress Requires Active Protein Synthesis

To determine whether the newly synthesized proteins were required for PCD or whether the entire cellular machinery for the execution of PCD was present before application of the H_2O_2 stimulus (and the post-translational activation of proteases was sufficient to execute the whole process), we tested whether inhibitors of protein synthesis could block H_2O_2-induced cell death. The concentration of H_2O_2 was chosen in preliminary experiments causing the death of 40 to 50% of the cells. This dose depended on the antioxidant activity of the culture, which was assayed by measuring the half-life of H_2O_2 (Levine et al., 1994). Two inhibitors of protein synthesis, puromycin and cycloheximide, were added to the cultures 30 min before stimulation, and the degree of cell death was measured by using Evan's blue staining.

As shown in Figure 2, puromycin decreased the amount of cell death induced by the H_2O_2 treatment in a concentration-dependent manner. The reduction in soybean PCD by puromycin corresponded to its effect on the inhibition of protein synthesis (Figure 2A, inset). Similarly, cycloheximide showed protection against soybean PCD (Figure 2B). Although cycloheximide is a more efficient protein synthesis inhibitor (98% protein synthesis inhibition at the applied concentration) than is puromycin, the prolonged treatment of soybean cells with cycloheximide, which takes 9 hr from the addition of inhibitors to the cell death assay, induced a very strong continuous oxidative burst by a mechanism that is unrelated to its effect on protein synthesis (Tenhaken and Rubel, 1998), resulting in extensive oxidation of cellular components and cell death, presumably by necrosis.

Our results indicate that unstimulated soybean cells contain a set of proteases in an inactive form and that oxidative stress treatment activated the enzymes via an indirect mechanism. Thus, the execution of PCD in response to H_2O_2 stimulus required either synthesis of new proteins or additional signaling events that were sensitive to puromycin. These results are in agreement with the data from Arabidopsis cell cultures, in which H_2O_2 activated a number of genes that were required for PCD (Desikan et al., 1998). These results are also in line with the observed induction of new proteins after stimulation with H_2O_2 (Figure 1B).

Substrate Specificity of Induced Proteases

To further characterize the proteases induced by oxidative stress with regard to the type of protease and the substrate

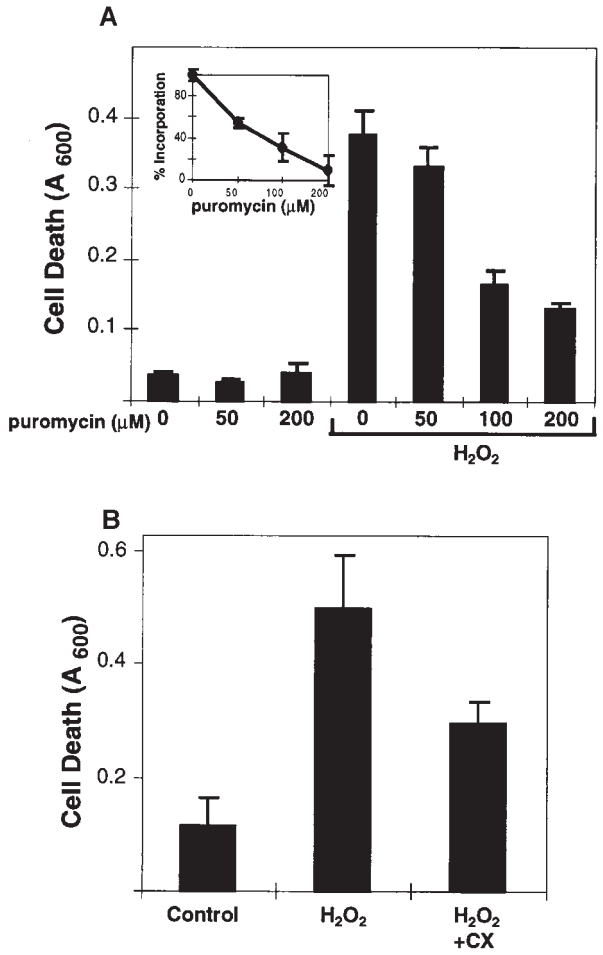

Figure 2. The Effect of Protein Synthesis Inhibitors on Soybean Cell Death.

Cells were pretreated with the indicated concentrations of puromycin or cycloheximide (CX) before challenge with 5 mM H_2O_2 for 9 hr. The indicated absorbance of 0.4 corresponds to ~45% of the dead cells, as determined by microscopic examination of Evan's blue–stained cells in three randomly selected fields (100 to 200 cells). A 40 μM cycloheximide concentration produced 99.5% inhibition of protein synthesis, and 200 μg/mL puromycin produced 94% inhibition. Cell death was assayed 9 hr later, according to Levine et al. (1994). The inset shows the percentage of incorporation of ^{35}S-cysteine into trichloroacetic acid–precipitable material in the presence of the indicated concentrations of puromycin for 2 hr.

(A) Pyromycin-treated cells.

(B) Cycloheximide-treated cells.

specificity, we fractionated extracts of H_2O_2-treated and control cells by anion exchange chromatography, and we assayed the activity of each fraction with a number of fluorogenic peptides. Substrates were chosen according to known activities against papain, a well-characterized plant cysteine protease (Katunuma and Kominami, 1995). Extracts prepared from the H_2O_2-treated cells had both stronger proteolytic activity and additional active fractions that were eluted at a higher ionic strength (Figure 3). Although the stronger activity detected in fractions 14 to 16 could have been due to increased activation of constitutively active proteases, the activity found in induced fractions 17 to 19 was virtually absent in the corresponding fractions from the uninduced cells, suggesting activation of novel proteases. Similar activity patterns also were obtained with other substrates, namely, benzyloxycarbonyl (Cbz) *Z*-Gly-Gly-Arg-AMC, *t*-butyloxycarbonyl (boc)-Gly-Lys-Arg-AMC, *Z*-Phe-Arg-AMC, and boc-Gln-Ala-Arg-AMC, which have arginine in the same position (P1). On the other hand, substrates with phenylalanine or tyrosine in the place of arginine (*Z*-Leu-Val-Tyr-AMC or Gly-Gly-Phe-AMC) showed stronger activity in corresponding fractions 11 to 13 from noninduced cells, and a substrate with proline in that position (*N*-succinyl-Ala-Ala-Pro-AMC) showed similar activities in induced and uninduced extracts (data not shown). We also tested unfractionated extracts with a peptide substrate of the ICE-like proteases, DABCYL-Tyr-Val-Ala-Asp-Ala-Pro-Val-EDANS (Howard et al., 1991), but no activity was detected, which is consistent with the inability of synthetic ICE inhibitors to block PCD in soybean cultures (Levine et al., 1996).

In general, the results of the chromatographically separated proteases (Figure 3) are in agreement with the gel activity data, which are shown in Figure 1, and confirm the activation of new proteases by oxidative stress. In view of the earlier work on PCD inhibition in this system, we wanted to determine whether the synthetic protease inhibitors that successfully inhibited PCD were effective against the induced proteolytic activity seen in fractions 15 to 19. The ad-

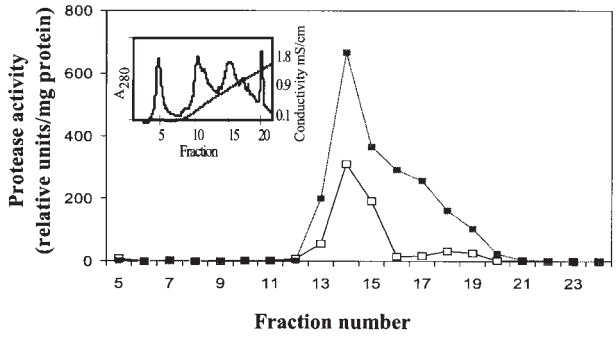

Figure 3. Fractionation of Soybean Proteases after Oxidative Stress.

Cells were treated with 5 mM H_2O_2 or water (control) for 40 min, and proteins were extracted as described in Methods. Proteins were chromatographed on a UNO-Q anion exchange column, with a continuous gradient of NaCl. Each fraction was tested with *Z*-Gly-Gly-Arg-AMC for activity. Open symbols, control extract; closed symbols, H_2O_2 treated. The inset shows the elution conditions and a typical profile of eluted proteins.

dition of AEBSF or leupeptin significantly inhibited protease activity in these fractions (data not shown). We also tested whether AEBSF inhibits papain by using the fluorogenic substrate *Z*-Gly-Gly-Arg-AMC and found strong inhibition at the recommended 0.5 mM concentration (data not shown), suggesting a possible involvement of cysteine proteases in oxidative stress–induced soybean PCD.

Ectopic Expression of an Endogenous Cysteine Protease Inhibitor Represses PCD of Soybean Cells

To analyze the role of specific types of proteases in soybean PCD, we took advantage of the high specificity in the protein–protein interactions established for a number of protease inhibitors. To this end, we transformed soybean cells with constructs carrying genes encoding three different protease inhibitors: (1) Sbcys soybean cystatin, which is a specific cysteine protease inhibitor (Hines et al., 1991); (2) Kunitz, an inhibitor of the trypsin-like proteases (Kido et al., 1992); and (3) CII, a Bowman-Birk–type inhibitor that is effective against chymotrypsin and elastase (Odani and Ikenaka, 1977). The genes were cloned behind the constitutively expressed 35S cauliflower mosaic virus promoter. For the cloning of Kunitz and CII genes, we used the published DNA sequences to design primers for amplification of the corresponding DNA fragments. However, because the sequence for only the mature protein encoded by the cystatin gene was available, we synthesized that cystatin gene on four overlapping oligonucleotides, according to soybean codon usage.

The resulting predicted amino acid sequence and its alignment with the R1 cysteine protease inhibitor (for comparison) are depicted in Figure 4. All constructs were verified by sequencing. As a control, cells also were transformed with the β-glucuronidase (*GUS*) gene that was cloned into an identical vector. The GUS transformation control also allowed us to estimate transformation efficiency, which was determined by microscopic examination of an aliquot stained with X-gluc. Expression was usually obtained in >70% of cells (Figure 5A). Transgene expression was tested by RNA gel blot analysis (Figure 5B). We did not analyze whether the transgene expression was transient or stable, because the experiment was completed within 4 days after inoculation.

After transformation, the majority of agrobacteria were removed by extensive washing, and the soybean cells were treated with H_2O_2 to induce PCD. As shown in Figure 6A, ectopic expression of soybean cystatin, but not of other protease inhibitor genes led to an effective block in H_2O_2-induced PCD, implicating the cysteine proteases in the H_2O_2-triggered pathway that leads to apoptosis of soybean cells. These data also argue against nonspecific oxidative damage, for example, via lipid peroxidation, which caused H_2O_2-induced membrane damage (detected by Evan's blue permeability) and subsequent cell death.

To test whether cysteine proteases also play an important role in hypersensitive response–associated cell death, we challenged the transformed cells with virulent and avirulent strains of *Pseudomonas syringae* pv *glycinea.* In this experiment, we used isogenic bacteria carrying either the *avrC* avirulence gene, which is virulent on cultivar Williams 82, or the *avrA* gene, which is avirulent and therefore causes hypersensitive response–related cell death (Keen, 1990). The results shown in Figure 6B demonstrate strong inhibition of cell death caused by the avirulent bacteria in soybean cells that constitutively expressed the *Sbcys* gene. These results argue that cysteine proteases play a key role in plant cell death. Interestingly, the cystatin-transformed cultures exhibited less cell death in the unchallenged (control) treatment and also after infection with the virulent strain of *P. syringae.* This basal level of cell death may be a result of the oxidative stress produced during the mechanical stress associated with cell manipulation (Yahraus et al., 1995). A weak oxidative burst also is produced by inoculation with virulent bacteria (Baker et al., 1991).

Expression of Cystatin Inhibits Induced Proteolytic Activity

To assess the activity of specific proteases in cells transformed with *Sbcys* cystatin in contrast to cells transformed with the *GUS* construct, we extracted cellular proteins 40 min after H_2O_2 treatment and fractionated the extracts on an anion exchange column, as described in the legend to Figure 3. All fractions were individually tested with *Z*-Phe-Arg-AMC and with boc-Gln-Ala-Arg-AMC. The activity of the H_2O_2-inducible proteases was strongly suppressed in the cystatin-transformed cells (Figure 7B). Interestingly, the basal (X-X-Arg-AMC–specific, where X stands for any amino acid) proteolytic activity was altered only slightly in these cells, indicating that overexpression of cystatin did not extensively affect the activity of the constitutively expressed proteases.

To test whether the inhibition was indeed due to the binding of cystatin to its target proteases, we preincubated the extract with 4 M urea and 0.5% SDS, and we separated the proteases from the inhibitor by electrophoresis on gelatin containing SDS-polyacrylamide, as described earlier. The released protease activity was tested in gel as described in the legend to Figure 1. A comparison of lanes with and without urea in Figure 8 shows that the inhibitory activity of

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                                                                *****
Sbcys:  MGS TDITGA QNSIDIENLA RFAVDEHNKK ENAVLEFVRV K SAKKQVVSG
R1:  MGGF TDITGA QNSIDIENLA RFAVDEHNKK ENAVLEFVRV I SAKKQVVSG TLYYITLEAN
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Figure 4. Alignment of Sbcys and R1 Amino Acid Sequences.

The translated sequence of the synthetic cystatin, Sbcys (Hines et al., 1991), is shown aligned with the R1 cysteine protease inhibitor from soybean (Botella et al., 1996). The asterisks indicate the QxVxG motif that is important for interaction with the protease.

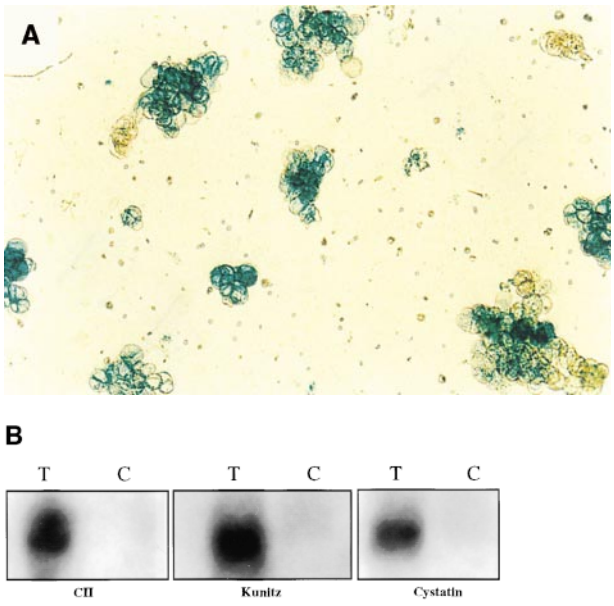

Figure 5. Transgene Expression in Soybean Cell Cultures.

Cells were transformed with binary vectors carrying three different protease inhibitor genes and the *GUS* gene as a control. Transformation was performed by coinoculation of soybean with Agrobacterium carrying the appropriate constructs for 48 hr.
(A) A representative field of the GUS-transformed cultures stained with X-gluc.
(B) RNA gel blot analysis of transgene expression. C, control, cells transformed with vector only; T, cells transformed with the indicated gene and probed with the corresponding complete cDNA (CII and Kunitz) or the synthetic Sbcys DNA (Cystatin).

cystatin was removed by urea. Interestingly, a much stronger protease activity was detected in the cystatin-transformed cells after urea treatment. This may have been due to overproduction of cysteine proteases in response to inhibition of basal protease activity by way of some kind of feedback regulation mechanism; however, further investigation is necessary to prove such regulation. Urea treatment had no effect on the GUS-expressing cells.

Isolation of Cysteine Proteinases Induced by Oxidative Stress That Are Sensitive to Inhibition by Cystatin

The above-mentioned results show that oxidative stress induced a number of proteases (Figure 1). Some of these had characteristics of the cysteine protease family (Figure 3), and a number of those were inhibited by cystatin (Figure 7). To focus on the cystatin-binding proteases that are induced by H_2O_2, we prepared a glutathione *S*-transferase (GST)–cystatin fusion construct and expressed it in *Escherichia coli.* The recombinant protein was purified by binding to glutathione beads and used for the final purification step. A higher capacity HighQ anion-exchange column and a stepwise elution protocol with small increments were used to obtain nonoverlapping fractions of eluted soybean proteins. To increase the separation of the proteases, specifically within the region of interest, we increased the initial binding stringency to the column to 200 mM NaCl, and we used extended elution volumes to fully separate the eluates from each increment (Figure 9A). In general, the pattern of protease activity eluting from the column was similar to that shown in Figure 3 with induced proteases eluting at higher salt concentration (Figure 9B). The active fractions were tested for sensitivity toward cystatin by preincubation of the

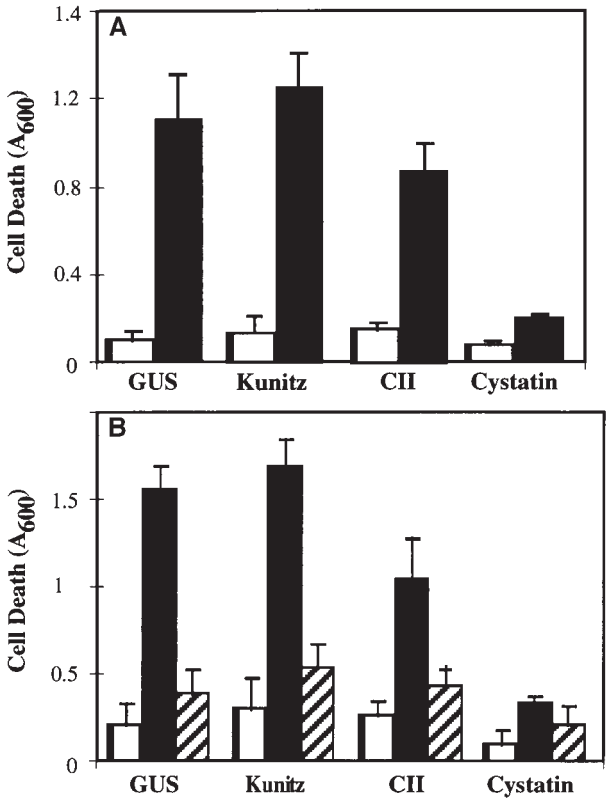

Figure 6. The Effect of Ectopic Expression of the Endogenous Protease Inhibitors on Soybean Cell Death.

The transformed cells were washed with medium and allowed to sit at 25°C with moderate shaking for 10 hr before challenge with a PCD-inducing stress.
(A) The transformed cultures were treated with water (control, white bars) or with 5 mM H_2O_2 (black bars). Cell death was determined 10 hr after H_2O_2 induction by staining with Evan's blue.
(B) Cultures were challenged with 5×10^7 cells per mL of *P. s. glycinea avrA* (black bars) or *avrC* (hatched bars), or with water (control, white bars). Cell death was assayed 28 hr after inoculation with *P. s. glycinea.* Each experiment was performed at least three times with similar results.

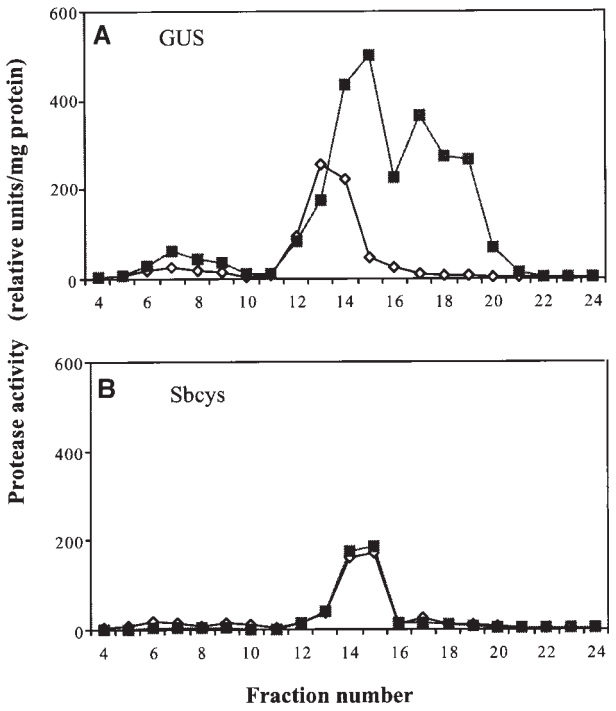

Figure 7. Fractionation of Soybean Proteases from Cells Transformed with Cystatin or GUS.

GUS- and cystatin-transformed cells were treated with 5 mM H_2O_2 for 40 min. Proteins were processed as given previously (see legends to Figures 3 and 4 for details) and chromatographed on a UNO-Q anion exchange column. Each fraction was tested with *Z*-Gly-Gly-Arg-AMC for activity. Open symbols, control extract; closed symbols, H_2O_2 treated.
(A) GUS-transformed cells.
(B) Cystatin-transformed cells.

eluted proteins with purified, recombinant GST–cystatin. Induced fraction 9 was strongly inhibited by cystatin (Figure 9C). The same fraction also was inhibited by another specific inhibitor of cysteine proteases, E-64 (Figure 9D), further supporting the designation of the induced eluted protease(s) as a member(s) of the cysteine protease family.

Fraction 9 from the HighQ column that showed activity only in induced cells and was sensitive to cystatin inhibition was collected and bound, in batch, to purified GST–cystatin. Measurements of protease activity toward *Z*-Gly-Gly-Arg-AMC performed before and after binding revealed that protease activity disappeared almost completely after incubation of fraction 9 with GST–cystatin and reappeared only after the addition of 2 M urea to the standard elution buffer (Figure 10A). These results indicate strong binding of the induced protease(s) to the protease inhibitor protein. Little inhibition of protease activity by cystatin was seen in fraction 7, and an intermediate level was seen in fraction 8. These results convincingly show that cystatin-sensitive proteases are activated by the H_2O_2 treatment.

The induced proteases eluted by urea from fraction 9 were separated by SDS-PAGE and analyzed by using the in-gel protease assay. Comparison of the molecular weights of the proteases on the SDS–polyacrylamide activity gel, shown in Figure 10B, with the proteases that were induced by H_2O_2 in total extracts (Figure 1) revealed that a number of the H_2O_2-induced proteases eluted in fraction 9. Not all of the induced proteases, however, interacted with cystatin. An example of an induced protease that did not interact with cystatin is indicated by the arrow in Figure 10B. Unfortunately, it is not possible to directly compare the results of protease activity measured by fluorogenic substrates and the in-gel assay. Nevertheless, our results show that H_2O_2 caused a rapid activation of cysteine and undoubtedly other proteases as well. The activation of the cysteine proteases was instrumental in the execution of PCD. Cloning the genes encoding these proteases is in progress, and we hope to directly analyze their function by transient transformation, as shown in Figures 5 and 6.

DISCUSSION

PCD constitutes the main form of cell death in animals, plants, and other organisms. Numerous stimulants can induce

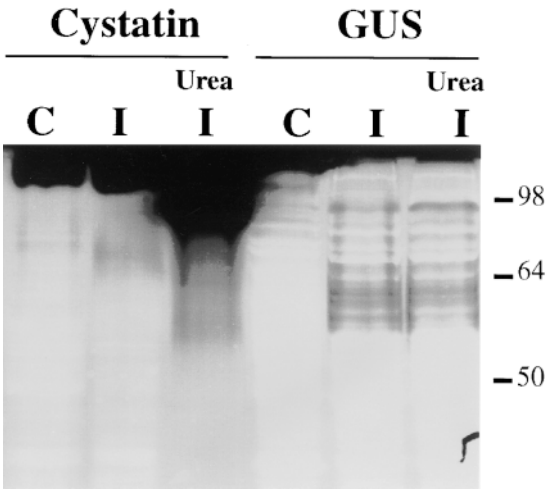

Figure 8. In-Gel Protease Activity Assay of Cystatin- and GUS-Transformed Cells.

Protein extracts from cells transformed with cystatin or *GUS* genes were treated with H_2O_2 and tested by using the in-gel assay. Where indicated, extracts were preincubated in 4 M urea and 0.5% SDS for 15 min, and the proteases were separated from the inhibitors by electrophoresis. C, control; I, induced with 5 mM H_2O_2 for 40 min. Numbers at right indicate molecular weight markers.

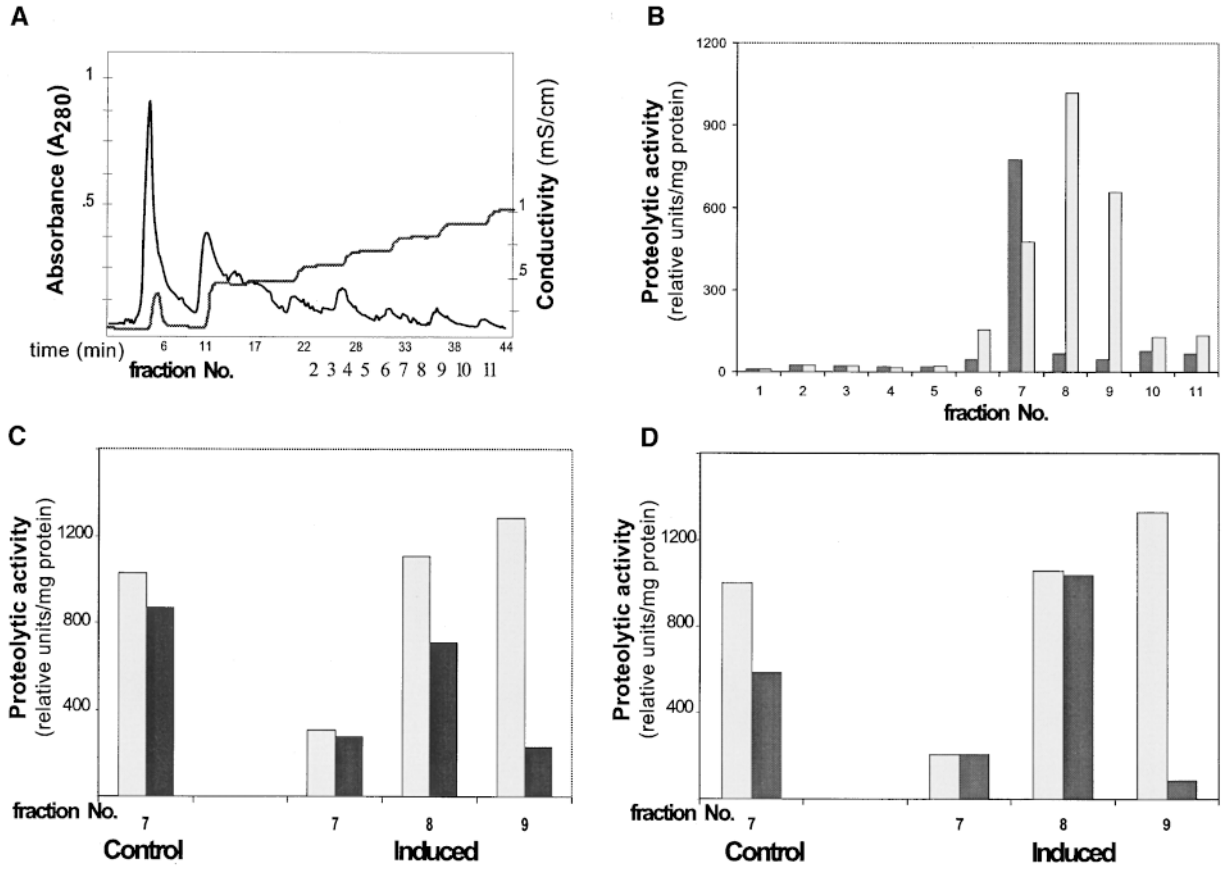

Figure 9. Purification of Cystatin Binding Proteases.

(A) Protein extracts from control and H_2O_2-treated cells were separated on a HighQ anion exchange chromatography column by using a stepwise elution protocol.

(B) Protease activity from the eluted fractions was determined with *Z*-Gly-Gly-Arg-AMC, (gray bars, induced; black bars, untreated).

(C) Inhibition of protease activity by GST–cystatin. Fractions that showed activity in **(B)** were preincubated with 0.4 μM purified GST–cystatin (black bars) for 10 min before the addition of the substrate or with water (control, gray bars).

(D) Inhibition of protease activity by cysteine protease inhibitor E-64. The same fractions as given in **(C)** were incubated in the presence of 10 μM E-64 (black bars) or water (gray bars).

PCD. In many systems, sublethal oxidative stress was found to be involved either directly or indirectly in the PCD process (reviewed in Korsmeyer et al., 1995; Payne et al., 1995; Lamb and Dixon, 1997). In plant systems, it was shown previously that H_2O_2 treatment induces PCD in soybean and Arabidopsis cell cultures (Levine et al., 1994, 1996; Desican et al., 1998). Interestingly, in Arabidopsis *lsd1* mutant plants, PCD was induced by superoxide rather than H_2O_2 (Jabs et al., 1996).

The mechanism of plant cell death is very similar to apoptosis described in animals, as has been determined by morphological and biochemical criteria. Specifically, the morphological hallmarks of apoptosis include cytoplasmic shrinkage, nuclear condensation, and membrane blebbing. The most discernible biochemical events involve calcium influx, protease activation, and DNA fragmentation, first to large 50-kb fragments and then to nucleosomal ladders (Oberhammer et al., 1993; Stewart, 1994; Ryerson et al., 1996). All of these phenomena were shown to occur during plant PCD; however, not all of the events were observed to occur within the same system (Mittler and Lam, 1995; Levine et al., 1996; Ryerson and Heath, 1996; Wang et al., 1996). Nevertheless, the conserved similarity of both morphological and biochemical hallmarks suggests a common cell death process in plants and animals (Greenberg, 1996; Ryerson et al., 1996). Moreover, cell death in plants is an active process that requires changes in gene expression (Desikan et al., 1998). In accord with the latter observation, we show

here that in soybean cells, oxidative stress induced an active cell death program that required biosynthesis of new proteins, as shown by the labeling of newly synthesized proteins after H_2O_2 treatment and by the inhibition of PCD with puromycin or cycloheximide. Future work will show whether these proteins are directly involved with the execution of PCD.

In the animal systems, activation of cysteine proteases has emerged as a key event in the regulation of apoptosis (Martin and Green, 1995). Molecular analysis of early events in these systems revealed a cascade of sequentially acting cysteine proteases with specificity toward aspartate (caspases) within the target sequence (Sleath et al., 1990). Functional analysis of these proteases established the key regulatory role of these proteases in the apoptosis pathway (Cohen, 1997). Mutations in the cysteine protease gene *Ced-3* prevent normal cell death in nematodes. Conversely, overexpression of the specific cysteine protease genes causes cell death in many cell types. Moreover, viral genes that inhibit specific cysteine proteases prevent apoptosis of host cells (Xue and Horvitz, 1995). In plant systems undergoing PCD, the induction of cysteine proteases was found during xylogenesis in Zinnia (Minami and Fukuda, 1995), during leaf and flower senescence, and after a drop in the level of cytokinins (Tournaire et al., 1996).

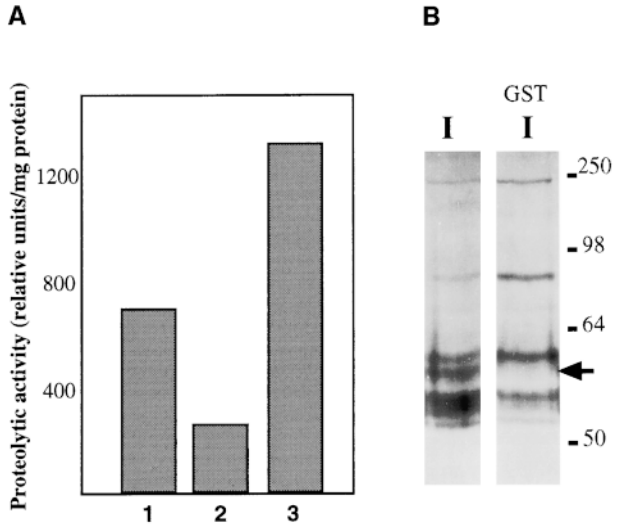

Figure 10. Analysis of GST–Cystatin Binding Protease Activity in Fraction 9.

Protease activity of GST–cystatin bound proteases assayed in vitro and in gel. Fraction 9 from the induced cell extract (see Figure 9) was incubated with GST–cystatin beads and eluted with urea.

(A) Proteolytic activity was measured before binding (bar 1), after binding (bar 2), and after elution with urea (bar 3).

(B) For the in-gel activity assay, proteins from fraction 9 of induced extract were concentrated by binding to GST–cystatin–Sepharose (GST I) or by ultrafiltration through 10,000 molecular weight cutoff membrane (I). Numbers indicate molecular weight markers.

Previous work has shown that in cultured soybean cells, the cell death process that is triggered by H_2O_2 could be inhibited by synthetic protease inhibitors, such as AEBSF and leupeptin (Levine et al., 1996). The inhibition of PCD by a small subset of different protease inhibitors that were tested indeed pointed to a possible role for specific proteases in H_2O_2-stimulated PCD. In plants, massive oxidative bursts that generate high levels of H_2O_2 (Legendre et al., 1993) have been observed in response to avirulent pathogens as part of the hypersensitive response (Lamb and Dixon, 1997). Generation of H_2O_2 and localized cell death also occur after UV irradiation (Murphy and Huerta, 1990), mechanical pressure (Yahraus et al., 1995), salt stress (Hernandez et al., 1995), and chilling stress (Prasad et al., 1994).

Interestingly, transcriptional induction of serine and cysteine proteases was found during xylogenesis in cultured Zinnia cells (Ye and Varner, 1996). This process is associated with both H_2O_2 generation and cell death. Data presented here show that oxidative stress induced the activity of a number of proteases by a post-translational mechanism. Some of these proteases belong to the cysteine class of proteases. The latter conclusion is based on the following results: the newly appearing protease activity digested papain-specific substrates; the activity was blocked by a specific cysteine protease inhibitor, E-64, and by the endogenous cysteine protease inhibitor, cystatin; the cleavage reaction required the presence of a reducing agent; and the proteases were fully active in the presence of EDTA, therefore excluding metalloproteases. The genetic approach of ectopic expression of the endogenous protease inhibitors also eliminated the major drawback of using synthetic inhibitors, that of nonspecific effects, because the added compounds may modify other signaling pathways that affect the PCD process.

Our results suggest that plants have the ability to control PCD by inhibiting cysteine proteases that regulate the expression of specific protease inhibitor genes, such as *Sbcys.* In plants, the proteases and the inhibitor proteins are regulated by different stimuli (Johnson et al., 1989; Doares et al., 1995; Koiwa et al., 1997). Expression of the protease inhibitor genes is usually limited to specific organs or to particular periods during plant development, for example, in seeds or during germination (Botella et al., 1996). Certain protease inhibitor genes are induced during different stresses, such as drought, wounding, and especially in response to insect attack (Waldron et al., 1993). In plants, protease inhibitor genes also are subject to regulation by intercellular signaling molecules, such as jasmonic acid (Farmer et al., 1992), salicylic acid (Doares et al., 1995), and systemin (Constabel et al., 1995). With respect to regulation of protease inhibitor expression, it is interesting that salicylic acid was found to repress expression of cystatin (Doares et al., 1995). Salicylic acid also was shown to act as a potentiator of cell death induced by direct oxidative stress or after pathogen attack by

a currently unknown mechanism (Shirasu et al., 1997). Results presented here suggest that salicylic acid may potentiate cell death when two parallel mechanisms work together: (1) increased generation of H_2O_2 (Shirasu et al., 1997), and (2) repressed cystatin expression. The latter mechanism would make the cells more responsive to peroxide, directing them toward PCD at lower H_2O_2 concentrations, perhaps in systemic leaves (Alvarez et al., 1998). Jasmonate and systemin, on the other hand, shift the balance toward "life," for example, in the face of H_2O_2 that may leak from mechanically damaged cells during insect attack. Our preliminary results with cultured soybean cells showed that methyl jasmonate treatment indeed raised the threshold of oxidative stress for PCD activation (M. Solomon, M. Delledonne, and A. Levine, manuscript in preparation). The purpose of PCD inhibition by jasmonate could be to downregulate the PCD response in cases in which PCD has no survival advantage, for example, during attack by herbivores. In these situations, it may act to prevent excess cell death caused by the release of peroxides from cells crushed by grazing.

In summary, our results show that plant cysteine proteases play an instrumental role in PCD triggered by oxidative stress. They also suggest a new role for protease inhibitor genes in addition to their role in defense against herbivores—that of preventing unwanted cell death. Such a situation may occur after wounding caused by insect chewing or during chilling-induced oxidative stress (Prasad et al., 1994). The control of cellular fate through regulation of the expression of specific proteases in combination with the associated protease inhibitor genes provides additional plasticity in regulating the responses to outside stimuli.

METHODS

Cell Growth and Protein Labeling

Cells were grown as described in Levine et al. (1994), except that 6 mL of cells was transferred into 40 mL of fresh medium every 7 days. All experiments were performed on day 2 after subculture. For labeling, cells were incubated with 0.5 μCi/mL of Pro-Mix (Amersham; ^{35}S-cysteine and ^{35}S-methionine) for 12 hr. H_2O_2 was added directly to the cultures, followed by the addition of 1 mM "cold" amino acids. For autoradiography, gels were impregnated in sulfur-35 enhancing solution (Amersham). Estimation of protein synthesis inhibition was performed by labeling proteins for 2 hr, followed by trichloroacetic acid precipitation.

In-Gel Protease Assay

Cells were harvested with a vacuum, washed with culture medium, and frozen in liquid nitrogen. Extracts were prepared by grinding the cells in buffer A (20 mM Tris, pH 7.8, 20 mM NaCl, 1 mM DTT, 1 mM EDTA and 0.6% polyvinylpolypyrrolidone). Cell walls and insoluble matter were removed by centrifuging twice at 4°C at 16,000*g* for 10 min, and extracts were concentrated by ultrafiltration through 10-kD cutoff cellulose membranes (Vivascience Ltd., Lincoln, UK). Laemmli loading buffer (Sigma) was added to the supernatant, and samples were incubated at 37°C for 4 min before loading on a 10% SDS–polyacrylamide gel containing 0.12% gelatin. Renaturation was done by two washes with 10 mM Tris, pH 7.5, and 0.25% Triton X-100 for 45 min each. The gel was incubated overnight at 30°C and stained with Coomassie Brilliant Blue R 250. Active proteases digested the gelatin and appear as white bands. After staining the gels were scanned in an Astra 1200S transparency scanner (UMAX, Taipei, Taiwan) in the RGB mode, converted to a grayscale mode, and inverted with Adobe PhotoShop (Adobe Systems Inc., San Jose, CA).

Chromatography

Total protein extracts were prepared by grinding frozen cells in liquid nitrogen, resuspending them in ice-cold buffer (20 mM Mes, pH 7.0, 50 mM NaCl, and 1 mM EDTA), and centrifuging them twice at 4°C at 21,000*g* for 15 min. Cleared extracts were loaded onto a Biologic (Bio-Rad) medium pressure chromatography system at 1 mL/min. Anion exchange columns of 1 mL UNO-Q with linear NaCl gradient or 5 mL HighQ (Bio-Rad) with a stepwise 50 mM NaCl elution were used.

Protease Activity Assay

Proteolytic activity was tested with 10 μM fluorogenic peptides (described in the text) in buffer (20 mM Mes, pH 6.5, 150 mM NaCl, 1 mM EDTA, and 0.5 mM DTT) in a 100-μL reaction volume. The reaction was followed for up to 0.5 hr in a FL500 fluorescence reader (BioTek, Winooski, VT), with excitation at 360 nm and emission at 460 nm. The blank fluorescence readings (minus substrate) were subtracted.

Binding of Proteases to Glutathione *S*-Transferase–Cystatin

Strain BL21 cells of *Escherichia coli* were transformed with the glutathione *S*-transferase (GST)–cystatin construct and grown overnight, diluted 1:100, and grown for 2 hr at 37°C before induction with 0.5 mM isopropyl β-D-thiogalactopyranoside for another 6 hr. The bacterial proteins were bound to GST and purified with a bulk GST purification module according to the manufacturer's instructions (Pharmacia). Ten-milliliter fractions from the HighQ column (Bio-Rad) were first precleaned with GST beads and then incubated with 0.2 mL of the purified GST–cystatin for 2 hr at room temperature, washed three times with PBS, and eluted with PBS plus 2 M urea. For preparation of the soluble GST–cystatin fusion protein, we used 10 mM glutathione in 50 mM Tris, pH 8.0.

Transformation of Soybean Suspension-Cultured Cells

A method similar to Forreiter et al. (1997) was used. Cells were coinoculated with 5×10^8 cells of *Agrobacterium tumefaciens* EHA105 carrying the appropriate constructs in 24-well culture plates with moderate shaking at 25°C. After 48 hr, the bacteria were removed by extensive washing over Miracloth (Calbiochem, San Diego, CA) and resuspended in the original volume of fresh medium. An aliquot of

the β-glucuronidase (GUS)–transformed cells was used to estimate transformation efficiency.

Constructs

The mature form of the published cystatin (SbcysN2) protein sequence (Hines et al., 1991) was converted to DNA, and the gene was synthesized as two cDNAs. The Kunitz (Kti3)- and CII-encoding genes were isolated by polymerase chain reaction from genomic DNA of soybean (*Glycine max*) by using the following primers: Kti forward (5′-GGATCCATGAAGAGCACCATCTTCTTTCTCTTTC-3′) and Kti reverse (5′-CAGCTGTCACTCACTGCGAGAAAGGCCATG-3′); and CII forward (5′-GGATCCATGGAACTGAACCTCTTCAAAAGTGAT-3′) and CII reverse (5′-GAGCTCCTAGTCATCATCTTCATCACTGGA-3′). Amplified DNA was cloned into the pGEM-T phagemid (Promega). Cystatin and CII transformation vectors were made by replacing the *GUS* gene in pBI121 (Clontech, Palo Alto, CA) by using the BamHI-SacI sites. Kti3 was cut out with BamHI and PvuII and inserted into a blunted SacI site of pBI121. Kunitz was cut out with BamHI and PvuII, filled with the Klenow fragment of DNA polymerase I, and ligated into a blunted SacI site of pBI121. GST–cystatin was prepared by cloning the cystatin gene into the BamHI-SalI sites of pGEX4 (Pharmacia). The correct frames were verified by sequencing.

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