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; Channongpol 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]-benzene sulfonylfluoride [AEBSF]), hydrochloride, and leupeptin) blocked PCD. No inhibition and in some cases even increased cell death were observed with other inhibitors (N-acetyl-
leucyl-
leucyl-
phenylanilide or tosyl-
leucyl-
leucyl-
benzamid), 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-(epoxy-
benzamid)leucyl-
leucyl-
leucine (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 critical sulfhydryls. However, in vitro treatment of extracts of H. parasitica plants infected with the nematode H. parasitica did not change the pattern of bands with proteolytic activity (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 proteases 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 (Sminoff, 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$_2$O$_2$ 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$_2$O$_2$. 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$_2$O$_2$ (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$_2$O$_2$, for example, by H$_2$O$_2$-dependent oxidation of critical sulfhydryls. However, in vitro treatment of extracts prepared from the uninduced cells with H$_2$O$_2$ did not change the pattern of bands with proteolytic activity (data not shown).
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 \[^{35}\text{S}\]-cysteine and \[^{35}\text{S}\]-methionine, chased the cultures with 1 mM "cold" cysteine and methionine, and immediately treated them with \(\text{H}_2\text{O}_2\) 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). Transient induction of a high molecular weight polypeptide was detected 1 hr after stimulation and disappeared 3 hr later. In brief, \(\text{H}_2\text{O}_2\) 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 \(\text{H}_2\text{O}_2\) stimulus (and the post-translational activation of proteases was sufficient to execute the whole process), we tested whether inhibitors of protein synthesis could block \(\text{H}_2\text{O}_2\)-induced cell death. The concentration of \(\text{H}_2\text{O}_2\) 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 \(\text{H}_2\text{O}_2\) (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 \(\text{H}_2\text{O}_2\) 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 \(\text{H}_2\text{O}_2\) 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 \(\text{H}_2\text{O}_2\) 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 \(\text{H}_2\text{O}_2\) (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...
The specificity, we fractionated extracts of H$_2$O$_2$-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$_2$O$_2$-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, benzoyloxy carbonyl (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-
induced PCD, implicating the cysteine proteases in the H
protease inhibitor genes led to an effective block in H
age, for example, via lipid peroxidation, which caused H
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triggered pathway that leads to apoptosis of soybean cells. 
induced membrane damage (detected by Evan’s blue per-

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 effec-
tive against chymotrypsin and elastase (Odani and Ikenaka, 1977). The genes were cloned behind the constitu-
tively 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 re-
moved by extensive washing, and the soybean cells were treated with H2O2 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 H2O2-induced PCD, implicating the cysteine proteases in the H2O2-triggered pathway that leads to apoptosis of soybean cells. These data also argue against nonspecific oxidative damage, for example, via lipid peroxidation, which caused H2O2-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 experi-
ment, 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 hyper-
 sensible 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 trans-
formed with Sbcys cystatin in contrast to cells transformed with the GUS construct, we extracted cellular proteins 40 min after H2O2 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 H2O2-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

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.
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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$_2$O$_2$, 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 5. Transgene Expression in Soybean Cell Cultures.

Cells were transformed by 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).

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$_2$O$_2$ (black bars). Cell death was determined 10 hr after H$_2$O$_2$ induction by staining with Evan's blue.

(B) Cultures were challenged with $5 \times 10^7$ 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.
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 H2O2 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 H2O2 in total extracts (Figure 1) revealed that a number of the H2O2-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 H2O2 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
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 \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) (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

Figure 9. Purification of Cystatin Binding Proteases.

(A) Protein extracts from control and \( \text{H}_2\text{O}_2 \)-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 \( \mu \text{M} \) 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 \( \mu \text{M} \) E-64 (black bars) or water (gray bars).
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$_2$O$_2$ 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).

Previous work has shown that in cultured soybean cells, the cell death process that is triggered by H$_2$O$_2$ 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$_2$O$_2$-stimulated PCD. In plants, massive oxidative bursts that generate high levels of H$_2$O$_2$ (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$_2$O$_2$ and localized cell death also occur after UV irradiation (Murphy and Huerta, 1990), mechanical pressure (Yahraus et al., 1995), salt stress (Hernandez et al., 1994), 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$_2$O$_2$ 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; Koiva 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 potentator of cell death induced by direct oxidative stress or after pathogen attack by

**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.
a currently unknown mechanism (Shirasu et al., 1997). Results presented here suggest that salicylic acid may potenti-
ate cell death when two parallel mechanisms work together: (1) increased generation of H$_2$O$_2$ (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$_2$O$_2$ concentrations, perhaps in systemic leaves (Alvarez et al., 1998). Jasmonate and sys-
tem toward PCD at lower H$_2$O$_2$ concentrations, perhaps in mechani-
cally damaged cells during insect attack. Our preliminary re-
results with cultured soybean cells showed that methyl jasmonate treatment indeed raised the threshold of oxida-
tive 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 advan-
tage, for example, during attack by herbivores. In these situa-
tions, 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 pro-
teases play an instrumental role in PCD triggered by oxida-
tive 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 chew-
ing 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 as-
associated protease inhibitor genes provides additional plastic-
ty 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 label-
ing, cells were incubated with 0.5 µCi/mL of Pro-Mix (Amerham;
$^{35}$S-cysteine and $^{35}$S-methionine) for 12 hr. H$_2$O$_2$, 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 (Amerham). 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% polyvinylpyrrolidone). Cell walls and insoluble
matter were removed by centrifuging twice at 4°C at 16,000g for 10
min, and extracts were concentrated by ultrafiltration through 10-kD
cutoff cellulose membranes (Vivascience Ltd., Lincoln, UK). Laemmlili
loading buffer (Sigma) was added to the supernatant, and samples
were incubated at 37°C for 4 min before loading on a 10% SDS-poly-
acrylamide 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 Agra 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
21,000g for 15 min. Cleared extracts were loaded onto a Biologic
(Bio-Rad) medium pressure chromatography system at 1 mL/min.
Anion exchange column 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 (de-
scribed 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 re-
action 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 glu-
tathione S-transferase (GST)-cystatin construct and grown over-
night, 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 coin-
ccultured with 5 × 10^5 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 sequence (Hines et al., 1991) was converted to DNA, and the gene was inserted 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. 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–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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The Involvement of Cysteine Proteases and Protease Inhibitor Genes in the Regulation of Programmed Cell Death in Plants
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