Activation of the Programmed Cell Death Pathway by Inhibition of Proteasome Function in Plants*

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Proteasomes constitute the major machinery to degrade or process proteins by ATP/ubiquitin-mediated proteolysis. Recent findings suggest a pivotal role of the ubiquitin/proteasome pathway in the regulation of apoptosis in animal cells. Here we show that virus-induced gene silencing of two different subunits of the 26 S proteasome, the α6 subunit of the 20 S proteasome and RPN9 subunit of 19 S regulatory complex, both activated the programmed cell death (PCD) program, accompanied by reduced proteasome activity and accumulation of polyubiquitinated proteins. These results demonstrate that disruption of proteasome function leads to PCD in plant cells. The affected cells showed morphological markers of PCD, including nuclear condensation and DNA fragmentation, accompanied by the 10-fold higher production of reactive oxygen species and increased ion leakage rate. Similar to apoptosis in animal system, mitochondrial membrane potential was decreased, cytochrome c released from mitochondria to cytosol, and caspase 9- and caspase 3-like proteolytic activities detected in the cells. Interestingly, this proteasome-mediated PCD stimulated the expression of only a subset of transcripts that are highly induced during pathogen-mediated hypersensitive response cell death, indicating that the two PCD pathways are differentially regulated. Taken together, these results provide the first direct evidence that proteasomes play a role in the regulatory program of PCD in plants. Controlled inhibition of proteasome activities may be involved in developmentally or environmentally activated plant cell death programs.

Programmed cell death (PCD) is a genetically defined process associated with distinctive morphological and biochemical characteristics (1), and it involves signaling pathways controlling the demolition of cells with the minimum damage to surrounding cells. PCD is an integral part of the life cycle in multicellular organisms including animals and plants (2) and can be induced by various stimuli, such as developmental and environmental cues (3, 4). PCD occurs in plants during developmental processes, such as senescence, embryogenesis, development of vascular tissues, and sex determination in unisexual plants, as well as during interactions with pathogens (5).

As a result of the extensive studies in the past decade, the basic blue print of the molecular control of apoptosis, the most widely studied PCD, has emerged in the animal system (1, 3, 6, 7). The key participants of the apoptotic cell death are the caspases, a family of cysteine proteases, which exist as dormant proenzymes in most cells. During apoptosis, a battery of caspases becomes activated through proteolytic processing at internal aspartic acid residues. The action of the active caspases on their substrates causes apoptotic morphological changes and leads to cell death. Two major pathways to caspase activation have been defined in mammals: the extrinsic death receptor pathway and the intrinsic or mitochondrial pathway (3). The intrinsic pathway is initiated through release of mitochondrial cytochrome c into the cytosol in response to cellular stresses. Cytochrome c release is a major checkpoint in the initiation of apoptosis, because this protein can induce assembly of the caspase-9 activating complex in the cytosol, termed apoptosome. Upon activation within the apoptosome, caspase-9 can propagate a cascade of further caspase activation events by direct processing of effector caspases.

Evidence is accumulating that the ubiquitin/proteasome pathway plays an important role in apoptosis (8, 9). The 26 S proteasome, consisting of two large subcomplexes, the 20 S proteasome and the 19 S regulatory complex, is a major cytoplasmic proteolytic enzyme complex, responsible for degradation of the vast majority of intracellular proteins in eukaryotes (10). In this pathway, ubiquitin becomes covalently attached to cellular proteins by an ATP-dependent reaction cascade, and then the ubiquitinated proteins are targeted for degradation by the proteasome (11). Proteasomal substrates include metabolic key enzymes, transcription factors, cyclins, inhibitors of cyclin-dependent kinases, and apoptotic regulators (11). In plants, the ubiquitin/proteasome pathway has been linked to cell cycle and to various signal transduction pathways including auxin signaling, photomorphogenesis, and jasmonic acid signaling (11). During apoptosis in animal cells, changes in the expression and activity of different components of the ubiquitin-proteasome system occur (9). Furthermore, proteasome inhibitors have been shown to induce apoptosis in most cell types, whereas in some cells, such as thymocytes and neural cells, these compounds were able to block apoptosis, revealing a complex mechanism of proteasome function in apoptosis (12). Proteasome-mediated steps in apoptosis in animal cells is located upstream.
of mitochondrial changes and caspase activation, and could be related with Bcl-2, Jun N-terminal kinase, heat shock protein, Myc, p53, and polyamines (8).

Studies of the involvement of proteasome in plant PCD lag behind those in animal systems. Among a few examples, application of protease inhibitor at an inhibition miazino mesophyll cell culture completely prevented differentiation of the transient element, whereas inhibition of proteasome activity following commitment to differentiation did not prevent formation of the organ but delayed the process (13). Additionally, overexpression of a mutant form of ubiquitin unable to form polyubiquitin chains induced formation of local lesion in response to mild stress, indicating that disruption of the ubiquitin pathway induces a HR-like cell death under certain conditions (14). However, no direct evidence of proteasome involvement in plant cell death has been provided. In this study, we demonstrate that disruption of proteasome function by gene silencing of the proteasome subunits activates programmed cell death in plant cells, revealing that proteasome is critically involved in cell death programs in plants. The proteasome-mediated PCD exhibited features of apoptotic cell death, such as involvement of reactive oxygen species (ROS), cytochrome c release from mitochondria, and activation of caspase-like protease activities. Interestingly, the gene expression profile during the PCD in this study was different from that of the HR cell death in response to pathogen infection, indicating that different pathways for PCD regulation might have evolved in plants. Signaling pathways of many plant PCD programs may include modulation of proteasome activities in response to the death signals.

EXPERIMENTAL PROCEDURES

Virus-induced Gene Silencing—The 0.3-kb N-terminal and 0.3-kb C-terminal fragments, and the full-length NbPAF cDNA were PCR-amplified and cloned into the pTV0 vector containing a part of the TRV genome (15) using BamHI and ApaI sites. The 0.5-kb NbRpn9 cDNA was also cloned into the pTV0 vector using the same sites. The recombinant pTV0 plasmids and the pBINTRAV6 vector containing RNA1 and 35S promoters of cauliflower mosaic virus were cotransfected into Nicotiana benthamiana (3 weeks old) was pressure-infiltrated with the Agrobacterium tumefaciens plasmid and the pBINTRA6 vector containing RNA1 and 5

Virus infection and silencing—Samples of leaves were detached from the infiltrated leaf within 2 h of infiltration and 15

Virus-induced Gene Silencing—Genomic DNA was isolated from the infiltrated leaf in the virus silencing (VIGS) lines using the Genome Isolation Kit (Qiagen) according to the instructions from the manufacturer. Five μg of genomic DNA was separated on a 1.2% agarose gel and transferred to Hybrid N membrane (Amersham Biosciences). As probes, 100 ng each of the total genomic DNA and chloroplast DNA of N. benthamiana were labeled with a random labeling kit (Bio-Rad). After hybridization, the membranes were washed with 0.2× SSC, 0.1% SDS at 60°C for 1 h.

In Vivo H2O2 Measurement—Protoplasts isolated from leaves of the VIGS lines were incubated in 2 μM 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA, Molecular Probes) for 30, 60, 90, 120, and 150 s. Protoplasts were transferred to wells on microscope slides and illuminated using a confocal microscope (Carl Zeiss, Axiovert) with a green laser (488-nm excitation, LP 505-nm emission) to visualize the oxidized green fluorescent probe. Quantitative images were captured and data were analyzed using the LSM 510 program (version 2.8).

Measurement of Peroxidase (POD) and Ascorbate Peroxidase (Apx) Activity—For POD activity, 0.2 g of leaves from TRV control and TRV:PAF lines were ground in liquid nitrogen, and suspended in 10 mm potassium phosphate buffer, pH 6.0. After centrifugation at 4°C, supernatants were taken, and measured for POD activity with 0.5 mm pyrogallol (Sigma) and 0.1 mm H2O2 using a spectrophotometer with 420-nm wavelength. For Apx activity, the leaves were homogenized in the homogenization buffer (50 mM HEPES, pH 7.0, 0.1 mM EDTA); 5 μg of supernatants were used for Apx activity with 0.03 mM ascorbate and 0.1 mm H2O2, using a spectrophotometer with 290-nm wavelength.

Ion Leakage—After Agrobacterium infiltration, the fourth leaf above the infiltrated leaf from the TRV control and TRV:PAF lines was collected and analyzed. Fifteen leaf discs (7 mm in diameter) were floated on the 0.4M sorbitol. The leaf discs were incubated in the darkness for 12 h, and this solution was measured for sample conductivity. Then the leaves were boiled in the same solution for 5 min, and the solution was measured for the subtotal conductivity. Membrane leakage is presented by the relative conductivity, which was calculated as sample conductivity divided by total conductivity (the sum of sample conductance and subtotal conductivity). Conductivity was measured with a conductivity meter (model 162, Thermo Orion, Beverly, MA).

Callose Staining and Autofluorescence Detection—For callose staining, leaves from TRV control and VIGS-NbPAF lines were fixed in 3:1 ethanol/acetic acid for 1 h, washed in distilled water for 15 min, and stained in 5 n sodium hydroxide at room temperature overnight. The leaves were then washed twice in distilled water and incubated in 0.1% aniline blue (Sigma) in 0.1% potassium phosphate buffer, pH 7.0, for 2 h in the darkness. The stained leaves were observed under a fluorescence microscope (Zeiss Axioskop). For autofluorescence detection, intact leaves were observed under a fluorescence microscope (Zeiss Axioskop).

Measurement of Mitochondrial Membrane Potential—Tritonx-100 (0.01%) and methyl ester (0.1% of total volume) was added into protoplasts isolated from leaves of the VIGS lines at the final concentration of 200 nM. After incubation for 10–15 min at 25°C, protoplasts were transferred to wells on microscope slides and illuminated using a confocal microscope (Carl Zeiss LSM 510) with optical filters (543-nm excitation, LP 556-nm emission) to visualize the oxidized red fluorophore. Quantitative images were captured and data were analyzed using the LSM 510 program (version 2.8).

Measurement of Caspase-like Activity—Leaves were ground and homogenized in caspase extraction buffer (50 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 1% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, 20% glycerol). Samples were incubated with shaking ice for 15 min, centrifuged, and filtrated. Samples (50 μl) were mixed with 50 μl of caspase assay buffer (caspase extraction buffer with 150 μM LEHD-AFC (R&D Systems) for caspase 9-like activity and DEVD-AFC (R&D Systems) for caspase 3-like activity, as peptide substrates). After incubation at 37°C for 1 h, the fluo-
rescence of AFC hydrolyzed from the peptide substrates was quantified in a spectrofluorometer (Shimadzu, RF-5000) using 490-nm excitation and 505-nm emission wavelengths. Enzymatic activity was normalized for protein concentration and expressed as percentage of activity present in control extracts. Each measurement was carried out with three independent VIGS plants.

**Cellular Fractionation and Detection of Cytochrome c Release**—Two grams of leaves from the TRV control and TRV-PAF line were ground in grinding buffer (0.4 M mannitol, 1 mM EGTAs, 20 mM 2-mercaptoethanol, 50 mM Tris, 0.1% bovine serum albumin, pH 7.8) for 1 min at 4 °C. Extracts were filtered through Miracloth, and the filtrates were centrifuged at 15,000 × g for 5 min at 4 °C. The supernatant was centrifuged at 16,000 × g for 15 min at 4 °C. Following this second centrifugation, the supernatants obtained were taken to represent the cytosol fraction, and the pellets were resuspended in grinding buffer to represent the mitochondria fraction. Fifty μg of proteins were electrophoresed on a 12% SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and probed with the monoclonal antibody against cytochrome c (1:1000 dilution; Pharmingen) and the monoclonal antibody (31HlL) against human voltage-dependent anion channel (VDAC/porin) (1:1000 dilution; Calbiochem). They were then reacted with secondary antibodies conjugated with horseradish peroxidase and ECL reagent (Amersham Biosciences) for detection.

**Measurement of Proteasome Activity**—Proteasome activity was assessed in cell extracts using synthetic peptide substrates (Sigma) linked to the fluorescent reporters, 7-amino-4-methylcoumarin (AMC) or β-naphthylamide (βNA) in the absence or presence of proteasome inhibitor MG132 (Calbiochem). Homogenized leaf extracts were cleared by centrifugation, and the supernatants used for determination of protein concentration and enzymatic activity. Fifty μl of the assay were assayed by addition of 50 μl of assay mixture (50 mM Tris-HCl, pH 7.5, 50 mM MgCl2, 1 mM dithiothreitol, 0.5 mM ATP, 2% glycerol), and incubation for 1 h at 37 °C. MG132 was added to the assay mixture at the final concentration of 100 μM. AMC and βNA hydrolyzed from the peptides were quantified in a spectrofluorometer (Shimadzu, RF-5000) using 380-nm excitation/460-nm emission wavelengths, and 342-nm excitation/460-nm emission wavelengths, respectively. Enzymatic activity was normalized for protein concentration and expressed as percentage of activity present in control extracts. Each measurement was carried out with three independent VIGS plants.

**Western Blot Analysis of the Levels of a Subunits**—Western blot analysis was carried out as described (16). Fifty μg of proteins were electrophoresed on a 10% SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and probed with the monoclonal antibody raised against the mixture of six α subunits (α1, α2, α3, α5, α6, and α7) (1:1000 dilution; Affiniti Research Products Ltd). They were then reacted with secondary antibodies conjugated with horseradish peroxidase and ECL reagent (Amersham Biosciences) for detection.

**RESULTS**

**Viruse-induced Gene Silencing of the Proteasome Subunits Activated Programmed Cell Death**—Phenomic analysis has been carried out to assess functions of signaling genes and hypothetical genes of N. benthamiana using TRV-based VIGS. VIGS is based on the phenomenon that gene expression is suppressed in a sequence-specific manner by infection with viral vectors carrying host genes (17). These screening led to suppression of the proteasome subunit genes.

**VIGS phenotypes and suppression of the endogenous transcripts.** A, the schematic drawing of the structure of NbPAF, and three VIGS constructs containing different regions of the NbPAF cDNA (marked by bars). The proteasome α-type subunits signature is indicated. B, VIGS phenotypes of the three NbPAF VIGS lines. N. benthamiana plants were infected with Agrobacterium containing the TRV control and three different forms of TRV-NbPAF constructs (TRV-PAF(N), TRV-PAF(C), and TRV-PAF(F)). The photographs of the plants were taken at 20 days after inoculation. C, semiquantitative RT-PCR analysis to examine the transcript levels of NbPAF. RNA was extracted from the fourth leaf above the infiltrated leaves from N. benthamiana plants infected with TRV, TRV-PAF(N), TRV-PAF(C), and TRV-PAF(F). The NbPAF-N and NbPAF-C primers were designed to detect the N- and C-terminal regions of the NbPAF cDNA, respectively. The NbPAF-N and NbPAF-C primers do not detect the viral genome transcripts from the TRV-PAF(N) and TRV-PAF(C) lines, respectively, but detect the TRV-PAF(F) viral genomic transcripts. As controls for RNA amount, the mRNA levels of actin and β subunit of the 20 S protease (NbPBC) were examined. D, the schematic drawing of the NbRpn9 structure and its VIGS phenotypes. N. benthamiana plants were infected with TRV control and TRV-Rpn9. The photographs of the plants were taken at 20 days after inoculation, exhibiting the same phenotype as the TRV-PAF VIGS line. The PCI domain (54) is indicated. The bar represents the NbRpn9 cDNA region used for VIGS. E, silencing of the NbRpn9 transcripts. Semi quantitative RT-PCR was carried out to examine the endogenous transcript levels of NbRpn9 using two individual VIGS plants. The primers were designed to exclude the cDNA region used in the VIGS construct. As a control for RNA amount, the mRNA levels of actin, NbPAF, and NbPBC were examined.

TRV-PAF(F) contains the full-length NbPAF cDNA. VIGS with the three constructs all resulted in the same phenotype of severe abnormality in plant development (Fig. 1B). Newly emerged leaves were small, severely curled, and wrinkled, making a cluster near the shoot apex, and the stem growth was completely arrested. Massive cell death soon followed, which resulted in premature death of the whole tissues of newly emerged leaves and flower buds. The effects of gene silencing on the endogenous amounts of the NbPAF mRNA were examined using semiquantitative RT-PCR (Fig. 1C), because the level of the transcript was low in the leaves. Primers for RT-PCR were designed to exclude the cDNA regions used in the VIGS constructs, and the transcript levels of the actin and the β subunit of the 20 S proteasome (NbPBC) were measured as controls. RT-PCR using NbPAF-N primers that detect the N-
terminal region of the NbPAF cDNA produced significantly reduced amounts of PCR products in the VIGS lines of TRV:PAF(C) compared with the TRV control, indicating that the endogenous level of the NbPAF transcripts is greatly reduced in those plants. The same primers detected high levels of viral genomic transcripts containing the N-terminal region of NbPAF in the TRV:PAF(N) and TRV:PAF(F) lines. In contrast, NbPAF-C primers that recognize the C-terminal region of the cDNA showed suppression of the endogenous NbPAF transcripts in the TRV:PAF(N) lines, whereas they detected the viral genomic transcripts in the TRV:PAF(C) and TRV:PAF(F) lines. The transcript levels of NbPBC and actin remained constant. These results demonstrate that expression of NbPAF was significantly reduced in the VIGS lines.

The NbRpn9 VIGS lines showed the same phenotypes as that of the NbPAF VIGS lines (Fig. 1D). Silencing of NbRpn9 was examined via semiquantitative RT-PCR using the primers that detect the cDNA region not covered in the VIGS construct. Compared with TRV control, the endogenous level of NbRpn9 transcripts was greatly reduced in the TRV::Rpn9 VIGS lines, whereas the transcript levels of NbPAF, NbPBC, and actin remained constant (Fig. 1E).

**Phenotypes of Programmed Cell Death—**We examined nuclear morphology of cells in the abaxial epidermal layer from the leaves of the VIGS lines by DAPI staining (Fig. 2A). In the epidermal cells of the TRV::PAF(N) leaves, condensation and margination of nuclear chromatin were evident, whereas chromatin was evenly distributed within the nucleus in the control lines. Furthermore, DNA laddering was observed in the genomic DNA of TRV::PAF(N) and TRV::Rpn9 lines (Fig. 2B).

DNA ladder is formed during PCD because of the activation of cell death-specific endonucleases that cleave the nuclear DNA into oligonucleosomal units. To visualize DNA laddering, the genomic DNA extracts from the VIGS lines were fractionated, transferred to nylon membranes, and hybridized with radiolabeled total genomic DNA and chloroplast DNA of *N. benthamiana*. DNA laddering was observed with the total genomic DNA probe, whereas the chloroplast DNA probe resulted in DNA degradation but without the laddering pattern, because the chloroplast genomic DNA is not packaged into nucleosomes. Because nuclear condensation and DNA laddering are the hallmark features of PCD, these results demonstrate that reduced expression of these proteasome subunits activates programmed cell death in plants. Interestingly, virus-induced gene silencing of other proteasome subunits (β1, β4, and Rpn3) also caused DNA laddering, indicating that inhibition of proteasome function by reduced availability of individual subunits is the reason for the PCD activation (Supplementary Fig. 1, available in the on-line edition of this article; Fig. 3). DNA laddering was also observed by gene silencing of ubiquitin, consistent with the previous report (14). In contrast, virus-induced gene silencing of other presumably essential genes of plants, such as cellulose synthase or glucan synthase, did not induce the PCD phenotypes, despite the fact that it resulted in severe morphological phenotypes including growth arrest and premature death of plants (Supplementary Fig. 1; Fig. 3). Thus, PCD appears to be activated by disrupted function of specific sets of genes such as ubiquitin and proteasome subunits.

**Fig. 2.** Phenotypes of programmed cell death. A, nuclear condensation. Fluorescent microscopic picture of the cells from the leaves of the TRV and TRV::PAF(N) VIGS lines after nuclear staining with DAPI (100 μg/ml). B, oligonucleosomal DNA fragmentation. Genomic Southern blot was carried out with DNA extracted from the leaves of the VIGS lines using the total genomic and chloroplast DNA of *N. benthamiana* as probes.

**Fig. 3.** PCD phenotypes induced by gene silencing of other proteasome subunits. Virus-induced gene silencing was carried out with *N. benthamiana* cDNAs encoding actin, β-tubulin, cellulose synthase, arginyl-tRNA synthetase, glucan synthase, RNA polymerase II subunit RPB10, myosin I, ubiquitin, and three subunits (β1, β4, and RPN3) of 26 S proteasome. RPB10 is an essential subunit of RNA polymerase II. To examine DNA laddering, genomic DNA was isolated from the fourth leaf above the infiltrated leaf in each VIGS line, and genomic Southern blot analysis was carried out using the total genomic DNA of *N. benthamiana* as a probe. Five μg of genomic DNA was used per lane.
Gene Silencing of the Proteasome Subunits Inhibits Proteasome Activity—To examine functional consequence of gene silencing of the proteasome subunits, we measured proteasome activities in cell extracts from TRV control, TRV:PAF(N), TRV:PAF(C), and TRV:PAF(F) lines using peptide substrates in the absence or presence of proteasome inhibitor MG132 (Fig. 4A). Previously, it has been shown that plant proteasomes possess classical chymotrypsin-like, peptidylglutamylpeptide hydrolyzing-like, and trypsin-like activities against fluorescent synthetic peptide substrates (18, 19). Indeed, compared with the control, reduced expression of the α6 subunit using the three VIGS constructs significantly reduced the hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC, Z-Leu-Leu-Leu-AMC, and Z-Gly-Gly-VIGS constructs significantly reduced the hydrolysis of Suc-H9262 extracts (50 μg of total protein) of TRV, TRV:PAF(N), TRV:PAF(C), and TRV:PAF(F) leaves using three peptide substrates of the 26 S proteasome in the absence (gray) or presence of proteasome inhibitor MG132 (black). Enzymatic activity was normalized for protein concentration and expressed as percentage of activity present in control extracts. Data represent mean values (±S.D.) of three independent measurements. B, cell extracts (50 μg of total protein) of TRV, TRV:PAF(N), TRV:PAF(C), and TRV:PAF(F) leaves were subjected to SDS-PAGE and Western blotting with the anti-ubiquitin monoclonal antibody and anti-β-tubulin monoclonal antibody. The protein levels of α6 subunit using the three VIGS constructs significantly reduced the hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC, Z-Leu-Leu-Leu-AMC, and Z-Gly-Gly-Arg-βNA, synthetic peptide substrates of the chymotrypsin-like, PGPH-like, and trypsin-like activities of the proteasome, respectively. The degree of reduced activity was comparable with or slightly more than that achieved by lactacystin or RNA interference of the proteasome subunits in animal cells (20, 21). These results demonstrate that VIGS-promoted depletion of the proteasome subunits decreased all three types of proteasome activity. Proteasome inhibitor MG132 significantly reduced all three types of proteasome activity in TRV control, demonstrating that most of the hydrolytic activity indeed comes from proteasome (Fig. 4A). However, the residual peptidase activity, particularly the trypsin-like activity, still remained in the extracts from TRV control as well as from TRV:PAF(N) leaves. These inhibitor-resistant residual activities likely represent other proteases besides the 26 S proteasome. The measured peptidase activity of all three types did not differ significantly in the absence or presence of MG132 in the TRV:PAF samples, indicating that VIGS abolished most of active 26 S proteasome complex. The reduced proteasome activity by gene silencing of PAF is likely caused by interference of proteasome assembly resulting from the reduced expression of the subunits. RNA interference of the individual proteasome subunit in Drosophila and trypanosome all resulted in disruption of proteasome assembly, and as a consequence, reduction of proteasome activity (20, 21).

Depletion of the Proteasome Subunits Leads to Accumulation of Polyubiquitinated Cellular Proteins—Most physiological substrates of the 26 S proteasome are cellular proteins covalently modified with a polyubiquitin chain. To determine the effect of gene silencing of the proteasome subunits on the degradation of such proteins, we carried out Western blotting with cell extracts of TRV control, TRV:PAF(N), TRV:PAF(C), and TRV:PAF(F) lines with a mouse anti-ubiquitin monoclonal antibody (Fig. 4B). It resulted in a smear of high molecular weight immunoreactive materials displaying many cellular proteins modified by polyubiquitin chains. Compared with the TRV control, gene silencing of the α6 subunit using the three different VIGS constructs greatly increased the level of polyubiquitinated proteins. Previously, inhibition of proteasome activity by proteasome inhibitors or by RNA interference of the proteasome subunits also increased the intensity of this smear, displaying accumulation of non-degraded cellular proteins in animal cells (20, 21). In plants, mutants of MCB1 (RPN10) and UBP14 (ubiquitin-specific protease) exhibited increased steady state levels of cellular ubiquitinated proteins (22, 23). These results mirrored corresponding effects of gene silencing on proteasome activity against peptide substrates.

Protein Levels of α6 and Other α Subunits in TRV:PAF Leaves—The protein levels of α6 and other α subunits in TRV:PAF plants were examined by Western blotting with the monoclonal antibody raised against the mixture of six α subunits (α1, α2, α3, α5, α6, and α7) and β-tubulin antibody as a control (Fig. 4C). In three different TRV:PAF lines, the α6 subunit...
Fig. 5. Involvement of ROS during cell death. A, protoplasts isolated from leaves of TRV or the mixture of TRV:PAF VIGS lines were incubated in 2 µM H2DCFDA for the indicated times (30–150 s). H2DCFDA is a ROS indicator that becomes fluorescent when oxidation occurs within the cell. The bar indicates 100 µm. B, fluorescence of protoplasts from control and the TRV:PAF VIGS lines was quantified as described under “Experimental Procedures.” Data points represent means ± S.D. of 9–12 individual protoplasts. FIV, pixel intensity values. C, relative ascorbate peroxidase activity; D, relative peroxidase activity. E, relative ion leakage.
aromatic polymers at infection sites. We examined the presence of the autofluorescent material and callose in the leaves of the TRV:PAF(N) and TRV:Rpn9 VIGS lines that undergo PCD. Fig. 8A demonstrates that leaves of both VIGS lines accumulated substantial amounts of autofluorescent products and callose. TRV control showed only small patches of those materials, primarily along the vein. Both TRV:PAF and TRV:Rpn9 VIGS lines also exhibited intense staining on the leaves with Evans blue indicating localized cell death, whereas TRV control showed no staining (Fig. 8A). These results demonstrate that some features of HR cell death are conserved in the PCD program induced by disruption of proteasome function.

We also examined whether the proteasome-mediated PCD induces expression of defense-related genes using semiquantitative RT-PCR (Fig. 8B). PR1a, PR1b, PR1c, PR2, PR4, PR5, S25-PR6, S25, HSR203J, HIN1, and 630 genes are all highly induced during HR cell death (28). NTCP-23 (cysteine protease) and p69d (serine protease) have been shown to be involved in pathogen-induced cell death (29), whereas the chloroplastic ClpP protease plays a role in chloroplast development but not in senescence or HR cell death (29). Among these genes, only PR2, PR5, HIN1, ClpP, and NTCP-23 genes were transcriptionally induced in both the TRV:PAF(N) and TRV:Rpn9 VIGS lines. Expression of SGT1, RAR1, and SKP1, recently identified signaling genes in plant defense (30–32), remained constant. Thus the proteasome-mediated PCD process promotes expression of only a subset of PR genes. Taken together, some features of HR cell death are conserved in the proteasome-mediated PCD program, but its gene expression profile is significantly different from the HR, indicating a possibility of differential regulation of each PCD pathway.

DISCUSSION

In this study, we present direct evidence that interruption of proteasome function activates programmed cell death in plant cells. When expression of proteasome subunits was suppressed by virus-induced gene silencing, the affected cells showed the characteristic features of apoptotic cell death, including nuclear condensation and DNA fragmentation. This proteasome-mediated cell death pathway involves ROS, cytochrome c release from mitochondria, activation of caspase-like activities, and transcriptional induction of a subset of defense-related genes. The role of the proteasome in PCD in animals has been extensively investigated in various cell lines using specific proteasomal inhibitors, such as lactacystin and peptide aldehydes...
already known to be substrates of the proteasome, and inhibitor of apoptosis proteins, which are critically involved in the negative regulation of apoptosis, have been shown to play an active role in the proteolytic inactivation of death executors (8, 9). The evidence of possible involvement of proteasome in PCD in plants has only begun to emerge. Recently, ubiquitin ligase-associated protein SGT1 has been found to be essential for R gene-mediated disease resistance (30, 35) and HR cell death elicited by multiple resistance interactions (35). Furthermore, SGT1 and RAR1 (SGT1-interacting protein) are co-immunoprecipitated with COP9 signalosome, demonstrating a direct interaction between COP9 signalosome and ubiquitin ligases (31). These results suggest that the ubiquitin protein degradation pathway regulates at least a subset of R-mediated defense responses and HR cell death (36).

Compared with animal system, relatively little information is available on the detailed mechanism of PCD in plants. However, some aspects of the molecular machinery of PCD seem to be conserved between plants and animals. Numerous mediators of disease resistance signaling in plants share conserved motifs with proteins that have similar roles in the defense response of animals (7). Furthermore, overexpression of Bax, which encodes a mammalian proapoptotic protein, induces PCD in plants and yeast (37, 38). In animal cells, mitochondria-mediated PCD acts through Bax family of proteins, which associates with the mitochondria membrane and forms an ion-conduction channel through which macromolecules and metabolites can pass (39). Although Bax plays a critical role in mitochondrion-mediated PCD in mammals, plants and yeast lack these proteins and many other regulators of mammalian PCD. Nevertheless, Bax-induced PCD in plant cells indicates common underlying mechanisms between animal and plant cell death programs.

In this study, we have demonstrated that proteasome-mediated cell death in plants involves the common components of apoptosis in animal cells, including decreased mitochondrial membrane integrity, ROS production, cytochrome c release, and activation of caspase-like protease activities. Although plant genome lacks direct homologues of caspase genes, caspase-like protease activities have been detected in the HR cell death and in PCD associated with other nonpathogenic responses, such as heat, menadione, and isopentenyladenosine treatment (40–42). Interestingly, HR cell death and the caspase-like activities were specifically inhibited by caspase inhibitors but not by other types of inhibitor including those targeting serine proteases, metalloproteases, calpain, and aspartate proteases (40, 43). The caspase 3-like activity found in barley embryonic cells also could be inhibited by the specific caspase 3 inhibitors, but not by general cysteine protease inhibitors (44). Recently, a family of caspase-related proteases (the metacaspases) has been identified in Arabidopsis based on homology searches (45). It remains to be seen whether the metacaspases are functionally equivalent to mammalian caspases in controlling cell-death activation. Several reports point to the importance of the mitochondria in the expression of HR cell death in plants, although it is not clear whether cytochrome c leakage occurs during the HR. Cytochrome c leakage and activation of caspase-like proteases was detected in our study, and during isopentenyladenosine- and heat-induced PCD, but not during petal senescence-associated PCD (41, 42, 46, 47). It remains to be investigated whether the released cytochrome c promotes assembly of the caspase-activating complex in the cytosol, as in the case of animal cells.

Disruption of two different proteasome subunits both induced transcription of defense genes, such as PR2, PR5, Hin1, and NTCP-23 (cysteine protease), and ClpP protease.

**Fig. 8. Accumulation of defense-related markers.** A, Autofluorescence, callose staining, and Evans blue staining of detached leaves from TRV control, TRV:PAF(N), and TRV:Rpn9 lines. Evans blue staining indicates dead cells without intact cellular membranes. B, semi-quantitative RT-PCR analysis to examine transcript levels of defense-related genes. RNA was extracted from the fourth leaf above the infiltrated leaves from several independent N. benthamiana plants infected with TRV, TRV:PAF(N), or TRV:Rpn9. As a control for RNA amount, actin mRNA levels were examined.
unidentified apoptosis regulators and death effectors. Stability of an individual proteasome subunit may be a target of regulation for controlling the PCD program. It will be important to investigate whether various developmental or environmentally activated cell death programs of plants involve modulation of proteasome function. In this scenario, some PCD pathways of plants may include signaling molecules that modify proteasome activity to activate the cell death process when death signals are perceived. To identify the important players in cell death activation in plants, reverse genetic approaches such as virus-induced gene silencing, coupled with bioinformatics approaches, will be useful to screen a large number of candidate regulators. Probing functional roles of the proteasome in PCD activation may give insight into how death signals, including developmental or pathogen-related signals, are perceived and translated into a cascade of changes leading to plant cell death. These efforts may reveal unique mechanisms of PCD program in plant cells, in addition to the conserved mechanisms between animals and plants. Finally, from an applied perspective, the ability to induce cell death by gene silencing of proteasome subunits may have useful applications in agriculture by providing a tool to selectively kill certain cells and tissues.

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