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Comparative microarray analysis of programmed cell death induced by proteasome malfunction and hypersensitive response in plants ☆

Moonil Kim [a,1], Sanghyeob Lee [b,1], Kyoungsook Park [a], Eun-Ju Jeong [a], Choong-Min Ryu [c], Doil Choi [b], Hyun-Sook Pai [d,*]

[a] *BioNanotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Republic of Korea*
[b] *Laboratory of Plant Genomics, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Republic of Korea*
[c] *Laboratory of Microbial Genomics, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Republic of Korea*
[d] *Department of Biology, Yonsei University, Seoul 120-749, Republic of Korea*

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Abstract

Programmed cell death (PCD) plays a pivotal role in the elimination of injured or unwanted cells during diverse physiological and developmental conditions in organisms. However in contrast to the animal system, signaling pathways and molecular mechanism of PCD are largely unknown in plants. We previously reported that silencing of *NbPAF* encoding the α6 subunit of 20S proteasome by virus-induced gene silencing activated programmed cell death in plants by inactivating proteasome function. In this study, we analyzed global gene expression profile of PCD induced by suppression of *NbPAF* expression, in comparison with that of hypersensitive response (HR)-induced PCD, using a cDNA microarray representing 4685 hot pepper genes. HR is a well-characterized PCD program in plants, which occurs in response to pathogen infection. The microarray analyses identified 247 genes whose gene expression was differentially modulated during PCD activated by NbPAF depletion or HR. Most of the genes that were up-regulated during the *NbPAF*-mediated PCD, including the ubiquitin/proteasome pathway-related genes, were down-regulated during HR cell death. In contrast, transcription of many defense-related genes, transcription factor genes, and photosynthesis-related genes remained unchanged or repressed during *NbPAF*-mediated PCD, while it was highly induced during HR cell death. Only a small number of genes including antioxidant-related genes and proteases were found to be up-regulated during induction of PCD by both proteasome inactivation and HR. Based on these results, these two PCD pathways appear to be differentially regulated, but some overlapping mechanism exists, which involves core regulators of plant PCD.

Keywords: α6 Subunit of 20S proteasome; cDNA microarray; Gene expression; Hot pepper; Hypersensitive response; Virus-induced gene silencing

Programmed cell death (PCD) is a genetically regulated biological process that plays a crucial role in the homeostasis of multicellular organisms [1]. PCD is essential for many developmental processes in plants, such as petal senescence, xylogenesis, aerenchyma formation, endosperm development, and for response of plants to pathogen infection as well [2]. While cell death pathways in animal cells have been well characterized, relatively little is known about the molecular mechanism of PCD in plants. Particularly, plant homologs of the key regulators of animal apoptosis, such as caspases and Bcl-2 family members, have not been identified yet.

The selective degradation or stabilization of intracellular proteins by ubiquitin-proteasome-dependent pathways is essential for the regulation of many cellular processes including development, cell cycle, cell growth, and apoptosis [3]. Studies have demonstrated that ubiquitin-proteasome

☆ *Abbreviations:* HR, hypersensitive response; *NbPAF*, *Nicotiana benthamiana* proteasome α6 subunit; PCD, programmed cell death; ROS, reactive oxygen species; VIGS, virus-induced gene silencing.

* Corresponding author. Fax: +82 2 312 5657.
E-mail address: hspai@yonsei.ac.kr (H.-S. Pai).

[1] These authors contributed equally to this work.

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pathway is involved in the regulation of PCD [4]. Many apoptosis regulatory proteins have been identified as target substrates of ubiquitination. Bax, a proapoptotic Bcl-2 family member, undergoes a conformation change through apoptosis signaling and caspase activation, which leads to the protein's dimerization and resistance to ubiquitin-mediated degradation [5]. In addition to being targets for ubiquitination, some apoptosis regulatory proteins such as IAPs (the inhibitor of apoptosis proteins) exhibit ubiquitin ligase activity [6]. The protein levels of p53 tumor suppressor and cell's susceptibility to apoptosis can be deregulated by Hdm2 E3 ligase, the human homolog of Mdm2 [7].

During apoptosis, changes in the expression and activity of different components of the ubiquitin-proteasome system occur in animal cells [8]. 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 [9]. Recently, we have shown that disruption of proteasome function leads to PCD in plants [10]. Virus-induced gene silencing (VIGS) of *NbPAF* or *NbRpn9*, respectively, encoding the α6 and RPN9 subunit of 20S proteasome, activated PCD program in plants. The affected cells exhibited morphological markers of PCD such as nuclear condensation, DNA fragmentation, and increased production of reactive oxygen species (ROS). The cells also showed several critical features of apoptosis, including cytochrome *c* release, disruption of mitochondrial membrane potential, and increased caspase-like proteolytic activity. These results suggest the existence of a cell death signaling cascade that is functionally conserved between animals and plants.

Interestingly, we found that expression of many pathogenesis-related (PR) genes remained at the basal level during PCD in *NbPAF*- and *NbRpn9*-silenced plants [10]. Transcription of those PR genes is highly induced during hypersensitive response- (HR) induced cell death caused by plant interaction with avirulent pathogen [11]. HR cell death is activated by perception of pathogen-derived molecules by the resistance (R) gene products, and it is associated with the massive accumulation of reactive oxygen species (ROS), salicylic acid (SA), and other pro-death signals such as nitric oxide [12]. The differences in gene expression profile between PCD induced by two different means, i.e., by proteasome malfunction and HR, indicate that mechanism of different types of PCD might be differentially regulated.

In this study, we analyzed the gene expression profiles during PCD activated by proteasome malfunction and HR in a large scale to investigate the cell death signaling pathway in plants and to identify novel genes related with plant PCD. We carried out comparative microarray analysis of PCD caused by proteasome suppression and HR in *Nicotiana benthamiana* by examining the expression profiles of 4685 genes. The results showed that 247 genes on the array, including transcription factor genes, ubiquitin/proteasome-related genes, defense-related genes, and photosynthesis-related genes, were differentially expressed between control and two types of PCD. We will discuss the similarities and differences between transcription profiles of the genes during PCD induced by two different treatments.

Materials and methods

Virus-induced gene silencing. Virus-induced gene silencing (VIGS) of *NbPAF* was carried out as described [10].

Induction of HR cell death. Leaves of the 4-week-old *N. benthamiana* plants were infiltrated with a bacterial pathogen *Pseudomonas syringae* pv. *syringae* 61 (*Pss*61) (1×10^8 colony forming unit ml^{-1} in 10 mM $MgCl_2$), using a 10 ml plastic syringe without a needle as previously described [13]. Control leaves were infiltrated with 10 mM $MgCl_2$. At 24 h after infiltration, the leaves were collected, frozen in liquid nitrogen, and stored at −70 °C for RNA extraction.

Preparation of fluorescent probes. Total RNA was extracted from the fourth leaf above the infiltrated leaf of three independent TRV control and TRV:NbPAF lines, using TRIzol™ reagent (Gibco/BRL, MD) following the instructions of manufacturer. Total RNA was also extracted from the mock-treated and the *Pss*61-infiltrated leaves of three independent *N. benthamiana* plants. The mRNA was isolated from the total RNA using the Oligotex mRNA Midi Kit (Qiagen, Valencia, CA), and 2 μg of mRNA was labeled by direct incorporation of Cy3- or Cy5-conjugated dUTP (Amersham Pharmacia Biotech, NJ) as described [14]. The labeled probes were combined and purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and concentrated to a final volume of 5 μl.

Hybridization. Microarrays were prehybridized by adding 30 ml prehybridizing solution (3× SSC, 2.5× Denhardt's Reagent, 1% (w/v) BSA, and 0.1% (w/v) SDS) for 40 min at 42 °C using a humidified hybridization cassette. Slides were washed in ddH_2O for 1 min and air-dried. For hybridization, the labeled probes were mixed with 10-μl formamide (Sigma, MO) and 5 μl 2× hybridization solution (Amersham Pharmacia Biotech, NJ). The whole mix was denatured at 95 °C for 3 min and applied to the microarray slides. Then the slides were covered with a cover slip and incubated in a 42 °C water bath for 16 h. After incubation, the slides were washed at 55 °C with 1× SSC/0.2% SDS, 0.1× SSC/0.2% SDS, and then 0.1× SSC for 10 min each.

Scanning and data analysis. The slide was scanned with an Axon GenePix 4000A scanner (Axon, CA) according to the manufacturer's instructions to capture the data. The PMT voltage was adjusted to yield Cy-3/Cy-5 signal intensity as close to 1.0 as possible. The spot intensities were measured using the Axon GenePix Pro 4.0 image analysis software, and global normalization was applied using the calculated ratio of median factor. Microarray experiments were repeated for three times. After the normalization, a number of quality control methods were applied. First, the spots flagged as "bad" or "not found" by image analysis software were removed from the analysis. Second, the spots smaller than 40 μm in diameter were discarded. Third, the spots that showed validated data at least three times were used. Fourth, the spots with standard deviation (SD) lower than 50% of the average ratio were used. These measures resulted in the selection of 1150 unique ESTs for data analysis. Ratio values were the average of three replicates.

Results and discussion

Phylogenetic analysis between N. benthamiana and hot pepper genes

In this study, we analyzed transcription profiles of two types of PCD in *N. benthamiana*, one induced by

depletion of proteasome function and the other by HR, by using a microarray representing 4685 hot pepper genes. Hot pepper (*Capsicum annuum*) has a close phylogenetic relationship with other members of the Solanaceae family, such as *N. benthamiana*, tobacco, tomato, and petunia. Since cross-species hybridization should occur during this microarray analysis, we first compared the nucleotide sequence identity between *N. benthamiana* and hot pepper genes. Since the full genome sequences are not available in either species, we randomly selected 608 *N. benthamiana* cDNA sequences (>1-kb length) from the NCBI database. Then the hot pepper homologs of the selected *N. benthamiana* sequences were identified using BLASTN program in the hot pepper unigene database in Korea Research Institute of Bioscience and Biotechnology (http://genepool.kribb.re.kr/). The search resulted in 90 hot pepper unigene sequences of >1-kb length (Fig. 1). The nucleotide sequence identity between the *N. benthamiana* cDNA sequences and the hot pepper homologs was analyzed using LALIGN program (http://www.ch.embnet.org/software/LALIGN_form.html). The average sequence identity between the hot pepper and *N. benthamiana* sequences was 90%. Previously, it has been shown that cross-species hybridization occurs when genes have >70–80% sequence identity [15]. Supporting this, gene expression of oilseed rape plants (*Brassica oleracea*) was recently analyzed by using an *Arabidopsis* cDNA microarray [15]. In the study, using probes derived from mRNAs of oilseed rape, the Arabidopsis arrays provided reliable data with only a minor loss in sensitivity. Based on these results, the sequence identity between hot pepper and *N. benthamiana* genes appeared to be high enough to allow cross-species hybridizations in cDNA microarray analysis. Supporting that, the expression profile of some *N. benthamiana* genes analyzed by the hot pepper cDNA microarray was consistent with the expression pattern determined by using semiquantitative RT-PCR (data not shown).

Functional classification of selected genes in hot pepper cDNA microarray during PCD

The 4685 genes included in hot pepper microarray were classified into functional categories according to the MIPS *A. thaliana* database (http://mips.gsf.de/projects/plants/) (Fig. 2). 58.9% of the genes had known or predicted function and 19.8% had unknown function. The rest of the genes showed no homology to any known genes. The genes with known or predicted functions were further classified into the functional groups: transcription, protein kinases, protein phosphatases, ubiquitin-proteasome pathway, proteases, defense-related, oxidative stress, photosynthesis, protein folding, hormone signaling, housekeeping, development, metabolism, protein synthesis, DNA processing, and unclassified.

Comparative microarray analysis of PCD induced by two different means

To make probes, total RNA was extracted from leaf tissues undergoing PCD by VIGS of *NbPAF* (ΔNbPAF) or by HR, and from control leaves. cDNA was synthesized by incorporating the fluorescent dye, Cy3 and Cy5 into the control and PCD samples. For each treatment, arrays were replicated for three times and the results from the three arrays were averaged. To select more significant fold changes in expression, we employed a *t* statistic method to rank the cDNAs in order of *p* value. After ranking the cDNAs based on *p* value, the *p* value cutoff was used to obtain a list of cDNAs that show significantly different expression under the two PCD conditions. The more stringent cutoff would result in selection of fewer genes, but would reduce false discovery rates of differentially expressed genes. Due to the variability in each microarray result, a higher *p* value cutoff ($p < 0.01$) was required in this study to identify the genes with significantly different expression patterns.

Comparison of the microarray data between NbPAF depletion-, and HR-induced PCD identified total 247 genes that were differentially expressed under the two conditions. The number of genes identified as significantly up-, or down-regulated under each condition was compared by using a Venn diagram (Fig. 3). Interestingly, as shown in Table 1, the changes in expression of many genes under one PCD condition appeared to be opposite to the other type of PCD. No gene was found to be commonly down-regulated, while total five genes were up-regulated in both proteasome-mediated and HR-induced PCD (Fig. 3).

Genes exhibiting significant changes in expression during PCD were assigned to groups according to their putative function (Table 1). Most of those genes had functions related with protein degradation, oxidative stress, photosynthesis, hormone signaling, and others. Several transcription factors, protein kinases, protein phosphatases, and defense-related genes were identified as significantly up-regulated under one PCD condition but repressed in response to the other PCD. In the proteasome-mediated PCD, a transcription factor gene encoding no apical meristem (NAM)-like protein was strongly induced, while the expression of the genes encoding a MYB family transcription factor, a bZIP transcription factor, a Golden2-like transcription factor, and a zinc finger transcription factor was either down-regulated or remained unchanged. However, expression of those genes was highly stimulated during HR cell death (Table 1). Interestingly, several protein kinase and phosphatase genes were transcriptionally induced, but also with different expression patterns in the two types of PCD. It has been previously shown that many receptor-like protein kinase genes are induced in response to infection by avirulent pathogen, suggesting their association with HR signaling pathway [16]. In agreement with the results, a receptor-like protein kinase

Nb contig ID	Nb Length	Ca contig ID	Ca Length	HSP (High Scoring Sequence Pairs)	HSP in Nb (%)	HSP in Ca (%)	Identity (%)	E-value
nbcn2195	1872	cn1819	1876	1870	100	100	90	1.75E-03
nbcn1741	1133	cn3750	1058	1054	93	100	88	1.00E-03
nbcn685	1179	cn1391	1159	1119	95	97	86	0.00E+00
nbcn2680	1586	cn1848	1579	1507	95	95	92	1.80E+00
nbcn2334	1494	cn1997	1469	1397	94	95	93	0.00E+00
nbcn1023	1760	cn8312	1833	1728	98	94	90	5.00E-20
nbcn3241	1210	cn5527	1238	1158	96	94	93	0.00E+00
nbcn2141	1438	cn5612	1400	1286	89	92	89	0.00E+00
nbcn2935	1260	cn1265	1222	1099	87	90	89	5.00E-30
nbcn98	1042	cn2937	1020	898	86	88	90	3.00E-05
nbcn906	1217	cn1695	1138	992	82	87	87	1.50E-22
nbcn2130	1063	cn12432	1136	977	92	86	89	5.00E-37
nbcn905	1125	cn1695	1138	974	87	86	87	4.00E-20
nbcn1616	2080	cn2632	2193	1872	90	85	89	5.65E-01
nbcn1167	1390	cn1599	1451	1233	89	85	90	0.00E+00
nbcn1217	1335	cn9449	1222	1032	77	84	91	0.00E+00
nbcn1428	1153	cn2605	1099	926	80	84	91	0.00E+00
nbcn901	1361	cn5561	1386	1166	86	84	91	0.00E+00
nbcn966	1477	cn275	1602	1330	90	83	93	5.00E-06
nbcn1588	1731	cn8072	1788	1482	86	83	89	0.00E+00
nbcn843	1623	cn3536	1705	1411	87	83	91	5.00E-08
nbcn1463	1746	cn6063	1917	1583	91	83	91	2.00E-04
nbcn900	1299	cn5561	1386	1144	88	83	91	0.00E+00
nbcn2399	1411	cn2776	1539	1259	89	82	91	4.00E-08
nbcn2136	1418	cn1435	1538	1254	88	82	90	5.00E-23
nbcn992	1522	cn96	1595	1298	85	81	89	2.83E-02
nbcn3416	1072	cn1261	1113	905	84	81	92	1.48E-02
nbcn548	1378	cn253	1372	1110	81	81	92	0.00E+00
nbcn2247	1203	cn129	1258	1001	83	80	90	2.27E-02
nbcn2067	1145	cn3086	1088	861	75	79	89	0.00E+00
nbcn363	1279	cn5472	1367	1076	84	79	91	4.00E-14
nbcn995	1805	cn3602	1770	1386	77	78	94	1.35E-01
nbcn1378	1054	cn6020	1118	875	83	78	89	3.00E-08
nbcn1377	1085	cn6020	1118	874	81	78	90	3.57E-10
nbcn815	1111	cn2942	1209	932	84	77	92	0.00E+00
nbcn1418	1542	cn11108	1472	1132	73	77	91	1.67E-03
nbcn2711	1334	cn1853	1433	1097	82	77	92	5.00E-16
nbcn3417	1058	cn1261	1113	850	80	76	91	5.00E-31
nbcn1130	1261	cn3475	1294	988	78	76	88	3.50E-02
nbcn1636	1052	cn3213	1054	797	76	76	89	0.00E+00
nbcn118	1832	cn2736	1722	1298	71	75	89	0.00E+00
nbcn1579	1340	cn293	1417	1068	80	75	89	1.85E-02
nbcn70	1150	cn8242	1117	838	73	75	90	1.30E+00
nbcn3176	1543	cn1258	1619	1213	79	75	90	4.30E-02
nbcn416	1180	cn5327	1197	895	76	75	87	0.00E+00
nbcn1484	1122	cn154	1053	786	70	75	89	0.00E+00
nbcn1599	1035	cn3562	1071	795	77	74	89	1.33E-06
nbcn1721	1353	cn5208	1446	1069	79	74	91	1.50E-02
nbcn1073	1143	cn7385	1146	844	74	74	88	4.95E-01
nbcn1553	1008	cn2247	1100	805	80	73	93	7.00E-03
nbcn1479	1565	cn5476	1581	1146	73	72	85	0.00E+00
nbcn844	1298	cn8130	1352	977	75	72	90	0.00E+00
nbcn69	1033	cn8242	1117	805	78	72	89	5.00E-07
nbcn25	1041	cn4936	1050	754	72	72	89	1.20E+00
nbcn872	1205	cn5927	1319	939	78	71	90	0.00E+00
nbcn3377	1311	cn8468	1203	846	65	70	91	2.25E+00
nbcn3252	1087	cn5618	1105	775	71	70	89	3.00E-33
nbcn862	1019	cn4103	1027	720	71	70	88	2.00E-06
nbcn1661	1005	cn64	1092	765	76	70	88	0.00E+00
nbcn3233	1096	cn5618	1105	773	71	70	88	5.00E-37
nbcn347	1176	cn10092	1266	885	75	70	87	1.50E-56
nbcn1472	1017	cn10509	1041	720	71	69	87	2.00E-15
nbcn736	1069	cn1054	1035	714	67	69	94	4.75E-03
nbcn1738	1002	cn9025	1061	728	73	69	92	1.70E+00
nbcn2779	1174	cn680	1137	772	66	68	86	2.18E-02
nbcn3013	1212	cn8326	1323	889	73	67	87	3.00E-33
nbcn1405	1383	cn3353	1288	864	62	67	89	0.00E+00
nbcn2848	1247	cn1217	1255	832	67	66	93	2.30E-02
nbcn1968	1568	cn3273	1698	1097	70	65	89	4.50E-08
nbcn949	1084	cn259	1151	740	68	64	92	2.50E-27
nbcn3391	1105	cn2964	1079	691	63	64	88	8.00E-04
nbcn304	1275	cn14580	1346	859	67	64	87	1.00E-69
nbcn1799	1157	cn10546	1265	791	68	63	90	5.00E-01
nbcn3197	1105	cn7734	1174	730	66	62	92	0.00E+00
nbcn3164	1172	cn5509	1120	679	58	61	89	1.50E-10
nbcn3228	1211	cn636	1267	722	60	57	90	0.00E+00
nbcn920	1704	cn2068	1672	937	55	56	88	1.46E+00
nbcn1560	1212	cn7946	1294	717	59	55	91	2.75E-01
nbcn1246	1044	cn2864	1067	590	57	55	92	4.90E-02
nbcn1360	1106	cn228	1035	545	49	53	89	2.33E-08
nbcn3278	1348	cn6085	1309	663	49	51	87	1.63E+00
nbcn1697	1766	cn9365	1834	861	49	47	89	0.00E+00
nbcn3277	1381	cn6085	1309	545	39	42	86	1.00E-136

Fig. 1. Phylogenetic analysis. Six hundred and eight *N. benthamiana* cDNA sequences (>1-kb length) were randomly selected from the NCBI database, and their hot pepper homologs were identified in the hot pepper unigene database in Korea Research Institute of Bioscience and Biotechnology. The search resulted in 90 hot pepper unigene sequences of >1-kb length. The nucleotide sequence identity between 90 *N. benthamiana* cDNA sequences and their hot pepper homologs is shown.

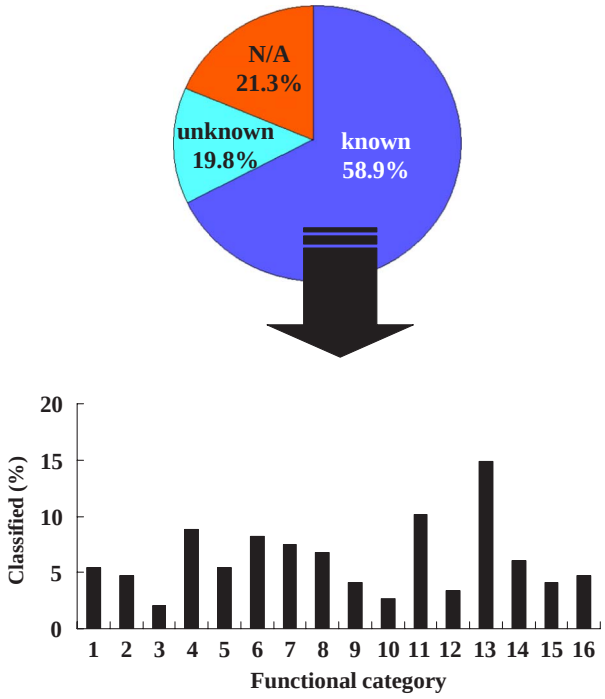

Fig. 2. Functional classification of hot pepper ESTs. Four thousand six hundred and eighty five hot pepper genes included in DNA chip are classified according to their putative function. The upper diagram represents the percentage of genes for known or predicted functions, unknown proteins, and that showed no homology to any known genes (N/A). The lower graph represents the distribution of genes among the hot pepper cDNAs with known or predicted functions. (1) Transcription; (2) protein kinases; (3) protein phosphatases; (4) ubiquitin-proteasome pathway; (5) proteases; (6) defense-related; (7) oxidative stress; (8) photosynthesis; (9) protein folding; (10) hormone signaling; (11) housekeeping; (12) development; (13) metabolism; (14) protein synthesis; (15) DNA processing; (16) others.

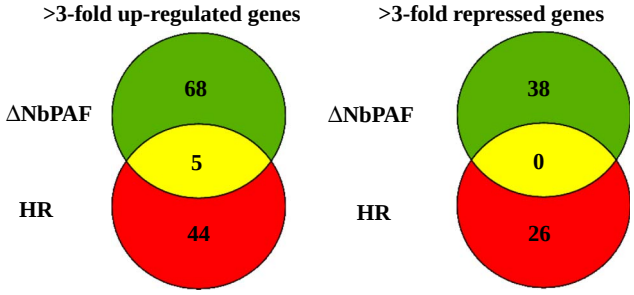

Fig. 3. Classification of the genes up-regulated or down-regulated during PCD induced by two different means based on Venn diagram. The number of genes classified in each region is indicated. The *t* test was used to identify the differentially expressed genes during each PCD condition.

was significantly up-regulated during HR cell death, but not during the proteasome-mediated PCD.

We also observed strong induction of defense-related genes that usually accumulate during resistance response. This group included PR-1, PR-4, disease resistance protein Bs2, defender against cell death 1 (DAD1), and beta-1,3-glucanase. Expression of *PR-1* and *PR-4* was up-regulated 3.7-, and 3.2-fold, respectively, during HR cell death, but not altered during the proteasome-mediated PCD. This finding is consistent with our previous gene expression analysis using semiquantitative RT-PCR [10]. In the study, VIGS of *NbPAF* caused accumulation of autofluorescent cell wall materials and callose, similar to the reaction caused by HR, but it stimulated the expression of only a subset of defense-related gene transcripts including *PR-5* and *Hin1*, but not *PR-1*, *PR-4*, and *PR-6*. These genes are all strongly induced during HR cell death. Moreover, the defender against cell death 1 (*DAD1*) gene, disruption of which has been shown to induce apoptosis in animal cells, was down-regulated during the proteasome-mediated PCD, but up-regulated during HR.

Transcript levels of the enzymes involved in oxidative stress were elevated in both types of PCD (Table 1). They included glutathione transferase, of which transcription is modulated in response to elevated H_2O_2 levels, and antioxidant enzymes such as catalase, several types of peroxidases, and oxidoreductase. We also observed up-regulation of several different protease genes including cysteine protease and serine protease genes, which were previously associated with developmentally regulated PCD, in both types of PCD (Table 1). These results indicate a possibility that oxidative stress-related proteins and proteases play an important role in the execution of all types of PCD in plants. This possibility is strengthened by the previous finding that antioxidant-related genes and cysteine proteases were up-regulated during PCD induced by both heat and senescence in *Arabidopsis* suspension cultures [17]. These genes may constitute general PCD regulators that underlie all PCD events in plants.

The genes related with ubiquitin-proteasome degradation pathway, which plays an important role in eliminating misfolded or aberrant proteins accumulated during stress, are up-regulated only in the proteasome-mediated PCD. These components included ubiquitin-specific protease UBP6, UBP12, ubiquitin-protein ligase, several catalytic subunits of the 20S proteasome, and AAA-type ATPase (Table 1). The ubiquitin-proteasome pathway is responsible for degradation of the majority of intracellular proteins in eukaryotes [18]. Recently, increasing evidence suggests that the ubiquitin-proteasome pathway plays an important role in apoptosis [19]. In plants, ubiquitin-proteasome-dependent protein degradation pathway has been related with heat stress, wounding, and senescence [19]. Additionally, transcription of the polyubiquitin genes and the ubiquitin extension protein gene was stimulated in response to mercuric chloride and dehydration, respectively [20]. Chilling has been shown to activate ubiquitin conjugation in *Clerodendrum speciosum* [21]. These results all indicate that there are increased demands for ubiquitin/proteasome-mediated proteolysis for plant cells to cope with diverse stresses. Our results in the microarray analysis suggest that ubiquitin/proteasome-dependent proteolysis may play a role in the defense response during the proteasome-mediated PCD.

In contrast to the up-regulation of the genes related with the ubiquitin-proteasome pathway, many genes encoding

Table 1
Gene expression profiles during cell death induced by NbPAF depletion and HR

Functional category	EST-ID	Description	Fold change	
			ΔNbPAF	HR
Transcription factor	KS01023H11	MYB family transcription factor	0.3	2.6
	KS01017A10	bZIP transcription factor	1.1	3.6
	KS01049E12	Golden2-like transcription factor	0.2	3.1
	KS01013E12	Zinc-finger transcription factor	0.2	3.7
	KS01060A05	NAM-like protein	8.2	1.1
Protein kinase	KS01028H03	Pyruvate kinase	4.6	0.3
	KS01051E06	ABA-inducible protein kinase	0.4	3.5
	KS01063B02	Receptor-like protein kinase	0.2	2.7
	KS01016D12	Phosphoglycerate kinase	1.1	3.2
Protein phosphatase	KS01054B01	Protein phosphatase 2C (PP2C)	3.4	1.1
	KS07007A03	Inorganic pyrophosphatase	0.6	3.4
Ubiquitin-proteasome pathway	KS07016B11	Ubiquitin-specific protease 6	5.1	0.6
	KS01043A12	Ubiquitin-specific protease 12	3.3	0.6
	KS01012B10	Ubiquitin-protein ligase	4.4	0.3
	KS01072E09	AAA-type ATPase	3.5	0.4
	KS01050C02	20S proteasome alpha subunit 3	4.1	0.1
	KS01058E04	20S proteasome beta subunit 2	3.3	0.2
	KS01041F05	20S proteasome beta subunit 5	3.3	0.3
Protease	KS01041G12	Cysteine protease	4.1	1.4
	KS07003B01	Serine protease	1.4	3.4
	KS01056H06	Cucumisin-like serine protease	5.7	1.6
	KS07008H03	Lipid transfer protein	4.7	1.8
Defense-related/cell death	KS01053A09	Pathogenesis-related protein-1 (PR-1)	1.2	3.2
	KS01007B02	Pathogenesis-related protein-4 (PR-4)	0.8	3.4
	KS01003D01	Disease resistance protein Bs2	4.9	0.8
	KS01056G02	Defender against cell death 1 (DAD1)	0.7	3.4
	KS01055F06	SGT1	3.4	0.9
	KS01038D06	Beta-1,3-glucanase	4.4	0.5
Oxidative stress	KS01057F06	Glutathione transferase	1.5	3.5
	KS01012E05	Catalase	3.1	3.2
	KS07015H02	Secretory peroxidase	1.9	4.9
	KS07012A09	Putative peroxidase	1.2	3.4
	KS01031B10	Oxidoreductase subunit	5.7	4.6
Photosynthesis	KS01050B02	Rubisco small subunit	0.1	2.8
	KS01030D08	PSI subunit IV precursor	0.1	3.4
	KS01049C10	PSI subunit XI precursor	0.2	2.3
	KS01012D01	PSII subunit X precursor	0.2	2.6
	KS01007F12	Chlorophyll a/b-binding protein	0.3	3.2
	KS01046G01	Chlorophyll a/b-binding protein 7	0.4	4.6
GA signaling	KS01011H04	Gibberellin-regulated protein	1.2	1.1
ABA signaling	KS01040B03	ABA-responsive protein	0.2	2.3
Housekeeping	KS01049E09	Pectin esterase	0.4	4.1
	KS08016E11	Pectin esterase-like protein	0.3	3.7
	KS01065C08	Cytochrome P450	0.6	3.5
	KS01043G07	Alpha-tubulin	1.4	4.7
	KS01048D08	Xyloglucan endotransglycosylase	0.3	2.9
	KS01006A11	Glycine dehydrogenase	0.2	3.5
	KS01060F06	RNA binding protein	3.2	0.8
	KS01039E01	Mitochondrial formate dehydrogenase	16.4	0.3
	KS01044A11	Heat shock protein 70	4.1	0.4
	KS01034E02	Heat shock protein 80	2.8	0.2
Development	KS01029E03	Cytochrome B-6	0.2	2.1
	KS07002E07	Thiazole biosynthetic enzyme	1.3	3.6
	KS07008H03	Nonspecific lipid transfer protein	5.7	3.0
Carbohydrate metabolism	KS01056A11	Beta amylase	3.8	0.4
	KS08013A07	Phosphoglucomutase	0.2	2.9

(*continued on next page*)

Table 1 (*continued*)

Functional category	EST-ID	Description	Fold change	
			ΔNbPAF	HR
	KS01057F05	Glyceraldehyde 3-phosphate dehydrogenase	5.1	0.1
	KS01012A04	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	0.3	3.3
	KS01057B09	Sugar epimerase	0.7	3.2
	KS01006A02	Phosphoenolpyruvate carboxylase	0.2	2.5
Protein synthesis	KS07009G10	40S Ribosomal protein S31	0.2	2.9
	KS01004B11	50S Ribosomal protein L12	0.3	2.6
	KS07001D03	Translation elongation factor EF-G	0.5	2.9
	KS01064G07	Phosphoenolpyruvate carboxykinase	4.7	1.6
Cellular metabolism	KS01049D01	Protochlorophyllide reductase	0.3	4.9
	KS01042A10	Endochitinase precursor	3.6	1.2
Cell cycle/DNA processing	KS08007C08	Cell division cycle 48 protein	3.4	0.8
	KS01040A04	Histone deacetylase	0.2	2.9
	KS01060D02	Prohibitin	2.8	0.2
	KS01005C03	Histone H2B	2.6	0.7
Others	KS01032H06	Hypothetical protein	3.2	0.2
	KS01057H07	Hypothetical protein	3.7	0.3
	KS01008F12	Unknown protein	3.1	0.3
	KS01047A12	Hypothetical protein	3.2	0.4
	KS01017H02	Unknown protein	3.7	0.4
	KS01040D09	Unknown protein	9.8	9.1
	KS01057A12	Unknown protein	5.6	8.9
	KS07001F04	Unknown protein	0.2	7.5
	KS01061E08	Unknown protein	0.3	5.2

photosynthesis-related proteins, such as Rubisco small subunit, several photosystem subunits, and chlorophyll a/b-binding proteins, were significantly down-regulated during the proteasome-mediated PCD, but up-regulated during HR cell death. This finding is not surprising, given that the VIGS phenotype of *NbPAF* includes chlorosis in leaves. Regarding hormone-related genes, expression of the gene encoding a gibberellin-regulated protein remained unchanged, whereas the gene encoding an ABA-responsive protein showed the opposite expression pattern between the two types of PCD (Table 1). Particularly, HR cell death positively regulated gene expression of cell wall modifying enzymes, such as pectin esterase and xyloglucan endotransglycosylase, which have not been previously associated with HR. Moreover, the genes related with development, carbohydrate metabolism, protein synthesis, cellular metabolism, and cell cycle/DNA processing were also differentially regulated between the two PCD pathways (Table 1). Finally, we observed differential expression of some housekeeping genes that are involved in basic cellular activities.

Taken together, the microarray analysis in this study showed global changes in transcription patterns during two different PCD induced by proteasome malfunction and HR in plants. We found that gene expression profiles of these two pathways were significantly different from each other. Many genes up-regulated during proteasome-mediated PCD were down-regulated during HR cell death, and in turn, the majority of the genes induced during HR were repressed during proteasome-mediated PCD. These results indicate that there are functional differences between the PCD pathways activated by proteasome malfunction and HR, likely involving distinct downstream components. Overall, only a small number of genes were commonly induced during both types of PCD, including oxidative stress-related genes, protease genes, and several unknown genes. These genes may function as a core regulator of plant PCD. Particularly, the genes with unknown function (KS01040D09 and KS01057A12; Table 1), which were highly up-regulated under both PCD conditions, were identified in this study as a potential cell death marker.

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