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

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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.

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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) ($1 \times 10^8$ 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