Gene Expression Profiles during the Hydrogen Peroxide Stress in Chinese Cabbage

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Objectives

Oxidative stress occurs when cellular redox homeostasis is altered. This imbalance may be due to either an excess of reactive oxygen species (ROS) or a deficiency in antioxidant systems (Morel et al. 1999). Slight alteration in the homeostatic set point of intracellular ROS has been shown to modulate cell metabolism, gene expression, and post-translational modification of proteins. On the other hand, when the level of ROS exceeds the cellular antioxidant capacity, ROS become detrimental and cause oxidative injuries. Therefore, cells have developed complex mechanisms to maintain redox homeostasis and/or to cope with the overproduction of ROS during oxidative stress (Arrigo et al. 1999). Of the ROS, H_2O_2, a well-known product of oxidative stress, plays multiple roles in plant physiology. Recent studies indicate that H_2O_2 may act as a secondary messenger to mediate cross-tolerance in response to various stresses in plants. Identification of the genes and proteins regulated by H_2O_2 is of great importance because H_2O_2 is thought to be a common factor regulating various signaling pathways.

In this study, we carried out a transcriptomic analysis of oxidative stress-regulated genes in Chinese cabbage using cDNA microarray technology. The technology enables measurement of expression of thousands of genes to identify changes in expression between different biological states. After 10 mM H_2O_2 stress at different time courses, we observed transcript expression patterns and conducted RNA-blot analyses on selected genes to verify the microarray data.

Materials and Methods

cDNA Clones and Microarray Preparation

A total of 3,058 unique expressed sequence tags (ESTs) were selected for microarray spotting from 6,000 Chinese cabbage ESTs present in the Chinese cabbage database (Genbank) (http://gcc.gsnu.ac.kr). An additional 208 clones, which served as positive, negative and non-specific hybridization check controls (LifeGrid Yeast Control 100-800 from IncyteGenomics and Human ESTs contributed by the Bank of Human Stromal of Cell cDNA in Kyungpook National University, Korea) were added to the set. Each of the 3,264 ESTs was amplified by the polymerase chain reaction (PCR) in 100 μL reaction volumes. At the end of each reaction, 5 μL of each product was electrophoresed on agarose gels to confirm amplification quality and quantity. To clean up PCR products and prepare the cDNA for printing, PCR products were precipitated in ethanol and resuspended in 14 μL of spotting solution. PCR products were arrayed onto CMT-GAPS amino silane coated slides by using a homemade microarrayer, equipped with 16 pins. After printing, PCR products were immobilized by UV cross-linking and baked at 80℃ for 2 hr. Non-bound DNA was removed with 0.2% SDS and double-distilled H_2O (ddH_2O); covalently bound DNA was denatured for 2 min in boiling water. Free aldehydes were reduced by soaking slides for 5 min in 68 mM sodium borohydride (dissolved in PBS containing 25% ethanol). Several washing steps were performed with 0.2% SDS and ddH_2O; slides were then dried by centrifugation at 800 rpm for 5 min and stored at room temperature for further hybridizations.

H_2O_2 Treatment and Total RNA Isolation

Imbibed Chinese cabbage seeds (maintained at 4℃ for seven days) were grown in soil for ten days in a growth chamber (22℃, 16 hr photoperiod). After the first leaves appeared, plants were incubated with a solution of 10 mM H_2O_2 in growth bottles for 30 min, 1 hr, 3 hr, 6 hr, 12 hr and 24 hr; reference plants were incubated with distilled water for 24 hr. Total RNA was extracted with 1:1 (v/v) extraction buffer: phenol solution, followed by two to three washes with chloroform and overnight precipitation with

an equal volume of ice-cold 6 M LiCl. Total RNA was sometimes further purified by CsCl centrifugation (sambrook et al. 2001). The RNA pellet was resuspended in RNase free-$H_2O$and precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol. The pellet was rinsed with 70% ethanol, air dried, and resuspended in RNase free-H_2O.

Preparation of Fluorescent Probes

mRNA from human and yeast clones was obtained following *in vitro* transcription. Total RNA from Chinese cabbage, yeast and human was mixed and reverse-transcribed with Cy3-dUTP or Cy5-dUTP. Each reaction was performed in a 40 μL volume containing 60 μg of total RNA, 5 × first strand buffer, 0.1 M DTT, ribonuclease inhibitors, dNTPs (10 mM each except for dTTP at 2.5 mM), and superscript reverse transcriptase. After incubation at 42°C for 1.5 hr, 5 μL of 0.5 M EDTA and 10 μL of 1 N NaOH were added to the labeled samples and then incubated for 10 min at 65°C to degrade the RNA. The probes were purified by passage through a Millipore YM-100 (Volketswil) and then precipitated with ethanol. The pellet was air dried, and resuspended in 10 μL of hybridization solution.

Hybridizations and Data Analysis

Hybridizations were performed at 62°C for 16 hr in a humidified chamber. Microarrays were washed twice for 40 min in wash solution (5 × SSC/0.05% SDS) at 62°C, rinsed in 0.05 × SSC for 5 min at room temperature and dried by centrifugation at 800 rpm for 5 min. Microarrays were scanned with a scanning laser microscope (ScanArray Lite; GSI Lumonics). Hybridizations were repeated two to four times at each time point (30 min, 2; 1 hr, 2; 3 hr, 4; 6 hr, 2; 12 hr, 2; and 24 hr, 3). To normalize the two channels with respect to signal intensity, we adjusted the photomultiplier and laser power settings such that the signal ratio of the human, yeast, and internal controls were as close to 1.0 as possible. Local background was subtracted from the value of each spot on the array. Normalization between the Cy3 and Cy5 fluorescent dye intensities was achieved by global normalization (Hardwick et al. 1999). Genes showing a signal value < 1000 (which was typically twice the mean background value) in both the Cy3 and Cy5 channels were not considered for the analyses. The results were analyzed with a QuantAarray program (GSI Lumonics), hierarchical clustering (Eisen et al., 1998), and self-organizing maps (SOMs, Tomayo et al. 1999).

RNA Gel Blot Analysis

Each 15 μg of total RNA was electrophoresed in a formaldehyde-containing 1.5% agarose gel and transferred to a nylon membrane (Hybond N+; Amersham). Hybridizations were performed at 65°C for 16 hr with randomly primed ^{32}P-labeled cDNA probes in church buffer (1% BSA, 1 mM EDTA, 7% SDS, and 0.5 M phosphate buffer). Membranes were washed twice in 2 x SSC, once in 0.1 x SSC/0.1% SDS at 60°C and exposed to X-ray films at -80°C (Sambrook et al., 2001).

Results and Discussion

To obtain a global picture of gene expression during oxidative stress, we constructed a cDNA microarray (BC-EST ver. 1.01) containing 3,058 ESTs from cDNA libraries of Chinese cabbage. Using the BC-EST ver. 1.01, we compared the intensities of Cy3- and Cy5-labeled targets to normalize variability (Figure 1). In this control, 97.7% of the spots were located within (± 2 log-2 expression ratios (LR), providing a measure of experimental and systematic errors. H_2O treated 24 hr RNA hybridized against another H_2O treated RNA showed profiles very similar to those of control versus control RNA, indicating that diurnal changes had little impact.

Altered regulation of gene expression after a 10 mM H_2O_2 shock was detected by comparing probes from control times to probes at six RNA collection times (30 min, 1 hr, 3 hr, 6 hr, 12 hr, and 24 hr) after the imposition of stress. The expression ratios of all transcripts (log2 Cy5/Cy3) from the different time point experiments were plotted against the printed order of the ESTs (X-axis) on the microarray (Figure 2). After 30 min and 1 hr of H_2O_2 stress, about 17.5% and 16.9% of the transcripts were up- or down- regulated more than 2-fold, respectively. After 3 and 6 hr, alteration of all transcripts decreased to about 7.3% and 9.9%, respectively. However, after 12 hr, transcription increased again to 15.3% before decreasing to 7.5% at the 24 hr time point.

The expression profiles from the different time course microarray hybridizations were categorized into several patterns using hierarchical clustering analysis (Eisen et al. 1998). Results of hierarchical clustering demonstrated that all of the 53 transcripts fell within two patterns (Figure 3). Thirty clones could be categorized into a pattern that showed increased transcription at -3 hr followed thereafter by a gradual loss of this induced level of transcription. The MYB transcription factor, calmodulin, the WRKY DNA-binding protein, and the Cys-proteinase inhibitor all occurred within this pattern. Another 23 clones displayed a pattern

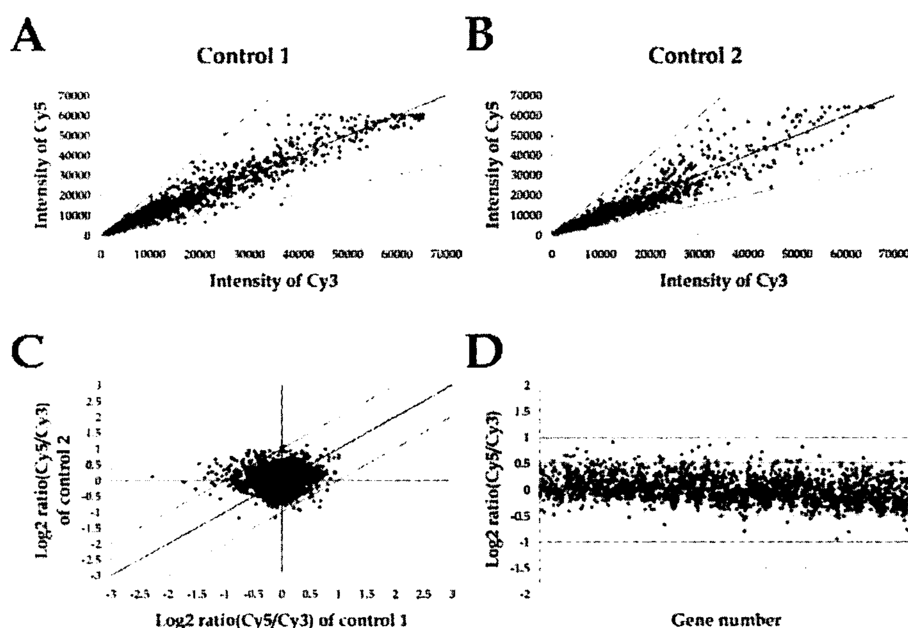

Figure 1. Comparison of expression ratios in replication experiments and application of the four-step data mining procedure. (A) Scatter plot of microarray hybridization with labeled cDNA probes obtained from total RNA of 24 hr H_2O-treated Chinese cabbages (control sample). (B) Scatter plot of replication microarray hybridization using total RNA of control samples from different samplings. About 0.3 to 0.8% of all transcripts displayed greater than 2-fold altered expression levels. (C) Variation of expression ratios between replication experiments. The Log2 values of the Cy5/Cy3 ratios were plotted for the two replicates. About 2.3% of the transcript levels differed by more than 2-fold. (D) Expression ratios of transcripts after application of the four-step data mining procedure. All transcripts were located within a 2-fold range. Expression ratios (log2 Cy5/Cy3) are plotted against the order of ESTs printed on the microarray. The red lines represent 2.0-fold induction/repression ratio cutoffs.

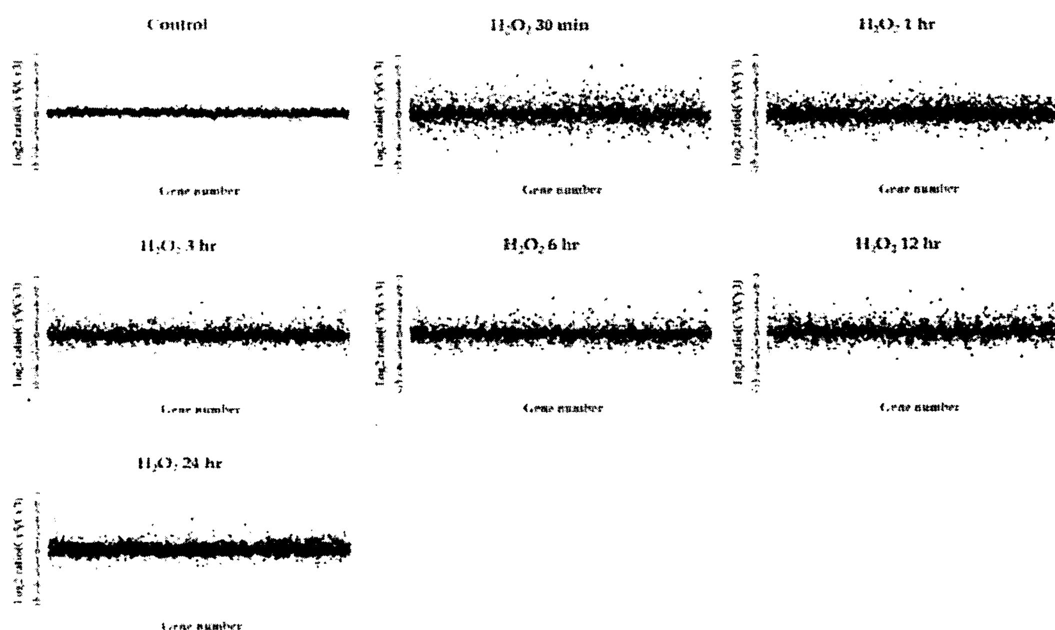

Figure 2. Expression of transcripts at different time courses after a 10 mM H_2O_2 treatment of Chinese cabbage. The expression ratios (log2 Cy5/Cy3) of all transcripts following the four-step data mining procedure for the different time course hybridizations (30 min, 1 hr, 3 hr, 6 hr, 12 hr, and 24 hr) are plotted against the order of ESTs printed on the microarray. The transcripts show 2-fold greater expression at the 30 min, 1 hr, and 12 hr time points.

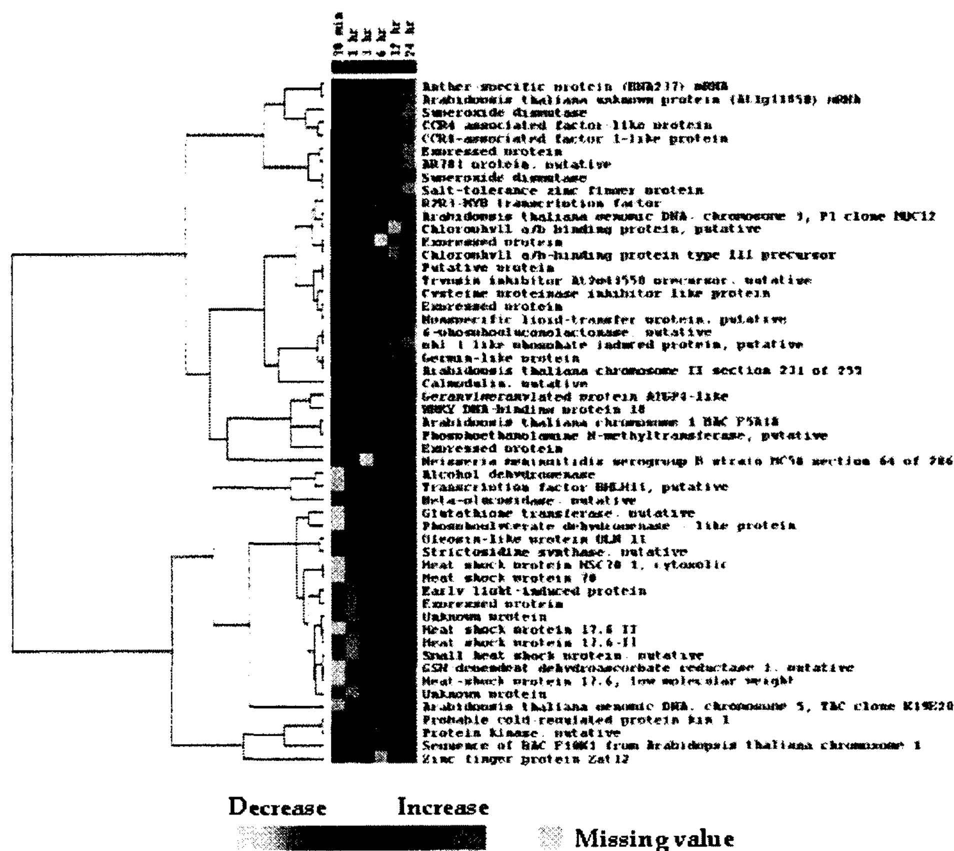

Figure 3. Clustergram of gene expression profiles during H_2O_2 stress. Gene expression profiles are shown as: red, transcriptional up-regulation; green, down-regulation; gray, missing value. The cluster tree illustrates the node of co-regulation of gene expression over all time courses.

opposite to that of the first pattern; transcription was reduced at ~ 3 hr compared to earlier times and was then followed by a period of increased transcription. Proteins categorized in this pattern include the heat shock protein, protein kinase, the zinc finger protein Zat 12, and the GSH-dependent dehydroascorbate reductase 1.

To obtain more information on the expression patterns, we performed self-organizing maps (SOMs) analysis (Tomayo et al., 1999). One hundred and seventy two of the transcripts were categorized into 16 expression groups (Figure 4). Cluster 4, comprising 13 clones, showed rapid induction of a number of genes, including the ethylene-responsive element binding factor (EREBP), calmodulin, the WRKY DNA-binding protein, nitrate reductase, and the salt-tolerance zinc finger protein. Cluster 16, containing 12 clones, showed gradually increased transcription of genes such as the bZIP transcription factor, the Bax inhibitor-like protein, ascorbate peroxidase, and phenylalanine ammonia-lyase. From the SOMs analysis, 172 ESTs were classified according to potential functions (based on the BLAST homology) to determine whether H_2O_2 regulates the differential expression of particular classes of genes. This functional categorization was performed according to the functional organization of the *Arabidopsis* genome (http://mips.gsf.de/proj/thal/db/index.html). The broad spectrum of gene functions depicted in Figure 5 is similar to that predicted for the Chinese cabbage EST functional distribution. However, as might be expected, the representation of ESTs involved in cell rescue & defense responses is increased.

In this microarray experiment, various ESTs encoding transcription factors were induced by H_2O_2. For example, EREBP, the salt-tolerance zinc finger protein, the WRKY DNA-binding protein 18, the transcription factor bHLH 11, the zinc finger protein Zat 12, the dehydration-responsive element binding protein 2A (DREB2A), heat shock transcription factor (HSF 4), and the bZIP transcription factor were induced by H_2O_2. The subsequent expression of additional genes is likely to occur at later time points. When cDNA microarray data and RNA gel blot analysis of selected H_2O_2-induced or -repressed transcripts were compared, a similar trend was observed (Figure 6).

The microarray analysis identified a number H_2O_2-regulated ESTs that are of potential importance in diverse stress responses. Particularly, induction or repression of various stress-related genes supports the hypothesis that H_2O_2 mediates cross-tolerance. Although this study was restricted to an analysis of ESTs that are responsive to H_2O_2, it identified genes that could be used as a basis for further studies using gene-specific sequences to analyze the

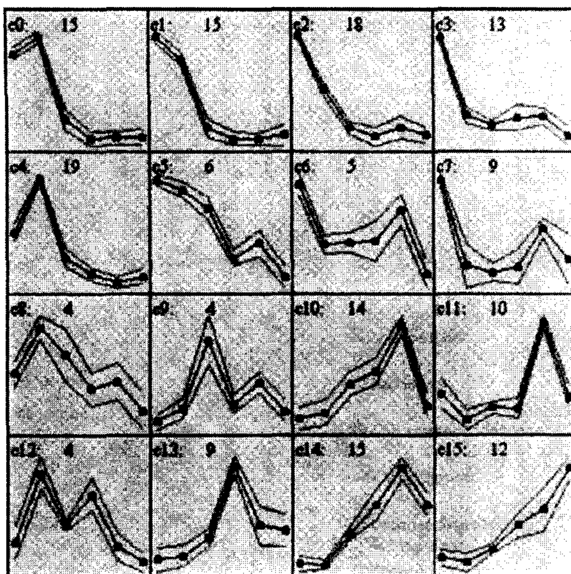

Figure 4. Self-organizing map clusters of expression profiles. One hundred and seventy two of the transcripts were categorized into 16 expression patterns. Blue lines indicate the mean expression profiles of the ESTs in the cluster. Red lines indicate standard deviation of average expression. The number in the top center of each box indicates how many genes are in each cluster, whereas the label in the upper left corner of each cluster indicates the cluster number. The x and y axis represent the time points and normalized gene expression levels (Mean = 0, SD = 1), respectively.

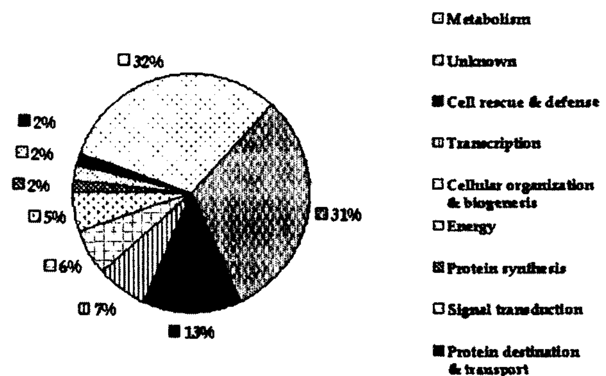

Figure 5. Functional distribution of oxidative stress-induced genes. The top BLASTX hits of the 172 ESTs were classified according to the functional organization of the *Arabidospis* genome.

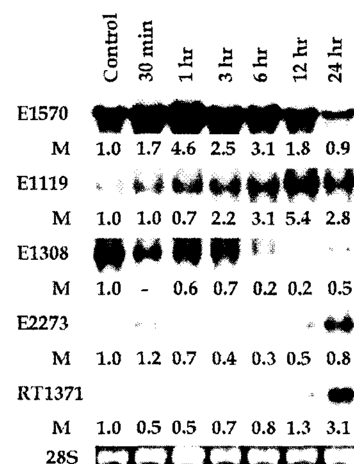

Figure 6. Comparison of RNA gel blot analysis and cDNA microarray data. Chinese cabbage seedlings were treated with H_2O_2 and total RNA was isolated at the indicated times and analyzed for S-adenosylmethionine synthetase (E1570), no match (E1119), hypothetical protein (E1308), catalase (E2273), EST (RT1371) and rDNA (28S). M indicates the ratio of each microarray analysis.

expression and function of genes that are sensitive to H_2O_2. Traditionally, the impairment caused by increased ROS is thought to result from random damage to proteins, lipids and DNA (Finkel et al., 2000). In this respect, ROS may be closely correlated with cell death. Thus, we hope to identify H_2O_2-related genes that suppress or rapidly advance Bax-induced lethality by obtaining from yeast DNA microarrays. Plant cells may contain redox sensors that detect and respond to signals such as H_2O_2. The information obtained from the analysis of the effects of H_2O_2 on gene expression will advance understanding of the complex mechanisms underlying gene regulation during various stresses.

Acknowledgment

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