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Temporal evolution of the *Arabidopsis* oxidative stress response

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Abstract

We have carried out a detailed analysis of the changes in gene expression levels in *Arabidopsis thaliana* ecotype Columbia (Col-0) plants during and for 6 h after exposure to ozone (O_3) at 350 parts per billion (ppb) for 6 h. This O_3 exposure is sufficient to induce a marked transcriptional response and an oxidative burst, but not to cause substantial tissue damage in Col-0 wild-type plants and is within the range encountered in some major metropolitan areas. We have developed analytical and visualization tools to automate the identification of expression profile groups with common gene ontology (GO) annotations based on the sub-cellular localization and function of the proteins encoded by the genes, as well as to automate promoter analysis for such gene groups. We describe application of these methods to identify stress-induced genes whose transcript abundance is likely to be controlled by common regulatory mechanisms and summarized our findings in a temporal model of the stress response.

Introduction

Plants react to abiotic and biotic challenges from the environment with rapid biochemical and molecular changes that trigger physiological adaptations and programmed cell death (Lamb and Dixon, 1994; Greenberg, 1996; Shirasu *et al*., 1996; Lamb and Dixon, 1997). This complex of biochemical, genetic and structural changes is termed the stress- or defense response, depending on whether the inciting agent is abiotic or biotic. The plant stress/defense response includes the production of reactive oxygen species (ROS), the biosynthesis of antimicrobial compounds, cell wall proteins, antioxidants, and signaling molecules, as well as elaboration of the apoptotic hypersensitive response (HR) and the development of systemic acquired resistance (SAR) (Lamb and Dixon, 1994; Baker and Orlandi, 1995; Baker *et al*., 1997; Lamb and Dixon, 1997; Baker and Orlandi, 1999). Ozone (O_3), a component of photochemical smog, represents an oxidative stress to living organisms and is a major atmospheric pollutant, damaging crops and forests (Runeckles and Chevonne, 1992). The plant's response to this oxidative stressor resembles components of the pathogen defense response, including the production of ROS and induction of the HR and SAR (Conklin and Last, 1995; Sharma *et al*., 1996; Sharma and Davis, 1997; Sandermann *et al*., 1998; Rao *et al*., 2000; Sandermann, 2000). Expression of ozone-responsive genes is modulated by inter-

actions among the second messenger salicylic acid and the plant hormones jasmonic acid and ethylene (Rao *et al*., 2000; Tamaoki *et al*., 2003; Tuominen *et al*., 2004).

While it is known that genes are expressed differentially during stress responses and are activated in response to different signals (Bowler and Fluhr, 2000; Overmyer *et al*., 2000; Desikan *et al*., 2001; Moeder *et al*., 2002; Vandenabeele *et al*., 2003, 2004), how the genetic response evolves in time is poorly understood and we know little about how changes in transcript and protein levels are interconnected and regulated. To gain a better understanding of the temporal evolution of the genetic response to abiotic stress, we carried out microarray expression profiling of *Arabidopsis* plants over a 12-h period commencing from the onset of a 6-h exposure to ozone. We used cDNA microarrays comprising 1391 stress-induced genes to assess the changes in transcript levels in four-week-old *Arabidopsis* plants exposed to 350 ppb O_3. We developed analysis and visualization tools to identify genes likely to be controlled by common underlying regulatory mechanisms in response to O_3 exposure. We present a model for the temporal evolution of the oxidative stress response.

Materials and methods

Plant growth conditions and treatments

Arabidopsis thaliana Col-0 plants were grown in soil (Scotts-Sierra Horticultural Products Company, Marysville, OH) in 5 cm pots (50 per flat) under fluorescent light with a 10-h photoperiod for 4 weeks. Plants were transferred to growth chambers for O_3 fumigation (with clean air control plants transferred to an adjacent chamber under identical conditions except for the O_3 treatment). For acute O_3 exposure, plants were transferred to the ozone chambers at 4 weeks after germination, and treated with 0.35–0.4 μl l^{-1} O_3 for 6 h. At the end of 6 h, the ozone generator was turned off and the growth chamber doors were opened for 5 min to let the remaining ozone out. Leaf samples were harvested during (0.5, 1, 1.5, 3, 4.5 h) and after (6, 9 and 12 h) exposure to O_3. Leaves (4–5/plant) were harvested from 5–6 plants avoiding the most immature leaves, cotyledons, and the first 1–2 true leaves. Leaves from control plants not exposed to O_3 were collected for each time-point to avoid confusion arising from diurnal rhythms in gene expression. There were three biological replicates of the O_3 time course and there were two technical replicates in each experiment. The efficacy of the treatment was checked using a cDNA clone (At1g56060) that represents a gene of unknown function whose transcript level increases rapidly to a very high level after O_3 treatment. The consistency of O_3 treatment was monitored by testing expression of this gene for each biological replicate before proceeding with microarray experiments (Figure 3).

RNA isolation and Northern blot hybridizations

Harvested leaves were flash-frozen in liquid N_2 immediately after removal from the plants and the tissue was stored at −80C. For microarray hybridizations, total RNA was isolated using RNEasy plant RNA isolation kit (Qiagen, Valencia, USA). Ten micrograms of total RNA from control and O_3-treated leaf tissue was fractionated on a 1.2% agarose/0.4 M formaldehyde RNA gel and transferred to Hybond N^+ nylon membrane (Amersham-Pharmacia, Buckinghamshire, England). Membranes were stained with methylene blue to visualize the rRNA to confirm equal loading. Probes were made from PCR-amplified fragments of SSH clones (Mahalingam *et al*., 2003) using the ReadyPrime DNA labeling kit (Amersham-Pharmacia, Buckinghamshire, England) with [α−^{32}P]dCTP (ICN Biomedicals Inc., Irvine, USA). Blots were hybridized and washed according to standard procedures (Sambrook *et al*., 1989).

Preparation of cDNA microarrays

Silanized glass slides were prepared following a procedure adapted from Beier and Hoheisel (1999). Glass microscope slides (Goldseal) placed in glass racks were soaked overnight in 10% NaOH on a shaking platform, rinsed thoroughly with nanopure H_2O, sonicated in H_2O for 15 min, soaked 15 min in 1% HCl, thoroughly rinsed and sonicated again in H_2O for 15 min. The cleaned slides were soaked in 5% triethoxysilane in methanol (Sigma–Aldrich, Saint Louis, USA) for

15 min on a shaking platform, followed by 5 min shaking in 100% methanol, spun on racks in a centrifuge at 500 rpm for 5 min, dried under a stream of N_2, and baked at 110° C for 20 min.

The derivation of the 1058 cDNAs used to prepare our stress microarrays was previously described (Mahalingam *et al.*, 2003). We also purchased an *Arabidopsis* EST collection containing 12, 000 clones from Michigan State University and selectively amplified 355 clones from this collection based on a literature survey for previously identified stress-responsive genes. The 1472 stress-associated cDNAs, together with 10 negative controls (AFGC control kit) and three spiking controls were spotted on silanized glass slides using a GeneMachines spotting robot in the PSU DNA microarray facility supplementary table 2. Clones from subtracted libraries prepared as previously described (Mahalingam *et al.*, 2003) were amplified using the nested primers and the clones from the EST collection were amplified using M13 primers in 50 μl reactions containing 1 μl bacterial culture, 0.5 μM each primer, 0.2 mM dNTPs, 1× REDTaq buffer, and 2.5 units of REDTaq DNA polymerase (Sigma–Aldrich, Saint Louis, USA). Products (2.5 μl) were visualized on 1.5% agarose gels to ensure amplification. Samples were purified using ArrayIt 96-well PCR purification filters (Telechem International), dried down, resuspended to a final concentration of 0.2–0.4 μg/μl in 5–10 μl 3 × SSC. The top six rows in each block of 12 rows and 16 columns were identical to the bottom six rows. Protocols for the slide processing, pre-hybridization and hybridizations are available at http://www.lsc.psu.edu/stf/dnama/home.html.

Preparation of spiking controls

Three 'spiking control' mRNAs were added to each RNA preparation prior to labeling. cDNAs corresponding to genes coding for the spiking control mRNAs were spotted 98 times (>30 spots/spike) and these spots were randomly distributed in different blocks throughout the array. The three spiking control genes were bacterial diaminopimelate decarboxylase (lysA) obtained from ATCC (GenBank Accession 090189), ATCC (87482), and two human I.M.A.G.E. clones obtained from Research Genetics, Inc., mysoin light chain (mlc, GenBank Accession AA962056), IMAGE ID (1592600), and insulin-like growth factor (igf, GenBank Accession AA870797), IMAGE ID (1576490).

PCR primers were made to amplify a 500-bp fragment of each of these sequences as follows: 5′-GGAGAAATATGGTACACC-3′ (lysA forward), 5′-TACTTGTTCAATGGCCCG-3′ (lysA reverse), 5′-TCGGGCCGAACAGAAGCG-3′ (mlc forward), 5′-GCACCCCAGGCTTTACAC-3′ (mlc reverse), 5′-TGGGATTGCAAGCGTTAC-3′ (igf forward), 5′-GCCATTCGGAACATTGGA-3′ (igf reverse). These primer sets were used to amplify the corresponding cDNA for printing microarray slides in order to avoid the use of vector-specific primers that include a polyA stretch that might interact with oligo-(dT)-primed fluorescent probe cDNAs.

A second set of PCR primers was prepared using the same sequences described above, but also including the T7 promoter sequence 5′-TAATA CGACTCACTATAGGGC-3′ at the 5′ end of the forward primer and an oligo(dT)22 at the 5′ end of the reverse primer. PCR products amplified from these primers were made RNAse-free and used as a template for polyA$^+$-RNA synthesis using the MEGAScript T7 RNA synthesis kit (Ambion Inc., Austin, USA), which yielded 70–100 μg RNA from 1 μg DNA template. Following RNA synthesis, RNA samples were extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) pH 4.5, once with chloroform:isoamyl alcohol (24:1), ethanol precipitated, and RNA pellets resuspended in RNAse-free water.

Probe preparation and microarray hybridization

Probes for microarray hybridizations were prepared using a two-step labeling process by reverse transcription of total RNA in the presence of amino-allyl-dUTP (Sigma–Aldrich, Saint Louis, USA) using Superscript II reverse transcriptase, followed by linking of amino-allyl-labeled cDNA with NHS-ester Cy-3 or Cy-5, as described at http://www.lsc.psu.edu/stf/dnama/home.html. Following dye conjugation and quenching reactions, Cy-3- and Cy-5-labeled probes were mixed together, purified on Qiagen PCR purification columns, eluted with 50 μl H_2O, dried by vacuum centrifugation, and resuspended in 15 μl of hybridization buffer consisting of 50% formamide, 5 × SSC, 10% Denhardt's solution, 10% salmon sperm DNA and 5% SDS. Probes were placed in boiling

H_2O for 2 min, allowed to cool to room temperature for 5–10 min, pipetted onto a processed microarray covered with a clean glass lifter slip (Erie Scientific Company, Portsmouth, NH). Microarrays were incubated in the dark at 42° C for 16–18 h in hybridization chambers (Corning, Pittsburgh, USA). Arrays were washed at room temperature for 3 min each in 0.1% SDS/2× SSC, two minutes in 1× SSC, 1 min in 0.2× SSC, and 15 s in 0.05× SSC, centrifuged in a glass rack for 5 min at 500 rpm and scanned immediately in a Axon confocal laser scanner. The resulting images were analyzed using GenePix Microarray analysis software (Axon Instruments, Union City, USA). Microarray hybridizations for the entire time course for each of the experiments were conducted on a single day to minimize variability.

Microarray data analysis

Stringent quality control measures were applied to determine the set of spots to be used for further statistical analysis. Spots with abnormal shapes, fused spots and spots with high local background were flagged manually. Spots with channel intensity values lower than mean background plus two standard deviations were flagged, as were spots that failed to show more than 80% mean–median correlation between the technical replicates of the spot. Two additional filters were applied at the whole time-course level. Genes that were flagged for all the time-points in one of the technical replicates were not considered for further analysis. If more than 12 of the 16 data points were flagged for a gene in the two technical replicates, such genes were also excluded from further analysis. Spiking control normalization using the three different mRNAs spiking controls was used to balance the channel intensities. Normalized, $\log_2$-transformed data were used to identify genes whose transcript levels showed significant changes in abundance in response to O_3 exposure of plants. In order to identify genes whose transcript abundance had changed significantly, we selected all genes for which the expression ratio of experimental to control values differed from the mean by 2 standard deviation units at one or more time points, calculating Z-scores using the formula: $Z = (X - \mu)/\sigma$, where X is the expression ratio for a gene, μ is the mean expression at that time point and σ is the standard deviation. This approach identifies genes in the tails of the ratio distribution for which the transcript level differences were the largest and least likely to occur by chance alone. For a normal distribution, a Z-score of 2 has the confidence level of 0.02, minimizing the number of false positives. We also applied a more conventional filter that identified genes whose transcript levels differed from control values by at least 2-fold after treatment.

To use K-means clustering for data analysis, we first determined the optimal number of clusters (K) in two different ways. We performed a stability analysis for values of K from 2 to 8 (Ben-Hur *et al.*, 2002). A set of 100 sub-samples of the data were created for each value of K by resampling 500 genes with replacement, followed by application of K-means clustering. A 'matching index' for these 100 partitions was then computed by dividing the number of gene-pairs that stay together in one cluster during subsequent partitions by the total number of gene pairs. The value of K for which the average matching index is highest or is distributed tightly around one is the optimal K. Using this method, we found the optimal number of clusters to be three. We also carried out a quality analysis using the smallest inter-cluster distance and the distribution of within-cluster distances for values of K from 2 to 8. We then chose the K value for which the most within-cluster distances are smaller than the smallest distance among clusters (Azuaje, 2002). The K value that maximized between-cluster distances was also three.

Functional category enrichment analysis

We used a modified version of CLENCH, a program for calculating cluster enrichment using the gene ontology (Shah and Fedoroff 2004). The new program accepts two lists of genes: (1) total-genes (which is the set of genes that each 'cluster' is to be compared with; all the genes on the chip in this study) and (2) changed-genes (a subset of genes, for example a cluster of genes, that is to be analyzed for enrichment of functional categories). It retrieves GO annotations and descriptions for both gene lists to calculate the number of genes (n and m) belonging to a particular GO category or having a particular description in both lists and then calculates the hypergeometric probability (P-value) for finding at least n genes belonging to that category or having that description in the

changed-genes list given that m genes were annotated to that category or description in the total-genes list. This *P*-value tells us how likely it is to find at least n genes of a particular category in the changed-genes list by chance alone, given the number of genes in that category in the total set. A category is called enriched if the *P*-value is less than 0.05. We performed a conversion of GO annotations to plant specific GO-slim annotations before making the *P*-value calculations. GO-slim terms are a high level view of the complete list of GO terms selected by database curators on the basis of annotation statistics and biological significance. We used slim term lists developed by TAIR and added terms that were of significance in the study of the stress response.

Promoter analysis

We analyzed promoters for the presence of 90 different promoter motifs from either the PLACE database of regulatory elements (Higo *et al.*, 1998) or extracted from the literature. To determine whether a particular motif is over-represented among the promoters of a given cluster of genes, we first determined the frequency of each motif in the promoters of the 1391 genes on our microarray using the 1000 bp upstream promoter files from TAIR (ftp://ftp.arabidopsis.org/Sequences/blast_onlydatasets/). We then calculated the probability of finding *m* promoter regions having one or more motifs in the set of *n* promoters in the cluster of interest. We considered a motif to be significantly over-represented if the probability of the observed frequency by chance alone was less than 0.05. These calculations were implemented using Perl scripts that are available at (http://www.personal.edu/nhs109/Programs/).

Results

Microarray hybridization

In previous work, we described the identification by suppression subtractive hybridization (SSH) PCR of genes whose transcript levels change in response to a variety of biotic and abiotic stresses, including oomycete and bacterial pathogens, O_3 exposure, as well as treatment with salycilic acid (SA) and jasmonic acid (JA) (Mahalingam *et al.*, 2003). We constructed microarrays using cDNAs for 1391 genes whose transcript levels changed in response to biotic and abiotic stresses. These comprised the approximately 1100 genes we identified by SSH PCR (Mahalingam *et al.*, 2003), supplemented by additional cDNAs representing genes identified from literature searches and microarray experiments carried out in other laboratories (see Methods). To analyze the temporal evolution of the oxidative stress response, we exposed Col-0 wild-type plants to 350 ppb O_3 for 6 h. This O_3 treatment is sufficient to evoke a physiological and transcriptional response, but causes little tissue damage to wild-type plants of the relatively O_3-resistant Col-0 ecotype, as judged either by the development of visible lesions or by enhanced ion leakage, a measure of cell membrane damage (Joo *et al.*, 2005). It should be noted that this level of O_3 is reached in some major metropolitan areas in summer and constitutes an oxidative stress for both vegetation and human health (Baldasano *et al.*, 2003; Sandermann Jr., 1996).

We collected leaves from several O_3-exposed and control plants for RNA extraction and microarray hybridization at various intervals during and after the O_3 treatment. The first three time-points were collected within the first 90 min (0.5, 1 and 1.5 h) during the treatment in order to identify primary response genes that are differentially expressed very early during oxidative stress. The next three time points were separated by 90-min intervals (3, 4.5 and 6 h) and were anticipated to be important for signal transduction processes. The last two time-points were collected after the end of ozone fumigation and were at 3-h intervals (9 and 12 h). We expected the identification of effector genes at these late time-points.

We analyzed the results of 32 independent microarray hybridization experiments, including three biological replicates of a time course extending over a 12-h period during and after O_3 treatment. All the microarray data are MIAME compliant (Brazma *et al.*, 2001) and available as supplementary data. Figure 1A shows the results of a 'control-control' experiment for biological replicates. In this experiment, only two of the 3072 cDNAs on the slide gave Cy3/Cy5 ratios >2, a common minimal value used as a criterion of

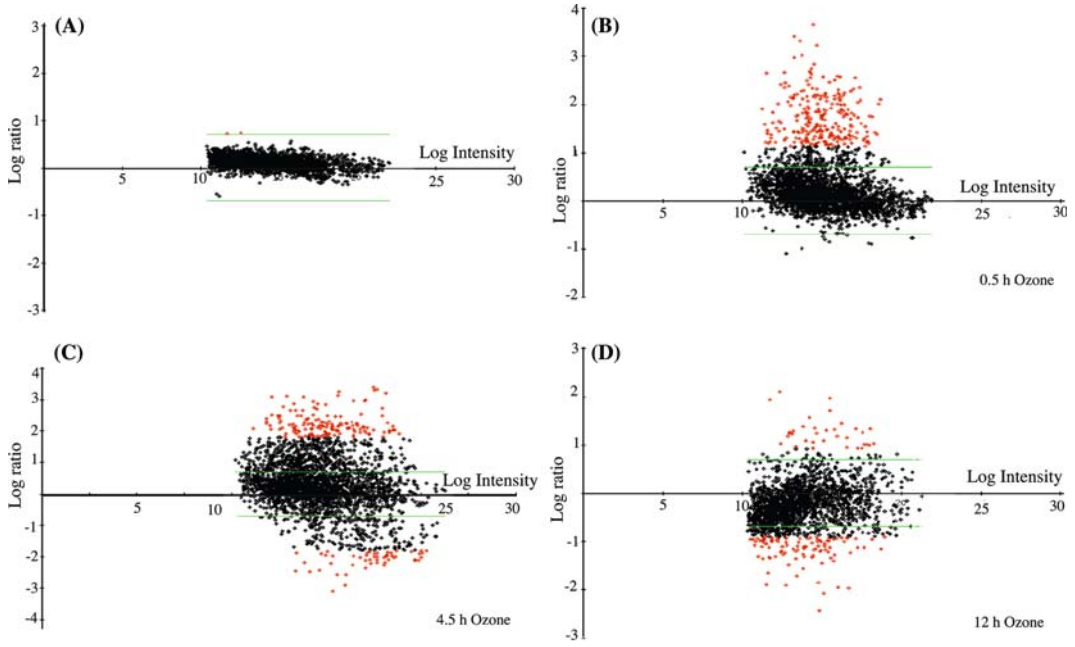

Figure 1. Ratio-intensity (*R–I*) plots. The $\log_2$ of the ratio is plotted against the $\log_2$ of the product of the two values. (A) Ratios for control samples from 2 biological replicates. (B, C and D) Ratios for 4 week-old Col0 plants exposed to ozone for the indicated times. Green lines mark experimental/control ratios of 2 and −2; experimental/control ratios having a Z value $> |2|$ are shown in red.

increased gene expression. The correlations for individual time-points between biological replicates was >0.8. Technical replicates from a single experiment, carried out by comparing Cy3 and Cy5 labeled aliquots of the same RNA sample were even less variable, giving correlations >0.9 (not shown). Hence both the technical and biological replicability of these experiments is high and the probability of false positives is low.

Transcriptional response to O_3

O_3 exposure alters transcript levels of many stress-responsive genes. Both the magnitude and direction of the change vary in a time-dependent way. Figure 1B shows that 30 min after the onset of ozone exposure, the distribution of experimental/control ratios has been substantially broadened and is skewed toward positive values (74 genes with Z-scores >2 and two genes with Z-scores <-2). Figure 1C shows that by 4.5 h after the onset of ozone exposure, there are also many genes whose transcript levels have fallen below those observed in control samples from leaves harvested at the same time from plants maintained in O_3-free air (35 and 42 genes with Z-scores >2 and $Z<-2$, respectively). Figure 1D shows that the distribution of values is returning to control values by 12 h after exposure. An interesting general feature of the temporal response to oxidative stress is that almost all of the early changes in transcript abundance are increases (Figure 1B). At 30 min after the onset of O_3 exposure, almost all responding genes exhibit increases in transcript levels, many quite substantial (>20-fold). Later in the response, transcript abundance is increased and decreased for roughly equal numbers of genes (Figure 1C), while at 12 h, the latest time-point in the present experiments, the number of genes showing differential gene expression decreased (Figure 1D; 31 and 25 genes with Z-scores >2 and $Z<-2$, respectively). The magnitude of later changes in transcript abundance is generally less than that observed for the most rapidly responding genes.

Although temporal profiles are particularly vulnerable to data loss, we were able to obtain statistically sound and biologically reproducible data over three replicates for almost 80% of the genes represented in our stress microarray (1083/

1391) even after stringent filtering and flagging steps (see Methods). Moreover, when all 8 time-points were considered, correlations between the biological replicates were in the range of 0.60–0.72. We selected the genes for which the ratio of experimental to control values differed from the mean by two standard deviation units for at least one time point (Z-scores >|2|, see Methods); this group consists of 200 genes representing 18% of the genes for which we had reliable data (supplementary table 1). Application of the more conventional criterion of >2-fold change in transcript abundance, indicated by green lines in Figure 1, identified a larger group of 533 genes. Thus, half of the genes for which we had statistically valid and biologically reproducible data were classified as exhibiting significant changes in transcript levels in O_3-exposed plants. In order to conduct a detailed comparison of a conventional, literature-based analytical approach and the automated, ontology-based approach described below, we focused our analysis on the group of 200 genes with $Z > |2|$.

Based on statistical tests (see Methods), the O_3 time-course data could most reliably be partitioned into three clusters. We therefore used the *K*-means algorithm (Cluster program) to generate three clusters and visualized them using the TREEVIEW program (Eisen *et al.*, 1998). The resulting three clusters for the 200 genes with *Z* scores >|2| are displayed in Figure 2A and the average expression profile of each cluster is shown in Figure 2B. Because the changes in

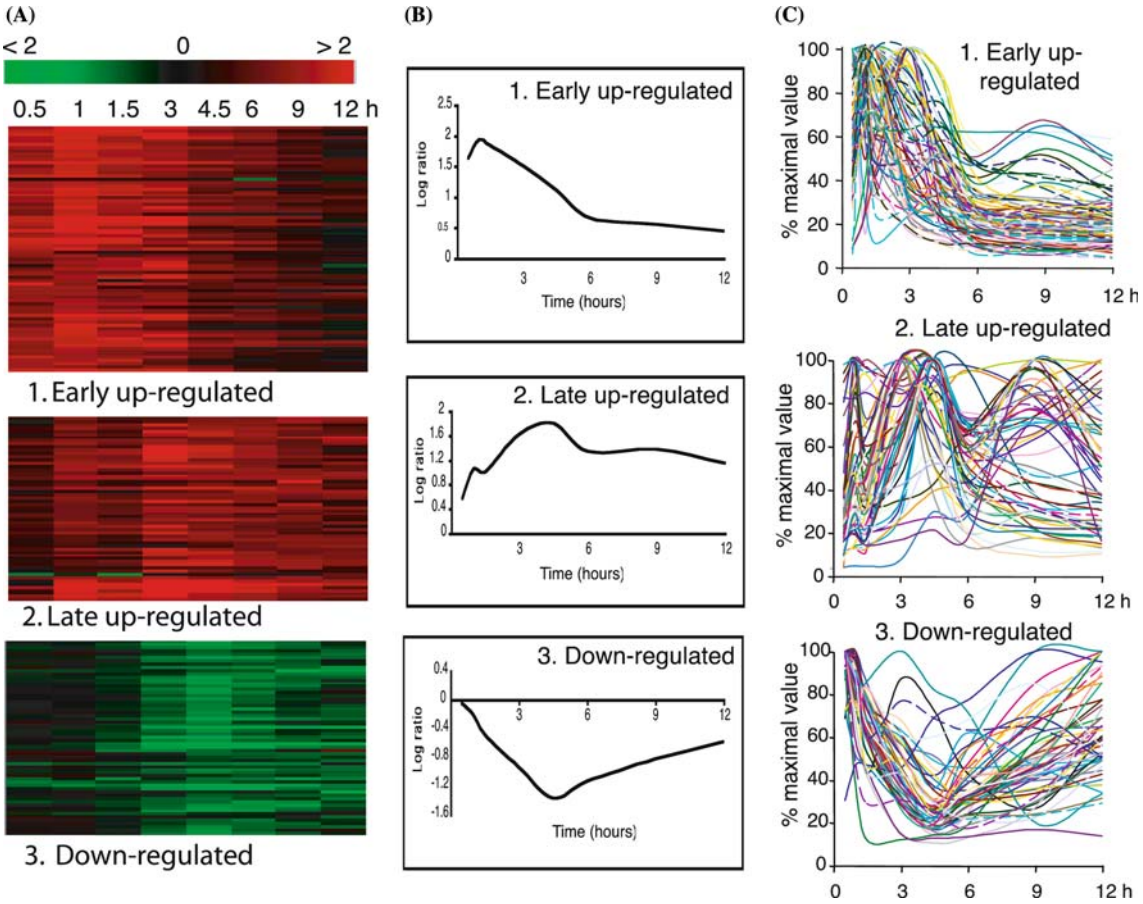

Figure 2. *K*-means clustering of genes with $Z > |2|$ in O_3-treated plants. (A) Color representation of the ratio of transcript abundance in experimental and control plants. The rows represent genes and each column represents a time-point. Experimental/control ratios >1, 1 and <1 are represented by red, black and green, respectively, and correspond to increased, unchanged, and decreased transcript abundance compared with controls. (B) The average expression profiles for each of the 3 clusters shown in (A). (C) All profiles normalized to peak expression value.

transcript abundance varied by an order of magnitude, we also normalized expression values to their maximum value in order to more readily compare the shapes of the expression profiles (Figure 2C).

The first cluster of 81 genes, designated 'early up-regulated,' comprises genes whose transcript levels begin to increase almost immediately after the onset of O_3 exposure, reaching their maximal amplitude within the first 1.5–3 h and declining thereafter. Most genes in the early up-regulated cluster show a single peak of elevated expression (Figure 2A1, B1, C1). However, some exhibit more complex profiles, either with two early peaks or a second peak of lower amplitude at about 9 h. The expression patterns of several early induced transcription factors and signaling genes were confirmed by conventional Northern blot analysis (Figure 3). Transcript levels of the second cluster of 60 genes, designated 'late up-regulated,' increased from pre-exposure levels more slowly, peaking at 3–4.5 h or later, with most returning to pre-exposure levels by 12 h (Figure 2A2, B2, C2). This cluster is quite heterogeneous, comprising simple profiles with a single peak, as well as more complex profiles with two or three peaks. Transcript abundance generally peaked at 3–4.5 h or at 9 h after the onset of O_3 exposure, but many profiles also had an early peak or shoulder at 1–1.5 h. Inspection of the normalized profiles for the whole cluster, shown in Figure 2C2, suggests that there are three major periods of enhanced transcript abundance during the 12-h time course, with peaks at about 1, 3–4.5 and 9 h. A third group of

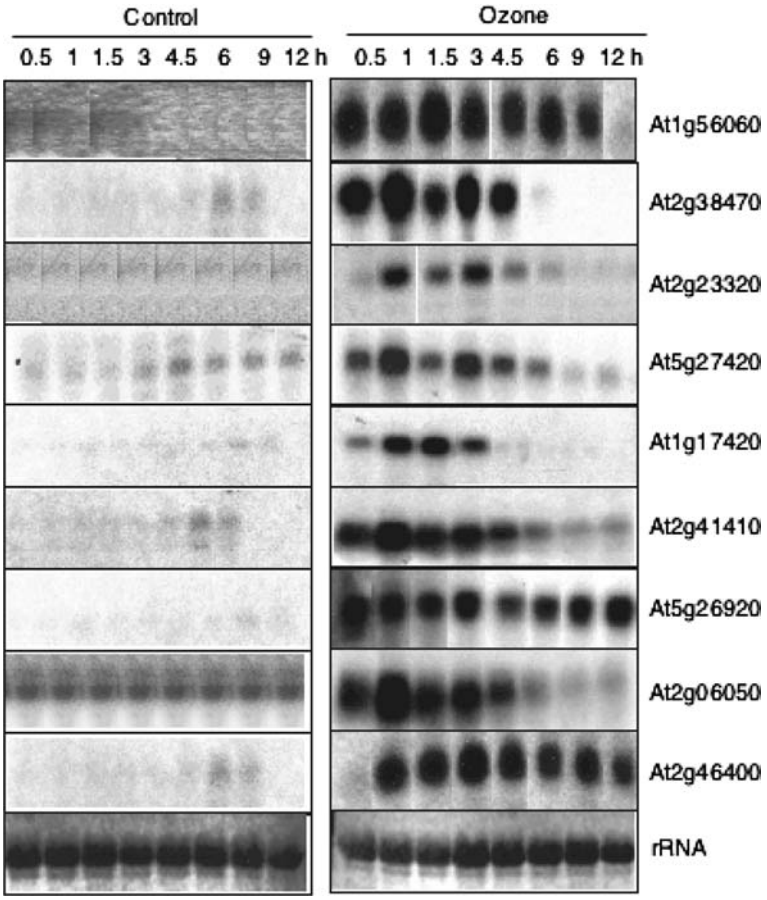

Figure 3. Analysis of early O_3 up-regulated genes by Northern blot hybridization. Ten micrograms of total RNA from control and O_3-treated plants harvested at the indicated times after the onset of O_3 treatment were fractionated on formaldehyde–agarose gels and transferred to nylon membranes. The membranes were hybridized with ^{32}P-labeled PCR fragments of the genes indicated by their MIPS identifiers.

59 genes, designated the 'down-regulated' genes, showed a transient decrease in transcript abundance from pre-exposure levels during the 12-h time course, generally returning to near pre-exposure levels by 12 h. Many profiles in this group reached a minimum value at about 4.5 h after the onset of O_3 exposure, then returning gradually to pre-exposure levels (Figure 2A3, B3 and C3).

All of the clusters shown in Figure 2 are quite heterogeneous and individual profiles differ both in time at which they show maximal transcript abundance and in the complexity of the profiles themselves. We took several approaches to asking whether this complexity could be reduced to identify gene groups likely to be subject to common regulatory mechanisms. We grouped profiles into smaller subsets based on similarity, time of maximal or minimal transcript abundance, on the characteristics of the proteins encoded by the genes, including both function and location within the cells, and on transcription factor (TF) binding motifs in their promoters. We automated the sub-grouping of genes by common functions using Gene Ontology (GO) annotations provided by TAIR. We developed a program called CLENCH (Cluster ENriCHment using the Gene Ontology) to extract GO annotations from the TAIR database using MIPS ID numbers (Shah and Fedoroff, 2004). CLENCH identifies groups of two or more genes with the same annotations in each of the three GO categories corresponding to the biological process in which the gene product participates, the cellular location (or destination) of the gene product, and its molecular function. CLENCH displays the profiles of genes in each functional group of more than two genes that it finds to be statistically over-represented within the cluster relative to all of the genes represented in our stress microarrays. The results of the automated analysis of the three gene clusters are included in supplementary table 2. In order to assess the accuracy of this automated approach to identifying sub-groups of genes, we also conducted a thorough manual analysis of this set of 200 genes, collecting information from other publicly available databases, such as the MIPS *Arabidopsis thaliana* database (http://mips.gsf.de/proj/thal/db/index.html) and Interpro (http://www.ebi.ac.uk/interpro/), as well as the published literature.

The down-regulated gene cluster

All three types of GO annotations used by TAIR proved useful in identifying genes likely to be regulated by a common molecular mechanism (supplementary data). For example, 34 of the 59 genes (58%) in the down-regulated cluster are annotated, either in the TAIR database or the MIPS *Arabidopsis thaliana* database, as chloroplast-targeted proteins. Figure 4A shows all of the profiles of the down-regulated nuclear genes coding for chloroplast-targeted proteins and Figure 4B shows the profiles of the remaining 25 genes which do not have plastid targeting motifs in the encoded proteins. The profiles of the chloroplast-targeted genes are much more similar to each other than are the profiles of the non-chloroplast targeted genes. Grouping of genes by the processes in which they participate identified further subsets of the chloroplast-targeted proteins as involved in photosynthesis (19 genes, Figure 4C), including seven light-harvesting proteins (Figure 4D) and seven proteins involved in photosynthetic electron transport (Figure 4E). The expression profiles of these sub-groups are similar in the rate and extent of decrease in transcript abundance, all reaching a minimum at 4.5 h, and returning to pre-exposure levels at similar rates. This was not true of the sub-group of eight genes coding for proteins targeted to the secretory pathway (Figure 4F).

A comparable enrichment for chloroplast protein genes was observed when the down-regulated genes in the larger group of 533 genes showing >2-fold changes in transcript abundance were analyzed using CLENCH. Of the 118 down-regulated genes, 57 (48%) were annotated as targeted to plastids. While this was somewhat less than the 58% in the smaller set of 200 genes, this is because the automated analysis based on the information in TAIR was supplemented for these genes with additional information from other databases and the literature (CLENCH identified 32 of the 34 chloroplast-targeted genes in the set of 200). There is substantial evidence that chloroplast signals regulate nuclear genes involved in photosynthesis during early development and acclimation responses to increased and decreased light levels (Jarvis, 2003; Pfannschmidt, 2003). The present observations suggest that the chloroplast signaling system is activated by oxidative stress to coordinately and transiently down-regu-

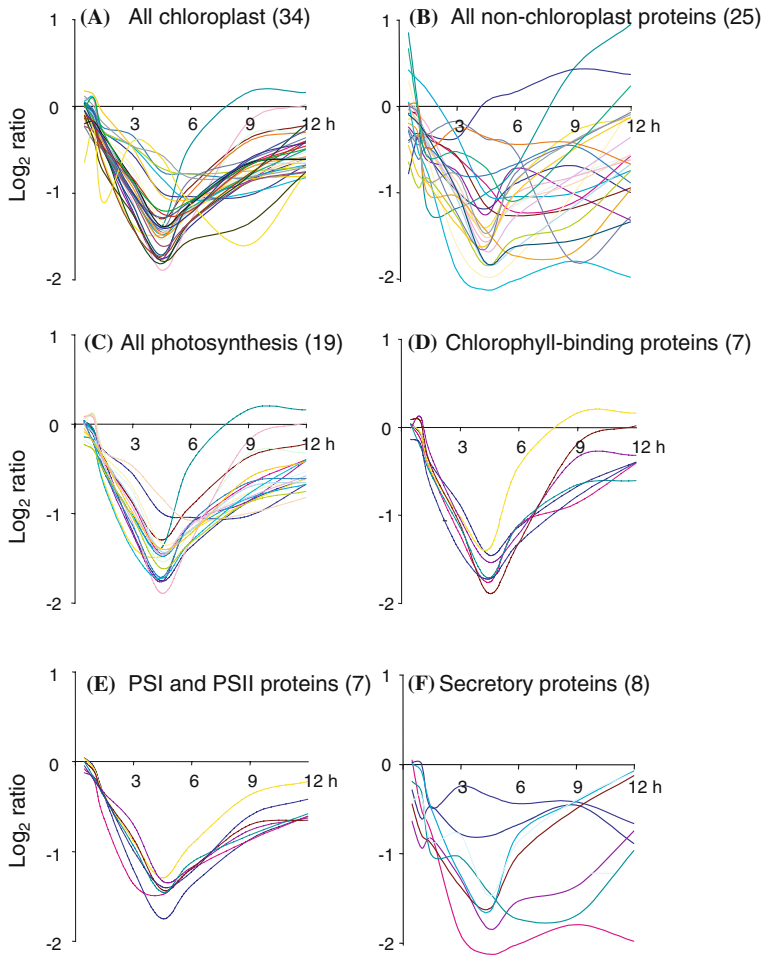

Figure 4. Down-regulated genes. CLENCH was used to identify genes in the down-regulated cluster that were chloroplast targeted (A, B) shows the profiles of all other genes in the cluster as well as further subgroups involved in a common cellular process (C, D) and having a common molecular function (E, F).

late expression of nuclear genes coding for proteins that function in chloroplasts, particularly those involved in photosynthesis. The marked similarity of the expression profiles of the nuclear genes coding for chloroplast proteins suggests the operation of a common molecular mechanism.

The early up-regulated gene cluster

Analysis of the early up-regulated gene cluster identified 36 of the 64 (56%) genes whose subcellular localization could be assigned with reasonable confidence as membrane proteins. These included six proteins with 2–9 trans-membrane domains known or likely to function in trans-membrane transport of small molecules. Among these were the ERD6 gene (At1g08930) encoding a putative sugar transporter (Kiyosue *et al*., 1998), the STP4 gene (At3g19930) encoding an integral plasma membrane sugar transporter (Fotopoulos *et al*., 2003), the AtPUP1 gene (At1g57990) coding for a member of the purine permease family having 9 trans-membrane domains (Gillissen *et al*., 2000), a MATE (multidrug and toxic compound extrusion) family gene (At5g44050) (Nawrath *et al*., 2002), and the chloroplast-targeted ATR2 gene (At4g30210) coding for a NADPH-cytochrome p450 reductase (Louerat-Oriou *et al*., 1998; Hull and Celenza, 2000). These proteins are integral membrane proteins involved in transporting small molecules across membranes and in detoxification reactions. The group of genes annotated as trans-

porters also includes the TCH4 gene (At5g57560) which codes for a xyloglucan endotransglycosylase believed to be a cell wall-modifying enzyme (Xu *et al*., 1995); the TCH4 protein has an N-terminal signal sequence that directs it to the secretory pathway (Campbell and Braam, 1998).

The early up-regulated cluster also contains 17 nuclear genes coding for chloroplast-targeted proteins (Figure 5A; supplementary data); these comprise 27% of the genes in the early up-regulated cluster for which a sub-cellular compartment is known or can be predicted with confidence, while nuclear genes coding for mitochondrial proteins comprised a substantially smaller subset (6%). All of the genes coding for chloroplast-targeted proteins showed extremely rapid initial increases in transcript abundance and all showed subsequent rapid declines in transcript levels. In addition, some genes showed either a shoulder or an additional peak at 3–4.5 h and two genes (At5g26920 and At4g18250) coding for a calmodulin-binding protein and a receptor-like kinase (RLK), respectively, exhibited a peak at 9 h after the onset of O_3 exposure in addition to either one or two early peaks. The profiles of the early up-regulated chloroplast protein genes were not as similar as those of down-regulated genes coding for chloroplast-targeted proteins (Figure 4A). Moreover, while the down-regulated genes were largely ones coding for proteins associated with photosynthesis, the up-regulated genes encode proteins involved primarily in transcription and signaling. The transcription factors (TFs) include a NAM-like NAC-family (At5g63790) and a WRKY family (Atg38470, WRKY33) TF (Duval *et al*., 2002; Eulgem *et al*., 2000). The signaling genes include several calmodulin-like and putative calmodulin-binding proteins, three TIR-NBS-LRR-like disease resistance and other receptor like proteins (Meyers *et al*., 1999; Ikura *et al*., 2002; Meyers *et al*., 2003;

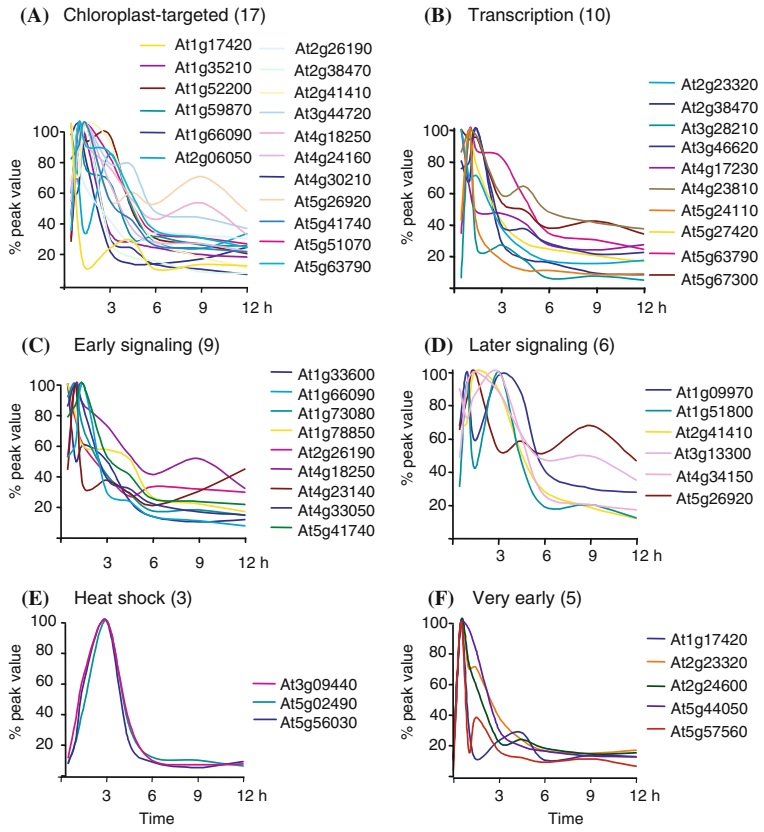

Figure 5. Early up-regulated genes. CLENCH was used to identify groups in the early up-regulated cluster based on their sub-cellular location (A) and function (B–E); (F) shows a group of genes exhibiting a very early expression peak and a rapid decline in transcript abundance.

Dievart and Clark, 2004). Two genes coding for JA biosynthetic enzymes, lipoxygenase (At1g17420, LOX), and 12-oxophytodienoic acid reductase (At2g06050, ODR3/DDE1), are among the most rapidly up-regulated chloroplast genes (Creelman and Mullet, 1997). JA signaling modulates the deleterious effect of O_3 (Overmyer *et al.*, 2000; Rao *et al.*, 2000). The initial steps in JA biosynthesis occur in the chloroplast and the final steps are likely to be peroxisomal, involving the peroxisome-targeted ACX1-encoded acyl-CoA oxidase (Bell *et al.*, 1995; Cruz Castillo *et al.*, 2004). Both of the genes coding for chloroplast-targeted JA biosynthetic enzymes show a second peak of transcript abundance coincident with the 3-h peak of ACX1 (At4g16760) transcript abundance (Figures 5A and 6A). There are also two genes in this early up-regulated group that code for TFs.

CLENCH also identified sub-groups within the early up-regulated gene cluster based on similar molecular functions or participation in a common cellular process. There are 13 up-regulated genes in the group of 200 analyzed that code either for known TFs or for proteins with homology to proteins involved in transcriptional regulation; 10 of the 13 (77%) were in the early cluster and all show extremely rapid increases in transcript levels, followed for most by equally rapid decreases in transcript abundance, suggesting activation of a degradation mechanism (Figure 5B). In addition to the chloroplast-targeted WRKY33 gene, the rapidly up-regulated cluster contains 3 WRKY family TFs (At2g23320, WRKY 15; At2g38470, WRKY 53; At5g24110, WRKY30), a MYB family TF (At5g67300). It also includes two RING finger proteins annotated as involved in transcriptional regulation, a scarecrow-like TF (SCL13), a NAM-family TF, and an AN1-like zinc finger protein (Pysh *et al.*, 1999; Duval *et al.*, 2002).

Nine of the 18 early up-regulated genes annotated as involved in signaling showed similar rapid increases in transcript levels, peaking in the first 1.5 h after the onset of O_3 exposure, followed by rapid decreases (Figure 5C). There were also several genes coding for signaling proteins that peaked later or showed several peaks (Figure 5D). By contrast, transcript levels of the three heat-shock proteins in the early up-regulated cluster showed very simple and coincident profiles, peaking at 3 h (Figure 5E). Figure 5F shows the profiles of several genes that exhibit particularly rapid and transient expression spikes, reaching maximal expression levels during the first 30 min and then declining by 80% or more within 2 h, suggesting a very brief mRNA half-life. Strikingly, of the 39 genes in the early up-regulated cluster for which a functional category could be assigned, 15 (39%) were involved in signaling and 10 (26%) in transcription. Thus 64% of the genes in the early up-regulated cluster code for either signaling proteins or TFs. Similarly, the two largest groups of genes CLENCH identified based on functional categories among the 175 genes in the larger early up-regulated cluster selected based on a 2-fold or greater increase in transcript abundance coded for proteins involved in transcription and signaling (supplementary table 2).

The late up-regulated gene cluster

Complex expression profiles with two or three peaks are common in the late up-regulated gene cluster and the cluster itself is more heterogeneous than the others. Inspection of the total set of profiles suggested that there were several discrete peaks and this cluster could indeed be sub-divided into sub-clusters with peaks at 3, 4.5 and 9–12 h after the onset of O_3 exposure (Figure 6A–C; supplementary table 1). While the sampling frequency in these experiments was sufficiently low so that it seems a priori unlikely that peaks as close as 3 and 4.5 h could be distinguished reliably, it is striking that a majority of profiles in Figure 6A have a single expression peak, while a majority of those in Figure 6B have an additional peak at 9 h. Moreover, most of the genes in the 9–12 h sub-cluster had two peaks of varying magnitude at 1–1.5 and 3–4.5 h, in addition to the major peak at 9–12 h. CLENCH was able to identify sub-groups within this cluster annotated as coding for membrane proteins or as functioning in the biotic or abiotic stress responses in both the late up-regulated clusters in the group of 200 genes initially analyzed (supplementary data) and in the larger group of 533 genes. However, the GO annotations in TAIR were markedly less uniform and complete for this group than for the down-regulated gene cluster, for example, and our ability to identify potentially co-regulated clusters improved upon supplementation with information from other databases and the literature.

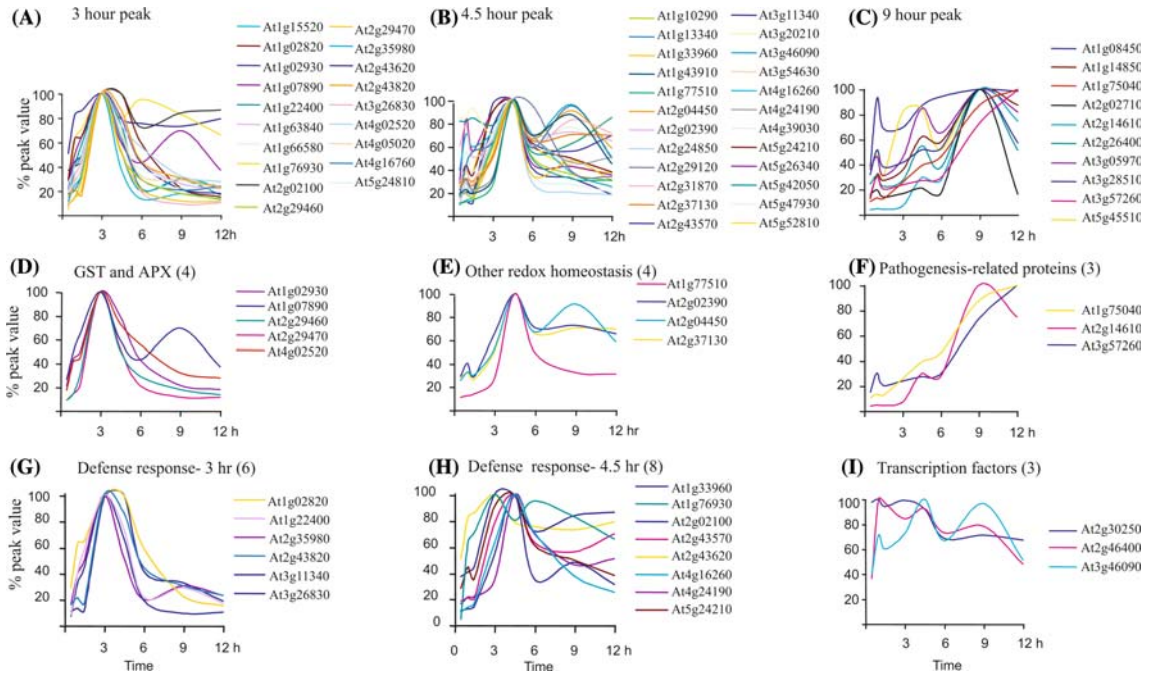

Figure 6. Late up-regulated genes. Subsets of the late up-regulated cluster grouped by expression peak (A, B, C) and by molecular function (D–I).

Of the 43 genes in the late up-regulated cluster coding for proteins whose sub-cellular location was known or could be predicted with some certainty, 27 (63%) were either membrane-associated (15) or secretory proteins (12), but only 7 were chloroplast-targeted. However, none of these categories identified genes whose profiles were more similar than those comprising the whole group. Subgroups with similar profiles emerged when gene groups were identified by participation in a common physiological process. For example, among the 200 genes analyzed in detail, there were 15 genes coding for proteins likely to be involved in redox homeostasis. Transcript abundance for 13 of these 15 genes was up-regulated and nine of the 13 up-regulated genes were expressed in a tightly coordinated manner in the late up-regulated cluster. These formed two groups with peaks at 3 and 4.5-h after the onset of O_3 exposure. The 3-h group included four of the five markedly up-regulated glutathione *S*-transferase (GST) genes and the cytoplasmic ascorbate peroxidase (APX1) gene (Figure 6D); the expression profiles of the four GST genes were quite similar, with a single peak at 3 h and a rapid decrease in transcript abundance thereafter. Two of these genes (At2g29460 and At2g29470) are adjacent to each other on chromosome 2 and probably arose as a result of a relatively recent duplication. However, the other two GST genes are on different chromosomes. Hence the coincidence of expression profiles is not likely to be explained by their proximity. The expression profile of the APX1 gene was similar to that of the three co-expressed GST genes, except that it had a second peak at 9 h.

The 4.5-h group included the fifth GST gene, a thioredoxin (Trx) gene, and another peroxidase (PER21) gene (Figure 6E), as well as a gene coding for a protein with homology to MutT, a bacterial enzyme that repairs oxidative damage to DNA (Lee *et al.*, 2004). Three of the four genes in this group showed a second expression peak at 9 h. The late up-regulated genes involved in the pathogen defense and abiotic stress response could be subdivided into three groups (Figure 6F–H), two of which show peaks coincident with those of the 3 and 4.5-h redox homeostasis groups and are shown directly below them in Figure 6. The third group consists of three genes that code for pathogenesis-related (PR) proteins; one of the PR protein genes peaks at 9 h and the other two show maximal expression at the latest time point taken, possibly

still increasing thereafter (Figure 6F). As observed with the 3 and 4.5-h redox homeostasis genes, the earlier group of defense/stress-response genes is dominated by profiles with a single peak, while the later group shows a predominance of profiles with a second peak or a shoulder at 9 h. Moreover, there are three genes in the 3-h defense/stress group that code for proteins with significant homology for UDP glucosyl transferases, all of which are encoded by genes on different chromosomes. Thus all of the genes coding for proteins involved in redox homeostasis and eight of the 11 defense/stress-response genes showed peaks at 3 and 4.5 h, while all three genes coding for PR proteins showed similar profiles, peaking at 9 h or later after the onset of O_3 exposure. Strikingly, the three TFs in the late up-regulated cluster, two belonging to the WRKY TF family and the Zat7 zinc finger protein, show complex expression patterns. All exhibit an early increase in transcript abundance and their transcript abundance remains elevated, beginning to decrease after 9 h. Moreover, all three profiles exhibit multiple peaks of relatively equal magnitude at 1, 3–4.5 and 9-h. (Figure 6I). By contrast, profiles of TFs in early up-regulated cluster exhibited a single peak at 1–1.5 h.

Promoter analysis

If the gene groups identified by clustering expression profiles and identifying sub-groups based on sub-cellular location and physiological and biochemical function are regulated at the transcriptional level, it should be possible to identify common regulatory elements in their promoters. We therefore automated identification of potential TF binding sites in the 1-kb sequence upstream of the groups of genes identified by CLENCH (supplementary data). We grouped the TF binding sites according to whether they were associated with developmental processes, or with gene regulation in response to light, phytohormones, or pathogen defense/stress response. We used the list of TF binding sites compiled in the PLACE database of regulatory elements (Higo *et al*., 1998), supplemented with several additional motifs extracted from the literature and used in characterizing the stress-induced genes identified in previous work from our laboratory (Mahalingam *et al*., 2003). The results are displayed by CLENCH as a matrix of binding sites and genes in each sub-group analyzed, with presence within the promoter indicated by a filled square (supplementary data). CLENCH also determines whether a particular motif is overrepresented in a given sub-group of genes relative to its frequency among all of the promoters of the 1391 genes represented on our stress microarray (see Methods). We previously reported that binding motifs for WRKY TFs were markedly enriched in the promoters of our group of stress up-regulated genes relative to all *Arabidopsis* promoter sequences, as were GCC-box binding sites for TFs belonging to the ethylene response element binding protein (EREBP) family, motifs common to salicylic acid (SA)-induced genes and binding sites for the TGA TF activated by NPR1 (Mahalingam *et al*., 2003).

We identified the TF binding motifs that are overrepresented in the promoters of gene groups showing expression peaks at 0.5–1, 3, 4.5 and 9 h after the onset of O_3 exposure (Table 1). CLENCH promoter analysis revealed that the related W-box, WRKY and WRKY-like TF binding sites were enriched relative to the promoters of all the genes represented in our stress microarray in the three early groups, but not the 9-h group. The core ABRE sequence (ACGT), as well as Myc and Myb TF consensus sequences was present in most of the genes in all four groups. However, genes whose peak expression occurred at 0.5–1 h after the onset of O_3 exposure showed a significant over-representation of the light-regulated T box motif (ACTTG) identified through analysis of the nuclear gene coding for the B sub-unit of the chloroplast glyceraldehyde-3-phosphate dehydrogenase gene (Chan *et al*., 2001). This is consistent with the observation that ROS signals emanating from chloroplasts are a critical component of the initial physiological response to O_3 exposure (Joo *et al*., 2005). Because only three of the genes in the group of 29 whose expression profiles peaked within the first hour code for chloroplast-targeted proteins, signals emanating from chloroplasts are likely to be involved in activating a larger group of stress-responsive nuclear genes. A second motif found to be significantly over-represented in the group of genes showing the most rapid increases in transcript levels is the TGTCTC auxin response element, an auxin response factor (ARF) binding site (Ulmasov *et al*., 1997; Ulmasov *et al*., 1995). It

Table 1. Over-representation of transcription factor binding motifs in genes expressed at different times during the O_3-induced oxidative stress response.

Time	Designation	Sequence	*P*-value
0.5 h	WRKY-like	BBWGACYT	0.03
	TBOXATGAPB	ACTTTG	0.002
	ARFAT	TGTCTC	0.035
3.0 h	WRKY	TTGACY	0.049
	CarG	$CC(A/T)_6GG$	0.032
4.5 h	WBOXATNPR1	TTGAC	0.041
	TGA1	TGACG	0.027
	UPRMOTIFIAT	CCACGTCA	0.023
	ACGTATERD1	ACGT	0.038
	SA-induced (LS7)	ACGTCA	0.009
9 h	MRE1	TGCRCNC	0.004

has been reported that auxin and salicylic acid analogs can activate the same promoter (Ulmasov *et al.*, 1994). It has also been reported that the production of ROS is essential for auxin action (Joo *et al.*, 2001; Schopfer *et al.*, 2002). This suggests that such genes either respond to multiple signals or that ROS signals are transmitted to a common subset of genes.

While some of the over-represented motifs in the promoters of the 3- and 4.5-h clusters were common to both groups of genes, others were over-represented in one group, but not the other, further indicating that the two groups are regulated differently at the transcriptional level. All of the promoters in both groups contain the W-box motif found in the NPR1 gene (TTGAC) and they commonly contain two or more closely spaced copies of the motif within the first 300 bp upstream from the transcription start site. Moreover, almost all of the promoters in both groups contain the short core ABRE sequence, as well as Myc and Myb consensus binding sites. However, the 3-h group is significantly enriched for the A/T-rich CArG motif identified as the binding site of the MADS-domain protein APETALA3 (Tilly *et al.*, 1998). The 4.5-h group is enriched for a ACGTCA motif common to SA-induced promoters (Shah and Klessig 1996), as well as the TGACG binding motif of the TGA1 protein known to interact with the redox-activated NPR1 protein (Zhou *et al.*, 2000; Despres *et al.*, 2003). Finally, while core ABREs occur in all of the late genes, binding motifs for WRKY TFs are less prevalent in this group. The metal response element (Stuart *et al.*, 1985) is highly enriched in the promoters of the late response genes.

Discussion

The analysis of detailed gene expression profiles of O_3-exposed *Arabidopsis* plants has revealed new patterns in the temporal evolution of the genetic response to oxidative stress. Identification of such regularities was facilitated by the development of analysis and visualization tools that permit expression profiles to be grouped by the biological properties of the proteins they encode and automate identification of TF binding motifs in the genes' promoters (Shah *et al.*, 2003; Shah and Fedoroff, 2004). Application of well-established clustering algorithms to the expression profiles provided a coarse grouping of genes into early up-regulated, late up-regulated, and down-regulated (Eisen *et al.*, 1998), but many of the expression profiles were complex and the groups were sufficiently heterogeneous so that genes likely to be subject to a common regulatory mechanism emerged only when addition information was used to identify sub-groups.

We used the GO annotations in the TAIR database to automate identification of genes coding for proteins having a common sub-cellular location, associated with the same physiological process or having a similar molecular function. A detailed manual analysis of the 200 genes showing the most marked changes in transcript abundance also identified aspects of the annotations themselves that limited their utility for automated grouping. The least serious of these were omissions, because annotations are constantly being updated. More serious problems arose from two sources: (1) the history of gene identification and (2) annotation by homology. Thus a gene might

originally have been identified as one induced in response to a pathogen and this remains part of its annotation, even though it is now recognized as a general stress-response gene, such as the genes coding for proteins involved in redox homeostasis, for example. The second weakness is that annotations can derive from a limited sequence homology identified between two proteins whose subsequent analysis shows them to be functionally unrelated. Despite these caveats, all three GO categories proved useful in identifying co-expressed and potentially co-regulated gene groups and automation of this process in the CLENCH program markedly accelerates detecting such gene groups in large complex datasets.

Chloroplast signaling in the oxidative stress response

Perhaps the most striking example of coordinate regulation is provided by the expression profiles of nuclear genes coding for proteins with chloroplast target sequences. The 59 nuclear chloroplast protein genes comprise 30% the 200 genes showing the most marked changes in transcript abundance over the 12-h time course of the present experiments. More than half of these genes were in the down-regulated profile cluster and this group comprised more than half of all O_3-down-regulated genes. The expression profiles of the down-regulated genes coding for chloroplast-targeted proteins were more similar to each other than the profiles of down-regulated genes whose proteins were not chloroplast-targeted (Figure 4A and B). The transcript abundance of the nuclear chloroplast protein genes declined at virtually the same rate, reached a minimum at 4.5 h, then rose again at a similar rate for many chloroplast protein genes, approaching pre-exposure abundance by 12 h. A majority of the genes in this group code for proteins known to be involved in photosynthesis, including several genes coding for chlorophyll a/b binding proteins and components of photosystems I and II. The average profile for these genes is shown in Figure 7, a composite diagram of the major themes in the temporal evolution of the genetic response to ozone stress.

The recent observation that transcript abundance levels for nuclear genes encoding chloroplast proteins vary coordinately with genetic backgrounds and environmental conditions, substantially amplified previous observations on a small number of genes and has led to the postulate of a master switch for nuclear chloroplast genes (Richly *et al.*, 2003). However, the present detailed temporal profiling suggests that there are likely to be several distinct regulatory mechanisms controlling the transcript abundance of nuclear genes coding for proteins that function in chloroplasts. The highly coordinate decline in the abundance of photosynthesis-associated gene transcripts may operate at the post-transcriptional level. Decreases in transcript abundance can be achieved either by transiently inhibiting transcription of highly unstable mRNAs, by accelerating the mRNA degradation rate, or both. The stability of mRNAs encoded by chloroplast genes is regulated by RNA-binding proteins encoded in the nucleus (Rochaix, 1996; Salvador and Klein, 1999; Barnes and Mayfield, 2003). However, relatively little is known about transcript stability of nuclear chloroplast genes, except that stability, like transcription, are affected

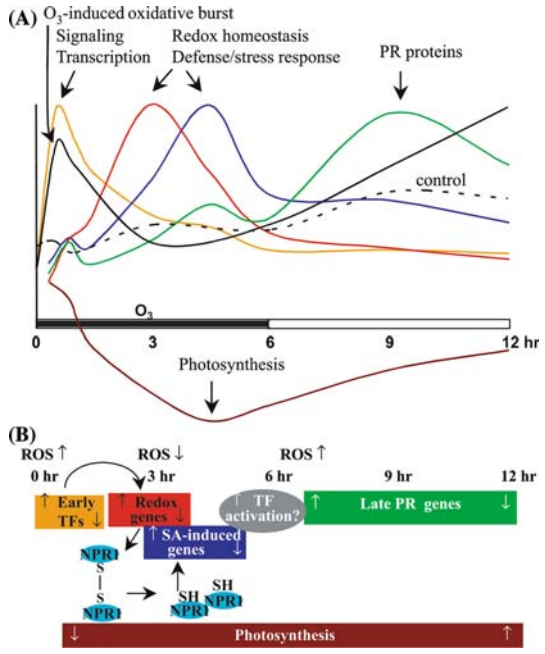

Figure 7. (A) Temporal profile of the oxidative stress response to O_3. The biphasic O_3-induced oxidative burst is represented in black, with the ROS control measurements shown as a broken line. Average transcript profiles are shown for early up-regulated genes (yellow, peaks at 0.5–1 h), and the 3 h (blue), 4.5 h (red) and 9–12 h (green) late up-regulated genes and for the down-regulated genes coding for photosynthesis proteins (brown). (B) Diagrammatic representation of redox regulation of the oxidative stress response.

by light and oxidative stress (Montane *et al.*, 1998; Pursiheimo *et al.*, 2001; Sullivan and Gray, 2002; Gray *et al.*, 2003).

Although the transcript abundance for down-regulated genes other than those coding for nuclear chloroplast-targeted proteins also declined only transiently, the rates of decrease and subsequent increase varied considerably from gene to gene. Nonetheless, as can be seen in Figure 4B, the 4.5-h time point was a common minimum for many genes. Although the temporal resolution of the current experiments is limited, this common nadir of transcript abundance significantly precedes the termination of O_3 exposure, indicating that it is likely to be controlled by internal biochemical processes triggered by the external stress, but not dependent on its duration.

There is substantial evidence that transcriptional activation of genes coding for chloroplast proteins is coordinately regulated by redox signals from chloroplasts, but little is known about how the signals are transmitted to the nucleus (Pfannschmidt, 2003). Chloroplast signals initiate the response to light stress (Mullineaux and Karpinski, 2002) and there are commonalities between light stress and pathogen defense responses (Mullineaux *et al.*, 2000; Karpinski *et al.*, 2003). Excess light activates chloroplast ROS production and activates nuclear genes, implicating redox signals from the chloroplast in nuclear gene regulation. We have recently reported that the early component of the O_3-elicited biphasic oxidative burst arises from guard cell chloroplasts and can be inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of chloroplast electron transport (Joo *et al.*, 2005). The early component of the oxidative burst coincides with the first transcript peak during the first hour of O_3 exposure (Figure 7). The finding that almost all of the promoters of the 28 most rapidly up-regulated genes in the group of 200 genes analyzed here contain TF binding motifs originally identified as necessary for light regulation of the nuclear gene coding for chloroplast glyceraldehyde-3-phosphate dehydrogenase gene (Chan *et al.*, 2001) suggests that chloroplast signals also activate genes in the early stress response (Figure 7).

Although the most rapid changes in gene expression, almost all of which increase transcript abundance (Figure 2B), may be triggered by chloroplast signals, they are not confined to genes coding for chloroplast-targeted proteins. Genes coding for proteins targeted to the chloroplast comprise only about a quarter of the most rapidly up-regulated gene group, exhibit similar expression profiles as genes whose proteins lack chloroplast targeting sequences, and code for the same kinds of proteins as other early up-regulated genes. By contrast, the genes coding for down-regulated chloroplast-targeted proteins appear to comprise a coordinately regulated sub-group within the down-regulated gene cluster. The kinetics of the changes in transcript abundance is also markedly different between these sub-groups of up- and down-regulated genes (Figures 4 and 5). Thus chloroplast signals appear capable of activating different regulatory mechanisms that have opposite effects on the transcript abundance of genes coding for chloroplast-targeted proteins and a larger group of stress-responsive genes, a subset of which code for chloroplast targeted proteins.

There is evidence that the connection between photosynthetic electron transport and nuclear gene expression is mediated by the redox state of the plastoquinone pool (Escoubas *et al.*, 1995; Sherameti *et al.*, 2002). The redox state of plastoquinone and plastoquinol bound to the cytochrome b_6/f complex control the activity of kinases that phosphorylate thylakoid proteins (Vener *et al.*, 1997, 1998). The phosphorylated proteins include those of photosystem II (PSII) core complex, the PSII light-harvesting proteins, the cytochrome b_6/f complex, and the kinases themselves (Aro and Ohad, 2003). Phosphorylation of the chlorophyll a/b binding proteins (LHCBs) of light-harvesting complex II (LHCII) by thylakoid kinases is extremely sensitive to environmental cues, such as changes in light level (Aro and Ohad, 2003). Changes in *lhcb* gene expression are correlated with LHCB phosphorylation: high light or cold stress both decrease phosphorylation and mRNA levels (Pursiheimo *et al.*, 2001). The LHCII kinase is activated by binding of plastoquinol to the cytochrome b_6/f complex and inactivated by chloroplastic thioredoxins, which are reduced in light by ferredoxin-thioredoxin reductase (Rintamaki *et al.*, 2000). However, the mechanisms that communicate the redox signal to the nucleus are not understood. The existence of redox regulated kinases and the recent identification of a protein that is released from the thylakoid membrane upon phosphorylation suggests the possibility that redox signals are transduced by protein phosphorylation (Carlberg *et al.*, 2003; Zer and Ohad 2003).

Redox regulation of gene expression in the stress response

It has long been known that pathogens and many kinds of stresses elicit a biphasic oxidative burst (Low and Merida, 1996; Scheel, 2002; Mahalingam and Fedoroff, 2003). Under the conditions used in the present experiments, the O_3-induced oxidative burst exhibits peaks at 1–1.5 h and at 20–24 h after the onset of the 6-h O_3 treatment (Joo *et al.*, 2005). The first peak of ROS production requires signaling through the heterotrimeric G protein and is initiated by guard cell chloroplasts, while the late peak is mediated by just the Gα subunit, which activates membrane-bound NADPH oxidases encoded by the *AtrbohD* and *AtrbohF* genes (Joo *et al.*, 2005). The three peak periods of transcript abundance identified in the present experiments are at about 1, 3–4.5, and 9 h after the onset of O_3 exposure. Down-regulated genes have a minimum in transcript abundance at 4.5 h that is common to many genes, after which transcript abundance begins to return to pre-exposure levels.

Because plants were exposed to a constant level of O_3 for 6 h, it appears likely that these patterns of gene expression are triggered by the onset of the oxidative stress, but unfold independently of it. Each peak (or minimum) is defined by the coordinate regulation of a different group of genes. Genes coding for proteins involved in signaling, especially membrane proteins, and transcription dominate the first peak, while genes coding for proteins involved in redox homeostasis, heat shock, and pathogen defense response dominate the second peak. Chloroplast-targeted proteins involved in photosynthesis comprise a significant fraction of all of the down-regulated genes. The average (normalized) profiles of these groups are shown in Figure 7, together with curves of ROS production during this period (Joo *et al.*, 2005). The expression levels of most of the genes represented on our microarrays that respond markedly to the O_3 treatment conditions used here have returned to nearly pre-exposure levels by 12 h. However, some genes, including those coding for PR proteins, achieve maximum expression levels at 9 h or later after the onset of exposure. This is likely to account for the minimal overlap between the results of the present study and those reported in an earlier study of O_3-responsive genes in *Arabidopsis* Col-0 plants exposed to 200 ppb of O_3 for 12 h (Tamaoki *et al.*, 2003). Direct comparisons with other studies on oxidative stress transcriptome (Desikan *et al.*, 2001; Matsuyama *et al.*, 2002; Vandenabeele *et al.*, 2003, 2004) are difficult because of differences in technical platforms and biological materials used for the experiments; nonetheless, common stress-responsive genes have been identified in all of these studies.

As discussed above, it is likely that chloroplast redox signals trigger the rapid early spike in transcript levels during the first 1.5 h. Moreover, intracellular redox changes may also drive the subsequent evolution of the stress response. Genes coding for proteins involved in redox homeostasis show transcript abundance peaks at 3 h, possibly in response to enhanced transcription by the TFs expressed during the first transcriptional response. The resulting enhanced capacity to reduce ROS coincides with and probably causes the ensuing decline in ROS to a minimum at 3–4.5 h (Figure 7B). Either the increasingly reducing environment or, more probably, the expression of specific redox-active proteins such as thioredoxins, activates expression of SA-dependent genes by the NPR1 protein. NPR1 protein exists in the cytoplasm in an oligomeric form interconnected by disulfide bonds (Mou *et al.*, 2003). Reduction of the intermolecular bonds releases NPR1 monomers, which then enter the nucleus and interact with TGA transcription factors to activate SAR genes (Fan and Dong, 2002). SA response elements and TGA binding sites are overrepresented in the genes whose transcript abundance peaks at 4.5 h, consistent with their activation by the NPR1-TGA complex. Thus, the first peak of transcript abundance is triggered by redox signals from chloroplasts, while the second peak is triggered by redox homeostatic genes expressed during the first peak (Figure 7B).

The rapid decline in transcript abundance of genes in the 3–4.5 h peak coincides both with the subsequent rise in ROS and the rise in transcript abundance both of up-regulated genes belonging to the 9 h PR protein peak and the genes that were down-regulated during the first 3 h of the stress response. The relationship between the ROS produced during the late oxidative burst and the changes in transcript levels can readily be defined using available inhibitors and mutants that affect the late oxidative burst (Joo *et al.*, 2005).

Post-transcriptional mechanisms of gene regulation in the oxidative stress response

A striking finding in the present experiments is the rapidity with which transcript levels rise and fall in stressed plants. Transcript levels for some genes increase by more than 20-fold in the first 30–60 min after the onset of O_3 exposure, then decline just as rapidly (Figure 4). Many of the genes expressed later in the response are likewise expressed for a very brief period. Preliminary studies on several genes, including the MAPK3 and WRKY33 genes, reveal that their transcripts are quite stable in unperturbed plants (S. Goud and N. Fedoroff, unpublished). This implies that the increase in transcript abundance is transcriptional, while the subsequent decrease occurs by an accelerated turnover mechanism. Recent reports of stress-induced increases in microRNA (miRNA) abundance suggests the possibility that the brief bursts of gene expression observed at various times during the stress response are controlled by activating an siRNA- or miRNA-mediated degradation mechanism (Jones-Rhoades and Bartel, 2004).

Acknowledgements

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