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Catalase deficiency drastically affects gene expression induced by high light in *Arabidopsis thaliana*

Steven Vandenabeele[1,†], Sandy Vanderauwera[1,‡], Marnik Vuylsteke[1,‡], Stephane Rombauts[1], Christian Langebartels[2], Harald K. Seidlitz[2], Marc Zabeau[1], Marc Van Montagu[1], Dirk Inzé[1,*] and Frank Van Breusegem[1]

[1]*Department of Plant Systems Biology, Flanders Interuniversity Institute for Biotechnology, Ghent University, Technologiepark 927, B-9052 Ghent, Belgium, and*

[2]*GSF-National Research Center for Environment and Health, Institute of Biochemical Plant Pathology and Department of Environmental Engineering, D-85764 Neuherberg, Germany*

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*For correspondence (fax +32 9 3313809; e-mail dirk.inze@psb.ugent.be).

†Present address: Laboratory of Plant Molecular Biology, The Rockefeller University, New York, NY 10021, USA.

‡Both authors contributed equally to the paper.

Summary

In plants, hydrogen peroxide (H_2O_2) plays a major signaling role in triggering both a defense response and cell death. Increased cellular H_2O_2 levels and subsequent redox imbalances are managed at the production and scavenging levels. Because catalases are the major H_2O_2 scavengers that remove the bulk of cellular H_2O_2, altering their levels allows *in planta* modulation of H_2O_2 concentrations. Reduced peroxisomal catalase activity increased sensitivity toward both ozone and photorespiratory H_2O_2-induced cell death in transgenic catalase-deficient *Arabidopsis thaliana*. These plants were used as a model system to build a comprehensive inventory of transcriptomic variations, which were triggered by photorespiratory H_2O_2 induced by high-light (HL) irradiance. In addition to an H_2O_2-dependent and -independent type of transcriptional response during light stress, microarray analysis on both control and transgenic catalase-deficient plants, exposed to 0, 3, 8, and 23 h of HL, revealed several specific regulatory patterns of gene expression. Thus, photorespiratory H_2O_2 has a direct impact on transcriptional programs in plants.

Keywords: catalase deficiency, *Arabidopsis thaliana*, microarray, hydrogen peroxide, high-light irradiation.

Introduction

A fine-tuned balance between hydrogen peroxide (H_2O_2) production and its removal is crucial for plant survival. However, not only does perturbed H_2O_2 management lead to cellular damage, but also moderately elevated H_2O_2 levels induce a defensive response during both biotic and abiotic stresses (Dat *et al.*, 2000). Such a defense response encompasses acclimation (Karpinski *et al.*, 1999; Prasad *et al.*, 1994), cross-tolerance (Bowler and Fluhr, 2000; Pastori and Foyer, 2002), and the induction of active cell death processes (Dat *et al.*, 2003; Levine *et al.*, 1994).

Like other eukaryotes, plants have an elaborate antioxidant system to control reactive oxygen species (ROS) concentrations during fluctuating environmental conditions. The different components of the enzymatic antioxidant system are diversified in their scavenging capacity not only in terms of specificity and affinity towards the different ROS species (e.g. superoxide, H_2O_2, singlet oxygen), but also by their appropriate subcellular localization in the vicinity of ROS production sites (Vranová *et al.*, 2002). An important source of H_2O_2 during light stress is the photochemical quenching of excess light by the Mehler reaction and by photorespiration. In C_3 plants, photorespiratory H_2O_2 production would account for the majority of total H_2O_2 formed (Karpinski *et al.*, 2003; Noctor *et al.*, 2002). This abundant H_2O_2 production is mainly counteracted by peroxisomal catalase, although other antioxidative enzymes are active in the leaf peroxisome (Corpas *et al.*, 2001). Catalase is a tetrameric iron porphyrin that catalyzes the dismutation of H_2O_2 to water and oxygen. Peroxisomal catalase, perturbed by mutation or gene silencing, results in decreased H_2O_2 scavenging during high-light (HL) irradiation or low CO_2 concentrations in different C_3 plants. These catalase-deficient plants were not only more sensitive to a variety of environmental stress factors, such as ozone and salt stress (Kendall *et al.*,

1983; Willekens *et al.*, 1997), but also became very useful tools to study the signaling role of photorespiratory H_2O_2 *in planta*. In catalase-deficient tobacco, elevated photorespiration-derived H_2O_2 is able to trigger both local and systemic defense responses and activate a cell death program that shares several features of programmed cell death (Chamnongpol *et al.*, 1998; Dat *et al.*, 2003). In a catalase-deficient barley mutant, Noctor *et al.* (2002) nicely demonstrated the ability of photorespiratory H_2O_2 to perturb antioxidant redox states in leaves. Such redox perturbations are inevitably potent drivers of signal transduction events at both the biochemical and the gene expression levels (Desikan *et al.*, 2001; Vandenabeele *et al.*, 2003).

In *Arabidopsis thaliana*, the catalase (*cat*) multigene family contains three genes (At4g35090, At1g20630, and At1g20620) with nucleotide and amino acid sequences 70–72% and 75–84% identity, respectively (Frugoli *et al.*, 1996). All three genes are highly expressed in inflorescences, but only the *cat2* and *cat3* genes are highly expressed in leaves and are both under the control of the circadian clock. Six different enzyme isoforms have been described in *Arabidopsis* (Michael and McClung, 2002; Zhong and McClung, 1996). Here, we report the first production of transgenic *Arabidopsis* plants that are perturbed in their most predominant leaf catalase (CAT2). The importance of peroxisomal catalase was confirmed because these transgenic plants were more sensitive to ozone stress and, more importantly, these plants allowed us to study comprehensively the transcriptomic variances induced by photorespiratory H_2O_2.

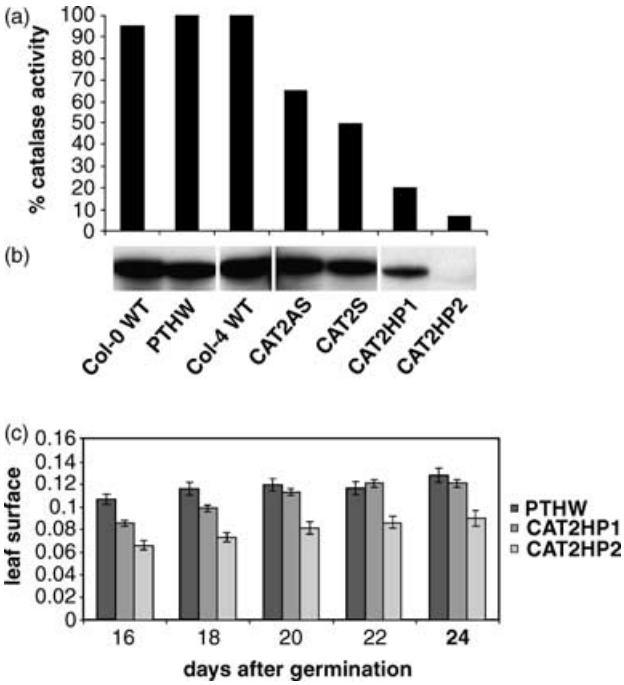


Figure 1. Catalase levels and growth characteristics of transgenic plants.
(a) Measurements of catalase activity with a spectrophotometric assay. Catalase activity was lower in the catalase-deficient lines. Activity levels were in agreement with protein levels shown in (b). Results are average of measurements on three plants of each line. The control line (PTHW) was used as a reference.
(b) Immunodetection of CAT2 isozyme by using antiserum against a cotton-seed catalase (Mullen *et al.*, 1997). Catalase protein levels correlated with the residual catalase activity in catalase-deficient plants.
(c) Surface area measurements of leaves 1 and 2, indicating reduced size of CAT2HP2 plants. Leaf surface is presented as average leaf surface ($n = 8$–10) $\pm$ SE.

Results and discussion

Transgenic Arabidopsis thaliana *with different levels of residual catalase are more stress sensitive*

Transgenic lines of *Arabidopsis* with various levels of decreased CAT2 activity were produced (for details, see Experimental procedures). Transcript, protein, and activity levels of CAT2, which are usually high in leaves, were reduced in four independent lines (CAT2AS, CAT2S, CAT2HP1, and CAT2HP2), retaining 65, 50, 20, and 7% of total residual catalase activity levels, respectively (Figure 1a,b). Under ambient growth conditions *in vitro* or in controlled growth chambers, no obvious phenotypic differences were noticeable between transgenic and control plants (wild-type or empty-vector control PTHW), except for CAT2HP2 plants, which showed significant growth retardation. These plants were on average 30% smaller than the control plants 24 days after germination (Figure 1c) and never reached a normal size at later developmental stages. DW and FW measurements confirmed the observed growth differences (see Experimental procedures and Supplementary Material).

Although the molecular mechanisms remain unresolved, elevated levels of ROS are known to affect both growth and productivity of plants. Other mutants and transgenic *Arabidopsis* plants, deficient in their antioxidative potential, were also affected in growth. Pnueli *et al.* (2003) reported knockout ascorbate peroxidase (Apx)1 plants with a suppressed growth and development phenotype. Even though in the vitamin C-deficient mutant *vtc1*, decreased ascorbate pools, and not increased H_2O_2 levels, were responsible for growth deficiencies, severe perturbations within the antioxidant machinery had apparently serious consequences for cell growth and/or division (Pignocchi *et al.*, 2003). CAT2HP2 was also able to undergo spontaneous cell death in individual plants. Spontaneous lesion formation in catalase-deficient plants has already been reported in a mutant barley line with 5–10% residual catalase activity (Smith *et al.*, 1984). Because of this spontaneous lesion formation and its differential growth capacities, the CAT2HP2 line was omitted from further analyses. To assess whether *cat1* and

cat3 transcript levels were affected in the transgenic plants, we performed a quantitative real-time PCR experiment. With gene-specific primers, we determined *cat1*, *cat2*, and *cat3* transcript levels in PTHW and in both CAT2AS and CAT2HP1. No significant differences were observed in *cat1* and *cat3* transcript levels within both the catalase-deficient lines and the control, whereas *cat2* transcript levels were clearly reduced in the catalase-deficient lines. However, because all *cat* genes could be involved in formation of peroxisomal CAT isoforms, we cannot exclude a decrease in general catalase activity levels because of a specific silencing of *cat2*. Therefore, we will refer to catalase-deficient instead of CAT2-deficient lines (Frugoli *et al.*, 1996).

Several adverse environmental conditions, such as heavy metal stress, drought, temperature stress, and air pollution, provoke oxidative stress, and consequently affect growth and productivity of plants (Van Breusegem and Inzé, 2002). To assess oxidative stress sensitivity of catalase-deficient lines, the lines were exposed for 5 days to elevated ozone concentrations (350 nl l^{-1}, 8 h per day). Cell death on the middle-aged leaves in a population of 100–150 plants per line and the percentage of damaged leaf surface on 10–15 plants per line were scored. Increased ozone sensitivity was inversely correlated with residual catalase activity (Figure 2). Columbia (Col-0) accessions are relatively resistant to ozone stress (Wohlgemuth *et al.*, 2002), and this resistance was clearly confirmed in this experiment because rarely cell death was scored in the control lines. Increased ozone sensitivity also correlated with increased diaminobenzidine (DAB) staining, which is indicative of increased H_2O_2 levels. No differences were observed in the nitro blue tetrazolium (NBT) staining patterns, showing no differential superoxide accumulation between transgenic and control lines (data not shown). These results indicate that catalase, like other antioxidants, is a determining factor in the ozone-resistance phenotype of *Arabidopsis* accession Col-0. Whether differences in catalase activity levels are also responsible for the variable sensitivity of different *Arabidopsis* accessions remains to be investigated.

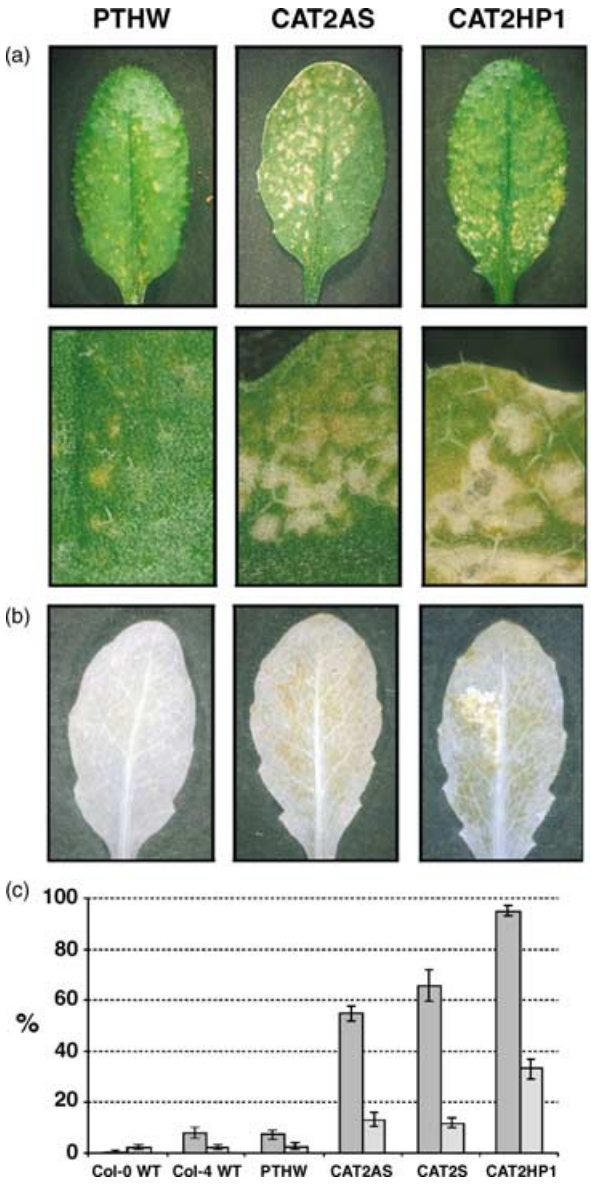


Figure 2. Cell death triggered by ozone stress in catalase-deficient plants.
(a) Cell death phenology after 3 days of ozone exposure.
(b) H_2O_2 accumulation after a 5 h ozone treatment (350 nl l^{-1}). Middle-aged leaves were stained with DAB.
(c) Quantification of cell death after 3 days of ozone (350 nl l^{-1}). Dark gray bars represent the percentage of plants with visually scored cell death events. Data are presented as average (n = 100–150) ± SE. Light gray bars indicate the percentage of the leaf area that was damaged. Data are presented as average (n = 10–15) ± SE. Control and catalase-deficient plants were exposed to ozone (350 nl l^{-1}) for 5 days.

HL-induced H_2O_2 and cell death in catalase-deficient Arabidopsis thaliana

Despite their extensive antioxidative capacities, peroxisomes are capable of generating and releasing signal molecules, such as H_2O_2, into the cytosol under specific conditions (Corpas *et al.*, 2001). H_2O_2 production is elevated during photorespiration. HL irradiation induces photorespiration and consequently strongly increases H_2O_2 levels in catalase-deficient plants (Chamnongpol *et al.*, 1998; Dat *et al.*, 2003). We assessed the response of the catalase-deficient *Arabidopsis* plants under HL irradiation conditions (1600 μmol m^{-2} sec^{-1}), which correspond to a bright, summer sunny day at mid-northern latitudes. Six-week-old control and catalase-deficient plants were exposed to HL for 23 h. Within such a long-term exposure, leaves from control plants colored brown-purple, probably because of the accumulation of anthocyanins (data not shown). In catalase-deficient plants, this

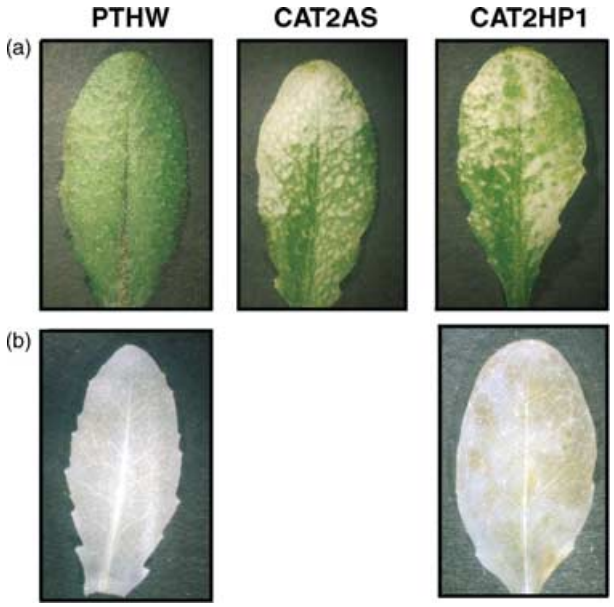


Figure 3. H_2O_2 accumulation and cell death triggered by HL irradiation in catalase-deficient plants.
(a) Control and catalase-deficient plants were exposed to HL (1600 μmol m^{-2} sec^{-1}) for 23 h. Cell death was visible after 8 h of HL only on the leaves of catalase-deficient lines. Middle-aged leaves of control plants (PTHW) and two catalase-deficient lines (CAT2AS and CAT2HP1) are shown.
(b) H_2O_2 accumulation. Control and catalase-deficient lines were illuminated at 1600 μmol m^{-2} sec^{-1} for 5 h, and middle-aged leaves were stained with DAB.

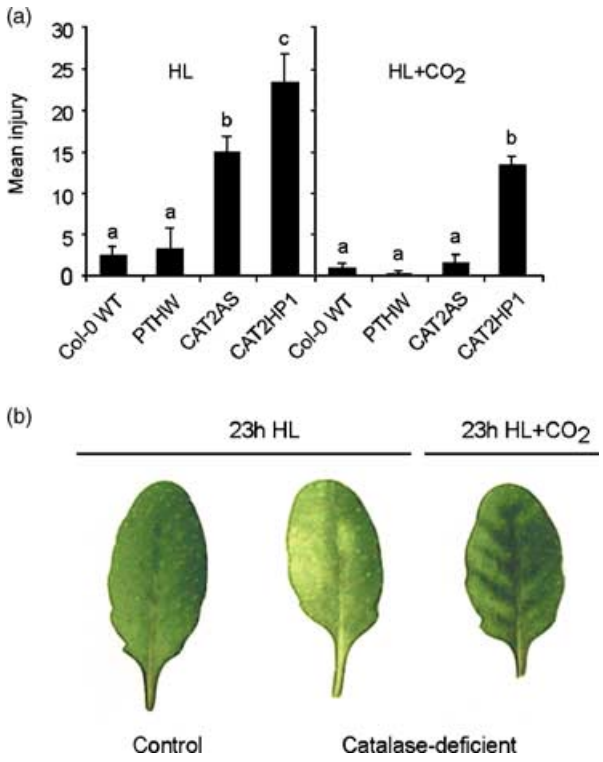


Figure 4. Suppression of cell death in catalase-deficient plants exposed to HL combined with high CO_2 levels.
(a) Quantification of cell death. Bars represent the mean injury of middle-aged leaves of five plants with visually scored cell death events in control plants (Col-0 WT and PTHW) and two catalase-deficient lines (CAT2AS and CAT2HP1). High CO_2 levels significantly reduce leaf injury.
(b) Representative middle-aged leaves of control and catalase-deficient line plants, were exposed to HL (1600 μmol m^{-2} sec^{-1}) for 23 h under ambient air conditions (400 p.p.m., 21% O_2) and under high CO_2 levels (1500 p.p.m., 21% O_2).

coloration was impaired and within 8 h of HL, cell death was visible. Both the timing and the extent of cell death were inversely correlated with the residual catalase activity in the different transgenic lines. Nomarski optics revealed that cell death occurred both in the mesophyll and palisade parenchyma layer, whereas trypan blue staining of leaves exposed for 24 h indicated its spatial distribution. Cell death was detected predominantly at the top of the leaf and in the vicinity of the veins (data not shown). Both catalase-deficient and control plants were also infiltrated with DAB and NBT after 5 and 23 h of HL. Whereas with NBT staining, no difference in superoxide accumulation was seen between control and catalase-deficient plants, DAB staining showed that cell death correlated spatially with increased H_2O_2 levels (Figure 3).

To validate the photorespiratory nature of the elevated H_2O_2 levels in HL-treated catalase-deficient plants, plants were exposed to HL under ambient air conditions (400 p.p.m., 21% O_2) and under high CO_2 levels (1500 p.p.m., 21% O_2). As expected, high CO_2 levels clearly impaired the induction of cell death in the catalase-deficient plants exposed to HL (Figure 4).

Thus, as shown before in catalase-deficient tobacco, a severe perturbation of catalase, combined with HL irradiance, allows H_2O_2 to accumulate, followed by the advent of cell death. Hence, these *Arabidopsis* lines could be used as tools to study the signaling role of H_2O_2.

Transcriptome analysis of HL-treated control and catalase-deficient plants

Previously reported H_2O_2 transcriptome studies relied on the use of cell suspensions and exogenously applied H_2O_2 and transcriptional changes were mostly assessed at only one time point during the stress treatment. Pnueli *et al.* (2003) described the transcriptome in *Arabidopsis* plants deficient in cytosolic Apx. The catalase-deficient transgenic lines allow us to follow transcriptional changes during a sustained H_2O_2 stress over time. By using two different transgenic lines (CAT2AS and CAT2HP1, each containing 65 and 20% residual catalase activity, respectively), we were also able to assess a potential relative responsiveness of the plant transcriptome toward increasing H_2O_2 levels. Twenty individual plants of the CAT2AS, CAT2HP1, and control lines were exposed continuously to a

1600 μmol m^{-2} sec^{-1} HL treatment for 0, 3, 8, and 23 h. Catalase-deficient plants developed visible cell death after 8 h of HL, while in the control plants, no cell death was evident even after 23 h of HL.

For the microarray study, a reference design was used that consisted of 12 dye-swap experiments in which pools of test samples were compared with a reference sample on a total of 24 cDNA microarrays. These microarrays had duplicated sets of 6008 cDNA clones of *Arabidopsis*. The 12 test samples hybridized on the microarrays were the pooled middle-aged leaves of CAT2AS, CAT2HP1, and the control line harvested at each of the four time points, while the reference sample represents a pool of all lines harvested at 0 and 23 h. None of the 6008 genes were uniformly below the signal threshold (see Experimental procedures). To select genes that are significantly differentially expressed over time between the three lines, we assessed for each gene, the significance of the interaction between genotype and time (the effect of primary interest) beyond that of the main effects genotype and time, which are of secondary interest. Therefore, we applied two sequentially mixed model analyses of variance (ANOVAs; Wolfinger *et al.*, 2001) to the base-2 logarithm of all the 'lowess'-transformed spot measurements with the residual maximum likelihood method (see Experimental procedures). As a measure of variability in expression levels among the effects, we used Wald statistics, which were tested against the χ^2 distribution. The *P*-value cut-off was set arbitrarily at the stringent level of 0.001. No further adjustments for multiple testing were performed. A significant difference in expression between catalase-deficient and control plants over time was found for 1552 genes. All expression data are available according to minimum information about a microarray experiment guidelines at http://www.psb.ugent.be/supplementary_data.

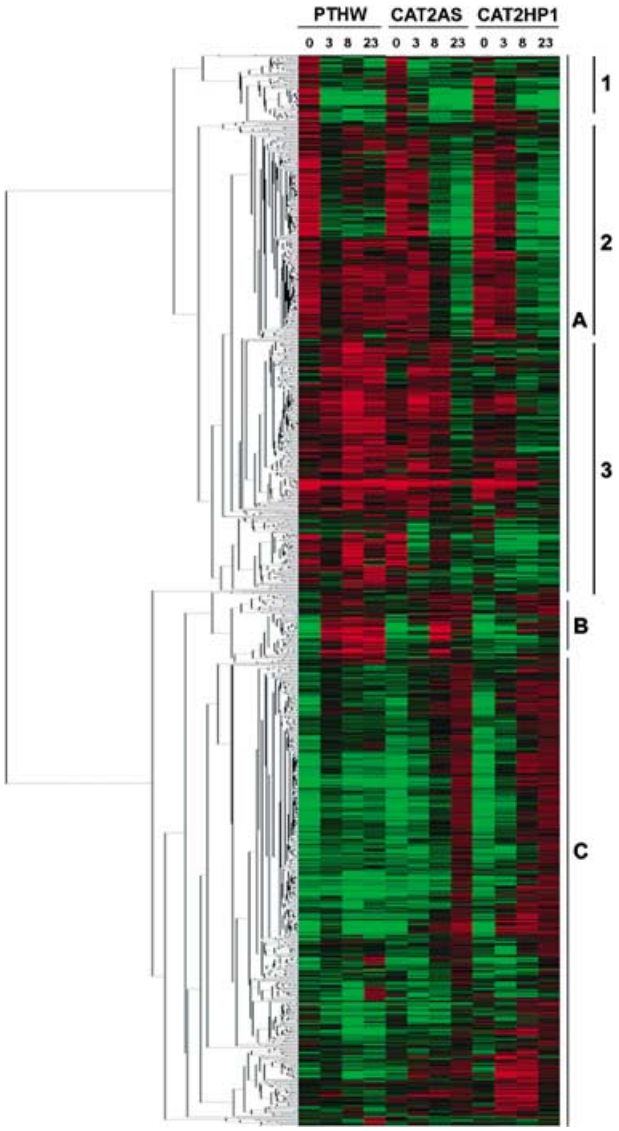


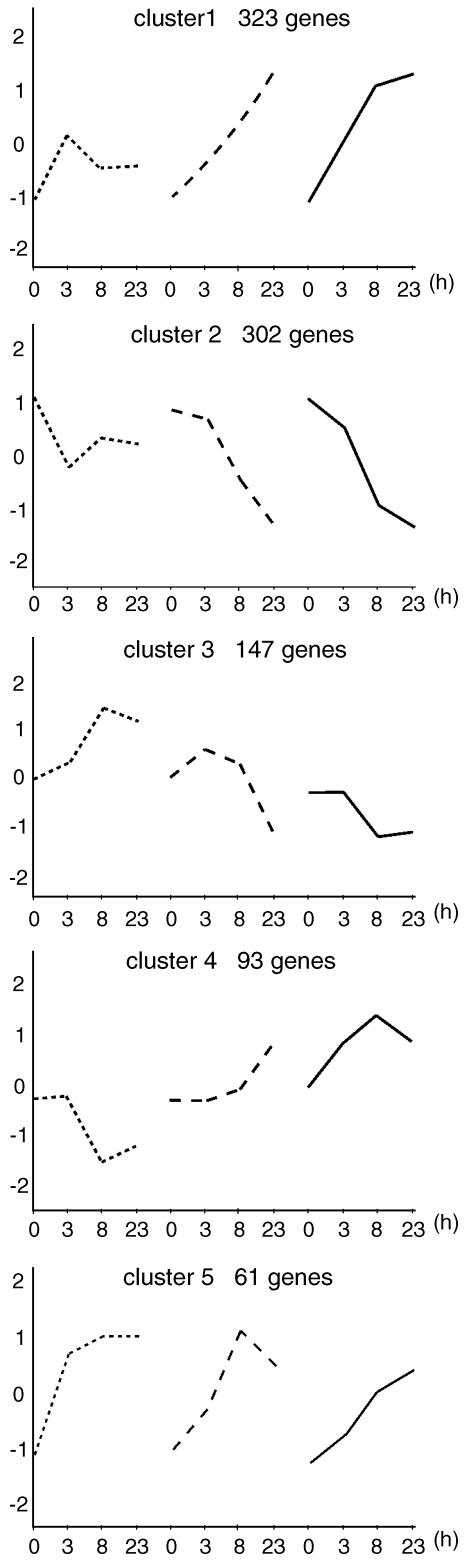
Figure 5. Hierarchical average linkage clustering of 1552 significantly differentially expressed genes between the catalase-deficient *A. thaliana* lines and the control line during the HL time course.
Each column represents the time point of sampling during HL treatment (0, 3, 8, and 23 h) in control (PTHW) and catalase-deficient plants (CAT2AS and CAT2HP1). Each row represents the expression profile of a transcript. Expression data were processed as described (see Experimental procedures). Three main clusters of expression (A, B, and C) are indicated on the right. Cluster 1 was divided into three subclusters (A1, A2, and A3).

Hierarchical and adaptive quality-based clustering reveal specific patterns of H_2O_2-regulated gene expression

For the 1552 genes selected as differentially expressed over time between the three lines, we used hierarchical average linkage clustering (HALC) and adaptive quality-based clustering (AQBC) to group genes with similar expression profiles (De Smet *et al.*, 2002; Eisen *et al.*, 1998). HALC of all differentially expressed genes resulted in three main clusters. Cluster A comprises all genes that are downregulated in the two catalase-deficient lines (Figure 5). They were further grouped based on their expression profile in the control line: rapidly downregulated (cluster A1); downregulated at 3 h, but upregulated again after 8 h (cluster A2); and maximally expressed after 8 h in the control line (cluster A3). Cluster B groups genes that were induced by HL stress in control plants, but whose induction was retarded or completely abolished in the catalase-deficient lines. Finally, the most predominant cluster C includes genes that were not or only transiently induced in the control line, but were sustainably upregulated in the two catalase-deficient lines during the HL treatment.

AQBC of the differentially expressed genes resulted in 31 different clusters (required probability, 0.97; minimal number of genes in a cluster, 5) containing a total of 1248 genes (Figure 6; http://www.psb.ugent.be/supplementary_data).

Clusters 1 and 4 included transcripts induced exclusively in the catalase-deficient lines and corresponded largely to cluster C of the HALC. Clusters 2 and 3 contain transcripts that are downregulated and/or expressed at a lower level from the start in the catalase-deficient lines (approximately cluster A of HALC). The induction of 64 genes belonging to cluster 5 was delayed or prevented in catalase-deficient lines (approximately cluster B of HALC). These five main clusters together contain 929 genes (75% of the genes that were included in an AQBC cluster with the above mentioned parameters).



The perturbation of H_2O_2-scavenging capacities has a significant effect on nuclear gene expression when plants are exposed to HL irradiation. Therefore, we wanted to define specifically in which of the 1552 differentially expressed genes, HL irradiation played an important role in combination to their H_2O_2 responsiveness. Exposure of *Arabidopsis* to HL intensities for 1 h led to a differential expression of at least 185 genes (Rossel *et al.*, 2002), and cyanobacterial acclimation to HL intensities was accompanied by changes in gene expression of at least 160 genes (Hihara *et al.*, 2001). Here, screening for genes differentially expressed by HL irradiation only in the control plant revealed 1561 genes responsive to HL (see http://www.psb.ugent.be/supplementary_data). Of these 1561 genes, 910 genes were affected in their responsiveness to HL in catalase-deficient plants. Consequently, the expression of 642 genes was only modulated by H_2O_2 in the catalase-deficient background, suggesting that these genes responded uniquely to H_2O_2. This responsiveness correlated with increasing levels of photorespiratory H_2O_2 because kinetics and amplitude of the response were stronger in the CAT2HP1 line, containing only 20% of residual catalase activity, than in the CAT2AS line with 65% of residual catalase activity (Figure 5). Table 1 presents the top 30 significantly upregulated transcripts with a minimal 10-fold upregulation in the catalase-deficient lines during HL exposure. The complete list of differentially expressed genes is available at http://www.psb.ugent.be/supplementary_data.

Our results confirm the presence of both an H_2O_2-dependent and -independent HL-triggered regulatory pathway (Kimura *et al.*, 2001). Interestingly, we can also note a modest transient induction or repression for most of the 642 strictly H_2O_2-modulated genes (clusters A2 and C; Figure 5) after 3 h of HL in control plants. This probably reflects responsiveness toward oxidative stress that rises transiently in control plants within the first hour of HL

Figure 6. Adaptive quality-based clustering.
The 1552 differentially expressed genes were clustered with the AQBC software (De Smet *et al.*, 2002). For each cluster, the number of genes and the trend line, representing the average profile, is shown: dotted, control (PTHW); dashed, CAT2AS (65% catalase activity); and solid, CAT2HP1 (20% catalase activity). Each trend line connects, for each plant line, the four time points during the HL treatment at which the samples were collected. Five main clusters are shown; the remaining smaller clusters are available at http://www.psb.ugent.be/supplementary_data.

Table 1 Top 30 of significantly ($P < 0.001$) upregulated genes in catalase-deficient plants (CAT2HP1) compared with control plants (PTHW) from HALC cluster C ranked according to their maximal fold change observed during HL exposure

EST accession number	Gene annotation	AGI code	Fold change	Time point (h)
AI998904	17.6-kDa class I small heat shock protein	At1g53540	85.9	8
AI992977	Expressed protein	At1g05340	44.3	8
AI998968	Heat shock protein 101	At1g74310	30.9	8
AW004348	Dihydroflavonol 4-reductase	At5g42800	28.0	3
AI998408	17.7-kDa class II heat shock protein 17.6 A	At5g12030	27.7	8
AI993358	Legume lectin family protein	At3g16530	25.9	8
AW004568	Putative mannitol dehydrogenase ELI3-2	At4g37990	22.2	23
AI996814	β-Fructosidase BFRUCT1/cell wall invertase	At3g13790	21.7	8
AI996408	Arabinogalactan protein AGP2	At2g22470	20.7	8
AI996535	Plastocyanin-like domain-containing protein	At5g20230	20.6	8
AI995531	18.1-kDa class I heat shock protein	At5g59720	19.9	8
AI993740	Expressed protein	At2g40000	19.8	8
AW004579	Mutator-like transposase	At1g33860	17.6	8
AI993868	Putative cytochrome P450	At2g45570	17.2	23
AI994052	Putative heat shock protein 70	At3g12580	16.6	8
AI993534	Heat shock protein 81-1	At5g52640	16.6	8
AI994585	Expressed protein	At4g39670	16.3	8
AI997508	Expressed protein	At2g18690	15.2	8
AI992420	Putative cytochrome P450	At2g30770	15.0	23
AW004360	Ribonuclease 1	At2g02990	15.0	23
AI996816	Arabinogalactan protein AGP1	At5g64310	14.6	8
AI998304	Trypsin and protease inhibitor family protein	At1g73260	13.6	8
AI994827	Glycosyl hydrolase family 17 protein	At4g16260	13.3	8
AI992663	Expressed protein	At2g27860	13.3	8
AI997082	Anthranilate synthase, α subunit, component I-1 ASA1	At5g05730	13.0	23
AI996685	Lateral organ boundaries domain protein 41	At3g02550	12.9	8
AI993083	Hevein-like protein	At3g04720	11.6	8
AI997494	Mitogen-activated protein kinase MPK17	At2g01450	11.1	8
AI998638	Protease inhibitor/seed storage/lipid transfer protein LTP family protein	At3g22600	10.7	23
AI993906	Class IV chitinase	At3g54420	10.5	8

Fold change refers to the maximal induction in catalase-deficient plants relative to the control plants observed at the time point at which the maximal fold change induction was observed. Complete tables of microarray results of all lines can be found at http://www.psb.ugent.be/supplementary_data.

exposure and is able to affect nuclear gene expression. Because these genes are clearly either continuously up- or downregulated in catalase-deficient plants, we can assume that in the control plants also, this transient H_2O_2 responsiveness is provoked by photorespiratory H_2O_2, but that it is probably efficiently counteracted by the antioxidant system. High levels of photorespiratory H_2O_2 can explain the abolishment of an adaptive response in the two catalase-deficient lines. In addition, cell death events at 8 h of HL in catalase-deficient plants will be able to amplify the signal, thereby sustaining the transcriptional response in these lines even more. Although we cannot discriminate between a direct responsiveness toward elevated photorespiratory H_2O_2 and a more integrated response via additional H_2O_2-steered oxidative stress at other subcellular locations, such as chloroplasts (i.e. photoinhibition) or mitochondria, our results clearly consolidate earlier reports on a potent early signaling role of peroxisomal H_2O_2 (Corpas *et al.*, 2001; Noctor *et al.*, 2002).

Functional categories of genes with H_2O_2-driven transcriptional changes

A complete overview of the H_2O_2-responsive genes is available at http://www.psb.ugent.be/supplementary_data. Based on the automatically derived functional categories as described by the Munich Information Center for Protein Sequences (MIPS; http://mips.gsf.de; Schoof *et al.*, 2002), the 1552 H_2O_2-responsive transcripts could be classified into 16 functional groups. A potential over-representation was assessed by comparing the number of genes of a specific functional category within a cluster with the total amount of genes present on the microarray classified within this category. These functional categories within the six largest AQBC clusters are presented in Table S1. In the AQBC cluster 1, genes that are involved in protein synthesis, protein fate, and energy are over-represented, whereas in AQBC cluster 4 (Figure 6), the group of cell rescue, defense, and virulence is dominant; AQBC clusters

5 and 6 contain mainly metabolic genes. Because these functional categories are automatically derived from the MIPS database, the use of this information is limited for the biological interpretation of genome-wide expression data. Therefore, we have additionally assessed specific functional categories, allowing us to analyze and interpret the microarray data into more detail.

H_2O_2 drives upregulation of the translational and proteolytic machinery

Together with an upregulation of several splicing-related components, the translational machinery is clearly transcriptionally upregulated because several cytosolic ribosomal proteins (60S components L30, L18A, and L6), and at least eight cytosolic translation initiation factors (EF1A, EF1Bγ, eIF3a, eIF3b, eIF3c, eIF3e, eIF5, eIF6, and eRF1) and a translation release factor are present in the AQBC cluster 1. Although active repression of protein synthesis protects cells against protein misfolding during oxidative stress, a long-term adaptation could involve an increase of proteins necessary for the translation of genes involved in this adaptation (Novoa *et al.*, 2003). In yeast and plants, Rausell *et al.* (2003) have recently shown that the translational factor eIF1A is an important determinator in salt stress tolerance. Proteolysis is also clearly upregulated by H_2O_2. Four (poly)ubiquitin genes, together with a ubiquitin-conjugating enzyme-like protein, are upregulated (Bachmair *et al.*, 2001). Within the AQBC cluster 1, four proteins contain an F-box motif, three a WD40 repeat, and six a RING-finger domain. Additionally, five different components of the 26S proteasome and three proteasome regulatory subunits are upregulated by H_2O_2. Whether H_2O_2-increased proteolysis within our system solely reflects the increased demand for removal of misfolded proteins or it plays a signaling role by targeted degradation of regulatory components within the defense response of programmed cell death remains to be elucidated (Estelle, 2001).

H_2O_2 reverses or mitigates HL-driven expression of antioxidant, hypersensitive response-related and anthocyanin biosynthetic genes

Whereas in the AQBC clusters 1 and 2, the expression of genes in catalase-deficient plants was mainly induced or repressed while their profile hardly changed in control plants, expression of genes in clusters 3–5 changed in the control line, where H_2O_2 exerted an additionally suppressive or inducing effect in the catalase-deficient lines. Cluster 3 contains genes that are induced in the control line and whose induction is delayed in the catalase-deficient lines. Surprisingly, four antioxidant genes belong to this cluster: one coding for a Cu/Zn superoxide dismutase-like protein and three for peroxidase. Cluster 4 groups genes that are downregulated by HL in the control plants, but are induced in the catalase-deficient plants by HL. Here, several genes are present that are classically linked with the hypersensitive response, including the oxidative burst (*A. thaliana respiratory burst oxidase homolog* (*atrbohC*); Torres *et al.*, 2002). The exact function of AtrbohC is unknown, but because of its H_2O_2-triggered upregulation, it probably plays a role in the induction of cell death and defense response. Interestingly, a hevein-like protein, which is an allergenic lectin from rubber latex that is able to activate oxidative burst of human neutrophils (Rojas *et al.*, 2001); four resistance (*R*) genes, of which two containing an NB-ARC domain (van der Biezen and Jones, 1998); and two β-1,3-glucanases are upregulated. Within the hypersensitive cell death response, Ca^{2+} fluxes are an important early signal (Blume *et al.*, 2000). In cluster 4, an anion channel protein, a Ca^{2+}-transporting ATPase, and a calmodulin domain protein kinase (Hrabak *et al.*, 1996) are present. Four other genes contain an EF hand motif (Day *et al.*, 2002). A concomitant downregulation of specific antioxidant enzymes and the upregulation of hypersensitive response-related genes can contribute to the observed cell death in catalase-deficient plants (Clarke *et al.*, 2002; Fath *et al.*, 2001; Király *et al.*, 2002). Cluster 5 contains at least three genes involved mainly in phenylpropanoid biosynthesis and, in particular, in anthocyanin biosynthesis (phenylalanine ammonia lyase, chalcone synthase, chalcone isomerase, and dihydroflavonol 4-reductase). The induction of these genes is delayed and reduced in the catalase-deficient plants. Leaves of control plants colored mostly brown-purple during long-term HL exposure. This coloration was not observed in catalase-deficient plants (data not shown).

H_2O_2-dependent masterswitch controls part of the HL-induced gene expression

Within the experimental set-up, we followed nuclear gene expression changes provoked by two primary perturbations: exposure to HL irradiation and peroxisomal catalase deficiency. The combination of these perturbations led to a third primary perturbation in the transgenic lines: elevated photorespiratory H_2O_2, which allowed us to assess transcriptomal changes provoked by HL and H_2O_2 only. We were able to identify not only a signaling role for H_2O_2 within the HL response, but also the negative impinging of elevated levels of photorespiratory H_2O_2 at the HL-modulated gene expression. Our results suggest that a masterswitch transcriptionally controls the HL response of plants, as already described for the nuclear chloroplast transcriptome (Richly *et al.*, 2003), and that the activity of this masterswitch can be reversed by elevated H_2O_2 levels originally produced in the peroxisomes.

H_2O_2 regulates nuclear-encoded chloroplastic and mitochondrial proteins

We addressed the potential interplay between different ROS-producing subcellular sites via the expression behavior of nuclear-encoded chloroplastic and mitochondrial proteins (Heazlewood *et al.*, 2003; Kruft *et al.*, 2001; Leister, 2003). The most abundant functional group of genes in the AQBC cluster 2 consists of nuclear-encoded genes involved in photosynthesis. In control plants, at least 17 different nuclear genes coding for photosystem I and II components drop to a basal expression level within 3 h of HL, while in the transgenic plants, this downregulation is delayed but finally more pronounced at 8 and 23 h of HL. This downregulation in control plants probably reflects an adaptation response to the sudden shift to HL intensities, thereby avoiding additional damage caused by the excessive energy influx (Allen and Pfannschmidt, 2000). A similar transcriptional regulation of photosynthetic activities during HL treatment was reported by Rossel *et al.* (2002). The modulation of photosynthetic components is a common reflex during stress, such as the combination of drought and heat shock (Rizhsky *et al.*, 2002). Nuclear-encoded plastid proteins are controlled by a transcriptional switch that functions in a binary mode, either by inducing or by repressing the same large set of genes (Richly *et al.*, 2003). The signals for this downregulation could originate from the chloroplasts themselves because chloroplasts not only provide energy but also are sensors for environmental information and, hence, are clearly involved in interorganellar signaling (Karpinski *et al.*, 1999; Pfannschmidt, 2003; Pfannschmidt *et al.*, 1999). Apparently, catalase perturbation initially impairs the downregulation in the transgenic lines. Possibly, these sustained high expression levels of photosynthetic components promote additional chloroplastic oxidative stress, fastening the induction of cell death, which, in turn, could release signals to decrease the expression of these genes. To obtain a more global view on the expression behavior of nuclear-encoded chloroplastic genes, we analyzed separately all the genes on the array that had been assigned to code for chloroplastic proteins (Leister, 2003). Of the 985 such genes, 23% present on the array had a differential expression pattern. The majority was downregulated or completely repressed and, hence, was present in the AQBC clusters 2 and 3.

An integrative role for mitochondria in oxidative stress signaling and, more specifically, cell death is not only consolidated in mammalian systems (Desagher and Martinou, 2000; Kaufmann and Hengartner, 2001), but is emerging in plants as well (Beers and McDowell, 2001; Dat *et al.*, 2003; Dutilleul *et al.*, 2003; Tiwari *et al.*, 2002). Here, we show that an increase in mitochondrial metabolism is also reflected at the transcriptional level: cytochrome *c*, a cytochrome *c*-like protein, a mitochondrial elongation factor G, chaperonin of heat shock protein 60, two mitochondrial processing peptidases, an uncoupling protein BCS1, and several genes involved in the citrate cycle were upregulated in catalase-deficient plants during HL stress. Our results demonstrate that photorespiratory H_2O_2 influences the expression of both photosynthetic and mitochondrial genes, providing additional evidence for the importance of intraorganellar communication within the plant's defense response.

Signal transduction

Of the 340 transcription factors (Riechmann *et al.*, 2000) present on our microarray, 85 were significantly differentially expressed in the catalase-deficient plants compared to control plants during the HL treatment. Most of them were affected within 3 h of HL, making them strong candidates to be part of the regulatory circuit that governs downstream H_2O_2 processes. As shown in Figure 7, these transcription factors are spread over the different clusters. Predominant categories are AP-2-related transcription factors (12), WRKY (8), Myb (9), and NAC (7) domain-containing factors and, interestingly, five CONSTANS-like transcription factors are also differentially expressed. Among the other transcription factors, we identified factors assigned to the bZIP (4), Zinc finger (4), bHLH (3), a MADS box, two NF-Y (CCA box binding), two SCARECROW (GRAS), two GARP, and one HSF4 family. The complexity of the H_2O_2-driven regulatory network is illustrated by the observation that transcription factors of the same family respond inversely to the same stress situations. AP-2/EREBP factors are not only up- and downregulated, but also present in the HALC cluster B, in which H_2O_2 mitigates the HL responsiveness. In addition to the transcription factors, potential upstream signal transducers are transcriptionally regulated. At least 36 different kinases, including LRR kinases, receptor-like protein kinases, wall-associated kinases, SNF1-like kinases, five members of the known MAPK cascades, calcium-dependent kinases, and several phosphatases are differentially regulated. Now that we have a comprehensive inventory of transcription factors and other signal transducers responsive to an initial H_2O_2 stress, a more detailed analysis will be necessary to clarify how these different factors interconnect with each other and how specific their regulatory role is within the management of the downstream response. Systematic perturbations of these factors and a more detailed kinetic study of their responsiveness toward H_2O_2 will be the initial steps to be taken to unravel the hierarchical structure of the genetic network that governs the H_2O_2 response in plants.

Figure 7. Hierarchical average linkage clustering.
HALC resulted in 127 differentially expressed genes involved in signal transduction. Transcription factors, kinases, and phosphatases were selected for HALC. Each line, representing a differentially expressed gene, contains the unique array spot number of the gene and its description as based on the MIPS database.

Regulatory elements in promoters

We used different approaches and algorithms to detect conserved motifs in the promoters of co-regulated genes. The clusters deduced from the combination of AQBC and HALC defined the potentially co-regulated genes. To reduce the unavoidable noise that might spoil the promoter analysis, we subclustered the larger clusters into smaller groups with gradually more stringent parameters. We analyzed 800 bp of sequence upstream of the ATG and screened for common motifs with the MOTIFSAMPLER, YMF, and the RSA tools (see Experimental procedures). Only one motif (GAAGnnGAwG) was found to be significantly over-represented in the promoters of one set of co-regulated genes, whose expression was repressed by H_2O_2 (AQBC cluster 2; Figure 6). This motif was not found in PlantCARE (Lescot *et al.*, 2002) and, to our knowledge, has not been described before, although, the motif GAAGAA might be recognized as part of motifs known to be involved in stress responses in *Brassica oleracea*. Because of the combination of several perturbations and late sampling, the analyzed data set represents a complex network of regulatory circuits and the presence of secondary effects within each response is not unrealistic. This combined response resulted probably in the introduction of a considerable amount of noise in the data sets and might explain why no other regulatory elements were significantly over-represented within other sets of co-regulated genes. Because different classes of transcription factors are spread over the different clusters, we have to be cautious when drawing conclusions from the identified regulatory elements. Also, despite significant progress, the current algorithms that look for regulatory elements have still significant drawbacks to unambiguously predict regulatory elements within linear promoter fragments. The available software is able to identify potentially good candidate motifs, but currently these predictions can only be confirmed by extensive experiments, which is the subject of a separate investigation (Rombauts *et al.*, 2003).

Experimental procedures

Production of transgenic Arabidopsis thaliana *with decreased CAT2 levels*

Sense and antisense cassettes were constructed by cloning the PCR-amplified (5′-GCGCCATGGTTCCTTACAAGTATCGTCCAG-3′; 5′-CGGGATCCTTAGATGCCTGGTCTCACGTTC-3′) open-reading frames of *cat2* in both sense and antisense directions into the *Nco*I–*Bam*HI sites of the vector pH35S, which resulted in pH35SCATS and pH35SCATAS (Hemerly *et al.*, 1995). Subsequently, the *Eco*RI–*Xba*I fragment of the pH35SCAT(A)S, encompassing the CaMV 35S promoter *cat2* and the 3′ nopaline synthase terminator, was cloned into the binary pBINPLUS vector

containing the neomycin phosphotransferase II (*NPTII*) gene as a selectable marker (van Engelen *et al.*, 1995). For the hairpin construct, a 515-bp PCR product of the 5′ coding sequence of CAT2 (5′-CGCGGATCCTTACAAGTATCG-3′; 5′-CGCGGATCCTCCAGTTCT-CTTGGATGTGAG-3′) was inserted as a *Bam*HI fragment into pH35SCATS and selected for inverted direction by PCR. A *Pst*I fragment was ligated into the *Pst*I-digested pthw142 vector, a binary vector containing the bialaphos resistance gene (*BAR*), which is a resistance gene for tranformant selection. Binary constructs were mobilized into *Agrobacterium tumefaciens* (pMP90) with the *Escherichia coli* helper strain HB101. *A. thaliana* (L) Heyhn. ecotype Col-0 plants were transformed by floral dip (Clough and Bent, 1998). For the hairpin construct, Col-4 plants were used. Residual catalase expression levels in T_1 and T_2 transformants were monitored by Northern analysis with the 3′ untranslated region of *cat2* as a probe and by Western blot analysis with rabbit antiserum against a cotton catalase (Mullen *et al.*, 1997).

Growth measurements, catalase activity measurements, Western blot analysis, and real-time PCR experiments

Seedlings were grown *in vitro* on Murashige and Skoog medium (2% sucrose) for 24 days under 16 h light/8 h dark conditions. Surface area of leaves 1 and 2 (8–10 plants per line) was determined with IMAGEJ software (publically available at http://rsb.info.nih.gov/ij) 18, 20, 22, and 24 days after germination, knowing that leaves 1 and 2 are fully grown 20–22 days after germination (De Veylder *et al.*, 2001).

FW and DW measurements were performed on 2-week-old plants. The plants were grown under a 16 h light/8 h dark photoperiod, with a relative humidity of 70% and temperature of 21°C. Fifteen individual plants of the CAT2AS, CAT2HP1, CAT2HP2, and control lines were weighed just after harvesting (FW) and after 48 h at 70°C (DW). Water content was calculated as follows: (FW − DW)/FW. Statistically significant differences were found in both FW and DW of the CAT2HP2 line, and in FW and nearly in DW of the CAT2HP1 line. No significant differences could be observed in the water content (see Table S2).

Leaf tissue was ground in extraction buffer (60 mM Tris (pH 6.9), 1 mM phenylmethylsulfonylfluoride, 10 mM DTT) on ice with 1 ml extraction buffer for 0.15 g tissue. The homogenate was centrifuged at 18 000 ***g*** for 15 min at 4°C. The supernatant was used for spectrophotometric catalase analysis and SDS–PAGE protein separation. Catalase activity assays were performed according to Clare *et al.* (1984). For Western blot analysis, 50 μg of leaf proteins were separated on a 12% SDS–PAGE gel and hybridized with a 1 : 1000 dilution of rabbit antiserum against cotton seed catalase (Mullen *et al.*, 1997). One sense line (CAT2S), one antisense line (CAT2AS), and two hairpin construct-containing lines (CAT2HP1 and CAT2HP2) were withdrawn for further analysis.

For the quantification of *cat1*, *cat2*, and *cat3* transcript levels by real-time PCR, plants were grown under the same conditions as above. Total RNA of 5-week-old plants was prepared with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). For each sample, 1 μg of total RNA was reverse-transcribed with Superscript II RNaseH$^-$ Reverse Transcriptase (Invitrogen). First-strand cDNA was generated with an oligo(dT)$_{12–18}$ primer. One microliter of the 1 : 25 diluted first-strand cDNA was used as a template in a subsequent PCR. The transcripts were amplified with gene-specific primers: 5′-TGCTCCTCTTACGGTTTGGTTTC-3′ and 5′-ACCCTTCTTTAAGCGTTTCATTGTC-3′ for *cat1*; 5′-GCTGGCAAGCCGTCTGAAC-3′ and 5′-AGCACAGAAGATCCACATGATGAAG-3′ for *cat2*; 5′-GGCCAATCTCCATATAAGCTCAGT-3′ and 5′-GGATTTAACGACCAAGCGATGATAG-3′ for *cat3*, and 5′-AACTTGTGCTCATCTGCCATTAGG-3′ and 5′-TGATTCTGCGGAAACACCACTTTAG-3′ for actin (At3g60830).

Plant growth conditions and stress treatments

Unless mentioned otherwise, the plants were grown in exposure chambers, which had been specially designed for plant studies (Thiel *et al.*, 1996). The light regime was 12 h/12 h (from 6:00 to 18:00) at 140 μmol m^{-2} sec^{-1}, the climate was adjusted to relative humidity of 70% and 22°C day/18°C night temperatures. Six-week-old plants were exposed for 6 days to ozone (350 nl l^{-1}) for 8 h (8:00–16:00) during the light period. The remainder of the time, ozone-free air was applied. For HL treatments, 6-week-old plants were transferred to a sun-simulator with identical growth conditions and exposed to continuous HL irradiation (photosynthetically active radiation 400–700 nm at approximately 1600 μmol m^{-2} sec^{-1}). For the transcriptome analysis, the middle-aged leaves of five plants per line were pooled after 0, 3, 8, and 23 h of HL irradiation.

ROS stainings

Leaves or whole plants were infiltrated with 0.1% DAB (Sigma-Aldrich, St Louis, MO, USA) in 10 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.5) for H_2O_2 staining. After a 45-min incubation, leaves were de-stained in ethanol. Leaves were infiltrated with 0.1% NBT (Sigma-Aldrich) in 50 mM potassium phosphate buffer (pH 6.4) for superoxide staining. After 10–30 min of incubation in the dark, leaves were de-stained in 95% ethanol.

Microarray preparation, target labeling, hybridizations, and data analysis

The *Arabidopsis* 6K microarray used in our study contained 6008 cDNA fragments from the Incyte Unigene collection (Arabidopsis Gem I, Incyte, Palo Alto, CA, USA) together with 520 positive and negative controls. For details, see http://www.microarrays.be. For each sample, 5 μg total RNA was amplified and hybridized as described previously by Puskás *et al.* (2002). Arrays were scanned with a Generation III scanner (Amersham BioSciences, Little Chalfont, UK) at 532 and 635 nm wavelengths for the two fluorescence dyes Cy3 and Cy5 (Amersham Biosciences), respectively. Image analysis was performed with ArrayVision (Imaging Research, St. Catharines, Ont., Canada). Spot intensities were measured as artifact-removed total intensities without correction for background. Variation in gene expression was assessed by using a two-step procedure essentially as outlined by Wolfinger *et al.* (2001). In a first step, a linear normalization ANOVA model accounts for experiment-wide systematic effects (i.e. array effects) that could bias inferences made on the data from the individual genes. The residuals from this model represent normalized values and serve as input data for the gene models. In a second step, the gene models were fit separately to the normalized data from each gene, allowing inferences to be made by using separate estimates of variability. Here, estimates of primary interest are genotype × time effects. So finally, as a measure of variability in expression levels among the effects, Wald statistics were calculated and significance was assigned to these effects for each gene (for detailed description of the statistical analysis, see Supplementary Material).

Promoter analysis

On the normalized data, both HALC and AQBC were used. To avoid noisy big clusters that would spoil the promoter analyses, each cluster was independently subclustered by using the same algorithms but with gradually more stringent parameters. The promoter analysis was performed on the 800 bp upstream of the start codon. As software YMF (Sinha and Tompa, 2000) and the RSA tools (van Helden, 2003) were used for word-counting methods and the MOTIFSAMPLER (Thijs *et al.*, 2002) as Gibbs sampler. The default parameters from the web site were used for the RSA tools. For the YMF software, version 2.0 was used locally and a specific *A. thaliana* background model was built by using curated intergenic sequences bordered at both sides by experimentally verified genes. On each cluster, different sets of parameters were run that mainly varied in the size of the gap (0–2, 3–5, 6–8) and the length of the words (up to 15). With the top 100 words returned by YMF 2.0, the software 'FIND EXPLANATORS' was used to extract the top five words. The MOTIFSAMPLER was run 100 times for each cluster and each combination of parameters. The background orders alternated between 2 and 3 while looking for three motifs of lengths 7–17 bp. To extract the relevant motifs from these 100 runs, MOTIFRANKING was used to present the top five words. Results from all three methods were then compared, expecting motifs to be found by at least two methods. Alternatively, motifs with distinctively high scores were also considered.

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2105/TPJ2105sm.htm

Statistical microarray data analysis

Table S1 Over- or under-represented functional categories of genes for the six largest AQBC clusters

Table S2 DW and FW measurements

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