A comprehensive analysis of hydrogen peroxide-induced gene expression in tobacco

Steven Vandenabeele*, Katrien Van Der Kelen*, James Dat*†, Ilya Gadjev*‡, Tom Boonefaes§, Stijn Morsa*, Pieter Rottiers§, Luit Slooten¶, Marc Van Montagu*, Marc Zabeau*, Dirk Inzé*‖, and Frank Van Breusegem*

*Department of Plant Systems Biology and §Department for Molecular Biomedical Research, Flanders Interuniversity Institute for Biotechnology, Ghent University, Technologiepark 927, B-9052 Ghent, Belgium; and ¶Laboratorium voor Biofysica, Vrije Universiteit Brussels, Pleinlaan 2, B-1050 Brussels, Belgium

Contributed by Marc Van Montagu, October 13, 2003

Hydrogen peroxide plays a central role in launching the defense response during stress in plants. To establish a molecular profile provoked by a sustained increase in hydrogen peroxide levels, catalase-deficient tobacco plants (CAT1AS) were exposed to high light (HL) intensities over a detailed time course. The expression kinetics of >14,000 genes were monitored by using transcript profiling technology based on cDNA-amplified fragment length polymorphism. Clustering and sequence analysis of 713 differentially expressed transcript fragments revealed a transcriptional response that mimicked that reported during both biotic and abiotic stresses, including the up-regulation of genes involved in the hypersensitive response, vesicular transport, posttranscriptional processes, biosynthesis of ethylene and jasmonic acid, proteolysis, mitochondrial metabolism, and cell death, and was accompanied by a very rapid up-regulation of several signal transduction components. Expression profiling corroborated by functional experiments showed that HL induced photoinhibition in CAT1AS plants and that a short-term HL exposure of CAT1AS plants triggered an increased tolerance against a subsequent severe oxidative stress.

Although reactive oxygen species (ROS) and, more particularly, hydrogen peroxide (H_2O_2) are produced during normal cell metabolism in plants, elevated ROS levels that cause oxidative stress are mostly associated with adverse environmental conditions (1, 2). H_2O_2 plays a dual role in plants: at low concentrations, it acts as a messenger molecule involved in acclimatory signaling, triggering tolerance against various abiotic stresses (3, 4), and, at high concentrations, it orchestrates programmed cell death (5). Knowledge on the molecular mechanisms involved in H_2O_2 signal transduction in plants remains scarce (6, 7) and the question remains unresolved as to how H_2O_2 levels can trigger two extreme responses. Comprehensive expression analyses provide insight into the transcriptional changes triggered by a specific stimulus or perturbation. Detailed monitoring of gene expression over time after application of a specific stress or signaling molecule is a first step necessary to understand the rules and dynamics that govern gene expression. In a mammalian cell line (8) and in yeast (9), global transcriptional responses provoked by H_2O_2 have been reported, but few genomic approaches to ROS levels in plants have been published (10, 11). *Arabidopsis thaliana* deficient in cytosolic ascorbate peroxidase had increased H_2O_2 levels and altered gene expression under normal growth conditions (12).

To study H_2O_2 signaling *in planta* (13), transgenic catalase-deficient tobacco plants (CAT1AS) are exposed to high light (HL) intensities (>800–1,000 μmol $m^{-2}{\cdot}s^{-1}$) to modulate H_2O_2 stress. Under these conditions, photorespiration is induced, and, because the CAT1AS plants only retain 10% of their residual catalase activity, photorespiratory H_2O_2 cannot be scavenged efficiently. During such an HL exposure, H_2O_2 accumulates as early as 45 min after the treatment in CAT1AS, but not in WT plants (5). Hence, CAT1AS plants are an ideal model system to study H_2O_2-derived molecular changes because perturbation in H_2O_2 homeostasis can be sustained over time, no invasive techniques are needed, and physiologically relevant levels of H_2O_2 are obtained. CAT1AS plants allowed us to study several signaling aspects of H_2O_2 from local and systemically acquired resistance to the induction of an active cell death program (5, 14).

Materials and Methods

Plant Growth Conditions and Stress Treatments. Eight-week-old CAT1AS and WT plants were grown under normal growth conditions and exposed to HL (1,000 μmol $m^{-2}{\cdot}s^{-1}$; ref. 5). For the acclimation experiments, leaves 6 and 7 were partially covered with aluminum foil before HL preexposure (800–1,000 μmol $m^{-2}{\cdot}s^{-1}$) that was removed before the plant were returned to normal growth conditions (150 μmol $m^{-2}{\cdot}s^{-1}$). Plants were reexposed for 24 h to HL and scored for cell death on leaves 6 and 7. Ion leakage was measured by using a K610 conductivity meter (Consort, Turnhout, Belgium; ref. 11) on leaf discs from CAT1AS plants preexposed for 0, 1, and 2 h, recovered for 1, 3, or 7 days, and again treated with HL for 24 h. Values were averaged from measurements on three different plants.

cDNA-Amplified Fragment Length Polymorphism (AFLP) Analysis and Data Processing. cDNA-AFLP and data were processed essentially as described (15). In the time course (TC) experiment, all time points were profiled (except for 0 min, 1 h, and 6 h HL for WT that were excluded because of minor mRNA quality) with 20 randomly chosen primer combinations. In the gene discovery (GD) experiment, all 128 *Bst*YI+1/*Mse*I+2 primer combinations were used. Differentially expressed transcript tags were excised from gels of the *Bst*YI+2/*Mse*I+2 expression pattern of the pooled time points and PCR reamplified with *Bst*YI+2/*Mse*I+0 primers. PCR fragments were directly sequenced. Data processing (15) was slightly modified (for details, see *Supporting Methods*, which is published as supporting information on the PNAS web site).

F_v/F_m Measurements. Details on how the measurements were conducted are presented in *Supporting Methods*.

Results

cDNA-AFLP Profiling of Gene Expression Induced by a Sustained Increase in H_2O_2 Levels. To establish a molecular phenotype during elevated H_2O_2 levels, we compared the transcriptome of

Abbreviations: AFLP, amplified fragment length polymorphism; CAT1AS, catalase1-deficient tobacco plants; F_v/F_m, measure for exciton trapping efficiency when all photochemical traps are open; HL, high light; LL, low light; PS*n*, photosystem *n*; ROS, reactive oxygen species; SA, salicylic acid; TC, time course; EREBP, ethylene-responsive element-binding protein.

†Present address: Département de Biologie et Ecophysiologie, Université de Franche-Comté, F-25030 Besançon Cedex, France.

‡Present address: Department of Plant Physiology and Molecular Biology, Plovdiv University, 24 Tsar Assen Street, 4000 Plovdiv, Bulgaria.

‖To whom correspondence should be addressed. E-mail: dirk.inze@psb.ugent.be.

CAT1AS and WT tobacco plants exposed to HL. The sixth leaves of individual transgenic CAT1AS and WT tobacco plants were harvested after 0, 10, 20, 30, 40, and 50 min, and 1, 2, 4, 6, 8, and 11 h of HL exposure. Cell death was visually detected after 8 h. Transcriptional changes were monitored with a modified cDNA-AFLP transcript profiling protocol (15).

In an initial TC experiment, 20 randomly chosen AFLP primer combinations with three selective nucleotides were used (a section of a typical cDNA-AFLP gel is presented in Fig. 5, which is published as supporting information on the PNAS web site). For each primer combination, 80–120 transcript fragments were detectable, varying in length from 50 to 600 bp. All lanes (one per time point) were identified with the AFLP-QUANTAR*PRO* software (Keygene Products, Wageningen, The Netherlands); oblique lanes were corrected, and 1,628 unique transcript fragments were quantified, resulting in individual intensities for each time point per transcript fragment. These expression data were further processed with a tailor-made, in-house-developed ARRAYAN cDNA-AFLP software package, allowing for an accurate and automated high-throughput handling of gene expression data, and 262 transcripts were selected as differentially expressed (see *Materials and Methods*).

A GD experiment was performed on selected time points: in the CAT1AS time series, three time points within the first hour (0, 30, and 50 min) allowed visualization of early induced genes and 2-, 4-, and 11-h targeting of the later responses; the WT time series was reduced to 30 min, 50 min, 2 h, and 11 h. The expression analysis of 13,752 transcripts, covering ≈50–60% of the tobacco transcriptome, was monitored with 128 primer combinations and revealed 1,207 differentially expressed fragments. Expression data were variance normalized (16) and analyzed by hierarchical average linkage clustering (17). For both the TC and GD experiments, clustering resulted in the same molecular profile (Fig. 1, and Fig. 6, which is published as supporting information on the PNAS web site). The hierarchical average linkage cluster analysis of the 262 differentially expressed transcripts of the TC experiment revealed two main clusters (Fig. 1): cluster A contains transcript tags that are initially repressed or down-regulated in CAT1AS plants and cluster B contains the other transcripts up-regulated specifically in CAT1AS by HL, and, hence, induced by elevated H_2O_2 levels. Cluster A can be subdivided into the following three subclusters: (*i*) subcluster A1 included transcripts that were already repressed in CAT1AS under normal growth conditions, but were down-regulated in WT plants only after 4 h of HL; (*ii*) subcluster A2 contained transcripts repressed in both lines; and (*iii*) subcluster A3 consisted of transcripts whose up-regulation is blocked in CAT1AS plants. By considering only the response in WT tobacco plants, we could monitor transcriptional changes in these plants. Three different groups of genes could be distinguished: those repressed within the first hour of HL, those repressed only after 2–4 h of HL, and those induced by HL. Original and normalized data sets and clustering results of the TC and GD experiments, are available at http://www2.psb.ugent.be:8080/BY2. Reproducibility of the cDNA-AFLP expression analysis was assessed by Northern blot analysis with RNA obtained from a biological repeat experiment. Seven hybridizations confirmed the cDNA-AFLP results, whereas three remaining blots had no or very weak signals and represented low-abundant transcripts that may only be detectable by the more sensitive cDNA-AFLP technique (Fig. 7, which is published as supporting information on the PNAS web site).

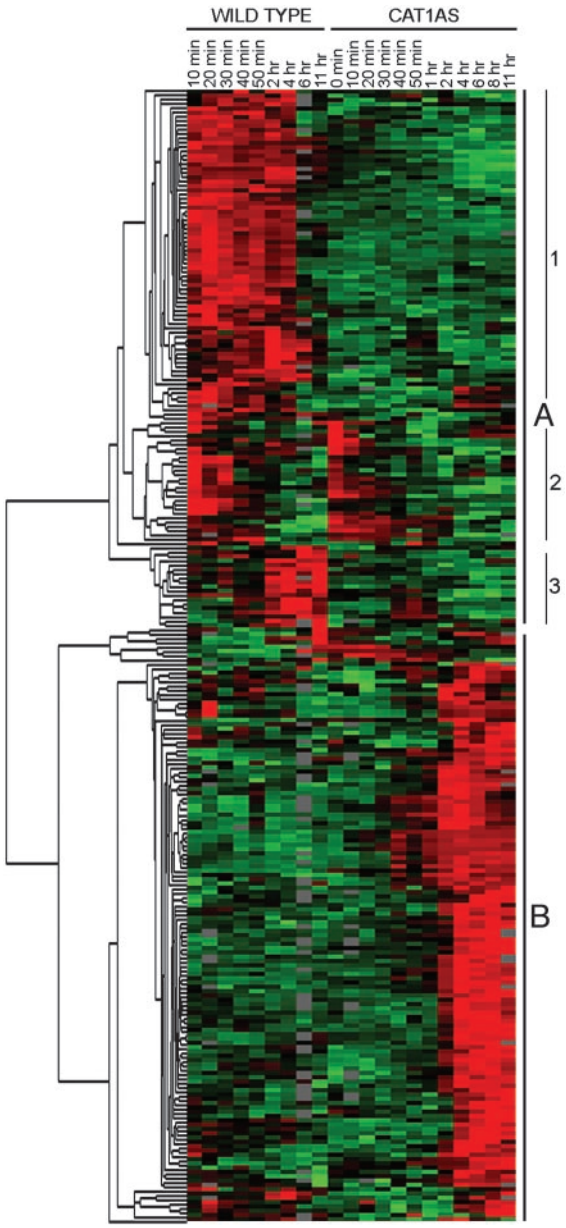

Fig. 1. Hierarchical average cluster image of transcript tags, which were differentially regulated in WT and/or CAT1AS plants during HL exposure. Each column represents the time point of sampling during HL treatment in both WT and CAT1AS plants, and each row represents the expression profile of an individual and nonredundant transcript fragment. Red and green indicate higher and lower expression values than those of the gene of this transcript over all time points, respectively. Gray indicates missing data.

Sequence Analysis. Sequences from 713 transcript tags were compared with those in publicly available databases. When no significant homology (*e* value $>10e^{-3}$) was found, the FASTA algorithm was used to find a corresponding gene index from The Institute for Genomic Research (TIGR) to retrieve additional homologous sequence information. With this TIGR gene index, the database was searched again, resulting in 389 fragments similar to genes with a known function, 109 to sequences without any function assigned, and 215 that were not homologous with any sequence in the public databases (see Table 1, which is published as supporting information on the PNAS web site). All sequences are publicly available at http://www2.psb.ugent.be:8080/BY2. Among the H_2O_2-up-regulated genes (cluster B), ≈15% of the genes were already induced within 50 min. Several genes are known to be involved in stress signal transduction. Heat shock factor 5 is up-regulated within 10 min, preceding the up-regulation of numerous heat shock proteins (HSPs) after 30 or 50 min. Two unknown protein kinases, a

SHAGGY-like kinase, an ethylene-responsive element-binding protein (EREBP), WRKY, MYB, and SCARECROW transcription factors were induced within 50 min. This rapid induction of signal transduction components was followed by up-regulation of genes involved in processes related to defense response and cell death. Other transcripts in cluster B could be attributed to different functional categories: lipid metabolism, proteolytic machinery, vesicles and protein transport, hormone interplay, hypersensitive response (HR), mitochondrial metabolism, and (a)biotic defense response, including antioxidants, phytoalexin production, mobile elements, and heat shock response.

In subcluster A1, transcript tags representing nuclear-encoded chloroplastic proteins involved in photosynthesis are clearly overrepresented: chlorophyll *a*/*b*-binding proteins of the light-harvesting complex, Mg protoporphyrin IX chelatase, an oxygen-evolving enhancer protein, the D1 CtpA C-terminal protease, cytochrome B6, and several photosystem (PS)I subunits. This transcriptional down-regulation of photosynthetic components was linked to a repression of genes from the Calvin cycle. Subcluster A2 also contained genes involved in photosynthesis, such as two chlorophyll *a*/*b*-binding proteins and a PSII reaction center protein, an ATPase, and three genes related to posttranscriptional processes, and, thus, potentially involved in the regulation of genes belonging to this cluster. Finally, the up-regulation of genes in subcluster A3 was impaired in CAT1AS plants, namely genes involved in ADP/ATP metabolism, such as a plastidic ATP-diphosphatase and an ATP/ADP transporter, and in defense response, such as a trehalose-6-phosphate synthase and a putative metallothionein protein.

In summary, we observe (*i*) a very rapid up-regulation of signal transduction components involved in stress resistance in CAT1AS, accompanied and followed by the induction of genes that had previously been associated with the onset of cell death, and (*ii*) the transcriptional down-regulation of nuclear genes, which code for photosynthetic components in WT after 4–6 h of HL exposure, which are repressed in CAT1AS already under standard growth conditions.

HL Causes Photoinhibition in CAT1AS Plants. To assess whether the decreased expression of the photosynthetic components influenced the stress tolerance of photosynthesis, the chlorophyll fluorescence ratio (F_v/F_m), which is the measure for exciton trapping efficiency when all photochemical traps are open, was measured at 0, 0.5, 2, 4, and 11 h of HL in WT and CAT1AS plants and in leaf discs after 0, 0.5, 1, 2, 4, 7, 11, and 17 h of HL. The shift from normal to HL conditions caused a decrease in F_v/F_m, followed by a partial recovery after 4 h in WT plants only. In catalase-deficient plants, F_v/F_m was comparable to that of WT plants at the start of the HL treatment, but decreased rapidly and continuously during HL exposure. Photorespiratory H_2O_2 was the cause of this sharp decrease in CAT1AS plants because F_v/F_m was unaffected by HL under nonphotorespiratory conditions (Fig. 2).

H_2O_2 Can Trigger Either Protection or Cell Death. The very rapid induction in CAT1AS plants of regulatory genes within the defense response led to the hypothesis that defense response and cell death could be uncoupled by terminating the H_2O_2 signal in a timely and dose-responsive manner. We assessed whether a short HL preexposure of CAT1AS plants could trigger an acclimatory effect that protected the plant against a subsequent stress treatment. Individual plants were preexposed to HL for 1, 2, 3, 4, 5, 6, 7, and 8 h of HL, whereas the middle leaf parts were covered from the HL. Immediately after the HL preexposure, plants were allowed to recover for 1, 3, or 7 days under low light (LL) conditions. In plants preexposed at least for 4 h to HL, cell death was apparent within 12 h under subsequent LL conditions.

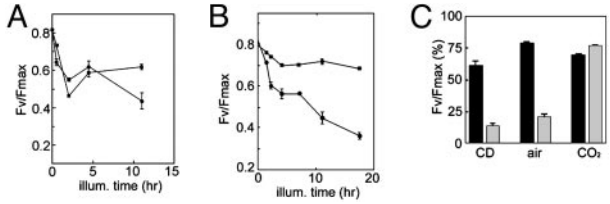

Fig. 2. F_v/F_m measurements on WT and CAT1AS plants during HL exposure. (*A*). Intact leaves illuminated for the indicated time at 22°C. (*B*) Leaf discs in closed Petri dishes. Data are presented as mean ± SEM ($n = 4$). ■, WT; ●, CAT1AS. (*C*) Leaf discs illuminated for 22 h at 22°C, in closed Petri dishes (CD), under compressed air (air), or 2% O_2 and 0.5% CO_2 in N_2 (CO_2). F_v/F_m is expressed as percentage of F_v/F_m in leaf discs that were not illuminated.

Dead cells were most prominent along the veins (data not shown). In plants exposed for <4 h to HL, no cell death was detectable either by visual scoring or by microscopic analysis several days after the treatment (5). After the above mentioned recovery periods, these plants were exposed to an additional 24 h of HL stress to initiate H_2O_2-induced cell death. Preexposure to HL for 1–2 h was sufficient to render the noncovered parts of the leaf more resistant against cell death (Fig. 3*A*). This observation was confirmed by a significant decrease in electrolyte leakage (Fig. 3*B*). Chamnongpol *et al.* (14) showed that a prolonged (2 days) HL exposure of CAT1AS plants resulting in cell death could trigger a defense response in systemic leaves. Here, no cell death was induced during the 1–2 h preexposure, and, surely, no systemic response because the cell death border between preexposed and nonpreexposed leaves was clearly separated and corresponded exactly with the borders of the aluminum foil used for coverage. We conclude (*i*) that a short H_2O_2 increase without the occurrence of cell death is unable to induce the systemic defense response that protects the preexposed leaf parts, and (*ii*) that H_2O_2-induced cell death can be uncoupled from H_2O_2-induced defense response, because, within 1–2 h, signals are triggered that are sufficient to initiate an acclimatory process that builds a long-lasting protective mechanism against H_2O_2-induced cell death.

Discussion

Lower Expression of Photosynthetic Components Does Not Influence PSII Activity Under LL But Causes Photoinhibition in CAT1AS Plants Under HL. We present a detailed time course of both the H_2O_2-dependent and -independent transcriptional changes provoked by a continuous HL exposure in WT and CAT1AS tobacco. By hierarchical clustering, two main clusters of gene expression

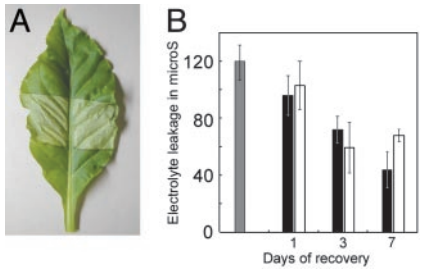

Fig. 3. Protection against H_2O_2-induced cell death in CAT1AS plants by a short-term HL treatment. (*A*) Leaf of a CAT1AS plant that had been covered partially with aluminum foil before a short-term 1,000 μmol $m^{-2}\cdot s^{-1}$ HL exposure (2 h). The foil was removed and the plant recovered for 24 h at 150 μmol $m^{-2}\cdot s^{-1}$ light intensity, followed by a subsequent exposure for 24 h to HL. (*B*) Ion leakage in CAT1AS plants not preexposed to HL (gray), preexposed for 1 h (white), and 2 h (black) followed by 1, 3, and 7 days of recovery and a subsequent 24 h of HL. Data are presented as means ± SEM ($n = 3$).

PLANT BIOLOGY

were visualized. Apparently, genes in cluster A1 respond to a signal that is also produced after continuous HL stress in WT plants, although it is already present in CAT1AS under LL conditions. Low levels of photorespiratory H_2O_2 in CAT1AS plants are probably able to trigger this response. A similar down-regulation accompanied the defense response during other oxidative stress situations (18), suggesting a common H_2O_2-dependent regulatory mechanism. In double antisense plants lacking catalase and ascorbate peroxidase, low photosynthetic activity was proposed as a possible mechanism for decreasing total ROS production, hence compensating for the lack of ascorbate peroxidase and catalase (19). Under LL conditions, F_v/F_m was similar in WT and CAT1AS plants, indicating that plants can maintain PSII activity even when the expression level of photosynthetic components is considerably decreased. In WT plants, the shift from LL to HL conditions caused a decrease in F_v/F_m, followed by a partial recovery after 4 h. The timing of this recovery coincided with the decrease in transcript levels coding for photosynthetic components, indicating that transcriptional down-regulation of photosynthetic components may serve as a protective mechanism against HL stress. In CAT1AS plants, PSII activity decreased rapidly under photorespiratory conditions. Damage to PSII was similarly enhanced by HL in a catalase-deficient *Cyanobacterium synechocystis* (20). High CO_2 conditions restored F_v/F_m in CAT1AS plants, indicating that photorespiratory H_2O_2 caused the drop in F_v/F_m. Whether photoinhibtion is caused directly by H_2O_2 that affects the photosynthetic apparatus or whether other mechanisms are involved needs to be further studied.

H_2O_2 Sensing and Early Signal Transduction. The most dominant cluster contains up-regulated transcripts that coincide with H_2O_2-induced acclimation and cell death. Fig. 4 gives a schematic overview of these transcripts and their potential involvement in H_2O_2-triggered defense responses and cell death, based on their functional annotation. How H_2O_2 is perceived and transmitted within plant cells remains elusive. We anticipate that among the H_2O_2-responsive transcripts, several candidate genes have been identified, which are involved in oxidative stress sensing and signal transduction. GenBank accession no. AJ538745 is homologous to the C-terminal domain of an *Arabidopsis* F-box protein containing a PAS domain, which was originally described as a bacterial oxygen and redox sensor (21). GenBank accession no. AJ538628 is homologous with the DNA-binding domain of *Arabidopsis* two-component cytokinine response regulators, ARR, which are up-regulated by temperature stress (22). In eukaryotes, two-component circuits provide an interface between environmental cue sensing and a downstream kinase-signaling cascade (23). An *S*-receptor-like kinase, as well as two different putative receptor kinases were up-regulated within 2 h. Their expression profile was nicely complemented with that of GenBank accession no. AJ538427, which is homologous to the Armadillo repeat-containing ARC1 protein. This protein is known to interact with the kinase domain of an *S*-receptor kinase in rapeseed (24). GenBank accession no. AJ538706 shows sequence similarity to the cytosolic domain of a cell wall-associated receptor-like kinase (WAK1). Interestingly, WAK1 is proposed to launch an overall antioxidant machinery during the HR, hereby protecting noninfected cells against the oxidative burst (25). In *Arabidopsis*, WAK1 aggregates with a glycine-rich extracellular protein (26) and a cytoplasmic-type 2C protein phosphatase (27). Transcript tags coding for both a cytoplasmic-type 2 protein phosphatase and a glycine-rich protein are also clearly up-regulated in CAT1AS plants. Finally, two different putative protein kinases and SHAGGY-like kinases were up-regulated. We have also identified at least four main classes of transcription factors, which are potential candidates for regulating downstream gene expression:

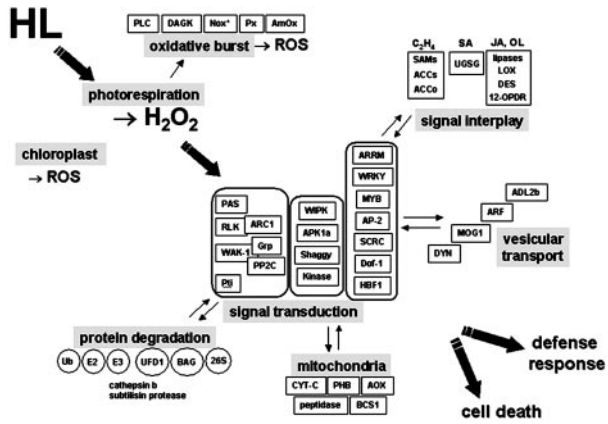

Fig. 4. Model for the role of H_2O_2 in the induction of defense and cell death and the relation of the genes identified in this expression analysis. HL intensities provoke an increase in photorespiratory H_2O_2 in CAT1AS plants. The oxidative burst and chloroplastic ROS amplify the initial ROS. Signal transduction components in close interaction with hormone signals, vesicular transport, protein degradation, and mitochondrial responses regulate the induction of the defense response and cell death. 12-OPDR, 12-oxophytodienoate reductase; 26S, 26S proteasome non-ATPase regulatory subunit; DYN, dynamin; ACCo, 1-aminocyclopropane-1-carboxylic acid oxidase; ACCs, 1-aminocyclopropane-1-carboxylic acid synthase; ADL2b, ADL2b dynamin; AmOx, amine oxidase; AOX, alternative oxidase; AP2, APETALA2 domain-containing protein; ARF, ADP-ribosylation factor; ARRM, two-component cytokinine response regulator; BAG, BAG-domain-containing protein; BCS1, ubiquinol-cytochrome reductase synthase; CYT-C, cytochrome C; DAGK, diaglycerol kinase; DES, divinyl ether synthase; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; Grp, glycine-rich protein; HBF1, bZIP DNA-binding protein, HBF1; HL; LOX, lipoxygenase; MYB, MYB transcription factor; Nox, NADPH oxidase; PAS, PAS-domain containing protein; PHB, prohibitin; PLC, phospholipase C; PP2C, protein phosphatase 2C; PSI/PSII; Pti, Pto-interacting protein; Px, peroxidase; RLK, receptor-like kinase; ROS; SAMs, *S*-adenosyl-L-methionine synthetase; SCRC, SCARECROW transcription factor; Shaggy, SHAGGY-like kinase; Ub, ubiquitin; UGSG, UDP-glucose:SA:glucosyltransferase; WAK1; WIPK, wound-induced kinase.

MYB family, WRKY, AP2, and SCARECROW. Four transcript tags represent MYB-related transcription factors. GenBank accession no. AJ538985 is homologous with the N-terminal MYB domain of a jasmonic acid-dependent and cell death-inducing MYB transcription factor (28); its peak of expression coincides with the minimal HL exposure time that is needed to trigger irreversible cell death in CAT1AS (4 h). Five independent transcript fragments encode plant-specific WRKY transcription factors that contain a redox-sensitive zinc-finger DNA-binding domain (29). Four EREBP/AP2 and the ethylene-responsive transcription factor (ERF1) are up-regulated together with a CEO1-like protein. CEO1 is a potential cofactor of EREBP transcription factors (30). Finally, the up-regulation of SCARECROW transcription factors, which are involved in root patterning, cell division, and phytochrome A signal transduction was not anticipated (31). Whether H_2O_2 plays a signaling role within these diverse biological processes or whether specific members of the SCARECROW family are involved in the oxidative stress response remains to be further elucidated. Nevertheless, the recently reported transcriptional up-regulation of SCARECROW transcription factors during Pto-dependent HR could support such a regulatory role (18). Other genes that are differentially expressed in response to H_2O_2 are capable of steering the H_2O_2 transcriptional response, such as glycine-rich proteins containing an RNA-binding region, which play an important role in posttranscriptional regulation and are affected during cold and salt stress (32). Interestingly, proteome analyses of H_2O_2 stress in yeast indicated drastic changes at the protein level (33). A translational control of the H_2O_2 response in

tobacco is reflected by the up-regulation of several transcript tags coding for elongation factor, EF-1α, ribosomal genes 40S and 60S, and the CAP-binding protein, eIF4E.

H_2O_2: A Common Denominator That Orchestrates the Molecular Response During Both Biotic and Abiotic Stresses. The signaling role of ROS during temperature stress has been thoroughly studied (34). In our study, the 16 heat shock genes follow the up-regulation of two heat shock transcription factors. This pronounced up-regulation probably reflects the refolding effort of the stressed cells toward oxidatively damaged proteins; however, it cannot be excluded that some specific HSPs play an essential role in the regulation of cell death (35). An oxidative stress-induced heat shock response in plants was evidenced before in *Arabidopsis* cell suspensions and plants deficient in cytosolic ascorbate peroxidase (10, 12). In addition, several genes previously associated with hypersensitive cell death are also up-regulated (see Table 1). One of these genes, Hsr203J, accelerates the development of hypersensitive cell death when suppressed in transgenic tobacco, suggesting a role in scavenging ROS-derived compounds (36). Similarly, transcripts coding for an arabinogalactan protein, a member of a hydroxyproline-rich glycoprotein family, which is involved in programmed cell death are up-regulated (37). Another tag, GenBank accession no. AJ538582, represents a protein with a membrane attack complex motif. Such proteins are able to form transmembrane pores in lipid bilayers, thereby provoking apoptosis in rat cells (38). Furthermore, two cytoplasmic resistance genes (Rx and I2C-1) are up-regulated by H_2O_2. Rx contains a NB-ARC domain, which is a signaling motif shared by plant resistance gene products and regulators of cell death in animals (39). Increased R gene products through oxidative stress-induced transcriptional up-regulation in cells surrounding the initial lesions would amplify the HR and help arrest pathogen invasion. In addition, an avirulence 9 elicitor response protein, an endo-1,3;1,4-β-D-glucanase precursor, and a chitinase are up-regulated early. A Pto kinase interactor-like protein, probably involved in a phosphorylation cascade downstream of Pto is also up-regulated (40). Recently (18), a transcriptome analysis of Pto-mediated host defense response to infection in tomato has been reported. The transcriptional changes mimic, to a large extent, those provoked by H_2O_2 in CAT1AS plants: down-regulation of the photosynthetic apparatus, up-regulation of markers of mitochondrial stress, a heat shock response, anthocyanin production, proteolysis, and the involvement of salicylic acid (SA), oxylipins, and ethylene. Most strikingly, signal transduction components also overlap: a receptor-like protein kinase, a NAC domain-containing transcription factor (GenBank accession no. AJ539065), WIZZ, MYB transcription factor (GenBank accession no. AJ538949), CEO1, and two different SCARECROW proteins.

Mitochondrial Integration, Proteolysis, and Vesicular Transport. Mitochondrial dysfunction during both biotic and abiotic stresses suggests that it may act as a target and/or sensor of these stresses (41). During H_2O_2-induced cell death in CAT1AS plants, a significant mitochondrial disruption correlates with oxidative stress, as evidenced by the increased manganese superoxide dismutase and alternative oxidase expression (5). In addition, cytochrome *c* transcripts, together with a putative mitochondrial carrier protein and prohibitins, are up-regulated within 2 h of HL. Prohibitins are evolutionarily conserved mitochondrial chaperones that bind directly to newly synthesized translation products and protect mitochondria against degradation by triple-A proteins (42). The AJ539054 sequence is similar to that of the BCS1-like protein, which is involved in the expression of a functional Rieske iron-sulfur protein in mitochondria of *Saccharomyces cerevisiae* (43). Ubiquitylation-dependent proteolysis is a major event during both the induction and execution of cell death (44). The up-regulation of both ubiquitin precursor proteins, ubiquitin-conjugating enzymes (E2), and candidate ubiquitin-protein ligases (E3), suggests that a similar proteolytic mechanism is involved during H_2O_2-induced cell death in plants. Proteins with ring finger (GenBank accession no. AJ538491), F-box (AJ538745 and AJ538627), and WD repeats (AJ538664 and AJ538479) are potential E3 ligases. Transcript tag AJ538779 shares homology to the rice 26S proteasome non-ATPase regulatory subunit (45). Transcript fragment AJ538945 is homologous to UFD1, which is a protein involved in the presentation of polyubiquitin-tagged proteins to the 26S proteasome (46). AJ538416 corresponds to a protein with a BAG domain. Human BAG-1 is a ubiquitin domain protein previously shown to act as a coupling factor between Hsc/HSP70 chaperones and the proteasome. This protein has antiapoptotic activity and increases the anticell death function of BCL-2 (47). Remarkably, all transcript tags involved in ubiquitylation and proteasomal degradation are up-regulated in our system within the first 2 h. This rapid up-regulation suggests a signaling role for the ubiquitin pathway in addition to removal of oxidatively damaged proteins.

Intracellular vesicle trafficking is involved in the execution of plant cell death (48). Vesicle traffic during H_2O_2-induced cell death is clearly regulated on a transcriptional level. A joint regulation of transcripts coding for transporting components (dynamins, COP, syntaxin, ARF-like protein, and Mog1-like protein) suggests that activation of secretory mechanisms partly induce defense and cell death by secreting PR genes, disturbing membrane homeostasis, or cargoing cell death-promoting components to the membrane, such as proteases or members of the NADPH oxidase complex.

Brothers in Arms: The Close Interplay of H_2O_2 with Stress Hormones Is Regulated at the Transcriptional Level. For the orchestration of both the defense response and cell death, H_2O_2 is not the only signal involved. A close interaction with other signaling molecules, such as oxylipins, ethylene, SA, jasmonic acid, and nitric oxide is envisaged (13). We show that an increase in peroxisomal H_2O_2 is able to trigger transcriptional changes of genes involved in the biosynthesis of other stress hormones. There is a very rapid and sustained up-regulation of transcript tags that are involved in the production of oxylipin signals. Transcript tags coding for *S*-adenosyl-L-methionine synthetase and 1-aminocyclopropane-1-carboxylate oxidase are up-regulated within 2 h, followed by an increase in ethylene-responsive proteins, such as EREBP/AP2 domain proteins. An UDP-glucose:SA:glucosyltransferase that converts SA to SA β-glucoside in tobacco is up-regulated after 30 min in CAT1AS and reflects the accumulation of ethylene and SA in HL-treated CAT1AS tobacco plants at a transcriptional level (14). Evidence indicates that nitric oxide plays an important signaling role in activating the resistance response in plants (49). Only recently, the source of nitric oxide was identified as a variant of the P protein of the glycine decarboxylase complex (50). The expression of the transcript for this protein (AJ539057) is lower in CAT1AS than in WT and hardly differs during the HL treatment in CAT1AS, but, in WT plants, it increases after 2 h of HL and is repressed after 11 h of HL, indicating that H_2O_2 could exert a negative regulatory role on the expression of this enzyme. Further analysis of the interaction between NO and H_2O_2 in CAT1AS plants has to clarify their mutual relation.

Conclusion

We present a detailed transcriptome analysis in tobacco during *in planta*-increased H_2O_2 concentrations. This study delivers a molecular profile that confirms the importance of H_2O_2 during both abiotic and biotic stresses and provides insights into earlier described stress responses. The dual face of H_2O_2 was clearly

confirmed by the induction of a defense response at lower concentrations, whereas cell death was triggered at elevated levels. Because CAT1AS plants treated with HL for 4 h were more resistant to pathogen infection (14), we anticipate that, among the genes up-regulated within the first 2 h of HL, potential key regulators responsible for this stress resistance are present. A direct comparison with published transcriptome analyses in plants assessing H_2O_2-induced gene expression is not straightforward (10, 12) because different technical platforms are used and the identification of orthologs within the tobacco and the *Arabidopsis* data sets is not always unambiguous. However, we notice that similar up- or down-regulated gene families are involved in defense, signal transduction, and photosynthesis related genes.

In our view, the most interesting findings are the induction of protein kinases and several transcription factors, such as two WRKYs, two SCARECROW proteins, an EREBP, and a NAM-like protein during the first hour of HL treatment. Future (functional) characterization of the identified genes will not only help to better understand the responses to H_2O_2, but also, more generally, to stress in plants.

We thank P. Breyne for helpful discussion; W. Ardiles-Diaz, C. Buysschaert, J. Gielen, and R. Villarroel for sequencing the transcript fragments; S. Rombauts for assistance in creating the web page; and M. De Cock for assistance in preparation of the manuscript. This work was supported by Research Fund of Ghent University (Geconcerteerde Onderzoeksacties) Grant 12051403. S.V., J.D., and I.G. acknowledge the Instituut voor de aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen for a predoctoral fellowship and the European Union for an Individual Marie Curie Fellowship and a Trans-European Mobility Program for University Students fellowship (Joint European Project no. 13168/98), respectively.

1. Mittler, R. (2002) *Trends Plant Sci.* **7,** 405–410.
2. Noctor, G., Veljovic-Jovanovic, S., Driscoll, S., Novitskaya, L. & Foyer, C. H. (2002) *Ann. Bot. (London)* **89,** 841–850.
3. Karpinski, S., Reynolds, H., Karpinska, B., Wingsle, G., Creissen, G. & Mullineaux, P. (1999) *Science* **284,** 654–657.
4. Dat, J., Vandenabeele, S., Vranová, E., Van Montagu, M., Inzé, D. & Van Breusegem, F. (2000) *Cell. Mol. Life Sci.* **57,** 779–795.
5. Dat, J. F., Pellinen, R., Beeckman, T., Kangasjärvi, J., Langebartels, C., Inzé, D. & Van Breusegem, F. (2003) *Plant J.* **33,** 621–632.
6. Kovtun, Y., Chiu, W. L., Tena, G. & Sheen, J. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 2940–2945.
7. Neill, S., Desikan, R. & Hancock, J. (2002) *Curr. Opin. Plant Biol.* **5,** 388–395.
8. Yoneda, K., Peck, K., Chang, M. M.-J., Chmiel, K., Sher, Y.-P., Chen, J., Yang, P.-C., Chen, Y. & Wu, R. (2001) *Am. J. Respir. Crit. Care Med.* **164,** S85–S89.
9. Chen, D., Toone, W. M., Mata, J., Lyne, R., Burns, G., Kivinen, K., Brazma, A., Jones, N. & Bähler, J. (2003) *Mol. Biol. Cell* **14,** 214–229.
10. Desikan, R., Mackerness, S. A.-H., Hancock, J. T. & Neill, S. J. (2001) *Plant Physiol.* **127,** 159–172.
11. Vranová, E., Atichartpongkul, S., Villarroel, R., Van Montagu, M., Inzé, D. & Van Camp, W. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 10870–10875.
12. Pnueli, L., Liang, H., Rozenberg, M. & Mittler, R. (2003) *Plant J.* **34,** 187–203.
13. Dat, J. F., Inzé, D. & Van Breusegem, F. (2001) *Redox Rep.* **6,** 37–42.
14. Chamnongpol, S., Willekens, H., Moeder, W., Langebartels, C., Sandermann, H., Jr., Van Montagu, M., Inzé, D. & Van Camp, W. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 5818–5823.
15. Breyne, P., Dreesen, R., Cannoot, B., Rombaut, D., Vandepoele, K., Rombauts, S., Vanderhaeghen, R., Inzé, D. & Zabeau, M. (2003) *Mol. Genet. Genomics* **269,** 173–179.
16. Tavazoie, S., Hughes, J. D., Campbell, M. J., Cho, R. J. & Church, G. M. (1999) *Nat. Genet.* **22,** 281–285.
17. Eisen, M. B., Spellman, P. T., Brown, P. O. & Botstein, D. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 14863–14868.
18. Mysore, K. S., Crasta, O. R., Tuori, R. P., Folkerts, O., Swirsky, P. B. & Martin, G. B. (2002) *Plant J.* **32,** 299–315.
19. Rizhsky, L., Hallak-Herr, E., Van Breusegem, F., Rachmilevitch, S., Barr, J., Rodermel, S., Inzé, D. & Mittler, R. (2002) *Plant J.* **32,** 329–342.
20. Nishiyama, Y., Yamamoto, H., Allakhverdiev, S. I., Inaba, M., Yokota, A. & Murata, N. (2001) *EMBO J.* **20,** 5587–5594.
21. Zhulin, I. B., Taylor, B. L. & Dixon, R. (1997) *Trends Biochem. Sci.* **22,** 331–333.
22. Sakai, H., Aoyama, T. & Oka, A. (2000) *Plant J.* **24,** 703–711.
23. Hwang, I., Chen, H. C. & Sheen, J. (2002) *Plant Physiol.* **129,** 500–515.
24. Gu, T., Mazzurco, M., Sulaman, W., Matias, D. D. & Goring, D. R. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 382–387.
25. He, Z.-H., He, D. & Kohorn, B. D. (1998) *Plant J.* **14,** 55–63.
26. Park, A. R., Cho, S. K., Yun, U. J., Jin, M. Y., Lee, S. H., Sachetto-Martins, G. & Park, O. K. (2001) *J. Biol. Chem.* **276,** 26688–26693.
27. Anderson, C. M., Wagner, T. A., Perret, M., He, Z.-H., He, D. & Kohorn, B. D. (2001) *Plant Mol. Biol.* **47,** 197–206.
28. Lee, M.-W., Qi, M. & Yang, Y. (2001) *Mol. Plant–Microbe Interact.* **14,** 527–535.
29. Eulgem, T., Rushton, P. J., Robatzek, S. & Somssich, I. E. (2000) *Trends Plant Sci.* **5,** 199–206.
30. Belles-Boix, E., Babiychuk, E., Van Montagu, M., Inzé, D. & Kushnir, S. (2000) *FEBS Lett.* **482,** 19–24.
31. Lim, J., Helariutta, Y., Specht, C. D., Jung, J., Sims, L., Bruce, W. B., Diehn, S. & Benfey, P. N. (2000) *Plant Cell* **12,** 1307–1318.
32. Sachetto-Martins, G., Franco, L. O. & de Oliveira, D. E. (2000) *Biochim. Biophys. Acta* **1492,** 1–14.
33. Godon, C., Lagniel, G., Lee, J., Buhler, J.-M., Kieffer, S., Perrot, M., Boucherie, H., Toledano, M. B. & Labarre, J. (1998) *J. Biol. Chem.* **273,** 22480–22489.
34. Iba, K. (2002) *Annu. Rev. Plant Biol.* **53,** 225–245.
35. Beere, H. M. & Green, D. R. (2001) *Trends Cell Biol.* **11,** 6–10.
36. Tronchet, M., Ranty, B., Marco, Y. & Roby, D. (2001) *Plant J.* **27,** 115–127.
37. Gao, M. & Showalter, A. M. (1999) *Plant J.* **19,** 321–331.
38. Nauta, A. J., Daha, M. R., Tijsma, O., van de Water, B., Tedesco, F. & Roos, A. (2002) *Eur. J. Immunol.* **32,** 783–792.
39. van der Biezen, E. A. & Jones, J. D. G. (1998) *Curr. Biol.* **8,** R226–R227.
40. Bogdanove, A. J. & Martin, G. B. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 8836–8840.
41. Lam, E., Kato, N. & Lawton, M. (2001) *Nature* **411,** 848–853.
42. Nijtmans, L. G. J., de Jong, L., Sanz, M. A., Coates, P. J., Berden, J. A., Back, J. W., Muijsers, A. O., van der Spek, H. & Grivell, L. A. (2000) *EMBO J.* **19,** 2444–2451.
43. Nobrega, F. G., Nobrega, M. P. & Tzagoloff, A. (1992) *EMBO J.* **11,** 3821–3829.
44. Estelle, M. (2001) *Curr. Opin. Plant Biol.* **4,** 254–260.
45. Shibahara, T., Kawasaki, H. & Hirano, H. (2002) *Eur. J. Biochem.* **269,** 1474–1483.
46. Bays, N. W. & Hampton, R. Y. (2002) *Curr. Biol.* **12,** R366–R371.
47. Thress, K., Song, J., Morimoto, R. I. & Kornbluth, S. (2001) *EMBO J.* **20,** 1033–1041.
48. Levine, A., Belenghi, B., Damari-Weisler, H. & Granot, D. (2001) *J. Biol. Chem.* **276,** 46284–46289.
49. Wendehenne, D., Pugin, A., Klessig, D. F. & Durner, J. (2001) *Trends Plant Sci.* **6,** 177–183.
50. Chandok, M. R., Ytterberg, A. J., van Wijk, K. J. & Klessig, D. F. (2003) *Cell* **113,** 469–482.