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Research Communication

Hydrogen Peroxide-induced Cell Death in Arabidopsis: Transcriptional and Mutant Analysis Reveals a Role of an Oxoglutarate-dependent Dioxygenase Gene in the Cell Death Process

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Summary

Hydrogen peroxide is a major regulator of plant programmed cell death (PCD) but little is known about the downstream genes from the H_2O_2-signaling network that mediate the cell death. To address this question, a novel system for studying H_2O_2-induced programmed cell death in Arabidopsis thaliana was used. The catalase inhibitor aminotriazole (AT) reduced the catalase activity and caused endogenous accumulation of hydrogen peroxide that eventually triggered cell death. Microarray analysis with a DNA chip representing 21500 genes and subsequent comparison with other PCD-related expression studies revealed a set of new H_2O_2-responsive genes that were highly regulated in a common fashion during different types of PCD. These included an oxoglutarate-dependent dioxygenase and various oxidoreductases, the transcription factors Zat11, WRKY75 and NAM, proteasomal components, a heterologous group of genes with diverse functions, and genes encoding proteins with unknown functions. Knockout lines of the oxoglutarate-dependent dioxygenase exhibited significantly reduced death symptoms and chlorophyll loss upon H_2O_2-induced cell death, indicating a role for this gene in the cell death network.

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Keywords Hydrogen peroxide; aminotriazole; PCD; oxoglutarate-dependent dioxygenase.

INTRODUCTION

Hydrogen peroxide, generated by various developmental and environmental stimuli, is a signaling molecule that regulates plant development, stress adaptation or programmed cell death (PCD) (*1, 2*). Low doses of H_2O_2 induce stress acclimation, while high doses trigger PCD (*3, 4*). H_2O_2-induced PCD itself is essential for developmental processes and environmental responses, including aleurone cell death, hypersensitive response to pathogens, and allelopathic plant-plant interactions (*1, 5, 6*). While the process is well studied in animals, little is known about the genes involved in plant PCD (*7*). A problem for achieving high H_2O_2 levels suitable for studying H_2O_2-induced PCD is the rapid H_2O_2 degradation by the plant antioxidant system. To study H_2O_2-induced PCD, we developed a novel system based on inhibiting catalase with the specific inhibitor aminotriazole (AT). Catalases are the main H_2O_2-detoxifying antioxidant enzymes in plants with paramount importance for stress tolerance (*4, 8*). AT is a potent catalase inhibitor that can be applied to the whole plant by spraying, or added to the plant growth media. A near-full genome-coverage microarray analysis of the Arabidopsis transcriptome revealed a significant percentage of previously unidentified H_2O_2-responsive genes. Comparison with AAL-toxin-induced cell death and other PCD-related studies discriminated further a set of genes highly regulated in a common fashion. The possible roles of these novel genes in the cell death process are discussed and the function of one of them encoding for an oxoglutarate-dependent dioxygenase is studied further by knock-out technology. We present evidence for a role of the oxoglutarate-dependent dioxygenase in the H_2O_2-induced cell death.

EXPERIMENTAL PROCEDURES

Plant Material and Induction of Cell Death

Arabidopsis thaliana ecotype Colombia was grown in soil under standard greenhouse conditions: 14 h light/10 h dark period, photosynthetic photon flux density 400 μmol m^{-2} s^{-1}, 22°C and relative humidity 70%. Four-week old plants were sprayed twice within one hour either with water or 20 mM AT

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for induction of PCD in the leaves as described (*3*). Seeds from the knockouts of the oxoglutarate-dependent dioxygenase (At3G13610.1) used in this study were obtained from the Arabidopsis Biological Resource Center (*9*), references SALK132418 (OG1) and SALK050137 (OG2). Homozygous plants from the two lines were identified by screening the population by PCR with primers CAGTTCTGGCCTGATATCTGC and ACAGTCGTCGACAGCGAATTC for the two genes and TGGACCGCTTGCTGCAAC for the left border of the T-DNA insert. The obtained fragments were then sequenced to verify the inserts. For induction of cell death in the seedlings, seeds from wild type plants and the two knockout lines were grown on standard MS plant growth media supplemented or not with 7 and 9 μM AT.

Protein Isolation and Catalase Activity Measurements

Protein preparations and catalase activity measurements were done as previously described (*3*). Enzyme activity was expressed as μmol H_2O_2. mg^{-1} protein. min^{-1}.

RNA Isolation and Microarray Experiments

RNA was isolated from leaf tissues of water- or AT-sprayed *Arabidopsis thaliana* ecotype Colombia with RNAeasy Plant Mini Kit (Qiagen) according to the instructions of the manufacturer and treated with DNAse free of RNAse (Promega). Microarray experiments were performed in compliance with the MIAME standards (*10*). Arabidopsis2 oligonucleotide array of Agilent Technologies was used, representing 21 500 genes. The quality and quantity of the RNA was assessed with an Agilent Bioanalyzer. The labeling, hybridization, and data extraction were done at ServiceXS (The Netherlands) according to the instructions of Agilent Technologies as previously described (11), except that 500 ng of total RNA was used for the linear amplification non-radioactive labeling procedure.

Cell Death and Chlorophyll Measurements

Cell death was determined as electrolyte leakage (increased conductivity) from the leaves (*11*). The increase in conductivity is expressed as percent of the total conductivity determined after boiling the samples to disrupt all cell membranes. The cell death of Arabidopsis grown on AT-supplemented media was scored as number of dead cotyledons (percent of total) twelve days after germination. Chlorophyll content was determined by reading chlorophyll absorbance in 80% acetone extracts as previously described (*12*).

Cytochemical Techniques and Microscopy

Hydrogen peroxide production was determined by cytochemically staining leaves with 3,3′-diaminobenzidine (DAB). DAB forms a brown precipitate when oxidized by H_2O_2. Leaves were infiltrated with 5 mM DAB 30 minutes before sampling. After sampling, leaves were fixed in ethanol for one hour and examined for DAB deposits under a microscope.

RESULTS

AT Triggers H_2O_2-dependent Cell Death

Spraying one-month-old Arabidopsis plants with the catalase inhibitor AT resulted in reduction of total catalase activity (Fig. 1A), rapid accumulation of H_2O_2 (Fig. 1B) and subsequent cell death (Fig. 1C). The first noticeable reduction in catalase activity was at 4 h, which coincided with accumulation of H_2O_2 in single mesophyll cells (Figs 1A and 1B). At 7 h and 24 h time points catalase reduction was much stronger, leading to an oxidative burst as visualized by the massive accumulation of H_2O_2 in many mesophyll cells. The first detectable signs of cell death were noticed one day after the AT treatment. The massive cell death, as estimated by increased electrolyte leakage, was observed two days after the AT treatment (Fig. 1C). Intensive bleaching and necrotic spots on the leaves were the common phenotype two days after the AT treatment (Fig. 1D). As AT inhibits all catalases and should be active at all developmental stages, we hypothesized that AT can induce cell death when added to the growth media and in this way to be an useful agent for identifying mutants

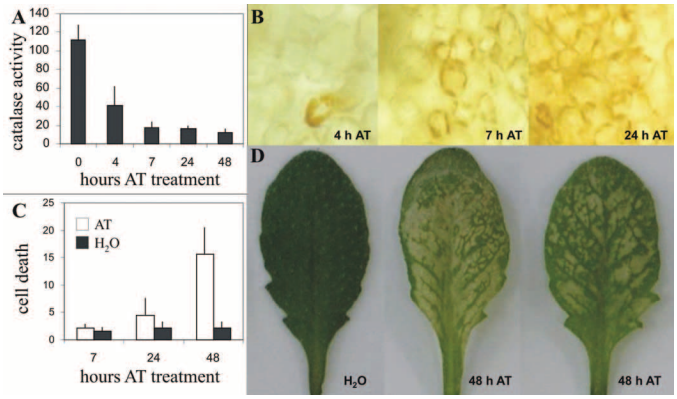

Figure 1. Catalase inhibitor AT triggers H_2O_2-induced cell death in Arabidopsis leaves. Plants were sprayed with either 20 mM AT or water (controls). (A) AT treatment reduces catalase activity. Catalase activity was measured at the time points indicated and the activity expressed in (μmol H_2O_2. $min.^{-1}$. mg $protein^{-1}$). (B) Accumulation of H_2O_2 in mesophyll cells of AT-treated plants. Leaves were stained for H_2O_2 by infiltration with DAB as described in Materials and Methods. DAB forms brown precipitates when oxidized by H_2O_2. Single cells can be seen already at 4 h while at 7 and 24 h the brown staining is much more intense. The staining persisted at 48 h (data not shown). (C) Cell death measurements expressed as increase in conductivity, percent of total conductivity obtained after boiling the samples. (D) Phenotype of H_2O_2-induced cell death recorded two days after AT treatment. On the left, a water-treated control. In the middle and on the right are two examples of AT-treated leaves. For (A) and (C) data are means $\pm$ SD, $n = 3$.

compromised in H_2O_2-induced cell death. Seedlings germinated on 7 and 9 μM AT developed extensive bleaching and necrotic lesions resulting in dead cotyledons twelve days after germination (Fig. 3).

Molecular Biology of H_2O_2-induced Cell Death

To gain more information about the nature of H_2O_2-induced cell death and to select genes for further PCD-related functional studies, we conducted a microarray analysis with a DNA chip representing 21500 genes 7 h after the AT treatment. This time point followed the H_2O_2 accumulation but preceded the cell death. The full dataset can be found on our web site: (http://www.rug.nl/biologie/onderzoek/onderzoekGroepen/molecularBiologyOfPlants/onderzoek/copyof-SupplementalTable1_H2O2.xls). The expression of about one thousand genes out of 21500 was altered more than 3.5 times. The expression pattern of selected genes was verified by northern blot analysis (data not shown). The list of upregulated genes included many glutathione-S-transferases (GSTs) and heat shock proteins, two regulons that are markers for H_2O_2 inducibility (*13*), many members of the proteasome pathway and the recently identified H_2O_2-relaying serine-threonine kinase oxi1 (*14*).

Using such a chip with wide-genome coverage allowed us to identify many new H_2O_2-responsive genes, some of them listed in Table 1. The novel genes included transcription factors, genes involved in calcium signalling, biotic and abiotic stress responses, as well as a heterologous group of genes with diverse functions and many genes encoding putative or unknown proteins. To further substantiate on these findings, our dataset was compared with the recently published comprehensive transcriptional analysis of AAL-toxin-induced PCD (*11*). More than 70% of the novel H_2O_2-responsive genes presented in Table 1 were also highly regulated by AAL-toxin, including the transcription factors, abiotic and biotic stress response genes, the heterologous group of genes with diverse functions and most of the genes with unknown function. Members from the proteasome pathway were the only proteolysis-related genes upregulated by both cell death stimuli. Photosynthesis-related, auxin-regulated and cold stress-induced genes were commonly repressed.

An Oxoglutarate-dependent Dioxygenase Gene is Involved in the H_2O_2-induced Cell Death Response

We reasoned that genes regulated early in the process by both H_2O_2 and AAL-toxin may not be merely a consequence of the global transcriptional reprogramming but can be involved in the PCD process itself. Preliminary studies with knockout lines from four of those genes: an oxoglutarate-dependent dioxygenase, a no-apical meristem (NAM) gene, a carbonic anhydrase and a gene encoding an unknown protein, indicated that the oxoglutarate-dependent dioxygenase (At3G13610.1) mutants exhibited less damage when grown on media with AT. This gene was therefore selected for further functional analysis. The oxoglutarate-dependent dioxygenase was induced 12-fold by H_2O_2 and 10.5-fold by the AAL-toxin. It has two exons of 512 and 1134 base pairs, respectively, intervened by a short 84 bp intron (Fig. 2). The gene encodes for a protein of 361 amino acids with predicted cytoplasmic localization. Two independent T-DNA knockout lines were isolated from seeds obtained from ABRC.

The first knockout line has a T-DNA insertion at the beginning of exon 1 and the second knockout line in exon 2 (Fig. 2). The identity of the knockouts was verified by PCR analysis and sequencing (data not shown). Both lines were phenotypically indistinguishable from the wild type when grown at standard non-stressful conditions. Seeds from these knockouts were germinated on media supplemented with 7 and 9 μM AT and the cell death was evaluated twelve days after germination. Wild type *Arabidopsis thaliana* ecotype Colombia, serving as control, showed significant percentage of cell death at 7 μM AT and all plants died at 9 μM AT, while both T-DNA knockout

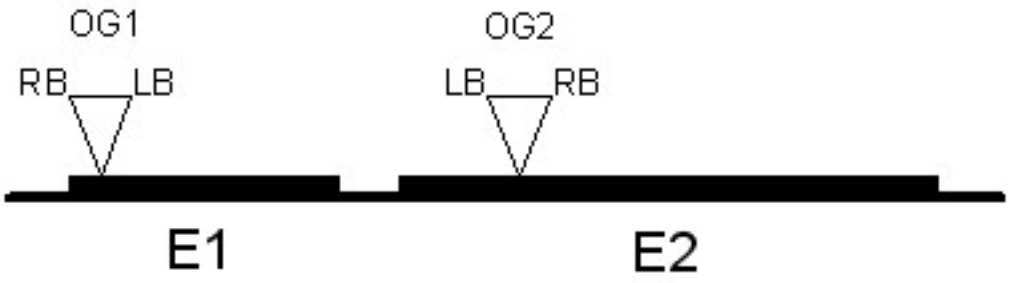

Figure 2. Schematic diagram of the gene structure of the oxoglutarate-dependent dioxygenase (At3G13610.1) indicating the positions and orientations of the insertions of the two independent T-DNA knockout lines used in this study. The gene has two exons of 512 and 1134 base pairs, respectively (depicted in bold), intervened by a short 84 bp intron. The first knockout line (OG1, SALK132418) is at the beginning of exon1 and the second (OG2, SALK050137) is in exon2.

Table 1.

Genes most altered in expression during H_2O_2-induced cell death. Leaves of wild type Arabidopsis plants were sprayed either with AT or water (controls) and samples collected for microarray analysis 7 hours after the treatment. Listed are some of the most regulated genes. The novel H_2O_2-responsive genes identified are marked with (N). Positive ratios indicate the genes induced by H_2O_2 and negative ratios indicate the repressed genes.

Function		Gene locus	Description	Ratio
Transcription factors	N	AT5G13080.1	WRKY family transcription factor	27.4
	N	AT2G47520.1	Putative AP2 domain transcription factor	25.9
	N	AT2G38250.1	Putative GT-1-like transcription factor	22.7
	N	AT5G08790.1	No apical meristem (NAM) family	17.8
	N	AT1G43160.1	AP2 domain protein RAP2.6	13.8
	N	AT5G64750.1	AP2 domain-containing transcription factor, putative	12.9
		AT4G36990.1	Heat shock transcription factor HSF4	11.4
	N	AT2G37430.1	Zat11	8.9
	N	AT1G07050.1	CONSTANS-like protein-related	−14.3
	N	AT1G74930.1	AP2 domain containing protein, putative	−10.5
Hormone responses		AT3G24500.1	Ethylene-responsive transcriptional coactivator, putative	17.9
		AT5G43450.1	1-aminocyclopropane-1-carboxylate oxidase	10.2
		AT1G04240.1	Putative auxin-induced protein AUX2-11	−9.1
ABA/abiotic stress	N	AT4G12400.1	Stress-induced protein sti1-like protein	28.2
		AT4G02380.1	Late embryogenesis abundant protein-related	19.2
	N	AT3G05500.1	Stress related protein, putative	12.8
	N	AT2G42540.1	Cold-regulated protein cor15a precursor	−12.5
		AT5G25610.1	Dehydration-induced protein RD22	−10.9
Heat shock	N	AT5G51440.1	Mitochondrial heat shock 22 kd protein-like	41.7
	N	AT2G29500.1	Putative small heat shock protein	22.4
		AT1G53540.1	17.6 kDa heat shock protein (AA 1-156)	21.9
		AT3G12580.1	Heat shock protein 70	19.4
	N	AT1G07400.1	Heat shock protein, putative	19.3
		AT1G74310.1	Heat shock protein 101 (HSP101)	13.2
Biotic stress/defense		AT4G16260.1	Glycosyl hydrolase family 17	52.5
	N	AT1G57630.1	Disease resistance protein (TIR class), putative	14.7
	N	AT2G43570.1	Glycosyl hydrolase family 19 (chitinase)	12.3
		AT3G13790.1	Glycosyl hydrolase family 32	11.2
Antioxidant metabolism	N	AT2G29460.1	Glutathione transferase, putative	66.1
	N	AT1G17170.1	Glutathione transferase, putative	33.9
	N	AT2G29470.1	Glutathione transferase, putative	27.8
	N	AT1G45145.1	Thioredoxin, putative	15.9
		AT5G03630.1	Monodehydroascorbate reductase, putative	12.3
		AT4G21960.1	Peroxidase, putative	−17.9
		AT1G20620.1	Catalase 3	−9.4
Photosynthesis	N	AT1G06680.1	Photosystem II oxygen-evolving complex 23 (OEC23)	−11.1
		AT4G27440.1	Protochlorophyllide reductase precursor	−9.5
Ca^{2+} signaling and response	N	AT4G20780.1	Calcium-binding protein-like	9.1
		AT2G38170.1	High affinity Ca^{2+} antiporter	−10.7
Kinases/phosphatases	N	AT4G17615.1	Calcineurin B-like protein 1	10.3
		AT4G25390.1	Receptor kinase-like protein	7.2
	N	AT5G58350.1	MAP kinase	6.4
		AT2G30020.1	Protein phosphatase 2C (PP2C)	−6.4
	N	AT4G15420.1	UFD1 like protein	22.9

(*continued*)

Table 1. (*continued*)

Function		Gene locus	Description	Ratio
Ubiquitin/proteasome pathway	N	AT5G55970.1	Zinc finger (C3HC4-type RING finger)	15.7
	N	AT5G20000.1	26S proteasome AAA-ATPase subunit RPT6a	11.8
		AT4G02890.1	Polyubiquitin (UBQ14)	10.7
	N	AT3G55380.1	E2, ubiquitin-conjugating enzyme 14 (UBC14)	8.9
Diverse	N	AT3G26830.1	Cytochrome p450 family	66.4
	N	AT3G49620.1	Oxidoreductase (din11), putative	64.1
	N	AT2G39030.1	GCN5-related N-acetyltransferase (GNAT) family protein	50.1
		AT1G22400.1	UDP-glucose glucosyltransferase, putative	47.4
	N	AT3G28210.1	Zinc finger protein (PMZ), putative	38.9
	N	AT2G41380.1	Putative embryo-abundant protein	36.3
		AT5G38900.1	frnE protein-like	30.9
	N	AT2G43820.1	Putative glucosyltransferase	30.0
	N	AT4G15120.1	VQ motif-containing protein	28.2
		AT3G11340.1	Glucosyl transferase, putative	26.9
		AT3G16530.1	Putative lectin	23.4
		AT4G01870.1	tolB protein-related	22.6
	N	AT1G32170.1	Xyloglucan endotransglycosylase (XTR4), putative	21.6
	N	AT3G09350.1	Armadillo/beta-catenin repeat family protein	21.4
	N	AT1G21310.1	Proline-rich extensin-like family protein	20.8
	N	AT3G15356.1	Similar to putative lectin	20.9
	N	AT3G13610.1	2-oxoglutarate-dependent dioxygenase family	12.1
Putative/unknown	N	AT2G39030.1	Expressed protein	50.3
	N	AT4G15120.1	Expressed protein	28.3
		AT5G14730.1	Putative protein	25.1
		AT4G01870.1	Hypothetical protein	22.6
	N	AT1G10585.1	Expressed protein	22.1
	N	AT3G09350.1	Expressed protein	21.4
	N	AT1G21310.1	Hypothetical protein	20.1
		AT4G28080.1	Putative protein	−16.9
	N	AT1G07050.1	Expressed protein	−14.1

lines exhibited no necrotic symptoms at 7 μM AT and much less cell death at 9 μM AT (Figs 3A and 3B) despite the fact that catalase activity was as reduced as in the wild type and there was DAB staining indicating H_2O_2 accumulation (data not shown). The cell death of the wild type was accompanied by extensive chlorophyll bleaching while little chlorophyll loss was observed in the two knockout lines (Fig. 2).

DISCUSSION

H_2O_2 can switch on either protective mechanisms or PCD depending on the endogenous levels and cellular context (*2, 3*). Reactive oxygen species-mediated cell death is a genetically controlled suicidal process as mutations in single genes can completely abolish the cell death despite the accumulation of reactive oxygen species (*15*). A problem for achieving high H_2O_2 levels suitable for studying H_2O_2-induced PCD is the rapid H_2O_2 degradation by the plant antioxidant system. As a consequence, only few mutants from the H_2O_2-signaling pathway have been identified so far despite the recent surge in H_2O_2 research (*14, 16*). The problem of rapid H_2O_2 decomposition can, therefore, be overcome by reducing the catalase activity, as recently demonstrated by the silencing of the photorespiratory catalase2 gene in tobacco and Arabidopsis (*17, 18*). AT is a potent inhibitor of catalases, the main plant H_2O_2–detoxifying enzymes, and its application leads to sustained accumulation of H_2O_2 that eventually initiates PCD. The phenotype of the AT-induced cell death in our experiments was similar to that obtained by the recent silencing of the photorespiratory catalase2 gene (*18*). An advantage of AT over the gene silencing approach is that the catalase inhibitor allows rapid functional analysis of PCD-related genes since no plant crosses are necessary. AT added to the

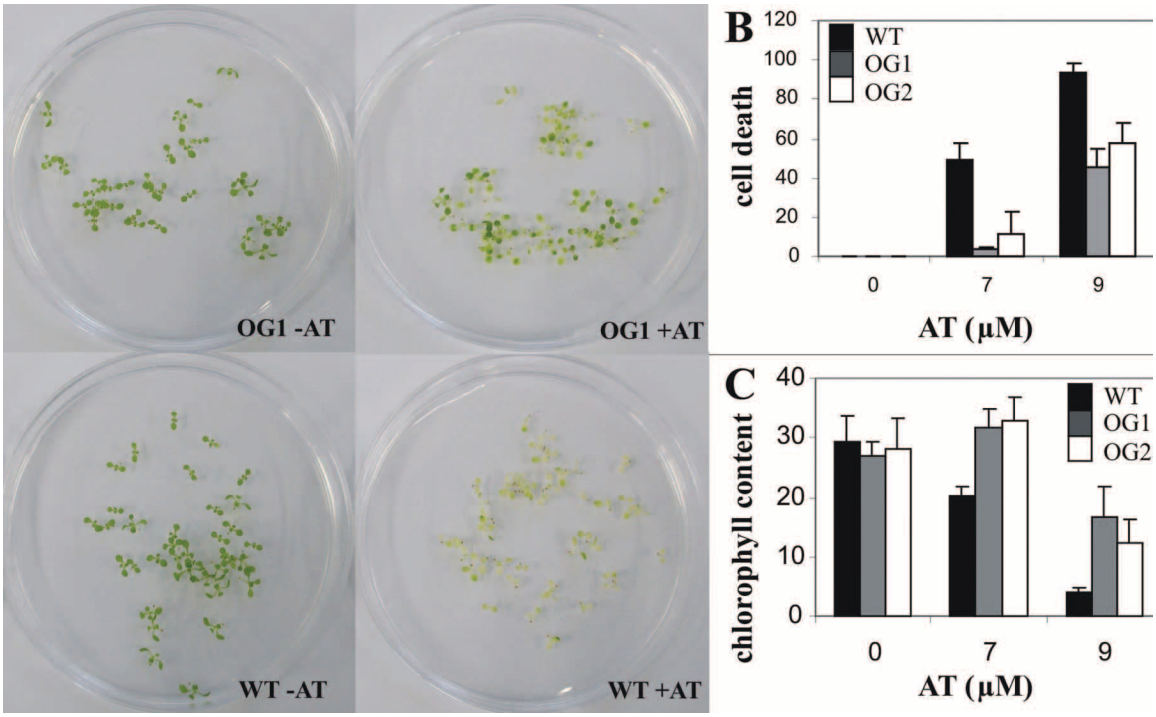

Figure 3. Oxoglutarate-dependent dioxygenase is required for full mounting of H_2O_2-induced cell death. (A) Seeds from wild type *Arabidopsis thaliana* ecotype Colombia and from the two oxoglutarate-dependent dioxygenase mutant lines were germinated on standard MS media with or without 9 μM AT and pictures taken twelve days after germination. (B) Seeds from the same plants germinated on 0, 7 and 9 μM AT and cell death scored as percent of dead or necrotic cotyledons. (C) Decrease of total chlorophyll in the same plants, chlorophyll content expressed as $\mu g.mg^{-1}$ fresh weight. Data are means $\pm$ SD, $n = 3$.

plant growth media is a powerful agent for identifying mutants compromised in H_2O_2-induced PCD. The lack of suitable screening systems for mutants altered in H_2O_2 responses was the main reason that a limited number of genes has been identified as components of the presumably extensive H_2O_2 network. Large collections of chemically or T-DNA-mutagenized plants can be screened or T-DNA knockout mutants of genes selected on the basis of PCD-induced alterations in gene expression can be tested on media with AT. Such an approach can therefore significantly advance the research on H_2O_2 signaling.

A microarray analysis of H_2O_2-triggered PCD with genome-wide gene coverage is an unbiased way to identify genes that are transcriptionally regulated by H_2O_2 treatment and may have important roles in PCD. In a previous study, H_2O_2-induced cell death was profiled with a set of 6000 genes, that is about a quarter of the genome (*18*). The genes identified in that paper as highly regulated were also highly regulated on our chip, which is consistent with the similar phenotype in the two studies and serves as an independent validation of our dataset. The present study reports a near genome-wide transcriptional analysis of H_2O_2-induced cell death and as a result of this broader coverage new genes have been identified. For example, in Table 1, representing some of the most regulated genes, more than 60% are new H_2O_2-responsive genes and most of them have not been implicated in PCD before. A role for some of the genes, especially the unknown proteins, in PCD is not that obvious, but for other genes like those related to biotic stress or Ca^{2+} signaling a role in PCD can easily be envisaged. For instance, calcium release is one of the earliest steps during various stress responses, including oxidative stress (*19*). Calcium is a second messenger with a crucial role in regulating plant PCD and Ca^{2+} interacting and responsive proteins are likely to modulate the cell death (*20, 21*). Consistent with the oxidative burst caused by AT, many H_2O_2-responsive and H_2O_2-producing genes were upregulated. Thus, the H_2O_2-induced PCD is an active process in which the H_2O_2 signal is overamplified and not just a consequence of catalase inhibition. This notion was further corroborated by the downregulation of the most abundant catalase at the transcriptional level, contributing to the overall reduction in catalase activity.

The AAL-toxin inhibits ceramide synthase, which leads to disruption of sphingolipid metabolism. Interestingly, AAL-toxin-induced cell death is associated with an H_2O_2 burst but the phenotype is different: a slower process with dark brown spots instead of bleaching (*11*). Comparison of our dataset with the transcriptome during AAL-toxin-induced cell death

revealed striking similarities in the expression patterns. Many of the newly identified H_2O_2-responsive genes are induced also by the AAL-toxin.

The group of upregulated transcription factors deserves a special interest as transcription factors determine the global transcriptional reprogramming occurring during PCD. WRKY transcription factors and disease resistance proteins participate in the recognition and response during the pathogen-induced hypersensitive response, a well-known form of PCD, as well as in different developmental processes that require PCD (*22–24*). Another highly induced gene, the C_2H_2 zinc finger Zat 11, is the most significantly regulated transcription factor by both singlet oxygen and superoxide radicals, two other types of reactive oxygen species that can trigger PCD (*25*). These findings suggest a more prominent role for this C_2H_2 zinc finger protein in reactive oxygen species–mediated PCD. A noticeable difference with the AAL-toxin was the H_2O_2-specific induction of the heat shock regulon (Table 1, ref. (*11*)).

Repression of photosynthesis-related, auxin-regulated and cold stress-induced genes were other common features of the two death systems. The decrease in chlorophyll content we observe is therefore not just a consequence of the chlorophyll degradation during the cell death but is also a result of switching off chlorophyll biosynthesis genes. In Arabidopsis, H_2O_2 can negatively regulate auxin responses through a MAPK signaling cascade, serving as a molecular link between oxidative stress and inhibition of photosynthesis and growth (*13*). The role of cold-regulated genes, however, is less clear. Cold stress can induce PCD with apoptosis-like features in plants (*26*). Following this line, cold-protective genes may be important in protecting the plant from the cell death and a downregulation of such genes may facilitate the switching on of the cell death programme. The large number of regulated genes with unknown function in these studies provides us with novel leads to search for plant-specific PCD key regulatory molecules.

The oxoglutarate-dependent dioxygenase is a novel H_2O_2-inducible gene belonging to a very large gene family that is part of the diverse cupin superfamily (*27*). Plant oxoglutarate-dependent dioxygenases are mainly implicated in the biosynthesis of flavonoids (*28*) but also various other secondary metabolites, hydroxyproline-rich proteins and gibberellin (*29–32*). Our data suggest an additional function of this particular oxoglutarate-dependent dioxygenase gene related with the regulation or execution of H_2O_2-induced PCD. The mutations in the oxoglutarate-dependent dioxygenase in our study significantly reduced the cell death symptoms and the chlorophyll loss upon AT treatment but the cell death was not completely abolished. No previous involvement of an oxoglutarate-dependent dioxygenase in plant cell death has been reported. How exactly this gene can be involved in the regulation of the AT-induced cell death? Enzyme activity measurements showed that the catalase activity in the oxoglutarate-dependent dioxygenase mutants is as reduced as in the wild type (data not shown), which means that the mutants are not limited in their ability to take up the catalase inhibitor AT. Another possible mode of action could be interference with the H_2O_2 accumulation. Although such a scenario cannot be ruled out completely, it is unlikely as we also found significant accumulation of H_2O_2 in the knockout mutants as well. It could be that the oxoglutarate-dependent dioxygenase gene may act more downstream and in concert with other genes to fine-tune the cell death or be partially redundant. Future work is needed to determine the biological substrates of the plant oxoglutarate-dependent dioxygenase gene and to elucidate its exact place in the cell death network.

In summary, a near genome-wide coverage transcriptional analysis of H_2O_2-induced cell death allowed us to identify novel H_2O_2-responsive genes and presents evidence for the involvement of one of them in the cell death process. The method for studying H_2O_2-induced PCD presented in this study should be suitable for rapid functional analysis of PCD-related genes and screening large collections of mutagenized plants. The latter approach gives opportunities to identify mutants that are compromised in H_2O_2-induced cell death and in this way to identify low-abundant or not transcriptionally altered components of the H_2O_2-induced cell death network. Given the complexity of the PCD network, more mutants await discovery and further functional characterization of the respective genes will bring new insights into the plant PCD.

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