Hydrogen Peroxide–Induced Gene Expression across Kingdoms: A Comparative Analysis

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Cells react to oxidative stress conditions by launching a defense response through the induction of nuclear gene expression. The advent of microarray technologies allowed monitoring of oxidative stress–dependent changes of transcript levels at a comprehensive and genome-wide scale, resulting in a series of inventories of differentially expressed genes in different organisms. We performed a meta-analysis on hydrogen peroxide (H_2O_2)–induced gene expression in the cyanobacterium *Synechocystis* PCC 6803, the yeast *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, the land plant *Arabidopsis thaliana*, and the human HeLa cell line. The H_2O_2-induced gene expression in both yeast species was highly conserved and more similar to the *A. thaliana* response than that of the human cell line. Based on the expression characteristics of genuine antioxidant genes, we show that the antioxidant capacity of microorganisms and higher eukaryotes is differentially regulated. Four families of evolutionarily conserved eukaryotic proteins could be identified that were H_2O_2 responsive across kingdoms: DNAJ domain–containing heat shock proteins, small guanine triphosphate-binding proteins, Ca^{2+}-dependent protein kinases, and ubiquitin-conjugating enzymes.

Introduction

All aerobic organisms frequently experience endogenous and environmental conditions that provoke the accumulation of reactive oxygen species (ROS). Hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$), and singlet oxygen are highly reactive molecules and, therefore, potentially harmful at higher concentrations. They can haphazardly assault proteins, lipids, DNA, and any other cellular component, thereby causing severe damage. Consequently, aerobic organisms have developed or adapted an efficient ROS scavenging machinery, involving enzymes such as superoxide dismutases (SODs) and catalases together with an extensive battery of nonenzymatic antioxidants (Halliwell 2006).

Compared with other ROS, H_2O_2 is a relatively long-lived molecule (1 ms) that is able to diffuse across cell membranes (Bienert et al. 2006). This characteristic is compatible with its role as a signaling molecule during growth and development (Finkel and Holbrook 2000; Sauer et al. 2001; Neill et al. 2002; Van Breusegem and Dat 2006). The transduction of H_2O_2 signals into biologically relevant information is governed by sensors or receptors, mitogen-activated protein kinases, and transcription factors and has been suggested to be evolutionarily conserved (Toone and Jones 1998; Georgiou 2002; Liu et al. 2005). The best-known example is the apoptosis signal-regulating kinase 1/c-jun N-terminal kinase cascade that activates the AP-1 transcription factor through the oxidation of cysteine residues (Abate et al. 1990; Delauney et al. 2000; Shen and Liu 2006). In yeast, different H_2O_2 levels trigger independent signaling pathways (Vivancos et al. 2006). H_2O_2 signaling in plants is coordinated via a complex network that involves multiple protein kinases and transcription factors (Mittler et al. 2004; Miller and Mittler 2006; Kaminaka et al. 2006). The coordinated action of 2 redox-regulated transcription factors, TGACG motif-binding factor 1 and non-expressor of pathogenesis-related genes 1, is required for defense gene expression and systemic acquired disease resistance in *Arabidopsis thaliana* (Després et al. 2003; Mou et al. 2003).

Since recently, transcriptional changes can be monitored on a genome-wide scale by using different technologies, such as differential display, expressed sequence tag sequencing, serial analysis of gene expression, cDNA-amplified fragment length polymorphism, microarrays, and deep sequencing technologies (Lockhart and Winzeler 2000; Donson et al. 2002; Vandenabeele et al. 2003; Emrich et al. 2007). Such analysis has led to comprehensive inventories of genome-wide H_2O_2-related gene expression in bacteria (*Escherichia coli* and *Bacillus subtilis*), the cyanobacterium *Synechocystis* sp. strain PCC 6803, 2 yeast species (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*), mouse (*Mus musculus*), fruit fly (*Drosophila melanogaster*), several human (*Homo sapiens*) cell lines, and 1 plant species (*A. thaliana*) (Gasch et al. 2000; Zheng et al. 2001; Chuang et al. 2002; Chen et al. 2003; Lee et al. 2003; Desaint et al. 2004; Girardot et al. 2004; Kobayashi et al. 2004; Li et al. 2004; Mostertz et al. 2004; Murray et al. 2004; Davletova et al. 2005; Kim et al. 2005; Vanderauwera et al. 2005). These gene expression studies clearly showed that increased cellular H_2O_2 levels have a considerable impact on the transcriptome of all species, by changing the expression of hundreds of genes. H_2O_2 not only affects genes involved in ROS detoxification but also drives the expression of genes involved in signal transduction, transcriptional regulation, and protein, carbohydrate or lipid metabolism, illustrating the complexity of the transcriptional response to H_2O_2.

We present a comparative transcriptome analysis that assesses, at a genome-wide scale, the similarity of the H_2O_2-dependent transcriptional response in evolutionarily distant species. Besides some species- or lineage-specific H_2O_2 responses, our analysis identified a confined set of similarly induced gene products in eukaryotes, with a strong conservation in yeast and *Arabidopsis*.

Key words: oxidative stress, hydrogen peroxide, microarray, comparative transcriptomics, *Synechocystis*, yeast, *Arabidopsis*, *H. sapiens*.

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Materials and Methods

Microarray and Protein Data Sets

Expression data of H_2O_2-induced genes were obtained from either Web sites or supplementary data of the corresponding articles: *Synechocystis* (Li et al. 2004; available at http://jb.asm.org); the complete data set of *S. cerevisiae* and *S. pombe* from http://www-genome.stanford.edu/yeast_stress/data/rawdata/complete_dataset.txt and from ftp://ftp.sanger.ac.uk/pub/postgenomics/s_pombe/wtaverage.txt, respectively; the complete microarray data set of the human HeLa cell line from http://microarray-pubs.stanford.edu/human_stress/Home.shtml; and a completely processed data set of the microarray analysis in *A. thaliana* (Kim et al. 2005; http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2295/TPJ2295sm.htm). A 2-fold change cutoff was used to identify genes that were differentially expressed.

The protein data set consisted of sequences from 1 cyanobacterium (*Synechocystis* sp. strain PCC 6803), 2 yeasts (*S. cerevisiae* and *S. pombe)*, 2 mammals (*H. sapiens* and *M. musculus*), and 2 plants (*A. thaliana* and rice [*Oryza sativa*]). Sequence information for all *Synechocystis* proteins was obtained from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov, release NC_0009111). Protein sequences from *S. cerevisiae* and *S. pombe* were retrieved from the *Saccharomyces* Genome Database (http://www.yeastgenome.org; release of August 2004) and Genome DataBase (http://www.genedb.org; release of November 2004), respectively. All protein sequences from *H. sapiens* (U25 NCBI 34 assembly) and *M. musculus* (U25 NCBI m33 assembly) were obtained from EnsEMBL (http://www.ensembl.org). Sequence information for *Arabidopsis* (release 5 January 2004; Wortman et al. 2003) and rice (release 2 April 2004; Yuan et al. 2003) was provided by The Institute for Genome Research. When multiple protein sequences were available for the same gene locus, the longest was retained.

Construction of Protein Families

Protein families were constructed by applying the Tribe-Markov clustering algorithm (Tribe-MCL) that uses graph-clustering methods and identifies clusters in a protein–protein similarity graph in a process that is sensitive to the density and the strength of the connections (Enright et al. 2002). A similarity matrix was generated from an all-against-all comparison using Blast (Altschul et al. 1997) with an *E* value threshold of 0.01. Clusters were formed with an inflation factor of 3.0. The original MCL algorithm was obtained from http://micans.org/mcl/, and more information concerning Tribe-MCl is also available at http://www.ebi.ac.uk/research/cgg/services/tribe/.

Significance Estimation Using Random Sampling

The significance of the number of stress-responsive gene families conserved between 2 species was estimated with random sampling. Briefly, for both species, the number of genes found in our analysis was randomly selected from all the genes present on the microarrays and the corresponding protein families were identified together with the number of conserved families. Based on 1,000 random sampling iterations, the significance of the observed overlap was estimated.

Results and Discussion

Identification of Homologous Gene Products across Kingdoms

We compared H_2O_2-driven gene expression in evolutionarily distant species by performing a meta-analysis on publicly available microarray data sets from 5 completely sequenced and annotated species (*Synechocystis* PCC 6803, *S. cerevisiae*, *S. pombe*, *H. sapiens*, and *A. thaliana*). These 5 species were selected because the relevant microarray studies were available and, with the exception of the protista, they cross the different biological kingdoms, hence spanning a broad evolutionary distance (Margulis 1992; Hedges and Kumar 2003).

A first necessary step was the identification of homologous gene products within the different organisms. Therefore, we clustered the protein sequences of the different species with Tribe-MCL. This algorithm allows a fast and accurate classification of large protein data sets into protein families and has multiple advantages over alternative protein clustering methods (Enright et al. 2002). Protein sequences of a second plant (*O. sativa*) and mammalian species (*M. musculus*) were included to improve the clustering outcome.

The clustering resulted in 16,207 protein families (containing more than 1 protein) encompassing 118,020 individual proteins in total. The different protein families were first evaluated according to size (protein number), species number, and species representation. Family size was opposite proportional to frequency of occurrence, with the majority (>95%) of all families smaller than 20 proteins (fig. 1*A*). Most protein families were restricted to 1 (3232; 20%) or 2 (9112; 56%) species, reflecting the large evolutionary distances between the species (fig. 1*B*). The species representation of all 16,207 protein families pointed to the existence of a limited set of highly conserved proteins (belonging to 244 protein families) that have a molecular function similar in *Synechocystis* and in eukaryotes, as well as to a substantial diversity among the different kingdoms (fig. 2). In addition, with this analysis, 1,244 conserved eukaryotic protein families were identified (fig. 2).

Quality of the Tribe-MCL was further assessed by manual inspection of the phylogenetic profiles of several genuine antioxidant enzymes: SODs, catalases, and peroxiredoxins, all known to have an evolutionarily conserved function during ROS detoxification (Touati 1988; Zámocký and Koller 1999; Rhee et al. 2005). As expected, protein sequences of SODs, catalases, and peroxiredoxins were contained within specific protein families (data not shown). Proteins with abundant domains, including protein kinase or DNA-binding domains were frequently found in larger, more divergent protein families containing more than 200 proteins (Riechmann et al. 2000; Wang et al. 2003).

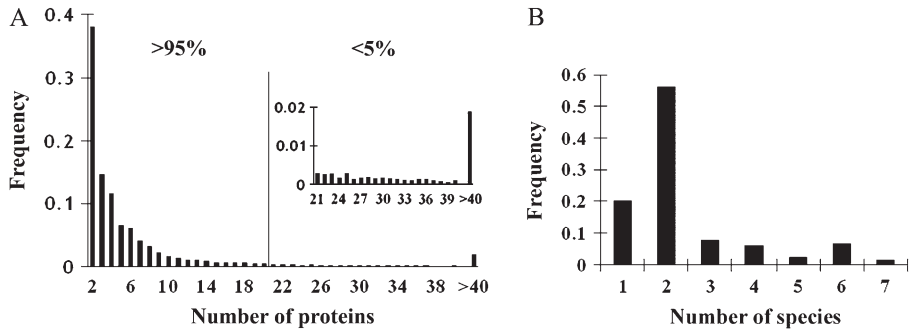

FIG. 1.—Frequency distribution of protein family size and species number for 16,207 protein families. (*A*) Histogram of protein family size, represented as protein number. The distribution of protein families with more than 20 proteins is blown up in the inset image. For sake of clarity, protein families containing more than 40 proteins (frequency <0.001) are not shown separately. (*B*) Frequency distribution of species number.

Because Tribe-MCL correctly grouped proteins with significant sequence similarity and almost identical functions, we concluded that the clustering in protein families was accurate and reliable for further analysis.

H_2O_2-Induced Gene Expression in Evolutionarily Distant Species

For prokaryotes, we selected a microarray experiment that followed the expression of 3,168 genes from *Synechocystis* sp. strain PCC 6803 after addition of 1.5 mM H_2O_2 to a cell culture (Li et al. 2004). In the yeast experiments, cDNA microarrays (containing ca. 5,200 and 6,000 *S. pombe* and *S. cerevisiae* genes, respectively) were used to monitor gene expression after addition of H_2O_2 (0.3–0.5 mM) to a cell culture (Gasch et al. 2000; Chen et al. 2003). To avoid an additional level of complexity related to tissue-specific responses in multicellular organisms, single-cell systems were also used to study H_2O_2-induced stress responses in animals and plants. For *H. sapiens*, we selected a microarray analysis of 25,802 genes in HeLa cells treated with different H_2O_2 concentrations (Murray et al. 2004). For the plant kingdom, we opted for an experiment in which microarrays (representing 25,636 genes) were used to monitor the transcriptional changes of 2-week-old, liquid-cultured *A. thaliana* seedlings that were treated with 5 mM H_2O_2 (Kim et al. 2005). More details on the selected microarray experiments can be found in table 1. Due to the heterogeneity of the experimental setups, we used relative expression data to identify differentially expressed genes. Figure 3 presents the kinetics of the transcription response, showing the number of genes with 2-, 3-, 4-, and more than 5-fold changes within these experiments. In all species, a significant up- and downregulation of transcript levels occurred, but we focused only on the inductive response because, in most cases, it starts earlier than the repressed response, enabling one to target upstream genes with minimal interference of secondary effects. Within the early time points, we selected those at which the strongest induction was observed: 30 min, 30 min, 1 h, 6 h, and 3 h for *Synechocystis*, *S. cerevisiae*, *S. pombe*, human HeLa cell lines, and *A. thaliana*, respectively. Genes with an H_2O_2-induced expression of at least 2-fold were retained for further analysis (161, 607, 578, 298, and 690 genes for *Synechocystis*, *S. cerevisiae*, *S. pombe*, human HeLa cell line, and *A. thaliana*, respectively).

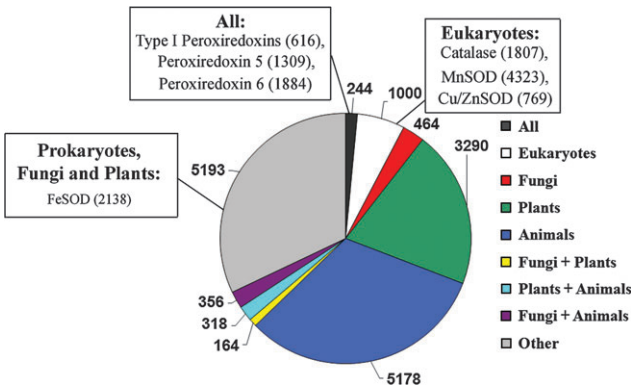

FIG. 2.—Evolutionary distribution of all protein families. All include protein families with representatives in all analyzed species. Eukaryotes group protein families with representatives in all species other than *Synechocystis*. *Fungi*, *Plants*, and *Animals* include protein families with only representatives for fungi, plants, and animals, respectively. The total number of protein families in each category is indicated. The evolutionary conservation of different SODs, catalases, and peroxiredoxin protein families is boxed. Family ID numbers are shown in parentheses.

H_2O_2-Response Matrix as a Tool for Comparing Gene Expression

First, we identified H_2O_2-responsive protein families. Within a species, a protein family was considered to be responsive when at least 1 of its members was more than 2-fold upregulated. A H_2O_2-response matrix was obtained that indicated how many gene products within each family were responsive. The data can be queried on our Web site (http://bioinformatics.psb.ugent.be/supplementary_data/strob/h2o2/). The number of H_2O_2-responsive families varied for each species and was proportional to the total number of H_2O_2-responsive genes. In *S. cerevisiae*, *S. pombe*, and *A. thaliana*, approximately 400 families were responsive to H_2O_2, in contrast to only 101 and 168 families in *Synechocystis* and *H. sapiens*, respectively (table 1). We used the H_2O_2-response matrix to determine the evolutionary conservation of the H_2O_2 response in all species (fig. 4*A*). The H_2O_2 responsiveness of 87% of the 1,253 families was restricted to 1 species, indicating a strong species-specific response (fig. 4*B*). Table 2

Table 1
Overview and Details of the 5 Selected Microarray Experiments

Species	Microarray Platform	Genes Represented	Treatment H_2O_2 (mM)	Time Points	Induced Genes[a]	Responsive Protein Families	Reference
Synechocystis	GST[b]	3,168	1.5	30 min	121	101	Li et al. (2004)
Saccharomyces cerevisiae	GST[b]	6,000	0.30	10, 20, 30, 40, 50, 60, 80, 90, 100, 120 min	504	403	Gasch et al. (2000)
Schizosaccharomyces pombe	GST[b]	5,269	0.50	15, 60 min	504	392	Chen et al. (2003)
Homo sapiens HeLa	cDNA	25,802	0.60	0.5, 1, 2, 8, 16, 24, 30 h	191	168	Murray et al. (2004)
Arabidopsis thaliana	GST[b]	25,636	5	1, 3, 6, 12 h	658	390	Kim et al. (2005)

[a] H_2O_2-induced genes with homologous gene products.
[b] GST, gene-specific tag.

presents the pairwise overlap of the H_2O_2 response between the different species. Not surprisingly, the overlap was the largest between *S. cerevisiae* and *S. pombe* with 107 common H_2O_2-responsive protein families (*P* value <0.001), revealing that the H_2O_2-induced transcriptional response is highly conserved between these 2 yeasts. Our data, together with the conserved core environmental stress responses of distant yeast species, indicate that stress responses in general are well conserved in yeast (Chen et al. 2003).

Four Protein Families within the Core Eukaryotic H_2O_2 Response

In addition to protein families that were H_2O_2 responsive in only 2 species, 31 protein families were responsive in at least 3 species (table 3). Remarkably, 23 families were responsive in *A. thaliana*, *S. cerevisiae*, and *S. pombe*, but only 6 families were responsive in *H. sapiens* and both yeasts (fig 4*C* and *D*). Although the low number of H_2O_2-responsive families in *H. sapiens* is partially responsible for this difference, the conservation between both yeasts and *A. thaliana* was significant (*P* value <0.05) and that with *H. sapiens* was not. These data demonstrate that the transcriptional response to increased H_2O_2 levels in yeast is more similar to that of plants than to that of animals.

Three protein families (representing guanine triphosphate [GTP]-binding proteins, protein kinases, or ubiquitin [Ub]-conjugating enzymes) were induced in yeast, *A. thaliana*, and *H. sapiens* but had no homologs in the prokaryote *Synechocystis*, restricting this conservation to eukaryotes. One protein family, representing DNAJ heat shock proteins (HSPs), was induced by H_2O_2 in all kingdoms. Together, these 4 protein families were defined as the "core eukaryotic H_2O_2 response." In the remaining protein families, no conserved response was found within 1 of the abovementioned species combinations.

It is known that HSPs, GTP-binding proteins, protein kinases, and Ub-conjugating enzymes function in evolutionarily conserved biological processes, such as heat shock response, cellular signaling, or protein metabolism. Therefore, they might also have an important and conserved role in responses to oxidative stimuli. This analysis suggests that this conserved functionality requires, at least to some extent, regulation at the transcriptional level.

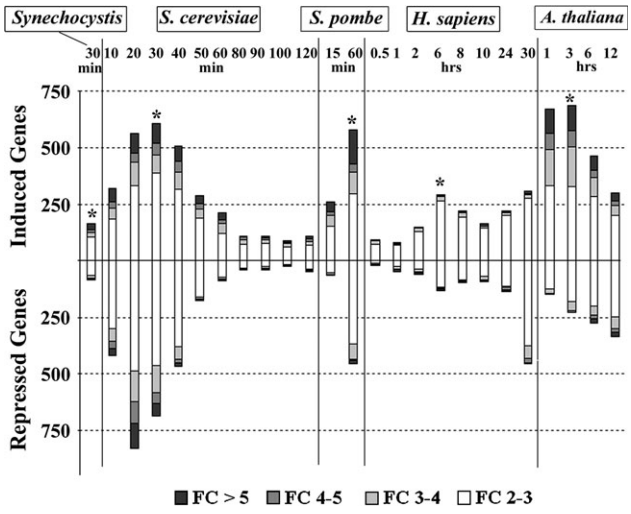

FIG. 3.—Kinetics of the transcriptional response within the individual ROS experiments. The number of transcripts with a 2- to 3-, 3- to 4-, 4- to 5-, and >5-fold increase or decrease in expression at the different time points are represented by white, light gray, dark gray, and black bars, respectively. Time points indicated with asterisks were used for this study. FC, fold change.

Evolutionarily Conserved H_2O_2-Induced Heat Shock Response

The proteins with the best evolutionarily conserved response to H_2O_2 are DNAJ HSPs, which are molecular chaperones defined by the presence of the conserved J domain (table 3). They can stimulate the substrate-binding activity of 70-kDa HSPs, thereby modulating accurate protein folding and transport (Walsh et al. 2004). Other HSPs (HSP90 and HSP20) were also, albeit less conserved, induced by H_2O_2 (table 3). In addition, H_2O_2 induction of HSPs has been reported in other species, such as tomato, rice, and *Drosophila* (Courgeon et al. 1990; Banzet et al. 1998; Lee et al. 2000). The conserved need for HSPs during oxidative stress might be explained by the chaperone function that HSPs can exert on oxidatively damaged and partially denatured proteins (Jakob et al. 1999). Alternatively, heat shock factors can act as direct sensors of H_2O_2, thereby regulating the expression of defense genes and subsequent protection during oxidative stress (Ahn and Thiele 2003; Volkov et al. 2006; Miller and Mittler 2006). Because of their protective function, loss of HSPs leads to increased

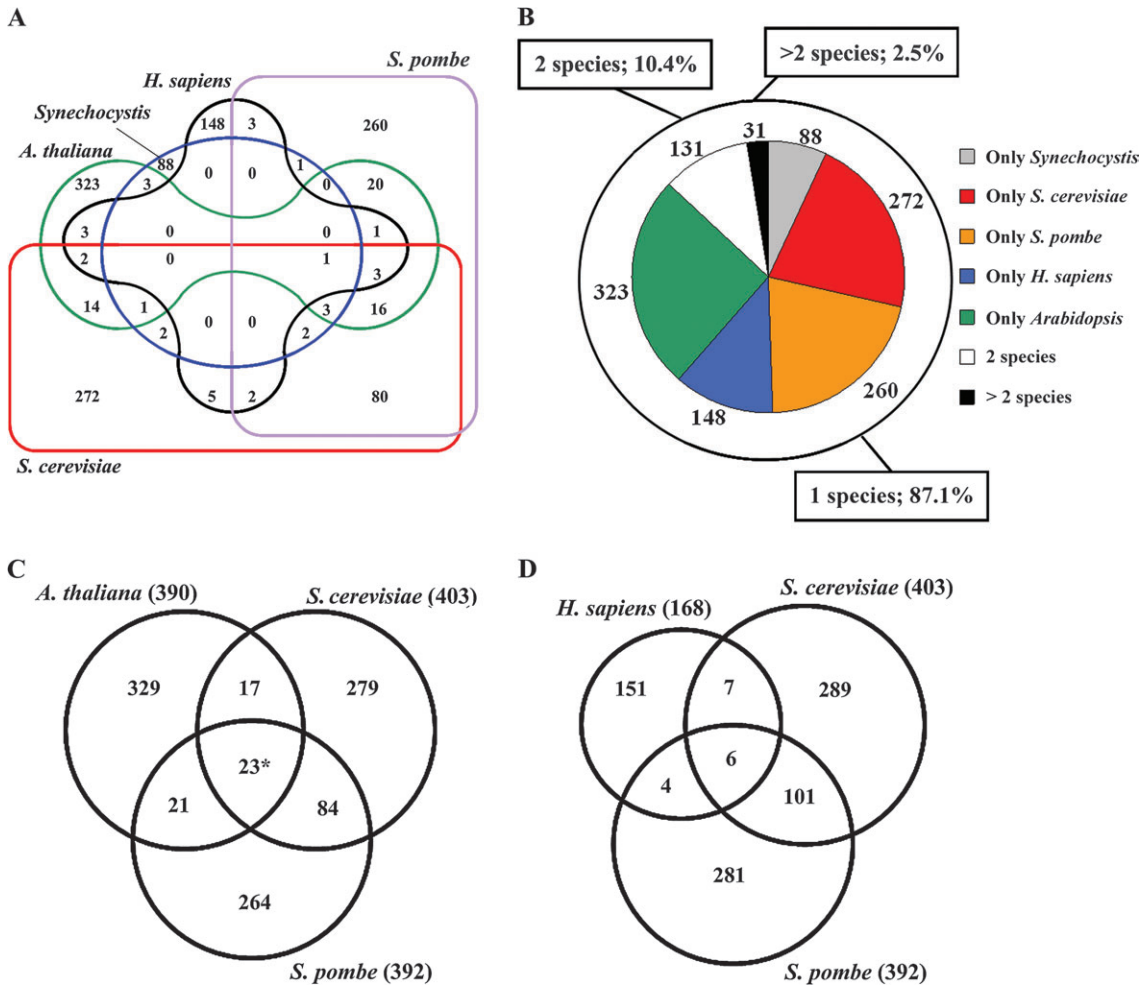

FIG. 4.—Evolutionary conservation of the H_2O_2-inductive response. Each value represents a number of responsive protein families. (*A*) Venn diagram illustrating the conservation between *Synechocystis*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Homo sapiens*, and *Arabidopsis thaliana*. (*B*) Pie diagram showing the number of families of which the response is conserved in only 1 species, in just 2 species, and more than 2 species. (*C*) Detailed Venn diagram demonstrating the overlap between *S. cerevisiae*, *S. pombe*, and *A. thaliana*. (*D*) Detailed Venn diagram showing the conservation in *S. cerevisiae*, *S. pombe*, and *H. sapiens*. *$P < 0.05$.

sensitivity, whereas constitutive expression of some HSPs (such as chloroplastic HSP21) enhances the tolerance toward heat and H_2O_2 stress (Härndahl et al. 1999; Jakob et al. 1999; Ahn and Thiele 2003; Neta-Sharir et al. 2005). Together, these observations suggest a significant overlap between the heat shock and oxidative stress response in all kingdoms.

Eukaryotic H_2O_2 Signaling Involves Induction of G Proteins and Ca^{2+}-Dependent Protein Kinases

We observed a conserved H_2O_2 induction in *S. pombe*, *S. cervisiae*, *A. thaliana*, and *H. sapiens* for 1 family of small, ras-like GTP-binding proteins (G proteins) and 1 protein family containing calcium (Ca^{2+})-dependent protein kinases (table 3). Both ras-like G proteins and protein

Table 2
Pairwise Comparisons of H_2O_2-Induced Transcriptional Responses

Species	*Synechocystis*	*Saccharomyces cerevisiae*	*Schizosaccharomyces pombe*	*Homo sapiens*	*Arabidopsis thaliana*
Synechocystis		407	412	446	867
S. cerevisiae	9		2,476	1,961	1,757
S. pombe	7	107*		2,067	1,864
H. sapiens	1	13	10		1,981
A. thaliana	8	40	44	10	

NOTE.—Above and under diagonal, numbers of protein families with genes from both species and observed numbers of common H_2O_2-induced protein families, respectively. *$P < 0.001$.

Table 3
Protein Families (31) with a Conserved H_2O_2 Expression Profile, Shown as Fraction of H_2O_2-Induced Genes, in At Least 3 Species

Family ID	Total Entries	Family Description	*Synechocystis*	*Saccharomyces cerevisiae*	*Schizosaccharomyces pombe*	*Arabidopsis thaliana*	*Homo sapiens*
All species							
38	197	DNAJ HSP	0.40	0.19	0.11	0.05	0.03
All eukaryotes							
8	361	Ras-related GTP-binding protein	N.R.	0.05	0.17	0.05	0.04
44	184	Ca^{2+}-dependent (S/T) protein kinase	N.R.	0.25	0.40	0.02	0.03
47	176	Ub-conjugating enzyme	N.R.	0.08	0.15	0.05	0.03
Unicellular organisms							
87	108	Short chain dehydrogenase/(oxido)reductases	0.12	0.20	0.50	0.00	0.00
616	25	Thioredoxin peroxidase (type II peroxiredoxins)	0.25	0.67	0.67	0.00	0.00
All species-*H. sapiens*							
28	231	ATPase, AAA family/FtsH protease	0.33	0.19	0.05	0.02	0.00
182	63	Small HSPs	1.00	1.00	0.50	0.24	N.R.
578	26	D-3-phosphoglycerate dehydrogenase	0.33	0.20	0.40	0.20	0.00
Yeast and *A. thaliana*							
13	329	Protein kinase/mitogen-activated protein kinase	N.R.	0.23	0.05	0.01	0.00
15	295	(Serine/threonine) protein kinase	0.00	0.23	0.22	0.04	0.00
20	259	Zinc finger, C3HC4 type (Really interesting new gene finger)	N.R.	0.50	0.50	0.06	N.R.
41	195	EF hand Ca^{2+}/Calmodulin-binding protein	N.R.	0.50	0.17	0.11	0.00
58	156	ABC transporter, subfamily G	0.00	0.10	1.00	0.02	0.00
130	80	Cinnamoyl-CoA/anthocyanidin reductase	0.00	0.25	1.00	0.12	N.R.
148	72	ABC transporter, subfamily C	0.00	0.14	0.25	0.19	0.00
151	72	Oxidoreductase, alcohol (aldo/keto) reductase family	0.00	1.00	0.83	0.20	0.00
154	71	Cation-transporting ATPase	0.00	0.20	0.33	0.20	0.00
199	60	Glutaredoxin	0.00	0.75	0.33	0.04	0.00
361	36	Trehalose-phosphatase/glycosyl transferase	0.00	0.75	0.60	0.09	N.R.
363	36	Oxidoreductase, zinc/nicotinamide adenosine dinucleotide phosphate-dependent dehydrogenase	N.R.	1.00	1.00	0.43	0.00
382	35	Heavy metal-transporting ATPase	0.00	0.50	1.00	0.12	0.00
1356	14	GTP cyclohydrolase	0.00	0.50	0.33	0.20	N.R.
1541	13	Ribonucleoside diphosphate reductase	0.00	0.50	1.00	0.330.00	
1818	11	Gluthatione peroxidase	0.00	0.67	1.00	0.25	N.R.
Yeast and *H. sapiens*							
1	1093	Zinc finger protein	N.R.	0.14	0.27	0.00	0.001
179	64	Ub-protein ligase	N.R.	0.25	0.33	0.00	0.05
Other[a]							
27	235	Serine/threonine protein kinase/MAPKKK	N.R.	0.00	0.06	0.02	0.04
55	165	Sugar transporter	0.00	0.17	0.00	0.04	0.13
331	38	Transcription factor/Jumonji/AT-rich interaction domain domain–containing protein	N.R.	0.50	0.00	0.10	0.20
311	40	HSP90	1.00	0.50	0.00	0.12	0.00

NOTE.—N.R., no representative protein found.
[a] Include protein families with a conserved H_2O_2 response in any combination of 3 species that is not represented in the other categories.

kinases have already been implicated in oxidative stress signaling in yeast, plants, and mammals, suggesting a conserved function for such proteins (Toone and Jones 1998; Finkel and Holbrook 2000; Essers et al. 2004; Rentel et al. 2004). Closer investigation of the G-protein family revealed that it represents Rab GTP–binding proteins, 1 of the 5 subfamilies of ras-like GTPases (Vernoud et al. 2003). Rab GTPases are mainly involved in cellular trafficking, but at least in plants, they might have evolved additional functions (Rutherford and Moore 2002). However, a role for Rab proteins during oxidative stress signaling has not been elucidated yet.

Environmental or cellular stimuli, including oxidative stress, can cause changes in Ca^{2+} patterns, which can be sensed by specific Ca^{2+}-dependent protein kinases and decoded into downstream effects, such as altered protein phosphorylation and gene expression (Cheng et al. 2002). In animals, it is well known that H_2O_2 can activate Ca^{2+}-dependent protein kinases to prevent oxidative stress–induced cell death (Franklin et al. 2006).

In addition to the importance of G proteins and Ca^{2+}-dependent protein kinases in controlling the eukaryotic response to oxidative stress, our data suggest the involvement of transcriptional regulation of these genes by H_2O_2, which might be essential for signal amplification and cross talk during oxidative stress. This hypothesis would be in agreement with the general function of ras-like G proteins and protein kinases in multiple, interconnected signaling cascades that control various biological processes (Matozaki et al. 2000).

Table 4
H_2O_2 Responsiveness of Antioxidant Genes, Shown as Fraction of H_2O_2-Induced Genes

Family ID	Family Description	*Synechocystis*	*Saccharomyces cerevisiae*	*S. pombe*	*Arabidopsis thaliana*	*Homo sapiens*
1807	CAT	N.R.	0.50	1.00	0.00	0.00
2138	Iron SOD	0.00	0.00	0.00	0.00	N.R.
4323	MnSOD	N.R.	1.00	0.00	0.00	0.00
769	Cu/ZnSOD	N.R.	0.50	0.50	0.00	0.00
413	GPX	0.00	1.00	1.00	0.00	0.00
812	APX/CAT peroxidase	0.00	1.00	N.R.	0.00	N.R.
1309	Peroxiredoxin 5 (type 2)	1.00	1.00	0.00	0.00	0.00
1884	Peroxiredoxin 6 (type 2)	0.00	1.00	N.R.	0.00	0.00
616	Peroxiredoxin 1, 2, 3, 4 (type 1)	0.25	0.67	0.67	0.00	0.00

NOTE.—APX, ascorbate peroxidase; CAT, catalase; GPX, glutathione peroxidase; N.R., no representative protein found.

Conserved H_2O_2-Induced Ubiquitination Response in Eukaryotes

Ub-conjugating enzymes act within proteasome-dependent proteolysis where they transfer Ub molecules, either directly or via an Ub ligase, to a substrate protein, a process known as ubiquitination (Pickart 2001). Transcripts of Ub-conjugating enzymes were induced by H_2O_2 in all 4 eukaryotes (table 3). A robust ubiquitination response and a transient increase in activity of the Ub-dependent pathway have been demonstrated to occur in lens cells exposed to oxidative stress, resulting in enhanced recovery after oxidative stress (Shang et al. 1997). This protection is probably a result of the targeted removal of oxidized or damaged proteins by Ub-conjugating enzymes or the Ub-dependent proteolytic pathway in general (Shang and Taylor 1995). The importance of the Ub-dependent pathway during oxidative stress is further highlighted by the requirement of a functional polyubiquitin gene to withstand toxic H_2O_2 levels in yeast (Cheng et al. 1994).

A Conserved Antioxidant Response in Unicellular Organisms

Besides 4 protein families with a conserved H_2O_2 induction in eukaryotes, we also observed a significant (P value <0.05) conservation within all the unicellular organisms, with 2 families showing a specific transcriptional induction in *Synechocystis*, *S. cerevisiae*, and *S. pombe*: short-chain dehydrogenases/reductases (SDR) and type I peroxiredoxins (table 3). Both SDR and peroxiredoxins are evolutionarily conserved proteins that are directly involved in the protection of cells against oxidative stress (Kallberg et al. 2002; Rhee et al. 2005). For example, constitutive expression of a SDR protein confers protection against oxidative stress-induced cell death via the detoxification of highly reactive xenobiotics (Botella et al. 2004). Peroxiredoxins are thioredoxin-dependent peroxidases that remove H_2O_2 and peroxinitrites (Rhee et al. 2005). The importance of peroxiredoxins as antioxidants is further illustrated by their capacity to prevent H_2O_2-induced apoptosis in human cells (Yuan et al. 2004).

In addition to SDR and type I peroxiredoxins, H_2O_2 induction of genuine antioxidant enzymes, such as catalases, SODs, glutathione peroxidases, ascorbate peroxidases, and type II peroxiredoxins, was also restricted to unicellular organisms (table 4). Catalases, copper/zinc (Cu/Zn) SODs, and glutathione peroxidases were induced in both yeast species but not in *Synechocystis*. Other antioxidant enzymes showed less conserved expression patterns in unicellular organisms. However, none of the antioxidant genes were induced by H_2O_2 in *A. thaliana* and *H. sapiens*. These data indicate that unicellular antioxidant systems are part of the oxidative stress-inducible adaptive responses, whereas higher eukaryotes carry a rather constitutive transcriptional antioxidant response during H_2O_2-induced oxidative stress (Storz and Imlay 1999). Although transcriptional control of antioxidant genes in specific (oxidative) stress situations cannot be excluded, antioxidant gene expression of animals and plants seems to be controlled at the posttranscriptional level. Manganese (Mn) SOD production in animal models, for example, is regulated via the binding of an unidentified MnSOD mRNA-binding molecule (Clerch 2000). In plants, posttranscriptional regulation of ascorbate peroxidase levels has been evidenced during programmed cell death and drought stress (Mittler and Zilinskas 1994; Mittler et al. 1998). Recently, a microRNA molecule (miR398) has been identified as a repressor of Cu/ZnSOD expression in *A. thaliana* and downregulation of miR398 is important for tolerance against oxidative stress (Sunkar et al. 2006). These data suggest that posttranscriptional control of antioxidant gene expression might be very important in mammals and plants.

Conserved H_2O_2 Induction of Protein Families with Unknown Function

To investigate the H_2O_2 response of genes with unknown function, we manually analyzed our set of 162 different protein families that were responsive in at least 2 species for the overrepresentation of unknown, expressed, or hypothetical proteins. Because most unknown proteins are species-specific, only 18 such protein families were found (Gollery et al. 2006). Eight of these families were highly H_2O_2 responsive but contained only yeast proteins, suggesting that they might be part of a yeast-specific response to H_2O_2 (data not shown). The 10 remaining protein families had homologs from at least 2 kingdoms and were retained for further analysis. For these 10 unknown protein families, the conservation of the H_2O_2 induction was restricted to yeast or to yeast and *A. thaliana*, again

Table 5
Conserved H_2O_2 Response of Unknown Protein Families, Shown as Fraction of H_2O_2-Induced Genes

Family ID	Family Description[a]	*Saccharomyces cerevisiae*	*Schizosaccharomyces pombe*	*Arabidopsis thaliana*
976	Unknown protein, putative choline transporter	1.0	1.0	0
1033	Unknown protein, putative glutamate binding, inner membrane localization	N.R.	1.0	0.2
2139	Unknown, contains RING-zinc finger	1.0	1.0	0
3312	Unknown protein, predicted membrane function, contains unknown DUF962 domain	1.0	1.0	0
3319	Unknown protein, ubiquitin-associated/ubiquitin regulatory X domain	1.0	1.0	N.R.
4314	Unknown, conserved eukaryotic protein with Lissencephaly-1 protein homology, C-terminal to LisH, and RING-zinc finger motif	1.0	1.0	0
5028	Unknown, pyridoxine 5′-phosphate oxidase-related	1.0	1.0	0
5899	Unknown	1.0	1.0	0
8617	Unknown, contains Ub, WLM metallopeptidase, and peptide N-glucanases and other putative nuclear UBA or UBX domains	N.R.	1.0	1.0
9064	Unknown, contains UbiE/COQ5 methylase/ methyltransferase domains	N.R.	1.0	0.5

NOTE.—N.R., no representative protein found.
[a] Family description is based on protein annotation and Blast homology searches.

demonstrating that the H_2O_2 response is better conserved between yeast and *A. thaliana* than between yeast and *H. sapiens* (table 5).

The term "protein with unknown function" is used broadly and is mostly based on lack of clear homology with known proteins. A better definition for unknown proteins is "proteins with obscure features" (POFs), which lack defined motifs or protein domains (Gollery et al. 2006). To identify POFs, the proteins within the different unknown families were subjected to Blast homology searches. In doing so, we were able to identify functional domains and could assign putative functions to 8 out of 10 unknown protein families. One family contained proteins with no functional domains and these are considered to be POFs. A second protein family represented proteins with only predicted membrane function and unknown DUF962 domains.

The conserved H_2O_2 induction of these POFs suggests an important role for them during oxidative stress in yeast or plants. Therefore, these proteins are maybe good candidates to study new aspects of stress signaling and it would be interesting to further analyze the function of these proteins, for example, to improve stress tolerance in these species.

Conclusions

The comparative analysis of H_2O_2-induced gene expression across kingdoms hints at a strongly specialized transcriptional response, besides a small core eukaryotic H_2O_2 response. In addition, this analysis clearly reveals that the inductive transcriptional response to H_2O_2 is highly conserved in yeasts and that this yeast response is more conserved in plants than in animals. Antioxidant gene expression is only induced in unicellular organisms and not in higher eukaryotes, indicating that some specific responses are only partially conserved. Furthermore, the presented approach was used for gene discovery by focusing on unknown proteins, hereby hypothesizing that genes with a conserved H_2O_2-induced transcription might have an important role during oxidative stress. As more sequence and transcriptome data of other species are expected in the future, sampling within 1 specific kingdom, phylum, or taxon will lead to new insights into the evolution and conservation of the transcriptional response to oxidative stress.

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