Hydrogen Peroxide-Responsive Genes in Stress Acclimation and Cell Death

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Abstract Reactive oxygen species (ROS) are key signalling molecules that regulate growth and development and coordinate responses to biotic and abiotic stresses. ROS homeostasis is controlled through a complex network of ROS production and scavenging enzymes. Recently, the first genes involved in ROS perception and signal transduction have been identified and, currently, we are facing the challenge to uncover the other players within the ROS regulatory gene network. The specificity of ensuing cellular responses depends on the type of ROS and their subcellular production sites. Various experimental systems, including catalase-deficient plants, in combination with genome-wide expression studies demonstrated that increased hydrogen peroxide (H_2O_2) levels significantly affect the transcriptome of plants and are capable of launching both defence responses and cell death events. A comparative analysis between H_2O_2-induced transcriptional changes and those provoked by different environmental stresses, not only consolidated a prominent role for H_2O_2 signalling in stress acclimation, but also allowed the identification of new candidate regulatory genes within the plant's abiotic stress response.

1 Dual Face of Reactive Oxygen Species

Reactive oxygen species (ROS) are partially reduced or activated derivatives of oxygen that are formed during normal cell metabolism in different subcellular compartments. Adverse environmental conditions lead to an increased production of ROS, resulting in oxidative stress. At higher concentrations, ROS cause oxidative injuries by reacting with different cellular components, including DNA, proteins and lipids, and become toxic to the cell. In order to avoid ROS-dependent cellular damage, a fine-tuned balance between ROS production and scavenging is essential. In *Arabidopsis thaliana*, this balance is governed by a complex network that comprises

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at least 152 genes encoding both ROS-producing and ROS-scavenging enzymes (Mittler et al. 2004). The different components of the antioxidant system are diversified in their scavenging capacity not only in terms of specificity and affinity towards the different ROS species, but also by their distinct subcellular location in the vicinity of the ROS production sites. Peroxisomes, chloroplasts and mitochondria are organelles with highly oxidising metabolic activities or with intense rates of electron flow and are, hence, major sources of ROS production in plant cells. Therefore, it is not surprising that these subcellular compartments contain various ROS-scavenging enzymes (Mittler et al. 2004). The tight regulation of ROS homeostasis also creates a baseline on which ROS spikes can signal in different cellular processes. Although the underlying signalling function of ROS has long been masked by the inherent toxic nature of these oxidants, their triggering ability during various developmental processes and environmental stress responses is now widely recognised (Foyer and Noctor 2005; Torres and Dangl 2005; Gapper and Dolan 2006; Mullineaux et al. 2006; Fig. 1). ROS with documented signalling functions include hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydroxyl radical (OH•), and superoxide anion radical ($O_2•^-$). How ROS and ROS-derived signals are perceived, integrated and transduced to eventually control a broad range of biological processes remains largely elusive. ROS might interact selectively with specific target molecules that thereby sense and translate increased ROS levels into a transcriptional response (Apel and Hirt 2004). Intensity, duration and subcellular localisation of ROS production are likely crucial in the cellular and intracellular transduction of the ROS signals (Bailey-Serres and Mittler 2006).

2 ROS Perception and Signal Transduction

Increased ROS levels in different subcellular compartments can result in changes in nuclear gene expression, indicating a ROS-dependent intraorganellar signalling (Vanderauwera et al. 2005; Rhoads et al. 2006; Koussevitzky et al. 2007). Recently,

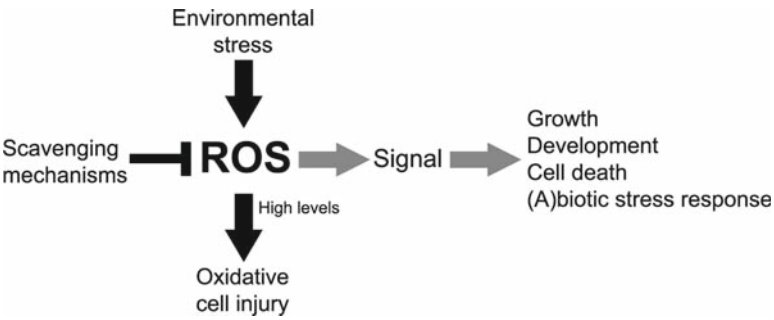

Fig. 1 The dual face of ROS. Unfavourable environmental conditions result in excessive ROS production that leads to oxidative cell injuries at high concentrations. To prevent ROS-dependent cellular damage, plants have a broad range of antioxidant mechanisms at their disposal to use ROS simultaneously as a signal within various biological processes, such as growth and development, defence responses to biotic and abiotic stimuli and programmed cell death

the first genes involved in ROS perception and signal transduction have been identified (Rentel et al. 2004; Kaminaka et al. 2006; Lee et al. 2007), but it remains a challenge to identify other players in the gene regulatory networks and to unravel their mode of action in ROS signal perception and transduction.

One likely mechanism for cells to perceive the presence of ROS is through ROS-dependent protein modifications. The oxidising nature of H_2O_2 and $O_2\bullet^-$ allows the direct modification of thiol groups in proteins, although other amino acids, such as tyrosine, tryptophan and histidine, may be oxidised as well. Oxidation of cysteine (Cys) residues might lead to disulphide bridging and subsequent conformational changes or, alternatively, to a variable degree of oxidation that depends on the residue's redox potential and the intracellular redox state (Hancock et al. 2006). The alteration of key Cys residues of transcription factors provides a direct and powerful means to influence nuclear gene expression. For example, in *Escherichia coli*, the transcription factor OxyR is activated by H_2O_2 (Zheng et al. 1998), whereas in *Saccharomyces cerevisiae*, oxidation of YAP1 following oxidative stress results in its nuclear accumulation and subsequent activation of antioxidant gene expression (Delaunay et al. 2000). In plants, redox regulation has been reported for the nonexpressor of pathogenesis-related 1 (NPR1) and the leucine zipper transcription factor TGA1 that are both important mediators of systemic acquired resistance during plant-pathogen interactions. Reduction of key Cys residues in these proteins relocates NPR1 to the nucleus and modulates the DNA-binding activity of the NPR1/TGA1 protein complex, thereby decisively affecting downstream gene expression (Després et al. 2003; Mou et al. 2003). The activity of the transcription factor Rap2.4a that controls plastid antioxidant gene expression is also redox regulated. Rap2.4a is activated by a dithiol/disulphide transition of regulatory Cys residues and subsequent changes in the quaternary structure (Shaikhali et al. 2008).

Transcriptional modifications may also require more upstream sensing and transduction of ROS and ROS-derived signals. In yeast, histidine kinases (HKs) of two-component signalling systems have been reported to function as sensors of oxidative stress (Singh 2000). In *Arabidopsis*, the HK of the ethylene receptor ETR1 appears to be essential for H_2O_2 perception leading to stomatal closure. Mutation of a Cys residue in the N-terminal region of ETR1 disrupted the H_2O_2 perception and diminished the stomatal closure in response to H_2O_2, indicating that this thiol group is important for H_2O_2 signalling. As the kinase domain of ETR1 was not required for H_2O_2 signalling, the H_2O_2-provoked signalling through ETR1 was seemingly unrelated to its well-described role as an ethylene receptor (Desikan et al. 2005). In addition, the *Arabidopsis* HK5 plays a crucial role in mediating H_2O_2-dependent processes in stomatal guard cells that are induced by both environmental and hormonal signals (Desikan et al. 2008).

A mitogen-activated protein kinase (MAPK) cascade and several protein phosphatases are involved in relaying H_2O_2 signals in *Arabidopsis*. Both ANP1, a MAPK kinase kinase, and OXI1, a serine/threonine protein kinase, are important for H_2O_2 sensing and the activation of a MAPK cascade (Kovtun et al. 2000; Rentel et al. 2004). H_2O_2 also strongly activates the nucleotide diphosphate kinase 2 (NDPK2) that specifically interacts with MPK3 and MPK6, thereby regulating the cellular redox state (Moon et al. 2003). MPK6 can also be activated via H_2O_2-mediated

inhibition of the protein tyrosine phosphatase PTP1 (Gupta and Luan 2003), indicating that protein phosphatases might act as negative regulators of ROS-induced MAPK pathways (Schweighofer et al. 2004).

Taken together, these studies give a glimpse of the complex signalling network that underlies the cellular response to elevated levels of ROS and, more particularly, H_2O_2. However, specific signalling effects have also been reported for singlet oxygen and superoxide anion, involving the two closely related chloroplastic proteins EXECUTER1 and EXECUTER2, and LESION SIMULATING DISEASE 1 (LSD1), respectively (Mateo et al. 2004; Wagner et al. 2004; Kaminaka et al. 2006; Lee et al. 2007).

3 ROS-Dependent Gene Expression

The integrated cellular response to oxidative stress is particularly suited for a genomic approach: ROS levels can be modulated relatively easily in an organism by exposing it to increased dosages of oxidants or by depleting or perturbing its antioxidant capacities (Table 1). Genome-wide expression tools provided the means to assess the overall effect of ROS signalling on gene expression in different organisms. Transcriptomic and proteomic surveys in bacterial, yeast and mammalian cell lines have provided significant insights into the cellular responses towards oxidative stress (Causton et al. 2001; Mostertz et al. 2004; Murray et al. 2004; Vandenbroucke et al. 2008). In plants, the first report on a genome-wide expression analysis was provided by Desikan et al. (2001). Using a cDNA microarray, at least 113 and 62 transcripts were shown to be induced and repressed, respectively, in *Arabidopsis* cell suspensions treated with a specific dose of H_2O_2. A substantial number of these genes were predicted to function within the defence response, cell signalling, transcription and cell death. In tobacco (*Nicotiana tabacum*), a combination of differential display and cDNA macroarray analysis identified 53 transcripts involved in stress acclimation responses (Vranová et al. 2002). The two experimental set-ups relied on the exogenous addition of H_2O_2 and a superoxide generator, respectively. An alternative approach to modulate ROS levels in planta is through the use of loss-of-function mutants in specific antioxidant genes. The cDNA-amplified fragment length polymorphism (AFLP) technology revealed that more than 700 transcripts were differentially expressed upon increase of photorespiratory H_2O_2 (Vandenabeele et al. 2003). Later, the advent of different microarray platforms that covered the majority of the annotated *Arabidopsis* genes allowed the assessment of the transcriptome of available mutants and transgenic plants perturbed in the antioxidant gene network (op den Camp et al. 2003; Rizhsky et al. 2003; Davletova et al. 2005a; Umbach et al. 2005; Vanderauwera et al. 2005) (Table 1). These surveys provided not only detailed inventories of ROS-dependent gene expression in plants, but also evidence for the specific signalling capacities of different ROS that allow them to act efficiently in various developmental processes and environmental stress responses. A meta-analysis of oxidative stress-responsive transcripts in *Arabidopsis* revealed both common and specific responses towards the different ROS signals

Table 1 Overview of studies describing the impact of different ROS on gene expression

	Major ROS	Localisation	Plant system	Experimental details	Expression profiling platform	Reference
Exogenous ROS						
H_2O_2	H_2O_2	Unknown	Cell suspension	1.5, 3 h 20 mM	cDNA microarray (8K)	Desikan et al. (2001)
H_2O_2	H_2O_2	Unknown	Cell suspension	5 h 10 mM	cDNA microarray (7K)	Takahashi et al. (2004)
H_2O_2	H_2O_2	Unknown	2-week-old plants	1, 3, 6, 12 h 5 mM (liquid medium)	cDNA microarray (26K)	Kim et al. (2005)
H_2O_2	H_2O_2	Unknown	5-day-old plants	1 h 20 mM (liquid medium)	Affymetrix ATH1 microarray (22K)	Davletova et al. (2005b)
Aminotriazol	H_2O_2	Peroxisomes	4-week-old plants	7 h 20 mM (spray)	Agilent Arab2 microarray (22K)	Gechev et al. (2005)
AAL toxin	H_2O_2	Unknown	4-week-old plants	7, 24, 48, 72 h 200 nM (infiltration)	Agilent Arab2 microarray (22K)	Gechev et al. (2004)
Ozone	O_3, $O_2^{\bullet-}$, H_2O_2	Apoplast	4-week-old plants	1, 4, 8, 12, 24 h 300 nL L^{-1} (6 h exposure)	Operon microarray (26K)	Mahalingam et al. (2006)
			4-week-old plants	3, 6, 12, 24 h 300 ppb (6 h exposure)	Affymetrix ATH1 microarray (22K)	Tosti et al. (2006)
			4-week-old plants	3, 6 h 350 ppb (6 h exposure)	Affymetrix ATH1 microarray (22K)	Ludwikow et al. (2004)
			Rosette stage	8-12 days free air concentration enrichment	Operon microarray (26K)	Li et al. (2006)
Endogenous ROS						
cat2 RNAi	H_2O_2	Peroxisomes	6-week-old plants	3, 8 h from 100 to 1,800 µmol m^{-2} s^{-1}	cDNA microarray (6K)	Vandenabeele et al. (2004)
				3, 8 h from 100 to 1,800 µmol m^{-2} s^{-1}	Affymetrix ATH1 microarray (22K)	Vanderauwera et al. (2005)

(continued)

Table 1 (continued)

	Major ROS	Localisation	Plant system	Experimental details	Expression profiling platform	Reference
cat2 KO	H_2O_2	Peroxisomes	5-week-old plants	1 week from 4,500 ppm CO_2 to ambient air	cDNA-AFLP (2K)	Queval et al. (2007)
apx1 KO	H_2O_2	Cytosol	2-week-old plants	0, 1, 48 h from 100 to 425 μmol m^{-2} s^{-1}	Affymetrix microarray (8K)	Pnueli et al. (2003)
			3-week-old plants	0, 0.25, 0.5, 1.5, 3, 6, 24 h from 25 to 250 μmol m^{-2} s^{-1}	Affymetrix ATH1 microarray (22K)	Davletova et al. (2005a)
flu mutant	$^1O_2$	Chloroplasts	3-week-old plants	from dark to 100 μmol m^{-2} s^{-1}	Affymetrix ATH1 microarray (22K)	op den Camp et al. (2003)
AOX1a AS	$O_2^{\bullet -}$, H_2O_2	Mitochondria	3-week-old plants	Standard growth conditions	Affymetrix ATH1 microarray (22K)	Umbach et al. (2005)
SOD KD	$O_2^{\bullet -}$, H_2O_2	Chloroplasts	3-week-old plants	Standard growth conditions	Affymetrix ATH1 microarray (22K)	Rizhsky et al. (2003)

Plants were treated with exogenously applied ROS and ROS-generating agents, whereas endogenous ROS accumulation was the result of perturbed levels of individual ROS-scavenging enzymes. *AS* antisense, *KD* knockdown, *KO* knockout

(Gadjev et al. 2006). Besides several hallmark transcripts of the general oxidative stress response, new candidate regulatory transcripts that could orchestrate specific transcriptomic signatures were detected as well as antagonistic effects triggered by the different ROS. Anthocyanin biosynthesis transcripts were induced in Cu/Zn superoxide dismutase (SOD) knockdown plants, while their induction during high-light (HL) exposure was compromised in catalase-deficient plants (Gadjev et al. 2006). This inverse correlation between the effect of $O_2^{\bullet -}$ and H_2O_2 on anthocyanin accumulation clearly demonstrates specific signalling capacities of particular ROS.

4 Photorespiratory H_2O_2-Dependent Gene Expression

Photorespiration is the process by which ribulose-1,5-bisphosphate carboxylase uses oxygen to oxygenate ribulose-1,5-biphosphate instead of carboxylating carbon dioxide (CO_2). This oxygenation results in two glycolates that are subsequently transported from the chloroplast to the peroxisomes and catalysed by glycolate oxidase, yielding H_2O_2. Photorespiration occurs at high rates in the leaves of C_3 plants (Noctor et al. 2002), most probably acts as an alternative electron sink, and increases under adverse environmental conditions that, either limit the CO_2 availability (for instance, through sustained stomatal closure during drought stress) or increase the light energy influx. The considerable energy used in photorespiratory carbon– and nitrogen-recycling lowers the quantum yield of photosynthesis, thereby utilising light less efficiently in CO_2 fixation. This effect could be physiologically advantageous because it reduces the probability of photoinhibition during abiotic stresses and, thereby, attenuates ROS production in the chloroplasts. A complementary function of increased photorespiratory flux during abiotic stress follows from the increased H_2O_2 production in the peroxisomes: photorespiratory H_2O_2 production was estimated to account for approximately 70% of the total H_2O_2 formed at any given irradiance intensity (Noctor et al. 2002). Increased levels of photorespiratory H_2O_2 are counteracted by peroxisomal catalases that are tetrameric heme-containing enzymes converting H_2O_2 into oxygen and water. Catalases are the principal peroxisomal H_2O_2-scavenging enzymes and are an important sink for photorespiratory H_2O_2 (Dat et al. 2001; Noctor et al. 2002; Mateo et al. 2004). Perturbation of catalase activities, by mutation or gene silencing, results in decreased H_2O_2 scavenging during HL irradiation or low CO_2 availability in different C_3 plants. These catalase-deficient plants were more sensitive to a variety of environmental stresses, such as chilling, ozone and salt stress (Kendall et al. 1983; Willekens et al. 1997; Vandenabeele et al. 2004), and revealed that photorespiratory H_2O_2 is able to trigger both local and systemic defence responses and activate a cell death programme that shares several features with programmed cell death. Cell death occurred both in the mesophyll and palisade parenchyma and spatially correlated with increased levels of H_2O_2 (Chamnongpol et al. 1998; Dat et al. 2003; Vandenabeele et al. 2004).

The importance of peroxisomal H_2O_2 scavenging by catalase was also corroborated in catalase-deficient *Arabidopsis* plants. Transgenic lines, containing antisense or

RNAi constructs that target the catalase 2 gene (*CAT2*), were more sensitive to HL irradiation and ozone, and subsequently provoked cell death (Fig. 2a). Both the timing and extent of cell death were inversely correlated to the residual catalase

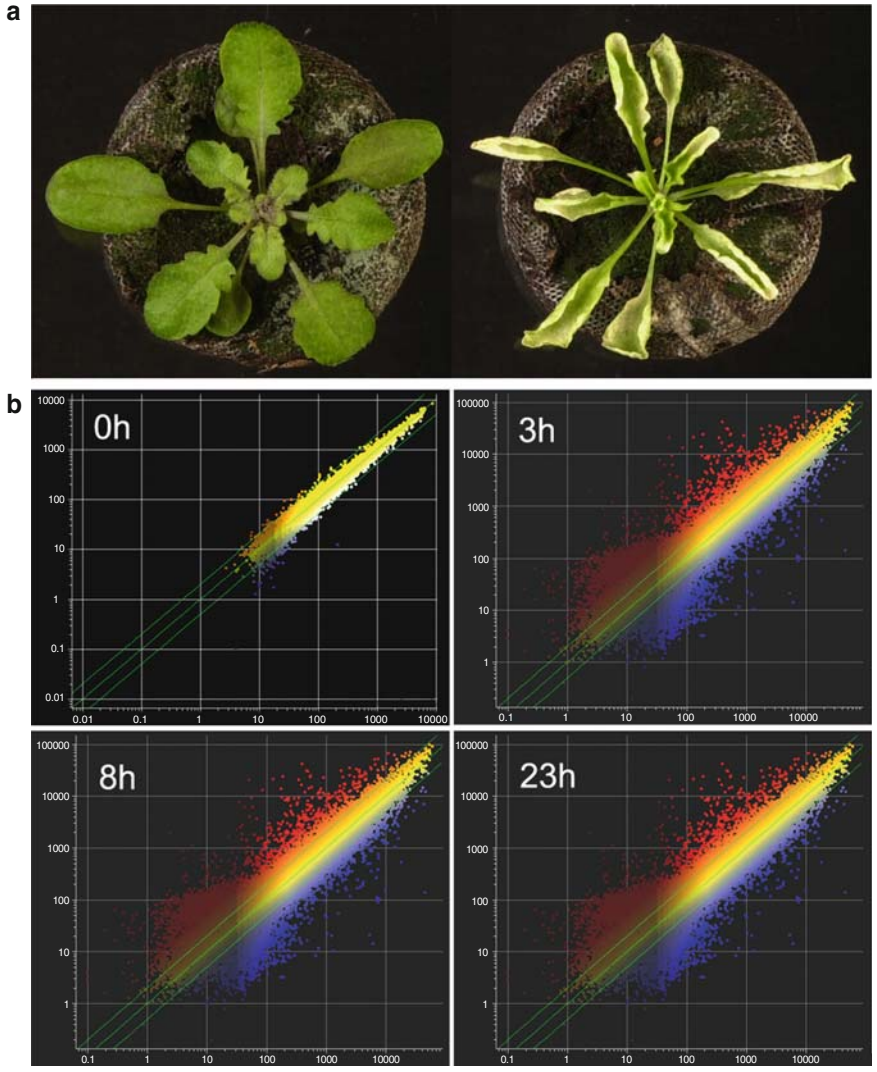

Fig. 2 Photorespiratory H_2O_2-induced transcriptional changes and cell death in catalase-deficient plants upon HL irradiation. (**a**) Control and catalase-deficient plants exposed to HL for 23 h. Leaves of catalase-deficient plants developed cell death after 8 h of HL, while in the control plants no cell death was visible even after 23 h of HL. (**b**) Scatter plots representing temporal expression changes in catalase-deficient versus control plants during a HL time course (0, 3, 8 and 23 h). Genes that fall above or below the diagonal are at least twofold upregulated (*red*) or downregulated (*blue*), respectively. Samples were analysed with Affymetrix MAS5.0 software and the Silicon Genetics GeneSpring version 5.1

activity (Vandenabeele et al. 2004). To validate the photorespiratory nature of the elevated H_2O_2 levels in HL-treated catalase-deficient plants, plants were exposed to HL under ambient air conditions (400 ppm, 21% O_2) and under high CO_2 levels (1,500 ppm, 21% O_2). As expected, leaf cell death could be prevented in a CO_2-enriched environment (Vandenabeele et al. 2004).

Recently, *Arabidopsis cat2* knockout mutants were used to define the photoperiod as an additional regulatory factor of H_2O_2-induced cell death and defence gene expression. By growing *cat2* at different daylengths in ambient air, H_2O_2-induced lesion development was found to depend on long days. Unexpectedly, perturbed intracellular redox state and oxidative signalling pathway induction were more prominent in short days than in long days, as evidenced by the glutathione status and the induction of defence genes and oxidative stress-responsive transcripts. These data indicated that photoperiod is a critical determinant in the acclimation versus cell death decision during stress (Queval et al. 2007). In a second work, the *cat2* mutant uncovered a new crosstalk between oxidative stress, cation homeostasis and ethylene signalling. While investigating the effects of catalase deficiency on cation homeostasis, *cat2* plants appeared to be more tolerant to lithium, as a result of lithium-induced insensitivity to the inhibitory ethylene and reduced capability for ethylene production. The accumulation of H_2O_2 during stress responses was postulated to alleviate the negative effects of excess ethylene generated (Bueso et al. 2007).

Catalase loss-of-function mutants are an ideal in planta system to examine the consequences of increased availability of endogenous H_2O_2, 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. Transcriptome analysis of catalase-deficient plants demonstrated that perturbation of the H_2O_2-scavenging capacity significantly affects nuclear gene expression after HL irradiation, altered photoperiod or low CO_2 levels (Vandenabeele et al. 2003, 2004; Vanderauwera et al. 2005; Queval et al. 2007) (Fig. 2b). By monitoring different time points after HL exposure, the dynamics of the transcriptional response could be visualised, and early expressed candidate receptor and signal transducing genes could be distinguished, from later expressed effector genes, such as those involved in defence responses and cell death (Vandenabeele et al. 2003, 2004; Vanderauwera et al. 2005).

Under non-stressed conditions, several stress-related genes, including glutathione S-transferases, heat shock proteins and UDP-glycosyltransferases, were upregulated in the catalase-deficient plants. Almost all the 51 induced transcripts, including a WRKY (At5g24110) and a bHLH (At1g10585) transcription factor, also responded highly to HL and to various other environmental stresses, such as wounding, heat, salt and osmotic and genotoxic stress (Vanderauwera et al. 2005). Both bHLH and WRKY transcription factors have been shown to be involved in abiotic stress responses in plants (Eulgem et al. 2000; Wang et al. 2003; Lorenzo et al. 2004). Therefore, these early responsive genes might be implicated in the primary cellular stress responses mediated by increased levels of H_2O_2.

Exposure of both control and catalase-deficient plants to HL allowed HL- and H_2O_2-responsive transcripts to be distinguished. Of the 906 transcripts that were exclusively regulated by HL, 379 were upregulated and 527 were downregulated.

In response to H_2O_2, 349 transcripts were induced and 88 were repressed in the catalase-deficient plants (Vanderauwera et al. 2005).

The Hsp17.6 class of small heat shock proteins and the heat shock transcription factors (HSFs) HsfA2, HsfB2b and HsfA7a were strongly responsive to photorespiratory H_2O_2. A rapid induction of two other HSFs, HsfA4a and HsfA8, in response to light stress has been reported in knockout ascorbate peroxidase 1 plants (Pnueli et al. 2003). HsfA4a and HsfA8 were, however, not induced during HL stress in the catalase-deficient plants, indicating that the induction of these HSFs is not part of a general response to oxidative damage but rather the outcome of a response to increased levels of specific ROS. HSFs also respond to many different biotic and abiotic stresses, and HsfA2 has indeed been reported as a key regulator in response to heat, HL and osmotic stress (Nishizawa et al. 2006; Ogawa et al. 2007). Recent studies in yeast and mammalian cells provided considerable evidence for redox-dependent regulation of HSFs (Kotak et al. 2007), further feeding the hypothesis that oxidative stress-responsive HSFs might also function in plants as H_2O_2 sensors involved in the regulation of oxidative stress-responsive genes (Miller and Mittler 2006).

As mentioned above, oxidative stimuli provoke large-scale changes in the transcriptome of plants. One approach to analyse this transcriptional response is to identify co-regulated genes by making intersections with other related datasets or by experimentally merging several perturbations and, thereby, trying to dissect the transcriptional response into smaller functional entities (Ma and Bohnert 2008). By combining a genetic (catalase deficiency) with an environmental (HL) perturbation, a transcriptional regulon was identified that was strongly and rapidly induced by HL in wild-type plants, but whose induction was impaired in catalase-deficient plants. This cluster comprised the complete anthocyanin biosynthetic and regulatory pathway, together with several genes of hitherto unknown function (Vanderauwera et al. 2005). This molecular phenotype also correlated with the observed phenology of the catalase-deficient plants under HL stress: wild-type plants accumulated significantly more anthocyanins than catalase-deficient plants after exposure to HL. Because anthocyanins have the ability to reduce the potential to oxidative damage via light attenuation (Steyn et al. 2002), failure of anthocyanin accumulation in the catalase-deficient plants might contribute to their increased sensitivity to HL irradiation (Hoch et al. 2003). How H_2O_2 impinges negatively on this HL-induced anthocyanin regulon remains currently elusive and is the subject of further investigations.

By assessing the expression of H_2O_2-induced transcripts within three important environmental stresses (cold, heat and drought), an important intersection was revealed that not only confirmed a prominent role for photorespiratory H_2O_2-dependent signalling but also allowed the identification of new candidate regulatory genes within the plant's abiotic stress response (Vanderauwera et al. 2005). Among the 20 genes that were commonly induced by H_2O_2 and at least two of the selected stresses, two transcription factors, DREB2A and ZAT12, were identified. DREB2A is known to be an important regulator of water- and heat-responsive gene expression (Shinozaki and Yamaguchi-Shinozaki 2000; Sakuma et al. 2006a,b, 2006), whereas ZAT12 is thought to be involved in cold and oxidative stress signalling (Rizhsky et al. 2004; Davletova et al. 2005b; Vogel et al. 2005). These examples illustrate the

importance of the systematic perturbation of H_2O_2-regulated transcription factors and subsequent functional analysis to unravel the hierarchical structure of the network that governs the H_2O_2 response in plants. Such an integrative survey of transcriptional footprints and stress tolerance characteristics of new transgenic plants and mutants will provide a view on how these different regulatory factors interconnect with each other and what their role is in the mechanisms by which ROS signals are integrated to eventually regulate specific biological processes.

5 Perspectives

Molecular phenotyping of different ROS-responsive mutants and transgenic plants have resulted in comprehensive inventories of transcripts involved in production, scavenging, perception and signal transduction of ROS. Not only was the signalling role of ROS during various environmental cues validated, but a better view was also gained on the shape of the complex regulatory network implicated in ROS signal transduction. Next, a more detailed analysis is required to clarify the function of each of these genes within the ROS signalling network. The most informative results are expected from in-depth functional analyses of transgenic plants perturbed in signal transducers and transcription factors, which will identify downstream targets and organise the oxidative stress response into various transcriptional regulons.

Currently, microarrays are the most popular technology for large-scale expression profiling because they allow the simultaneous detection of tens of thousands of transcripts at a reasonable cost. Most microarrays used in today's research are based on spotted oligonucleotides that are designed to question the levels of individual transcripts and standard sets covering various parts of the genome are available (CATMA, ATH1). The major limitation of such microarrays is that they rely on current genome annotations, which precludes the identification of novel or very small transcription units. Tiling arrays, on which the entire genome (including intergenic regions) is represented by evenly spaced probes, provide a novel and unbiased means of transcript identification (Rensink and Buell 2005). Tiling arrays will make it possible to identify novel transcription units involved in H_2O_2-induced responses, including antisense transcripts, small non-coding RNAs and previously unidentified genes (Busch and Lohmann 2007; Gregory et al. 2008). Recent breakthroughs in sequencing technologies have led to innovations, such as pyrosequencing (Margulies et al. 2005), sequencing by synthesis (Steemers and Gunderson 2005), SOLiD sequencing by oligonucleotide ligation (Cloonan et al. 2008) and true single molecule sequencing (Harris et al. 2008). The unprecedented number of base pairs that can be read in one run by high-throughput sequencers based on these techniques allows hypothesis-free, quantitative analysis of the entire transcriptome. As a result, so-called digital gene expression analysis will soon become an attractive alternative for microarrays.

Both tiling arrays and deep-sequencing approaches can be used to detect small RNAs, a class of non-coding transcripts that have been shown to control different aspects of plant development as well as plant stress responses (Sunkar et al. 2007).

An interesting example is the upregulation of two Cu/ZnSOD genes that depended on changes in the levels of miR398 (Sunkar et al. 2006). Hence, small RNAs may prove to be yet another level in the regulatory networks that orchestrate ROS-directed gene expression.

Integration of transcriptional networks with other "omics" disciplines, such as proteomics and metabolomics will become increasingly important in the near future. Such "poly-omics" approaches are well suited to analyse the consequences of ROS-provoked changes at both the cellular tissue and whole plant levels, and, for example, allow the comparison of the relative importance of transcriptional and post-transcriptional regulation of the plant's response to ROS. Eventually, this will provide us with a holistic view on the networks involved in various ROS-mediated plant processes.

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