Catalase function in plants: a focus on Arabidopsis mutants as stress-mimic models

Amna Mhamdi1,2, Guillaume Queval1, Sejjir Chaouch1, Sandy Vanderauwera3, Frank Van Breusegem3 and Graham Noctor1,∗

1 Institut de Biologie des Plantes, UMR CNRS 8618, Université de Paris sud, F-91405 Orsay cedex, France
2 Département de Biologie, Faculté des Sciences de Tunis, Université de Tunis El Manar, Campus Universitaire, 2092 Tunis, Tunisia
3 Department of Plant Systems Biology, Flanders Institute for Biotechnology, 9052 Gent, Belgium; Department of Plant Biotechnology and Genetics, Gent University, 9052 Gent, Belgium

∗ To whom correspondence should be addressed: E-mail: graham.noctor@u-psud.fr

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Abstract

Hydrogen peroxide (H2O2) is an important signal molecule involved in plant development and environmental responses. Changes in H2O2 availability can result from increased production or decreased metabolism. While plants contain several types of H2O2-metabolizing proteins, catalases are highly active enzymes that do not require cellular reductants as they primarily catalyse a dismutase reaction. This review provides an update on plant catalase genes, function, and subcellular localization, with a focus on recent information generated from studies on Arabidopsis. Original data are presented on Arabidopsis catalase single and double mutants, and the use of some of these lines as model systems to investigate the outcome of increases in intracellular H2O2 are discussed. Particular attention is paid to interactions with cell thiol-disulphide status; the use of catalase-deficient plants to probe the apparent redundancy of reductive H2O2-metabolizing pathways; the importance of irradiance and growth daylength in determining the outcomes of catalase deficiency; and the induction of pathogenesis-related responses in catalase-deficient lines. Within the context of strategies aimed at understanding and engineering plant stress responses, the review also considers whether changes in catalase activities in wild-type plants are likely to be a significant part of plant responses to changes in environmental conditions or biotic challenge.

Key words: Arabidopsis thaliana, glutathione, H2O2, mutants, oxidative stress, pathogens, redox signalling.

Introduction

Increasingly refined and powerful methods of plant breeding and selection produced substantial increases in potential and actual crop yields throughout the last century. Oftentimes, however, environmental factors impose constraints on genetic potential so that actual yields are significantly below the theoretically possible yields (Bray et al., 2000). Such limiting factors include the capture of light, water, and nutrients, the driving forces of plant growth (Ahrens et al., 2010). Even when these factors are non-limiting, yield can be compromised by the existence of other factors. While the word ‘stress’ is widely used in plant biology, interpretations vary as to its exact meaning. Perhaps the simplest definition is a physiological condition caused by any environmental constraint that limits growth, reproductive success, yield, quality, or other traits desirable to humans. In many cases, the effects of stress persist for a certain time even after the removal of the environmental constraint(s). Among the possible causes of such persistent effects are (i) an enduring effect on biomass production following temporary diversion of resources to defence; (ii) deleterious modifications to cell components that take time to repair; and (iii) insufficiently rapid reversibility of developmental programmes (e.g. arrest of cell division, cell death) that are engaged to favour survival over growth and biomass production. All these mechanisms may contribute to what are perceived by humans as the negative effects of stress conditions on plant performance. If insight into the way plants work is to enable crop improvement through the development of
rationally designed strategies, it will be necessary to develop accurate concepts of the mechanisms that underlie the effects of stress.

**Stress and reactive oxygen**

Examples of stress conditions are supra-optimal light, insufficient water supply, atmospheric and soil pollutants, excessive salt, and pathogen attack. In the field, plants often experience more than one of these conditions simultaneously. Depending on the species or variety, plants have the capacity to acquire resistance to stress within the life cycle of the individual (acclimation). Such processes include cold-hardening, adjustment to different growth light intensities and systemic acquired resistance to certain pathogens. Plants exposed to one type of stress condition can also acquire a certain resistance to other stress conditions (cross-tolerance). Inversely stress-specific mechanisms may also compromise responses to other stresses. These observations imply the existence of common and/or antagonistic factors that are involved in responses to different stress conditions. For example, the key stress phytohormone, abscisic acid, is not only important in dormancy and drought stress but also in pathogen responses (Asselbergh et al., 2008), while antagonism has been described between salicylic acid (SA)- and jasmonic acid (JA)-dependent responses to biotic stress (Browse, 2009). A central theme in many stress responses is the accumulation of reactive oxygen species (ROS) and ROS-induced changes in cellular redox state (Foyer and Noctor, 2000; Dat et al., 2001; Pastori and Foyer, 2002).

**Changing views of ROS function in plants**

Originally considered of little or no importance in biology, then subsequently as rapidly metabolized by-products in reactions such as chloroplast pseudocyclic electron transport (also called the water–water cycle: Asada, 1999), ROS gained importance during the 1980s as key players in both abiotic and biotic stress responses. Until ten years ago, the predominant view on ROS was that they were toxic molecules that cause damage, leading to the notion that (cross-)resistance to stress could be engineered in plants by enhancing antioxidative capacity. This view of ROS action persists within the literature. Indeed, the reactivity of ROS may inevitably lead to some incidental modifications to cell components (Møller et al., 2007). However, since the beginning of this century, it has been established that the most important physiological effects of ROS are not mediated via indiscriminate damage. Key observations were the ROS-driven activation of gene expression, specific protein kinases and calcium signatures, the ‘programmed’ production of ROS by NADPH oxidases, the roles of ROS production in sustaining cell growth, the importance of ROS in hormone signal transduction, and the requirement of specific gene modulation for ROS-induced cell death (Kovtun et al., 2000; Desikan et al., 2001; Foreman et al., 2003; Kwak et al., 2003; Vandenabeele et al., 2003; Wagner et al., 2004; Vandenbroucke et al., 2008). Among the many current questions in the field are the nature of the components that perceive ROS, the specificity of the effects of different ROS, the importance of the (sub)cellular location of ROS production, the roles of changes in redox-homeostatic components, such as glutathione, in transmitting or adjusting ROS signals, and the importance of environmental and physiological contexts in determining the outcome of ROS-related redox signalling.

**Generation and metabolism of H₂O₂ in plants**

The term ROS includes any derivative of molecular oxygen (O₂) that is considered more reactive than O₂ itself. Thus, ROS refers to free radicals such as superoxide (O₂⁻) and the hydroxyl radical (OH), but also to non-radicals like singlet oxygen (¹O₂) and H₂O₂. Because of its relative stability, H₂O₂ has received particular attention as a signal molecule involved in the regulation of specific biological processes such as plant–pathogen interactions. H₂O₂ is generated by a two-electron reduction of O₂, catalysed by certain oxidases or indirectly via reduction or dismutation of O₂⁻ that is formed by oxidases, peroxidases, or by photosynthetic and respiratory electron transport chains (Foyer and Noctor, 2000; Mittler et al., 2004; Bindschedler et al., 2006; Sagi and Fluhr, 2006). These reactions generate H₂O₂ at several subcellular compartments of the cell (Fig. 1), and the impact of H₂O₂ will be strongly influenced by the extent to which the potent antioxidative system allows its accumulation. Moreover, oxidative perturbation of components of the antioxidative system is likely to play a part in the initial transmission of H₂O₂ signals. The peroxisomes are important sites of ROS production. In this organelle, H₂O₂ can be formed directly from O₂ by photosynthetic glycolate oxidase (Foyer et al., 2009) or by other enzyme systems such as xanthine oxidase coupled to superoxide dismutase (del Rio et al., 2006; Corps et al., 2008).

Genomic information and approaches have greatly advanced our understanding of plant antioxidant systems (Mittler et al., 2004). Plants contain several types of enzymes that are able to metabolize peroxides such as H₂O₂. These include catalases, ascorbate peroxidases (APX), various types of peroxiredoxins (PRX), glutathione/thioredoxin peroxidases (GPX), and glutathione S-transferases (GST) (Willekens et al., 1995; Asada, 1999; Wagner et al., 2002; Dietz, 2003; Mittler et al., 2004; Iqbal et al., 2006). In all cases, these enzymes are encoded by multiple genes. Catalases are most notably distinguished from the other enzymes in not requiring a reductant as they catalyse a dismutation reaction. Together with APX, catalases are also distinguished from many other peroxide-metabolizing enzymes by their high specificity for H₂O₂, but weak activity against organic peroxides. Catalases have a very fast turnover rate, but a much lower affinity for H₂O₂ than APX and PRX, which have Kₘ values below 100 μM (Mittler and Zilinskas, 1991; König et al., 2002). Although it is difficult to determine the precise kinetic characteristics for catalase, most estimates of the apparent Kₘ for H₂O₂
are in the range 40–600 mM (Chelikani et al., 2004). A value of 190 mM was measured in crude extracts of pea (del Rı´o et al., 1977). This indicates that catalase functions in vivo well below its maximal capacity, but that its activity accelerates even if H2O2 concentrations increase to relatively high values. Catalases are highly expressed enzymes, particularly in certain plant cell types, and are thus an integral part of the plant antioxidative system. In this review, an overview of catalase function is provided and new results on single and double Arabidopsis T-DNA mutants are included as part of a discussion of how studying plants deficient in certain catalases can provide insights into the functional outcome of stress-linked H2O2 signalling in plants.

Plant catalases

Biochemistry

Catalase was the first antioxidant enzyme to be discovered and characterized. In giving the enzyme its name, Loew (1900) noted that ‘there seems to exist no plant and no animal which is without that particular enzyme’ (Kirkman and Gaetani, 2007). Even some anaerobes are known to contain catalase (Zamocky et al., 2008). The typical catalase reaction is the dismutation of two molecules of H2O2 to water and O2. Although some bacterial catalases use manganese as the redox-active co-factor, all known eukaryotic forms are haem-based (Zamocky et al., 2008). The best-characterized type of haem-dependent catalase is found in diverse organisms, including prokaryotes, fungi, animals, and plants, and is composed of ‘typical’ or ‘monofunctional’ catalases (Chelikani et al., 2004). These enzymes consist of polypeptides of 50–70 kDa in mass that are organized into tetramers, with each monomer bearing a haem prosthetic group (Regelsberger et al., 2002). A second type of haem-dependent catalase is bifunctional catalase-peroxidases that are structurally distinct proteins found in some fungi and prokaryotes (Mutsada et al., 1996; Regelsberger et al., 2002). The division between monofunctional and bifunctional catalasases is not absolute, because the first type can also catalyse some H2O2-dependent peroxidation of organic substrates (Zamocky et al., 2008). On the other hand, bifunctional catalase-peroxidases are more similar to the haem-containing peroxidases such as APX and fungal cytochrome c peroxidase (Regelsberger et al., 2001). They also have much higher affinities for H2O2 than monofunctional catalases and can be distinguished by their relative insensitivity to the inhibitor 3-amino-1,2,4-triazole (3-AT) (Margoliash and Novogrodsky, 1960; Regelsberger et al., 2002). They are the most common type of catalase in cyanobacteria (Zamocky et al., 2008).

Isotope labelling studies have established that O2 generated in the catalatic (dismutation) reaction comes from a single H2O2 molecule (Kato et al., 2004), with the other H2O2 being reduced to two water molecules that are formed in distinct steps (Fig. 2). Both haem-dependent catalases and peroxidases reduce H2O2 by splitting the O–O bond to generate a first molecule of water with the concomitant production of an oxy-ferryl intermediate (compound I) and a porphyrin cation radical. The specificity of the catalase reaction involves the oxidation of a second H2O2 to O2 as compound I is reduced back to the initial state (Fig. 2B). This entails the release of the bound O, originating from the first H2O2, as a second molecule of water (Regelsberger et al., 2001; Alfonso-Prieto et al., 2009). Whereas catalase compound I is very rapidly reduced by two electrons from H2O2 (albeit probably in a stepwise manner: Kato et al., 2004; Alfonso-Prieto et al., 2009), reduction of compound I in peroxidases involves successive single-electron oxidations.
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et al.

Kato

products are indicated in blue and red. Although not shown, some catalase subunit polypeptides (Kirkman

less tightly depending on the species, to each of the four

involves one molecule of NADPH, which is bound, more or

compound II formation and/or reduction to the initial state

Simplified scheme of the catalytic mechanism of catalase.

Fig. 2. (A) Overall substrates and products of the classical dismutation reaction. (B) Scheme of the four-step redox reaction. The roman numerals indicate oxidation state of the haem iron, while Por indicates an oxidizing equivalent accumulated as a porphyrin cation radical. The two different H2O2 substrates and their respective products are indicated in blue and red. Although not shown, some of the reactions are reversible. Based on Regelsberger et al. (2001), Kato et al. (2004), and Alfonso-Prieto et al. (2009).

of organic compounds to generate two free radicals (e.g. monodehydroascorbate radicals in the case of APX).

As noted above, even monofunctional catalases can catalyse some peroxidation of reducing substrates. In this reaction, the second, reducing H2O2 is replaced by small compounds such as ethanol that provide the two electrons required for the reduction of compound I (Zamocky et al., 2008). Rates are slow compared with the dismutation reaction, but this reaction has been suggested to be significant or even predominant when H2O2 concentrations in mammalian cells are low (Kirkman and Gaetani, 2007). Catalase-associated peroxidation has also been reported in plants. In partially purified protein extracts from maize, barley, and tobacco leaves, ethanol peroxidase activities have recently been reported in Arabidopsis (Hu et al., 2010). Catalase nomenclature is a potential source of confusion when comparing studies on different plant species. Willekens et al. (1995) proposed a classification based on the naming of the tobacco genes. According to this grouping, Class I catalases are strongly expressed in photosynthetic tissues, while Class II catalases are associated with vascular tissues. Class III catalases are notably expressed in reproductive tissues. Although the three catalases may not exactly correspond between species, there is considerable evidence that functional specialization is quite well conserved, and that this classification is useful. Conserved features include tissue and cellular expression, and day–night rhythms in transcript abundance. Comparisons of gene structure (e.g. between maize and rice catalase genes; Iwamoto et al., 2000) also support the division of catalases into three classes (Table 1).

Catalase genes and isoforms

Phylogenetic classification of the numerous available catalase sequences has recently been performed by Zamocky et al. (2008). Two catalase genes are found in Escherichia coli: KatE encodes a monofunctional catalase while KatG encodes a catalase-peroxidase. Available genomic information suggests that most animals, including mammals, contain a single catalase gene. Angiosperm species studied to date all contain three catalase genes. This includes monocots and dicots such as tobacco, Arabidopsis, maize, pumpkin, and rice (Willekens et al., 1995; Frugoli et al., 1996; Guan and Scandalios, 1996; Esaka et al., 1997; Iwamoto et al., 2000). Information from genome sequencing has confirmed the presence of three catalase genes in Arabidopsis, two located on chromosome 1 (CAT1, CAT3) and one located on chromosome 4 (CAT2) (Frugoli et al., 1996). All three translation products consist of 492 amino acids, with high similarity between the sequences (Fig. 3). While nucleotide sequences are sufficiently different to allow construction of gene-specific primers, the similarity of the three polypeptides makes it difficult to produce isoform-specific antibodies, though CAT1-specific antibodies have recently been reported in Arabidopsis (Hu et al., 2010). In-gel analysis with antibodies can be performed after electrophoretic separation of CAT2 and CAT3 isoforms (Zimmermann et al., 2006; Smykowska, 2010).

Available evidence from expression patterns and functional analysis suggests that Arabidopsis CAT1, CAT2, and CAT3 correspond to Class III, Class I, and Class II catalases, respectively (Table 1). The CAT1 gene is mainly expressed in pollen and seeds, CAT2 in photosynthetic requirement for NADPH may be the main cause of erythrocyte malfunction in the genetic disease caused by loss of glucose-6-phosphate dehydrogenase activity in humans (Kirkman and Gaetani, 2007). However, comparative structural analyses suggest that the NADPH-binding site is present only in some clade III catalases, such as the human enzyme, but is not found in clade I catalases, the evolutionary group to which most plant catalases belong (Chelikani et al., 2004; Zamocky et al., 2008).
tissues but also in roots and seeds, while CAT3 is associated with vascular tissues but also leaves (Fig. 4A). All three transcripts can be detected in mature Arabidopsis rosettes, though CAT3 and CAT2 transcripts are much more abundant than those of CAT1 (Frugoli et al., 1996; McClung, 1997; Fig. 4A). Class I and Class II catalases show a contrasting day–night rhythm in transcript abundance. Thus, in maize, CAT3 transcripts (encoding Class II catalase) show a circadian rhythm that is opposite to many genes involved in photosynthesis-related metabolism, which have peak expression at the night/day transition (Redinbaugh et al., 1990). Subsequent work in Arabidopsis revealed a marked photosynthetic-type rhythm for CAT2 transcripts (Zhong et al., 1994) and an opposite rhythm for CAT3 (Fig. 4B). Class III catalases like Arabidopsis CAT1 show less significant day-night variation in transcript abundance (McClung, 1997). Although some evidence of a day–night rhythm in CAT3 transcripts was found in tobacco leaves, variations were less marked than for the Class I and II catalases (Dutilleul et al., 2003).

Subcellular localization

Questions remain concerning the subcellular localization of catalases in plants. Of the two genes found in yeast, one encodes a cytosolic isoform, the other an enzyme found mainly in peroxisomes (Petrova et al., 2004). The single gene found in mammals is considered to encode a solely or predominantly peroxisomal enzyme, but some activity may be localized in mitochondria (Zamocky et al., 2008). Unlike most animals studied so far, the worm Caenorhabditis

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**Table 1.** Probable classification of the three catalases found in different plant species

The division into three classes is based on the classification introduced by Willekens et al. (1995) and is shown for five species in which a complete trio of catalase genes have been identified and studied.

<table>
<thead>
<tr>
<th>Species</th>
<th>Class I</th>
<th>Class II</th>
<th>Class III</th>
</tr>
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<tbody>
<tr>
<td>Tobacco</td>
<td>Cat1</td>
<td>Cat2</td>
<td>Cat3</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>CAT2</td>
<td>CAT3</td>
<td>CAT1</td>
</tr>
<tr>
<td>Maize</td>
<td>Cat2</td>
<td>Cat3</td>
<td>Cat1</td>
</tr>
<tr>
<td>Pumpkin</td>
<td>cat2</td>
<td>cat3</td>
<td>cat1</td>
</tr>
<tr>
<td>Rice</td>
<td>CatC</td>
<td>CatA</td>
<td>CatB</td>
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**Fig. 3.** Alignment of Arabidopsis catalase protein sequences. The amino acid sequences of CAT1, CAT2, and CAT3 were aligned using BioEdit software (Hall, 1999). Regions of identical amino acids are shown in grey. Carboxy terminal regions that have been implicated in determining import into peroxisomes are boxed.
*elegans* was reported to contain three genes, one of which encodes a major peroxisomal form while a second encodes a minor cytosolic enzyme (Petriv and Rachubinski, 2004). In plants, classical subcellular fractionation studies as well as *in situ* activity staining have established that peroxisomes contain high catalase activity and import of catalase into these organelles has been experimentally demonstrated (Mullen et al., 1997). However, questions concerning the nature of the import mechanism remain. Two principal pathways have been identified for the entry of proteins into peroxisomes. The most common is the Peroxisomal Targeting Sequence 1 (PTS1) pathway (Brown and Baker, 2008; Kaur et al., 2009). Depending on the protein, the PTS1 pathway is completely or partly governed by non-cleaved tripeptide sequences at the extreme C-terminus of the polypeptide (Reumann et al., 2007; Brown and Baker, 2008; Kaur et al., 2009). While one classical PTS1 motif (S-R-L) is found in both *Arabidopsis* CAT2 and CAT3, and CAT1 contains a similar T-R-L sequence at the same position (Fig. 3, box 1), these tripeptides are not located at the extreme C-terminus but at –7 to –9 upstream. Such internally located motifs may enhance import into peroxisomes but are not in themselves considered sufficient (Kaur et al., 2009). Although known C-terminus PTS1 tripeptides show considerable variability between different peroxisomal proteins (Reumann et al., 2007), the corresponding sequences of *Arabidopsis* catalases (Fig. 3, box 2) are not considered to be among them (Kaur et al., 2009). Despite this, the C-terminal tripeptide sequence (Fig. 3, box 2) was necessary for efficient import of cottonseed catalase into tobacco BY-2 cell peroxisomes (Mullen et al., 1997).

As well as the internally located S/T-R-L, a third motif has been implicated in catalase import into peroxisomes (Kamigaki et al., 2003). This is a Q-K-L sequence located further upstream of the S/T-R-L motif (Fig. 3, box 3). This sequence was reported to influence the interaction between CAT1 and the PTS1 receptor protein, Pex5p (Kamigaki et al., 2003). Based on current understanding of PTS1 pathway mechanisms (Lanyon-Hogg et al., 2010), it seems unlikely that the internal Q-K-L sequence interacts directly with Pex5p. Another study concluded that accumulation of pumpkin CAT1 in peroxisomes requires several components of the PTS1 pathway machinery (Oshima et al., 2008). Together, these observations suggest that several motifs within the 13 C-terminal amino acids may act to allow and/or enhance import of catalases into peroxisomes via the PTS1 pathway, although further work is required to resolve this question.

The presence of significant catalase activity in other subcellular compartments is less well established. In the literature, catalase activity in the cytosol and mitochondria has been frequently described. Indeed, catalase activity can be detected in chloroplasts isolated by classical fractionation techniques. It has been accepted for many years,
however, that this activity can be attributed to the presence of peroxisomes in the chloroplast preparation or adhesion of contaminating enzyme to the exterior of the chloroplasts (Allen and Whatley, 1977, and references therein for a discussion of concepts prior to the discovery of chloroplast APX in 1979). In cyanobacteria, which are considered to be the closest extant relatives of the earliest oxygen-evolving organisms, both monofunctional catalases and catalase-peroxidases exist, although this varies between cyanobacterial species (Mutsada et al., 1996; Regelsberger et al., 2002). The model unicellular eukaryotic green alga (Chlamydomonas reinhardtii), contains several isoforms of catalase. These differ from higher plant catalases because they are dimeric (rather than tetrameric) and localized in the mitochondria (Kato et al., 1997) rather than in peroxisomes, which are generally negligible or absent in this organism. Significant catalase activity has been detected in washed mitochondria from maize primary leaves (in which Cat3 is the major isoform), and was partly retained in subsequently prepared submitochondrial particles (Scandalios et al., 1980). Interestingly, the putative mitochondrial maize catalase isoform was reported to have higher peroxidase activity than the other maize isoforms and to be relatively sensitive to 3-AT (Havir and McHale, 1989). Proteomic analysis of highly purified mitochondria from Arabidopsis cells identified CAT2 and CAT3 peptide sequences (Heazlewood et al., 2004). This finding was interpreted with some caution since catalase activity, used as a marker for peroxisomal contamination, showed a progressive decline throughout the mitochondrial purification procedure, along with the plastid marker enzyme, alkaline pyrophosphatase (Heazlewood et al., 2004). In yeast, peroxisomal catalase was co-localized to mitochondria in a manner that depended on nutritional conditions (Petrova et al., 2004). Dual peroxisomal/mitochondrial targeting of catalases cannot yet be ruled out. However, to date there have been no demonstrations of catalase import into angiosperm mitochondria using either in vitro or in vivo approaches, and it is possible that contamination may account for reports of catalase activity in this organelle.

Whether some cytosolic catalase activity originates from incomplete import of catalases into the peroxisomes also remains unclear. At least for some peroxisomal proteins, it is known that they can enter peroxisomes as preformed oligomers or as monomers following oligomer disassembly (Kamigaki et al., 2003; Brown and Baker, 2008). Thus, cytosolic catalase activity could possibly arise from assembled tetramers awaiting import. Studies in human cell lines suggest that catalase may be imported less efficiently than typical PTS1-dependent peroxisomal proteins (Koecke et al., 2007). While the issue of cytosolic catalase in plants remains to be resolved, no substantive conclusion can be drawn from the detection of the enzyme in the soluble fraction produced following removal of intact organelles by high-speed centrifugation. Even if such soluble fractions were organelle-free, the activity detected could reflect the presence of enzymes released from organelles during disruption of the original tissue or cells.

Genetic manipulation of catalase

Overexpression and ectopic expression of catalase

Catalase capacities in leaf peroxisomes are already very high in many plants, particularly C₃ species. Introduction of the maize CAT2 gene into tobacco did cause some increase in leaf activity and the lines were used to analyse responses to bacterial infection (Polidoros et al., 2001). An O₂-resistant tobacco mutant line was identified that showed a significant increase in leaf catalase activity (Zelitch, 1992). Using both this mutant line and catalase underexpressors, an inverse correlation was reported between catalase capacities and the leaf compensation point, the ambient CO₂ concentration at which photosynthetic CO₂ uptake equals (photo)respiratory CO₂ release (Brisson et al., 1998). This inverse correlation was explained by catalase-dependent control of H₂O₂-dependent decarboxylation of glyoxylate. Most glyoxylate formed in the peroxisomes during photorespiration is transaminated to glycine, but oxidative decarboxylation triggered by H₂O₂ could also occur. Any such effect would increase the CO₂ yield per glycolate carbon above the accepted value of 0.25. Less direct effects on photosynthesis are also conceivable through inhibition of the Calvin cycle caused by less efficient glycolate recycling. Little evidence of increased glyoxylate decarboxylation was reported in the barley mutant with less than 10% leaf catalase capacity, even though increased decarboxylation was measurable in barley serine:glyoxylate aminotransferase (SGAT) mutants (Leegood et al., 1995, and references therein). In view of the mutant lines and other tools available, this question might be worth revisiting in Arabidopsis.

Expression of E. coli KatE in the chloroplast improved paraquat and drought tolerance in tobacco and protected thiol-regulated chloroplast enzymes from oxidative inactivation (Shikanai et al., 1998). Introduction of the same catalase gene into rice was reported to increase resistance to salt (Moriwaki et al., 2007). Thus, even though APX and thioredoxin peroxidases are considered to be the main enzymes involved in H₂O₂ metabolism in the chloroplast, ectopically introduced catalase appears to be able to make a significant contribution. Characteristics such as efficacy at low H₂O₂ concentrations might explain why peroxidases have replaced catalases during chloroplast evolution.

Catalase mutants and knockdowns

In humans, the autosomal recessive peroxisomal disorder acatalasemia (also known as Takahara’s disease) is caused by a complete deficiency in catalase. While this can have several adverse consequences on health, symptoms are confined to specific tissues or physiological conditions (Zamocky et al., 2008). Knockout mice with negligible catalase activity only show differences from wild-type mice under conditions of oxidative challenge (Ho et al., 2004). Likewise, the first plant catalase mutants, isolated in the C₄ plant maize, did not show obvious phenotypes (reviewed in Scandalios, 1994). Subsequently, however, a photorespiratory screen of a mutant collection in the C₃ plant barley
identified a stable line with only about 10% wild-type leaf catalase activity (Kendall et al., 1983). When grown under standard conditions (moderate irradiance in air), this mutant displayed leaf bleaching and marked perturbation of glutathione pools, both of which could be prevented by growth at 0.2% CO₂ (Kendall et al., 1983; Smith et al., 1984). Studies using antisense technology confirmed the importance of a specific catalase isoform in C₃ photosynthetic metabolism: tobacco CAT1 but not CAT2 knockdowns show spontaneous lesion formation and oxidative perturbation when grown above a threshold irradiance (Chamnongpol et al., 1996, 1998; Willeksens et al., 1997).

In contrast to the observations in barley, no photosynthetic catalase mutants were identified using a forward genetics approach in Arabidopsis (Somerville, 1986). However, analysis of RNAi lines using a CAT2-based sequence revealed phenotypes and changes in gene expression that were dependent on irradiance and that could be opposed by high CO₂ (Vandenabeele et al., 2004). A study of gene-specific T-DNA knockouts confirmed that CAT2 encodes the major leaf catalase isoform and that the function of this enzyme is closely linked to photorespiration (Queval et al., 2007).

In cat2 knockouts, leaf catalase activity is only about 10% of Col-0 wild-type plants (Fig. 5) and root catalase activity is also decreased (Bueso et al., 2007). Knockout lines for cat1 and cat3 (Fig. 5A) show much less decrease in leaf activity than cat2 (Fig. 5B). In leaves from 6-week-old plants, catalase activity in cat3 is reduced about 20%, while in cat1 it is similar to Col-0. This is consistent with the very low abundance of CAT1 transcripts in leaves (Fig. 4A). Double cat2 cat1 and cat2 cat3 mutants have similar decreases in leaf catalase activity to those in cat2.

Figure 5 provides further evidence that CAT2 and CAT3 are the major isoforms in Arabidopsis rosette tissue. While the relative contribution of the different genes to overall leaf catalase activity changes with the developmental stage of the plant (Zimmermann et al., 2006), the approximately additive nature of cat2 (90% decrease in leaf activity) and cat3 (20% decrease in leaf activity) mutations suggests that the formation of hetero-oligomeric proteins from more than one catalase gene product is a minor phenomenon in vivo.

An irradiance of 200 μmol m⁻² s⁻¹ at the Arabidopsis leaf surface can be considered moderate since it drives photosynthesis at about 50% of the maximal rate (Veljovic-Jovanovic et al., 2001). When grown in these conditions, cat2 shows a dwarf phenotype linked to redox perturbation. Phenotypes of cat2 are apparent at growth irradiances of 50–100 μmol m⁻² s⁻¹ and become more evident with increasing irradiance, demonstrating a close link to the rate of photorespiratory H₂O₂ production (Queval et al., 2007). By contrast, cat2 is aphenotypic when grown at high CO₂ or at low light (below 50 μmol m⁻² s⁻¹), consistent with studies of tobacco CAT1 antisense lines (Willeksens et al., 1997).

Catalase-deficient barley show leaf bleaching (Kendall et al., 1983) while tobacco CAT1 antisense lines showed necrotic lesions linked to the activation of certain pathogen responses (Chamnongpol et al., 1996; Takahashi et al., 1997). In Arabidopsis cat2 grown in short days, a dwarf phenotype is observed but no lesions (Fig. 6A). However, when grown in a 16 h photoperiod (long days), Arabidopsis cat2 shows spreading necrotic lesions (Fig. 6B). The cat2 phenotypes are accompanied by intracellular redox perturbation as evidenced by the accumulation of oxidized glutathione (GSSG; Fig. 7). However, this perturbation is at least as marked in short-day as in long-day growth conditions (Queval et al., 2007, 2009), indicating that lesion formation in cat2 in long days is not simply caused by greater oxidative stress than in short days (Chaouch et al., 2010). Thus, increased intracellular oxidation caused by H₂O₂ availability is not sufficient to trigger cell death. Additional signals are required (discussed further below).

In contrast to cat2, neither cat1 nor cat3 show any obvious rosette phenotype (Fig. 6) or perturbation of glutathione status (Fig. 7) during the vegetative growth stage. Both cat2 cat3 and cat2 cat1 double mutants have a slightly exacerbated lesion phenotype in long days (Fig. 6B) but quite similar glutathione status compared to cat2 (Fig. 7). None of the lines show lethality, and all can survive and set seed, at least when grown under moderate irradiance. Most of these observations on catalase activity

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**Fig. 5.** Catalase transcript abundance and extractable enzyme activity in Arabidopsis catalase single and double T-DNA mutants. (A) RT-PCR was performed using gene-specific primers (see Supplementary Table 1 at JXb online) and cDNA obtained from duplicate RNA extracts of 6-week-old plants grown in a 8/16 h light/dark regime. (B) Catalase activity was measured according to Queval et al. (2007) in leaf extracts from plants sampled in the same conditions as (A). Data are means ±SE of three different extracts. Percentage catalase activity relative to Col-0 (dotted line) is indicated at the top of the frame. T-DNA mutants from the SALK collections were obtained from Nottingham Arabidopsis Stock Centre (NASC), Nottingham University, UK. The polymorphisms were SAIL_760_D05 for cat1, SALK_076998 for cat2 (cat2-1; Bueso et al., 2007; Queval et al., 2009, previously named cat2-2 (Queval et al., 2007)) and SALK_092911 for cat3.
and phenotype in the knockouts are in agreement with those of the study of Hu et al. (2010). These authors reported that cat1 and cat3 knockouts show no phenotype whereas cat2 rosette growth was decreased. They also nicely showed that CAT2 function is determined by regulation of expression rather than any biochemical specificity, as cat2 could be complemented by either CAT2 or CAT3 expressed under the control of the cat2 promoter (Hu et al., 2010). Production of the cat1 cat3 T-DNA combination is not feasible by crossing because of the contiguous position of the two genes. Therefore, it remains to be seen whether plants can survive without any catalase activity at all.

Recently, novel CAT2 mutant alleles have been identified in a screen of a mutagenized Arabidopsis collection for resistance to hydroxyurea, an inhibitor of ribonucleotide reductase (Juul et al., 2010). Seventeen allelic lines were identified with between 15% and 59% wild-type activity, and it was concluded that resistance was probably linked to loss of catalase-dependent activation of hydroxyurea (Juul et al., 2010). Catalase-dependent pre-drug activation is well known in the pathogenic bacterium Mycobacterium tuberculosis, where the catalase-peroxidase katG activates the anti-tubercular compound, isoniazid (Lei et al., 2000).

Exploiting catalase-deficient plants to understand stress responses

Numerous studies over the last two decades have adopted the strategy of overexpressing antioxidative enzymes with the ultimate aim of enhancing stress resistance in plants. These studies have produced variable effects, though there are many reports of enhanced resistance to artificially induced and relatively short-term oxidative stress generated, for example, by the exogenous addition of ROS or ROS-producing agents. It is less clear whether enhanced expression of antioxidative enzymes would produce desirable effects under agronomically relevant conditions. For example, while overexpression of glutathione reductase (GR) was reported...
to enhance resistance to paraquat, ozone, and photoinhibitory conditions (Aono et al., 1993; Broadbent et al., 1995; Foyer et al., 1995), it did not lead to improved performance in field conditions (Korniyeyev et al., 2005). There is increasing recognition of the complexity and redundancy of the antioxidative system, as well as the importance of ROS as essential signal molecules whose effects, whether perceived as negative or beneficial, are embedded in cellular and intercellular interactions. These notions are possibly important for attempts to engineer responses to ROS in all complex multicellular organisms. For instance, while mouse life span was increased by the expression of catalase in mitochondria (Schriner et al., 2005), several studies on mammalian cells indicate that catalase overexpression can have undesirable consequences, for example, on wound healing (Zamocky et al., 2008).

In plants, the situation is further complicated by the multiplicity of ROS-producing pathways and the constitutively high capacity of the antioxidative system (Foyer and Noctor, 2009). These factors perhaps make it unlikely that attempts to reinforce front-line metabolic ‘defences’ will markedly enhance broad-spectrum stress resistance in plants. An alternative approach is to search for ROS-dependent control components (e.g. transcription factors) that act as key nodes in stress responses. Within this objective, plant systems that allow inducible, endogenous components (e.g. transcription factors) to act as key nodes in stress responses. Within this objective, plant systems that allow inducible, endogenous control components (e.g. transcription factors) to be expected to accumulate high levels of H$_2$O$_2$. Estimations of photorespiratory rates under typical controlled environment growth conditions point to a H$_2$O$_2$ generation rate of around 30 µmol g$^{-1}$ FW h$^{-1}$ through this pathway alone (Table 2). Assuming no compensatory metabolism of H$_2$O$_2$ by components other than Class I catalase, this would represent a theoretical increase in mean tissue H$_2$O$_2$ concentration of about 30 mM h$^{-1}$. Despite this, available data suggest that H$_2$O$_2$ accumulation is relatively minor or undetectable in CAT1-deficient tobacco or plants treated with 3-AT (Ferguson and Dunning, 1986; Willekens et al., 1997; Rizhsky et al., 2002). In Arabidopsis, increased diaminobenzidine (DAB) staining was reported in a cat2 mutant grown at relatively low light (Bueso et al., 2007) while a recent study reported a 2-fold increase in extractable H$_2$O$_2$ in cat2 and cat2 cat3 knockouts (Hu et al., 2010). Increased DAB staining was also observed in cat2 RNAi lines exposed to short-term high light stress or ozone (Vanderauwera et al., 2004). While no difference was found in the absence of infection, DAB staining of leaves 24 h after fertilization with avirulent bacteria was about 40% more intense in cat2 compared with the wild type (Simon et al., 2010). In Arabidopsis expressing glycolate oxidase in the chloroplast (in which H$_2$O$_2$ production should also be influenced by RuBP oxygenation rates), increased DAB staining was detected above a threshold irradiance (Fahnenstich et al., 2008). However, using DAB staining or assays of extractable H$_2$O$_2$ with luminol, no increased signal was observed in cat2 exposed to moderate irradiances, despite the clearly perturbed cell redox state under these conditions (Chaouch et al., 2010; Mhamdi et al., 2010a).

Visualization of ROS in the mesophyll cells showed only a slight increase in dichlorofluorescein (DCF) fluorescence in cat2 compared with Col-0 (Mhamdi et al., 2010a). Secondary signals produced by high light, ozone or pathogens may be required to trigger appreciable increases in ROS contents in catalase-deficient plants. Consistent with the notions that secondary production of ROS is important

**Modification of cell redox state in catalase-deficient plants**

**H$_2$O$_2$ contents in catalase-deficient plants**

Given the high flux through the photorespiratory glycolate oxidase reaction, plants deficient in Class I catalase might be expected to accumulate high levels of H$_2$O$_2$. Estimations of photorespiratory rates under typical controlled environment growth conditions point to a H$_2$O$_2$ generation rate of around 30 µmol g$^{-1}$ FW h$^{-1}$ through this pathway alone (Table 2). Assuming no compensatory metabolism of H$_2$O$_2$ by components other than Class I catalase, this would represent a theoretical increase in mean tissue H$_2$O$_2$ concentration of about 30 mM h$^{-1}$. Despite this, available data suggest that H$_2$O$_2$ accumulation is relatively minor or undetectable in CAT1-deficient tobacco or plants treated with 3-AT (Ferguson and Dunning, 1986; Willekens et al., 1997; Rizhsky et al., 2002). In Arabidopsis, increased diaminobenzidine (DAB) staining was reported in a cat2 mutant grown at relatively low light (Bueso et al., 2007) while a recent study reported a 2-fold increase in extractable H$_2$O$_2$ in cat2 and cat2 cat3 knockouts (Hu et al., 2010). Increased DAB staining was also observed in cat2 RNAi lines exposed to short-term high light stress or ozone (Vanderauwera et al., 2004). While no difference was found in the absence of infection, DAB staining of leaves 24 h after fertilization with avirulent bacteria was about 40% more intense in cat2 compared with the wild type (Simon et al., 2010). In Arabidopsis expressing glycolate oxidase in the chloroplast (in which H$_2$O$_2$ production should also be influenced by RuBP oxygenation rates), increased DAB staining was detected above a threshold irradiance (Fahnenstich et al., 2008). However, using DAB staining or assays of extractable H$_2$O$_2$ with luminol, no increased signal was observed in cat2 exposed to moderate irradiances, despite the clearly perturbed cell redox state under these conditions (Chaouch et al., 2010; Mhamdi et al., 2010a).

Visualization of ROS in the mesophyll cells showed only a slight increase in dichlorofluorescein (DCF) fluorescence in cat2 compared with Col-0 (Mhamdi et al., 2010a). Secondary signals produced by high light, ozone or pathogens may be required to trigger appreciable increases in ROS contents in catalase-deficient plants. Consistent with the notions that secondary production of ROS is important

**Table 2. Calculation of approximate typical rates of photorespiratory H$_2$O$_2$ production**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Net CO$_2$ uptake rate</td>
<td>7 µmol m$^{-2}$ s$^{-1}$ a</td>
</tr>
<tr>
<td>RuBP oxygenation rate</td>
<td>2 µmol m$^{-2}$ s$^{-1}$ b</td>
</tr>
<tr>
<td>Glycolate production</td>
<td>2 µmol m$^{-2}$ s$^{-1}$ c</td>
</tr>
<tr>
<td>H$_2$O$_2$ production</td>
<td>2 µmol m$^{-2}$ s$^{-1}$ c</td>
</tr>
<tr>
<td>Fresh weight/leaf area</td>
<td>235 g m$^{-2}$</td>
</tr>
<tr>
<td>H$_2$O$_2$ production per second</td>
<td>8.5 mmol g$^{-1}$ FW</td>
</tr>
<tr>
<td>per minute</td>
<td>511 mmol g$^{-1}$ FW</td>
</tr>
<tr>
<td>per hour</td>
<td>30.6 µmol g$^{-1}$ FW</td>
</tr>
<tr>
<td>per 8 h day</td>
<td>245 µmol g$^{-1}$ FW</td>
</tr>
<tr>
<td>per 16 h day</td>
<td>490 µmol g$^{-1}$ FW</td>
</tr>
</tbody>
</table>

a Typical rate for Arabidopsis leaves growing in air at moderate irradiance (Veliopu-Jovanovic et al., 2001).
b The carboxylation:oxygenation ratio is taken as four, a conservative estimate of oxygenation (Keys, 2000).
c Assuming that all glycolate produced is oxidized by glycolate oxidase.
d Measured in expanded Arabidopsis rosette leaves.
and that catalase can act as a sink for H₂O₂ produced outside the peroxisomes (Willekens et al., 1997), more intense DAB staining can be detected in cat2 compared to Col-0 when exogenous H₂O₂ is supplied to excised leaves (A Mhamdi, unpublished results).

Minor or undetectable increases in H₂O₂ under conditions in which catalase deficiency triggers significant effects on gene expression and cell redox state may appear surprising. A possible explanation is that H₂O₂ increases in cat2 are either local and/or transient and that H₂O₂ produced in catalase-deficient plants is rapidly scavenged by other pathways. In the barley catalase mutant, slightly increased H₂O₂ was observed within the first few hours after transfer from high CO₂ to air (Noctor et al., 2002). Nevertheless, extractable leaf H₂O₂ contents measured in this mutant did not exceed 200 nmol g⁻¹ FW. Measurements in Arabidopsis Col-0 and the cat2 mutant are also within this range (Chaouch et al., 2010; Mhamdi et al., 2010a). When comparing these values with the estimated rate of photorespiratory H₂O₂ production (Table 2), it becomes evident that the majority of H₂O₂ must be metabolized, even in the absence of the major leaf catalase isoform. This view is consistent with the obvious effect of catalase deficiency on glutathione status in C₃ plants (see next section).

Measuring H₂O₂ in plants is not trivial and is further complicated by several interfering factors (Veljovic-Jovanovic et al., 2002; Queval et al., 2008). Most of the currently available methods may preferentially measure H₂O₂ that is sequestered within compartments such as the apoplast, vesicles or other compartments where redox buffering could be relatively low (Leshem et al., 2006; Foyer and Noctor, 2009). Furthermore, the accuracy of many of the techniques commonly used to quantify H₂O₂ is uncertain. One issue is chemical specificity, notably due to interference from other reactive oxidants or from reductants (Wardman, 2007). Additional to this uncertainty are difficulties in the extraction of H₂O₂. Some of these issues may contribute to the very high variability in reported H₂O₂ contents, even in the absence of stress and between studies using the same technique (Queval et al., 2008). If sufficiently specific, the use of in vivo probes and emerging nanotechnologies could provide more incisive information on compartment-specific H₂O₂ concentrations in planta.

The close link between intracellular H₂O₂ and thiol-disulphide status

Tissue glutathione status is markedly and reproducibly perturbed in catalase-deficient plants, as shown for cat2 in Fig. 7. This effect is presumably a consequence of the enhanced engagement of catalase-independent pathways to metabolize intracellular H₂O₂ and was first reported in barley (Smith et al., 1984). Very similar effects have been observed in catalase-deficient tobacco and Arabidopsis (Willekens et al., 1997; Rizhsky et al., 2002; Queval et al., 2007) and are the most dramatic effects of catalase deficiency on cellular redox state described to date. In cat2, perturbation of glutathione redox state occurs within hours after the onset of photorespiratory H₂O₂ production and continues over the following days, with little or no change in global leaf redox states of functionally associated redox compounds through which GSH can be oxidized (ascorbate/dehydroascorbate) or GSSG can be reduced (NADPH/NADP⁺) (Queval et al., 2007; Mhamdi et al., 2010a, b). This suggests that reductive pathways appear to compensate quite rapidly for catalase deficiency, leading to a new, more oxidized cellular redox state, notably reflected in adjustments of thiol-disulphide status.

An outstanding question is the functional impact of H₂O₂-triggered changes in glutathione. Despite the association of GSSG accumulation with dormancy and cell death (Creissen et al., 1999; Kranmer et al., 2006), this phenomenon does not in itself appear to be part of the signal that leads to lesion formation in catalase-deficient plants (Queval et al., 2007). Extremely dramatic accumulation of GSSG can occur in cat2 gr1 double mutants without necessarily leading to either cell death or bleaching (Mhamdi et al., 2010a). Cell death in cat2 is reverted by extinction of SA synthesis or by exogenously supplying myo-inositol, but reversion is associated with an unchanged or more oxidized glutathione pool (Chaouch and Noctor, 2010; Chaouch et al., 2010). Current evidence from work on catalase-deficient plants suggests that glutathione oxidation may be more important in the decreased growth phenotype rather than in processes associated with cell death. This would be consistent with the role of thiol-disulphide systems in cell cycle and meristem function (Reichheld et al., 1999, 2007; Vernoux et al., 2000; Frendo et al., 2005; Bashandy et al., 2010).

One approach to determine whether the changes in glutathione status provoked by catalase deficiency are part of H₂O₂-triggered signal transduction is to compare responses in cat2 with those observed in lines in which glutathione contents or reduction states are altered independent of changes in H₂O₂ availability. Mutants in which one of the two Arabidopsis GR genes is knocked out (gr1) are aphytontypic and show no evidence of oxidative stress. However, this mutation triggers changes in gene expression that partly recapitulate those observed in cat2 (Mhamdi et al., 2010a). Moreover, introduction of the gr1 mutation into the cat2 background causes marked modulation of H₂O₂-associated transcript profiles (Mhamdi et al., 2010a). This observation points to a significant role for glutathione status in transmitting signals derived from intracellular H₂O₂, though further work is required to resolve this issue fully.

The accumulation of glutathione when catalase is deficient very likely involves up-regulation of biosynthetic pathways. The amount of glutathione that is produced represents a significant increase in sulphur sinks (Queval et al., 2009) and work on barley revealed a marked stimulation of sulphate incorporation into organic compounds when catalase was genetically decreased or biochemically inhibited (Smith et al., 1985). H₂O₂-triggered oxidative accumulation of glutathione possibly involves...
several mechanisms of sulphate reduction and synthesis of cysteine and glutathione, including enhanced gene expression and post-transcriptional activation (Bick et al., 2001; Queval et al., 2009).

**Dissecting the interplay between different antioxidative systems**

In systems where catalase is no longer sufficient, H$_2$O$_2$ or its derivatives such as organic peroxides must primarily be metabolized by peroxidases, which all require reductants. Catalase-deficient plants therefore provide (i) a means of penetrating the apparent redundancy of some of the different reductant-dependent pathways, and (ii) a genetic background in which the impact of perturbing specific components of these pathways can be evaluated. Recent demonstration of the usefulness of catalase-deficient plants has come from the analysis of gr1 mutants. Growth of *Arabidopsis* seedsling under non-physiological oxidative stress conditions (H$_2$O$_2$ dissolved in agar) produced marked effects in wild-type and in gr1 plants (Marty et al., 2009). As gr1 mutants are phenotypically indistinguishable from the wild type in optimal growth conditions (Marty et al., 2009; Mhamdi et al., 2010a), this observation provides little evidence for an important role for GR1, even under conditions of oxidative stress. However, introduction of the gr1 mutation into a cat2 background drastically exacerbates the cat2 phenotype, modulates the cat2 transcriptome, and causes a dramatic acceleration of the onset of leaf bleaching (Mhamdi et al., 2010a). As the phenotype of the single cat2 mutant is less severe compared with wild-type plants grown on H$_2$O$_2$, the difference in both studies cannot simply be explained by the dose of H$_2$O$_2$ or the severity of oxidative stress. It probably reflects the importance of the location of H$_2$O$_2$ production and the fact that in cat2 and other catalase-deficient lines, the oxidative signal is produced through a physiologically relevant intracellular pathway. Interestingly, a peroxisomal GR activity has been characterized in pea (Romero-Puertas et al., 2006), and studies in *Arabidopsis* show that GR1 is addressed to the peroxisomes as well as the cytosol (Kataya and Reumann, 2010). However, the peroxisomes probably contain only a very minor part of the GR1 gene product (Romero-Puertas et al., 2006), which accounts for 30–60% of the overall leaf enzyme activity in *Arabidopsis* (Marty et al., 2009; Mhamdi et al., 2010a).

Differences between cat2 and cat2 gr1 transcript profiles could be linked to differences in glutathione redox state. Alternatively, they could be caused by more indirect effects of the gr1 mutation on H$_2$O$_2$ metabolism in cat2. Analysis of several oxidative stress factors in cat2 and cat2 gr1 double mutants suggest a quite specific effect of H$_2$O$_2$ on glutathione redox status (Mhamdi et al., 2010a).

One outstanding issue concerns the reductive pathways that metabolize H$_2$O$_2$ when catalase is down-regulated (Fig. 8). Ascorbate redox state is generally much less perturbed than that of glutathione in catalase-deficient plants. In part, this could be due to the redox potential of the ascorbate/dehydroascorbate (DHA) couple, which is about 200–300 mV more positive (less reducing) than the GSH/GSSG couple (Noctor, 2006). This predicts that intracellular ascorbate pools should remain highly reduced, even at low GSH/GSSG ratios, as long as both couples are close to redox equilibrium. This situation is observed in double cat2 gr1 mutants, where whole leaf GSH/GSSG ratios (typically 20 or above in unstressed wild-type plants) can fall well below 0.1 while ascorbate:DHA ratios remain close to wild-type values (Mhamdi et al., 2010a).

Increases in intracellular H$_2$O$_2$ may impact more on total ascorbate pools than on ascorbate redox states. Decreases in total ascorbate are sometimes observed in cat2, although these are relatively minor compared with the marked increase in total glutathione (Queval et al., 2007; Mhamdi et al., 2010a; Chaouch and Noctor, 2010; Chaouch et al., 2010). Total ascorbate pools in wild-type plants are highly influenced by growth irradiance (Grace and Logan, 1996; Gatzek et al., 2002). In tobacco Cat1 knockdowns, increased H$_2$O$_2$ stress was triggered by transferring plants from low to moderate light (Willekens et al., 1997). While this treatment caused ascorbate contents to increase 4-fold within 2 d in wild-type tobacco plants, no increase was observed in the catalase-deficient lines (Willekens et al., 1997). Evidence that enhanced availability of intracellular H$_2$O$_2$ opposes certain other responses to increased irradiance has been presented in studies of *Arabidopsis CAT2* knockdowns (Vandenabeele et al., 2004; Vanderauwera et al., 2005).

Electron flow between glutathione and ascorbate (Fig. 8) can occur chemically but is also catalysed by dehydroascorbate reductases (DHAR) (Foyer and Mullineaux, 1998). Enhanced activity of the ascorbate–glutathione pathway when catalase is deficient was evidenced by induction of APX and DHARs at both the transcript level and by enzyme activity (Willekens et al., 1997; Vanderauwera et al., 2005; Mhamdi et al., 2010a). Changes at transcript level were specific to cytosolic forms of these enzymes in *Arabidopsis* cat2 and cat2 gr1 mutants (Mhamdi et al., 2010a), despite the presence of at least one and up to three peroxisome-associated APXs and a dual cytosol/peroxisomal location for GR1 (Narendra et al., 2006; Nyathi and Baker, 2006; Kaur et al., 2009). This points to a close coupling of increased peroxisomal H$_2$O$_2$ availability and cytosolic antioxidiant systems, consistent with the demonstrated importance of cytosolic APX1 in *Arabidopsis* (Davletova et al., 2005a).

H$_2$O$_2$-triggered oxidation of glutathione in cat2 may also be mediated through ascorbate-independent pathways (Fig. 8). Catalase-deficient tobacco shifted to high light to induce photorespiration showed the accumulation of GPX proteins alongside APX (Willekens et al., 1997) and GPX transcripts are induced in *Arabidopsis* cat2 mutants (Queval et al., 2007; Mhamdi et al., 2010a). However, thioredoxins are more efficient reductants for annotated GPXs than glutathione (Iqbal et al., 2006). A more significant route for
direct peroxidation of GSH could be through glutathione S-transferases (GSTs), many of which have peroxidase activity (Dixon et al., 2009). Several GSTs are strongly induced in catalase-deficient lines (Vanderauwera et al., 2005; Queval et al., 2007).

The effects of the gr1 mutation on the cat2 phenotype contrast intriguingly with the ameliorated phenotype observed when cytosolic APX activity is down-regulated in tobacco CAT1 knockdowns (Rizhsky et al., 2002). Interestingly, the ameliorated phenotype of Arabidopsis mutants that are deficient in both CAT2 and cytosolic APX is correlated with the induction of a network of DNA repair, cell cycle control, and ER cell death pathways (Vanderauwera et al., 2010). Different effects of APX and GR1 deficiency in catalase-deficient backgrounds imply that oxidation of glutathione in catalase-deficient plants occurs only partly through the APX/DHAR route and more direct peroxidation may be catalysed by enzymes such as GSTs that are linked to GR1 but independent of APX1 (Fig. 8). This conclusion is consistent with the observed gene expression patterns. Further work is required to establish which glutathione-associated peroxidases are most important in ascorbate-independent reduction of H$_2$O$_2$ or derived peroxides.

**Physiological significance of studies of catalase mutants**

A major advantage of using C$_3$ plants deficient in Class I catalases to modify cell redox state is that the rate of intracellular H$_2$O$_2$ production can be readily modulated by irradiance and switched off in the light by high CO$_2$. This feature enables redox state to be conditional and controllably perturbed. Growth of Arabidopsis cat2 at high CO$_2$ prevents redox perturbation and hence the marked phenotype observed during growth in air (Fig. 9A). Transferring cat2 back to air causes the onset of measurable oxidative stress within hours to days, as evidenced by changes in glutathione status (Fig. 9A). However, within a time-scale of several days after transfer, little effect of the oxidative stress on the plant phenotype is observed. Thus, catalase-deficient plants allow early and late events in oxidative signalling pathways to be studied, within the overall aim of distinguishing between H$_2$O$_2$-dependent and -independent components involved in stress responses (Fig. 9B). Furthermore, the conditional photorespiratory nature of cat2 predicts that any effects of secondary mutations should be annulled by growth at high CO$_2$. For example, cat2 gr1 double mutants show a phenotype that is much more severe than observed in the single mutants.
than cat2 grown in air. However, when cat2 and cat2 gr1 are grown at high CO2, they display similar phenotypes to wild-type and gr1 single mutants (Mhamdi et al., 2010).

Two related but distinct questions arise from studies of catalase-deficient plants: (i) How relevant are effects observed in such systems to those occurring during stress? (ii) Does regulation of catalase activity itself play a significant role in H2O2-linked signalling in wild-type plants?

How relevant are observations in catalase-deficient models to events occurring during stress conditions?

Considerable information on H2O2-induced changes in gene expression has been generated by studies of catalase-deficient tobacco and Arabidopsis lines (Vandenabeele et al., 2003, 2004; Vanderauwera et al., 2005; Gadjev et al., 2006; Queval et al., 2007). Analysis of transcription factors that responded rapidly on shifting Arabidopsis CAT2 knockdowns to high light revealed that several were also affected by abiotic stresses such as cold, heat, and drought (Vanderauwera et al., 2005). Several of these H2O2-induced transcription factors are now established regulators of abiotic stress responses (Shinozaki and Yamaguchi-Shinozaki, 2000; Davletova et al., 2005b; Vogel et al., 2005; Nishizawa et al., 2006; Ogawa et al., 2007). Furthermore, many of the genes that were strongly induced in Arabidopsis cat2 knockouts after transfer from high CO2 to air (Queval et al., 2007) are also induced by abiotic and biotic stresses (Table 3), consolidating the notion that increased availability of H2O2 is an important signal in abiotic stress-induced gene expression (Vanderauwera et al., 2005). Furthermore, a complex pattern of different ROS-responsive genes has been identified during various abiotic stresses, suggesting that different abiotic stress conditions provoke the production of different ROS and that transcriptome profiling analyses could predict the degree of involvement of a specific ROS during a specific stress condition (Gadjev et al., 2006).

Phenotypes of tobacco and Arabidopsis lines suggest that catalase deficiency mimics biotic stress particularly strongly. Under conditions favouring photorespiration, responses...
include lesion formation, accumulation of SA and induction of PR genes (Chamnongpol et al., 1996, 1998; Du and Klessig, 1997; Takahashi et al., 1997; Dat et al., 2003; Chaouch et al., 2010). In Arabidopsis cat2, the formation of lesions is daylength-dependent (Fig. 6), as are SA accumulation, PR gene induction, activation of camalexin and its synthesis pathway, and enhanced resistance to bacterial challenge (Chaouch et al., 2010). All these responses are absent when oxidative stress occurs under short-day conditions, even though the intracellular thiol-disulphide state is at least as perturbed in short days as in long days. However, responses can be induced in short days by supplementation of exogenous SA and reverted in long days by extinction of SA synthesis through the isochorismate pathway (Chaouch et al., 2010), which is responsible for SA production in response to biotrophic pathogens (Wildermuth et al., 2001). Together, these observations show that cat2 grown in long-day conditions is a constitutive defence mutant in which peroxisomal H2O2 triggers SA-dependent hypersensitive response (HR)-like lesion formation (Chaouch et al., 2010).

While most of the focus on pathogen-associated ROS production has focused on apoplastic events (Bindschedler et al., 2006; Sagi and Fluhr, 2006; Torres et al., 2006), analysis of cat2 provides further evidence that intracellular ROS play an important role in programmed cell death (Dat et al., 2003; Vlot et al., 2009), particularly the HR, and points to a potentially crucial role for the peroxisomes. These organelles play several potentially important roles in response to stress (Del Rio et al., 2006; Nyathi and Baker, 2006; Palma et al., 2009). Besides the production of ROS such as superoxide and H2O2, they harbour the terminal steps of JA and SA synthesis. Key peroxisomal biogenesis genes are induced by stresses, including H2O2 (Lopez-Huertas et al., 2000) while peroxisome congregation at the invasion site was an early event in cells in response to fungi (Lipka et al., 2005). Indications of a role for photorespiratory H2O2 in pathogen responses come from studies of plants with altered serine:glyoxylate aminotransferase activity (Taler et al., 2004) and from work on the lsd1 lesion-mimic mutant (Mateo et al., 2004).

As discussed above, perturbation of glutathione is one of the clearest responses to increased H2O2 availability. Environmental stresses also trigger qualitatively similar changes to those observed in catalase-deficient plants, i.e. decreases in the GSH:GSSG ratio and increases in total glutathione (Vanacker et al., 2000; Bick et al., 2001; Gomez et al., 2004b). Redox-sensitive in vivo probes revealed perturbation of glutathione redox state in Arabidopsis subjected to water stress (Jubany-Mari et al., 2010). Glutathione and related thiols are well known to be involved in pathogen responses, including induction of PR genes and phytoalexin synthesis (Edwards et al., 1991; May et al., 1996; Vanacker et al., 2000; Gomez et al., 2004a; Parsy et al., 2007). In Arabidopsis, the expression of some PR genes is under the control of the SA-dependent signalling component, NPR1, which is regulated by cytosolic thiol-disulphide components involving thioredoxins and, potentially, glutathione (Tada et al., 2008). Studies of SA responses in cat2 backgrounds suggest that catalase deficiency recapitulates at least some of the pathogen-related responses involving cytosolic NADP-dependent thiol-disulphide systems (Chaouch et al., 2010; Mhamdi et al., 2010a, b). Thus, cat2 and similar systems are likely to be particularly useful in the evaluation of in vivo interactions between H2O2 and thiol components.

As shown in Fig. 6, growth photoperiod influences the functional outcome of oxidative stress in cat2. The absence of an HR-like response in cat2 in short days is not linked to insufficient oxidative stress. Accumulation of both glutathione (Fig. 7) and H2O2-inducible transcripts is at least as pronounced in short days as in long days (Queval et al., 2007; Chaouch et al., 2010). Recovery of lesion formation and a range of other pathogen responses in cat2 in short days by SA treatment suggests that either daylength or the duration (rather than the intensity) of oxidative stress governs a switch between H2O2 responses that do not require SA accumulation in short days and SA-dependent HR-like programmes in long days (Chaouch et al., 2010). Such an interpretation is consistent with observations of other lesion-mimic mutants (Dietrich et al., 1994; Meng et al., 2009), although in many of these mutants the link between oxidative stress and phenotype is less clear than in cat2. Links between phytochrome signalling and SA accumulation and between SA, flowering and defence reactions have been reported (Genoud et al., 2002; Martinez et al., 2004).

Table 3. Examples of genes that are conditionally induced in cat2 and a summary of their responses to environmental stresses or hormones

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene product</th>
<th>Stress condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>At1g05560</td>
<td>UGT75B1</td>
<td>1 2 3 4 5 6 7 8 9 10 11 12 13</td>
</tr>
<tr>
<td>At1g17170</td>
<td>GSTU24</td>
<td>+++++</td>
</tr>
<tr>
<td>At2g15480</td>
<td>UGT73B5</td>
<td>+++++</td>
</tr>
<tr>
<td>At2g29600</td>
<td>HSP17.6</td>
<td>+++++</td>
</tr>
<tr>
<td>At2g30750</td>
<td>Cyt. P450, put.</td>
<td>+++++</td>
</tr>
<tr>
<td>At2g37770</td>
<td>Aldo/keto reductase</td>
<td>+++++</td>
</tr>
<tr>
<td>At2g43820</td>
<td>UGT74F2</td>
<td>+++++</td>
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<tr>
<td>At2g47730</td>
<td>GSTF8</td>
<td>+++++</td>
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<tr>
<td>At3g25250</td>
<td>OX1 protein kinase</td>
<td>+++++</td>
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<tr>
<td>At3g55430</td>
<td>put. β-1,3-glucanase</td>
<td>+++++</td>
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<tr>
<td>At4g11600</td>
<td>GPX6</td>
<td>+++++</td>
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<tr>
<td>At4g16690</td>
<td>Esterase/liope thoiest.</td>
<td>+++++</td>
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<tr>
<td>At4g33540</td>
<td>Lactamase</td>
<td>+++++</td>
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<tr>
<td>At5g14730</td>
<td>Unk. Prot.</td>
<td>+++++</td>
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<tr>
<td>At5g22300</td>
<td>Nitrilase4</td>
<td>+++++</td>
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<tr>
<td>At5g43450</td>
<td>ACC oxidase, put.</td>
<td>+++++</td>
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Ozone-induced leaf damage was shown to be influenced by the photoperiod context in which plants were subjected to equal-time treatments (Vollines et al., 2009). In both animals and plants, ROS have been implicated in ageing and catalase may play a role in senescence. However, transgenic mice in which the single mammalian catalase gene is knocked out show normal development (Ho et al., 2004). Knockout of peroxisomal catalase in C. elegans causes premature ageing (Petrit and Rachubinski, 2004) while overexpression in mice mitochondria or cardiac-specific expression was reported to alleviate symptoms of ageing (Schriner et al., 2005; Wu et al., 2007). In humans, catalase deficiency has recently been implicated in greying of hair (Wood et al., 2009) and another interesting observation is that ageing of human cells can be alleviated by engineering improved peroxisomal import of catalase (Koepeke et al., 2007). Several studies implicate peroxisomes and ROS in senescence in plants (for a review, see del Río et al., 1998). In Arabidopsis, CAT2 expression decreases after flowering and this decrease has been proposed to be an integral part of H₂O₂-triggered leaf senescence (Zimmerman et al., 2006; Smykowski et al., 2010). Treatment of non-senescent Arabidopsis with 3-AT induced the expression of H₂O₂-responsive genes, including PRI and GPX6, that were also highly expressed in senescent leaves (Navabpour et al., 2003). However, the senescence marker SAG12 was not among the genes induced by catalase inhibition (Navabpour et al., 2003).

Genes induced by photorespiratory H₂O₂ in cat2 include some that are induced during senescence (Table 3). Despite this, the cat2 phenotypes do not provide strong evidence for a co-ordinated activation of senescence programmes by peroxisomal H₂O₂, and this view is supported by other data from transcriptomic profiling. For instance, WRKY53 is a transcription factor involved in senescence that is induced in leaves within hours after spraying with H₂O₂ and that can interact with the promoters of CAT1, CAT2, and CAT3 (Miao et al., 2004, 2007). Analysis of transcription factors rapidly induced (within 8 h) of transferring cat2 RNAi lines to high light identified several WRKY genes, but not WRKY53 (Vanderauwera et al., 2005). Experiments over a longer time-scale (2–4 d after transfer of cat2 knockouts from high CO₂ to air) also show that WRKY53 is not significantly induced in response to increases in peroxisomal H₂O₂ (Mhamdi et al., 2010a; authors’ unpublished results). On the contrary, WRKY53 was found to be strongly and commonly up-regulated in KD-SOD, KO-Apx1, and flu mutants, and upon treatment with ozone or methyl viologen (Gadjev et al., 2006). The lesion phenotype and other responses of cat2 in long days are clearly more similar to HR-like processes than to senescence, while in short days cat2 plants have a decreased growth rather than altered senescence. Furthermore, CAT3 transcripts are not increased in cat2 (Queval et al., 2007; Fig. 5), although expression of CAT3 is enhanced in older Arabidopsis leaves (Zimmermann et al., 2006). The growth restraint observed in cat2 possibly involves mechanisms common to many environmental stresses, reflecting the centrality of redox state in these conditions. The link between H₂O₂ and senescence appears to be complex and future studies will further unravel the roles of catalase regulation in this process.

How important is catalase regulation in response to stress?

Numerous studies of plant responses to various stresses have included data on changes in catalase activity or expression. A detailed analysis of this literature is beyond the scope of the present review. The following discussion focuses on potential mechanisms by which regulation of catalase could contribute to oxidative signalling, with a particular focus on increases in H₂O₂ availability that could result from down-regulation of catalase.

A first mode of possible regulation is through changes in transcript abundance. Potato Class II catalase was induced in roots exposed to nematodes and bacteria and by SA in stem tissue (Niebel et al., 1995). Similar induction of Class II catalase was observed in tobacco leaves treated with tobacco mosaic virus or fungal elicitor (Dorey et al., 1998). Intriguingly, in view of the observations in catalase-deficient plants, Class I catalase transcripts were down-regulated by these treatments (Dorey et al., 1998). CAT1 expression was strongly induced by treatment of Arabidopsis seedlings with H₂O₂ (Xing et al., 2008), though a similar effect on CAT1 transcripts has not been observed in cat2 mutant backgrounds, whether measured by qPCR (Queval et al., 2007), semi-quantitative RT-PCR (Fig. 5) or microarray analysis (Mhamdi et al., 2010a). This discrepancy may reflect the importance of developmental stage or the (sub)cellular localization of H₂O₂ signals. CAT1 transcripts were also induced by abscisic acid through a pathway dependent on mitogen-activated protein kinase-dependent signalling (Xing et al., 2008). As well as potential catalase interactions with WRKY53 (Miao et al., 2004), a G-box binding factor (GBF1) was found to interact with the CAT2 promoter in yeast 1-hybrid experiments and this interaction may play a role in regulating the onset of leaf senescence (Smykowski et al., 2010). However, as discussed above, phenotypic and other effects observed in cat2 knockouts do not point to a simple relationship between CAT2 expression and senescence.

A second potential mode of regulation of catalase activity is at the post-transcriptional level. Selective degradation of catalase was reported to be a trigger of autophagic cell death in animal cells (Yu et al., 2006). One well-described mechanism in plants is diminished re-synthesis of catalase in stress conditions. Catalase is a light-sensitive protein that has a high turnover rate, and stresses such as cold, salt, and high light can cause decreases in the total protein through accelerated inactivation or decreased capacity to replace the protein (Volk and Feierabend, 1989; Hertwig et al., 1992; Streb and Feierabend, 1996). Re-synthesis of rye Cat1 is post-transcriptionally controlled by mRNA methylation, which is stimulated by blue light and peroxides (Schmidt et al., 2002, 2006).
Other potential mechanisms that could down-regulate catalase activity include SA and nitric oxide (Vlot et al., 2009), as well as an unknown inhibitor that accumulated in the medium of Arabidopsis cells treated with fusicoccin (Beffagna and Lutz, 2007). As noted above, it is also possible that ROS homeostasis is affected by catalase targeting efficiency and potential changes in subcellular distribution. In Chlamydomonas, which lack peroxisomes, mitochondrial catalase activity is inhibited by light via a redox-dependent mechanism involving chloroplast thioredoxins (Shao et al., 2008).

Several proteins have been identified that could interact with catalase polypeptides. Arabidopsis CAT3 has been reported to bind calmodulin (Yang and Poovaiah, 2002). Calmodulin was shown to be present in peroxisomes and to increase the activity of tobacco catalase but not catalases from a bacterium, a fungus, or humans (Yang and Poovaiah, 2002). Catalases have also been found to interact with nucleoside diphosphate kinase 1 (NDK1), a cytosolic protein (Fukamatsu et al., 2003). This interaction may also increase catalase activity (Fukamatsu et al., 2003). Another cytosolic protein reported to interact with both CAT2 and CAT3 is SOS2, a SNF-related kinase (Verslues et al., 2007). Both associations could modulate the activity of any catalase that may be found in the cytosol. However, it remains unclear whether these interactions reflect in vivo phenomena because NDK2, which was also found to interact with SOS2 (Verslues et al., 2007), was reported to be located in the stroma rather than in the cytosol (Bölt et al., 2007).

It remains unclear whether natural genetic variability in catalase could contribute to differences in stress responses, though there is sufficient allelic variability at the maize Cat3 locus to encode electrophoretically distinguishable proteins (Scandalios et al., 1980). A maize line showing increased resistance to Aspergillus flavus infection had a Cat3 allele with a deletion of 20 amino acids compared to the control line (Magbanua et al., 2007).

### Conclusions and perspectives

Concepts of ROS as toxic compounds have been qualified over recent years by the realization that they are also important signal molecules. It is often considered that concentration is a key factor in determining which of these modes ROS operate through. However, observations that singlet oxygen-induced bleaching can be genetically reverted (Wagner et al., 2004) have been complemented by the finding that plants deficient in the major catalase isoform not only recapitulate a wide range of pathogen responses, but that these responses, including cell death, can also be reverted (Chaouch et al., 2010). It is therefore likely that ROS signalling is a more useful conceptual paradigm than damage, even when this involves marked intracellular redox perturbation that results from the loss of a highly active H$_2$O$_2$-metabolizing enzyme. Further, analysis of biochemical markers of redox state in cat2 and cat2 gr1 mutants points to a rather specific perturbation of glutathione, suggesting that altered status of this key redox buffer may act as an important channel for H$_2$O$_2$-triggered signal transduction.

To exploit processes in which ROS act through widespread damage, preferred strategies would be to minimize ROS accumulation or the probability of interaction with sensitive components (Fig. 10A), because it would presumably be too energetically costly or too difficult to engineer directly resistance against a plethora of indiscriminate modifications. However, if the physiological effects of ROS work primarily through a more limited number of signalling mechanisms, other approaches could be feasible, such as the selection of gain-of-function or loss-of-function mutants (Fig. 10B). Although there could be overlap and interdependence between the two approaches (enhancing defence or adjustment of signalling), the choice of experimental strategies will inevitably be influenced by which concept is dominant. Hence, whether ROS act primarily through ‘damage or signalling’ is more than a merely semantic issue. Whichever view is favoured, it is not likely to be simple to achieve the desired modifications in plant performance without less attractive trade-off effects. However, this objective will probably be aided by improved knowledge on how redox signalling interfaces and interacts with the network of signalling pathways through components such as phytohormones. The growing awareness of the crucial role of redox-dependent signalling in plant development and function suggests that conditional catalase-deficient mutants are likely to continue to be useful tools to this end.

### Supplementary data

Supplementary data can be found at JXB online.

**Supplementary Table S1.** PCR and RT-PCR primers used to characterize cat1 and cat3 T-DNA mutants.
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