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Review

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Hydrogen peroxide metabolism and functions in plants

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Contents

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Summary

Hydrogen peroxide (H_2O_2) is produced, via superoxide and superoxide dismutase, by electron transport in chloroplasts and mitochondria, plasma membrane NADPH oxidases, peroxisomal oxidases, type III peroxidases and other apoplastic oxidases. Intracellular transport is facilitated by aquaporins and H_2O_2 is removed by catalase, peroxiredoxin, glutathione peroxidase-like enzymes and ascorbate peroxidase, all of which have cell compartment-specific isoforms. Apoplastic H_2O_2 influences cell expansion, development and defence by its involvement in type III peroxidase-mediated polymer cross-linking, lignification and, possibly, cell expansion via H_2O_2-derived hydroxyl radicals. Excess H_2O_2 triggers chloroplast and peroxisome autophagy and programmed cell death. The role of H_2O_2 in signalling, for example during acclimation to stress and pathogen defence, has received much attention, but the signal transduction mechanisms are poorly defined. H_2O_2 oxidizes specific cysteine residues of target proteins to the sulfenic acid form and, similar to other organisms, this modification could initiate thiol-based redox relays and modify target enzymes, receptor kinases and transcription factors. Quantification of the sources and sinks of H_2O_2 is being improved by the spatial and temporal resolution of genetically encoded H_2O_2 sensors, such as HyPer and roGFP2-Orp1. These H_2O_2 sensors, combined with the detection of specific proteins modified by H_2O_2, will allow a deeper understanding of its signalling roles.

I. Introduction

In recent years, considerable attention has been paid to the involvement of hydrogen peroxide (H_2O_2) and associated reactive oxygen species (ROS) in plant function. Consequently, the topic has been extensively reviewed. This review is distinct in attempting to assess our knowledge of H_2O_2 function in the context of its metabolism and signalling roles. To do this, we focus as specifically as possible on H_2O_2 rather than on other forms of ROS. ROS comprise a set of chemically distinct species (Fig. 1), but a large proportion of the literature refers to the involvement of unspecified ROS in physiological processes. This situation is caused by the difficulty in measuring specific ROS and, consequently, our understanding is hampered. Therefore, we critically assess H_2O_2 measurement methods with a focus on genetically encoded probes with improved specificity (Section II). H_2O_2 is relatively stable in biological systems compared with its usual precursor superoxide ($O_2^{\cdot-}$); hence, it can be used as a substrate and signalling molecule in a relatively controllable manner. $O_2^{\cdot-}$ and H_2O_2 production are therefore entangled, a situation further complicated by the production and reactions of nitric oxide ($NO^{\cdot}$), a radical signalling molecule (Fig. 1). At the same time, the problem of specificity is complicated by 'downstream' radical formation. Figure 1 illustrates the pathways of H_2O_2 production and the array of consequences that arise in terms of radical production and modifications of cellular components. These modifications include oxidative damage, but are also potentially signals that affect protein function and gene expression. To critically assess the functions of H_2O_2, the review covers potential targets of H_2O_2 and $O_2^{\cdot-}$ toxicity (Section III), the production, transport and removal of peroxide (Sections IV–VI) and its functions in terms of metabolism and signalling (Sections VII, VIII).

II. Measurement and imaging of H_2O_2

Critical to an understanding of H_2O_2 metabolism and signalling is reliable measurement with high temporal and spatial resolution (Gilroy *et al.*, 2016). The widely used methods for the measurement of H_2O_2 and other ROS have been extensively criticized for their lack of chemical specificity and insufficient spatial and temporal resolution (Winterbourn, 2014). Surprisingly, these warnings have been largely ignored and methods are often applied without critical evaluation or sufficient controls. This situation is largely a pragmatic response to a lack of practical alternatives. All commonly used fluorescent or coloured reagents, such as 3,3′-diaminobenzidine (DAB), Amplex Red and fluorescein-based compounds, should be used with care for the quantification or imaging of H_2O_2 (Kristiansen *et al.*, 2009; Šnyrychová *et al.*, 2009; Schmitt *et al.*, 2014; Noctor *et al.*, 2016). The fluorescein probes, for example dihydrofluorescein diacetate (H_2FDA), 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and 5- (and 6-)chloromethyl-2′,7′ dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), react with a wide range of radical-based reactive species, including peroxinitrite (Winterbourn, 2014) and

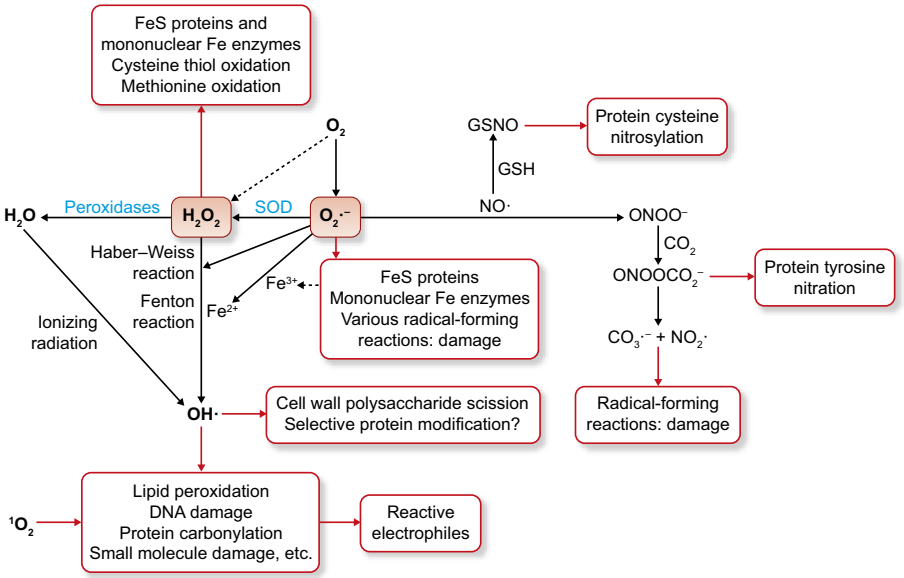

Fig. 1 Reactions of superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) and their interaction with nitric oxide. $O_2^{\cdot-}$ is produced by oxidases (e.g. NADPH oxidase) and electron transport processes. H_2O_2 is produced by $O_2^{\cdot-}$ dismutation (spontaneous and catalysed by $O_2^{\cdot-}$ dismutase (SOD)), type III peroxidases (not shown) and also directly released by some oxidases (Fig. 3). $O_2^{\cdot-}$ exerts toxic effects by damaging Fe- and FeS-containing proteins, reacting with various cellular constituents to form reactive radicals (e.g. thiyl radicals from thiols). Further reactive species are generated by the fast reaction between $O_2^{\cdot-}$ and nitric oxide (NO). H_2O_2 is relatively unreactive at cellular concentrations, but damages Fe- and FeS-containing proteins and can oxidize methionine residues. It is essentially unreactive with ascorbate and most thiols, except when catalysed by peroxidases. The final reactive species, hydroxyl radicals, are generated from $O_2^{\cdot-}$ and H_2O_2 (with the aid of redox-active metals) and react with almost anything at their site of production. Some of the resulting reactive electrophiles have a signalling role. The rate constants for some of the reactions are shown in Table 1. Red arrows and outlined boxes, targets. $CO_3^{\cdot-}$, carbonate radical; GSH, glutathione; GSNO, nitrosoglutathione; $^1O_2$, singlet oxygen; $OH^{\cdot}$, hydroxyl radical; $ONOO^-$, peroxinitrite; $ONOOCO_2^-$, nitrosoperoxycarbonate.

reactive sulfur species (DeLeon *et al.*, 2016). A useful critical assessment of CM-H_2DCFDA has been made (Kristiansen *et al.*, 2009). Amplex Red and DAB are fairly specific for H_2O_2, but require peroxidase activity to provide fast reaction rates, and DAB is not suitable for live cell imaging. The use of these probes has been critically evaluated (Šnyrychová *et al.*, 2009). Cerium chloride is a potentially useful probe for H_2O_2. Cerium (Ce^{3+}) forms insoluble Ce(IV) perhydroxide which can be visualized by electron microscopy (Voothuluru & Sharp, 2013), but confocal reflectance microscopy might provide an alternative visualization. Luminol, which produces chemiluminescence when oxidized, has been widely used to measure the extracellular 'oxidative burst', but has a complex chemistry (Winterbourn, 2014). Finally, even the apparently simple task of measuring the H_2O_2 concentration in tissue extracts is fraught with technical difficulty, leaving no consensus on the average concentrations in plants, which range from 50 to 5000 nmol g^{-1} FW (Noctor *et al.*, 2016). Moreover, the extraction of tissue samples does not reveal the cellular and organelle specificities for H_2O_2 production and scavenging.

Genetically encoded probes, in which fluorescent proteins have been engineered to detect H_2O_2, have a high potential to solve some of the problems associated with small-molecule probes. The first widely used green fluorescent protein (GFP)-based sensors (roGFP1 and roGFP2) were modified with a cysteine pair to respond to the glutathione redox state (GSH) in a ratiometric manner, and have been well validated in plants in different subcellular compartments (Schwarzlander *et al.*, 2008; Bratt *et al.*, 2016). To provide improved coupling to GSH/GSSG, the latest version of roGFP2 is attached to glutaredoxin, producing Grx1-roGFP2 (Gutscher *et al.*, 2008). The environment of cysteines in proteins determines their accessibility to substrates and redox potential, providing some specificity to their oxidation. HyPer was the first genetically encoded probe with a high specificity for H_2O_2 (Belousov *et al.*, 2006), and new versions, such as HyPer-3 and HyPerRed, have been developed (Bilan *et al.*, 2013; Ermakova *et al.*, 2014). HyPer was constructed by incorporating OxyR into circularly permuted yellow fluorescent protein (cpYFP). OxyR is an *Escherichia coli* H_2O_2-sensitive transcription factor, which contains a cysteine pair with high specificity for oxidation by H_2O_2, resulting in a disulfide bond. The thiol–disulfide interconversion changes the fluorescence excitation ratio of the attached YFP, allowing the probe to be used ratiometrically. HyPer and its variants have been widely used in various systems, including plants (Costa *et al.*, 2010; Exposito-Rodriguez *et al.*, 2013, 2017; Hernández-Barrera *et al.*, 2015; Rodrigues *et al.*, 2017; Mullineaux *et al.*, 2018). However, it has a disadvantage in being pH sensitive, requiring the use of a control, such as SypHer, in which one of the cysteines has been replaced, removing its ability to respond to H_2O_2, but maintaining pH response (Matlashov *et al.*, 2015; Exposito-Rodriguez *et al.*, 2017). In some cases, HyPer is prone to silencing beyond the cotyledon stage in Arabidopsis (Exposito-Rodriguez *et al.*, 2013), which could limit its use in plants. However, it has been successfully targeted to peroxisomes (Costa *et al.*, 2010) and multiple subcellular compartments by transient expression in *Nicotiana benthamiana* (Exposito-Rodriguez *et al.*, 2017). Following from HyPer, new sensors based on redox relays are being designed. A fusion of roGFP2 with the yeast thiol peroxidase (TPX) Orp1 (roGFP2-Orp1) allows H_2O_2-dependent oxidation of roGFP2 (Gutscher *et al.*, 2009; Scuffi *et al.*, 2018). roGFP2-Orp1 is promising because, unlike HyPer, it is not pH sensitive. Other variants of H_2O_2 sensors not yet reported in plants are HyPerRed and a roGFP2-peroxiredoxin (Prx)-based probe (Ermakova *et al.*, 2014; Morgan *et al.*, 2016).

Although genetically encoded sensors provide specificity and temporal information on H_2O_2, they do not measure absolute concentrations, as the oxidation state of the probe is dependent on the rate of H_2O_2 production vs the capacity and rate of the thiol system to reduce the probe. The use of external H_2O_2 additions can calibrate HyPer (Huang & Sikes, 2014) and the observation of the kinetics of HyPer re-reduction after H_2O_2 exposure can provide information on the capacity of the thiol system. roGFP-Orp1 also reacts with lipid hydroperoxides and peroxinitrite (Muller *et al.*, 2017), and so the extent to which these species interfere with H_2O_2 measurement *in vivo* requires investigation. A potential disadvantage of constitutively expressed probes is that they could disturb H_2O_2 concentration, as observed for *E. coli* (Lim *et al.*, 2014), and could cause longer term changes in the activity of the antioxidant system. This problem could be avoided by inducible expression. The current genetically encoded proteins are unlikely to work well in more oxidizing compartments, such as the apoplast and endoplasmic reticulum (ER) lumen, where the probe is likely to become fully oxidized. A recent modification of HyPer (TriPer) is able to operate in the ER lumen of mammalian cells (Melo *et al.*, 2017).

III. H_2O_2 and $O_2^{\cdot-}$ toxicity

Arabidopsis growth is inhibited by 1 mM H_2O_2 (Claeys *et al.*, 2014). In mammals and yeast, toxicity occurs at micromolar, but not low millimolar, concentrations (Nakamura *et al.*, 2003; Semchyshyn & Valishkevych, 2016). The data of Claeys *et al.* (2014) also provide a hint of this complex response curve. Estimates for resting intracellular H_2O_2 concentrations in *E. coli* and mammalian cells (20–35 nM) are remarkably similar (Seaver & Imlay, 2001; B. K. Huang *et al.*, 2016). The requirement for the removal of $O_2^{\cdot-}$ and H_2O_2 is made clear by the impaired function of mutants with compromised antioxidant systems, particularly when pushed to the limit by environmental stresses. A striking natural example of the importance of defence against H_2O_2 is illustrated by the pico-cyanobacterium *Prochlorococcus*, which lacks catalase-peroxidase (KatG). It cannot grow unless naturally occurring H_2O_2 is removed by 'helper' bacteria (Morris *et al.*, 2011). Specific targets of $O_2^{\cdot-}$ and H_2O_2 toxicity in plants have not been extensively studied and their effects are hidden within the blanket term 'oxidative stress' (Fig. 1). In some cases, programmed cell death or autophagy of organelles (Section VI) is induced and has physiological significance in pollen–stigma incompatibility (Dat *et al.*, 2003; Wilkins *et al.*, 2011). It is not known whether there are specifically sensitive targets for $O_2^{\cdot-}$ and H_2O_2 toxicity in plants. Proteins containing FeS clusters and mononuclear iron centres are susceptible to demetallation by both $O_2^{\cdot-}$ and H_2O_2, leading to amino acid auxotrophy in *E. coli* and yeast $O_2^{\cdot-}$

dismutase (SOD) mutants (Imlay, 2013) (Table 1). SODs are a diverse set of enzymes containing iron (Fe), manganese (Mn) or copper/zinc (Cu/Zn), and are present in the cytosol, chloroplasts, mitochondria, peroxisomes (Pilon *et al.*, 2011) and the apoplast (Kim *et al.*, 2008). SOD mutants in yeast, flies and mice are usually more sensitive to oxidative stress and sometimes have a decreased lifespan, although not in *Caenorhabditis elegans* (Van Raamsdonk & Hekimi, 2009). Arabidopsis lacking Cu/ZnSOD activity grows normally under laboratory conditions (Chu *et al.*, 2005). Of the three FeSODs in Arabidopsis, two are chloroplastic (FSD2 and FSD3), whereas FSD1 is cytosolic/nuclear. Knockouts of the two chloroplast isoforms cause light sensitivity and severe bleaching, whereas the cytosolic FSD1 knockout shows no obvious phenotype (Myouga *et al.*, 2008; Zhang *et al.*, 2011). Mitochondrial MnSOD antisense lines have somewhat impaired growth and show inhibition of the tricarboxylic acid (TCA) cycle enzymes aconitase and isocitrate dehydrogenase (Morgan *et al.*, 2008), the former at least being consistent with FeS targeting by $O_2^{\cdot-}$. The characterization of knockout mutants showed that mitochondrial MnSOD is essential for female gametogenesis (Martin *et al.*, 2013). Ascorbate supplementation rescues oxygen sensitivity, lifespan and amino acid auxotrophy of yeast SOD mutants (Zyracka *et al.*, 2005), presumably because of its appreciable rate constant for $O_2^{\cdot-}$ dismutation to H_2O_2 (Table 1). It is possible that high ascorbate concentration in plants (Wheeler *et al.*, 2015) buffers them against the more severe symptoms of SOD deficiency.

The reaction between methionine and H_2O_2, forming methionine sulfoxide, has a relatively large rate constant (Table 1) and, consistent with this, *c.* 400 proteins containing methionine sulfoxide have been identified in Arabidopsis catalase (*cat2*) mutants that accumulate excess H_2O_2 when exposed to high light and ambient CO_2 (Jacques *et al.*, 2015). Methionine sulfoxide reductase regenerates methionine. Arabidopsis contains at least five type A peptide methionine sulfoxide reductases and nine type B isoforms which use thioredoxin or glutaredoxin as reductant (Laugier *et al.*, 2013). Mutants in the various isoforms are more sensitive to high light and show increased metabolic disruption (Bechtold *et al.*, 2009; Laugier *et al.*, 2013). Although methionine sulfoxide production is generally seen as damage, two points are worth bearing in mind. First, proteins contain sufficient methionine residues for them to be used catalytically via methionine sulfoxide reductase as an H_2O_2-removing system (Levine *et al.*, 1996). Second, serine and threonine phosphorylation is influenced by the oxidation of nearby methionine residues, and there is an over-representation of oxidation-susceptible methionine residues near phosphorylation sites in the human proteome, giving rise to the possibility of effects on metabolic activity or signalling (Veredas *et al.*, 2017). However, the physiological significance of methionine oxidation remains to be established.

$O_2^{\cdot-}$ can react with cysteine thiols to produce reactive and undesirable thiyl radicals (Winterbourn, 2015) (Table 1), but their significance *in vivo* is unknown. H_2O_2 is poorly reactive, except with TPXs. H_2O_2 can give rise to the highly reactive hydroxyl radical in the Fenton reaction or Haber–Weiss reaction (Fig. 1). The former reaction requires free Cu^+ or Fe^{2+}. Fe^{2+} could be generated by the release of Fe^{3+} from proteins, followed by its reduction with $O_2^{\cdot-}$ or ascorbate. Hydroxyl radicals initiate lipid peroxidation and cause protein carbonylation and DNA damage, as evidenced by mutagenesis in *E. coli* SOD mutants (Imlay, 2008). Lipid peroxidation results in a range of reactive electrophile species (RES), which are signalling molecules (Farmer & Mueller, 2013).

Table 1 Rate constants ($M^{-1}\,s^{-1}$) for reactions of superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) with selected cellular components.

	$O_2^{\cdot-}$	H_2O_2	References
Ascorbate	10^5	2 (pH 7.5), 6 (pH 6)	Polle & Junkermann (1994); Buettner & Schafer (2003)
Thiols (cysteine/GSH/thioredoxin)	7×10^5	1–2	Winterbourn (2015)
Methionine	–	30 (pH 2–6)	Yin *et al.* (2004)
Phenolic compounds	10^3–10^7	[a]	Taubert (2003)
Fe enzymes	10^6–10^7	10^3	Winterbourn (2015); Anjem & Imlay (2012)
Peroxiredoxins/GPX/OxyR	–	10^7	Winterbourn (2015)
Haem peroxidases/catalase	–	10^7	Winterbourn (2015)
$NO^{\cdot}$	10^{10}	–	Nauser & Koppenol (2002)
$O_2^{\cdot-}$	10^5; 10^9 (SOD)	[b]	Imlay (2008)

Conditions for rate constant determination are described in the references, but are usually pH 7–7.5 at 20–25°C. *In vivo* reaction rates will of course depend on the reactant concentrations and pH. The reactivity of phenolic compounds (e.g. cinnamic acid derivatives and flavonoids) is largely determined by hydroxyl groups: *ortho*-dihydroxy, $10^{5/6}\,M^{-1}\,s^{-1}$; *ortho*-trihydoxy, $10^7\,M^{-1}\,s^{-1}$. Amongst the compounds tested, quercetin, gallic acid, epicatechin gallate and oligomeric proanthocyanidins were the most active. Because of its relatively high concentration in plant cells, ascorbate could be a contender for the effective removal of $O_2^{\cdot-}$ via the formation of the relatively stable and easily regenerated monodehydroascorbate radical and H_2O_2. However, the reaction of $O_2^{\cdot-}$ with thiols potentially gives rise to thiyl radicals which are reactive and can damage other molecules. Nitric oxide ($NO^{\cdot}$) is a very strong sink for $O_2^{\cdot-}$ giving rise to peroxinitrite (Fig. 1). GSH, glutathione, GPX, glutathione peroxidase; SOD, $O_2^{\cdot-}$ dismutase.
[a]Type III peroxidase catalysed oxidation of phenolic compounds by H_2O_2.
[b]Haber–Weiss reaction producing hydroxyl radical.

IV. Production of H_2O_2: enzymes and subcellular locations

H_2O_2 forms spontaneously from the interaction between water, organic matter and light. Consequently, nanomolar to low micromolar concentrations are found in the environment (Li & Imlay, 2018). Photochemical reactions producing $O_2^{\cdot-}$ and H_2O_2 are also a potential concern in microscopy when ROS probes are being used. Illumination of dihydrofluorescein dyes oxidizes them, and so investigations involving light and ROS production must be carefully controlled. Flavin-containing proteins are potentially able to reduce oxygen in a blue light-dependent manner. ROS (H_2O_2) production from cryptochrome, a blue light photoreceptor, occurs in Arabidopsis and Drosophila (Consentino *et al.*, 2015; Arthaut

et al., 2017). Other flavin-containing enzymes could also generate $O_2^{\cdot-}$ and then H_2O_2 in the light, and it has been shown that a yeast mutant in peroxisomal acyl-CoA oxidase is impaired in light-induced H_2O_2 production and downstream signalling (Bodvard *et al.*, 2017). As yeast lacks conventional photoreceptors, another flavin-dependent protein must provide light–dark or circadian cues. The routes of H_2O_2 production are covered in the following sections and summarized in Fig. 2.

1. Peroxisomes and glyoxysomes

Peroxisomes and glyoxysomes are organelles bound by a single membrane and specialized for the compartmentalization of oxidase enzymes which produce $O_2^{\cdot-}$ and H_2O_2 as a 'side reaction' (del Rio & Lopez-Huertas, 2016). Flavin-containing oxidases and dehydrogenases vary considerably in the relative amounts of $O_2^{\cdot-}$ and H_2O_2 produced during catalysis, depending on the redox potential and degree of solvent exposure of the active site (Messner & Imlay, 2002). In plants, $O_2^{\cdot-}$/H_2O_2-forming oxidases are involved in photorespiration (glycolate oxidase), fatty acid oxidation (acyl-CoA oxidase), polyamine and purine catabolism, and the synthesis of some hormones. Large fluxes of H_2O_2 are particularly likely for photorespiration (Queval *et al.*, 2007) and fatty acid utilization during germination of oilseeds in glyoxysomes, a specialized type of peroxisome. Peroxisomes appear to be a source of $NO^{\cdot}$ as well, providing the possibility of peroxinitrite formation.

2. Chloroplasts and mitochondria

Chloroplasts and mitochondria are sites of intense electron transport activity, coupled with the production of large electrochemical gradients across energy transducing membranes. These conditions provide opportunities for H_2O_2 generation via $O_2^{\cdot-}$ dismutation. In chloroplasts, there are multiple potential sites of $O_2^{\cdot-}$ production, such as photosystems I and II (PSI and PSII) and the electron transport chain (ETC). Estimates of the proportion of electrons reducing oxygen to form $O_2^{\cdot-}$ at PSI (Mehler reaction) vary from 1% to 5% (Mullineaux *et al.*, 2018) Oxygen photoreduction producing $O_2^{\cdot-}$ and H_2O_2 has been documented *in situ*, in isolated chloroplasts and in thylakoid membranes (Mubarakshina Borisova *et al.*, 2012). Recently, the use of stroma-targeted HyPer has shown that most of the H_2O_2 production is blocked by the photosynthetic electron transport inhibitor DCMU (3-(3,4-Dichlorophenyl)-1,1-dimethylurea), suggesting that it is formed

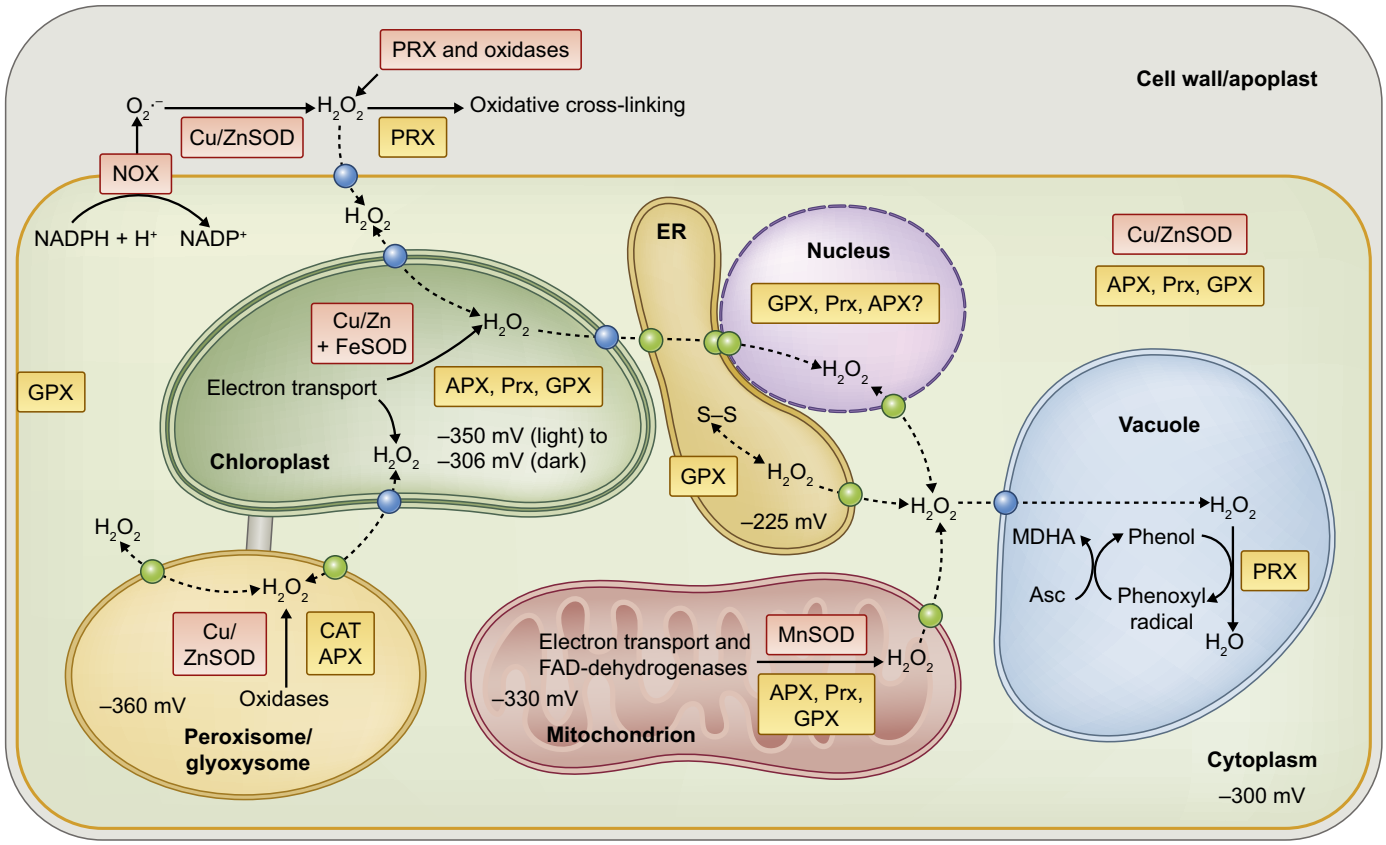

Fig. 2 Sites of hydrogen peroxide (H_2O_2) production, scavenging and transport. The diagram shows the main sites of H_2O_2 production and scavenging in a typical plant cell. H_2O_2 transport from chloroplast to nucleus is shown via the endoplasmic reticulum (ER), but could be more direct. The normal glutathione redox potential (mV) in each compartment is also indicated (Schwarzlander *et al.*, 2008). Asc, ascorbate; APX, ascorbate peroxidase; CAT, catalase; FAD, flavin adenine dinucleotide; GPX, glutathione peroxidase; MDHA, monodehydroascorbate radical; NOX, NADPH oxidase; PRX, type III peroxidase; Prx, peroxiredoxin; SOD, superoxide dismutase. Yellow boxes, H_2O_2-removing enzymes; red boxes, H_2O_2-producing enzymes; blue circles, H_2O_2-transporting aquaporins; green boxes, H_2O_2 transporters not confirmed; grey rectangle, chloroplast–peroxisome tether; solid lines, reactions; dashed lines, transport.

downstream of PSII, most likely in the Mehler reaction (Exposito-Rodriguez *et al.*, 2017). Stromal and thylakoid membrane (stromal side)-located SOD assists conversion to H_2O_2, followed by reduction to water by peroxidases (Section VI). Although it is tacitly assumed that the Mehler reaction does not involve specific proteins, this may repay consideration. Alternative routes of oxygen photoreduction have been proposed which could act as 'valves' to decrease H_2O_2 production. Plastid terminal oxidase (PTOX) accepts electrons from plastoquinol and reduces oxygen to water but, more recently, a role for flavodiiron proteins (FDPs) in oxygen photoreduction has been identified in cyanobacteria, green algae and bryophytes (Chaux *et al.*, 2017; Shimakawa *et al.*, 2017). FDPs take electrons from PSI and reduce oxygen to water instead of H_2O_2. FDPs are lacking in angiosperms, where it is suggested that cyclic electron transport (CET) has a similar protective function (Chaux *et al.*, 2017). Relevant to this, H_2O_2 production activates NADPH-dependent CET (Strand *et al.*, 2015). Mitochondria generate $O_2^{\cdot-}$ and H_2O_2 through the ETC, particularly through respiratory complexes I, II and II and Flavin-containing dehydrogenases (Schwarzlander *et al.*, 2009; Riemer *et al.*, 2015; S. Huang *et al.*, 2016).

3. Plasma membrane and apoplast

There are numerous routes to H_2O_2 production in the apoplast and the complexity is illustrated in Fig. 3. A prominent source is NADPH oxidase (NOX). Plant NOXs are named respiratory burst oxidase homologues (RBOHs) after the mammalian enzyme in the neutrophil microbial defence system (Segal, 2016). NOXs are plasma membrane enzymes which use electrons from cytosolic NADPH to reduce oxygen to $O_2^{\cdot-}$ in the apoplast. Models predict six membrane-spanning domains with cytosolic N- and C-termini. The C-terminus has binding sites for NADPH and flavin adenine dinucleotide (FAD). Reduced FAD transfers electrons to cytochromes located in a channel formed by the transmembrane domains (Fig. 3). $O_2^{\cdot-}$ production is followed by its rapid dismutation to H_2O_2. This proton-requiring reaction is favoured by low apoplastic pH, but there is also evidence for apoplastic SOD activity, perhaps using germin-like proteins. The proton consumption by $O_2^{\cdot-}$ dismutation could increase apoplastic pH, and the RBOH-mediated transport of electrons to the extracellular space will depolarize the membrane, so that NOX function might not just be about $O_2^{\cdot-}$ (Segal, 2016). Mutants are important for understanding NOX functions, but many studies have used the inhibitor diphenyleneiodonium (DPI), which is not specific to NOX but is a general flavoprotein inhibitor (Riganti *et al.*, 2004), and so caution and corroborative evidence are needed. Given that $O_2^{\cdot-}$ is both short lived and poorly membrane permeable, the proposed signalling roles for NOXs either require aquaporin-mediated H_2O_2 transport to the cytosol or interaction of its products with plasma membrane sensor kinases. Localization in lipid rafts could facilitate their interaction with potential regulatory proteins (Hao *et al.*, 2014; Nagano *et al.*, 2016). Under some conditions, for example salt stress, $O_2^{\cdot-}/H_2O_2$ production occurs in cytoplasmic vesicles derived from the plasma membrane or ER (Leshem *et al.*, 2006), and RBOHD is internalized into plasma membrane vesicles (Hao *et al.*, 2014). The N-terminus of NOX has Ca^{2+}-binding EF hand motifs, and Ca^{2+}-dependent activation has been demonstrated *in vivo* (Potocky *et al.*, 2012). NOX-dependent ROS production in the apoplast is induced by a plethora of stimuli, such as extracellular ATP, hormones (abscisic acid (ABA), ethylene) and pathogen- and damage-associated molecular patterns (PAMPs and DAMPs). Molecular mechanisms activating NOX via its regulatory N-terminal domain have been extensively reviewed (Qi *et al.*, 2017) and involve receptor-like kinases (RLKs), phosphatidic acid, calcium influx-activating calcium-dependent protein kinases (CPKs), protein kinases (e.g. BIK1) and small GTPases (RAC/ROP). Examples and references are given in Table 2. Arabidopsis has 10 NOX isoforms with distinct expression patterns and, to some extent, they are specialized for specific functions in a wide range of processes. For example, RBOHC and RBOHH/J function in the polar growth of root hairs and pollen tubes, respectively (Foreman *et al.*, 2003; Boisson-Dernier *et al.*, 2013; Kaya *et al.*, 2014), and RBOHD in wound responses and systemic signalling (Miller *et al.*, 2009). RBOHD and RBOHF are also variously involved in stomatal function and pathogen responses (Kadota *et al.*, 2015). The role of NOX in polar growth is conserved across brown seaweeds, fungi and mammals, but, following the seminal work on the role of NOX in root hair growth (Foreman *et al.*, 2003), the biochemical events for which NOX is required still seem obscure. Cell wall remodelling (Section VII) or signalling (Rentel *et al.*, 2004) are candidates. Consistent with these multiple functions, it is not surprising that NOX mutants are relatively susceptible to environmental changes. Antisense suppression of the tomato RBOH family produced plants with multiple developmental defects as well as pathogen sensitivity (Sagi *et al.*, 2004), and more influences on root development are still coming to light (Orman-Ligeza *et al.*, 2016).

NOX has received high profile attention, but there are other sources of apoplastic H_2O_2. The cell wall contains numerous type III peroxidase isoenzymes with functions in the cross-linking of cell wall polymers (Section VII). However, peroxidases have been reported to variously generate $O_2^{\cdot-}$, H_2O_2 and hydroxyl radicals, depending on the chemical environment (Fig. 3) (Chen & Schopfer, 1999; Kawano, 2003; Daudi *et al.*, 2012; Kimura *et al.*, 2014). The H_2O_2-generating reaction is not fully understood, but is favoured by high pH and requires a reductant which has not been identified. The use of mutants suggests a role for peroxidases in the generation of H_2O_2 or other ROS during the immune response (Daudi *et al.*, 2012), stomatal defences (Khokon *et al.*, 2010; Arnaud *et al.*, 2017) and root hair growth (Sundaravelpandian *et al.*, 2013; Mangano *et al.*, 2017). The H_2O_2 production reaction described by Bolwell's group (Daudi *et al.*, 2012) is favoured by high pH, consistent with the pH increases reported during elicitor/PAMP responses. In potato, comparison of various PAMPs and DAMPs shows that only those causing a pH increase also elicit 'ROS' production (Moroz *et al.*, 2017). Is it feasible that NOX, together with the suppression of H^+-ATPase activity could contribute to the high pH to favour type III peroxidase-derived H_2O_2 production.

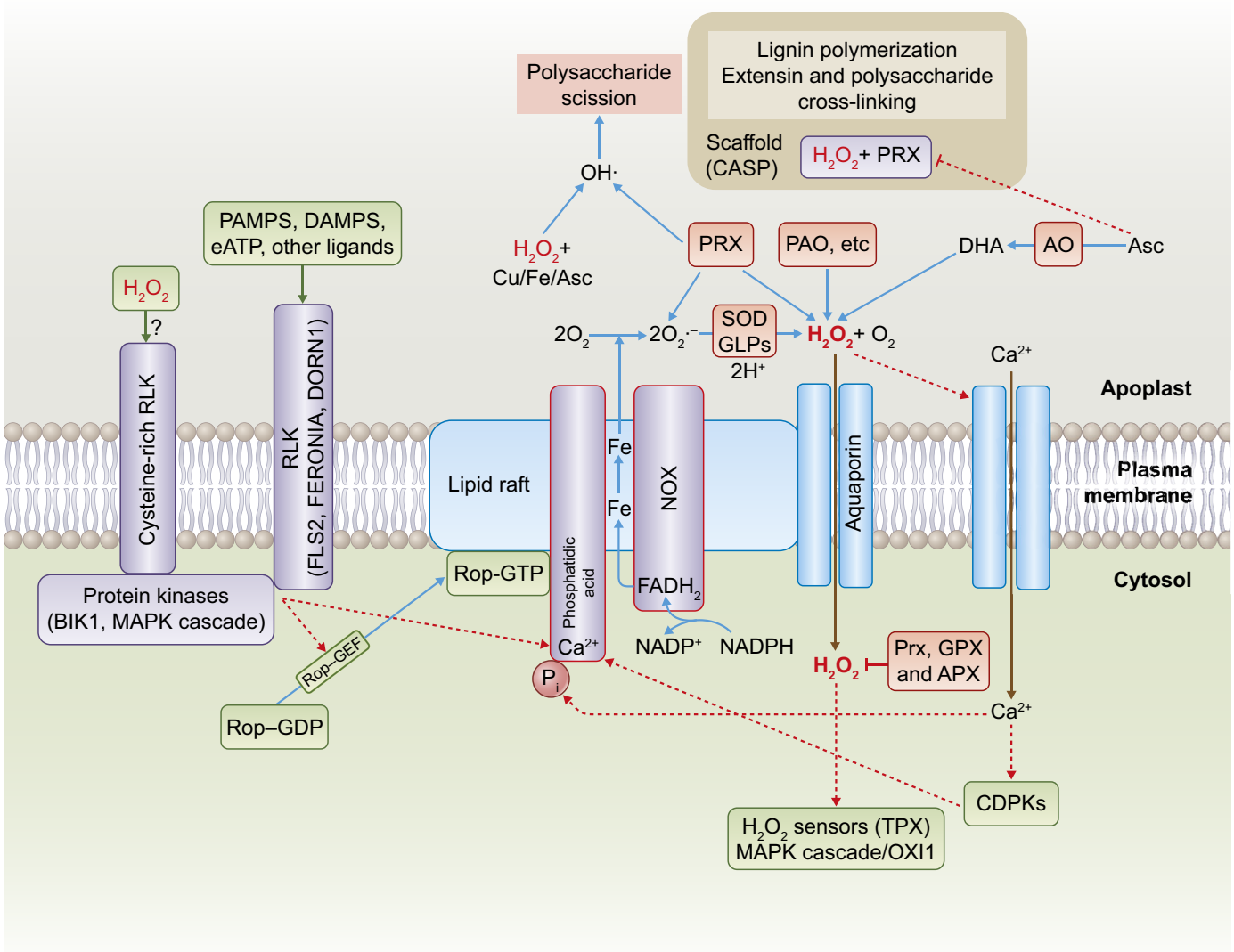

Fig. 3 Superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) production and utilization in the apoplast and its relationship with cytosolic H_2O_2. $O_2^{\cdot-}$ and H_2O_2 are produced by NADPH oxidase (NOX, RBOH), other oxidases (e.g. copper (Cu) amine oxidases, polyamine oxidases and oxalate oxidase) and by type III peroxidase (PRX). The PRX reaction activated in response to pathogen-associated molecular patterns (PAMPs) depends on the apoplast alkalinization that follows PAMP perception and an unknown reductant. In other chemical environments, PRX can produce $O_2^{\cdot-}$ and $OH^{\cdot}$. Non-enzymatic dehydroascorbate (DHA) breakdown generates H_2O_2. Interaction of Cu^+ or Fe^{2+} with H_2O_2 (Fenton reaction) generates $OH^{\cdot}$ and is facilitated by the reduction of Cu^{2+} or Fe^{3+} by ascorbate (Asc). $O_2^{\cdot-}$ dismutation to H_2O_2 is catalysed by Cu/ZnSOD and, possibly, germin-like proteins. PRX uses H_2O_2 in various cross-linking reactions involved in cell wall organization and pathogen defence. On the other hand, $OH^{\cdot}$ breaks polysaccharides, allowing cell expansion. $O_2^{\cdot-}$, $OH^{\cdot}$ and H_2O_2 might attack invading organisms and, in the case of H_2O_2, initiate long-distance signalling. A key question is how the system is organized to provide reaction specificity. Attachment of PRX to scaffold proteins (Casparian strip domain proteins in the endodermis) or wall polymers could direct reactions and influence the peroxidatic vs hydroxylic reactions, whereas the localization of redox-active metals could direct $OH^{\cdot}$ localization. Not all the depicted reactions occur to the same extent in different cells, and we do not know whether PRX or NOX isoenzymes expressed in different cells have significantly different catalytic or regulatory properties. Polyamine oxidase (PAO) activity depends on polyamine transport from the cytosol. How the $O_2^{\cdot-}$- and H_2O_2-forming activity is activated on stimulation is unknown, although pH could be a factor. NOX activity depends on activation by Ca^{2+}, phosphatidic acid (produced by phospholipase D), phosphorylation and Rop-GTPase binding (see Table 2 for references). Spatial localization of NOX and its interactors, including signalling receptors and aquaporins, may be facilitated by location in lipid rafts. Glutathione, ascorbate peroxidase (APX) and ascorbate recycling enzymes have also been measured in apoplastic fluid. Hormones (e.g. abscisic acid (ABA), cytokinin) variously activate NOX and PRX, but these interactions are not shown in the diagram. Red dashed lines show activation, inhibition or other interactions. AO, ascorbate oxidase; CDPK, Ca^{2+}-dependent protein kinase; DAMP, damage-associated molecular pattern; eATP, extracellular ATP; FAD, flavin adenine dinucleotide; GEF, guanine nucleotide exchange factor; GLP, germin-like protein; GPX, glutathione peroxidase; MAPK, mitogen-activated protein kinase; SOD, superoxide dismutase; P_i, phosphate; Prx, peroxiredoxin; RLK, receptor-like kinase; Rop, Rho-GTPase of plants in GDP or GTP-bound form; TPX, thiol peroxidase.

A number of other oxidases are located in the apoplast, including copper amine oxidases (CuAOs), FAD-dependent polyamine oxidases (PAOs) and oxalate oxidase. CuAOs (*c.* 10 genes in Arabidopsis) are largely apoplastic, whereas PAOs (five genes in Arabidopsis) are largely peroxisomal, but also apoplastic (PAO1). They have wide substrate specificity for polyamines (Tavladoraki

Table 2 Examples of modulation of NADPH oxidase activity by a variety of interacting factors.

Isoforms	Interacting factors	Effect on activity	Physiological processes	References
AtRBOHC/RHD2	Calcium	Activation through EF-hand binding	Root hair growth	Takeda *et al*. (2008)
AtRBOHD	BIK1	Activation by phosphorylation	Immunity	Kadota *et al*. (2014); Li *et al*. (2014)
	CPK5	Activation by phosphorylation	Immunity	Dubiella *et al*. (2013)
	XLG2	Activation	Immunity	Liang *et al*. (2016)
	Nitric oxide	Inhibition of FAD binding by *S*-nitrosylation	Immunity	Yun *et al*. (2011)
	DORN1	Activation by phosphorylation	ATP-mediated stomatal immunity	Chen *et al*. (2017)
	Phosphatidic acid	Activation	Stomatal closure	Zhang *et al*. (2009)
AtRBOHD AtRBOHF	Calcium	Activation through EF-hand binding		Ogasawara *et al*. (2008); Kimura *et al*. (2012)
AtRBOHF	CIPK26	Activation by phosphorylation	Guard cell ABA signalling?	Drerup *et al*. (2013)
	OST1/SnRK2.6	Phosphorylation	Guard cell ABA signalling	Sirichandra *et al*. (2009)
	Calmodulin 4	?	Senescence, programmed cell death (PCD)	Koo *et al*. (2017)
AtRBOHH AtRBOHJ	Calcium	Activation through EF-hand binding	Pollen tube growth	Kaya *et al*. (2014)
OsRbohB	OsRac1	Activation through EF-hand binding	Immunity	Wong *et al*. (2007); Nagano *et al*. (2016)
	OsRACK1	Activation?	Immunity	Nakashima *et al*. (2009)
StRBOHB	StCDPK5	Activation by phosphorylation	Immunity	Kobayashi *et al*. (2007)

These mechanisms provide a rapid means of activating or inhibiting superoxide/H_2O_2 formation via NADPH oxidase activity in response to environmental and developmental cues. See Fig. 3 for a summary of NADPH oxidase-related processes in the apoplast. FAD, flavin adenine dinucleotide.

et al., 2016). Products of amine and polyamine oxidation are H_2O_2 and an aldehyde or amino aldehyde. Mutants or the over-expression of various apoplastic amine oxidases confirms an increase or decrease in reactive species. The use of mutants and polyamine feeding suggests that they contribute to ABA and H_2O_2-dependent stomatal closure (An *et al*., 2008; Gémes *et al*., 2016) and H_2O_2 production in pollen tubes (Wu *et al*., 2010). The potential signalling roles of the reactive aldehydes and their further products have not been considered.

Some plants, for example cereals, contain apoplastic oxalate oxidase (germin) which produces H_2O_2 and CO_2 (Le Deunff *et al*., 2004). Arabidopsis has a family of germin-like proteins, which do not have oxalate oxidase activity (Membré *et al*., 2000), but which may have SOD activity. Oxalate is a product of ascorbate degradation in the cell wall, but it is also potentially relevant that oxidation products of ascorbate (dehydroascorbate (DHA) and 2,3-diketogulonate) can be degraded under apoplastic conditions with non-enzymatic H_2O_2 production (Kärkönen *et al*., 2017). Very interestingly, a currently unidentified oxidation product of ascorbate also inhibits peroxidase activity (Kärkönen *et al*., 2017). Cell wall ascorbate oxidase maintains ascorbate in a relatively oxidized state, and its activity could therefore influence H_2O_2 production. Overall, a complex picture of H_2O_2 production (NOX, peroxidase, PAO, CuAO, oxidized ascorbate) and removal (peroxidase) is emerging, together with consequences for growth, lignification, pathogen defence and signalling (Fig 3). Extracellular H_2O_2 production by nectaries and trichomes (Peiffer *et al*., 2009) may contribute to defence against insects and microbes. Nectar H_2O_2 (*c*. 30 μM) is formed via glucose oxidase and a germin-like protein, which are secreted into the nectar, and by a nectary NOX (Bezzi *et al*., 2010; Harper *et al*., 2010).

4. Endoplasmic reticulum

The ER is an oxidizing compartment for glutathione and thiol groups which facilitates the protein folding processes that depend on the formation of disulfide bonds. The two means of oxidative cross-linking via protein disulfide isomerases (PDIs) both involve H_2O_2 (Bulleid, 2012). In the first, a FAD-containing ER oxidase (ERO) produces an endo-disulfide by reducing oxygen to H_2O_2. Oxidized ERO then oxidizes PDI, which, in turn, transfers the disulfide bond to target proteins to induce correct folding. This process therefore generates H_2O_2. By contrast, the second mechanism uses H_2O_2 to produce the disulfide form of a 2-cysteine Prx or the sulfenic acid form of a glutathione peroxidase (GPX), which then oxidize PDI. In plants, it is not known whether both pathways operate. Although the Arabidopsis genome contains two ERO and 13 PDI proteins, there is no evidence for an ER-localized Prx (Aller & Meyer, 2013). However, there is strong evidence for the ERO pathway in developing soybean seedlings, where storage protein synthesis involves the extensive formation of disulfide links (Matsusaki *et al*., 2016). The possibility of a GPX-like (GPXL) pathway in plants is supported by the observation that rice mutants in OsGPXL5, which is ER/chloroplast localized, show noticeably decreased grain filling, consistent with a role in storage protein synthesis (Matsusaki *et al*., 2016). Arabidopsis GPXL3 is attached to the ER membrane (Attacha *et al*., 2017). GPXLs or Prxs could be involved in the removal of H_2O_2 produced by the ERO

system. Finally, but not investigated, the presence of DHA, the oxidized form of ascorbate, in the ER could also facilitate disulfide bond formation. Further investigation of the oxidative processes involved in protein folding in plants is required.

V. H_2O_2 transport

Like water, H_2O_2 is relatively poor at permeating membranes and its transport is facilitated by channel proteins of the aquaporin type. Plant aquaporins are present on the plasma membrane (PIPs) and tonoplast (TIPs) and most likely in the chloroplast inner envelope (Mubarakshina Borisova *et al.*, 2012; Bienert & Chaumont, 2014). Their presence on other membranes is not well characterized. A mouse aquaporin 3 mutant is impaired in NOX2/H_2O_2-mediated signalling, which activates the redox-controlled transcription factor NF-κB (Hara-Chikuma *et al.*, 2015), providing evidence for the aquaporin dependence of an entire H_2O_2 signalling system. Most likely, the same aquaporins used by water are involved in H_2O_2 transport, but some isoforms may have greater selectivity for H_2O_2 or may be impermeable to H_2O_2 (Almasalmeh *et al.*, 2014). AtPIP1;4 facilitates H_2O_2 movement from the apoplast to the cytosol during PAMP-triggered immunity. Decreased H_2O_2 movement in the mutant allows increased bacterial growth, presumably by interfering with intracellular H_2O_2 signalling (Tian *et al.*, 2016). *Atpip2;1* mutants are impaired in ABA- and PAMP-induced stomatal closure and the mutants apparently have decreased intracellular ROS, suggesting a role in both water and H_2O_2 transport (Grondin *et al.*, 2015; Rodrigues *et al.*, 2017). Interestingly, AtPIP2;1 is phosphorylated by OST1 in response to ABA (Grondin *et al.*, 2015). The differences between maize plasma membrane aquaporins in H_2O_2 permeability was demonstrated by expression in yeast (Bienert *et al.*, 2014). High H_2O_2 concentration (*c.* 30 mM) decreases the water permeability of maize roots, implying that a possible oxidative modification to aquaporins decreases their water permeability (Ye & Steudle, 2006). Interestingly, a lower concentration of H_2O_2 (0.5 mM) causes AtPIP2;1 to be internalized in endosomes (Wudick *et al.*, 2015). It appears that the distribution and permeability of aquaporins is very dynamic and could influence H_2O_2 signalling. The exit of photosynthetic electron transport-sourced H_2O_2 from isolated chloroplasts is blocked by acetazolamide, a membrane-impermeable reagent that blocks aquaporins (Mubarakshina Borisova *et al.*, 2012). As chloroplast-sourced H_2O_2 influences nuclear H_2O_2 and gene expression (Exposito-Rodriguez *et al.*, 2017), it is possible that the expression or gating of specific chloroplast envelope aquaporins could influence light signalling. Although the plasma membrane aquaporins have been investigated in this regard, little is known of their role in other membranes. H_2O_2 transport between ER and cytosol is most likely facilitated by aquaporins in mammalian cells (Appenzeller-Herzog *et al.*, 2016), but has not been investigated in plants. Furthermore, in mammals, NOX2 and the aquaporin AP3 are physically associated, which would facilitate the movement of H_2O_2 into the cytosol for signalling (Hara-Chikuma *et al.*, 2015). The function of aquaporins in H_2O_2 transport requires further confirmation in plants and will require the careful use of mutants and inhibitors.

VI. Control of H_2O_2 concentration: how and where?

Most organisms contain multiple enzymes to remove H_2O_2 with two distinct reaction mechanisms: haem peroxidases and thiol-based peroxidases. In plants, the haem peroxidases are catalase (CAT), ascorbate peroxidase (APX) and type III peroxidases (PRXs). APXs are strongly restricted to photosynthetic organisms but, together with ascorbate, are absent from cyanobacteria (Wheeler *et al.*, 2015). Compared with animals, where ascorbate is not considered as a major player in H_2O_2 removal, plants can develop very high ascorbate concentrations in photosynthetic tissues. The TPXs include Prxs (peroxiredoxins) and GPXL (Glutathione peroxidase-like)enzymes, which use thioredoxin as reductant (Iqbal *et al.*, 2006; Navrot *et al.*, 2006; Attacha *et al.*, 2017). In addition to H_2O_2, GPXLs also react with organic hydroperoxides and phospholipid hydroperoxides. TPXs are also candidates for H_2O_2 sensing (Section VIII). The subcellular locations, properties and functions of the peroxidases have been extensively reviewed (Dietz, 2016; Maruta *et al.*, 2016). APX and TPXs have numerous isoforms and are found in all subcellular compartments, whereas CAT is most likely restricted to peroxisomes. In a nutshell, knockout mutants are generally more sensitive to H_2O_2 and stresses that are expected to increase H_2O_2 load. Transcripts of some, but not all, increase in response to stress and, in some cases, control of their enzyme activity by post-translational modification (PTM) has been reported. There are numerous reports of increased resistance to stress as a result of the over-expression of peroxidases, particularly APX (Dietz, 2016; Maruta *et al.*, 2016).

1. Peroxisomes and glyoxysomes

Catalase is present in very high concentration in peroxisomes, to the extent that it is often associated with crystalline structures (Kleff *et al.*, 1997).This high concentration is perhaps required because CAT does not have a high affinity for H_2O_2. However, CAT is 'ideal' in the sense that a large flux of H_2O_2 is removed without perturbing the cellular redox state (i.e. GSH and NADPH are not needed as the ultimate reductant). Peroxisomal CAT mutants have been extensively studied in Arabidopsis and tobacco, and show its key role in H_2O_2 removal (Queval *et al.*, 2007). In addition to CAT, APX is also associated with peroxisomes. In Arabidopsis, a peroxisomal APX3 mutant shows no obvious phenotype at low light intensity at which photorespiratory H_2O_2 production would be low (Narendra *et al.*, 2006). The single report of Prx in plant peroxisomes (Corpas *et al.*, 2017) requires corroboration. Despite the assumption that peroxisomes evolved to remove or contain H_2O_2, it has been suggested that they could also function as H_2O_2 sources, particularly for signalling (del Rio & Lopez-Huertas, 2016). Kinetic modelling of H_2O_2 production predicts that 5% of H_2O_2 produced would leak from peroxisomes (Poole, 1975) and, in broad support of this prediction, isolated rat liver peroxisomes actively oxidizing fatty acids release H_2O_2, albeit at very low concentration (Mueller, 2000). The peroxisomal membrane has pores large enough to allow H_2O_2 loss, but, surprisingly, it appears to be relatively impermeable based on CAT latency assays (Heupel

et al., 1991). Therefore, retention in the peroxisome would be aided by channelling as a result of close interaction of enzymes. In support of this hypothesis, there is evidence for physical association of spinach leaf peroxisomal enzymes (Heupel & Heldt, 1994). These results suggest that close enzyme association allows metabolite channelling and is also supported by physical association between CAT and glycolate oxidase in rice (Zhang *et al.*, 2016). Salicylic acid (SA) disrupts this association and causes a small increase in H_2O_2, detected by DAB staining in intact leaves. However, this evidence is weak because its source from peroxisomes was not determined. Contrary to peroxisomes acting as a source of H_2O_2, antisense or co-suppression of *CAT1*, the major CAT isoform in tobacco, provided evidence that peroxisomes are a sink for H_2O_2 (Willekens *et al.*, 1997). Leaf discs of wild-type plants floating on H_2O_2 solution depleted H_2O_2 more rapidly than did *CAT1*-deficient mutants. Therefore, CAT in peroxisomes could act as a sink for extra-peroxisomal H_2O_2, even when the peroxisomes are producing photorespiratory H_2O_2. Given the contradictory nature of the evidence so far, it will be important to determine whether peroxisomes are always net sinks or whether they can also be sources. Interestingly, strong evidence for a specific signalling role for peroxisome-sourced H_2O_2 has been demonstrated in yeast (Bodvard *et al.*, 2017). Other features of peroxisome activity that will impinge on this question are the proliferation and increased mobility under oxidative stresses and the formation of peroxule extensions (Rodríguez-Serrano *et al.*, 2016). Salt stress causes peroxisome proliferation, but overexpression of PEX11, promoting peroxisome proliferation, does not increase salt tolerance (Mitsuya *et al.*, 2010); therefore, perhaps proliferation is necessary, but not sufficient, for salt tolerance. Do these peroxisomal responses change their function as H_2O_2 sources or sinks, and can channels for H_2O_2 in the peroxisomal membrane be gated? A subset of peroxisomes is also attached to chloroplasts (Gao *et al.*, 2016) and the potential for channelled movement of H_2O_2 and other metabolites needs to be addressed. CAT is sensitive to photoinactivation via blue light absorption. It is continuously degraded and synthesized in a light-dependent manner in leaves (Engel *et al.*, 2006), and a cytosolic chaperone protein (NCA1), which interacts with Arabidopsis CAT2, maintains CAT activity (Li *et al.*, 2015). Under conditions of severely high H_2O_2 production, for example in *cat2*, peroxisomes become aggregated and are degraded by autophagy (Shibata *et al.*, 2013). CAT and APX are inhibited by SA, suggesting the possibility that H_2O_2 will increase during defence responses. However, it seems that physiological concentrations of SA would be too low for direct inhibition (Ruffer *et al.*, 1995), but indirect interaction may occur (Yuan *et al.*, 2017). Arabidopsis CAT1, CAT2 and CAT3 interact with LSD1, a zinc finger protein which is involved in cell death in a potentially SA-dependent manner (Li *et al.*, 2015). CAT3 is phosphorylated and activated by the calcium-dependent protein kinase CPK8, although both proteins interact in the nucleus and cytosol, but not in the peroxisomes, as determined by bimolecular fluorescence complementation (BiFC) assay (Zou *et al.*, 2015). A *cpk8* mutant had a somewhat higher H_2O_2 level, as determined by DAB and fluorescein-based probes. It is therefore likely that CAT activity can be modulated by a number of interactions and modifications, which could result in controlled H_2O_2 release from peroxisomes (Costa *et al.*, 2010; Kneeshaw *et al.*, 2017).

2. Chloroplasts

The key components are APX (and a high concentration of its substrate ascorbate) and TPXs which, together, remove H_2O_2 using NADPH and photosynthetic electron transport via ferredoxin as the ultimate reductant. APX has isoforms in the stroma (sAPX) and attached to the thylakoid membrane (tAPX) (Maruta *et al.*, 2016). 2-Cys Prx, GPXL1 and GPXL7 are prominent TPXs in Arabidopsis (Dietz, 2016; Attacha *et al.*, 2017). APX- and ascorbate-deficient (*vtc*) mutants in Arabidopsis have somewhat increased sensitivity to photo-oxidative stress, while a double mutant of the two 2-Cys Prx proteins in chloroplasts is sensitive to photo-oxidative stress, and a triple mutant with tAPX is synergistically more sensitive (Awad *et al.*, 2015). This provides a multi-layered H_2O_2 removal system. Involvement of the TPXs also allows a potential signalling/chaperone element through PTMs and gene expression (Dietz, 2016). It is possible that inactivation of Prx by over-oxidation and of sAPX by H_2O_2, which is potentiated by a specific amino acid loop (Kitajima *et al.*, 2010), could enable transient H_2O_2 accumulation, allowing it to act as a signal. As with peroxisomes, severe oxidation of chloroplasts in high light, UVB radiation and in a tAPX mutant can result in autophagic destruction (chlorophagy) (Izumi *et al.*, 2017), and is presumably beneficial in removing the potential for damaged chloroplasts to act as sources of singlet oxygen.

3. Mitochondria

The enzymes removing H_2O_2 in mitochondria and their relationship with signalling (in the case of TPXs) have been well reviewed (Riemer *et al.*, 2015; S. Huang *et al.*, 2016), and only some key points are summarized here. Prxs, GPX and APX are present, together with ascorbate and GSH, in the matrix (Jiménez *et al.*, 1997). Plant mitochondria are intimately involved in photosynthesis, both in glycine metabolism as one of the sites of photorespiration and in the oxidation of reducing equivalents produced by chloroplasts. To accommodate these fluxes and to prevent $O_2^{\cdot-}$ and H_2O_2 production by over-reduction of the ETC, there are two key features: the alternative oxidase (AOX), which diverts electrons to oxygen with the production of water (analogous to chloroplast PTOX and flavodiiron reactions, Section IV), and uncoupling proteins (UCPs), which allow the dissipation of the proton gradient. Mutants in these processes have increased $O_2^{\cdot-}$/H_2O_2 and compromised photosynthesis (Sweetlove *et al.*, 2006; Morgan *et al.*, 2008).

4. Vacuoles as a H_2O_2 sink

In fully expanded cells, the vacuole comprises *c.* 90% of the cell volume and accumulates secondary compounds (via ABC transporters), inorganic ions, as well as sugars, amino acids and organic acids. Ascorbate occurs in vacuoles as does a large proportion of the type III peroxidase activity. Vacuoles could comprise an H_2O_2 sink

with uptake facilitated by tonoplast aquaporins. A phenolic substrate is oxidized by H_2O_2 using type III peroxidase. The resulting phenoxyl radical is reduced by ascorbate. The resulting DHA could be transported to the cytosol for reduction in exchange for ascorbate (Fig. 2) (Zipor & Oren-Shamir, 2013). A detailed study of peroxidase and phenolic substrates in *Catharanthus roseus* identified all the components of this system (Ferreres *et al.*, 2011). Consistent with this proposal, peroxidase activity and phenolic substrates increase during drought and high light (Sultana *et al.*, 2015; Tattini *et al.*, 2015). Although it is an attractive idea that vacuoles could act as an H_2O_2 buffer, the extent of their capacity so far lacks evidence, and the investigation of mutants lacking vacuole-localized peroxidase or with altered phenolic composition is required in this context.

VII. Metabolic functions of H_2O_2

Remarkably, the Arabidopsis genome encodes *c.* 65 expressed type III haem peroxidases, targeted to the cell wall or vacuole (Valério *et al.*, 2004). They are generally N-glycosylated and, like cell wall extensin and arabinogalactan proteins, contain hydroxyproline residues (Nguyen-Kim *et al.*, 2016). Some of these generate ROS (Section IV), but they also oxidize a wide range of substrates and have their most obvious function in modifying the cell wall during development or in response to pathogens. Lignification involves the oxidation of monolignols in the cell wall to form radicals, which then react with each other to produce polymerized lignin. The mechanism of monolignol radical formation potentially involves O_2-dependent laccases (copper oxidase enzymes) or H_2O_2-dependent type III peroxidases (Marjamaa *et al.*, 2009; Berthet *et al.*, 2011). PRX2, PRX25 and PRX71 mutants have impaired stem lignification (Shigeto *et al.*, 2015). Lignification is inhibited by the H_2O_2 scavenger potassium iodide in spruce cell cultures (Laitinen *et al.*, 2017), indicating an important role for peroxidase. The source of H_2O_2 for xylem lignification is not established. NOX activity could contribute via $O_2^{\cdot-}$-derived H_2O_2, but the involvement of specific isoforms is not established. As peroxidase can generate H_2O_2 under appropriate conditions (Section IV), it is tempting to speculate that the same (or different) isoenzymes could provide H_2O_2, as well as catalysing monolignol oxidation. Effects on lignin in mutants could derive from either activity. The other H_2O_2-producing apoplastic oxidases and ascorbate could also be involved (Section IV). As well as the xylem, the endodermis lignifies using peroxidase (PRX64) and NOX (RBOHF) as the $O_2^{\cdot-}/H_2O_2$ source (Lee *et al.*, 2013). Critically, this process involves CASP1 which is needed for the localization of PRX64. Casparian strip domain proteins are endodermal proteins which provide a platform for endodermal cell wall modifications. Therefore, it is possible that the scaffolding of enzymes producing and consuming H_2O_2 occurs in other cell types and responses, such as defence. In spruce, the cationic peroxidase binds to negatively charged pectins associated with the polymerization of lignin and possibly with the lignin itself, and this may contribute to the characteristic pattern of cross-linking (Laitinen *et al.*, 2017). Extensin is a structural cell wall protein which forms a cross-linked network with itself and pectins during wall development and pathogen responses. Oxidative cross-linking via isodityrosine uses extensin-specific peroxidases (Price *et al.*, 2003). Although H_2O_2-dependent cross-linking will restrain cell expansion, under some circumstances, apoplastic hydroxyl radicals could take part in directed reactions, particularly in polysaccharide scission, leading to cell wall loosening and enhanced growth (Richards *et al.*, 2015).

VIII. H_2O_2 signalling

1. Intracellular signalling

Being moderately long lived *in vivo* (half-life of milliseconds to seconds), H_2O_2 can accumulate transiently and even form gradients on a cellular scale given a localized source (Marinho *et al.*, 2014). However, it will be scavenged by the antioxidant system when its production slows down. These features make it a useful signalling molecule, and it is well established that H_2O_2 influences gene expression across all groups of organisms. ABA signalling in guard cells, root hair and pollen tube growth, programmed cell death and pathogen responses are well-studied processes involving H_2O_2 in various ways. However, speculation on how H_2O_2 sensing and signalling operate in plants far exceeds the available data. In bacteria, fungi and mammals, the emerging paradigm for H_2O_2 sensing involves TPXs, which contain low-pK_a cysteine thiols in a suitable chemical environment to react with H_2O_2 (Fig. 4). *Escherichia coli* OxyR is directly oxidized by H_2O_2, and the disulfide form is an active transcription factor, so that this protein is both a sensor and transducer. Yeast uses a cascade in which a TPX (Orp1) acts as the sensor and is initially oxidized on one cysteine, forming a sulfenic acid. This reacts with its target protein, Yap1, forming inter-protein disulfide bonds, which then resolve to produce Yap1 with an intra-protein disulfide. Yap1 disulfide enters the nucleus where it acts as a transcription factor (Boronat *et al.*, 2014). Plants have a wide range of TPXs in most subcellular compartments, and it has been suggested that these could act as H_2O_2 sensors. However, at this point, there is limited evidence for a complete TPX-based sensing and signal transduction system in plants (Mullineaux *et al.*, 2018). A potential example is GPXL3, which interacts with ABI2, a type 2C protein phosphatase involved in ABA signalling, causing its oxidation and inactivation (Miao *et al.*, 2006). GPXL3 is most likely a transmembrane protein located in the secretory system (Attacha *et al.*, 2017), and so its function in this context is contradictory regarding the cytosolic interaction of GPXL3 with ABI2 (Miao *et al.*, 2006). GPXLs could also act as sensors of lipid hydroperoxides. Detection of increased protein sulfenylation using a Yap1 pulldown system, or a chemical trap for sulfenic acid (DYn-2), shows increased sulfenylation of proteins, including TPX and transcription factors, following H_2O_2 challenge (Waszczak *et al.*, 2014; Akter *et al.*, 2015), indicating potential H_2O_2 sensors. Recently, sulfenylation of tryptophan synthetase during the response to *Pseudomonas syringae* has been detected and may have a functional role in inhibiting indole-3-acetic acid (IAA) synthesis during infection (Yuan *et al.*, 2017).

OXI1 is a kinase that influences root hair growth and pathogen resistance. It is induced by H_2O_2 and has its kinase activity activated

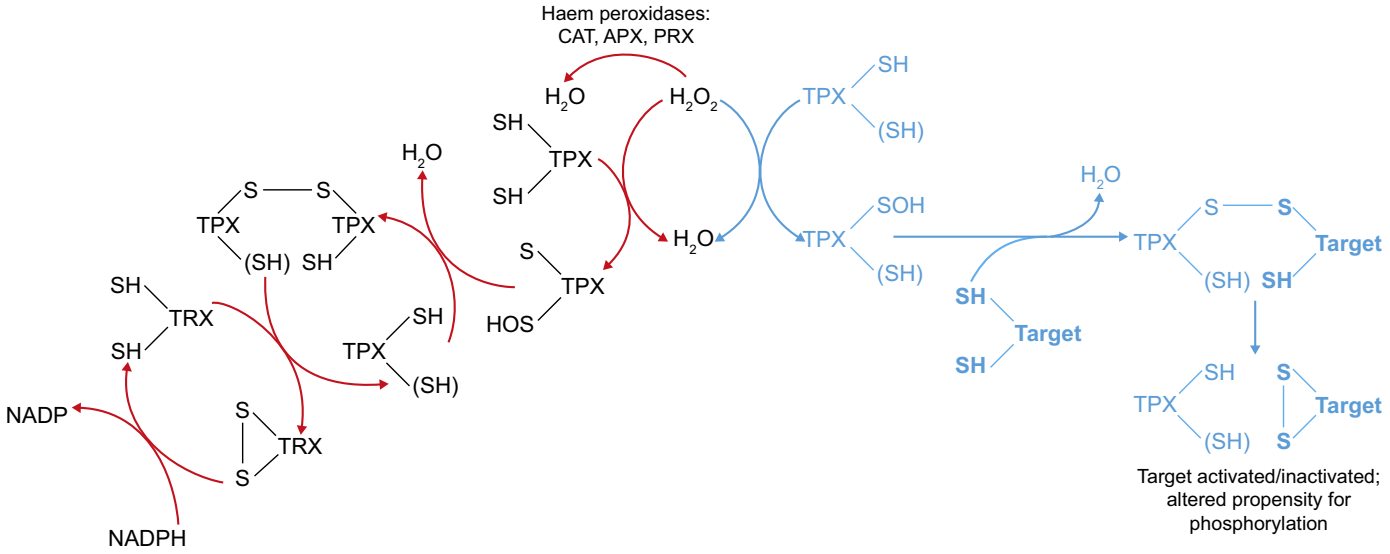

Fig. 4 Dual role of thiol peroxidases (TPXs) as hydrogen peroxide (H_2O_2) scavengers (red arrows) and sensors (blue arrows). TPXs include peroxiredoxins and glutathione peroxidase-like enzymes. The sulfenic acid form of a TPX interacts with a target protein via a disulfide bond, which then resolves to release a target protein, which could be a transcription factor, a protein kinase/phosphatase (for example, mitogen-activated protein (MAP) kinases) or other enzymes whose activity or subcellular location changes between the thiol and disulfide state. This paradigm is conserved across eukaryotes but, as in the bacterial OxyR transcription factor, target proteins could be oxidized directly. Thioredoxin (TRX, or glutaredoxin/glutathione) could reduce target protein disulfides to terminate signalling. Specificity in sensing and signalling can be achieved by the subcellular location of sensors, propensity to interact with targets and the chemical environment of cysteines within proteins which modulates their reactivity with H_2O_2. Cysteine and glutathione (GSH) are relatively poorly reactive with H_2O_2 (see Table 1). Haem peroxidases form a parallel H_2O_2 removal system and, in the case of type III peroxidase (PRX), utilize H_2O_2 to oxidize a wide range of substrates in the apoplast and vacuoles. TPX, thiol peroxidases (e.g. 2-cysteine peroxiredoxin (2-Cys Prx); 1-Cys Prx; PrxII, PrxQ and glutathione peroxidase-like; the reaction mechanism of 2-Cys Prx is shown in this example); CAT, catalase; APX, ascorbate peroxidase.

by H_2O_2. H_2O_2 activation of mitogen-activated protein (MAP) kinases (MPK3 and MPK6), previously known to be involved in H_2O_2 responses, is dependent on OXI1 (Rentel *et al.*, 2004). Ultimately, for H_2O_2 to influence gene expression, transcription factors must be modified, for example by phosphorylation, cysteine oxidation and, more speculatively, methionine oxidation (Jacques *et al.*, 2015). Redox-sensitive transcription factors and their target genes have not been extensively studied in plants, but a number of candidates have been proposed. Rap2.4a is involved in the light responsiveness of a chloroplast-located 2-Cys Prx. The disulfide form binds to 2-Cys Prx promoter (Shaikhali *et al.*, 2008). Heat shock proteins (HSPs) are induced by H_2O_2 and heat shock transcription factors (HSFs) can also be redox active and have a wide range of target genes (Miller & Mittler, 2006; Jung *et al.*, 2013; Perez-Salamo *et al.*, 2014). However, it should be borne in mind that HSP induction could be caused by a protein unfolding response to excessive H_2O_2. Apoplastic H_2O_2 sensing may be achieved by cysteine-rich RLKs (CRKs) localized on the plasma membrane (Fig. 3) (Idänheimo *et al.*, 2014; Bourdais *et al.*, 2015; Lu *et al.*, 2016; Kimura *et al.*, 2017). This would be coupled to the activation of cytosolic kinases. Currently, there is no biochemical detail, but it is assumed that H_2O_2 oxidizes specific cysteine residues and that there is a route to reduce oxidized cysteines or otherwise to recycle oxidized CRKs. Cysteines are also modified by gluthionylation and *S*-nitrosylation. These modifications interact with H_2O_2 signalling (Kovacs *et al.*, 2016).

For H_2O_2 to act as an effective signal, there must be specificity, and this could be generated by the thiol reactivity of specific proteins, the propensity of sensor and target proteins to interact (facilitating the oxidation of the target) and the subcellular location of H_2O_2 production and sensors. Isoforms of plant GPXL proteins, which are potential sensors, are present in various cellular locations, including membrane anchored (Attacha *et al.*, 2017). Modelling and the use of HyPer tethered to membranes and the cytoskeleton show that H_2O_2 concentration gradients can form (Warren *et al.*, 2015). In poppy pollen tubes, cell death induced by self-incompatibility proteins involves reactive oxygen formation in the shank of the tube, whereas H_2O_2 production at the tip, required for pollen tube growth, does not cause cell death (Wilkins *et al.*, 2011). Attempts to generate H_2O_2 in specific compartments to address spatial specificity include the redirection of glycolate oxidase expression to chloroplasts (Fahnenstich *et al.*, 2008). Although these approaches are useful to identify H_2O_2-responsive genes, the problem posed by mutants is that extrapolation to function in wild-type plants is problematic. CAT mutants exemplify this point. They show that CAT is needed to control H_2O_2 and that excess H_2O_2 causes changes in gene expression and cell death. The problem is two-fold. First, mutants in ROS scavenging or producing enzymes may have pleiotropic effects, and the plants adapt to these perturbations by remodelling their transcriptome. Second, the mutation may place H_2O_2 so far outside its physiological range that pathological effects are observed. More subtle approaches are needed to unravel H_2O_2 sensing and signalling, and are likely to involve the use of new genetically encoded probes to follow H_2O_2 in space and time (Exposito-Rodriguez *et al.*, 2017), combined with the

identification of redox modifications to candidate sensor/target proteins.

2. Which genes are influenced by H_2O_2 and is there an acclimatory response?

Meta-analyses of transcriptome data have attempted to identify groups of genes with specificity for singlet O_2, $O_2^{\cdot-}$ and H_2O_2 responses (Willems *et al.*, 2016). Sets of H_2O_2-specific genes have been proposed, but it is difficult to detect any coherence over a range of treatments and tissues. The types of genes most widely induced are diverse in function and are also associated with responses to pathogens, UV-B and UV-C radiation, ozone and other toxic chemicals, implying that these conditions increase H_2O_2 production (which has been verified in some cases) or cause other forms of damage that induce a similar set of genes, including HSPs and glutathione *S*-transferases likely involved in repair processes. More focused studies show that the expression of APX and TPX is induced by H_2O_2 and that these genes are also controlled by other conditions that cause increased H_2O_2, such as high light and extreme temperatures (Mullineaux *et al.*, 2018).

As H_2O_2 changes gene expression and directly affects the function of specific proteins by cysteine oxidation, we must suppose that this results in acclimation to potentially toxic H_2O_2 exposure and related stresses. Various studies with APX and CAT mutants, most recently in rice, suggest that, under some conditions, these are more resistant to oxidative stress, consistent with acclimation (Bonifacio *et al.*, 2016). Specifically, H_2O_2 pretreatment increased tobacco resistance to CAT inhibition and high light (Gechev *et al.*, 2002). Likewise, ascorbate-deficient *vtc* mutants have higher H_2O_2 concentration and greater basal resistance to pathogens (Mukherjee *et al.*, 2010). Results of this kind are contrary to the widespread assumption that increasing antioxidant defences is universally beneficial, and more studies under realistic environmental conditions are needed to assess the actual benefit.

3. Systemic signalling

Systemic acquired resistance (SAR) in response to pathogen infection is well known. Systemic signalling of high light resistance, termed 'systemic acquired acclimation', was identified and proposed to be dependent on H_2O_2 (Karpinski *et al.*, 1999). Since then, further evidence for systemic signalling in response to wounding, heat, cold, salt and high light has been produced (Miller *et al.*, 2009). Exposure of a target leaf to the various conditions influences gene expression in remote leaves. The involvement of $O_2^{\cdot-}$ or H_2O_2 in transmitting the signal is suggested by the attenuated response of the NOX mutant *rbohD*. Recently, it has been shown that a local application of high light stress can induce a systemic stomatal closure to the whole canopy (Devireddy *et al.*, 2018). This systemic response was dependent on an RBOHD-mediated 'ROS wave'. The possible role of cell-to-cell signalling mediated by NOX-generated H_2O_2, the activation of Ca^{2+} channels and the activation of NOX via a Ca^{2+}-dependent protein kinase has been proposed, together with the involvement of ABA (Dubiella *et al.*, 2013; Evans *et al.*, 2016; Devireddy *et al.*, 2018). The possibility of H_2O_2-mediated systemic acquired acclimation is interesting, and future work should determine how it interacts with other potential signals (e.g. jasmonic acid) and the extent to which plants grown in natural fluctuating conditions benefit from systemic signalling.

IX. Where next?

H_2O_2 plays a prominent role in plants, particularly because photosynthesis provides an extra source relative to non-photosynthetic organisms, together with a specialized APX. H_2O_2 is one of the signals for photosynthetic status and for stomatal movements. High light signalling and stomatal guard cells will continue to serve as useful model systems. Although the dominant molecular genetics approach has provided a powerful means of identification of the 'parts', to move forward we need to better understand the biochemical details of H_2O_2 and signalling, as well as its role in cell wall biochemistry. This will be aided by the sensitive detection of oxidative protein modifications and by new H_2O_2 probes able to provide the necessary chemical, temporal and spatial resolution. At the same time, more incisive physiological measurements than undertaken hitherto are needed to assess the properties of mutants and transgenic plants. A particular concern is the interpretation of the large number of studies (not reviewed here) in which the overexpression of single antioxidant genes improves 'stress resistance'. These effects are revealed under laboratory conditions, whereas plants grown under fluctuating light and temperature conditions, together with exposure to pest and pathogen attack, may have a very different physiology, making translation to crop improvement problematic.

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