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Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context

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ABSTRACT

While the chemical nature of reactive oxygen species (ROS) dictates that they are potentially harmful to cells, recent genetic evidence suggests that *in planta* purely physicochemical damage may be much more limited than previously thought. The most potentially deleterious effect of ROS under most conditions is that at high concentrations they trigger genetically programmed cell suicide events. Moreover, because plants use ROS as second messengers in signal transduction cascades in processes as diverse as mitosis, tropisms and cell death, their accumulation is crucial to plant development as well as defence. Direct ROS signal transduction will ensue only if ROS escape destruction by antioxidants or are otherwise consumed in a ROS cascade. Thus, the major low molecular weight antioxidants determine the specificity of the signal. They are also themselves signal-transducing molecules that can either signal independently or further transmit ROS signals. The moment has come to re-evaluate the concept of oxidative stress. In contrast to this pejorative or negative term, implying a state to be avoided, we propose that the syndrome would be more usefully described as 'oxidative signalling', that is, an important and critical function associated with the mechanisms by which plant cells sense the environment and make appropriate adjustments to gene expression, metabolism and physiology.

Key-words: ascorbate; glutathione; ozone; programmed cell death; reactive oxygen species; redox signalling; thiol-disulphide interactions

Abbreviations: ABA, abscisic acid; AO, ascorbate oxidase; AOBP, ascorbate oxidase gene binding protein; BSO, buthionine sulphoximine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DHA(R), dehydroascorbate (reductase); Dof, DNA-binding with one finger; γ-ECS, γ-glutamylcysteine synthetase; (PH)GPX, (phospholipid hydroperoxide) glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSH-S, glutathione synthetase; GSSG, glutathione disulphide; GST, glutathione-*S*-transferase; HR, hypersensitive response; JA, jasmonic acid; MDHA(R), monodehydroascorbate (reductase); MetSO, methionine sulphoxide; NCED, 9-*cis*-epoxycarotenoid dioxygenase; NPR1, nonexpressor of PR proteins1; PARP, poly(ADPribose)polymerase; PCD, programmed cell death; PMSR, protein methionine sulphoxide reductase; PR, pathogenesis-related; ROS, reactive oxygen species; SA, salicylic acid; SAR, systemic acquired resistance; SOD, superoxide dismutase; TRX, thioredoxin.

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INTRODUCTION

Plant metabolism must be highly regulated in order to allow effective integration of a diverse spectrum of biosynthetic pathways that are reductive in nature. This regulation does not completely avoid photodynamic or reductive activation of molecular oxygen to produce reactive oxygen species (ROS), particularly superoxide, H_2O_2 and singlet oxygen (Halliwell 1981; Fridovich 1998). However, in many cases, the production of ROS is genetically programmed, induced during the course of development and by environmental fluctuations, and has complex downstream effects on both primary and secondary metabolism. Plant cells produce ROS, particularly superoxide and H_2O_2, as second messengers in many processes associated with plant growth and development (e.g. Schroeder, Kwak & Allen 2001a; Schroeder *et al*. 2001b; Foreman *et al*. 2003). Moreover, one of the major ways in which plants transmit information concerning changes in the environment is *via* the production of bursts of superoxide at the plasma membrane (Doke *et al*. 1994). Situations which provoke enhanced ROS production have in the past been categorized under the heading of 'oxidative stress', which in itself is a negative term implying a harmful process, when in fact it is probably in many cases quite the opposite, enhanced oxidation being an essential component of the repertoire of signals that plants use to make appropriate adjustments of gene expression and cell structure in response to environmental and developmental cues. Rather than involving simple signalling cassettes, emerging concepts suggest that the relationship between metabolism and redox state is complex and subtle.

Numerous redox components can be sensed by the cell to effect acclimatory changes that are mediated at diverse levels of regulation, and it is becoming apparent that redox

modulation of protein function is a much more widespread phenomenon than previously considered. The extensive literature on ROS and antioxidants has previously been reviewed by ourselves (Noctor & Foyer 1998; Foyer & Noctor 2000, 2003) and others (Dat *et al*. 2000; Mittler 2002; Mahalingam & Federoff 2003; Conklin & Barth 2004; Mittler *et al*. 2004; Baier *et al*. 2005). Since it is not our intention to reiterate this information, we will concentrate on how plants might use and sense ROS and the two major redox buffers, ascorbate and glutathione (GSH) to regulate gene expression and plant function.

DO REACTIVE OXYGEN SPECIES CAUSE DAMAGE OR IS THIS JUST SIGNALLING IN DISGUISE?

The oxidative stress paradigm

Oxygenic photosynthesis evolved in the early proterozoic era and it has long been accepted that the subsequent atmospheric accumulation of the by-product, dioxygen, had a profound effect on living organisms (Falkowski *et al*. 2004). Superoxide dismutase (SOD) was discovered in the late 1960s and is now thought to be present in all organisms except strict anaerobes. The discovery of SOD was a crucial development leading to the widespread acknowledgement that univalent reduction of oxygen to superoxide occurred in biological systems alongside tetravalent reduction to water. ROS have since become accepted as important molecules in many aspects of biology, and are produced by numerous enzyme systems as well as low potential electron transport components. Nevertheless, the consensus view persists in seeing them as an inevitable cost associated with the benefits of oxygen as a respiratory electron acceptor. It is conventionally considered that ROS must not be allowed to react with lipids, proteins or nucleic acids; otherwise, oxidative 'damage' will be inflicted on vital cellular components. Such oxidative changes have come to be collectively characterized as symptoms of the syndrome termed 'oxidative stress'.

The oxidative stress concept in choroplasts and mitochondria

The harmful effects of photoxidative stress, defined as the generation of ROS by light-dependent processes, have been extensively studied since it has long been appreciated that reactions associated with photosynthesis and photorespiration are major sources of ROS within plant cells (Foyer & Noctor 2003). It is only relatively recently that mitochondrial ROS generation and protein oxidation have been perceived as contributing factors to the 'oxidative stress' syndrome in plants (Sweetlove *et al*. 2002; Møller & Kristensen 2004; Kristensen *et al*. 2004). The extreme sensitivity of the thiol-modulated enzymes of the Benson–Calvin cycle to oxygen and ROS has been known since the 1970s (Kaiser 1979). Much more recently, sensitivity to lipid peroxides has been described for plant mitochondrial proteins, in particular for those that contain lipoic acid. In mitochondria isolated from stressed pea plants, a loss of glycine oxidation capacity was correlated with the disappearance of the reactivity of the H protein of glycine decarboxylase to an antibody specific to lipoic acid (Taylor, Day & Millar 2002). Further work is required to establish whether inhibition of glycine decarboxylase capacity by oxidants has an impact on photorespiratory metabolism *in vivo*. Recent work on a tobacco mutant deficient in Complex I, the major NADH dehydrogenase of the mitochondrial electron transport chain, has highlighted the potential role of leaf mitochondria in integrating information on cellular redox state. Loss of this important redox component in the mutant is not associated with evidence of oxidative stress; on the contrary, the mutant shows up-regulation of components of the antioxidative system and enhanced resistance to viral infection and ozone (Dutilleul *et al*. 2003).

Although oxidation of proteins and other molecules might contribute directly to a lowering of overall plant vigour, it is becoming increasingly evident that oxidation of target or signal molecules is an intrinsic part of how plants perceive and respond to environmental and developmental triggers. Oxylipin production, recognition of damaged DNA by the enzyme poly(ADPribose)polymerase (PARP) and the oxidation of specific proteins such as glyceraldehyde-3-phosphate dehydrogenase are all linked to cell signalling events, initiating structural changes and isoform replacement. Interestingly, recent results have shown that down-regulation of PARP provides substantial protection against a range of abiotic stresses (De Block *et al*. 2004).

Protein oxidation and its regulation

Among the most important components in any response to increased cellular oxidation will be proteins, whether these be enzymes, structural proteins, signal transduction components, ion channels, transporters, transcription factors, or other type. Amino acid residues vary in their susceptibility to oxidative modification, the most vulnerable including Cys, Tyr, Trp and His (Dröge 2002). The maintenance of sulphur-containing amino acids is particularly important. Methionine is readily oxidized to the sulphoxide (MetSO) and oxidation of this amino acid on proteins has been considered to be a 'last chance' antioxidant defence (Levine *et al*. 1999). The *S*-stereoisomer of MetSO is reduced back to Met by type A protein MetSO reductase (PMSR), enzymes that use thioredoxins (TRX) as reductant (Romero *et al*. 2004). Type B PMSRs are proteins that reduce the *R*-stereoisomer of MetSO, and one has recently been found to be a target for CDSP32, a chloroplast drought-induced protein that includes TRX domains (Rey *et al*. 2005). There are several copies of both types of PMSR in the *Arabidopsis thaliana* genome (Sadanandom *et al*. 2000; Rey *et al*. 2005).

Various mechanisms may act to regenerate protein Cys, including protein disulphide reductases such as TRXs and glutaredoxins (Schürmann & Jacquot 2000; Lemaire 2004). More oxidized Cys sulphur states occur in the catalytic

cycle of important antioxidative enzymes such as peroxiredoxins (Horling *et al*. 2003; Baier *et al*. 2005), which are regenerated by specific TRXs (Collin *et al*. 2004), glutaredoxins (Rouhier *et al*. 2001), or proteins such as CDSP32 (Broin *et al*. 2002). Such Cys sulphenic acids (SOH) can be hyperoxidized to Cys sulphinic acid (SO_2H), causing inactivation of the enzyme. A new class of enzymes discovered in yeast, sulphiredoxins, is capable of reducing sulphinic acid back to sulphenic acid in an ATP-dependent manner (Biteau, Labarre & Toledano 2003). In the case of the mammalian sulphiredoxin, the regenerating reductant may be TRX or glutathione (Chang *et al*. 2004). The *Arabidopsis* genome contains one putative sulphiredoxin sequence, likely encoding a protein targeted to the chloroplast (E. Issakidis-Bourguet, pers. comm.).

Glutathionylation, either by reaction of GSH with protein thiyl radicals or via exchange between protein thiols and glutathione disulphide (GSSG), may be important both in protecting protein Cys groups from further oxidation and in oxidative signalling, but as yet little is known about this for plants (Foyer, Trebst & Noctor 2005). Two enzymes of the Calvin cycle have been shown to undergo glutathionylation in cultured *Arabidopsis* cells (Ito, Iwabuchi & Ogawa 2003), and studies in yeast and mammals strongly suggest that other glutathionylated proteins await identification in plants. In humans, the removal of glutathione from glutathionylated proteins is performed by glutaredoxins linked to either glutathione or the TRX system (Johansson, Lillig & Holmgren 2004).

Programmed production of ROS, an essential process in plant development and defence

The concept of oxidative stress has come a long way since Halliwell (1981) posed the question of why oxygen is toxic, and drew the conclusion, later reiterated by Fridovich (1998), that the culprits were oxidative reactions involving ROS. Over the last 20 years, a large body of evidence has demonstrated unequivocally that H_2O_2 is a key signalling molecule in plants, as it is in other eukaryotes (Dröge 2002; Neill, Desikan & Hancock 2002; Ermak & Davies 2002), and that dedicated ROS-producing NADPH oxidases and peroxidases are activated to control processes as diverse as gene expression, stomatal closure, root growth and programmed cell death (PCD). The ability of the plant to launch a genetically programmed cell suicide programme is a central feature of development and defence. For example, the hypersensitive response (HR) to pathogen attack involves rapid PCD of single cells or groups of cells accompanied by the elaboration of systemic acquired resistance (SAR), and ROS are an essential component in this signal transduction cascade. Exposure to ozone (Conklin & Last 1995; Sandermann 2000) or singlet oxygen (Leisinger *et al*. 2001) induces ROS signalling, HR and SAR (Kangasjärvi *et al*. 2005). However, in contrast to ozone, which enters the leaf through open stomata and is perceived firstly by the plasma membrane, singlet oxygen is mainly generated in the lipid interior of the thylakoid membrane.

REACTIVE OXYGEN SPECIES, MODULATION OF THE TRANSCRIPTOME, AND CELL DEATH

Death by singlet oxygen is genetically programmed

Investigations of the effects of singlet oxygen on gene expression have used different strategies to induce production of this ROS. It is important to consider the site of singlet oxygen formation in these studies. In *Chlamydomonas reinhardtii* singlet oxygen production was induced by either class I photosensitizers such as neutral red or class II photosensitizers such as rose bengal (Leisinger *et al*. 2001). Neutral red acts by inhibiting photosynthetic electron transport, whereas photodynamic singlet oxygen production by rose bengal is independent of photosynthesis. Singlet oxygen is generated at photosystem II (PSII) during photosynthesis (Foyer *et al*. 2005), but can also be generated *in planta* by photosensitization of intermediates in the biosynthesis of cytochromes and chlorophylls, such as protoporphyrin IX. In these conditions, excitation of the porphyrin molecules by light causes bleaching through exacerbated rates of ROS generation. Precursors of chlorophyll synthesis, notably protochlorophyllide, are accumulated in the *Arabidopsis flu* mutant, which has a defect in the regulation of porphyrin synthesis that prevents shutdown of the pathway in the dark (Meskauskiene *et al*. 2001). Although the *flu* mutant can survive in continuous light because protochlorophyllide is photoreduced in these conditions, accumulation of this intermediate in the dark leads to photosensitized production of singlet oxygen on subsequent illumination. Under intermittent light conditions, mature *flu* mutants stop growing and seedlings bleach and die (Op den Camp *et al*. 2003). In the *flu* mutant, singlet oxygen is thought to be produced peripherally on the stromal face of the thylakoid membrane.

Elegant analysis of the *flu* mutant by Apel and colleagues has highlighted the potential role of singlet oxygen as a signal (Apel & Hirt 2004). Their work has shown that the bleaching response does not result from direct oxidative damage but rather from the activation of distinct cell suicide programmes. Molecular genetic screens have shown that inactivation of a single gene, *EXECUTER1* (*EX1*), is sufficient to prevent singlet oxygen-induced *flu* seedling death and growth inhibition in mature plants (Wagner *et al*. 2004). Inactivation of the *EX1* protein not only suppresses the induction of death in *flu* seedlings but it also prevents death in wild type plants treated with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). However, the mechanism might not necessarily be the same as that operating in the *flu* mutant, because the relationship between DCMU and singlet oxygen production is unclear (Foyer *et al*. 2005). However, herbicides whose primary site of action is the chloroplast cause a form of plant cell death that is typified by apoptosis-like features (Chen & Dickman 2004). This apoptosis-like cell death requires light, involves ROS and is inhibited in transgenic tobacco plants expressing animal *Bcl-2* family antiapoptotic genes that produce proteins localized within

the chloroplasts as well as mitochondria (Chen & Dickman 2004).

Although cell death is repressed in the double *flu ex1* mutant, the majority of the singlet-oxygen-induced changes in gene expression observed in the single *flu* mutant are not. Thus, singlet oxygen production activates at least two distinct signal transduction pathways, one that requires *EX1* and one that is independent of *EX1* (Lee *et al*. 2003; Op den Camp *et al*. 2003; Apel & Hirt 2004; Wagner *et al*. 2004). Interestingly, a specific intermediate of the chlorophyll biosynthetic pathway, Mg-protoporphyrin XI, has itself been proposed as a plastid-derived signal (Strand *et al*. 2003). Evidence for such a role came from analysis of a group of nuclear recessive mutants called *gun* (*genomes uncoupled*). The *gun* mutants de-repress transcription of nuclear genes involved in photosynthesis (Rodermel 2001). Like *flu* (Op den Camp *et al*. 2003) the large changes in gene expression (affecting about 4% of the total *Arabidopsis* genome) brought about by release of the plastid signalling factor include many known stress-responsive genes (Strand *et al*. 2003). Mg-protoporphyrin IX and singlet oxygen may therefore use similar signalling pathways.

Do different ROS produce different signals?

Like ozone, singlet oxygen rapidly produces other ROS on contact with water. Thus, it remains to be conclusively demonstrated whether or not singlet oxygen is a unique signal. It is unclear if its effects on gene expression act, at least in part, through production of other ROS. In the case of the *flu* mutant singlet oxygen is probably the source of the signal because quenching prevents initiation of the cell suicide programme, but this question remains to be definitively resolved. Approximately 5% of the total *Arabidopsis* mRNAs are modified in the *flu* mutant within the first 30 min after release of singlet oxygen (Op den Camp *et al*. 2003). However, the overall pattern of differential gene expression observed upon singlet oxygen generation bears striking similarity to that obtained by applying high light or paraquat (Kimura *et al*. 2001; Vranova *et al*. 2002). The latter treatment increases production of superoxide and H_2O_2. In contrast to singlet oxygen, which is a very short-lived molecule ($t_{1/2}$ about 200 ns; Gorman & Rodgers 1992), H_2O_2 is relatively stable. It is thus easier to imagine a role for this molecule in simple signal transduction circuits (e.g. direct interaction of oxidants with a *trans*-acting factor) compared to superoxide or singlet oxygen. However, less direct pathways coexist, in which even short-lived ROS can be sensed through their interaction with other components, such as specific proteins or antioxidants, and we discuss some of these below.

More than 20 years ago, it was reported that barley mutants deficient in catalase show a phenotypic response (severe leaf bleaching) reminiscent of that observed more recently in the *flu* mutant, and that this occurs specifically in conditions favouring H_2O_2 production through photorespiration (Kendall *et al*. 1983). It was subsequently shown that catalase deficiency in tobacco is associated with induction of pathogenesis-related (PR) proteins (Chamnongpol *et al*. 1996). Such plants have recently been exploited in transcriptomic studies of H_2O_2 signalling (Vandenabeele *et al*. 2002), complementing other studies in which the influence of this ROS on the transcriptome was explored by exogenous application to cultured cells (Desikan *et al*. 2001). Sustained increases in H_2O_2 provoked a transcriptional response that mimicked both biotic and abiotic stresses, including expression of HR and PCD genes as well as genes involved in ethylene and jasmonic acid signalling (Vandenabeele *et al*. 2002).

It is very difficult to compare array data from different laboratories effectively as growth and oxidant applicant conditions vary greatly. In particular, it is virtually impossible to determine the amount of active oxidant that reaches key sites within the cell and moreover the extent to which each oxidant perturbs the redox balance of the cell is never measured. Although it is not known how an extremely short-lived molecule like singlet oxygen can give rise to a signal that is transmitted to the nucleus to regulate gene expression, it is clear that any singlet oxygen sensor would have to be located in close proximity to the source of generation in the thylakoid membrane. However, reaction products arising from chlorophyll or D1 protein degradation could relay the signal. Lipid peroxides produced as a result of singlet oxygen production act as signals in mammals (Polte & Tyrrell 2004). In plants, the activation of lipoxygenases, which produce fatty acid hydroperoxides from polyunsaturated fatty acids, leads to the formation of the bioactive compounds called oxylipins which have diverse roles in signalling in biotic and abiotic stresses (Porta & Rocha-Sosa 2002).

INTERACTIONS BETWEEN REACTIVE OXYGEN SPECIES AND GLUTATHIONE

Glutathione is an abundant metabolite in plants that has many diverse and important functions (Noctor & Foyer 1998), including signal transduction (Noctor *et al*. 2002a; Gomez *et al*. 2004a). In many reactions involving GSH, the Cys thiol group is oxidized to yield GSSG, and the reverse reaction is catalysed by glutathione reductase (GR) using NADPH. The highly reduced glutathione pool maintained by GR is necessary for active protein function and avoids unspecific formation of mixed disulphide bonds that cause protein inactivation or aggregation. Stable protein disulphide bonds are relatively rare except in quiescent tissues such as seeds, where GSSG is allowed to accumulate. In metabolically active tissues, millimolar concentrations of GSH act as a key redox buffer, forming a barrier between protein Cys groups and ROS. Moreover, as discussed below, GSH is a substrate for several reductive enzymes, including enzymes that reduce peroxides.

Stress and glutathione metabolism

Apart from the well documented roles of antioxidative enzymes and antioxidants in removing H_2O_2 and other

ROS, there is also a strong interaction between oxidants and antioxidants at the level of gene expression and translation. Exposure to ROS can result in either enhanced antioxidant capacity (if ROS are genenerated as a result of metabolic or environmental perturbations) or decreased antioxidant capacity (if ROS are generated around cells undergoing death responses). Activation of GSH synthesis and accumulation of glutathione are a general feature of enhanced oxidation of the cytosol. Exposure to ozone causes marked decreases in GSH:GSSG, followed by accumulation of glutathione (Sen Gupta, Alscher & McCune 1991). A similar response precedes leaf bleaching in maize subjected to chilling (Gomez *et al*. 2004b), and the correlation between perturbation of glutathione status and leaf death is particularly clear in catalase-deficient plants (Smith *et al*. 1984; Willekens *et al*. 1997; Noctor *et al*. 2002a). Recent evidence suggests that the enzymes of GSH synthesis and metabolism are induced together in response to stress (Mittova *et al*. 2003). This implies that there is considerable overlap in the signal transduction cascades that induce GSH synthesis and those involved in defence functions that use GSH, such as glutathione-*S*-transferases (GST) and glutathione peroxidase (GPX), some of which show a particularly strong response to ROS (Levine *et al*. 1994). The H_2O_2-induced expression of *GST1* involves glutathione and a biphasic elevation of cell calcium (Rentel & Knight 2004). A ROS-responsive gene (*Gpxh*) with GPX homology in *C. reinhardtii* (Leisinger *et al*. 2001) is more strongly induced by singlet oxygen than by superoxide and H_2O_2. The possible roles of plant GSTs in cell signalling are discussed below. In animals, this function is well established as GSTs regulate kinase activity during oxidative stress (Yin *et al*. 2000).

Glutathione-*S*-transferases in detoxification, transport and signalling

In contrast to GR, which catalyses a reaction with very restricted substrate specificity, GSTs constitute a complex family of proteins, grouped into six classes, with a large range of functions, many of which no doubt remain to be discovered (Frova 2003). The primary biochemical function of many GSTs is conjugation, either of xenobiotics or of intermediates and secondary metabolites (Frova 2003). In addition, certain GSTs play roles as peroxidases or in regenerating ascorbate from dehydroascorbate (DHA; Dixon, Davis & Edwards 2002; Cummins, Cole & Edwards 2003) and some of the zeta class of GSTs are maleoylacetoacetate isomerases that function in the catabolism of tyrosine (Thom *et al*. 2001).

Glutathione conjugates of xenobiotics are formed as stable end-products by GSTs. However, this is not the case for GSTs that are primarily involved in endogenous metabolism. These GSTs appear to act as binding proteins for bioactive ligands. Some act as flavonoid binding and transport proteins in *Arabidopsis,* for example, in the anthocyanin synthesis pathway. Certain GSTs bind linear tetrapyrroles or porphyrins (Lederer & Boger 2003). One important consequence of this type of GST binding is a decrease in the rate of spontaneous oxidation of protoporphyrinogens to protoporphyrins, whose toxic action has been discussed above (Lederer & Boger 2003). The early stages of porphyrin synthesis occur in the plastid but protoporphyrinogen IX is exported from the chloroplast to other organelles, particularly the mitochondrion, for futher metabolism to haem and for incorporation into cytochromes (Vavilin & Vermaas 2002). It is probable therefore that GSTs fulfil essential roles in intracellular transport during the synthesis and catabolism of tetrapyrrole pigments. There is therefore considerable potential for roles of GSTs in cell signalling, particularly as Mg-protoporphyrin IX is strongly linked to chloroplast-nucleus signalling (Strand *et al*. 2003). The translocation mechanism for the protoporphyrin IX signal is not known (Strand *et al*. 2003) but specific GST ligandins are potential selective mechanisms for the transport of signalling proteins.

Glutathione peroxidases in the plant response to stress

Some GPX genes are strongly induced by ROS (Levine *et al*. 1994; Willekens *et al*. 1997; Leisinger *et al*. 2001). Most identified plant GPX genes were shown to have high homology to the mammalian phospholipid hydroperoxide glutathione peroxidases (PHGPX), which have a higher affinity to lipid hydroperoxides than to H_2O_2. However, at least two plant PHGPXs probably represent novel isoforms of TRX peroxidase, which are generally more active against H_2O_2 than lipid peroxides (Herbette *et al*. 2002). Animal GPXs can have high activity against peroxides, due to the presence of a highly nucleophilic selenocysteine at the active site. In contrast, plant GPXs probably make a very small contribution to overall peroxide metabolism, compared to catalases, ascorbate peroxidases and peroxiredoxins. Despite this, overexpression of PHGPX in transgenic plants has been reported to enhance stress tolerance (Ursini *et al*. 1995) and transgenic tobacco plants expressing a PHGPX-like protein in either the cytosol (TcGPX) or chloroplasts (TpGPX) showed suppressed stress-induced production of malondialdehyde, compared to wild-type plants (Yoshimura *et al*. 2004). Some of these effects may be linked to control of lipid signalling strength through organic peroxides.

GLUTATHIONE BIOSYNTHESIS AND SIGNALLING

Key insights from studies of mutations in glutathione synthesis

The pathway of glutathione synthesis is conserved in all organisms and involves two enzymes, γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GSH-S). In *Arabidopsis* γ-ECS is encoded by a single gene, *GSH1,* with a plastid target signal (May & Leaver 1994). A number of GSH-deficient mutants have been described at this locus

(*rootmeristemless1* (*rml1*), *cadmium sensitive2* (*cad2*), *regulator of apx2 1-1*(*rax1-1*)). Analysis of these mutants has highlighted the complexity of glutathione function. The *cad2-1* mutant has a 6-bp deletion leading to loss of two amino acids and substition of another in a region within the active site of the γ-ECS protein (Cobbett *et al*. 1998). This leads to a decrease of about 70% in GSH contents, rendering *cad2-1* cadmium-sensitive but otherwise morphologically indistinguishable from WT plants (Howden *et al*. 1995). The *rml1* mutant has a point mutation causing Asn to replace Asp in a position that is central to the active site, and as a result has only about 2% of the leaf GSH of the wild-type (Vernoux *et al*. 2000). The *rml1* mutant shows extreme sensitivity to cadmium and cannot form a normal root system even under optimal conditions (Vernoux *et al*. 2000). Complementation studies between *rml1* and *cad2-1* and later cloning of the sequences confirmed that the two genes are allelic (Vernoux *et al*. 2000). Another γ-ECS mutant, *rax1-1*, was identified by its constitutive expression of luciferase under the control of the promoter for *APX2*, a gene encoding a cytosolic ascorbate peroxidase isoform that is normally only expressed in stresses such as high light. The *rax1-1* mutant is also allelic to *gsh1*, but has a substitution of Arg to Lys in a region of the primary sequence close to the *cad2-1* deletion though external to the likely active site (Ball *et al*. 2004). Consequently, *rax1-1 has* a systemic decrease in glutathione of about 60%; that is, similar to the decrease in *cad2-1* (Ball *et al*. 2004). This elegant study implicates glutathione as a key player in stress-related signalling. Interestingly, however, while both *cad2-1* and *rax 1-1* lack γ-ECS activity and have similar glutathione contents, they have different leaf transcriptome profiles (Ball *et al*. 2004). Furthermore, the severe decrease in glutathione in *rml1* is also observed in *Arabidopsis* carrying an antisense *gsh1* construct, though root growth is much less affected in this case (Xiang *et al*. 2001). Questions remain concerning the mechanisms that link glutathione content and synthesis to gene expression and the regulation of development.

Regulation and compartmentation of glutathione synthesis

There has been much speculation concerning the presence of a regulatory γ-ECS subunit in plants (May *et al*. 1998). In mammals, a smaller regulatory subunit acts to increase the catalytic potential of the larger catalytic subunit by increasing the K_i for GSH and decreasing the K_m for glutamate, thereby alleviating feedback control and allowing the enzyme to operate effectively under *in vivo* conditions (Huang *et al*. 1993). Although the plant enzyme is inhibited by GSH, no conclusive evidence has been produced that a regulatory subunit exists. Another level of control in mammals may occur through phosphorylation (Sun, Huang & Lee 1996) but this has not yet been found in studies on γ-ECS from plants. Novel regulatory mechanisms may well await discovery. In wheat, γ-ECS activity extractable from purified chloroplasts is higher than that which can be extracted from whole leaves, and specific activity increases markedly with protein concentration (Noctor *et al*. 2002b). Competitive inhibition of the rat enzyme by GSH is favoured by treatment with dithiothreitol (Huang *et al*. 1993), and γ-ECS from tobacco and *Arabidopsis* is also inhibited by dithiols (Hell & Bergmann 1990; Jez, Cahoon & Chen 2004). Thus, transient oxidation of an inhibited enzyme could be one way in which glutathione synthesis is kick-started in oxidative conditions. It remains to be established whether thiol–disulphide exchange is an important mechanism in regulating the *in vivo* activity of the enzyme.

A key question in glutathione function is compartmentation. Biochemical studies of subcellular localization have found γ-ECS and GSH-S activities both inside and outside the chloroplast (reviewed by Noctor *et al*. 2002b) yet only one gene encoding each enzyme has been described in *Arabidopsis*. Sequence information suggests that while the *A. thaliana* γ-ECS protein is located largely in the chloroplasts, GSH-S has a predominantly cytosolic location. Very recently, through a detailed study using transient expression of reporter gene fusion constructs as well as immunocytochemistry, it has been demonstrated that the *Arabidopsis GSH1* gene codes for two populations of transcripts, both of which encode a plastidic γ-ECS (Wachter *et al*. 2005). GSH-S was also encoded by transcripts differing in length, but in this case the protein product was directed to both plastids and cytosol, with the cytosolic enzyme being translated from shorter transcripts that lacked the plastid target sequence (Wachter *et al*. 2005). Such a localization implies γ-EC export from the chloroplast in *Arabidopsis* leaves, and it is possible that this could occur partly in exchange for cytosolic glutathione. Indeed, wheat chloroplasts are able to import glutathione and this activity is not affected by either light or ATP, suggesting that it may in part reflect the activity of an exchange transporter, for example, with γ-EC (Noctor *et al*. 2002b). In this case, the high concentrations of GSH found in the chloroplast could be to some extent dependent on import from the cytosol, although the contributions of the two compartments to glutathione formation may well vary during development, between different cells, or as a function of physiological status. Transporters with high specificity to glutathione have recently been cloned in *Arabidopsis* and transcripts for one of them have been shown to be induced by xenobiotic exposure, though not by H_2O_2 or cadmium (Cagnac *et al*. 2004). Important information concerning the regulation of glutathione concentration and compartmentation in response to stress is likely to come from studies of the physiological role of these proteins and of others able to transport GSH, GSSG or GS-conjugates (Foyer, Theodoulou & Delrot 2001).

Work with transgenic plants has shown that the abundance of γ-ECS is a major factor controlling GSH accumulation (Noctor *et al*. 1996), in agreement with the studies of the mutants discussed above. Another key player is Cys availability (Noctor *et al*. 1997; Harms *et al*. 2000; Droux

2004). Although the concentration of Cys is a limiting factor for γ-ECS activity, it is unclear how quantitatively important glutathione synthesis is in the context of the sulphur budget of the plant. Even when its synthesis is induced, glutathione probably contains no more than 5% of the total Cys of the cell. Nevertheless, it has been shown that ozone exposure leads to up-regulation of sulphur assimilation in response to decreases in GSH:GSSG, via activation of adenosine 5′-phosphosulphate reductase (Bick *et al*. 2001). Furthermore, total tissue glutathione is typically five to 20 times more abundant than free Cys and is highly mobile throughout the plant (Herschbach & Rennenberg 1995). Together with metabolites such as *O*-acetylserine (Hirai *et al*. 2003), GSH may act as an indicator of sulphur status. A simple regulatory mechanism has been proposed with regard to the regulation of demand-driven sulphur assimilation by positive signals such as *O*-acetylserine and negative signals such as GSH (Kopriva & Rennenberg 2004).

How do oxidants such as ozone lead to increases in glutathione?

There is little evidence as yet that oxidative up-regulation of glutathione synthesis occurs at the transcriptional level, although certain metals that favour ROS production can induce transcripts for the enzymes of glutathione synthesis, as can cold treatments. The abundance of *GSH1* and *GSH2* transcripts was increased by cadmium in *Brassica juncea* (Schäfer, Haag-Kerwer & Rausch 1998), by both cadmium and copper in *Arabidopsis* (Xiang & Oliver 1998), and *GSH1* transcripts accumulated in response to chilling in maize (Gomez *et al*. 2004b). Neither exogenous H_2O_2, diamide nor methyl viologen induced *GSH1* or *GSH2* (Xiang & Oliver 1998). Moreover, when *Arabidopsis* cell cultures were exposed to oxidative stress (by the addition of aminotriazole, menadione or fenchlorazole) cellular γ-ECS activity and glutathione content increased but γ-ECS mRNA levels were unchanged. Ozone and catalase deficiency are both known to trigger production of jasmonic acid (JA), which has been shown to increase *GSH1* and *GSH2* transcripts (Xiang & Oliver 1998; Harada, Kusano & Sano 2000) and a common signal transduction pathway may be involved. Glutathione synthesis may also be up-regulated by oxidation-induced increases in translation (Xiang & Oliver 1998). The 5′-untranslated region of the *gsh1* gene was found to interact with a repressor-binding protein that was released upon addition of H_2O_2 or changes in the GSH/GSSG ratio (Xiang & Bertrand 2000). Increases in Cys availability may also contribute. A further possibility is that ROS-induced oxidation of regulatory cysteines on γ-ECS and/or decreases in GSH temporarily alleviate inhibition, allowing a burst of γ-ECS activity to boost glutathione concentrations (as discussed above). This mechanism was proposed to explain transient decreases in GSH followed by accumulation of both forms of glutathione in response to ozone (Sen Gupta *et al*. 1991) and may also explain, at least to some extent, analogous changes during incompatible plant–pathogen interactions (Vanacker, Carver & Foyer 2000; Noctor *et al*. 2002b).

THE GSH/GSSG COUPLE AND REDOX SENSING

As an indicator of the general cellular thiol–disulphide redox balance, the GSH/GSSG couple is well suited to the role of redox sensor. Application of exogenous glutathione to leaves induces transcription of genes encoding cytosolic CuZnSOD, GR, 2-cys peroxiredoxins and PR proteins (Hérouart, Van Montagu & Inzé 1993; Wingsle & Karpinski 1996; Baier & Dietz 1997; Gomez *et al*. 2004a). Supplying external glutathione, either as GSH or GSSG, can induce calcium release into the cytosol (Gomez *et al*. 2004a). Furthermore, transcripts for a calcium/proton antiporter were among those modified in γ-ECS mutants described above (Ball *et al*. 2004), while oxidant-induced changes in calcium signatures are modified in such mutants and also by treatment with buthionine sulphoximine (BSO), a specific γ-ECS inhibitor (Rentel & Knight 2004).

Thiol–disulphide exchange in the pathogen response

The cytosolic thiol–disulphide status appears to be important in regulating the expression of PR proteins (Fig. 1). These are induced in response to stress and by salicylic acid (SA), their expression being tightly correlated with the onset of localized and systemic resistance. The mechanisms through which the SA signal pathway functions are not completely understood but the ankyrin repeat protein NPR1 (non-expressor of PR protein 1) is one of the key regulators of SA-dependent gene expression (Cao *et al*. 1994; Delaney, Friedrich & Ryals 1995). It has recently been demonstrated that NPR1 is converted from an inactive oligomer to an active monomer as a result of cellular redox changes induced by SA during SAR (Mou, Fan & Dong 2003). Monomers move into the nucleus where they activate expression of defence genes such as *PR1* via redox interaction with TGA transcription factors (Després *et al*. 2003). Both monomerization of NPR1 oligomers and reduction of monomeric NPR1 may involve glutathione and specific forms of proteins such as TRX (Vanacker *et al*. 2000; Mou *et al*. 2003; Laloi *et al*. 2004). The redox dependence of the pathway suggests that any biotic or abiotic stimulus that can perturb the cellular redox state could up-regulate the same set of defence genes via the NPR1 pathway (Mou *et al*. 2003). Redox-linked effects explain, for example, PR gene expression in response to UV-B exposure (Green & Fluhr 1995), in catalase-deficient mutants (Chamnongpol *et al*. 1996) or in chloroplastic γ-ECS overexpressors (Creissen *et al*. 1999). In the last two cases, extensive oxidation of the glutathione pool was shown to be concomitant with greatly enhanced glutathione accumulation. Further work is required to clarify the exact role of changes in glutathione in signalling the induction of PR proteins.

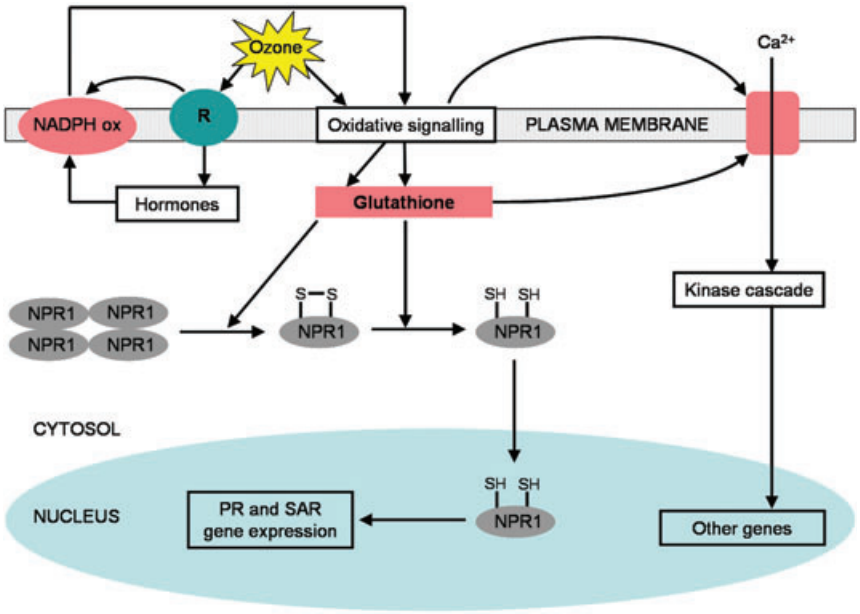

Figure 1. Model schematizing some of the relationships between components in modulation of gene expression by ozone, which shares features with other responses involving oxidative signalling. The central roles of ROS-triggered changes in GSH/GSSG and thiol–disulphide exchange around NPR1 are emphasized (Mou *et al*. 2003). Ozone can induce oxidative signalling by direct production of ROS on contact with water or by redox-sensitive receptor-mediated activation of NADPH oxidase. The strength of NADPH oxidase activation probably depends on interplay between hormone or signal molecules such as salicylic acid, jasmonic acid and ethylene. Key features of oxidative signalling appear to be (1) monomerization of NPR1 (2) changes in NPR1 thiol–disulphide status (3) ROS-induced increases in cytosolic calcium due to influx from the apoplast or intracellular stores, which cause activation of kinase cascades. Accumulating evidence suggests that changes in glutathione status may mediate or influence all three of these processes (Gomez *et al*. 2004a; Rentel & Knight 2004). Definition of the cause–effect relationships between the various components involved in oxidative signalling requires further work. Elucidation of these relationships may be complicated by the existence of reinforcement feedback loops and amplification mechanisms (Shirasu *et al*. 1997). PR, pathogenesis-related; R, receptor; SAR, systemic acquired resistance.

INTERACTIONS BETWEEN ASCORBATE AND REACTIVE OXYGEN SPECIES

Ascorbate content modulates stress perception and signalling

In addition to the role of glutathione in the the induction of PR proteins and SAR, the abundance of leaf ascorbate can also modulate PR gene expression and SAR (Pastori *et al*. 2003; Kiddle *et al*. 2003; Barth *et al*. 2004; Fig. 2). The *Arabidopsis* mutants, *vtc1* and *vtc2*, which have constitutively low ascorbate, show constitutive expression of PR transcripts. It has been suggested that the induction of PR proteins and enhanced pathogen resistance in *vtc1* are related to SA accumulation linked to earlier senescence in the mutants than the wild type (Barth *et al*. 2004). In fact, the *vtc* mutants grow more slowly and flower later than the wild type (Veljovic-Jovanovic *et al*. 2001). The smaller leaf size observed in the *vtc1* mutant is caused by a much reduced cell size, presumably because limiting ascorbate prevents full cell expansion (CHF, unpublished results). Moreover, the *vtc1* mutant shows micro-lesions made up of individual dead cells. It may therefore be that the induction of PR proteins and enhanced pathogen resistance observed in *vtc1* is due to enhanced local PCD and SAR rather than an earlier developmental senescence programme. The *vtc1* mutant is very sensitive to atmospheric ozone and shows increased sensitivity to other abiotic stresses such as freezing and UV-B irradiation (Conklin, Williams & Last 1996). These results suggest that low ascorbate decreases the threshold for sensing stress and triggers PCD and SAR responses even under optimal growth conditions (Fig. 2; see also Kangasjärvi *et al*. 2005). Consistent with this view, vtc1 has a small but significant increase in glutathione.

Specific effects of ascorbate on the expression of 2-Cys peroxiredoxin and other genes have been described (Baier *et al*. 2000; Horling *et al*. 2003). Low ascorbate leads to the activation of a suite of genes that might be considered to provide a molecular signature of ascorbate deficiency in plants (Pastori *et al*. 2003) while high ascorbate not only leads to repression of these transcripts but can result also in changes in other transcripts (Kiddle *et al*. 2003; Pastori *et al*. 2003). The patterns of gene expression observed at low and high ascorbate are distinct from those observed on adding H_2O_2. Hence ascorbate-mediated gene transcription cannot be ascribed simply to a reversal of ROS-mediated effects. However, through its role as an antioxidant, ascorbate can impede processes regulated through ROS-mediated signalling such as stomatal closure, which we now discuss.

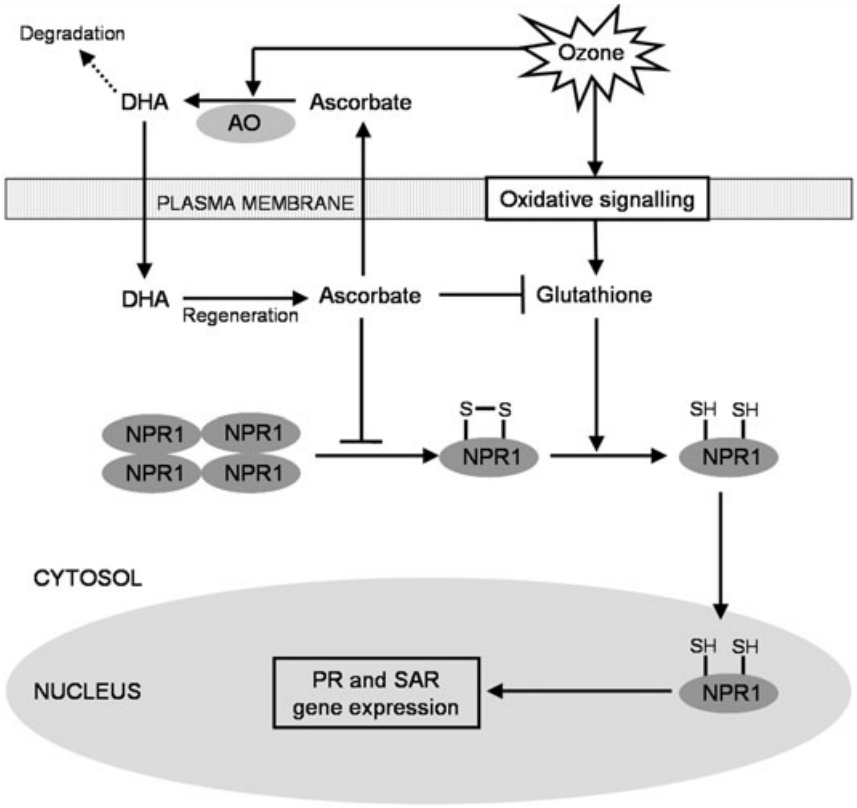

Figure 2. Role of ascorbate in modulation of oxidative signalling. Ascorbate is a key player in resistance to ozone, as shown by the identification of ascorbate-deficient *Arabidopsis* mutants by virtue of their ozone sensitivity (Conklin *et al*. 1996). Such mutants show small but significant increases in glutathione (Veljovic-Jovanovic *et al*. 2001) and constitutive expression of defence proteins, including PR1 (Pastori *et al*. 2003). The apoplastic ascorbate redox state depends on the balance between ascorbate oxidation to DHA (via MDHA, catalysed by AO) and cytosolic regeneration by reduction of DHA (notably by glutathione). Although not explicitly shown, membrane-bound MDHA reductases may also play a role (Berczi & Møller 1998). Ozone tips the balance towards accumulation of DHA, whose chemical instability leads to net ascorbate depletion. It is proposed that the resulting low ascorbate concentrations (constitutively present in ascorbate-deficient plants) favour both monomerization of NPR1 and reduction of the monomerized protein, the latter effect being linked to compensatory increases in glutathione. Thus, ascorbate status modulates the intensity and outcome of oxidative signalling, which is otherwise assumed to occur through the pathways shown in Fig. 1 (simplified here for clarity). AO, ascorbate oxidase; DHA, dehydroascorbate; MDHA, monodehydroascorbate. Other abbreviations as in Fig. 1.

Ascorbate interacts with H_2O_2 and abscisic acid in the regulation of stomatal function

In guard cells, H_2O_2 participates in the activation of plasmamembrane-localized anion channels that lead to stomatal closure (Schroeder *et al*. 2001a,b). Although the significance of H_2O_2 in stomatal regulation has been questioned (Köhler, Hills & Blatt 2003), two genes encoding subunits of an NADPH oxidase (*AtrbohD* and *AtrbohF*) appear to be involved in stomatal closure, as well as in other abscisic acid (ABA) responses such as seed dormancy (Kwak *et al*. 2003). Double mutants in which both genes are inactivated show impaired closure of stomata, an effect which can be rescued by exogenous H_2O_2 (Kwak *et al*. 2003). H_2O_2-induced stomatal closure was reversed by the application of exogenous ascorbate, presumably due to peroxidase-dependent H_2O_2-scavenging (Zhang *et al*. 2001). Moreover, high constitutive expression of dehydroascorbate reductase (DHAR) in transgenic plants increased the amount of ascorbate relative to DHA in leaves and guard cells (Chen *et al*. 2003) and significantly affected guard cell signalling and stomatal movement. The leaves of the DHAR overexpressors contained less H_2O_2 in the guard cells and had a higher percentage of open stomata and increased stomatal conductance (Chen & Gallie 2004). A further interaction between ABA and ascorbate is that the latter is a cofactor for 9-*cis*-epoxycarotenoid dioxygenase (NCED), a key enzyme in ABA synthesis. The abundance of NCED mRNA is modulated by ascorbate, such that transcripts are increased when ascorbate is low and decreased when ascorbate is high (Pastori *et al*. 2003).

Ascorbate is more important in stomatal regulation than glutathione, consistent with the quasi-absence of the latter from the apoplast. About 4–10% of the leaf ascorbate pool resides in the apoplast (Noctor & Foyer 1998; Veljovic-Jovanovic *et al*. 2001), giving an ascorbate concentration in the low millimolar range. This fraction depends on the over-

all ascorbate content of the leaf since, for example, feeding the ascorbate precursor L-galactono-1,4-lactone enhanced apoplastic ascorbate as well as total leaf ascorbate (Maddison *et al*. 2002) whereas no apoplastic ascorbate was detectable in the ascorbate-deficient *vtc1 Arabidopsis* mutant, which has only 30% of the total leaf ascorbate of the wild-type (Veljovic-Jovanovic *et al*. 2001). The apoplastic ascorbate pool is also thought to have important functions in defence, particularly in the protection against ozone injury (Burkey, Eason & Fiscus 2003; Conklin & Barth 2004; Kangasjärvi *et al*. 2005).

Ascorbate oxidase and ascorbate status in the apoplast

While the pathway of ascorbate synthesis is distributed between the cytosol and the mitochondrion (Smirnoff, Running & Gatzek 2004; Foyer 2004), the pathway of ascorbate degradation appears to reside in the apoplast (Green & Fry 2005). In particular, ascorbate oxidases (AO), which produce MDHA and DHA, are located exclusively in the apoplast and begin the pathway of ascorbate degradation (Pignocchi *et al*. 2003; Pignocchi & Foyer 2003; Green & Fry 2005). *Arabidopsis* has two genes that encode AO and it is probable that their expression is regulated by DNA-binding with one finger (Dof)-type transcription factors. The pumpkin Group II Dof protein, called ascorbate oxidase gene binding protein (AOBP) transcription factor, binds to a silencer region of the ascorbate oxidase promoter and regulates AO gene expression (Kisu *et al*. 1998). The Dof transcription factor proteins, which are only found in plants, have a highly conserved DNA-binding domain housing four conserved Cys residues that form a single Cys2/Cys2 zinc finger, which is essential for DNA binding. It is therefore not surprising that the function of these transcription factors can be modulated in response to redox changes, along with other transcription factors that have been implicated in oxidative signalling networks (Mittler *et al*. 2004).

The abundance and activity of AO greatly impacts on the redox state of the apoplastic ascorbate pool (Pignocchi *et al*. 2003; Pignocchi & Foyer 2003). High constitutive overexpression of AO in tobacco decreased the apoplastic ascorbate redox state from more than 50% to only 3% reduced without any effect on total leaf ascorbate accumulation or redox state (Pignocchi *et al*. 2003). The decrease in apoplastic ascorbate greatly enhanced leaf injury upon chronic ozone exposure (Sanmartin *et al*. 2003). An ascorbate gradient can be maintained across the plasma membrane such that the ascorbate redox state of the apoplast can be much more oxidized than that of the cytosol, which is largely reduced under most conditions. This gradient arises because of the very low capacity of the apoplast to reduce monodehydroascorbate (MDHA) and DHA and is linked to transport across the plasma membrane (Horemans, Foyer & Asard 2000). The apoplastic ascorbate pool exerts control over plant growth and defence responses (Pignocchi & Foyer 2003).

REDOX REGULATION OF PLANT GROWTH AND DEVELOPMENT

In addition to triggering the pathway of degradation (Fig. 2), AO has long been considered to have a role in cell elongation (Takahama & Oniki 1994). Both oxidants and antioxidants play roles in the regulation of the cell cycle and extension growth. In particular, apoplastic ROS, ascorbate and MDHA have functions in the control of extension growth through regulation of cell wall cleavage, peroxidases and other enzymes involved in cell wall synthesis (Pignocchi & Foyer 2003). In mammalian cell cultures, low levels of ROS are synchronously generated, indicating that ROS are closely connected with cell cycle progression. Moreover, perturbation of these ROS bursts prevented normal progression, indicating that they play a key role in regulation of the cell cycle (Meijer & Murray 2001; Dewitte & Murray 2003). Ascorbate and glutathione also regulate cell division (Vernoux *et al*. 2000; Potters *et al*. 2002, 2004). Synchronized tobacco BY-2 suspension cells are sensitive to GSH depletion by BSO, leading to specific arrest at the G1 checkpoint (Vernoux *et al*. 2000). Depletion of ascorbate also causes the cessation of cell division (Potters *et al*. 2002), but the requirement for ascorbate and glutathione appears to be different and complementary, i.e. one cannot compensate for the absence of the other (Potters *et al*. 2004).

Root growth appears to be strongly regulated by ROS (Foreman *et al*. 2003) and glutathione (Vernoux *et al*. 2000), although roots appear to be much less influenced by ascorbate, in line with the observation that this antioxidant is very strongly correlated with photosynthesis and light. However, maintenance of the root quiescent centre is correlated with high AO and low ascorbate contents. Although the *rml1* mutant, described above, cannot maintain a root meristem, shoot development is unaffected (Cheng, Seeley & Sung 1995). The *rml1* mutant phenotype can be rescued by addition of exogenous γ-EC or GSH (but not other reducing agents), indicating the importance of GSH in cell division in the root meristem. Cell division is inhibited in the roots of both *rml1* mutants and WT plants treated with BSO (Vernoux *et al*. 2000).

ROS are also necessary for the development of symbiosis and nodule development (Becana *et al*. 2000; Baudouin *et al*. 2004; Frendo *et al*. 2005). Recent evidence shows that nodules will not form on roots if GSH synthesis is blocked by addition of BSO, suggesting that like the root meristem, the nodule meristem is unable to develop in the absence of GSH (Frendo *et al*. 2004). Lastly, it is worth noting here that roles for ascorbate (Barth *et al*. 2004) and glutathione (Ogawa *et al*. 2002) in the developmental senescence programme have also been proposed.

CONCLUSIONS AND PERSPECTIVES

Increased ozone is one of many biotic and abiotic stresses that lead to enhanced local or systemic oxidation. The traditional characterization of this condition as 'oxidative

stress' hides the important integrating role of ROS and associated oxidants such as lipid peroxides and oxylipins in cell signalling. It is becoming increasingly difficult to reconcile the notion of passive 'oxidative stress' with observations that it is often genetically programmed systems that are responsible for ROS production. For example, the ROS generation that is a key feature of the HR and PCD responses following pathogen recognition is a genetically controlled response. In mammals the mitochondria-controlled PCD response involves the pro-apoptotic Bax family of proteins and anti-apoptotic Bcl-2 and Bcl-XL family. Although Bax, Bcl-2 and Bcl-XL homologues have not yet been found in plants, expression of mammalian Bax causes death while that of mammalian Bcl-XL or Bl-1 suppresses cell death in plant cells challenged with elicitors, suggesting that elements of mammalian PCD processes are also found in plants (Matsumura *et al*. 2003). Moreover, mammalian Bcl-2 family members localize to the mitochondrial, chloroplast and nuclear fractions when expressed in plants, where they prevent herbicide and ROS-induced apoptosis (Chen & Dickman 2004).

Plant plasma membrane receptors such as MLO, which is part of the innate immune network, also control ROS production and the HR response. The MLO protein dampens the cell wall-restricted H_2O_2 burst at points of attempted fungal penetration of the epidermal cell wall, and in subtending mesophyll cells, suppressing the prolonged oxidative burst and PCD (Piffanelli *et al*. 2002).

Traditionally, ROS have been associated with inactivation (of proteins or of gene function). In fact, their reactivity dictates that they are highly energetic compounds, able to undertake catalytic functions in the absence of enzymes (e.g. in cell wall cleavage and resynthesis). This property means that they are very well suited to activation of signalling cascades. We propose that this function and its consequences can perhaps be usefully viewed, in a physiological context, as a ripple effect, analogous to events that follow dropping a stone into a pool of water (Fig. 3). Further work is required to establish how interactions between ROS and the antioxidative system act to set signal intensity, to relay the oxidative message, and to determine its physiological outcome.

Key questions concern the perception of ROS. Although many proteins (particularly those containing thiol groups) are known to react directly with H_2O_2, to date no complete ROS signal transduction pathways have been described (Mahalingam & Federoff 2003). Two component circuits provide an interface between environmental cue sensing and a downstream kinase signalling cascades in eukaryotes (Hwang, Chen & Sheen 2002). Heterotrimeric G protein signalling to membrane bound NADPH oxidases has been implicated in the developmental of disease resistance and HR in the rice apoplast (Suharsono *et al*. 2002). An oxidative signal-induced kinase OXI1 has been shown to be upstream of two mitogen activated protein kinases (AtMPK3 and AtMPK6) in *Arabidopsis* (Rentel *et al*. 2004: Mittler *et al*. 2004).

The extracellular matrix (ECM)-plasma membrane cytoskeleton continuum is considered to play important roles in the perception of environmental signals (Baluska *et al*. 2003). In animals, heterodimeric plasma membrane proteins known as integrins anchor the cytoskeleton to the ECM. These transmembrane linker proteins can also function as bi-directional signal transduction molecules in processes such as apoptosis (Hynes 2002). Some integrins bind to an RGD (Arg-Gly-Asp) motif found on substrate adhesion molecules localized in the ECM. To date, analysis of the *Arabidopsis* genome has failed to reveal any true homologues of known animal adhesion proteins. The plasma membrane of *Arabidopsis* protoplasts contains high affinity

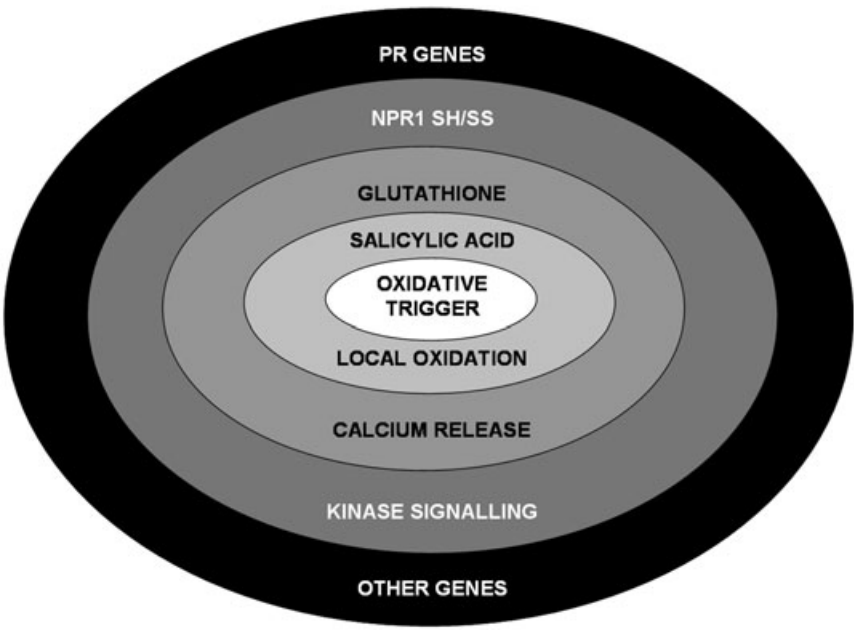

Figure 3. ROS-induced signalling mediates physiological responses through a ripple effect operating in space or time (or both). The scheme suggests that local and/or transient burst of reactive oxygen causes local and/or short-lived changes in the parameters shown, ultimately causing transient or more sustained alterations in expression of defence and regulatory genes. Ascorbate status may modulate signal strength by altering the amplitude and/or duration of the inner rings (Fig. 2).

sites for binding of peptides containing the RGD motif (Canut *et al*. 1998). Evidence from immunological studies, as well as from effects of RGD peptides on plant defence responses (Meinhardt *et al*. 2002), points to the existence in plants of proteins with integrin-like roles. This may be one example of parallel evolution in plants and animals of components with similar structural domains or cellular functions, encoded by divergent primary sequences. Although highly informative, primary sequence comparisons are nevertheless limited in the functional information they provide, since similar protein structural domains may be encoded by different primary sequences and homologous sequences and isoforms can have different physiological roles, notably because of localization. For example, glyceraldehyde 3-phosphate dehydrogenase, a well-defined redox-sensitive glycolytic enzyme, was found to be a surface-associated fibronectin and laminin-binding protein in *Streptococcus pyrogenes*, *Schistosoma mansoni* and *Candida albicans* (Gozalbo *et al*. 1998). Information generated by postgenomic high-throughput protein structure programmes will be crucial in elucidating functions that escape identification through analysis of primary sequences. There is no information to date on whether the orchestration of oxidative signalling events follows or is coupled to ECM-plasma membrane cytoskeleton adjustments in plants. However, there is now no doubt that oxidative signalling is central to the mechanisms by which plants cells sense the environment and make appropriate adjustments to gene expression, metabolism and physiology. Therefore, we suggest that 'oxidative stress' has outgrown its usefulness as a term to denote plant responses to environmental and metabolic fluctuations, and has become something of a misnomer that might be phased out of current nomenclature.

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