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EDITORS’ FORWARD LOOK

Oxidative stress and antioxidative systems: recipes for successful data collection and interpretation

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Statistics

Abstract: 197 words

Main text excluding title page, references, figure legends, and boxes: 10743 words

Number of tables: 1

Number of figures: 6

Number of boxes: 11

Number of references: 171

Running head: Methods in oxidative stress research
Abstract

Oxidative stress and reactive oxygen species (ROS) are common to many fundamental responses of plants. Enormous and ever-growing interest has focused on this research area, leading to an extensive literature that documents the tremendous progress made in recent years. As in other areas of plant biology, advances have been greatly facilitated by developments in genomics-dependent technologies and the application of interdisciplinary techniques that generate information at multiple levels. At the same time, advances in understanding ROS are fundamentally reliant on the use of biochemical and cell biology techniques that are specific to the study of oxidative stress. It is therefore timely to revisit these approaches with the aim of providing a guide to convenient methods and assisting interested researchers in avoiding potential pitfalls. Our critical overview of currently popular methodologies includes a detailed discussion of approaches used to generate oxidative stress, measurements of ROS themselves, determination of major antioxidant metabolites, assays of antioxidative enzymes, and marker transcripts for oxidative stress. We consider the applicability of metabolomics, proteomics, and transcriptomics approaches, and discuss markers such as damage to DNA and RNA. Our discussion of current methodologies is firmly anchored to future technological developments within this popular research field.

Key words: Reactive oxygen species (ROS), hydrogen peroxide, glutathione, ascorbate, NAD(P), antioxidative enzymes, redox proteomics, lipid peroxides, DNA damage

Summary statement

Oxidative stress and related redox processes have become integrated into many parts of plant biology research. Here, we provide a critical methodological evaluation of some of the approaches that are used to monitor, gauge and dissect oxidative stress and related redox signaling in plants. Our Forward Look review discusses current obstacles to progress and foreseeable technological developments that are likely to promote ever faster advances within this intensely studied area.
Introduction

Current concepts of oxidative stress and related redox signalling in plants depend on accurate, unambiguous measurement of a portfolio of relevant factors. These notably include reactive oxygen species (ROS), redox metabolites, enzyme activities, protein modifications, and transcript levels. While no single measurement gives a complete picture, each one contributes specific and potentially useful information. Figure 1 highlights some of the major points to be considered before undertaking analysis of redox-related factors. Many of these issues are generally applicable to experimental plant biology but some of them are particularly crucial in studies of redox processes. As discussed in more detail below, crucial issues include sampling, extraction, and assay specificity. This is because redox-linked factors can be highly reactive and vulnerable to chemical reaction and alteration during isolation and assay. A further complicating factor is that many of the enzymes that produce and process redox compounds in plants have a very high capacity. As a general rule, the key players in determining redox state (e.g., H$_2$O$_2$, ascorbate, NADPH, thioredoxins) may have turnover times on the order of seconds. The culture of due care and attention to this last point is perhaps less widely developed in oxidative stress research than in other related areas such as photosynthesis.

It is beyond doubt that the routine incorporation of molecular biological techniques has hugely advanced the oxidative stress field in plants. Nevertheless, the decreased emphasis on basic biochemistry has perhaps led to less attention being placed on the precautions necessary for precise and accurate measurement of metabolites and enzymes. These measurements require just as much attention to detail as techniques such as qPCR if reliable data are to be produced. Inaccurate conclusions may arise from problems with the measurements themselves, or a failure to understand the limitations of the procedures or the information contained within the results, even if the data are robust (Fig. 1).

Our aim here is discuss commonly employed approaches and techniques, highlighting some of the potential problems and pitfalls, and proposing best-practice solutions. Where appropriate, we draw attention to some relevant latest developments in oxidative stress research. The intention is not to provide an exhaustive overview of individual assays but rather to focus on general principles that should guide the user in obtaining reliable data.
Approaches for assessing oxidative stress responses

Oxidative stress is part of many responses to the environment (Miller et al. 2008), and a range of approaches has been applied specifically to assess tolerance to enhanced cellular oxidant production and to elucidate the mechanisms underlying the responses. Approaches include direct addition of relatively stable ROS such as H$_2$O$_2$, use of ROS-generating reagents, and exploitation of genetic backgrounds lacking antioxidant functions. Each of these approaches has advantages and drawbacks.

Elevated ozone

This is probably one of the most environmentally relevant ways of inducing oxidative stress, given that plants often have to cope with increased ozone levels in natural or agricultural contexts. In addition to studies seeking to establish the importance of components involved in redox homeostasis (eg, Conklin et al. 1996; Dizengremel et al. 2009; Brosché & Kangasjärvi 2012; Dghim et al. 2013), observations on ozone-exposed plants were crucial in demonstrating the role of oxidative bursts in pathogenesis responses (Sandermann et al. 1998; Vainonen & Kangasjärvi 2015). Ozone treatment generates a range of ROS within the apoplast, and is probably the only pure oxidative stress that has been thoroughly tested in the field, using open-top chambers in which environmentally relevant increases in ozone concentration are imposed (Kats et al. 1985; Ainsworth et al. 2012). While elevated ozone is an excellent approach for mimicking oxidative burst conditions, studies in controlled environment chambers often require rather high concentrations of the gas to elicit effects in relatively ozone-tolerant plants. For example, whereas peak tropospheric concentrations rarely exceed 100 ppb, lesions only rapidly appear on the leaves of the Columbia ecotype of Arabidopsis under acute exposure to ozone if concentrations approach 300 ppb (eg, Dghim et al. 2013).

Direct addition of H$_2$O$_2$ to cells and tissues

This is a very common approach that has been widely employed, particularly in cells in culture. It has the advantage of simplicity but complications can arise because of the rapid metabolism of H$_2$O$_2$ even before it enters the cell (Desikan et al. 2001). Like ozone, this treatment probably largely mimics an apoplast-localized oxidative burst, and is less likely to simulate the action of metabolically generated H$_2$O$_2$ inside the cell. This point may be even more relevant when H$_2$O$_2$ is applied to whole tissues. One-time or intermittent spraying may produce relatively little effect unless concentrations
are high (>1 mM), and elevated H$_2$O$_2$ concentrations experienced by the plant tissue are likely to be transient.

Another common approach involves the inclusion of H$_2$O$_2$ in the agar plates on which plants are grown *in vitro*. If the H$_2$O$_2$ concentration is high enough, this may provide a sustainable oxidative stress, and can clearly inhibit germination, root development and plant growth. Again, however, these effects may be due to relatively localized extracellular events, and the primary site of action is very likely restricted to the roots. Moreover, interpretation may be complicated by the presence of metal ions in the media and apoplastic space, which will generate hydroxyl radicals through Fenton chemistry, potentially producing a more severe oxidative stress. Addition of transition metals like iron or copper together with H$_2$O$_2$ and a metal reductant such as ascorbate can be used to enhance production of the hydroxyl radical, if this is the desired effect (eg, Foreman *et al.* 2003).

**ROS-generating reagents**

Many approaches of this type benefit from the advantage that ROS production depends on cellular metabolism, and therefore oxidative stress can be more appropriately targeted to intracellular locations. Paraquat (methyl viologen) is one of the most reliably targeted because its action is closely associated with photosynthetic electron transport. Although it is also exploited in studies on animal cells, and can stimulate superoxide production by the mitochondrial electron transport chain, its main effect in plants (at least at irradiances sufficient to drive photosynthetic electron transport) is to catalyze reduction of oxygen at the level of the highly reducing acceptor side of photosystem I. This produces an oxidative stress whose origin is light-dependent and considered to be largely localized to the chloroplast. Menadione (vitamin K$_3$) is another redox-cycling superoxide-generating reagent, which can be reduced by various cellular dehydrogenases, including mitochondrial complex I. Antimycin A, an inhibitor of ubiquinol oxidation by the cytochrome bc$_1$ complex (complex III) has been applied to promote superoxide generation in the mitochondria (Maxwell *et al.* 1999; Rhoads *et al.* 2006). Although all these reagents promote the production of superoxide, this initially generated ROS should be rapidly converted to H$_2$O$_2$ by superoxide dismutase (SOD).

Another convenient way of generating H$_2$O$_2$ is by using enzymatic systems, such as added glucose oxidase/glucose. Unless the glucose oxidase is taken up by the cell, production of H$_2$O$_2$ by this system will be extracellular. However, transgenic model systems expressing glucose oxidase have been exploited, for example, to study pathogenesis responses (Kazan *et al.* 1998). Ectopic expression of...
peroxisomal glycolate oxidase in the chloroplast has also been developed as a targeted system to study the effect of increased H$_2$O$_2$ production in the stroma (Fahnenstich et al. 2008).

Singlet oxygen

Unlike production of the other major ROS, generation of singlet oxygen is a physical rather than chemical reaction, ie, it is the result of the transfer of energy rather than electrons to the ground-state (triplet) dioxygen molecule. Several reagents can drive increases in singlet oxygen by stimulating this process in a light-dependent manner (Fischer et al. 2013). These include rose bengal, methylene violet, neutral red, and indigo carmine. While all produce singlet oxygen following their photoactivation, they do so with different efficiencies and also accumulate in different parts of the cell (Kovacs et al. 2014; Gutierrez et al. 2014). Some of these reagents may have other effects as well as direct photosensitization of singlet oxygen production. For instance, rose bengal can inhibit photosynthetic electron transport (Kovacs et al. 2014). The most physiologically relevant feature of these reagents may be in mimicking endogenous photosensitizers (eg, chlorophyll precursors and degradation products) that can promote singlet oxygen formation.

The Arabidopsis fluorescence (flu) mutant, which conditionally produces singlet oxygen due to accumulation of the chlorophyll precursor, protochlorophyllide, is a widely exploited genetic tool (Meskauskiene et al. 2001; op den Camp et al. 2003; Ramel et al. 2012). Despite the profound interest of studies on this system, it should be noted that singlet oxygen is probably not generated within photosystem II (PSII) in the flu mutant but rather at other sites within chloroplasts. Finally, any inhibitor of the photosynthetic electron transport chain that acts downstream of PSII (eg, DCMU, DBMIB) will increase the probability of triplet chlorophyll formation and hence be expected to favour singlet oxygen production through back- and side-reactions within PSII.

Knocking down antioxidative defenses

Numerous studies have been conducted on plants deficient in antioxidative enzymes with the primary aim of evaluating the functional importance of the targeted enzyme. In addition, some plants with compromised antioxidative systems can be useful as a model oxidative stress background. Perhaps the most intensively studied systems are conditional mutants deficient in catalase (Dat et al. 2001). Advantages of using such plants are that H$_2$O$_2$ production can be switched...
on and off by manipulating light intensity or CO₂ levels, and that as long as photorespiratory glycolate oxidase remains active, the H₂O₂ production can be sustained (Dat et al. 2001).

Catalase is one of two major routes for removing intracellular H₂O₂, the other being reactions dependent on several types of peroxidase, each of which is encoded by several genes and associated with many other reductant-producing pathways that show considerable redundancy. This makes catalase-deficient plants particularly appropriate tools with which to unravel the function of other genes involved in the metabolism of peroxide produced inside the cell (Mhamdi et al. 2010a). One example is that glutathione reductase (GR)-deficient gr1 mutants show a wild-type phenotype when grown on H₂O₂ in agar, suggesting that the enzyme plays little role in oxidative stress (Marty et al. 2009). In contrast, the gr1 mutation greatly exacerbates both glutathione oxidation and the phenotype associated with the cat2 background (Mhamdi et al. 2010b). This difference underlines the importance of choosing the appropriate system to explore the effects of H₂O₂ since it shows that producing this ROS through a physiologically relevant pathway (photorespiration) generates different effects to stressing plants by simply adding H₂O₂ externally (Marty et al. 2009; Mhamdi et al. 2010b).

One pharmacological tool that mimics genetic loss of catalase function is 3-aminotriazole (eg, May & Leaver 1993), although effects may be somewhat more severe because this compound can inhibit catalase activity almost completely and can also inhibit other enzymes.

Which approach is the best? There is no simple answer to this question because the approach(es) to be chosen will depend on the scientific problem under investigation. An important point is to be aware of available knowledge on each approach (eg, site of action, nature of ROS produced, anticipated effects on antioxidant status, dose-dependence of effects, specificity, stability). Taking all these issues into account, we consider that (1) elevated ozone is the most environmentally relevant approach; (2) procedures that involve site-specific generation of ROS are particularly useful, and (3) genetic systems with compromised antioxidant enzymes or conditional ROS production may offer greater specificity than pharmacological approaches.

The challenges of measuring ROS in plants: Occam’s razor can be a blunt tool

Occam’s razor refers to an influential explanatory principle outlined by the 14th Century scholar, William of Occam. It states that among competing hypotheses equally able to explain phenomena, the one that requires the fewest assumptions should be favoured. While this simple principle can often aid interpretation, it may not always be applicable in the biochemically complex environment of the cellular redox network. Oxidative stress is often simply equated to changes in ROS levels. This
concept is debatable, because ROS probably do not accumulate uniformly across the cell and may increase at sites whose function requires oxidation (Foyer & Noctor 2016). Hence, ROS accumulation may not actually be stressful. Apart from the relatively oxidized status of compartments such as the intrathylakoid space, endoplasmic reticulum lumen, vacuole, and apoplast, recent work has reported that the chloroplast stroma extensions known as stromules may play an important role in the spatial transfer of oxidizing molecules and, hence, in oxidative signaling (Caplan et al. 2015).

Measurements of ROS are often required for publication of papers that study oxidative stress. However, questions remain concerning the data that are produced by many of the available methods. Such methods can be divided into three broad categories: (1) monitoring of ROS produced in the solution surrounding the system, most often applied to the study of cell cultures; (2) staining methods that can be used in situ on plant tissues; and (3) procedures that aim to quantify ROS contents in tissue through extraction and in vitro assay. Within all these categories, many methods are available, and their number is increasing all the time. However, most if not all of these approaches are subject to artefacts and/or limitations, particularly if not employed correctly, and caution must always be exercised in data interpretation. While newly reported probes often show encouraging increases in specificity, potential problems persist relating to the stability of ROS, interference by other metabolites and processes, and the specificity of the reaction in the assay.

Monitoring ROS release into the medium

Such methods are particularly applicable to the analysis of ROS generated by cells in suspension. Although difficult to use on whole plants or some plant organs, they have been applied to seeds or to pieces of tissue such as leaf discs. Examples are the H$_2$O$_2$ assay based on oxidation of scopoletin catalyzed by exogenous peroxidase, and oxidation of 2’,7’-dichlorofluorescin (H$_2$DCF) to fluorescent 2’,7’-dichlorofluorescein (DCF; Schopfer et al. 2001; Chong et al. 2002). While a general principle of metabolite assays is that enzyme-dependent reactions ensure a degree of specificity, attention has nonetheless been drawn to interference in the scopoletin-peroxidase assay (Corbett 1989). It should also be noted that such measurements do not provide precise information on either tissue contents of ROS or on their rates of production. Rather, the signal will depend on the amount of oxidant that escapes into the solution. Hence, the assay should only detect those ROS that elude the antioxidant system. Even if this is only a small fraction of the ROS produced, the data are informative as a relative measure if the signal is proportional to the rate of production.
Examples of common probes for in situ analysis of ROS

This approach is attractive because, in theory, it enables visualization of *in vivo* processes. Many probes are available but, unfortunately, few of them are specific. Moreover, a number of the commonly used compounds, such as luminol, nitroblue tetrazolium (NBT), and the sensor green probe for singlet oxygen (SOSG), can actually promote production of the reactive species they are assumed to merely be detecting (Fridovich 1997; Kim *et al.* 2013). Based largely on the exploitation of *in situ* stains, there are many reports of patterning of accumulation of specific ROS in different cell types (e.g., bundle sheath), developmental zones, or tissues within roots, buds, and nodules (Fryer *et al.* 2002; Dunand *et al.* 2007; Tsukagoshi *et al.* 2010; Meitha *et al.* 2015). While these findings are very exciting, and sometimes based on results obtained with more than one probe, it is still necessary to exercise caution in data interpretation. The suitability of both NBT and 3,3'-diaminobenzidine (DAB) is often justified by the argument that they are commonly employed probes. Nevertheless, some possible complicating factors that should be considered are shown in Figure 2.

One apparently valid argument that is often advanced in justification of such approaches is that the detected signal can be abolished by addition of enzymes known to remove the ROS of interest. Even here, however, the interpretation may not always be simple. One example that has been discussed for many years concerns the application of NBT to detect superoxide in cells, which has been described as “widespread misuse” by the co-discoverer of SOD (Fridovich 1997). While this criticism seems somewhat forthright, it is clear that NBT is not specific to superoxide. It has been used successfully to screen for ascorbate-deficient mutants (Conklin *et al.* 1996), and it can also be reduced by many dehydrogenases (Fridovich 1997). Further, abolition of the signal by the addition of exogenous SOD may be an indirect effect and not a proof that colour formation is reflecting superoxide production that is independent of the presence of the probe (Fig. 3; Fridovich 1997). This is just one example of how the complex redox environment of plant tissues might lead to problems that are sometimes overlooked in data interpretation.

In addition to colour stains, many fluorescent probes are available for measuring ROS (Wardman 2007; Kalyanaramana *et al.* 2012; Winterbourn 2014). As mentioned above, H$_2$DCF has been employed to monitor ROS release from cells or tissues: this probe has also become accepted as an *in situ* stain for ROS. Two points must be considered in using such approaches. The first is that the oxidation that leads to the signal is not mediated by H$_2$O$_2$ directly. It can be achieved by the hydroxyl radical produced from H$_2$O$_2$ or, like NBT and DAB, by enzyme-dependent reactions (Kalyanaramana *et al.* 2012). A second point is that the diacetate form is considered to aid uptake of H$_2$DCF-DA into
cells, where conversion to DCF is considered to report on intracellular oxidants. However, plant cells
contain high activities of apoplastic esterases (Haslam et al. 2001; Cummins & Edwards 2004), and
their action may mean that the DCF signal is largely extracellular, which can be problematic if the
analytical resolution is not sufficient to distinguish signals at the subcellular level. The H$_2$DCF-DA
probe may be a semi-quantitative *in situ* indicator of ROS accumulation in organs such as roots.
Based on our experience, it is very difficult to use reproducibly in leaves, even with confocal
microscopy to enable subcellular resolution.

Other similar probes, such as 3′-O-Acetyl-6′-O-pentafluorobenzenesulfonyl-2′-7′-difluorofluorescein
(BES-H2O2-Ac), may be more specific for H$_2$O$_2$ and have been employed in plants (Maeda 2008;
Biswas & Mano 2015). Fluorescent probes have also been described for specific live-cell imaging
of lipid peroxides (Soh et al. 2007), a group of compounds that play important roles in
signalling downstream from primary ROS production (Farmer & Mueller 2013; Biswas & Mano 2015).
These compounds, and some of the others mentioned above, may generate important information. If
the aim is to assess oxidative stress intensity, however, it is probably advisable to compare at least
two of them, and to corroborate the signals by at least one of the other approaches discussed below
(eg, antioxidants, marker transcripts).

*Extraction of ROS for in vitro quantification*

The primary ROS (superoxide, H$_2$O$_2$, hydroxyl radical, singlet oxygen) are difficult to quantify reliably
in plant tissue extracts. Spin-traps, in which electron paramagnetic resonance (EPR) signals are
captured *in planta*, have been applied for the more reactive forms (Müller et al. 2009; Fischer et al.
2013). H$_2$O$_2$ is the most stable of these four compounds and, as a non-radical that is a substrate for
relatively well-defined enzymes, it is probably the only one that is considered to be quantifiable after
direct extraction. Nevertheless, the quantification of extracted H$_2$O$_2$ is far from trivial. As is the case
for other metabolites, the extraction procedure itself requires careful attention. We emphasize that
H$_2$O$_2$ should not be extracted into water or neutral buffer because this will allow enzymes to remain
active during the sample preparation. This point should be obvious given that such media are used to
extract highly active antioxidative enzymes like catalase. Extraction into acid or other media that
inhibit enzyme activities as well as many chemical redox reactions is essential, and experiments
should be carried out to check that H$_2$O$_2$ added at the early stage of extraction is recoverable in the
assay.
Other compounds present in plants can interfere with assays of extractable H$_2$O$_2$ (Veljovic-Jovanovic et al. 2002; Queval et al. 2008). For this reason, it is advisable to check the linearity of the assay response using a standard amount of H$_2$O$_2$ added together with the extract. Simply observing a linear standard curve for H$_2$O$_2$ prepared in water or buffer is not sufficient, unless it is established that the response is not affected by the presence of the extract. Such issues are clearly recognized in metabolomics techniques such as LC-MS, where alteration of the detector response by the chemical context is referred to as a « matrix effect ». Effects such as these, as well as an influence of the milieu on stability during the extraction, are sometimes overlooked in apparently simpler targeted analysis of specific compounds.

Another important point is that the activity be shown to be dose-responsive to the volume of extract added in the assay. In our hands, using two different methods to measure H$_2$O$_2$, little effect of extract volume on the signal was observed, meaning that the calculated tissue content was inversely proportional to the amount of extract added to the assay (Queval et al. 2008). Since different studies often employ different tissue mass:volume ratios during the extraction, this could be one of the factors leading to the enormous range of basal tissue H$_2$O$_2$ contents in the literature, which might be considered highly unusual for an important signalling compound. Other issues relating to the contents and assays of H$_2$O$_2$ in plants have been recently discussed in more detail elsewhere (Noctor et al. 2015; Foyer & Noctor 2016).

Box 1 describes methods we have employed for luminol and the peroxidase-coupled techniques for quantification of H$_2$O$_2$ and peroxides in extracts. Other methods, such as Amplex Red or peroxidase-coupled fluorescence, may also be applicable. However, as noted above, the key issue may not be related to the assays but rather to the extraction and sample preparation. We also note that many of the kits available for measuring ROS and related factors have been developed for use on organisms other than plants, and may not take account of plant-specific problems related to extraction and interference. One interfering compound in some peroxide assays is ascorbate, which is present in high levels in tissues such as leaves (Veljovic-Jovanovic et al. 2002; Queval et al. 2008). For this reason, we routinely treat extract supernatant with ascorbate oxidase to minimise concentrations of this redox-active compound in the assay (Box 1).

Lipid peroxides are key indicators of oxidative stress and related signalling, and can be profiled by LC-MS, with different signatures supplying some information on the source and location of ROS (Davoine et al. 2006; Triantaphylides et al. 2008). Because of its simplicity, the assay of thiobarbituric acid-reactive substances (TBARS) remains an assay of choice for many laboratories. This assay gives
information on the breakdown products issuing from lipid peroxidation. Malondialdehyde (MDA) is often used as a standard but the assay can detect a variety of reactive aldehydes. It usually gives contents that are substantially higher than more specific assays of MDA (eg, Moselhy et al. 2013). Moreover, MDA can be produced from sources other than lipid peroxides. Signals should therefore more accurately be described as “TBARS” rather than “lipid peroxidation” or “MDA”.

Measuring extractable enzyme activities in vitro

An overview of the usefulness of extractable enzyme activity assays has recently been provided elsewhere (Stitt & Gibon 2014). Here, we focus our discussion specifically on ROS-related enzymes, although generally applicable principles are emphasized at certain points.

Measurements of the maximal extractable activities of antioxidative enzymes have long been recognized as indicators of cellular redox status. This approach is facilitated by the high activities of the core antioxidative systems based around catalase and the ascorbate-glutathione pathway, a feature that allows them to be readily measured using simple spectrophotometric techniques (Fig. 4; Boxes 2-6). These enzymes have high capacities because they are involved in ROS-linked redox cycling. As ROS production is an integral part of metabolism and can occur at high rates even in the absence of stress, SOD and H$_2$O$_2$-processing enzymes are quite strongly expressed even under optimal conditions. Lower-capacity enzymes, such as those involved in glutathione and ascorbate synthesis, are more difficult to measure and require specialized techniques (Hell and Bergmann 1990; Noctor et al. 1998; Dowdle et al. 2007). Moreover, developments over the last two to three decades have led to the realization that enzymes exist whose primary physiological function is to generate ROS, showing that these oxidants are not simply the product of “imbalances” in metabolism. As discussed below, some of these activities are more difficult to reliably quantify than major antioxidative redox-cycling enzymes.

ROS-producing enzymes

Over recent years, the best studied ROS-producing components have been the respiratory burst oxidase homologue (RBOH) type of enzyme that oxidize NADPH to reduce O$_2$ to superoxide at the plasmalemma and, possibly, other sites (Desikan et al. 1996; Torres et al. 2002; Foreman et al. 2003). Despite extensive focus on these enzymes, their biochemical activities and capacities remain quite poorly characterized. They are highly regulated by a plethora of post-translational modifications involving such factors as protein phosphorylation, calcium ions, lipids, and interactions with other
proteins, e.g., receptor-like kinases (Marino et al. 2012; Kadota et al. 2015). Moreover, AtRbohD, perhaps the most studied of the plant NADPH oxidases, is S-nitrosylated on a specific cysteine residue, leading to a loss of activity (Yun et al. 2011). Because most of these modifying factors will be diluted or altered during sample preparation, it is difficult to relate NADPH oxidase activity in extracts to what is happening in vivo. Another problem is that specific analysis of membrane-bound RBOH-type NADPH oxidases requires lengthy preparation to remove other enzymes that could contribute to the measurement in a non-specific way. Because of these issues, particular caution is required in interpreting data obtained from in vitro measurements of NADPH oxidases.

In addition to the NADPH oxidase family, plant cells contain a battery of other ROS-producing enzymes. Some of these are largely soluble and relatively high-capacity, and so they are easier to measure. One example is glycolate oxidase. This enzyme may have several functions in plants, but it acts most notably in the photorespiratory glycolate-recycling pathway. Unlike NADPH oxidases, the primary function of such enzymes is not considered to be in ROS production. Rather, they generate H$_2$O$_2$ as a by-product, although some contribution to ROS-signalling pathways cannot be discounted (Foyer et al. 2009). Glycolate oxidase is the primary source of H$_2$O$_2$ in photorespiratory catalase mutants. Other ROS-producing enzymes include xanthine oxidases, amine oxidases, and class III (guaiacol-type) peroxidases (del Rio et al. 2006; Moschou et al. 2008; Angelini et al. 2010; O’Brien et al. 2012). Enzymes of the last type may also have an antioxidant (H$_2$O$_2$-consuming) function and, classically, were measured as such using model substrates such as guaiacol or pyrogallol. However, over 70 genes are thought to encode these enzymes in Arabidopsis (Cosio & Dunand 2009), making it rather difficult to link changes in measured activities to specific isoforms or gene products.

**Antioxidative enzymes**

Because their extractable activities are easy to quantify accurately (if correct procedures are followed), assays of the major antioxidative enzymes can provide insight into plant responses to ROS such as superoxide and H$_2$O$_2$. However, it is important to be clear on the limitations of the information that is generated. It is well documented that oxidative stress triggers increases in the total extractable activities of most major antioxidative enzymes, albeit usually to a modest extent (1.5- to 5-fold are typical values). Increases in the extractable activities of these enzymes can, therefore, be taken as indirect evidence of increases in ROS and/or oxidative stress in the plant at some point prior to sampling. However, relative increases in enzyme activities cannot be used to infer relative increases in ROS. Neither can in vitro activities easily be related to flux through the corresponding reactions in vivo. There are several reasons for this. One is that the protocols are
designed to allow maximal activities to be observed, notably by using high or near-saturating substrate concentrations. For example, GR is routinely assayed using 0.1 mM NADPH and 0.5 mM GSSG (Box 6). Both concentrations are well above the respective $K_M$ values of the enzyme (Smith *et al.* 1989; Edwards *et al.* 1990) and, at least for GSSG, probably well above the *in vivo* concentrations that the enzyme is likely to experience under most conditions (Meyer *et al.* 2007; Schwarzländer *et al.* 2008). A second complicating factor is that, in nearly all cases, the *in vitro* activity of a given antioxidative enzyme is a composite of the contributions of several isoforms encoded by a gene family. Although the gene families of these enzymes are not very large, they do encode isoforms present in multiple subcellular compartments, with the possible exception of catalase (Table 1).

Another issue concerns possible post-translational modifications that may modulate the activity *in vivo* but whose influence is not detected in the measurement, either because the modification is not stable throughout the sample preparation or because the assay is not able to detect its effect.

Despite the interest of these assays in probing oxidative stress and the cellular response, the literature unfortunately contains many examples of values for extractable enzyme activities that are obviously, or very likely, erroneous. While some aberrant values may be explained by typing or production errors, others are probably caused by issues such as inappropriate experimental procedures or poor data processing. It is obviously important to apply the correct extinction coefficient to convert an absorbance change per unit time to moles per unit time. With specific regard to catalase, it is worth noting that the absorbance of $H_2O_2$ at 240 nm is relatively weak, and so the extinction coefficient (about 40 M$^{-1}$cm$^{-1}$) is around 100 times lower than those of substrates monitored in other assays (eg, ascorbate or NAD(P)H; Fig. 4). One consequence is that conventional techniques are not suited to assaying catalase at physiological concentrations of $H_2O_2$ (probably sub-mM; Foyer & Noctor 2016). However, most assays performed on extracts of plant tissues are basically giving a measure of capacity, not *in vivo* activity. Hence, a concentration is routinely used that is both easily detectable in a spectrophotometer and high enough to drive catalase activities at appreciable rates (eg, 40 mM; Box 2). An added advantage of using such high concentrations is that any contribution to $H_2O_2$ removal in the assay from peroxidases will be a negligible proportion of the overall rate. Even if such enzymes were working at maximal rates (which should not be the case, if extracts are desalted and $H_2O_2$ is the only added substrate), their capacities are orders of magnitude below that of catalase (Fig. 4).

Model calculations for three commonly measured enzymes are shown in Fig. 4, based on typical rates observed in Arabidopsis leaf extracts. Using the procedures described in Boxes 2, 3, and 6, extractable catalase activity is about 100 times higher than APX which is in turn about 5-10 times
higher than GR. Similar ratios can be observed in leaf extracts from other C_{3} plants such as tobacco (eg, Dutilleul et al. 2003). In studies where several enzymes are measured, comparing the relative values obtained for the activities is an appropriate first check for identifying potential problems. While the relationship may vary somewhat between tissues and plants, or be influenced by growth conditions, an approximate order of rates obtained in leaf extracts using typical protocols is as follows: catalase>>>APX>MDHAR>DHAR>GR (Fig. 4 and Boxes 2-6). For example, if leaf activities are being measured in wild-type plants and the calculated APX activity is 10 times higher than the catalase activity, there is probably an error. Although the protocols detailed in boxes 2-6 are for conventional spectrophotometers, they can be adapted for plate-readers (eg, Murshed et al. 2008).

In-gel activity stains can be used to assess individual isoforms of antioxidative enzymes after separation by isoelectric focusing or other electrophoretic techniques (eg, Zimmermann et al. 2006). In theory, this approach allows closer definition of the antioxidative system, albeit in a less quantitative way than assays of extractable activities. In practice, for many enzymes, the information is often compromised by poor resolution on gels and diffuse staining. When such analyses do achieve relatively high resolution, they often reveal multiple activity bands, sometimes far more than would be expected from the number of predicted protein products. Thus far, there is little information that allows the functional relevance of these observations to be assessed.

An alternative or additional approach is to measure protein abundance, although immunoblotting does not provide information on activity and specific antibodies may not be easy to obtain if gene sequences within a given family are highly similar. Particularly in cases where different isoforms are difficult to resolve at the protein or enzyme level, quantification of transcripts may provide information on the responses of the different members of the antioxidative system. Measuring transcripts can provide greater sensitivity because they sometimes show a greater relative change than the encoded enzyme activity, especially if the transcript is only one of several encoding the proteins that contribute to an extractable activity. It should also be noted, however, that some antioxidative enzymes are regulated at both transcriptional and post-transcriptional levels (Mittler & Zilinskas 1994). Oxidative stress transcriptomics has revealed that quantification of responsive transcripts encoding proteins outside the core high-capacity ROS-processing system may be a sensitive approach to assessing the intensity of the oxidative stress response, a point we discuss further below.

The antioxidative system is by no means restricted to the enzymes mentioned above. Superoxide dismutase (SOD) is a key player and many other enzymes may also have important roles. These
include peroxiredoxins (PRX) and related enzymes that are, probably misleadingly, named glutathione peroxidases (GPX; Iqbal et al. 2006; Navrot et al. 2006; Tripathi et al. 2009). Based on genetic evidence, it is clear that at least some PRX play major roles in ROS metabolism in vivo (Awad et al. 2015). While several PRX and GPX have been characterized as recombinant proteins in vitro, there is much less information on how their extractable activities change in response to oxidative stress. This is because many of these activities require interactions with regenerating proteins that are largely prohibited in diluted extracts. To take the chloroplast stroma as an example, protein concentrations in vivo are of the order of 200-400 mg ml⁻¹, based on a leaf protein content of 10-20 mg g⁻¹ FW, 50% of which is in packed into a stromal volume of about 25 µl g⁻¹FW (Heldt 1980). In contrast, the protein concentrations of extracts prepared as in Box 2 will not greatly exceed 1 mg ml⁻¹.

Using assays such as peroxide-dependent NADPH oxidation in the presence of glutathione and GR, “glutathione peroxidase” activity can be measured in extracts. However, this activity is usually somewhat lower than that of APX and the enzymes responsible are not clearly identified. As well as some contribution from GPX isoforms, which in vivo are probably preferentially coupled to thioredoxins, glutathione oxidation may be the result of glutathione S-transferase (GST)-linked peroxidase activity (Dixon et al. 2009). Total GST activity can be measured using model substrates such as 1-chloro-2,4-dinitrobenzene (CDNB) to probe the conjugase function. Here, there are similar problems to those associated with classical assays of class III peroxidases. First, it is difficult to infer physiologically important information from an activity measured against a model substrate. Second, GSTs are encoded by a large gene family (55 in Arabidopsis; Dixon et al. 2009). Nevertheless, as detailed in the section below on marker transcripts, specific genes for GSTs are among those that are most strongly induced, on a fold-change basis, in response to oxidative stress.

Apart from substrate concentrations and allosteric regulation by metabolites, post-translational regulation of enzymes through covalent modification is a key mechanism controlling their activities in vivo. To date, there have been relatively few in-depth reports of post-translational regulation of antioxidative enzymes in plants. In Chlamydomonas, regulation of catalase activity has been linked to the thioredoxin system (Shao et al. 2008). Several other studies have detected antioxidative enzymes among proteins that interact with thioredoxins or that can undergo thiol modifications such as S-nitrosylation (Balmer et al. 2003, 2004; Romero-Puertas et al. 2007, 2008; Rosenwasser et al. 2014; Waszczak et al. 2014). In many cases, the in vivo significance of these processes remains to be more closely defined. Although assays of extractable enzyme activities have long been adapted to assess activation state related to post-translational modifications such as thiol-disulphide exchange and...
phosphorylation (e.g., Charles & Halliwell 1981; Kaiser & Huber 1997), little attention has as yet been paid to such issues in relation to major antioxidative enzymes in plants. It is also important to note that post-translational modifications may have biologically crucial effects that are independent of any impact on enzyme activity. One example is phosphorylation of the yeast CuZnSOD, which drives its relocation to the nucleus where it acts as a transcription factor (Tsang et al. 2014).

One antioxidant-linked enzyme that is clearly post-translationally regulated is γ-glutamylcysteine synthetase (γ-ECS, also known as glutamate-cysteine ligase), which catalyses the first step of glutathione synthesis and which is activated by oxidation of protein thiols (Hell and Bergmann 1990; Hicks et al. 2007; Gromes et al. 2008). This mode of regulation presumably allows the enzyme to rapidly achieve rates of glutathione synthesis that are appropriate to the prevailing cellular redox state, for example, to boost glutathione production under conditions of oxidative stress. In crude plant extracts, this enzyme is not easy to measure. The activity of the highly purified recombinant enzyme can be accurately measured by a conventional coupled assay that monitors ATP hydrolysis. Although data obtained by performing this assay on crude extracts can be found in the literature, the values are orders of magnitude higher than reliable, specific assays of γ-ECS production using HPLC-fluorescence. This is probably because the measured activity is overwhelmingly due to other ATP-hydrolyzing enzymes that are abundant in plant extracts and cannot, therefore, be described as “γ-ECS activity”.

Antioxidants and metabolite markers

Major antioxidant metabolites are key compounds that interact directly with ROS. With regard to physiologically produced singlet oxygen at photosystem II, the most important are tocopherols and carotenoids, which are located at high levels in plastid membranes, notably the thylakoids. While they make some contribution to light harvesting, carotenoids are particularly important in avoiding excessive accumulation of singlet oxygen through photodynamic quenching of excited chlorophyll states (Ruban et al. 2012; Fischer et al. 2013). They can also chemically react with this ROS to yield characteristic breakdown products that may be important in signalling (Ramel et al. 2012). Like lipid peroxide profiles, these products may be useful as markers for singlet oxygen-induced oxidation.

The most abundant antioxidant metabolites in the soluble phase of cells are ascorbate and glutathione. These compounds can chemically scavenge certain ROS and, as noted above, are co-factors for several antioxidative enzymes that ensure peroxide processing (Foyer & Noctor 2011;
As well as acting as reductants that regenerate certain PRX (Tripathi et al. 2009), ascorbate is the reducing co-factor for APX while glutathione is a substrate for some peroxidases and may also contribute to ROS processing indirectly by participating in ascorbate regeneration from dehydroascorbate (DHA), either chemically or in the reaction catalyzed by DHARs (Box 5). In addition to work specifically focused on ascorbate or glutathione metabolism, numerous studies have measured the stable oxidized and reduced forms of these two compounds as markers for cellular redox state. This approach is more reliable and has some advantages over direct assays of ROS. First, the major forms of ascorbate and glutathione are relatively stable during extraction, providing appropriate sample preparation techniques are followed. Second, they can be specifically measured using enzyme-dependent assays. Third, because they are in the frontline of ROS-processing, changes in their redox states and/or contents can be taken as indicators of oxidative stress inside the cell. The primary product of ascorbate oxidation (monodehydroascorbate, MDHA) is an unstable radical that rapidly dismutates to ascorbate and DHA, and thus cannot be easily measured. However, EPR techniques have been used to demonstrate increased MDHA signal in stress conditions (Veljovic-Jovanovic et al. 1998).

Characteristic responses of the major antioxidants to enhanced intracellular H$_2$O$_2$ in leaves are accumulation of total glutathione, mainly as glutathione disulphide (GSSG), and decreases in total ascorbate (Noctor et al. 2015). However, stress-induced changes in ascorbate redox state can also be observed, although often under different conditions from those that greatly influence the glutathione pool (Marquez-Garcia et al. 2015). Although these pools are functionally coupled under some conditions, they may also work more independently in other circumstances (Foyer & Noctor 2011). This presumably reflects the number of pathways potentially involved in the oxidation and reduction of each of these antioxidants, and their relative engagement in different conditions. Hence, one should not necessarily expect the status of ascorbate and glutathione to change in the same way.

Like the measurement of antioxidative enzymes, assays of glutathione and ascorbate on whole tissue extracts provide only a composite picture. The data do not necessarily report on the status of these compounds in the compartments where ROS are produced. Both compounds are heterogeneously localized within the cell, and oxidative stress can differentially affect their distribution. This has become apparent through the application of techniques such as redox-sensitive green fluorescent proteins (roGFPS) that provide compartment-specific information on the glutathione redox potential, as well as in situ detection of ascorbate and glutathione by immunolabelling (Meyer et al. 2007; Schwarzländer et al. 2008; Zechmann et al. 2008; Zechmann 2011). With respect to glutathione, the
studies using roGFP suggest that much of the GSSG that is detected in extracts do not reflect redox states in the cytosol, chloroplasts, or mitochondria (Meyer et al. 2007; Schwarzländer et al. 2008). This is probably the case even for the relatively low levels of GSSG found in samples from unstressed plants. Marked accumulation of GSSG in oxidative stress conditions partly reflects its sequestration in the vacuole (Queval et al. 2011), probably due to import from the cytosol by tonoplast transporters (Noctor et al. 2013).

Good oxidative stress markers are likely to be factors that have relative stability because they are the result of modifications that are not rapidly reversed. As noted above, ROS will only accumulate strongly if they are located in compartments with low antioxidative activity. This situation may be rare in many intracellular locations, although more common in the vacuole and endoplasmic reticulum. Accumulation of GSSG in compartments like the vacuole, as a secondary product of enhanced ROS production, may be a key process allowing glutathione to be used as a marker. Without such sequestration, GSSG would be recycled by the action of GR and its accumulation would be much more difficult to detect. A similar consideration may apply to ascorbate. The oxidized form, DHA, is thought to be largely located in the apoplast, where ascorbate oxidase is found and ascorbate-regenerating capacity is lower than inside the cell (Pignocchi & Foyer 2003). The best characterized ascorbate degradation pathway proceeds from DHA and is also localized in the apoplast (Parsons & Fry 2012). GSSG present in the vacuole will also be degraded (Grzam et al. 2007), although this seems not to be fast enough to prevent its accumulation during oxidative stress (Queval et al. 2011).

Similarly to transcriptomics (discussed further below), non-specific metabolite profiling using techniques such as gas chromatography-mass spectrometry (GC-MS) can reveal a signature indicative of an “oxidative stress metabolome”. This signature includes accumulation of several compounds implicated in ascorbate and glutathione synthesis and degradation pathways, as well as phytohormones that are known to be involved in oxidative stress signaling, such as salicylic acid and jasmonic acid (Noctor et al. 2015). It is noteworthy that the signature also includes several compounds that are not obviously connected to antioxidant metabolism or redox homeostasis (Noctor et al. 2015). For example, the accumulation of certain amino acids, respiratory intermediates, and aromatic compounds is indicative of the switch in metabolism that underpins the oxidative stress syndrome, perhaps triggered in part by post-translational redox regulation of enzymes (Rosenwasser et al. 2014) as well as by processes such as enhanced proteolysis. Based on studies that have manipulated the glutathione synthesis pathway directly by altered γ-ECS expression
or indirectly by oxidative stress, glutathione status may be important in linking oxidative stress to altered amino acid contents (Noctor et al. 1998; Han et al. 2013).

Pyridine nucleotides are central to both pro-oxidant and antioxidant metabolism (Foyer & Noctor 2009), and several studies over recent years have explored the roles of specific NADP-dependent dehydrogenases in ROS metabolism and related conditions (Valderrama et al. 2006; Mhamdi et al. 2010c; Voll et al. 2012; Li et al. 2013). As these enzymes produce NADPH, they can be readily measured using spectrophotometric techniques. One example of a method for measuring a typical dehydrogenase is given in Box 7.

In general, total tissue NAD and NADP reduction states are less clearly indicative of oxidative stress than those of ascorbate and glutathione. Redox states are generally more oxidized even in optimal conditions, with total tissue NAD pools being largely oxidized and NADP pools being about 50% reduced. This reflects their essential role in energy metabolism, which requires redox poising so that both oxidized and reduced forms are always sufficiently available. Nevertheless, the abundance of total pyridine nucleotide pools can be influenced by oxidative stress through effects on synthesis and degradation (Noctor et al. 2011). It can be useful to have data on these compounds alongside ascorbate and glutathione. Validated methods that allow analysis of all these compounds have been developed (Fig. 5), and are described in Boxes 8-10.

Assays using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) or ferric ion reduction capacity have been used to measure “total antioxidant capacity”. However, the nature of the factors that are monitored by these assays is unclear. These techniques may be useful in the food and associated industries, or in medical diagnostics, but they are of limited value for dissecting the biological complexity of oxidative stress and plant antioxidant systems.

Oxidation of proteins and nucleic acids

Protein oxidation is likely to be a key process in signalling downstream of ROS. The various possible oxidant-induced modifications have been reviewed in-depth elsewhere (Møller et al. 2007, 2011; Møller & Sweetlove 2010). In some cases, protein oxidation may be rapidly reversible, because of the action of thioredoxins or protein methionine sulfoxide reductases (Tarrago et al. 2009). More stable modifications include carbonylation, which can occur at several amino acid residues within proteins and is not reversible by known enzymes. Protein carbonylation is relatively easy to measure using a simple chemical stain, and has been employed as a convenient oxidative stress marker (Kingston-
Smith & Foyer 2000; Davletova et al. 2005a). Even here, the interpretation should take into account that the assay is only providing information on proteins that are allowed to accumulate following oxidation. Protein oxidation is clearly implicated in degradation of enzymes such as Rubisco and glutamine synthetase, either by protease-dependent mechanisms or, possibly, direct cleavage mediated by hydroxyl radicals (Desimone et al. 1996; Ishida et al. 2002).

There are now extensive lists of proteins that are potential targets for thiol-disulphide regulation. These have been produced using techniques such as binding of proteins to columns containing mutated thioredoxins (Balmer et al. 2003; Buchanan & Balmer 2005; Michelet et al. 2008; Montrichard et al. 2009). Methods have also been described for analyzing S-glutathionylated proteins (Gao et al. 2009). Although considered a key signalling molecule, H$_2$O$_2$ itself is not very reactive towards most cellular components. Apart from its high rates of metabolism through heme-containing enzymes such as catalases and peroxidases, it can react with protein thiols (SH) to produce sulphenic acid groups (SOH). Such a reaction is the first step in the catalytic cycle of PRX (Dietz 2003), and may occur on other proteins, if they contain a sufficiently reactive thiol. Affinity purification with a fusion protein containing the yeast transcription factor YAP1 has recently been used to identify more than 100 proteins that undergo this modification after exposure of Arabidopsis cell suspensions to H$_2$O$_2$ (Waszczak et al. 2014). It should be noted that protein thiol oxidation can also be catalyzed by specific enzymes such as plant cysteine oxidases, which play roles in oxygen sensing (Weits et al. 2014). As yet, the importance of such enzymes in ROS signalling is not clear.

Keen interest has focused on protein cysteine S-nitrosylation (Romero-Puertas et al. 2007, 2008). However, few studies have thus far reported on quantitative changes in S-nitrosylation of proteins as a result of oxidative stress in planta. One issue is that it can be difficult to preserve in vivo protein modifications throughout the sample preparation. Likewise, high background signals may be a problem using current techniques. Issues such as these may compromise reproducibility between biological replicates. As noted above for other types of measurement, this may relate to the complexity of the redox network of plant tissues, with high levels of ascorbate (which can efficiently reduce protein-SNO groups) being one example of an interfering compound. For these reasons, unicellular algae or cell cultures have generally been the preferred material for these kinds of studies (Astier et al. 2012).

The difficulty of quantifying the proportion of a given protein that is undergoing post-translational oxidation may be resolved by the use of differential labelling of oxidized and reduced cysteines using ICAT (isotope coded affinity tag). This approach was recently used to quantify oxidised cysteines in
more than 300 different proteins (Rosenwasser et al. 2014). As in many other studies, most of the proteins were involved in primary metabolism and, therefore, relatively abundant. The inability to detect low-abundance proteins is a key issue hindering the application of proteomics techniques to identify redox-sensitive signalling proteins. Nevertheless, future technical advances in these areas are likely to be a key development in understanding cellular signalling triggered by oxidative stress. For the moment, such approaches remain rather expensive and laborious. While they are crucial to pierce the complexity of oxidative stress signalling, they are not as yet well suited as convenient markers for oxidative stress in plant tissues.

In addition to proteins, DNA and RNA can also undergo oxidative modifications (Dizdaroglu et al. 2002). Guanine bases can be oxidised to 7,8-dihydro-8-oxoguanine (8-oxo-G), promoting mismatched pairing with adenine. One study reported accumulation of 8-oxo-G during seed ageing, and that overexpression of the repair enzyme, OGG1, mitigated the effects of ageing and also conferred enhanced resistance of seeds to oxidative stress (Chen et al. 2012). Double mutants lacking OGG1 and another enzyme involved in 8-oxo-G repair showed increased DNA damage, and both single and double mutants also lost germination capacity more quickly than wild-type seeds during ageing (Cordoba-Canero et al. 2014). Guanine bases in RNA can also undergo this modification. Dry seeds accumulate stored mRNA, facilitating rapid production of proteins on dormancy breakage (Rajjou et al. 2004). Interestingly, specific stored transcripts can undergo oxidation in sunflower seeds, leading to altered translation efficiency, an effect that may be involved in the regulation of germination capacity during seed storage (Bazin et al. 2011). Accumulation of 8-oxo-G increased with oxidative stress intensity when transcripts were exposed in vitro to conditions generating the hydroxyl radical, and analyses of extracted mRNAs from seeds showed the 8-oxo-G content was inversely correlated with dormancy (Bazin et al. 2011).

A small increase in 8-oxo-G was also reported in leaves of catalase-deficient plants grown in oxidative stress conditions, but no increase was observed in a marker for DNA double strand breaks that can accompany this modification (Vanderauwera et al. 2011). Accurate estimation of products of oxidative modification of DNA is not trivial, and high basal levels of 8-oxo-G have been reported in plants as in other organisms, possibly due to DNA oxidation during extraction (Dizdaroglu et al. 2002; Bazin et al. 2011; Vanderauwera et al. 2011; Cordoba-Canero et al. 2014).

One enzyme associated with DNA repair that has received some attention is poly(ADP-ribose)polymerase (PARP), which modifies nuclear proteins using NAD$^+$ as a source of ADP-ribose units (De Block et al. 2005). Mutants lacking AtPARP2 showed decreased oxidative stress responses
at the transcript level, allied to up-regulation of other stress-associated pathways such as those
linked to abscisic acid (ABA; Vanderauwera et al. 2007). It was suggested that these effects were due
to a decreased drain on cellular NAD(H) pools with a resultant increase in the production of cyclic
ADP-ribose and associated calcium-dependent signalling (Vanderauwera et al. 2007). Another study
has drawn attention to possible redox regulation of PARP function by interactions with nuclear
glutathione status (Pellny et al. 2009). It should be noted that measurements of PARP and related
modifications are challenging, and thus the literature does not contain extensive documentation of
the role of these factors in oxidative stress in plants.

ROS reporter transcripts

Since 2001 there have been numerous reports on ROS-responsive transcriptomes, with initial studies
of the effect of H$_2$O$_2$ on Arabidopsis cell cultures (Desikan et al. 2001) followed by in planta studies
using ROS-generating agents, the singlet oxygen-generating flu mutant, or catalase-deficient plants
(op den Camp et al. 2003; Vandenabeele et al. 2003; Vanderauwera et al. 2005; Gadjev et al. 2006;
Vandenbrouke et al. 2008; Mhamdi et al. 2010b; Queval et al. 2012). One advantage of this approach
is that it provides a more comprehensive picture of the cellular response to oxidative stress than
metabolite profiling or proteomics. The most obvious limitation is that transcriptomics does not
provide direct insight into function. Nevertheless, oxidative stress transcriptomes have allowed
identification of several transcription factors and enzymes that have subsequently been shown to
play key roles in stress responses (Davletova et al. 2005b; Tognetti et al. 2010). They have also
allowed identification of useful marker transcripts for assessing oxidative stress intensity in plants
under different conditions.

Several studies have drawn attention to the possibility that each form of ROS produces a specific
transcriptome. For example, different sets of genes were found to be induced in conditions of singlet
oxygen accumulation compared to those in which oxidative stress is linked to superoxide or H$_2$O$_2$ (op
den Camp et al. 2003; Gadjev et al. 2006). Assuming this is the case, specific transcript markers
would be very useful tools for assessing the relative contribution of different ROS forms to stress
responses. Catalase-deficient plants, in which the oxidative trigger is primarily peroxisomal H$_2$O$_2$, do
not produce the same signature as the flu mutant, which overproduces singlet oxygen in the
chloroplast (Gadjev et al. 2006). It is less clear whether this is a qualitative difference since at least
some stress-related genes are strongly induced in both systems (Noctor et al. 2014). Several factors
could make it difficult to unambiguously assign markers that are specific for each ROS form based
purely on comparison of model systems. The most obvious is possible differences in the intensity of
oxidative stress that is achieved in each system. In addition, subcellular location of oxidant production and possible site-specificity of signalling pathways could be at least as important, even for a given ROS type (Gutierrez et al. 2014). Another complicating factor could be plant history, meaning that transcriptomes would have to be compared in plants that are grown side-by-side under identical conditions. One factor is growth day length, which modulates the phenotype and transcriptome signature of the cat2 mutant (Queval et al. 2012), and also influences the response of wild-type Arabidopsis to equal-time exposure to ozone (Dghim et al. 2013). This may reflect the fine-tuning of oxidant-induced gene expression by the prevailing metabolic status of the cell. In other words, oxidative signalling does not occur in a vacuum, and will be influenced by a host of nutritional and environmental inputs.

Despite these uncertainties, the expression of several genes at the transcript level can be measured as markers for oxidative stress in plants. Such genes are weakly expressed in optimal conditions and strongly induced by increased oxidation. Three transcripts we regularly measure as indicators of oxidative stress are detailed in Figure 6. All three of these transcripts are induced more than 10-fold in microarray analyses of catalase-deficient mutants compared to wild-type under photorespiratory conditions (Fig.6). An even stronger fold increase is usually detectable using RT-qPCR.

Few of the genes most strongly induced by oxidative stress on a fold-change basis encode members of the core H$_2$O$_2$-processing system (Table 1). This presumably reflects the relatively strong expression of these genes under all conditions. Nevertheless, many of them become even more strongly expressed under oxidative stress. Enhanced APX1 transcripts, encoding a cytosolic APX shown to be crucial in redox homeostasis (Davletova et al. 2005a), is a particularly reproducible response to oxidative stress and probably explains at least part of the increase in extractable APX activity in these conditions. Finally, transcripts involved in DNA damage repair (DDR) have been used as markers to assess this process in plants because, as discussed above, direct measurement of DNA modification remains challenging (Vanderauwera et al. 2011).

Concluding remarks and outlook

In this text, we intended to present an overview of many of the commonly employed and available methods to assess oxidative stress, antioxidative systems, and redox homeostasis in plants. Our aim was to provide a user guide, highlighting what we consider to be best practice, outlining validated methods, and explaining where some of the pitfalls lie with regard to accurate assay and interpretation. For this reason, we have provided detailed protocols of convenient and reproducible
methods. In our view, seeking to justify a particular procedure and data interpretation by emphasizing previous and extensive use is not in itself very convincing, and inadequate to allay concerns about specificity and accuracy. We therefore provide a few basic recommendations (Box 11). Although this advice is not intended to be exhaustive, following it can help avoid ambiguities in data analysis and interpretation.

The approaches one chooses to analyze oxidative stress will depend on the aims of the study. The reasons why plant biology researchers should be interested in oxidative stress and related processes are increasingly numerous. Just three examples of study aims are: (1) to investigate processes involved in regulating a specific component of cell redox state; (2) to gauge oxidative stress intensity (or the intensity of the plant response) to establish whether and to what extent a given genetic modification or condition is indeed generating oxidative stress; (3) to provide new insight into the workings of oxidative stress and redox regulation in plants. Such divergent albeit potentially overlapping study aims may call for different subsets of techniques. Hence, it is difficult to prescribe specific assays that are to be preferred as a universal “oxidative stress indicator”. We note that factors that are most central to oxidative stress may not necessarily be the best markers. One example is pyridine nucleotides, whose role at the very heart of pro-oxidant and antioxidant metabolism, as well as their importance in other cellular functions, may require stability. In our experience, factors that track phenotypic evidence of oxidative stress in a relatively reproducible fashion and that are readily quantifiable are ascorbate and glutathione status, extractable antioxidative enzyme activities, and marker transcripts. Using currently available techniques, changes in ROS themselves may be more difficult to detect and, especially, to quantify (Noctor et al. 2015).

We are keenly aware of the areas where technological advances are essential to drive the field forward. Many researchers are looking for user-friendly, inexpensive, and accurate assay systems, hence the popularity of kits. Unfortunately, many of the most difficult measurements can only be performed with specialized and expensive equipment. The challenge is to develop convenient kit-type procedures and probes for metabolites like lipid peroxides, post-translational modifications such as S-nitrosylation and S-glutathionylation, and ROS, without sacrificing accuracy or specificity. Alongside these developments, the emergence of new in vivo probes able to provide information at the subcellular level is crucial. So far, these have notably included the roGFPs that report on glutathione redox potential and the HyPer probe for H$_2$O$_2$ (Meyer et al. 2007; Schwarzländer et al. 2008; Esposito-Rodriguez et al. 2013). These approaches are useful but undoubtedly have their limitations, necessitating further refinements and the generation of innovative solutions that allow in vivo function to be probed in real time. One example of an emerging technique may be in-cell
nuclear magnetic resonance (NMR) analysis, which has recently been applied to probe the redox state of three proteins in human cell cultures in a non-invasive fashion (Mercatelli et al. 2015). Limitations related to extract-based proteomics may to some extent be circumvented by intensified use of bait proteins in situ. Such approaches may avoid some of the problems related to extraction or even protein abundance, although quantification of post-translational modifications remains a thorny issue. As yet, no master-switches have been defined in ROS-dependent oxidative stress signalling. However, recent developments in oxygen sensing (Gibbs et al. 2014) suggest that, if they exist, their identification is only a matter of time.

The continuing intensive research focus on ROS and related factors, together with advances in bioinformatics and artificial intelligence, is likely to allow context-specific transcript, protein and metabolite patterns to be closely defined. This will further boost the rapid development of new analytical methods. For example, lipid peroxide signatures and marker transcripts, if they are specific enough, may be a more reliable and convenient approach than attempting to measure singlet oxygen directly. Appropriate marker transcripts are widely used to probe signalling through several phytohormone pathways and, given the information now at our disposal, can be applied in a similar way to assessing oxidative signalling intensity. High coverage RNA sequencing (RNA seq) is likely to significantly advance the field. For example, identification of allelic differences in transcripts offers the possibility of discovering protein domains that may be important in ROS signalling. Together with analysis of epigenetic regulation of transcription, differences in DNA-binding proteins could be key to understanding ROS-linked redox control of gene expression. Further, RNA seq may help to elucidate as yet hypothetical interacting protein modules that could be important in sensing and interacting ROS-triggered redox signals (Foyer & Noctor 2016).

Another foreseeable development is in nanotechnology, perhaps leading to redox-sensitive in vivo reporters that can be used to sense specific components at organellar or even sub-organellar level. At the other end of the scale, it is increasingly likely that remote sensing approaches could be developed to measure the intensity of stress experienced by a crop in the field or plant in its natural environment. As we have emphasized here, reliable and informative markers are crucial to understanding how plants exploit and respond to oxidative stress in laboratory conditions, and translational application of such markers should greatly aid stress diagnostics in agriculture and environmental science. In a physiological rather than diagnostic setting, however, it is unlikely that any single measurement, however precise and sensitive it might be, will be sufficient in studies that are aiming to dissect the impact of oxidative stress on cellular function. This is because “oxidative stress” is a blanket term covering a nexus of cellular changes occurring at multiple levels. Research
over the coming years should identify the key nodes and more closely identify the interactions within this complex network.

Acknowledgments

CHF thanks BBSRC (UK; BB/M009130/1) for financial support. Current work in this area in the laboratory of GN is partly funded by the French Agence Nationale de la Recherche project ‘Cynthiol’ project no. ANR-12-BSV6-0011.
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the UDPglucosyltransferase UGT74E2 modulates Arabidopsis architecture and water stress tolerance.


Table 1. Genes encoding major antioxidative enzymes in Arabidopsis.

<table>
<thead>
<tr>
<th>Protein</th>
<th>AGI code</th>
<th>Subcellular localisation</th>
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<td><strong>Superoxide dismutase</strong></td>
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<td>Chloroplast/mitochondrion(^4,5)</td>
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</table>

Box 1. Extractable H$_2$O$_2$

A. Peroxidase-based assay

Grind freshly harvested material (60 mg) in liquid nitrogen and 5% PVP and extract in 2 ml 1M HClO$_4$. Once thawed, centrifuge at 4°C and 14000 rpm for 10 minutes. Add 100 µl of 0.2M phosphate buffer pH 5.6 to an aliquot of 0.5 ml of the supernatant and adjust to pH 5 using 3 M K$_2$CO$_3$. Centrifuge for 30 seconds to remove insoluble KClO$_4$. Incubate 50 µl of the neutralised extract for 10 minutes with 1 unit of ascorbate oxidase (AO) to oxidise ascorbate. 3-(dimethylamino) benzoic acid (DMAB) and 3-methyl-2-benzothiazoline hydrazone (MBTH) are prepared freshly each day. Add 870 µl of 0.1 M phosphate buffer (pH 6.5), 20 µl of 165 mM DMAB, 50 µl of 1.4 mM MBTH and 50 ng of peroxidase to the cuvette. Initiate the reaction by adding 50 µl of the extract. Monitor changes in A$_{590}$ at 25°C.

Prepare and read H$_2$O$_2$ standards ranging from 0 to 2 nmoles for each experiment. Measure at least in triplicates for each extract or H$_2$O$_2$ standards.

B. Luminol

Grind freshly harvested material (50-100 mg) in liquid nitrogen 1 ml of 0.2M HCl. On thawing, centrifuge at 4°C and 14000 rpm for 10 minutes. Add 100µl of 0.2M phosphate buffer pH 5.6 to an aliquot of 0.5ml supernatant and adjust to pH 5 using 0.2 M NaCl. Incubate 50 µl of the neutralised extract for 10 minutes with 1 unit AO (10 µl) to remove ascorbate. Prepare luminol and K$_3$Fe(CN)$_6$ reagents using NH$_3$ pH 9.5. In the luminometer tube, add the AO-treated extract (60 µl) to 490 µl of 0.2 M NH$_3$ pH 9.5 and 50 µl of 0.5 mM luminol and vortex. Add 100 µl of 0.5 mM K$_3$Fe(CN)$_6$, mix rapidly and read immediately for 2s. Perform assay in triplicates. Include H$_2$O$_2$ standards ranging from 0 to 5 nmoles for each experiment.

Remarks: Worthwhile basic checks for H$_2$O$_2$ assays are that the signal is proportional to extract volume in the cuvette, that it can be abolished by pre-treatment with catalase prior to assay, and that the response to H$_2$O$_2$ standards is not greatly altered by the presence of the extract.
Box 2. Catalase

Co-factor: Heme

Number of genes: 3 (Arabidopsis, rice, maize, tobacco, others)

Subcellular location: Peroxisomes, cytosol(?)

Sample preparation: Use freshly harvested tissue or samples or stored at -80°C for no longer than several months. Grind 100-150 mg fresh weight tissue in liquid nitrogen then add approx. 50 mg insoluble polyvinylpyrrolidone followed by 1.5 mL 0.1 M phosphate buffer, 1 mM EDTA (pH 7.5). Protease inhibitors may be included but in our hands do not affect obtained activities in several plant species. Continue to grind during thawing until a homogenous suspension is obtained. Samples may be withdrawn for chlorophyll determination if desired. Centrifuge at 4°C and 15000 rpm. Desalt 0.5 ml supernatant on NAP-5 columns pre-equilibrated with 0.1 M phosphate buffer, 1 mM EDTA (pH 7.5). Collect 1 ml as final preparation. Measure protein content in crude and/or desalted extract.

Assay (Figure 4): Add 20 µl H₂O₂ to 780-930 µl of 0.1 M phosphate buffer, 1 mM EDTA (pH 7.5) in a quartz cuvette at 25°C to give a final H₂O₂ concentration of 40 mM (the initial A₂₄₀ should be about 1.6 as ε₂₄₀ H₂O₂ at 240 nm is around 40 M⁻¹ cm⁻¹). Start reaction by addition of 50-200 µl desalted extract and monitor decrease in A₂₄₀ for 1-2 minutes.

Baseline leaf rates in unstressed (C₃) plants: 100-200 µmol.mg prot⁻¹ min⁻¹

Remarks: The activity is typically measured at supra-physiological H₂O₂ concentrations (Foyer and Noctor 2016), producing a rapid reaction that should be monitored promptly to ensure initial rates are obtained. Monitoring over the first 30 seconds should give enough data-points to obtain a reproducible rate. This may limit adaptation of catalase assays to plate readers. Otherwise, the reaction dies off as H₂O₂ is depleted. At longer assays times, bubbles can cause assay noise as O₂ comes out of solution. Alternative method: monitoring oxygen evolution from H₂O₂ polarographically. In theory, this allows a more specific measurement as H₂O₂ removal can also be performed by peroxidases. In practice, however, if the spectrophotometric assay is performed in desalted extracts at H₂O₂ concentrations that are super-saturating for peroxidase, their contribution is negligible compared to catalase.
Box 3. Ascorbate peroxidase

Co-factor: Heme

Number of genes: 9 (Arabidopsis)

Subcellular location: Chloroplast, Cytosol, Mitochondria, Peroxisomes

Sample preparation: As for catalase except that extraction medium and desalting buffers should contain least 1 mM ascorbate.

Assay (Figure 4): Add 50 µl of desalted extract and 50 µl of 10 mM ascorbate to 890 µl of 0.1 M phosphate buffer, 1 mM EDTA (pH 7.5) in a quartz cuvette at 25°C. Start reaction by addition of 10 µl of 20 mM H₂O₂ and monitor decrease in A₂₉₀ for 1-2 minutes ($\varepsilon_{290}$ ascorbate = 2800 M⁻¹cm⁻¹). Perform control experiment (with no extract) to correct for chemical oxidation by H₂O₂ and subtract from rates obtained with extracts.

Baseline leaf rates in unstressed plants: 200-2000 nmol.mg prot⁻¹ min⁻¹

Remarks: Ascorbate is included throughout the extraction and sample preparation to avoid inactivation of chloroplastic isoforms (Amako et al. 1994). By omission of ascorbate and pre-incubation with H₂O₂ for a defined period, much of the chloroplast activity can be removed, thereby providing information on activities in other compartments such as the cytosol (eg, Veljovic-Jovanovic et al. 2001).
Box 4. Monodehydroascorbate reductase

Co-factor: FAD

Number of genes: 5 (Arabidopsis)

Subcellular location: Chloroplast, Cytosol, Mitochondria, Peroxisomes

Sample preparation: Prepare extraction buffer freshly each day. Grind 250 mg of leaves in liquid nitrogen then add approx. 50 mg insoluble polyvinylpyrrolidine followed by 1 mL 50 mM MES/KOH buffer, pH6, 1 mM ascorbate, 40 mM KCl and 2 mM CaCl$_2$. Centrifuge at 14000 rpm and 4°C for 10 minutes. Immediately use the supernatant for enzyme activity assay.

Assay: Perform assay at 25°C. Mix 925 µl 50 mM HEPES pH 7.6, 10 µl 25 mM NADH (or NADPH), 10 µl 250 mM ascorbate and 50 µl extract. Start the reaction by adding 0.4 units of ascorbate oxidase (5 µl). Monitor the decrease in A$_{340}$ for 2-3 minutes. $\varepsilon_{340}$NAD(P)H = 6200 M$^{-1}$cm$^{-1}$.

Baseline leaf rates in unstressed plants: 200-500 nmol.mg prot$^{-1}$ min$^{-1}$

Remarks: MDHAR can use both NADH and NADPH. In our hands, NADPH-dependent rates are higher in Arabidopsis leaf extracts.
**Box 5. Dehydroascorbate reductase**

Co-factor: Cysteines

Number of genes: 3 (Arabidopsis)

Subcellular location: Chloroplast, Cytosol, Peroxisomes (Mitochondria?)

**Sample preparation:** As for catalase.

**Assay:** Add 50 µl 4 mM DHA and 25 µl 100 mM GSH to 905 µl 0.1 M phosphate buffer, 1 mM EDTA (pH 7.0) in a quartz cuvette at 25°C. Start reaction by addition of 20 µl of desalted extract and monitor decrease in $A_{265}$ for 2-3 minutes. $\varepsilon_{265}$ ascorbate $= 14000 \, M^{-1} \, cm^{-1}$. Perform control experiment (with no extract) to correct for chemical reduction of DHA by GSH by and subtract from rates obtained with extracts. A second control can be performed to estimate GSH-independent DHA reduction in the presence of extract, but rates of this reaction are normally very low in desalted extracts.

**Baseline leaf rates in unstressed plants:** 100-300 nmol.mg prot$^{-1}$ min$^{-1}$

**Remarks:** The chemical reaction is rapid and must be controlled for. It is substantially accelerated as pH increases because deprotonation of the glutathione thiol group to produce the reactive thiolate anion is favoured. Hence, the relative contribution of the enzymatic reaction can be increased by lowering the assay pH (eg, to 6.5).
Box 6. Glutathione reductase

Co-factor: FAD, cysteines

Number of genes: 2 (Arabidopsis)

Subcellular location: Chloroplast, Cytosol, Mitochondria, Peroxisomes

Sample preparation: As for catalase.

Assay (Figure 4): Add 10 µl of 10 mM NADPH and 100 µl of desalted extract 880 µl of 0.1 M phosphate buffer, 1 mM EDTA (pH 7.5) in disposable plastic cuvette at 25°C. Start reaction by addition of 10 µl of 50 mM GSSG and monitor decrease in $A_{340}$ for 2-3 minutes. $\varepsilon_{340}$NADPH = 6200 M$^{-1}$cm$^{-1}$.

Baseline leaf rates in unstressed plants: 50-200 nmol.mg prot$^{-1}$ min$^{-1}$

Remarks: This enzyme and its assay are generally stable and very reproducible. Because some plant groups have glutathione homologs rather than glutathione (eg, homoglutathione in legumes or hydroxymethylglutathione in grasses), low rates may be obtained when using classical GSSG (γ-Glu-Cys-Gly disulfide).
Box 7. NADPH-generating enzymes

Co-factor: Various

Example of enzymes: Ferredoxin-NADP⁺ reductase, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, NADP-malic enzyme

Subcellular locations: Chloroplast, Cytosol, Mitochondria, Peroxisomes

Example: G6PDH

Number of genes: 6 (Arabidopsis)

Subcellular location: Chloroplast, Cytosol, Peroxisomes

Sample preparation: About 100 mg of leaf tissue, harvested freshly or stored at -80°C, are ground in liquid nitrogen in the presence of 20 mg insoluble polyvinylpyrrolidine. Add 1 ml extraction media containing 0.05 M Tris-HCl buffer pH 8, 10 mM MgCl₂, 5 mM EDTA and 1 mM DTT. After thawing, clarify the sample by centrifugation at 4°C and 14000 rpm for 10 min.

Assay: Add 12 µl 10 mM NADP⁺, 50 µl 100 mM MgCl₂ and 100 µl extract to 808 µl 0.05 M Tris-HCl buffer pH 8, in disposable plastic cuvette at 25°C. Start reaction by addition of 30 µl of 100 mM G6P and monitor increase in A_340 for 5 minutes. εₑ₃₄₀NADPH = 6200 M⁻¹ cm⁻¹.

Baseline leaf rates in unstressed plants: 25-100 nmol.mg prot⁻¹ min⁻¹

Remarks: In our hands no difference was observed between desalted and undesalted extracts of Arabidopsis. Because chloroplastic isoforms are inhibited by disulfide reduction, the samples can be treated with 10-20 mM DTT for 30 minutes to separate chloroplastic activities from those in other compartments.
Box 8. Ascorbate

Sample extraction (acid extraction): Grind about 100 mg of leaf tissue to a fine powder in liquid nitrogen and extract into 1 ml 0.2 M HCl. Continue to grind continuously when the slurry starts to thaw and clarify by centrifugation at 4°C and 14000 rpm for 10 min.

Sample neutralization. Add an aliquot of 0.5 ml of supernatant to 100 µl 0.2 M phosphate buffer pH 5.6 and vortex. Adjust pH to 4~5 by adding small volumes of 0.2 M NaOH, vortexing each time. Neutralisation of 0.5 ml of leaf extract from Arabidopsis requires approximately 400 µl of 0.2 NaOH. The neutralised extract can be used to assay both ascorbate and glutathione. The assay is performed in triplicate using 96 well UV plates and a microplate reader.

Enzyme preparation: Prepare ascorbate oxidase in 0.2 M NaH$_2$PO$_4$ pH 5.6 to 40 U.ml$^{-1}$. Use 5µL of this preparation for assay.

Assays (Figure 5): Add 100 µl of 0.2 M phosphate buffer pH 5.6, 55 µl H$_2$O and 40 µl of neutralised extract to the plate wells, mix twice by programmed shaking and measure absorbance at A$_{265}$. After the first read add 5 µl ascorbate oxidase (40 unit.ml$^{-1}$) to the mixture, mix and read for 5 min. This procedure gives the ascorbate present as such in the plant (reduced form). To measure ascorbate and its oxidized form dehydroascorbate (DHA) as total ascorbate, treat separate extract aliquots with a DHA-reducing compound such as dithiothreitol. Mix an aliquot of 0.1 ml neutralized extract with 0.14 ml 0.12 M phosphate buffer pH 7.5 and 10 µl 25 mM DTT, and incubate at room temperature for 30 min. Assay triplicate aliquots of 40 µl incubated extracts as described for ascorbate.

Baseline leaf contents in unstressed plants: 2-10 µmol.g$^{-1}$ fresh weight, 80-95% reduced form.

Remarks: Contents can vary as a function of environmental conditions, most notably growth irradiance. Higher light promotes higher total ascorbate (Dowdle et al. 2007). Although we find acceptable recovery rates of added antioxidants in Arabidopsis (Queval & Noctor 2007), the extraction protocol may require modification for some material, eg, those containing high contents of phenolic compounds.
**Box 9. Glutathione**

**Sample extraction and neutralization:** As for ascorbate (Box 8)

Enzyme preparation: To prepare GR from the concentrated enzyme preparation, centrifuge an aliquot of ammonium sulphate suspension for 5 min at 4°C and resuspend the pellet in 0.2 M phosphate buffer, 10 mM EDTA pH 7.5 to a concentration of 20 U.ml\(^{-1}\).

**Assays (Figure 5):** Total glutathione (reduced glutathione (GSH) and oxidized glutathione GSSG) is directly measured using the extract prepared as for ascorbate (Box 8). Specific assay of GSSG requires removal of GSH, for example, by incubating extract aliquots with a GSH-complexing reagent such as 2-vinylpyridine (VPD). To measure GSSG, pre-incubate extracts (200 µl) as well as GSSG standards (0 to 80 pmol) with 2 µl VPD for 30 min at room temperature. Next, centrifuge the mixture twice at 4°C and 14000 rpm for 15 min. To measure total glutathione add 10 µl of neutralized extract to plate wells containing 0.1 ml 0.2 M phosphate buffer, 10 mM EDTA (pH 7.5), 60 µl H\(_2\)O, 10 µl 10 mM NADPH and 10 µl 12 mM 5,5-dithiobis 2-nitro-benzoic acid (DTNB, Ellman’s reagent), pH 7.5. Mix by automatic shaking of plate twice and add 10 µl glutathione reductase (GR, 0.2U) to the wells. Monitor increase in \(A_{412}\) for 5 min. \(\varepsilon_{412}\)DTNB is about 13600 M\(^{-1}\)cm\(^{-1}\) but rates are calculated relative to standards run concurrently. GSH standards (0 to 1 nmol GSH) should be run in triplicates. GSSG is measured using the same protocol used for total glutathione assay, but triplicate samples of 20 µl supernatant (produced after the final centrifugation following incubation with VPD) are assayed and the volume of water is adjusted to 50 µl. Pre-treatment of GSSG standards alongside extract aliquots, as described above, improves accuracy because it corrects, to some extent, for a progressive inhibitory influence of residual VPD in the assay on the reaction rate.

**Baseline leaf contents in unstressed plants:** 200-1000 nmol.g\(^{-1}\)fresh weight, 90-95% reduced form.

**Remarks:** Contents can be affected by growth irradiance albeit not usually as strongly as ascorbate. See Box 8 for comment on extraction method.
**Box 10. Pyridine nucleotides**

**Sample extraction**

Acid extraction: The oxidized forms NAD$^+$ and NADP$^+$ are extracted as described for ascorbate (Box 8), into 1 ml 0.2 M HCl. Basic extraction: About 100 mg leaf tissue is ground to a fine powder in liquid nitrogen and extracted into 1 ml 0.2 M NaOH. In both cases, the sample is homogenized continuously until thawing and clarified by centrifugation at 4°C and 14000 rpm for 10 min.

**Sample preparation**

Acid extract: Boil 0.2 ml extract supernatant for 1 min and cool rapidly on ice then add 35 µl of 0.2 M phosphate buffer (pH 5.6) and vortex. Adjust pH to 6-7 by addition of small volumes of 0.2 M NaOH, vortexing each time. Neutralisation of 0.2 ml leaf extract from Arabidopsis requires about 200 µl 0.2 M NaOH. Use the neutralised extract to assay oxidized forms (NAD$^+$ and NADP$^+$). Basic extract: Boil 0.2 ml extract supernatant for 1 min and cool rapidly on ice then add 35 µl of 0.2 M phosphate buffer (pH 5.6) and vortex. Adjust pH to 6-7 by addition of small volumes of 0.2 M HCl, vortexing each time. Neutralisation of 0.2 ml leaf extract from Arabidopsis requires approximately 200 µl 0.2 M HCl. Use the neutralised extract to assay reduced forms (NADH and NADPH).

**Enzyme preparation:** Prepare alcohol dehydrogenase (ADH) freshly each day by resuspending the powder in 0.1 M HEPES, 2 mM EDTA (pH 7.5) to 2500 U ml$^{-1}$. Prepare glucose-6-phosphate dehydrogenase (G6PDH) by centrifugation of an ammonium sulphate aliquot and by resuspending the pellet in 0.1 M HEPES, 2 mM EDTA pH 7.5 to 200 U ml$^{-1}$.

**Assays (Figure 5):** Assay NAD$^+$ and NADH in the acid and basic extracts, respectively. Add 20 µl of each neutralized extract to a plate well containing 100 µL 0.1 M HEPES, 2 mM EDTA (pH 7.5), 20 µL 1.2 mM 2,6-dichlorophenolindophenol (DCPIP), 10 µL 20 mM phenazine methosulphate (PMS), 10 µL ADH, and 25 µL H$_2$O. Mix by automatic shaking and start the reaction by addition of 15 µL of absolute ethanol. Monitor decrease in A$_{600}$ for 5 min. To assay NADP$^+$ and NADPH, add 20 µl aliquots of the neutralized extract (acid extraction for NADP$^+$ and basic extraction for NADPH) to plate wells containing 100 µL 0.1 M HEPES, 2 mM EDTA (pH 7.5), 20 µL 1.2 mM DCPIP, 10 µL 20 mM PMS, 10 µL 10 mM glucose 6-phosphate, and 30 µL H$_2$O. After automatic shaking, start the reaction by addition of 10 µL G6PDH. Monitor decrease in A$_{600}$ for 5 min.

**Baseline leaf contents in unstressed plants:** NAD(H), 20-80 nmol.g$^{-1}$ fresh weight, 10-20% reduced form. NADP(H), 10-60 nmol.g$^{-1}$ fresh weight, 40-60% reduced form.

**Remarks:** See Box 8 for comment on extraction method.
Box 11. Basic does and don’ts

GENERAL
While technical repeats are always advisable, where possible, independent biological replicates are required. Variation is usually greater between biological replicates than technical repeats. The contrary may indicate some problem in the assay. Statistical analysis should be performed on single values derived from technical repeats for each biological replicate. For example, if three technical repeats are done on three independent biological samples, analysis of significant difference should use n=3, not n=9.

If measuring a factor for the first time, consult reliable work in the literature to verify whether the values are realistic.

Rapid and appropriate sampling is needed to preserve the factor in quantities as close as possible to those found in vivo.

Avoid non-specific assays where possible.

ROS and REDOX METABOLITES
Standard curves, where employed, should be designed to cover the range found in the sample.

Perform recovery experiments, especially on new species or tissues (addition of known amounts of the measured metabolite to parallel samples).

Do not extract metabolites that can be produced or consumed by highly active enzymes in neutral buffers or solvents that are not sufficient to inactivate these enzymes.

Perform assays rapidly after extraction.

ENZYMES
Use an appropriate buffer for extraction and assay.

Do not assume that an assay that is appropriate for a highly purified enzyme can be used to measure the enzyme in a crude extract. Specificity is a particularly important issue for the measurement of low capacity enzymes.

Assays using boiled protein extract provide a classical control that the signal is fully enzyme-dependent, and should be used if in doubt.
Figure legends

**Figure 1.** Checkpoints before starting. Some of the issues to be considered in measuring factors associated with redox processes and the antioxidative system. Many of these general principles are widely applicable to many kinds of measurement, but are particularly pertinent to the measurement of oxidants, antioxidants, and related factors.

**Figure 2.** Potential difficulties in interpretation of zonal distribution of reactive oxygen species using popular *in situ* cell staining techniques such as nitroblue tetrazolium (NBT) and 3,3'-diaminobenzidine (DAB). As well as the specific complicating factors indicated on the right, differences in uptake and access at different zones could also hinder interpretation, particularly in the case of NBT, which can actually lead to superoxide generation, as shown in Figure 3.

**Figure 3.** One example of how *in planta* complexity may obscure the interpretation of cell staining procedures commonly used to detect ROS. Reduction of NBT to a blue formazan occurs via an intermediate radical (NBTH) from which the coloured compound is produced by dismutation. Although many enzymes and metabolites can reduce NBT, inhibition of colour formation by superoxide dismutase (SOD) is usually advanced as a proof that superoxide is the causative agent. However, the complexity of cellular redox pathways may distort interpretation, as noted by Fridovich (1997). For example, reduction of NBT by cellular compounds other than superoxide could produce the blue formazan via NBTH (top). Oxygen competes with conversion of NBTH to the formazan dye, a reaction that produces superoxide in a manner that is dependent on the presence of NBT (middle). Added SOD can favour this competing pathway by displacing the equilibrium towards superoxide production and, therefore, NBT formation. Hence, inhibition of colour formation by SOD does not necessarily indicate that superoxide is the primary agent driving formazan formation or even that this ROS is accumulating to significant levels in the absence of NBT. Further, any superoxide that is artefactually generated may reduce NBT and contribute to the colour formation.

**Figure 4.** Back to basics. Examples of simple spectrophotometric assays of three major antioxidative enzymes highlighting the principles of measurement and calculation. Note the relationship between the extinction coefficients and the typical activities of the three enzymes.

**Figure 5.** Principles of assays of ascorbate, glutathione, thiols, and pyridine nucleotides. Assays are shown in simplified form. Acetald, acetaldehyde AO, ascorbate oxidase. ADH, alcohol dehydrogenase. ASC, ascorbate (reduced form). DCPIP, dichlorophenolindophenol. DHA,

**Figure 6.** Validated examples of H$_2$O$_2$-inducible transcripts. Data are from the microarray analyses reported by Queval *et al.* (2012). Primers that can be used for qRT-PCR are given for each gene. Numbers above the bars indicates fold-change in the catalase-deficient cat2 mutant relative to Col-0.
Figure 1. Checkpoints before starting. Some of the issues to be considered in measuring factors associated with redox processes and the antioxidative system. Many of these general principles are widely applicable to many kinds of measurement, but are particularly pertinent to the measurement of oxidants, antioxidants, and related factors.

<table>
<thead>
<tr>
<th>Factor to be measured</th>
<th>Plant material and sampling</th>
<th>Sample preparation</th>
<th>Assay procedures</th>
<th>Data processing and validation</th>
<th>Interpretation</th>
<th>Conclusions</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>- Are growth conditions appropriate for the question to be addressed?</td>
<td>- Is the extraction medium appropriate (pH, temperature, compounds required for stability)?</td>
<td>- Is the assay sufficiently specific, accurate and sensitive?</td>
<td>- Are the calculations correct? Check extinction coefficients, dilution factors, etc.</td>
<td>- Are statistics used appropriately? Avoid treating technical and biological repeats as on the same level.</td>
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<td>- Are there enough biological replicates and not just technical repeats?</td>
<td>- Does the sample preparation procedure remove enzymes or metabolites that may interfere with the assay?</td>
<td>- Is optimization required (eg, recovery experiments for metabolites)?</td>
<td>- Are the data expressed relative to some physiologically relevant factor (tissue mass, protein, chlorophyll, etc)?</td>
<td>- Have the limitations of the data being adequately taken into account? Compartmentation, differences between enzyme activity in vitro and in planta, between pool size and rates of synthesis, between transcript abundance and protein level, etc</td>
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<td></td>
<td>- Is sampling rapid enough to prevent alteration of the factor from its state in the plant?</td>
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<td>- Is the response linear to the amount of extract in the assay?</td>
<td>- How will the validity of the values be verified? Check against reliable literature values if these are available.</td>
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</table>

- Biological systems are complicated and interpretation of biological data is far from trivial: Occam’s razor can be a very blunt instrument!
<table>
<thead>
<tr>
<th>Tissue zones</th>
<th>Common interpretation</th>
<th>Complicating factors</th>
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<tbody>
<tr>
<td>NBT</td>
<td>Zone of superoxide accumulation</td>
<td>NBT not specific for superoxide (e.g., could be differences in ascorbate or dehydrogenases). Different oxygen concentrations could also influence the reaction.</td>
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<tr>
<td>DAB</td>
<td>Zone of H$_2$O$_2$ accumulation</td>
<td>Higher peroxidase activity could favour increased staining even at the same H$_2$O$_2$ concentration, depending on which of the two factors is rate-limiting</td>
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</tbody>
</table>

**Figure 2.** Potential difficulties in interpretation of zonal distribution of reactive oxygen species using popular *in situ* cell staining techniques such as nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB). As well as the specific complicating factors indicated on the right, differences in uptake and access at different zones could also hinder interpretation, particularly in the case of NBT, which can actually lead to superoxide generation, as shown in Figure 3.
Figure 3. One example of how in planta complexity may obscure the interpretation of cell staining procedures commonly used to detect ROS. Reduction of nitroblue tetrazolium (NBT) to a blue formazan occurs via an intermediate radical (NBTH) from which the coloured compound is produced by dismutation. Although many enzymes and metabolites can reduce NBT, inhibition of colour formation by superoxide dismutase (SOD) is usually advanced as a proof that superoxide is the causative agent. However, the complexity of cellular redox pathways may distort interpretation, as noted by Fridovich (1997). For example, reduction of NBT by cellular compounds other than superoxide could produce the blue formazan via NBTH (top). Oxygen competes with conversion of NBTH to the formazan dye, a reaction that produces superoxide in a manner that is dependent on the presence of NBT (middle). Added SOD can favour this competing pathway by displacing the equilibrium towards superoxide production and, therefore, NBT formation. Hence, inhibition of colour formation by SOD does not necessarily indicate that superoxide is the primary agent driving formazan formation or even that this ROS is accumulating to significant levels in the absence of NBT. Further, any superoxide that is artefactually generated may reduce NBT and contribute to the colour formation.
**Figure 4.** Back to basics. Examples of simple spectrophotometric assays of three major antioxidative enzymes highlighting the principles of measurement and calculation. Note the relationship between the extinction coefficients and the typical activities of the three enzymes.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction</th>
<th>Extinction Coefficient</th>
<th>Activity Calculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>2 H₂O₂ + O₂</td>
<td>ε₂₄₀ = 40 M⁻¹cm⁻¹</td>
<td>Activity = 0.2 x 10⁶/40 x 1/0.1 x 1.5/0.5 x 10/9 = 167 µmol mg⁻¹ prot min⁻¹</td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td>2 GSH + NADP⁺</td>
<td>ε₃₄₀ = 6200 M⁻¹cm⁻¹</td>
<td>Activity = 0.02 x 10⁶/6200 x 1/0.1 x 1.5/0.5 x 10/9 = 108 nmol mg⁻¹ prot min⁻¹</td>
</tr>
<tr>
<td>Ascorbate peroxidase</td>
<td>2 H₂O₂ + 2 MDHA</td>
<td>ε₂₉₀ = 2800 M⁻¹cm⁻¹</td>
<td>Activity = 0.03 x 10⁶/2800 x 1/0.05 x 1.5/0.5 x 10/9 = 714 nmol mg⁻¹ prot min⁻¹</td>
</tr>
</tbody>
</table>

**Typical calculation**

100 mg Arabidopsis leaves, soluble protein content = 9 mg.g⁻¹FW. Extracted in 1.5 ml buffer and desalted as described in Box 2. Assayed in 1 ml final volume in a Cary spectrophotometer (path length = 1 cm).
Figure 5. Principles of assays of ascorbate, glutathione, thiols, and pyridine nucleotides (Queval & Noctor 2007). Assays are shown in simplified form. Acetald, acetaldehyde. AO, ascorbate oxidase. ADH, alcohol dehydrogenase. ASC, ascorbate (reduced form). DCPIP, dichlorophenolindophenol. DHA, dehydroascorbate. DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid). DTT, dithiothreitol. EtOH, ethanol. GSH, glutathione (reduced form). GR, glutathione reductase. GSSG, glutathione disulphide. 6-PGgL, 6-phosphoglucono-γ-lactone. G6P(DH), glucose-6-phosphate (dehydrogenase). PMS, phenazine methosulphate. VPD, 2-vinylpyridine. More details are given on methods in boxes 8-10.
Figure 6. Validated example of $\text{H}_2\text{O}_2$ inducible transcripts. Data are from the microarray analyses reported by Queval et al. (2012). Primers that can be used for qRT-PCR are given for each gene. Numbers above the bars indicates fold-change in the catalase-deficient cat2 mutant relative to Col-0.