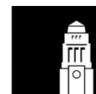
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Noctor, G, Mhamdi, A and Foyer, CH (2016) Oxidative stress and antioxidative systems: recipes for successful data collection and interpretation. Plant, Cell and Environment, 39 (5). pp. 1140-1160. ISSN 0140-7791

https://doi.org/10.1111/pce.12726

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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.

34 **Introduction**

35

36 Current concepts of oxidative stress and related redox signalling in plants depend on accurate,
37 unambiguous measurement of a portfolio of relevant factors. These notably include reactive oxygen
38 species (ROS), redox metabolites, enzyme activities, protein modifications, and transcript levels.
39 While no single measurement gives a complete picture, each one contributes specific and potentially
40 useful information. Figure 1 highlights some of the major points to be considered before undertaking
41 analysis of redox-related factors. Many of these issues are generally applicable to experimental plant
42 biology but some of them are particularly crucial in studies of redox processes. As discussed in more
43 detail below, crucial issues include sampling, extraction, and assay specificity. This is because redox-
44 linked factors can be highly reactive and vulnerable to chemical reaction and alteration during
45 isolation and assay. A further complicating factor is that many of the enzymes that produce and
46 process redox compounds in plants have a very high capacity. As a general rule, the key players in
47 determining redox state (eg, H_2O_2, ascorbate, NADPH, thioredoxins) may have turnover times on the
48 order of seconds. The culture of due care and attention to this last point is perhaps less widely
49 developed in oxidative stress research than in other related areas such as photosynthesis.

50

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

59

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

65

66

67 **Approaches for assessing oxidative stress responses**

68

69 Oxidative stress is part of many responses to the environment (Miller *et al*. 2008), and a range of
70 approaches has been applied specifically to assess tolerance to enhanced cellular oxidant production
71 and to elucidate the mechanisms underlying the responses. Approaches include direct addition of
72 relatively stable ROS such as H_2O_2, use of ROS-generating reagents, and exploitation of genetic
73 backgrounds lacking antioxidant functions. Each of these approaches has advantages and drawbacks.

74

75 *Elevated ozone*

76

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

91

92 *Direct addition of H_2O_2 to cells and tissues*

93

94 This is a very common approach that has been widely employed, particularly in cells in culture. It has
95 the advantage of simplicity but complications can arise because of the rapid metabolism of H_2O_2
96 even before it enters the cell (Desikan *et al.* 2001). Like ozone, this treatment probably largely mimics
97 an apoplast-localized oxidative burst, and is less likely to simulate the action of metabolically
98 generated H_2O_2 inside the cell. This point may be even more relevant when H_2O_2 is applied to whole
99 tissues. One-time or intermittent spraying may produce relatively little effect unless concentrations

100 are high (>1 mM), and elevated H_2O_2 concentrations experienced by the plant tissue are likely to be
101 transient.
102
103 Another common approach involves the inclusion of H_2O_2 in the agar plates on which plants are
104 grown *in vitro*. If the H_2O_2 concentration is high enough, this may provide a sustainable oxidative
105 stress, and can clearly inhibit germination, root development and plant growth. Again, however,
106 these effects may be due to relatively localized extracellular events, and the primary site of action is
107 very likely restricted to the roots. Moreover, interpretation may be complicated by the presence of
108 metal ions in the media and apoplastic space, which will generate hydroxyl radicals through Fenton
109 chemistry, potentially producing a more severe oxidative stress. Addition of transition metals like
110 iron or copper together with H_2O_2 and a metal reductant such as ascorbate can be used to enhance
111 production of the hydroxyl radical, if this is the desired effect (eg, Foreman *et al*. 2003).
112
113 *ROS-generating reagents*
114
115 Many approaches of this type benefit from the advantage that ROS production depends on cellular
116 metabolism, and therefore oxidative stress can be more appropriately targeted to intracellular
117 locations. Paraquat (methyl viologen) is one of the most reliably targeted because its action is closely
118 associated with photosynthetic electron transport. Although it is also exploited in studies on animal
119 cells, and can stimulate superoxide production by the mitochondrial electron transport chain, its
120 main effect in plants (at least at irradiances sufficient to drive photosynthetic electron transport) is to
121 catalyze reduction of oxygen at the level of the highly reducing acceptor side of photosystem I. This
122 produces an oxidative stress whose origin is light-dependent and considered to be largely localized to
123 the chloroplast. Menadione (vitamin K_3) is another redox-cycling superoxide-generating reagent,
124 which can be reduced by various cellular dehydrogenases, including mitochondrial complex I.
125 Antimycin A, an inhibitor of ubiquinol oxidation by the cytochrome bc_1 complex (complex III) has
126 been applied to promote superoxide generation in the mitochondria (Maxwell *et al*. 1999; Rhoads *et*
127 *al*. 2006). Although all these reagents promote the production of superoxide, this initially generated
128 ROS should be rapidly converted to H_2O_2 by superoxide dismutase (SOD).
129
130 Another convenient way of generating H_2O_2 is by using enzymatic systems, such as added glucose
131 oxidase/glucose. Unless the glucose oxidase is taken up by the cell, production of H_2O_2 by this system
132 will be extracellular. However, transgenic model systems expressing glucose oxidase have been
133 exploited, for example, to study pathogenesis responses (Kazan *et al*. 1998). Ectopic expression of

134 peroxisomal glycolate oxidase in the chloroplast has also been developed as a targeted system to
135 study the effect of increased H_2O_2 production in the stroma (Fahnenstich *et al*. 2008).
136
137 *Singlet oxygen*
138
139 Unlike production of the other major ROS, generation of singlet oxygen is a physical rather than
140 chemical reaction, ie, it is the result of the transfer of energy rather than electrons to the ground-
141 state (triplet) dioxygen molecule. Several reagents can drive increases in singlet oxygen by
142 stimulating this process in a light-dependent manner (Fischer *et al*. 2013). These include rose bengal,
143 methylene violet, neutral red, and indigo carmine. While all produce singlet oxygen following their
144 photoactivation, they do so with different efficiencies and also accumulate in different parts of the
145 cell (Kovacs *et al*. 2014; Gutierrez *et al*. 2014). Some of these reagents may have other effects as well
146 as direct photosensitization of singlet oxygen production. For instance, rose bengal can inhibit
147 photosynthetic electron transport (Kovacs *et al.* 2014). The most physiologically relevant feature of
148 these reagents may be in mimicking endogenous photosensitizers (eg, chlorophyll precursors and
149 degradation products) that can promote singlet oxygen formation.
150
151 The Arabidopsis *fluorescence* (*flu*) mutant, which conditionally produces singlet oxygen due to
152 accumulation of the chlorophyll precursor, protochlorophyllide, is a widely exploited genetic tool
153 (Meskauskiene *et al*. 2001; op den Camp *et al*. 2003; Ramel *et al*. 2012). Despite the profound
154 interest of studies on this system, it should be noted that singlet oxygen is probably not generated
155 within photosystem II (PSII) in the *flu* mutant but rather at other sites within chloroplasts. Finally, any
156 inhibitor of the photosynthetic electron transport chain that acts downstream of PSII (eg, DCMU,
157 DBMIB) will increase the probability of triplet chlorophyll formation and hence be expected to favour
158 singlet oxygen production through back- and side-reactions within PSII.
159
160 *Knocking down antioxidative defenses*
161
162 Numerous studies have been conducted on plants deficient in antioxidative enzymes with the
163 primary aim of evaluating the functional importance of the targeted enzyme. In addition, some
164 plants with compromised antioxidative systems can be useful as a model oxidative stress
165 background. Perhaps the most intensively studied systems are conditional mutants deficient in
166 catalase (Dat *et al*. 2001). Advantages of using such plants are that H_2O_2 production can be switched

on and off by manipulating light intensity or CO_2 levels, and that as long as photorespiratory glycolate oxidase remains active, the H_2O_2 production can be sustained (Dat *et al*. 2001).

Catalase is one of two major routes for removing intracellular H_2O_2, 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 H_2O_2 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 H_2O_2 since it shows that producing this ROS through a physiologically relevant pathway (photorespiration) generates different effects to stressing plants by simply adding H_2O_2 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

202 concept is debatable, because ROS probably do not accumulate uniformly across the cell and may
203 increase at sites whose function requires oxidation (Foyer & Noctor 2016). Hence, ROS accumulation
204 may not actually be stressful. Apart from the relatively oxidized status of compartments such as the
205 intrathylakoid space, endoplasmic reticulum lumen, vacuole, and apoplast, recent work has reported
206 that the chloroplast stroma extensions known as stromules may play an important role in the spatial
207 transfer of oxidizing molecules and, hence, in oxidative signaling (Caplan *et al*. 2015).
208
209 Measurements of ROS are often required for publication of papers that study oxidative stress.
210 However, questions remain concerning the data that are produced by many of the available
211 methods. Such methods can be divided into three broad categories: (1) monitoring of ROS produced
212 in the solution surrounding the system, most often applied to the study of cell cultures; (2) staining
213 methods that can be used *in situ* on plant tissues; and (3) procedures that aim to quantify ROS
214 contents in tissue through extraction and *in vitro* assay. Within all these categories, many methods
215 are available, and their number is increasing all the time. However, most if not all of these
216 approaches are subject to artefacts and/or limitations, particularly if not employed correctly, and
217 caution must always be exercised in data interpretation. While newly reported probes often show
218 encouraging increases in specificity, potential problems persist relating to the stability of ROS,
219 interference by other metabolites and processes, and the specificity of the reaction in the assay.
220
221 *Monitoring ROS release into the medium*
222
223 Such methods are particularly applicable to the analysis of ROS generated by cells in suspension.
224 Although difficult to use on whole plants or some plant organs, they have been applied to seeds or to
225 pieces of tissue such as leaf discs. Examples are the H_2O_2 assay based on oxidation of scopoletin
226 catalyzed by exogenous peroxidase, and oxidation of 2',7'-dichlorofluorescin (H_2DCF) to fluorescent
227 2',7'-dichlorofluorescein (DCF; Schopfer *et al*. 2001; Chong *et al*. 2002). While a general principle of
228 metabolite assays is that enzyme-dependent reactions ensure a degree of specificity, attention has
229 nonetheless been drawn to interference in the scopoletin-peroxidase assay (Corbett 1989). It should
230 also be noted that such measurements do not provide precise information on either tissue contents
231 of ROS or on their rates of production. Rather, the signal will depend on the amount of oxidant that
232 escapes into the solution. Hence, the assay should only detect those ROS that elude the antioxidant
233 system. Even if this is only a small fraction of the ROS produced, the data are informative as a relative
234 measure if the signal is proportional to the rate of production.
235

236 *Examples of common probes for* in situ *analysis of ROS*

237

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

250

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

263

264 In addition to colour stains, many fluorescent probes are available for measuring ROS (Wardman
265 2007; Kalyanaramana *et al*. 2012; Winterbourn 2014). As mentioned above, H_2DCF has been
266 employed to monitor ROS release from cells or tissues: this probe has also become accepted as an *in*
267 *situ* stain for ROS. Two points must be considered in using such approaches. The first is that the
268 oxidation that leads to the signal is not mediated by H_2O_2 directly. It can be achieved by the hydroxyl
269 radical produced from H_2O_2 or, like NBT and DAB, by enzyme-dependent reactions (Kalyanaramana *et*
270 *al.* 2012). A second point is that the diacetate form is considered to aid uptake of H_2DCF-DA into

271 cells, where conversion to DCF is considered to report on intracellular oxidants. However, plant cells
272 contain high activities of apoplastic esterases (Haslam *et al.* 2001; Cummins & Edwards 2004), and
273 their action may mean that the DCF signal is largely extracellular, which can be problematic if the
274 analytical resolution is not sufficient to distinguish signals at the subcellular level. The H_2DCF-DA
275 probe may be a semi-quantitative *in situ* indicator of ROS accumulation in organs such as roots.
276 Based on our experience, it is very difficult to use reproducibly in leaves, even with confocal
277 microscopy to enable subcellular resolution.
278
279 Other similar probes, such as 3'-*O*-Acetyl-6'-*O*-pentafluorobenzenesulfonyl-2'-7'-difluorofluorescein
280 (BES-H2O2-Ac), may be more specific for H_2O_2 and have been employed in plants (Maeda 2008;
281 Biswas & Mano 2015). Fluorescent probes have also been described for specific live-cell imaging
282 of lipid peroxides (Soh et al. 2007), a group of compounds that play important roles in
283 signalling downstream from primary ROS production (Farmer & Mueller 2013; Biswas & Mano 2015).
284 These compounds, and some of the others mentioned above, may generate important information. If
285 the aim is to assess oxidative stress intensity, however, it is probably advisable to compare at least
286 two of them, and to corroborate the signals by at least one of the other approaches discussed below
287 (eg, antioxidants, marker transcripts).
288
289 *Extraction of ROS for* in vitro *quantification*
290
291 The primary ROS (superoxide, H_2O_2, hydroxyl radical, singlet oxygen) are difficult to quantify reliably
292 in plant tissue extracts. Spin-traps, in which electron paramagnetic resonance (EPR) signals are
293 captured *in planta*, have been applied for the more reactive forms (Müller *et al.* 2009; Fischer *et al.*
294 2013). H_2O_2 is the most stable of these four compounds and, as a non-radical that is a substrate for
295 relatively well-defined enzymes, it is probably the only one that is considered to be quantifiable after
296 direct extraction. Nevertheless, the quantification of extracted H_2O_2 is far from trivial. As is the case
297 for other metabolites, the extraction procedure itself requires careful attention. We emphasize that
298 H_2O_2 should not be extracted into water or neutral buffer because this will allow enzymes to remain
299 active during the sample preparation. This point should be obvious given that such media are used to
300 extract highly active antioxidative enzymes like catalase. Extraction into acid or other media that
301 inhibit enzyme activities as well as many chemical redox reactions is essential, and experiments
302 should be carried out to check that H_2O_2 added at the early stage of extraction is recoverable in the
303 assay.
304

305 Other compounds present in plants can interfere with assays of extractable H_2O_2 (Veljovic-Jovanovic
306 *et al.* 2002; Queval *et al.* 2008). For this reason, it is advisable to check the linearity of the assay
307 response using a standard amount of H_2O_2 added together with the extract. Simply observing a linear
308 standard curve for H_2O_2 prepared in water or buffer is not sufficient, unless it is established that the
309 response is not affected by the presence of the extract. Such issues are clearly recognized in
310 metabolomics techniques such as LC-MS, where alteration of the detector response by the chemical
311 context is referred to as a « matrix effect ». Effects such as these, as well as an influence of the milieu
312 on stability during the extraction, are sometimes overlooked in apparently simpler targeted analysis
313 of specific compounds.
314
315 Another important point is that the activity be shown to be dose-responsive to the volume of extract
316 added in the assay. In our hands, using two different methods to measure H_2O_2, little effect of extract
317 volume on the signal was observed, meaning that the calculated tissue content was inversely
318 proportional to the amount of extract added to the assay (Queval *et al*. 2008). Since different studies
319 often employ different tissue mass:volume ratios during the extraction, this could be one of the
320 factors leading to the enormous range of basal tissue H_2O_2 contents in the literature, which might be
321 considered highly unusual for an important signalling compound. Other issues relating to the
322 contents and assays of H_2O_2 in plants have been recently discussed in more detail elsewhere (Noctor
323 *et al.* 2015; Foyer & Noctor 2016).
324
325 Box 1 describes methods we have employed for luminol and the peroxidase-coupled techniques for
326 quantification of H_2O_2 and peroxides in extracts. Other methods, such as Amplex Red or peroxidase-
327 coupled fluorescence, may also be applicable. However, as noted above, the key issue may not be
328 related to the assays but rather to the extraction and sample preparation. We also note that many of
329 the kits available for measuring ROS and related factors have been developed for use on organisms
330 other than plants, and may not take account of plant-specific problems related to extraction and
331 interference. One interfering compound in some peroxide assays is ascorbate, which is present in
332 high levels in tissues such as leaves (Veljovic-Jovanovic *et al.* 2002; Queval *et al.* 2008). For this
333 reason, we routinely treat extract supernatant with ascorbate oxidase to minimise concentrations of
334 this redox-active compound in the assay (Box 1).
335
336 Lipid peroxides are key indicators of oxidative stress and related signalling, and can be profiled by LC-
337 MS, with different signatures supplying some information on the source and location of ROS
338 (Davoine *et al*. 2006; Triantaphylidès *et al.* 2008). Because of its simplicity, the assay of thiobarbituric
339 acid-reactive substances (TBARS) remains an assay of choice for many laboratories. This assay gives

340 information on the breakdown products issuing from lipid peroxidation. Malondialdehyde (MDA) is
341 often used as a standard but the assay can detect a variety of reactive aldehydes. It usually gives
342 contents that are substantially higher than more specific assays of MDA (eg, Moselhy *et al.* 2013).
343 Moreover, MDA can be produced from sources other than lipid peroxides. Signals should therefore
344 more accurately be described as "TBARS" rather than "lipid peroxidation" or "MDA".

345

346 **Measuring extractable enzyme activities *in vitro***

347

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

351

352 Measurements of the maximal extractable activities of antioxidative enzymes have long been
353 recognized as indicators of cellular redox status. This approach is facilitated by the high activities of
354 the core antioxidative systems based around catalase and the ascorbate-glutathione pathway, a
355 feature that allows them to be readily measured using simple spectrophotometric techniques (Fig. 4;
356 Boxes 2-6). These enzymes have high capacities because they are involved in ROS-linked redox
357 cycling. As ROS production is an integral part of metabolism and can occur at high rates even in the
358 absence of stress, SOD and H_2O_2-processing enzymes are quite strongly expressed even under
359 optimal conditions. Lower-capacity enzymes, such as those involved in glutathione and ascorbate
360 synthesis, are more difficult to measure and require specialized techniques (Hell and Bergmann 1990;
361 Noctor *et al*. 1998; Dowdle *et al*. 2007). Moreover, developments over the last two to three decades
362 have led to the realization that enzymes exist whose primary physiological function is to generate
363 ROS, showing that these oxidants are not simply the product of "imbalances" in metabolism. As
364 discussed below, some of these activities are more difficult to reliably quantify than major
365 antioxidative redox-cycling enzymes.

366

367 *ROS-producing enzymes*

368

369 Over recent years, the best studied ROS-producing components have been the respiratory burst
370 oxidase homologue (RBOH) type of enzyme that oxidize NADPH to reduce O_2 to superoxide at the
371 plasmalemma and, possibly, other sites (Desikan *et al*. 1996; Torres *et al*. 2002; Foreman *et al*. 2003).
372 Despite extensive focus on these enzymes, their biochemical activities and capacities remain quite
373 poorly characterized. They are highly regulated by a plethora of post-translational modifications
374 involving such factors as protein phosphorylation, calcium ions, lipids, and interactions with other

375 proteins, eg, receptor-like kinases (Marino *et al.* 2012; Kadota *et al.* 2015). Moroever, AtRbohD,
376 perhaps the most studied of the plant NADPH oxidases, is *S*-nitrosylated on a specific cysteine
377 residue, leading to a loss of activity (Yun *et al.* 2011). Because most of these modifying factors will be
378 diluted or altered during sample preparation, it is difficult to relate NADPH oxidase activity in extracts
379 to what is happening *in vivo*. Another problem is that specific analysis of membrane-bound RBOH-
380 type NADPH oxidases requires lengthy preparation to remove other enzymes that could contribute
381 to the measurement in a non-specific way. Because of these issues, particular caution is required in
382 interpreting data obtained from *in vitro* measurements of NADPH oxidases.
383
384 In addition to the NADPH oxidase family, plant cells contain a battery of other ROS-producing
385 enzymes. Some of these are largely soluble and relatively high-capacity, and so they are easier to
386 measure. One example is glycolate oxidase. This enzyme may have several functions in plants, but it
387 acts most notably in the photorespiratory glycolate-recycling pathway. Unlike NADPH oxidases, the
388 primary function of such enzymes is not considered to be in ROS production. Rather, they generate
389 H_2O_2 as a by-product, although some contribution to ROS-signalling pathways cannot be discounted
390 (Foyer *et al.* 2009). Glycolate oxidase is the primary source of H_2O_2 in photorespiratory catalase
391 mutants. Other ROS-producing enzymes include xanthine oxidases, amine oxidases, and class III
392 (guaiacol-type) peroxidases (del Rio *et al*. 2006; Moschou *et al.* 2008; Angelini *et al.* 2010; O'Brien *et*
393 *al.* 2012). Enzymes of the last type may also have an antioxidant (H_2O_2-consuming) function and,
394 classically, were measured as such using model substrates such as guaiacol or pyrogallol. However,
395 over 70 genes are thought to encode these enzymes in Arabidopsis (Cosio & Dunand 2009), making it
396 rather difficult to link changes in measured activities to specific isoforms or gene products.
397
398 *Antioxidative enzymes*
399
400 Because their extractable activities are easy to quantify accurately (if correct procedures are
401 followed), assays of the major antioxidative enzymes can provide insight into plant responses to ROS
402 such as superoxide and H_2O_2. However, it is important to be clear on the limitations of the
403 information that is generated. It is well documented that oxidative stress triggers increases in the
404 total extractable activities of most major antioxidative enzymes, albeit usually to a modest extent
405 (1.5- to 5-fold are typical values). Increases in the extractable activities of these enzymes can,
406 therefore, be taken as indirect evidence of increases in ROS and/or oxidative stress in the plant at
407 some point prior to sampling. However, relative increases in enzyme activities cannot be used to
408 infer relative increases in ROS. Neither can *in vitro* activities easily be related to flux through the
409 corresponding reactions *in vivo*. There are several reasons for this. One is that the protocols are

410 designed to allow maximal activities to be observed, notably by using high or near-saturating
411 substrate concentrations. For example, GR is routinely assayed using 0.1 mM NADPH and 0.5 mM
412 GSSG (Box 6). Both concentrations are well above the respective K_M values of the enzyme (Smith *et*
413 *al*. 1989; Edwards *et al*. 1990) and, at least for GSSG, probably well above the *in vivo* concentrations
414 that the enzyme is likely to experience under most conditions (Meyer *et al*. 2007; Schwarzländer *et*
415 *al.* 2008). A second complicating factor is that, in nearly all cases, the *in vitro* activity of a given
416 antioxidative enzyme is a composite of the contributions of several isoforms encoded by a gene
417 family. Although the gene families of these enzymes are not very large, they do encode isoforms
418 present in multiple subcellular compartments, with the possible exception of catalase (Table 1).
419 Another issue concerns possible post-translational modifications that may modulate the activity *in*
420 *vivo* but whose influence is not detected in the measurement, either because the modification is not
421 stable throughout the sample preparation or because the assay is not able to detect its effect.
422
423 Despite the interest of these assays in probing oxidative stress and the cellular response, the
424 literature unfortunately contains many examples of values for extractable enzyme activities that are
425 obviously, or very likely, erroneous. While some aberrant values may be explained by typing or
426 production errors, others are probably caused by issues such as inappropriate experimental
427 procedures or poor data processing. It is obviously important to apply the correct extinction
428 coefficient to convert an absorbance change per unit time to moles per unit time. With specific
429 regard to catalase, it is worth noting that the absorbance of H_2O_2 at 240 nm is relatively weak, and so
430 the extinction coefficient (about 40 $M^{-1}cm^{-1}$) is around 100 times lower than those of substrates
431 monitored in other assays (eg, ascorbate or NAD(P)H; Fig. 4). One consequence is that conventional
432 techniques are not suited to assaying catalase at physiological concentrations of H_2O_2 (probably sub-
433 mM; Foyer & Noctor 2016). However, most assays performed on extracts of plant tissues are
434 basically giving a measure of capacity, not *in vivo* activity. Hence, a concentration is routinely used
435 that is both easily detectable in a spectrophotometer and high enough to drive catalase activities at
436 appreciable rates (eg, 40 mM; Box 2). An added advantage of using such high concentrations is that
437 any contribution to H_2O_2 removal in the assay from peroxidases will be a negligible proportion of the
438 overall rate. Even if such enzymes were working at maximal rates (which should not be the case, if
439 extracts are desalted and H_2O_2 is the only added substrate), their *capacities* are orders of magnitude
440 below that of catalase (Fig. 4).
441
442 Model calculations for three commonly measured enzymes are shown in Fig. 4, based on typical
443 rates observed in Arabidopsis leaf extracts. Using the procedures described in Boxes 2, 3, and 6,
444 extractable catalase activity is about 100 times higher than APX which is in turn about 5-10 times

445 higher than GR. Similar ratios can be observed in leaf extracts from other C_3 plants such as tobacco
446 (eg, Dutilleul *et al*. 2003). In studies where several enzymes are measured, comparing the relative
447 values obtained for the activities is an appropriate first check for identifying potential problems.
448 While the relationship may vary somewhat between tissues and plants, or be influenced by growth
449 conditions, an approximate order of rates obtained in leaf extracts using typical protocols is as
450 follows: catalase>>>APX>MDHAR>DHAR>GR (Fig. 4 and Boxes 2-6). For example, if leaf activities are
451 being measured in wild-type plants and the calculated APX activity is 10 times higher than the
452 catalase activity, there is probably an error. Although the protocols detailed in boxes 2-6 are for
453 conventional spectrophotometers, they can be adapted for plate-readers (eg, Murshed *et al*. 2008).
454
455 In-gel activity stains can be used to assess individual isoforms of antioxidative enzymes after
456 separation by isolectric focusing or other electrophoretic techniques (eg, Zimmermann *et al.* 2006).
457 In theory, this approach allows closer definition of the antioxidative system, albeit in a less
458 quantitative way than assays of extractable activities. In practice, for many enzymes, the information
459 is often compromised by poor resolution on gels and diffuse staining. When such analyses do achieve
460 relatively high resolution, they often reveal multiple activity bands, sometimes far more than would
461 be expected from the number of predicted protein products. Thus far, there is little information that
462 allows the functional relevance of these observations to be assessed.
463
464 An alternative or additional approach is to measure protein abundance, although immunoblotting
465 does not provide information on activity and specific antibodies may not be easy to obtain if gene
466 sequences within a given family are highly similar. Particularly in cases where different isoforms are
467 difficult to resolve at the protein or enzyme level, quantification of transcripts may provide
468 information on the responses of the different members of the antioxidative system. Measuring
469 transcripts can provide greater sensitivity because they sometimes show a greater relative change
470 than the encoded enzyme activity, especially if the transcript is only one of several encoding the
471 proteins that contribute to an extractable activity. It should also be noted, however, that some
472 antioxidative enzymes are regulated at both transcriptional and post-transcriptional levels (Mittler &
473 Zilinskas 1994). Oxidative stress transcriptomics has revealed that quantification of responsive
474 transcripts encoding proteins outside the core high-capacity ROS-processing system may be a
475 sensitive approach to assessing the intensity of the oxidative stress response, a point we discuss
476 further below.
477
478 The antioxidative system is by no means restricted to the enzymes mentioned above. Superoxide
479 dismutase (SOD) is a key player and many other enzymes may also have important roles. These

480 include peroxiredoxins (PRX) and related enzymes that are, probably misleadingly, named
481 glutathione peroxidases (GPX; Iqbal *et al*. 2006; Navrot *et al*. 2006; Tripathi *et al.* 2009). Based on
482 genetic evidence, it is clear that at least some PRX play major roles in ROS metabolism *in vivo* (Awad
483 *et al*. 2015). While several PRX and GPX have been characterized as recombinant proteins *in vitro*,
484 there is much less information on how their extractable activities change in response to oxidative
485 stress. This is because many of these activities require interactions with regenerating proteins that
486 are largely prohibited in diluted extracts. To take the chloroplast stroma as an example, protein
487 concentrations *in vivo* are of the order of 200-400 mg ml^{-1}, based on a leaf protein content of 10-20
488 mg.g^{-1} FW, 50% of which is in packed into a stromal volume of about 25 μl g^{-1}FW (Heldt 1980). In
489 contrast, the protein concentrations of extracts prepared as in Box 2 will not greatly exceed 1 mg ml^{-1}.
490
491
492 Using assays such as peroxide-dependent NADPH oxidation in the presence of glutathione and GR,
493 "glutathione peroxidase" activity can be measured in extracts. However, this activity is usually
494 somewhat lower than that of APX and the enzymes responsible are not clearly identified. As well as
495 some contribution from GPX isoforms, which *in vivo* are probably preferentially coupled to
496 thioredoxins, glutathione oxidation may be the result of glutathione *S*-transferase (GST)-linked
497 peroxidase activity (Dixon *et al.* 2009). Total GST activity can be measured using model substrates
498 such as 1-chloro-2,4-dinitrobenzene (CDNB) to probe the conjugase function. Here, there are similar
499 problems to those associated with classical assays of class III peroxidases. First, it is difficult to infer
500 physiologically important information from an activity measured against a model substrate. Second,
501 GSTs are encoded by a large gene family (55 in Arabidopsis; Dixon *et al*. 2009). Nevertheless, as
502 detailed in the section below on marker transcripts, specific genes for *GSTs* are among those that are
503 most strongly induced, on a fold-change basis, in response to oxidative stress.
504
505 Apart from substrate concentrations and allosteric regulation by metabolites, post-translational
506 regulation of enzymes through covalent modification is a key mechanism controlling their activities *in*
507 *vivo*. To date, there have been relatively few in-depth reports of post-translational regulation of
508 antioxidative enzymes in plants. In *Chlamydomonas*, regulation of catalase activity has been linked to
509 the thioredoxin system (Shao *et al*. 2008). Several other studies have detected antioxidative enzymes
510 among proteins that interact with thioredoxins or that can undergo thiol modifications such as *S*-
511 nitrosylation (Balmer *et al*. 2003, 2004; Romero-Puertas *et al*. 2007, 2008; Rosenwasser *et al*. 2014;
512 Waszczak *et al*. 2014). In many cases, the *in vivo* significance of these processes remains to be more
513 closely defined. Although assays of extractable enzyme activities have long been adapted to assess
514 activation state related to post-translational modifications such as thiol-disulphide exchange and

515 phosphorylation (eg, Charles & Halliwell 1981; Kaiser & Huber 1997), little attention has as yet been
516 paid to such issues in relation to major antioxidative enzymes in plants. It is also important to note
517 that post-translational modifications may have biologically crucial effects that are independent of
518 any impact on enzyme activity. One example is phosphorylation of the yeast CuZnSOD, which drives
519 its relocation to the nucleus where it acts as a transcription factor (Tsang *et al*. 2014).
520
521 One antioxidant-linked enzyme that is clearly post-translationally regulated is γ-glutamylcysteine
522 synthetase (γ-ECS, also known as glutamate-cysteine ligase), which catalyses the first step of
523 glutathione synthesis and which is activated by oxidation of protein thiols (Hell and Bergmann 1990;
524 Hicks *et al.* 2007; Gromes *et al*. 2008). This mode of regulation presumably allows the enzyme to
525 rapidly achieve rates of glutathione synthesis that are appropriate to the prevailing cellular redox
526 state, for example, to boost glutathione production under conditions of oxidative stress. In crude
527 plant extracts, this enzyme is not easy to measure. The activity of the highly purified recombinant
528 enzyme can be accurately measured by a conventional coupled assay that monitors ATP hydrolysis.
529 Although data obtained by performing this assay on crude extracts can be found in the literature, the
530 values are orders of magnitude higher than reliable, specific assays of γ-EC production using HPLC-
531 fluorescence. This is probably because the measured activity is overwhelmingly due to other ATP-
532 hydrolyzing enzymes that are abundant in plant extracts and cannot, therefore, be described as "γ-
533 ECS activity".
534
535 **Antioxidants and metabolite markers**
536
537 Major antioxidant metabolites are key compounds that interact directly with ROS. With regard to
538 physiologically produced singlet oxygen at photosystem II, the most important are tocopherols and
539 carotenoids, which are located at high levels in plastid membranes, notably the thylakoids. While
540 they make some contribution to light harvesting, carotenoids are particularly important in avoiding
541 excessive accumulation of singlet oxygen through photodynamic quenching of excited chlorophyll
542 states (Ruban *et al*. 2012; Fischer *et al*. 2013). They can also chemically react with this ROS to yield
543 characteristic breakdown products that may be important in signalling (Ramel *et al.* 2012). Like lipid
544 peroxide profiles, these products may be useful as markers for singlet oxygen-induced oxidation.
545
546 The most abundant antioxidant metabolites in the soluble phase of cells are ascorbate and
547 glutathione. These compounds can chemically scavenge certain ROS and, as noted above, are co-
548 factors for several antioxidative enzymes that ensure peroxide processing (Foyer & Noctor 2011;

549 Smirnoff 2011). As well as acting as reductants that regenerate certain PRX (Tripathi *et al*. 2009),
550 ascorbate is the reducing co-factor for APX while glutathione is a substrate for some peroxidases and
551 may also contribute to ROS processing indirectly by participating in ascorbate regeneration from
552 dehydroascorbate (DHA), either chemically or in the reaction catalyzed by DHARs (Box 5). In addition
553 to work specifically focused on ascorbate or glutathione metabolism, numerous studies have
554 measured the stable oxidized and reduced forms of these two compounds as markers for cellular
555 redox state. This approach is more reliable and has some advantages over direct assays of ROS. First,
556 the major forms of ascorbate and glutathione are relatively stable during extraction, providing
557 appropriate sample preparation techniques are followed. Second, they can be specifically measured
558 using enzyme-dependent assays. Third, because they are in the frontline of ROS-processing, changes
559 in their redox states and/or contents can be taken as indicators of oxidative stress inside the cell. The
560 primary product of ascorbate oxidation (monodehydroascorbate, MDHA) is an unstable radical that
561 rapidly dismutates to ascorbate and DHA, and thus cannot be easily measured. However, EPR
562 techniques have been used to demonstrate increased MDHA signal in stress conditions (Veljovic-
563 Jovanovic *et al*. 1998).
564
565 Characteristic responses of the major antioxidants to enhanced intracellular H_2O_2 in leaves are
566 accumulation of total glutathione, mainly as glutathione disulphide (GSSG), and decreases in total
567 ascorbate (Noctor *et al*. 2015). However, stress-induced changes in ascorbate redox state can also be
568 observed, although often under different conditions from those that greatly influence the
569 glutathione pool (Marquez-Garcia *et al*. 2015). Although these pools are functionally coupled under
570 some conditions, they may also work more independently in other circumstances (Foyer & Noctor
571 2011). This presumably reflects the number of pathways potentially involved in the oxidation and
572 reduction of each of these antioxidants, and their relative engagement in different conditions.
573 Hence, one should not necessarily expect the status of ascorbate and glutathione to change in the
574 same way.
575
576 Like the measurement of antioxidative enzymes, assays of glutathione and ascorbate on whole tissue
577 extracts provide only a composite picture. The data do not necessarily report on the status of these
578 compounds in the compartments where ROS are produced. Both compounds are heterogeneously
579 localized within the cell, and oxidative stress can differentially affect their distribution. This has
580 become apparent through the application of techniques such as redox-sensitive green fluorescent
581 proteins (roGFPs) that provide compartment-specific information on the glutathione redox potential,
582 as well as *in situ* detection of ascorbate and glutathione by immunolabelling (Meyer *et al*. 2007;
583 Schwarzländer *et al*. 2008; Zechmann *et al*. 2008; Zechmann 2011). With respect to glutathione, the

584 studies using roGFP suggest that much of the GSSG that is detected in extracts do not reflect redox
585 states in the cytosol, chloroplasts, or mitochondria (Meyer *et al*. 2007; Schwarzländer *et al*. 2008).
586 This is probably the case even for the relatively low levels of GSSG found in samples from unstressed
587 plants. Marked accumulation of GSSG in oxidative stress conditions partly reflects its sequestration in
588 the vacuole (Queval *et al*. 2011), probably due to import from the cytosol by tonoplast transporters
589 (Noctor *et al*. 2013).
590
591 Good oxidative stress markers are likely to be factors that have relative stability because they are the
592 result of modifications that are not rapidly reversed. As noted above, ROS will only accumulate
593 strongly if they are located in compartments with low antioxidative activity. This situation may be
594 rare in many intracellular locations, although more common in the vacuole and endoplasmic
595 reticulum. Accumulation of GSSG in compartments like the vacuole, as a secondary product of
596 enhanced ROS production, may be a key process allowing glutathione to be used as a marker.
597 Without such sequestration, GSSG would be recycled by the action of GR and its accumulation would
598 be much more difficult to detect. A similar consideration may apply to ascorbate. The oxidized form,
599 DHA, is thought to be largely located in the apoplast, where ascorbate oxidase is found and
600 ascorbate-regenerating capacity is lower than inside the cell (Pignocchi & Foyer 2003). The best
601 characterized ascorbate degradation pathway proceeds from DHA and is also localized in the
602 apoplast (Parsons & Fry 2012). GSSG present in the vacuole will also be degraded (Grzam *et al*. 2007),
603 although this seems not to be fast enough to prevent its accumulation during oxidative stress
604 (Queval *et al*. 2011).
605
606 Similarly to transcriptomics (discussed further below), non-specific metabolite profiling using
607 techniques such as gas chromatography-mass spectrometry (GC-MS) can reveal a signature indicative
608 of an "oxidative stress metabolome". This signature includes accumulation of several compounds
609 implicated in ascorbate and glutathione synthesis and degradation pathways, as well as
610 phytohormones that are known to be involved in oxidative stress signaling, such as salicylic acid and
611 jasmonic acid (Noctor *et al*. 2015). It is noteworthy that the signature also includes several
612 compounds that are not obviously connected to antioxidant metabolism or redox homeostasis
613 (Noctor *et al*. 2015). For example, the accumulation of certain amino acids, respiratory
614 intermediates, and aromatic compounds is indicative of the switch in metabolism that underpins the
615 oxidative stress syndrome, perhaps triggered in part by post-translational redox regulation of
616 enzymes (Rosenwasser *et al*. 2014) as well as by processes such as enhanced proteolysis. Based on
617 studies that have manipulated the glutathione synthesis pathway directly by altered γ-ECS expression

618 or indirectly by oxidative stress, glutathione status may be important in linking oxidative stress to
619 altered amino acid contents (Noctor *et al*. 1998; Han *et al*. 2013).
620
621 Pyridine nucleotides are central to both pro-oxidant and antioxidant metabolism (Foyer & Noctor
622 2009), and several studies over recent years have explored the roles of specific NADP-dependent
623 dehydrogenases in ROS metabolism and related conditions (Valderrama *et al*. 2006; Mhamdi *et al*.
624 2010c; Voll *et al*. 2012; Li *et al*. 2013). As these enzymes produce NADPH, they can be readily
625 measured using spectrophotometric techniques. One example of a method for measuring a typical
626 dehydrogenase is given in Box 7.
627
628 In general, total tissue NAD and NADP reduction states are less clearly indicative of oxidative stress
629 than those of ascorbate and glutathione. Redox states are generally more oxidized even in optimal
630 conditions, with total tissue NAD pools being largely oxidized and NADP pools being about 50%
631 reduced. This reflects their essential role in energy metabolism, which requires redox poising so that
632 both oxidized and reduced forms are always sufficiently available. Nevertheless, the abundance of
633 total pyridine nucleotide pools can be influenced by oxidative stress through effects on synthesis and
634 degradation (Noctor *et al*. 2011). It can be useful to have data on these compounds alongside
635 ascorbate and glutathione. Validated methods that allow analysis of all these compounds have been
636 developed (Fig. 5), and are described in Boxes 8-10.
637
638 Assays using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) or ferric ion reduction
639 capacity have been used to measure “total antioxidant capacity”. However, the nature of the factors
640 that are monitored by these assays is unclear. These techniques may be useful in the food and
641 associated industries, or in medical diagnostics, but they are of limited value for dissecting the
642 biological complexity of oxidative stress and plant antioxidant systems.
643
644 **Oxidation of proteins and nucleic acids**
645
646 Protein oxidation is likely to be a key process in signalling downstream of ROS. The various possible
647 oxidant-induced modifications have been reviewed in-depth elsewhere (Møller *et al*. 2007, 2011;
648 Møller & Sweetlove 2010). In some cases, protein oxidation may be rapidly reversible, because of the
649 action of thioredoxins or protein methionine sulfoxide reductases (Tarrago *et al*. 2009). More stable
650 modifications include carbonylation, which can occur at several amino acid residues within proteins
651 and is not reversible by known enzymes. Protein carbonylation is relatively easy to measure using a
652 simple chemical stain, and has been employed as a convenient oxidative stress marker (Kingston-

653 Smith & Foyer 2000; Davletova *et al*. 2005a). Even here, the interpretation should take into account
654 that the assay is only providing information on proteins that are allowed to accumulate following
655 oxidation. Protein oxidation is clearly implicated in degradation of enzymes such as Rubisco and
656 glutamine synthetase, either by protease-dependent mechanisms or, possibly, direct cleavage
657 mediated by hydroxyl radicals (Desimone *et al*. 1996; Ishida *et al*. 2002).
658
659 There are now extensive lists of proteins that are potential targets for thiol-diulphide regulation.
660 These have been produced using techniques such as binding of proteins to columns containing
661 mutated thioredoxins (Balmer *et al*. 2003; Buchanan & Balmer 2005; Michelet *et al*. 2008;
662 Montrichard *et al*. 2009). Methods have also been described for analyzing *S*-glutathionylated
663 proteins (Gao *et al*. 2009). Although considered a key signalling molecule, H_2O_2 itself is not very
664 reactive towards most cellular components. Apart from its high rates of metabolism through heme-
665 containing enzymes such as catalases and peroxidases, it can react with protein thiols (SH) to
666 produce sulphenic acid groups (SOH). Such a reaction is the first step in the catalytic cycle of PRX
667 (Dietz 2003), and may occur on other proteins, if they contain a sufficiently reactive thiol. Affinity
668 purification with a fusion protein containing the yeast transcription factor YAP1 has recently been
669 used to identify more than 100 proteins that undergo this modification after exposure of Arabidopsis
670 cell suspensions to H_2O_2 (Waszczak *et al*. 2014). It should be noted that protein thiol oxidation can
671 also be catalyzed by specific enzymes such as plant cysteine oxidases, which play roles in oxygen
672 sensing (Weits *et al.* 2014). As yet, the importance of such enzymes in ROS signalling is not clear.
673
674 Keen interest has focused on protein cysteine *S*-nitrosylation (Romero-Puertas *et al*. 2007, 2008).
675 However, few studies have thus far reported on quantitative changes in *S*-nitrosylation of proteins as
676 a result of oxidative stress *in planta*. One issue is that it can be difficult to preserve *in vivo* protein
677 modifications throughout the sample preparation. Likewise, high background signals may be a
678 problem using current techniques. Issues such as these may compromise reproducibility between
679 biological replicates. As noted above for other types of measurement, this may relate to the
680 complexity of the redox network of plant tissues, with high levels of ascorbate (which can efficiently
681 reduce protein-SNO groups) being one example of an interfering compound. For these reasons,
682 unicellular algae or cell cultures have generally been the preferred material for these kinds of studies
683 (Astier *et al*. 2012).
684
685 The difficulty of quantifying the proportion of a given protein that is undergoing post-translational
686 oxidation may be resolved by the use of differential labelling of oxidized and reduced cysteines using
687 ICAT (isotope coded affinity tag). This approach was recently used to quantify oxidised cysteines in

688 more than 300 different proteins (Rosenwasser *et al*. 2014). As in many other studies, most of the
689 proteins were involved in primary metabolism and, therefore, relatively abundant. The inability to
690 detect low-abundance proteins is a key issue hindering the application of proteomics techniques to
691 identify redox-sensitive signalling proteins. Nevertheless, future technical advances in these areas
692 are likely to be a key development in understanding cellular signalling triggered by oxidative stress.
693 For the moment, such approaches remain rather expensive and laborious. While they are crucial to
694 pierce the complexity of oxidative stress signalling, they are not as yet well suited as convenient
695 markers for oxidative stress in plant tissues.

696

697 In addition to proteins, DNA and RNA can also undergo oxidative modifications (Dizdaroglu *et al*.
698 2002). Guanine bases can be oxidised to 7,8-dihydro-8-oxoguanine (8-oxo-G), promoting mis-
699 matched pairing with adenine. One study reported accumulation of 8-oxo-G during seed ageing, and
700 that overexpression of the repair enzyme, OGG1, mitigated the effects of ageing and also conferred
701 enhanced resistance of seeds to oxidative stress (Chen *et al*. 2012). Double mutants lacking OGG1
702 and another enzyme involved in 8-oxo-G repair showed increased DNA damage, and both single and
703 double mutants also lost germination capacity more quickly than wild-type seeds during ageing
704 (Cordoba-Canero *et al.* 2014). Guanine bases in RNA can also undergo this modification. Dry seeds
705 accumulate stored mRNA, facilitating rapid production of proteins on dormancy breakage (Rajjou *et*
706 *al*. 2004). Interestingly, specific stored transcripts can undergo oxidation in sunflower seeds, leading
707 to altered translation efficiency, an effect that may be involved in the regulation of germination
708 capacity during seed storage (Bazin *et al*. 2011). Accumulation of 8-oxo-G increased with oxidative
709 stress intensity when transcripts were exposed *in vitro* to conditions generating the hydroxyl radical,
710 and analyses of extracted mRNAs from seeds showed the 8-oxo-G content was inversely correlated
711 with dormancy (Bazin *et al*. 2011).

712

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

719

720 One enzyme associated with DNA repair that has received some attention is poly(ADP-
721 ribose)polymerase (PARP), which modifies nuclear proteins using NAD^+ as a source of ADP-ribosyl
722 units (De Block *et al*. 2005). Mutants lacking AtPARP2 showed decreased oxidative stress responses

723 at the transcript level, allied to up-regulation of other stress-associated pathways such as those
724 linked to abscisic acid (ABA; Vanderauwera *et al*. 2007). It was suggested that these effects were due
725 to a decreased drain on cellular NAD(H) pools with a resultant increase in the production of cyclic
726 ADP-ribose and associated calcium-dependent signalling (Vanderauwera *et al*. 2007). Another study
727 has drawn attention to possible redox regulation of PARP function by interactions with nuclear
728 glutathione status (Pellny *et al*. 2009). It should be noted that measurements of PARP and related
729 modifications are challenging, and thus the literature does not contain extensive documentation of
730 the role of these factors in oxidative stress in plants.
731
732 **ROS reporter transcripts**
733
734 Since 2001 there have been numerous reports on ROS-responsive transcriptomes, with initial studies
735 of the effect of H_2O_2 on Arabidopsis cell cultures (Desikan *et al*. 2001) followed by *in planta* studies
736 using ROS-generating agents, the singlet oxygen-generating *flu* mutant, or catalase-deficient plants
737 (op den Camp *et al*. 2003; Vandenabeele *et al*. 2003; Vanderauwera *et al*. 2005; Gadjev *et al*. 2006;
738 Vandenbrouke *et al*. 2008; Mhamdi *et al*. 2010b; Queval *et al*. 2012). One advantage of this approach
739 is that it provides a more comprehensive picture of the cellular response to oxidative stress than
740 metabolite profiling or proteomics. The most obvious limitation is that transcriptomics does not
741 provide direct insight into function. Nevertheless, oxidative stress transcriptomes have allowed
742 identification of several transcription factors and enzymes that have subsequently been shown to
743 play key roles in stress responses (Davletova *et al*. 2005b; Tognetti *et al*. 2010). They have also
744 allowed identification of useful marker transcripts for assessing oxidative stress intensity in plants
745 under different conditions.
746
747 Several studies have drawn attention to the possibility that each form of ROS produces a specific
748 transcriptome. For example, different sets of genes were found to be induced in conditions of singlet
749 oxygen accumulation compared to those in which oxidative stress is linked to superoxide or H_2O_2 (op
750 den Camp *et al*. 2003; Gadjev *et al*. 2006). Assuming this is the case, specific transcript markers
751 would be very useful tools for assessing the relative contribution of different ROS forms to stress
752 responses. Catalase-deficient plants, in which the oxidative trigger is primarily peroxisomal H_2O_2, do
753 not produce the same signature as the *flu* mutant, which overproduces singlet oxygen in the
754 chloroplast (Gadjev *et al*. 2006). It is less clear whether this is a qualitative difference since at least
755 some stress-related genes are strongly induced in both systems (Noctor *et al*. 2014). Several factors
756 could make it difficult to unambiguously assign markers that are specific for each ROS form based
757 purely on comparison of model systems. The most obvious is possible differences in the intensity of

758 oxidative stress that is achieved in each system. In addition, subcellular location of oxidant
759 production and possible site-specificity of signalling pathways could be at least as important, even for
760 a given ROS type (Gutierrez *et al*. 2014). Another complicating factor could be plant history, meaning
761 that transcriptomes would have to be compared in plants that are grown side-by-side under identical
762 conditions. One factor is growth day length, which modulates the phenotype and transcriptome
763 signature of the *cat2* mutant (Queval *et al*. 2012), and also influences the response of wild-type
764 Arabidopsis to equal-time exposure to ozone (Dghim *et al*. 2013). This may reflect the fine-tuning of
765 oxidant-induced gene expression by the prevailing metabolic status of the cell. In other words,
766 oxidative signalling does not occur in a vacuum, and will be influenced by a host of nutritional and
767 environmental inputs.

768

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

775

776 Few of the genes most strongly induced by oxidative stress on a fold-change basis encode members
777 of the core H_2O_2-processing system (Table 1). This presumably reflects the relatively strong
778 expression of these genes under all conditions. Nevertheless, many of them become even more
779 strongly expressed under oxidative stress. Enhanced *APX1* transcripts, encoding a cytosolic APX
780 shown to be crucial in redox homeostasis (Davletova *et al*. 2005a), is a particularly reproducible
781 response to oxidative stress and probably explains at least part of the increase in extractable APX
782 activity in these conditions. Finally, transcripts involved in DNA damage repair (DDR) have been used
783 as markers to assess this process in plants because, as discussed above, direct measurement of DNA
784 modification remains challenging (Vanderauwera *et al*. 2011).

785

786 **Concluding remarks and outlook**

787

788 In this text, we intended to present an overview of many of the commonly employed and available
789 methods to assess oxidative stress, antioxidative systems, and redox homeostasis in plants. Our aim
790 was to provide a user guide, highlighting what we consider to be best practice, outlining validated
791 methods, and explaining where some of the pitfalls lie with regard to accurate assay and
792 interpretation. For this reason, we have provided detailed protocols of convenient and reproducible

793 methods. In our view, seeking to justify a particular procedure and data interpretation by
794 emphasizing previous and extensive use is not in itself very convincing, and inadequate to allay
795 concerns about specificity and accuracy. We therefore provide a few basic recommendations (Box
796 11). Although this advice is not intended to be exhaustive, following it can help avoid ambiguities in
797 data analysis and interpretation.
798
799 The approaches one chooses to analyze oxidative stress will depend on the aims of the study. The
800 reasons why plant biology researchers should be interested in oxidative stress and related processes
801 are increasingly numerous. Just three examples of study aims are: (1) to investigate processes
802 involved in regulating a specific component of cell redox state; (2) to gauge oxidative stress intensity
803 (or the intensity of the plant response) to establish whether and to what extent a given genetic
804 modification or condition is indeed generating oxidative stress; (3) to provide new insight into the
805 workings of oxidative stress and redox regulation in plants. Such divergent albeit potentially
806 overlapping study aims may call for different subsets of techniques. Hence, it is difficult to prescribe
807 specific assays that are to be preferred as a universal "oxidative stress indicator". We note that
808 factors that are most central to oxidative stress may not necessarily be the best markers. One
809 example is pyridine nucleotides, whose role at the very heart of pro-oxidant and antioxidant
810 metabolism, as well as their importance in other cellular functions, may require stability. In our
811 experience, factors that track phenotypic evidence of oxidative stress in a relatively reproducible
812 fashion and that are readily quantifiable are ascorbate and glutathione status, extractable
813 antioxidative enzyme activities, and marker transcripts. Using currently available techniques, changes
814 in ROS themselves may be more difficult to detect and, especially, to quantify (Noctor *et al*. 2015).
815
816 We are keenly aware of the areas where technological advances are essential to drive the field
817 forward. Many researchers are looking for user-friendly, inexpensive, and accurate assay systems,
818 hence the popularity of kits. Unfortunately, many of the most difficult measurements can only be
819 performed with specialized and expensive equipment. The challenge is to develop convenient kit-
820 type procedures and probes for metabolites like lipid peroxides, post-translational modifications
821 such as *S*-nitrosylation and *S*-glutathionylation, and ROS, without sacrificing accuracy or specificity.
822 Alongside these developments, the emergence of new *in vivo* probes able to provide information at
823 the subcellular level is crucial. So far, these have notably included the roGFPs that report on
824 glutathione redox potential and the HyPer probe for H_2O_2 (Meyer *et al*. 2007; Schwarzländer *et al*.
825 2008; Esposito-Rodriguez *et al*. 2013). These approaches are useful but undoubtably have their
826 limitations, necessitating further refinements and the generation of innovative solutions that allow *in*
827 *vivo* function to be probed in real time. One example of an emerging technique may be in-cell

828 nuclear magnetic resonance (NMR) analysis, which has recently been applied to probe the redox
829 state of three proteins in human cell cultures in a non-invasive fashion (Mercatelli *et al*. 2015).
830 Limitations related to extract-based proteomics may to some extent be circumvented by intensified
831 use of bait proteins *in situ*. Such approaches may avoid some of the problems related to extraction or
832 even protein abundance, although quantification of post-translational modifications remains a
833 thorny issue. As yet, no master-switches have been defined in ROS-dependent oxidative stress
834 signalling. However, recent developments in oxygen sensing (Gibbs *et al*. 2014) suggest that, if they
835 exist, their identification is only a matter of time.
836
837 The continuing intensive research focus on ROS and related factors, together with advances in
838 bioinformatics and artificial intelligence, is likely to allow context-specific transcript, protein and
839 metabolite patterns to be closely defined. This will further boost the rapid development of new
840 analytical methods. For example, lipid peroxide signatures and marker transcripts, if they are specific
841 enough, may be a more reliable and convenient approach than attempting to measure singlet oxygen
842 directly. Appropriate marker transcripts are widely used to probe signalling through several
843 phytohormone pathways and, given the information now at our disposal, can be applied in a similar
844 way to assessing oxidative signalling intensity. High coverage RNA sequencing (RNA seq) is likely to
845 significantly advance the field. For example, identification of allelic differences in transcripts offers
846 the possibility of discovering protein domains that may be important in ROS signalling. Together with
847 analysis of epigenetic regulation of transcription, differences in DNA-binding proteins could be key to
848 understanding ROS-linked redox control of gene expression. Further, RNA seq may help to elucidate
849 as yet hypothetical interacting protein modules that could be important in sensing and interacting
850 ROS-triggered redox signals (Foyer & Noctor 2016).
851
852 Another foreseeable development is in nanotechnology, perhaps leading to redox-sensitive *in vivo*
853 reporters that can be used to sense specific components at organellar or even sub-organellar level.
854 At the other end of the scale, it is increasingly likely that remote sensing approaches could be
855 developed to measure the intensity of stress experienced by a crop in the field or plant in its natural
856 environment. As we have emphasized here, reliable and informative markers are crucial to
857 understanding how plants exploit and respond to oxidative stress in laboratory conditions, and
858 translational application of such markers should greatly aid stress diagnostics in agriculture and
859 environmental science. In a physiological rather than diagnostic setting, however, it is unlikely that
860 any single measurement, however precise and sensitive it might be, will be sufficient in studies that
861 are aiming to dissect the impact of oxidative stress on cellular function. This is because "oxidative
862 stress" is a blanket term covering a nexus of cellular changes occurring at multiple levels. Research

863 over the coming years should identify the key nodes and more closely identify the interactions within
864 this complex network.
865
866 **Acknowledgments**
867
868 CHF thanks BBSRC (UK; BB/M009130/1) for financial support. Current work in this area in the
869 laboratory of GN is partly funded by the French Agence Nationale de la Recherche project 'Cynthiol'
870 project no. ANR-12-BSV6-0011.
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Table 1. Genes encoding major antioxidative enzymes in Arabidopsis.

Protein	AGI code	Subcellular localisation
Superoxide dismutase		
CSD1	At1g08830	Cytosolic
CSD2	At2g28190	Chloroplast
CSD3	At5g18100	Peroxisome
FSD1	At4g25100	Plastid[1]
FSD2	At5g51100	Chloroplast
FSD3	At5g23310	Chloroplast
MSD1	At3g10920	Mitochondrion
MnSOD-like	At3g56350	Mitochondrion
Catalase		
CAT1	At1g20630	Peroxisomes
CAT2	At4g35090	Peroxisomes
CAT3	At1g20620	Peroxisomes
Ascorbate peroxidase		
APX1	At1g07890	Cytosol
APX2	At3g09640	Cytosol
APX3	At4g35000	Peroxisome[2]
APX4	At4g09010	Chloroplast[3]
APX5	At4g35970	Peroxisome
APX6	At4g32320	Cytosol
APX7	At1g33660	Pseudogene?
Stromal-APX	At4g08390	Chloroplast/mitochondrion[4,5]
thylakoid-APX	At1g77490	Chloroplast
Monodehydroascorbate reductase		
MDAR1	At3g52880	Chloroplast/peroxisome
MDAR2	At5g03630	Cytosol
MDAR3	At3g09940	Cytosol
MDAR4	At3g27820	Peroxisome
MDAR6	At1g63940	Chloroplast/mitochondrion[4,5]
Dehydroascorbate reductase		
DHAR1	At1g19570	Cytosol/peroxisome[6,7]
DHAR2	At1g75270	Cytosol
DHAR3	At5g16710	Mitochondria[5] (Chloroplast?)
Glutathione reductase		
GR1	At3g24170	Cytosol/peroxisomes[8]
GR2	At3g54660	Chloroplast/mitochondrion[4,5]

Information is given for superoxide dismutase and the five enzymes for which methods are described in Boxes 2-6. Subcellular localisation is based on annotations in databases or studies using GFP-fusion proteins, numbered as follows: 1. Kuo *et al*. (2013). 2. Narendra *et al*. (2006). 3. Wang *et al*. (2014). 4. Xu *et al*. (2013). 5. Chew *et al*. (2003). 6. Grefen *et al*. (2010). 7. Reumann *et al*. (2009). 8. Kataya & Reumann (2010).

Box 1. Extractable H_2O_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_2CO_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 hydazone (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_2O_2 standards ranging from 0 to 2 nmoles for each experiment. Measure at least in triplicates for each extract or H_2O_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 $K_3Fe(CN)_6$ 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 $K_3Fe(CN)_6$, mix rapidly and read immediately for 2s. Perform assay in triplicates. Include H_2O_2 standards ranging from 0 to 5 nmoles for each experiment.

Remarks: Worthwhile basic checks for H_2O_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_2O_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 polyvinylpyrrolidine 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 H_2O_2 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 H_2O_2 concentration of 40 mM (the initial A_{240} should be about 1.6 as ε_{240} H_2O_2 at 240 nm is around 40 M^{-1} cm^{-1}). Start reaction by addition of 50-200 µl desalted extract and monitor decrease in A_{240} for 1-2 minutes.

Baseline leaf rates in unstressed (C_3) plants: 100-200 µmol.mg $prot^{-1}$ min^{-1}

Remarks: The activity is typically measured at supra-physiological H_2O_2 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 H_2O_2 is depleted. At longer assays times, bubbles can cause assay noise as O_2 comes out of solution. Alternative method: monitoring oxygen evolution from H_2O_2 polarographically. In theory, this allows a more specific measurement as H_2O_2 removal can also be performed by peroxidases. In practice, however, if the spectrophotometric assay is performed in desalted extracts at H_2O_2 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 H_2O_2 and monitor decrease in A_{290} for 1-2 minutes (ε_{290} ascorbate = 2800 $M^{-1}cm^{-1}$). Perform control experiment (with no extract) to correct for chemical oxidation by H_2O_2 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 H_2O_2 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. ε_{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. ε_{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. ε_{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 $MgCl_2$, 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 $MgCl_2$ 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. ε_{340}NADPH = 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 thawing 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_2PO_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_2O 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 $\mu mol.g^{-1}$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_2O, 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. ε_{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_2O. 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_2O. 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$^{\cdot}$) 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$^{\cdot}$ (top). Oxygen competes with conversion of NBTH$^{\cdot}$ 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,

dehydroascorbate. DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid). DTT, dithiothreitol.EtOH, ethanol. GSH, glutathione (reduced form). GR, glutathione reductase. GSSG, glutathione disulphide. 6-PG, 6-6-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 examples of H_2O_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.

Factor to be measured

Measurement

Plant material and sampling

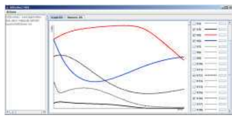
- Are growth conditions appropriate for the question to be addressed ?
- Are there enough biological replicates and not just technical repeats?
- Is sampling rapid enough to prevent alteration of the factor from its state in the plant?

Sample preparation

- Is the extraction medium appropriate (pH, temperature, compounds required for stability)?
- Does the sample preparation procedure remove enzymes or metabolites that may interfere with the assay?

Assay procedures

- Is the assay sufficiently specific, accurate and sensitive?
- Is optimization required (eg, recovery experiments for metabolites)?
- Is the response linear to the amount of extract in the assay?



Data

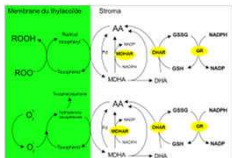
Interpretation

Data processing and validation

- Are the calculations correct? Check extinction coefficients, dilution factors, etc.
- Are the data expressed relative to some physiologically relevant factor (tissue mass, protein, chlorophyll, etc)?
- How will the validity of the values be verified? Check against reliable literature values if these are available.

Interpretation

- Are statistics used appropriately? Avoid treating technical and biological repeats as on the same level.
- 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
- Biological systems are complicated and interpretation of biological data is far from trivial: Occam's razor can be a very blunt instrument!



Conclusions

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.

Tissue zones	Common interpretation	Complicating factors
NBT	Zone of superoxide accumulation	NBT not specific for superoxide (eg, could be differences in ascorbate or dehydrogenases). Different oxygen concentrations could also influence the reaction.
DAB	Zone of H_2O_2 accumulation	Higher peroxidase activity could favour increased staining even at the same H_2O_2 concentration, depending on which of the two factors is rate-limiting

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.

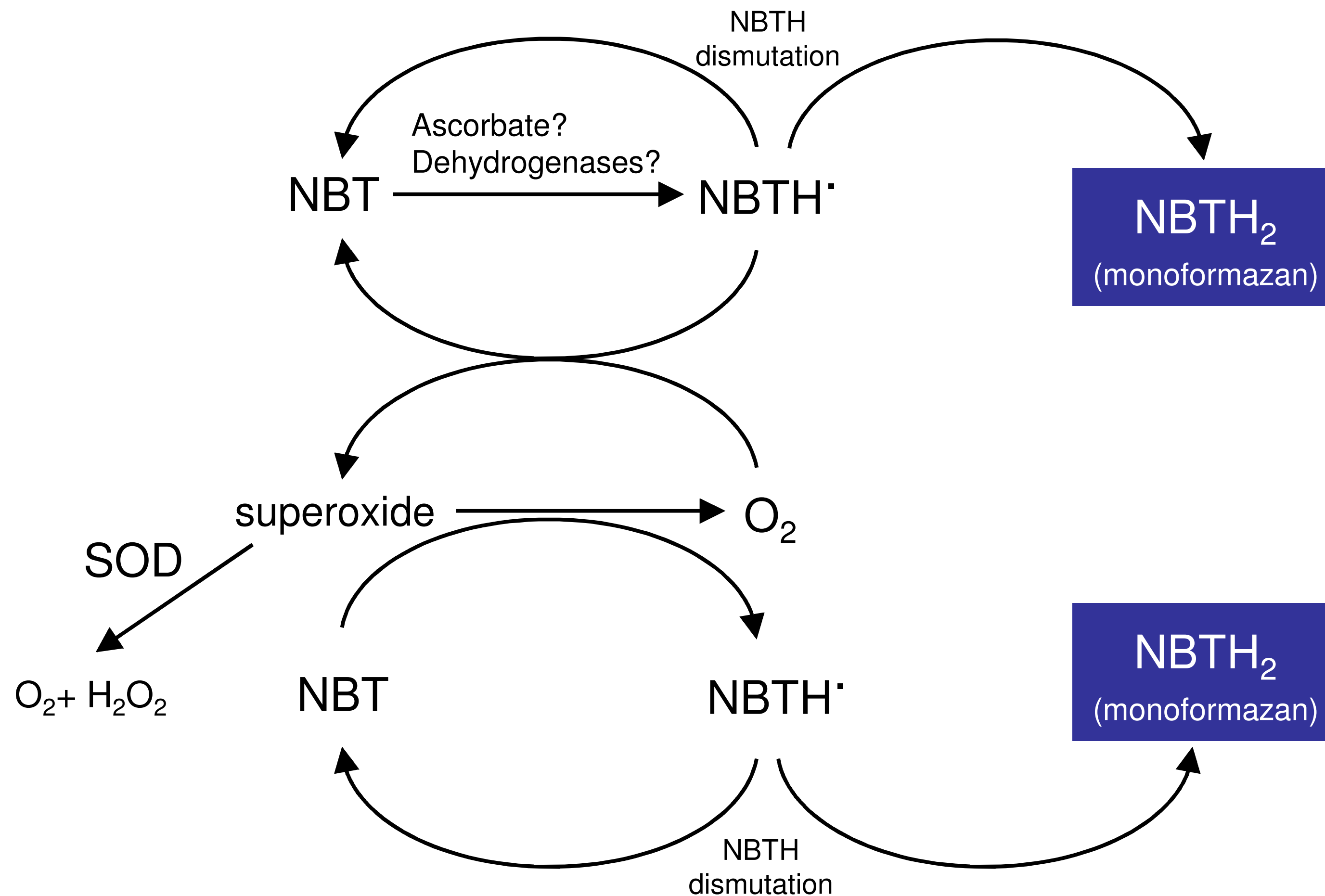
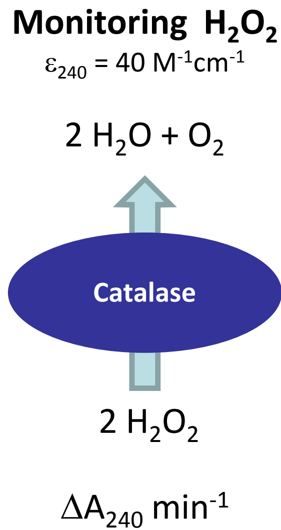
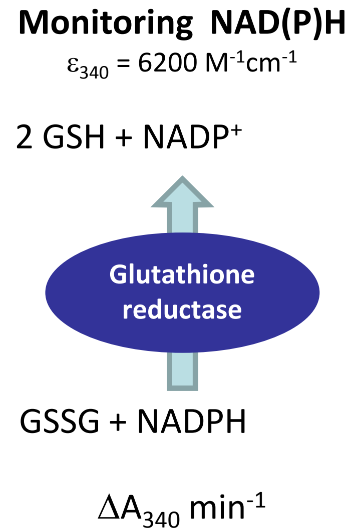
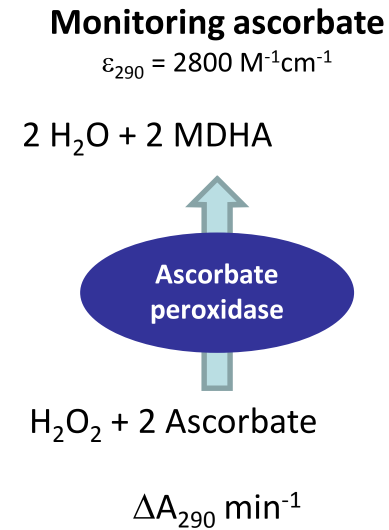


Figure 3. One example of how *in planta* complexity may obscure the interpretation of cell staining procedures commonly used to detect ROS. Reduction of nitroblue tetrazolium (NBT) to a blue formazan occurs *via* an intermediate radical (NBTH·) from which the coloured compound is produced by dismutation. 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.







Typical calculation

100 mg Arabidopsis leaves, soluble protein content = 9 mg.g^{-1}FW. 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).

Catalase
100 µl extract/assay
Measured ΔA_{240} min^{-1} = 0.2

Activity = 0.2 x 10^3/40 x 1/0.1 x 1.5/0.5 x 10/9
= 167 µmol mg^{-1} prot min^{-1}

Glutathione reductase
100 µl extract/assay
Measured ΔA_{340} min^{-1} = 0.02

Activity = 0.02 x 10^6/6200 x 1/0.1 x 1.5/0.5 x 10/9
= 108 nmol mg^{-1} prot min^{-1}

Ascorbate peroxidase
50 µl extract/assay
Measured ΔA_{290} min^{-1} = 0.03

Activity = 0.03 x 10^6/2800 x 1/0.05 x 1.5/0.5 x 10/9
= 714 nmol mg^{-1} prot min^{-1}

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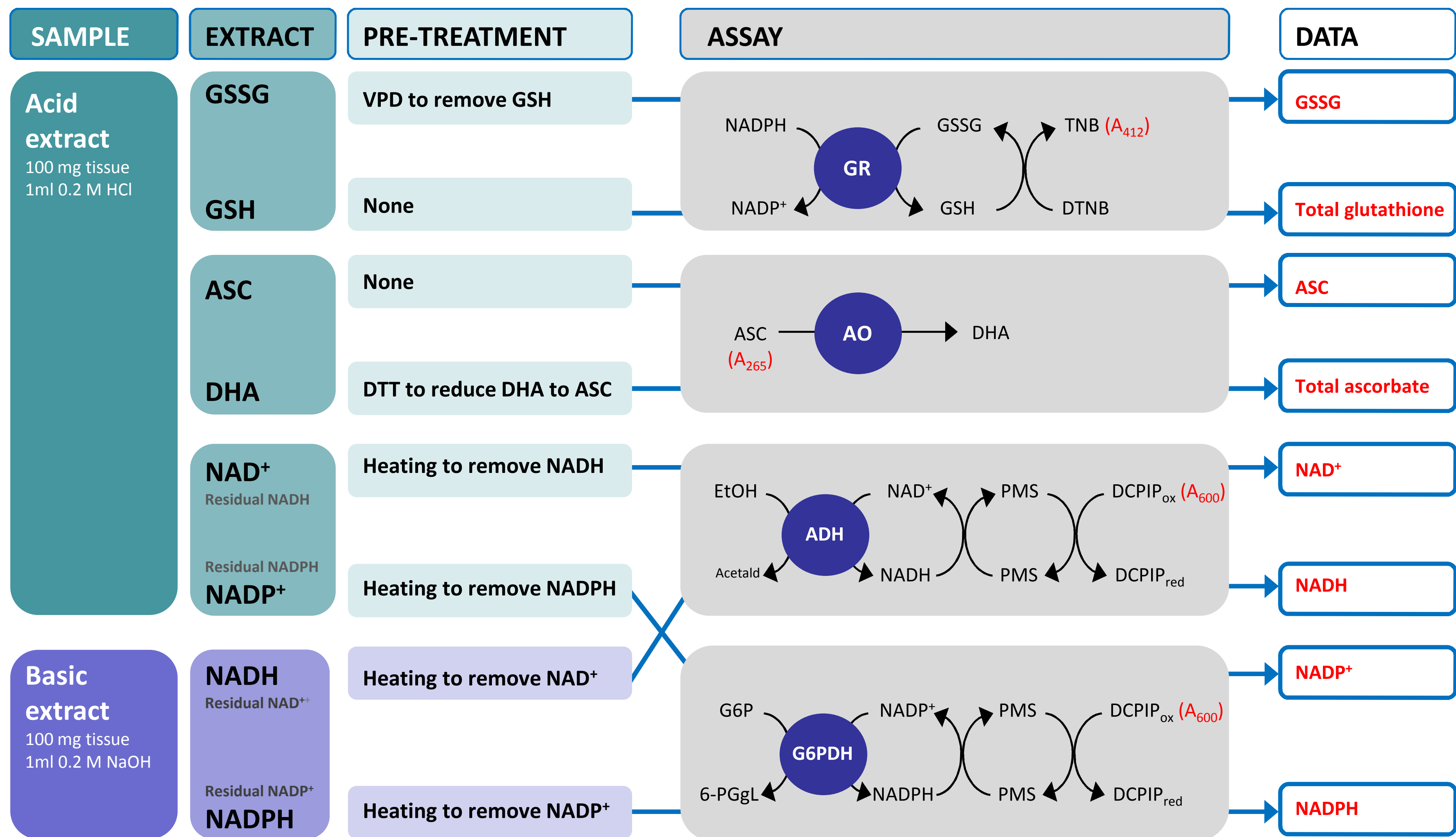

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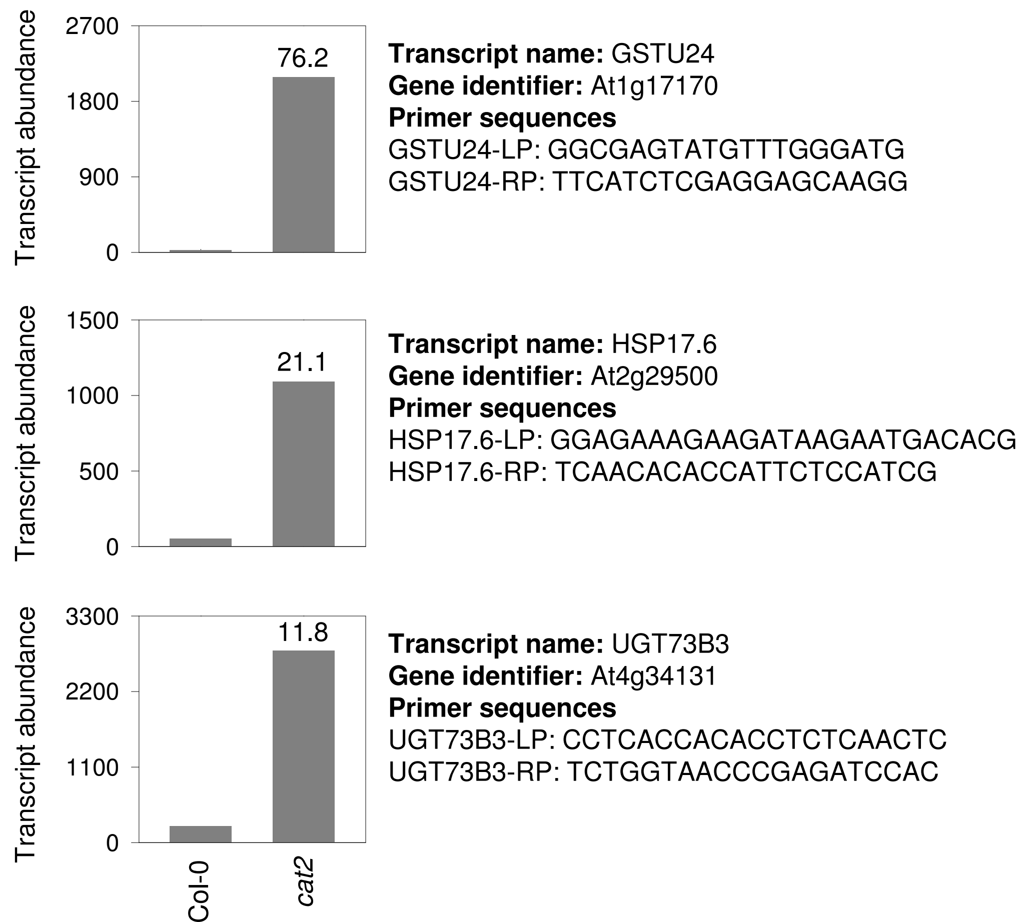

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