
Vladimir Shulaev* and David J. Oliver

Virginia Bioinformatics Institute, Blacksburg, Virginia 24061 (V.S.); and Department of Genetics, Development, and Cell Biology, Iowa State University, Ames, Iowa 50010 (D.J.O.)

Reactive oxygen species (ROS) play a significant role in plant growth, development, and interaction with biotic and abiotic environments (Alvarez et al., 1998; Blokhina et al., 2003). ROS have also been implicated as important regulatory and signaling elements in a variety of cellular processes (Foyer and Noctor, 2005). ROS are constantly produced during the course of photosynthesis and respiration, whereas redox homeostasis in the cell is tightly controlled by redundant protective mechanisms. Disruption of these protective mechanisms can cause oxidative stress, leading to oxidative damage and cell death. Measuring oxidative stress in the cell requires sensitive and robust assays for ROS detection, accurate quantitation, and measurements of intrinsic cell defense responses.

Measurement of ROS in living organisms carries a significant analytical challenge. Most ROS are highly reactive and short lived and therefore hard to detect in complex biological matrices. Additionally, ROS often are produced and/or detoxified in subcellular compartments, which requires detection methods directed to specific subcellular localization. ROS can be measured either directly or indirectly following the formation of oxidative by-products of lipids, proteins, or nucleic acids (a technique often called fingerprinting). Techniques to measure these reactive intermediates have been extensively reviewed (for a recent review, see Halliwell and Whiteman, 2004; Tarpey et al., 2004). Here we mainly focus on recent applications of these techniques to measure ROS in plants.

DIRECT MEASUREMENTS OF ROS BY ELECTRON PARAMAGNETIC RESONANCE

ROS can be detected directly by electron paramagnetic resonance (EPR; or electron spin resonance), which can also be used to monitor changes in the chemical forms of the oxidizable transition metal ions implicated in ROS generation (Khan and Swartz, 2002; Jackson et al., 2004). Because of the low sensitivity of EPR, it is extremely difficult to measure highly reactive radicals directly in vivo. To overcome this sensitivity issue, a technique called spin trapping is often used. In spin-trapping experiments, ROS are allowed to react with specially selected trap molecules to produce less reactive and more stable species that can be readily detected by EPR (Khan et al., 2003). EPR is being widely used to detect ROS in plants (for a recent review, see Bacic and Mojovic, 2005). Generally, overlap between different signals in the EPR spectrum makes it difficult to quantitatively measure individual ROS in plants and therefore EPR is often used to assess total free radical formation (Muckenschnabel et al., 2002).

The major advantage of EPR is its ability to measure and localize ROS in vivo. The latest progress in EPR techniques combined with the development of new spin traps (for review, see Halliwell and Whiteman, 2004) allows for noninvasive mapping of ROS in vivo in the whole animal. In vivo mapping using EPR spectroscopy with nitroxyl probes generating stable nitroxyl radicals, combined with other tomographic techniques, is an example of the new tools available for noninvasive detection and real-time monitoring of ROS formation, studying tissue localization and analyzing the effects of antioxidants on ROS detoxification and prevention of oxidative damage (Utsumi and Yamada, 2003; Utsumi et al., 2006). These exciting new EPR techniques are yet to be fully exploited by plant researchers.

The major limitation for using EPR with spin trapping to measure ROS in plants is the necessity to infiltrate spin trap molecules into the cells. In many cases, cytotoxicity and cellular permeability of the trapping agents have not been studied sufficiently in plant systems and their infiltration into plant tissues can cause additional stress and affect the levels of the ROS and other signaling compounds in the cell. One way to overcome this limitation is to engineer plants that can synthesize EPR-compatible ROS-trapping molecules in vivo to allow for truly noninvasive monitoring of ROS formation.

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* Corresponding author; e-mail vshulaev@vbi.vt.edu; fax 540–231–2606.

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MEASURING SUPEROXIDE

The detection of superoxide can be achieved by EPR with superoxide-specific spin probes (i.e. 5,5-dimethylpyrroline-N-oxide, 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide, or triaryl methyl radical Otx063), using assays based on the superoxide’s ability to reduce cytochrome c, nitroblue tetrazolium (NBT), and other compounds, or with chemiluminescence or fluorescence-based methods (i.e. lucigenin assay). The NBT assay can also be used for histochemical localization of superoxide. Infiltration of leaves with NBT leads to the formation of a dark blue insoluble formazen compound that can be detected microscopically to localize superoxide generation in plant tissues (Flore and Otting, 1984). This assay was used, for example, to localize superoxide production in Arabidopsis (Arabidopsis thaliana) leaves subjected to light stress (Fryer et al., 2002, 2003).

The majority of superoxide detection methods is subject to artifacts and should be interpreted with caution (for review, see Halliwell and Whiteman, 2004). Major drawbacks of both in vitro and histochemical assays are their low specificity and sensitivity. Many compounds besides superoxide can reduce cytochrome c or NBT, skewing the superoxide levels measured with these techniques. On the other hand, oxidation of lucigenin by some compounds can cause artificial superoxide production. Murphy et al. (1998) surveyed several assays to measure superoxide levels in different subcellular fractions of rose (Rosa damascena) cells and concluded that none of the assays can be used to compare superoxide production in different cellular compartments. Generally, superoxide measurement should be done with multiple controls and must be validated by alternative techniques.

MEASURING HYDROGEN PEROXIDE

Several methods have been developed to measure hydrogen peroxide (H$_2$O$_2$) in living tissues (for review, see Halliwell and Gutteridge, 1999). Common horseradish (Armoracia lapathifolia) peroxidase assays rely on H$_2$O$_2$-dependent oxidation of a nonfluorescent substrate to form a fluorescent product that can be easily detected (Andreae, 1955). A variety of substrates, including 4-aminoantipyrine, scopoletin, Amyplex Red (N-acetyl-3,7-dihydrophenoxazine), dichlorofluorescin diacetate (DCFDA), and homovanillic acid, are employed in these assays. Ease of use and availability of commercial kits have made peroxidase assays a popular technique for estimating H$_2$O$_2$ levels in isolated subcellular fractions. This approach was used to measure H$_2$O$_2$ concentration in soluble fractions, mitochondria, and peroxisomes purified from pea (Pisum sativum) leaves during senescence (Jimenez et al., 1998) and H$_2$O$_2$ released from germinating radish (Raphanus sativus) seeds controlled by light, gibberellin, and abscisic acid (Schoepfer et al., 2001). Orozco-Cárdenas and Ryan (2002) used a commercially available Amplex Red H$_2$O$_2$ detection kit for H$_2$O$_2$ quantitation in tomato (Lycopersicon esculentum) leaf extracts.

Histochemical staining methods provide an advantage over other assays because they allow for subcellular localization of H$_2$O$_2$, although, in many cases, these assays are semiquantitative. Additionally, probes used for histochemical detection of ROS have different permeability and may accumulate in a particular cellular compartment (i.e. DCFDA accumulates preferentially in the cytosol), complicating interpretation of the results. Leaf infiltration with 3,3-diaminobenzidine (DAB) is a common technique used to localize H$_2$O$_2$ in plants (Thordal-Christensen et al., 1997). DAB reacts rapidly with H$_2$O$_2$ in the presence of peroxidase, forming a brown polymerization product. The DAB assay was used to detect H$_2$O$_2$ in leaves interacting with the powdery mildew fungus (Thordal-Christensen et al., 1997) to localize H$_2$O$_2$ production caused by high light stress in Arabidopsis (Fryer et al., 2002), to detect H$_2$O$_2$ generated in response to wounding (Orozco-Cárdenas and Ryan, 1999), and to monitor H$_2$O$_2$ accumulation in knockout Apx1 plants (Davletova et al., 2005). Another widely used cytochemical assay is based on the H$_2$O$_2$ reaction with cerium chloride to produce electron-dense precipitates of cerium perhydroxides (Bestwick et al., 1997). An example of the successful application of this technique is the localization of H$_2$O$_2$ accumulation during the hypersensitive reaction of lettuce (Lactuca sativa) cells to Pseudomonas syringae pv phaseolicola (Bestwick et al., 1997) and in the plasma membrane and apoplast of the anoxia-tolerant iris pseudacorus and rice (Oryza sativa) and the anoxia-intolerant wheat (Triticum aestivum) and Iris germanica plants during anoxia and reoxygenation (Blokhina et al., 2001).

As with superoxide detection, many methods commonly used to measure H$_2$O$_2$, including DAB and DCFDA assays, suffer from low specificity and therefore often do not measure a particular ROS but rather generalized oxidative stress in the cell (Halliwell and Whitman, 2004).

MEASURING LIPID PEROXIDATION

Measuring the end products of lipid peroxidation is one of the most widely accepted assays for oxidative damage and has been extensively used in plants. ROS cause peroxidation of polyunsaturated fatty acids, producing α,β-unsaturated aldehydes such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA; Hartley et al., 1999). These aldehydic secondary products of lipid peroxidation are generally accepted markers of oxidative stress (Del et al., 2005). Several analytical techniques can be used to assay for lipid peroxidation (for review, see Halliwell et al., 1992; Halliwell and Whitman, 2004).

A popular thiobarbituric acid (TBA) assay for MDA is based on its reaction with TBA followed by measuring A$_{352}$ (Draper and Hadley, 1990; Hodges et al., 1999).
The TBA assay and its modifications were used to measure lipid peroxidation in plants exposed to UV irradiation (Hodges et al., 1999), cotton (Gossypium hirsutum) seedlings exposed to temperature stress (Mahmood and Maugel, 2005), and transgenic tobacco (Nicotiana tabacum) expressing glutathione S-transferase/glutathione peroxidase under stress conditions (Roxas et al., 2000). Results of the simple TBA assay should be interpreted with caution because it was shown that, in many cases, TBA-reactive substances are not related to lipid peroxidation (for review, see Halliwell and Whitman, 2004).

Recent progress in mass spectrometry (MS) has prompted the development of more accurate and sensitive methods for 4-HNE, MDA, and other lipid peroxidation products (Deighton et al., 1997; Liu et al., 1997). A method developed by Deighton et al. (1997) is based on the highly sensitive liquid chromatography-MS detection of the 2,4-dinitrophenyl (DNP) hydrazine (DNPH) derivatives of 4-HNE and MDA. The limits of detection for the 4-HNE and MDA DNP derivatives were approximately 5 pmol and 0.1 pmol, respectively, with linear MS response in the range from 2 to 200 μM for DNP-MDA and 0.02 to 10 μM for DNP-4-HNE. This method was validated by measuring the formation of 4-HNE and MDA in differentiated calus cultures of carrot (Daucus carota; Deighton et al., 1997) and in the spreading soft-rot lesions caused by the fungal pathogen Botrytis cinerea in leaves of beans (Phaseolus vulgaris; Muckenschnabel et al., 2001).

One considerable advantage of MS-based methods is the ability to identify individual lipid species targeted by ROS and to detect the various oxidative products formed (Byrdwell and Neff, 2002). Following the formation of oxidized intermediates and stable oxidized molecular species can help to demonstrate the molecular mechanisms of lipid oxidation and to identify new physiological biomarkers of oxidative stress. The development of sensitive MS methods for measuring lipid peroxidation marks significant progress in the field.

MEASURING NUCLEIC ACID OXIDATION

The chemistry of ROS-induced DNA damage has been extensively studied in vitro and in vivo (for review, see Beckman and Ames, 1997). ROS can cause DNA strand breaks or modification to deoxyribose sugar and bases. Several common DNA adducts resulting from oxidative damage have been characterized (Gedik et al., 2002). Formation of the 8-hydroxy-2'-deoxyguanosine, 8-hydroxyguanine, and DNA-MDA adducts are the most characteristic features of DNA oxidation (Bruskov et al., 2002). These metabolic markers form the basis for the majority of assays aimed at measuring oxidative DNA damage.

Despite the fact that measuring oxidative DNA damage is one of the most widely used approaches to quantify oxidative stress in animals and humans, application of this approach in plants is very limited. In one of a few studies on plants, Bialkowski and Olinski (1999) measured the level of 8-hydroxy-2'-deoxyguanosine in the DNA of Cardamine pratensis plants to estimate the level of total oxidative DNA damage.

CHEMISTRY OF PROTEIN OXIDATION BY ROS

Early studies using ROS generated by ionizing radiation suggested that activated oxygen has the potential to react with nearly all amino acid side groups as well as cleaving the polypeptide backbone (for review, see Garrison, 1987). The most studied types of protein oxidations are those that result in the formation of reactive carbonyl groups (ketones and aldehydes) because these are the most easily tracked experimentally. Carbonyl groups can be the product of a reaction between amino acid side groups (usually Lys, Arg, Pro, or Thr) and hydroxyl radicals. They can also result when the products of the reaction between ROS and lipids (4-HNE and MDA) or carbohydrates (ketoamines and ketoaldehydes) react with amino acid side chains (for review, see Berlett and Stadtman, 1997).

For illustrative purposes, Figure 1 shows the mechanism for ROS-driven conversion of a Lys residue to an α-aminoacidic semialdehyde residue as proposed by Stadtman and Oliver (1991). In this model, a ferric (or cupric) ion is reduced by superoxide to form a ferrous ion that binds to a cation binding site on a protein where one residue is a Lys. The bound metal then reacts with H2O2 to form a hydroxyl radical that in turn leads to the production of a carbon radical. This radical can lead to cleavage of the polypeptide backbone, cross-linking of two polypeptide chains, or, as illustrated, hydrolysis of the ε-amino group to leave an aldehyde.

MEASURING PROTEIN OXIDATION

The reason that protein carbonyl groups have been the most studied signature of protein oxidation by ROS is because they can be readily detected by their reaction with DNPH. Total protein oxidation levels can be determined spectrophotometrically in whole organ extracts (Levine et al., 1994). This system is straightforward, quantitative, and readily adaptable for high-throughput analyses. The technique has been used to determine oxidative stress during UVB exposure in Arabidopsis (Landry et al., 1995) and bean leaves (Shi et al., 2005), chilling in maize (Zea mays) seedlings (Prasad, 1996), and aluminum exposure in maize root tips (Boscolo et al., 2003) following H2O2 treatment of Arabidopsis plants (D.J. Oliver, unpublished data), and during periods of high H2O2 production during the breaker stage of tomato ripening (Jimenez et al., 2002).

Bulk measurements of protein oxidation provide a simple independent measure of oxidative stress that can confirm conclusions based on determining the formation of lipid oxidation products. The DNPH
system, however, becomes much more powerful when coupled with the commercially available anti-DNP antibodies (Shacter et al., 1994). These antibodies allow both immunodetection and immunoenrichment to be applied to protein oxidation studies. Standard spectrophotometric determinations of DNP have a lower detection limit of about 1 nmol/mg protein. This sensitivity can be increased at least 10-fold by using an antibody-based ELISA (Buss et al., 1997). Western blots, although only semiquantitative, can detect as little as 1 pmol of DNP-modified carbonyl groups per milligram of protein (Keller et al., 1993; Shacter et al., 1994).

More qualitative data are available by combining the anti-DNP antibody with one- or two-dimensional gel electrophoresis. A commercial kit is available for this analysis (OxyBlot kit; Chemicon International; Rizhsky et al., 2004; Davletova et al., 2005; Job et al., 2005). Once the proteins have been separated on gels and the oxidized proteins tagged by western blotting, MS can be used to identify the specific individual proteins that are oxidized (Johansson et al., 2004; Kristensen et al., 2004; Davletova et al., 2005). Some of the problems associated with two-dimensional gels (limited number of proteins, difficulty in solubilizing membrane proteins for the first dimension, poor resolution of some protein groups, and recallibration to high-throughput applications) can be addressed by using immunopurification methods to enrich the DNP-derivatized proteins (Kristensen et al., 2004; Davletova et al., 2005). Antibody-based column purification can be used before the gel electrophoresis systems or proteins from the immunoaffinity columns can be analyzed directly by liquid chromatography-tandem MS. Due to the difficulties of getting quantitative data from MS experiments, the use of techniques like isotope-coded affinity tags (Gygi et al., 1999) allows analysis of the amount of proteins oxidized in different samples. Medical researchers are attempting to correlate the oxidation of a group of proteins with a specific disease. It will be interesting to see whether protein oxidation patterns are diagnostic of particular stress states in plants.

ABILITY TO LOCALIZE OXIDATIVE STRESS

One of the unique advantages of incorporating studies on oxidative stress of protein is that it maintains some spatial information on the localization of the stress. Whereas H2O2 can cross cell membranes, superoxides and hydroxide radicals cannot, so such information is useful in localizing where ROS is produced and oxidative stress is experienced. This type of spatial information can be obtained in several ways. Organelles can be isolated by traditional biochemical techniques and then oxidized proteins within those preparations can be determined, or if proteomics techniques are used and the specific oxidized proteins are identified, bioinformatics methods can be used to determine the subcellular localization of a protein from a whole cell extract (Davletova et al., 2005). DNP derivatization and immunological tagging can also be done on intact organs (Smith et al., 1998) and these microscopy techniques have been extended to plants (Y. Xiong and D.C. Bassham, unpublished data).

CONCLUDING REMARKS

The production and scavenging of ROS is central to a broad range of stress and physiological responses in plants. Techniques capable of directly measuring ROS in vivo and tracking lipids, nucleic acids, and protein oxidation can augment these studies by providing, among other things, a spatial component to the localization of stress at the tissue, cellular, and subcellular level. In addition, the tools for studying protein oxidation and protein turnover may uncover new mechanisms for regulating protein activities.

Because ROS are highly reactive and short-lived species that do not accumulate to high levels, it is not possible to measure them directly; rather, one must measure either the accumulation of biomolecules or the exogenously added indicators that are modified by ROS. All of these assays are prone to numerous artifacts resulting from sample preparation and storage or from the analytical method itself, and all are limited in their ability to differentiate between different ROS molecules (for review, see Halliwell and Whiteman, 2004). The choice of which assay will be used, therefore, is a compromise between ease, ability to collect real-time data, need for spatial information, and instrumentation available. At present, real-time measures of ROS and oxidative stress are limited and there are no truly noninvasive methods. Even spin trapping alters cellular ROS levels during the assay. Measurements...
of oxidative damage to lipids, nucleic acids, and proteins are endpoint measures and have not been adapted to collecting real-time data. Whereas studies on lipid peroxidation might provide some information on the structure of the initial lipid, which can contribute information on the subcellular localization of stress, protein oxidation studies using either MS, cytotoxic methods, or ROS tracer dyes (Fryer et al., 2002; Davletova et al., 2005) make it possible to localize stress to the cellular or subcellular level. Real-time imaging of the redox changes using redox-sensitive green fluorescent proteins, which has been used in animal studies (Dooley et al., 2004), may provide additional information on subcellular location of ROS production, although novel radical-specific proteins or other probes will be required to identify individual ROS species responsible for alterations in redox balance.

Recent advances in analytical techniques, especially EPR and MS, already provide more accurate and quantitative ways to measure ROS in the cell. Measurements of the oxidative stress response and cellular redox status can provide a broader view of the impact of ROS formation on plants. Dynamic imaging of redox changes with redox-sensitive green fluorescent protein (Dooley et al., 2004) and the use of DNA microarrays to dissect global transcriptional effects of oxidative stress (Davletova et al., 2005) are examples of new approaches for studying cellular responses to ROS damage. Future progress in genomics, metabolomics, proteomics, and systems biology will result in more studies on global cellular responses to oxidative stress on transcript, protein, and metabolite levels, providing data for mathematical modeling of the biochemical networks involved.

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