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REVIEW ARTICLE

Detection of Reactive Oxygen Species in Higher Plants

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Abstract Formed during the reduction of molecular oxygen or water oxidation, reactive oxygen species (ROS) are produced by a variety of enzymes and redox reactions in almost every compartment of the plant cell. In addition to causing cellular damage, these ROS play a role in signaling networks. Many factors contribute to and, simultaneously, control their metabolism, and it is difficult to detect individual ROS accurately. This is due to several challenges inherent to ROS—their relatively short half-lives, low intracellular concentrations, enzymatic and non-enzymatic scavenging capacity of the cells, and the absence of absolutely selective probes for ROS. Here, we describe the common approaches taken for detecting primary ROS, singlet oxygen, superoxide, and hydrogen peroxide as we discuss their advantages and limitations. We can conclude that using two or more independent methods that yield similar results for detection is a reliable means for studying ROS in intact plant tissues.

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Introduction

Reactive oxygen species (ROS) are chemically reactive molecules produced from molecular ground-state oxygen (Fig. 1a). When higher plants are subjected to severe stress, ROS production is elevated in sub-cellular organelles. These damaging molecules include highly reactive singlet oxygen (Fig. 1b) (Kearns 1971), the superoxide anion radical (Fig. 1c), and hydrogen peroxide, or H_2O_2 (Fig. 1d) (Fridovich 1997). Under many biotic and abiotic stress conditions, these ROS are accumulated due to an imbalance between their generation and scavenging, which results in damage to cells and sub-cellular organelles near the sites where ROS are formed (Hideg et al. 2002). Even though they are scavenged by diverse antioxidative defense substances, including antioxidant enzymes (e.g., superoxide dismutase (SOD); catalase; peroxidase) and antioxidants (ascorbate, tocopherol, and glutathione) (Foyer and Noctor 1999; Mittler 2002), ROS levels often rise rapidly following environmental changes (Blokhina et al. 2003). Due to their highly reactive nature, ROS interact with a wide range of molecules in biological organisms; the consequences of such damage can be fatal to the cell or even the plant (Jabs 1999).

Although the main source of ROS in plant cells is the electron transport chain in chloroplasts, their generation site differs depending on the stress applied (Foyer and Noctor 1999; Laloi et al. 2004; Mittler et al. 2004). For example, singlet oxygen, a byproduct of photosynthesis, is mainly

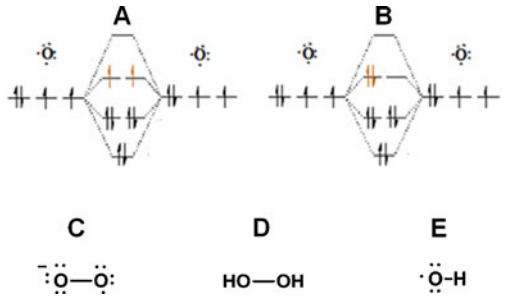

Fig. 1 Chemical structure of molecular oxygen and its reactive derivatives. **a** Molecular oxygen; **b** singlet oxygen; **c** superoxide anion radical; **d** hydrogen peroxide; **e** hydroxyl radical

formed at photosystem II (PSII) (Telfer et al. 1994) but can also arise at other locations where triplet chlorophyll molecules are produced. Generally, three different sites within the photosynthetic apparatus are associated with singlet oxygen production—the PSII reaction center, antennae of the light harvesting complex, or the photosystem I (PSI) acceptor site (Niyogi 1999). Singlet oxygen can be destructive to D1 protein within the reaction center of PSII (Keren et al. 1997; Trebst 2003), but little is known about how it affects other components of the thylakoid membrane. For example, the singlet oxygen produced in *flu* mutants of *Arabidopsis* strongly influences ATP synthase activity and alters non-photochemical quenching, although its production site differs from those mentioned above (Mahler et al. 2007). The water–water cycle includes the site of superoxide production on the reducing side of PSI, and it helps plants dissipate excess light energy by increasing the rate of electron transport and lowering the luminal pH (Asada 1999, 2006; Sonoike 2006). Generation of superoxide within PSII from the cyt b559 site (Pospíšil et al. 2006) and the Q_A site (Cleland and Grace 1999) has also been reported. It is rapidly dismutated to the more stable H_2O_2 by SOD (Fridovich 1997). The hydroxyl radical (Fig. 1e) is another potent ROS, being derived from H_2O_2 or superoxide in the presence of transition metals such as iron or copper (Halliwell and Gutterdge 1989; Nappy and Vass 2000). However, it is not included in our review because it interacts with cell components only at the site where it is produced due to its very short half-life and high reactivity (Asada and Takahashi 1987).

For obtaining better insight into the complex roles of ROS in plants, sensitive methods and techniques are required for their analysis. However, accurate detection of ROS is difficult for several reasons: a relatively short half-life for most of them, low intracellular concentrations, enzymatic and non-enzymatic scavenging capacity of the cells, and the absence of absolutely selective probes for ROS. Therefore, in this review, we will focus on approaches widely used to detect ROS, discussing both their advantages and limitations. Certain attention will be paid to methods for in vivo and in planta determinations.

Singlet Oxygen

Fluorescence Probes

Dansyl-2,2,5,5-tetramethyl-2,5-dihydro1*H*-pyrrole (DanePy) is a fluorescence probe as well as an electron spin resonance (ESR) probe specific for detecting singlet oxygen (Hideg et al. 1998, 2002). The amount of singlet oxygen present in a reaction mixture is estimated by a decrease in the initial fluorescence of DanePy (Fig. 2). For localization within plant tissues, infiltration is achieved by either floating leaf segments or holding roots in DanePy solution. A commercially available fluorescence probe—singlet oxygen sensor green (SOSG)—is used to detect the production of singlet oxygen in diatoms and leaves (Flors et al. 2006). However, SOSG is reported to produce singlet oxygen by itself when exposed to ultraviolet and visible radiation (Ragàs et al. 2009), and its chemical structure is a trade secret. This property makes SOSG less useful for quantification of singlet oxygen in leaves. With these fluorescence probes, researchers can also investigate the sub-cellular localization of singlet oxygen via confocal microscopy. In the case of DanePy, its fluorescence is observed by using 364-nm laser excitation with channel-mode detection to record emissions from 505 to 550 nm (Hideg et al. 2002).

Electron Spin Trapping

Singlet oxygen is a non-magnetic molecule that cannot be detected directly by ESR. However, the reaction of singlet oxygen with a stable molecule can generate a moderately long-lived free radical, which can then be detected by ESR. Spin-label 2,2,6,6-tetramethyl-4-piperidone (TEMP) is used as a spin-trapping probe. As shown in Fig. 3, the reaction of singlet oxygen with TEMP leads to the production of a free radical—2,2,6,6-tetramethyl-4-piperidone-*N*-oxyl (Lion et al. 1976). Another spin-trapping probe, DanePy, has also

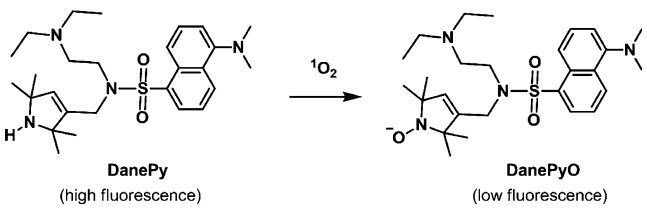

Fig. 2 Conversion of highly fluorescent dansyl-2,2,5,5-tetramethyl-2,5-dihydro1*H*-pyrrole (*DanePy*) to less-fluorescent dansyl-2,2,5,5-tetramethyl-2, 5-dihydro1*H*-pyrrol-1-yloxyl (*DanePyO*) by singlet oxygen

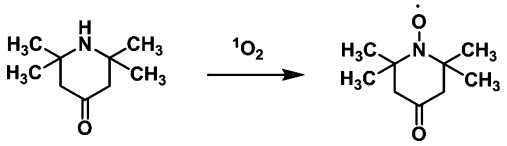

Fig. 3 Conversion of 2,2,6,6-tetramethyl-4-piperidone to 2,2,6,6-tetramethyl-4-piperidone-*N*-oxyl by singlet oxygen

been proven to detect singlet oxygen specifically (Hideg et al. 2006). Nonetheless, the high cost of ESR spectrophotometers, the lack of spin-trap specificity, probe instability, and the existence of cellular reductants make this method of superoxide detection less suitable.

Direct Measurements of Near Infrared Luminescence

Singlet oxygen can also be directly measured through its extremely weak luminescence at 1,270 nm (Schweitzer and Schmidt 2003) or by sensitive laser spectroscopy (Földes et al. 2009).

Superoxide

Colorimetric Probes

The reduction of ferricytochrome *c* to ferrocytochrome *c* has been used to measure superoxide production (McCord and Fridovich 1968; Azzi et al. 1975; Landmesser et al. 2003).

$$Fe^{3+}\text{cyt c} + O_2{}^{-*} \rightarrow Fe2^{3+}\text{ cyt c} + O_2$$

This reaction can be followed by monitoring the increase in absorbance at 550 nm (McCord and Fridovich 1968). However, this reaction is not absolutely specific for superoxide. Cellular reductants such as ascorbate, glutathione, and numerous reductases can also induce cytochrome *c* reduction. In addition, reduced quinones and redox-active dyes are capable of directly reducing cytochrome *c*, which can then be re-oxidized by cytochrome oxidases, cellular peroxidases, and other oxidants (Thomson et al. 1995). Therefore, these conflicts limit the applicability of this method for in vivo superoxide detection.

Leaf samples can be histochemically stained with a nitroblue tetrazolium (NBT) (Fig. 4) solution (Fryer et al. 2002; Kariola et al. 2005; Mahalingam et al. 2006). NBT is reduced by superoxide via a one-electron transfer reaction (Tarpey and Fridovich 2001). This reduction to diformazan proceeds in two steps, thereby yielding the partially reduced monoformazan as a stable intermediate. The first step occurs through a one-electron process to form the NBT radical, which can then be dismutated with another NBT radical. However, numerous substances, including P680 of PSII, can donate an electron to NBT to form the NBT

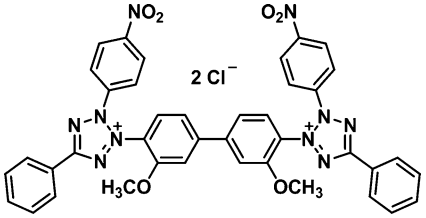

Fig. 4 Chemical structure of nitroblue tetrazolium

radical. Such quantification of superoxide production may be inaccurate because the NBT radical also reacts with environmental oxygen to generate superoxide under aerobic conditions (Tarpey and Fridovich 2001), leading to an overestimation of superoxide production. Finally, absorbance of NBT at 560 nm can be measured for superoxide detection in cell extracts, including chloroplasts and pigment–protein complexes, as described by Auclair and Voisin (1985).

Fluorescence Probes

Dihydroethidium (DHE) (Fig. 5) can detect superoxide in both intact cells and isolated sub-cellular fractions (Georgiou et al. 2005; Robinson et al. 2006; Peshavariya et al. 2007). Its suitability for this task has been proven by demonstrating that its fluorescence increases dose-dependently (Georgiou et al. 2005). DHE can easily penetrate cells, which results in two-electron oxidation to form fluorophore ethidium bromide or structurally similar products (Benov et al. 1998). Although this reaction is relatively specific for superoxide, H_2O_2 can also induce minimal DHE oxidation (Benov et al. 1998). DHE is useful for the detection of superoxide in plants because it is more sensitive than colorimetric methods and because confocal microscopy can facilitate sub-cellular localization (Georgiou et al. 2005).

There is little capacity for artificial formation of superoxide by DHE due to redox cycling, especially when the source of superoxide is localized in the chloroplasts or mitochondria. Because both organelles are rich in redox compounds that undergo such cycling, this quantification method may be inaccurate when the DHE concentration is relatively high and enhancement of superoxide dismutation is the result (Benov et al. 1998).

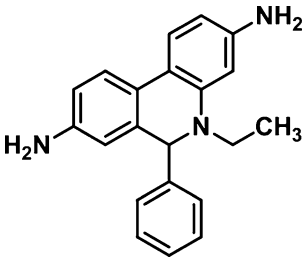

Fig. 5 Chemical structure of dihydroethidium

Chemiluminescence Probes

Most probes for superoxide are limited by membrane permeability. Because of their potential for access to intracellular sites of superoxide production, chemiluminescence probes are frequently used. The most widely applied compound is lucigenin (Fig. 6). Its chemiluminescence, the most sensitive for superoxide detection, is useful for detecting low superoxide concentrations (Afanas'ev 2001). However, lucigenin-amplified chemiluminescence can possibly overestimate the rate of superoxide production due to the redox cycling of lucigenin (Tarpey and Fridovich 2001). Because the presence of NADH or NADPH in the reaction media influences both lucigenin-dependent redox cycling and chemiluminescence intensities (Janiszewski et al. 2002), the use of lucigenin at a low concentration can lead to qualitative information being obtained (Tarpey and Fridovich 2001). In addition, to avoid redox cycling, other non-redox cycling chemiluminescent compounds, e.g., coelenterazine, can serve as probes for superoxide detection (Nakano 1990).

Electron Spin Trapping

In ESR spectroscopy, one adds exogenous spin traps, which are molecules that react with primary ROS to form longer-lived carbon radicals with characteristic ESR signatures. These traps, including 5,5-dimethylpyrroline-*N*-oxide (DMPO) (Fig. 7a), have long been used for detecting superoxide (Makino et al. 1990). However, the ability of ferric ions to oxidize DMPO (Makino et al. 1990) has led to the development of a more selective spin trap, 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) (Fig. 7b) (Frejaville et al. 1995). DEPMPO is useful for measuring superoxide production in cell and tissue extracts (Dambrova et al. 2000). However, the presence of cellular reductants often leads to an underestimation of superoxide production. By contrast, cyclic hydroxylamines are oxidized by superoxide to form stable radicals that can be detected by ESR and which are less reactive to those reductants (Dikalov et al. 1997).

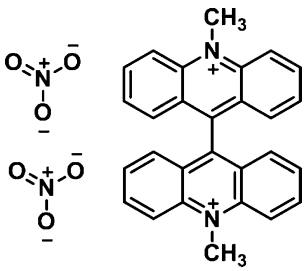

Fig. 6 Chemical structure of lucigenin

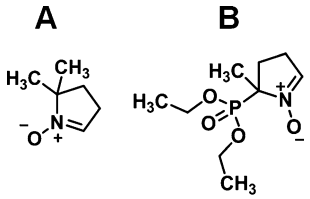

Fig. 7 Chemical structure of 5,5-dimethylpyrroline-*N*-oxide and 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide

Electrochemical Oxidation

Several methods are available for superoxide detection via electrochemical oxidation. One involves direct oxidation on a carbon microelectrode with an Ag counter-electrode (Tanaka et al. 1991). Similarly, a Clark-type platinum microelectrode can be coated with SOD-immobilized polypyrrole film so that the H_2O_2 generated from superoxide dismutation can be estimated (Lvovich and Scheeline 1997). However, a system that depends on the formation of hydrogen peroxide cannot differentiate between processes that are primarily associated with superoxide and those that are directly connected to H_2O_2 production.

Hydrogen Peroxide

Colorimetric Probes

Hydrogen peroxide can be detected by histochemical staining, using a 3,3′-diaminobenzidine (DAB) (Fig. 8) solution (Fryer et al. 2002; Kariola et al. 2005; Mahalingam et al. 2006). For cell extracts containing chloroplasts or pigment–protein complexes, H_2O_2 production can be gauged by measuring the absorbance of DAB at 450 nm (Geerts and Roels 1981).

Fluorescence Probes

In the presence of H_2O_2, hydrogen donors are oxidized by horseradish peroxidase (Boveris 1984), which can be trapped by fluorescence probes. The amount of hydrogen peroxide present in a reaction mixture is then estimated according to the decrease in fluorescence of initially fluorescent probes, such as scopoletin (Boveris et al. 1977). It can also be determined by monitoring the increase in fluorescence from previously non-fluorescent

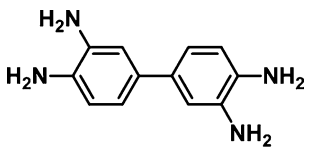

Fig. 8 Chemical structure of diaminobenzidine

probes, e.g., 2′,7′-dichlorofluorescein (DCF) (Fig. 9a) and 2′,7′-dichlorofluorescein diacetate (DCFDA) (Fig. 9b) (Hinkle et al. 1967). However, the presence of many biological compounds, including ascorbate, thiols (which serve as substrates for horseradish peroxidase), or endogenous catalase, may lead to an underestimation of H_2O_2 production when using a horseradish peroxidase assay system (Tarpey and Fridovich 2001).

The oxidation of DCF was initially thought to be relatively specific to H_2O_2 (Keston and Brandt 1965). Because its diacetate form DCFDA can be taken up in intact cells and degraded to DCF by intracellular esterases, DCFDA has been used more frequently to measure levels of intracellular H_2O_2 (Hempel et al. 1999). However, the presence of intrinsic peroxidase and lipid peroxides can lead to oxidation of DCFDA, thereby resulting in an overestimation of H_2O_2 production (Tarpey and Fridovich 2001). Components of the electron transport chain can also directly reduce the oxidized detector molecule, as has been demonstrated with mitochondria and its several different detector molecules (Staniek and Nohl 1999). Such a phenomenon might also be a limitation when employing this assay in chloroplasts.

Fluorescence probes are useful for detecting H_2O_2 in plants because they are more sensitive than colorimetric methods. Confocal microscopy also allows for the sub-cellular localization of H_2O_2.

Measurements of the Conjugated Hydrogen Peroxide–Catalase Complex

During the reaction of catalase with H_2O_2, a stable hydrogen peroxide–catalase complex is formed. Steady-state monitoring of this complex during catalase-dependent metabolism allows for direct detection (Sies 1981).

$$\text{Catalase} + 2H_2O_2 \rightarrow H_2O_2 - \text{catalase complex}$$

$$\rightarrow \text{catalase} + 2H_2O_2 + O_2$$

This complex can be evaluated by dual-wavelength spectrophotometry at 660 to 640 nm (Tarpey and Fridovich 2001). Intracellular H_2O_2 is then estimated by using aminotriazole, an inhibitor of that complex. However, one limitation to this method is that the fraction of catalase depends not only on the rate of H_2O_2 production but also on the total concentration of catalase (Sies 1981).

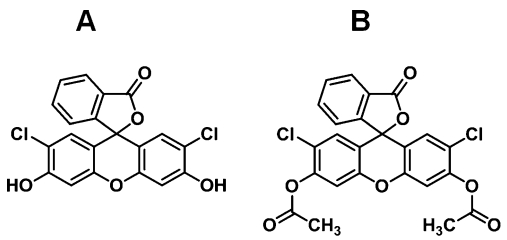

Fig. 9 Chemical structure of 2′,7′-dichlorofluorescein and 2′,7′-dichlorofluorescein diacetate

Conclusions

We have presented several methods for detecting the presence of various types of reactive oxygen species. However, the quantitative rates of ROS production under all circumstances cannot be accurately determined by a single technique. Each of these methods may be associated with potential artifacts, such as the often-overlapping capacity of ROS to react with detector molecules as well as the occurrence of other cellular substances that may react with detector molecules. Therefore, we recommend that at least two of these techniques should be applied, so that those yielding similar results can provide the most reliable approach when studying ROS in intact plant tissues.

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