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Fluorescent in vivo imaging of reactive oxygen species and redox potential in plants

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ABSTRACT

Reactive oxygen species (ROS) are by-products of aerobic metabolism, and excessive production can result in oxidative stress and cell damage. In addition, ROS function as cellular messengers, working as redox regulators in a multitude of biological processes. Understanding ROS signalling and stress responses requires methods for precise imaging and quantification to monitor local, subcellular and global ROS dynamics with high selectivity, sensitivity and spatiotemporal resolution. In this review, we summarize the present knowledge for in vivo plant ROS imaging and detection, using both chemical probes and fluorescent protein-based biosensors. Certain characteristics of plant tissues, for example high background autofluorescence in photosynthetic organs and the multitude of endogenous antioxidants, can interfere with ROS and redox potential detection, making imaging extra challenging. Novel methods and techniques to measure in vivo plant ROS and redox changes with better selectivity, accuracy, and spatiotemporal resolution are therefore desirable to fully acknowledge the remarkably complex plant ROS signalling networks.

1. ROS in plants: origin, homeostasis and functions

Reactive oxygen species (ROS) are partially reduced oxygen molecules produced in aerobic organisms [1]. These reactive oxygen derivatives form free radicals with one or more unpaired electrons, e.g. superoxide ($O_2^{\bullet-}$), hydroperoxyl ($HO_2^{\bullet}$), hydroxyl ($HO^{\bullet}$), peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$), or non-radicals, e.g. hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). Highly reactive atomic oxygen radical or ozone only play a role under very specific conditions and will therefore not be considered in this review. ROS co-exist with reactive nitrogen species (RNS), and interplay between these groups of reactive molecules occurs. $O_2^{\bullet-}$ and nitric oxide ($^{\bullet}NO$) can for example generate peroxynitrite anion ($ONOO^-$) that acts both as ROS and RNS [2]. The reactivity of these molecules towards biological cell components, and the accumulation of subsequent reaction products, can cause oxidative burst and stress, involving lipid peroxidation, protein damage, nucleotide degradation and ultimately cell death [2,3]. However, ROS are also important signalling molecules able to induce a multitude of responses depending on their concentration, sub-cellular localization and lifetime [4]. Thus, ROS production in plants is receiving increased

Abbreviations: 2-OH-E^+, 2-hydroxyethidium; $^{\bullet}NO$, nitric oxide radical; $^{\bullet}NO_2$, nitrogen dioxide radical; $^1O_2$, singlet oxygen; $^3O_2$, triplet oxygen; Al, aluminium; Ala, alanine; APX, ascorbate peroxidase; ASC, ascorbate; CAT, catalase; Ca^{2+}, calcium; Cd, cadmium; CFP, cyan fluorescent protein; CM-DCFH-DA, chloromethyl DCFH-DA; $CO_3^{\bullet-}$, carbonate radical; Cys, cysteine; DAB, 3,3´-diaminobenzidine; DCF, 2,7-dichlorofluorescein; DCFH, 2,7-dichlorodihydrofluorescein; DCFH-DA, 2,7-dichlorodihydrofluorescein diacetate; $DFC^{\bullet-}$, DCF's semiquinone radical; DanePy, (3-[N-(β-diethylaminoethyl)-N-dansyl] aminomethyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole); DHE, dihydroethidine; DHR, dihydrorhodamine 123; $DHR^{\bullet}$, dihydrorhodamine one-electron radical; DNA, deoxyribonucleic acid; DPI, diphenylene iodonium; E^+, ethidium; FMN, flavin mononucleotide; FP, fluorescent protein; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; GPX, glutathione peroxidase; GRX, glutarredoxin; GSH, reduced glutathione; GSSG, oxidised glutathione; $GS^{\bullet}$, thiyl radical; H_2O_2, hydrogen peroxide; HClO, hypochlorous acid; Hg, mercury; $HO^{\bullet}$, hydroxyl radical; $HO_2^{\bullet}$, hydroperoxyl radical; HRP, horseradish peroxidase; K, potassium; LC^{2+}, lucigenin; $LC^{\bullet+}$, lucigenin radical; Mn, manganese; MnTMPP, Mn-5,10,15,20-tetrakis(1-methyl-4-pyridyl)21H,23H-porphin; MTT, 3-(4,5´-dimethyl thiozolyl-2)-2,5-diphenyl tetrazolium bromid; NADPH, nicotinamide adenine dinucleotide phosphate; NADPHox, NADPH oxidase; NBT, nitroblue tetrazoluim; $O_2^{\bullet-}$, superoxide radical; Oba-Q, Oxidation balance sensed Quenching; $ONOO^-$, peroxynitrite anion; PG1, Peroxy Green 1; PO1, Peroxy Orange 1; POX, peroxidase; PRX, peroxiredoxin; PSI, photosystem I; PSII, photosystem II; PY1, Peroxy Yellow 1; QSAR, Quantitative Structure-Activity Relationship; RFP, red fluorescent protein; RNS, reactive nitrogen species; $RO^{\bullet}$, alkoxyl radical; $ROO^{\bullet}$, peroxyl radical; ROS, reactive oxygen species; SOD, superoxide dismutase; SOGS, Singlet Oxygen Sensor Green; TRX, thioredoxin; TTC, 2,3,5-triphenyl tetrazolium chloride; VIS, visible light; VPE, vacuolar processing enzyme; XO, xanthine oxidase; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)–2*H*-tetrazolium-5-carboxanilide; YFP, yellow fluorescent protein

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attention for its regulatory role during development and plant stress responses [5,6].

Redox chemistry of ground state molecular oxygen (triplet oxygen, $^3O_2$) facilitates the acceptance of an electron from different cellular sources (e.g. chloroplasts and mitochondria electron transfer chains and Fe-containing molecules), generating $O_2^{\bullet-}$ [7]. Enzymes such as NADPH-oxidases (NADPHox), peroxidases (POXs), lipo- and cyclo-oxygenases, cytochrome P450s and xanthine oxidases (XO) can also participate in electron transfer and ROS production [8–11]. $O_2^{\bullet-}$ is subsequently converted into H_2O_2, spontaneously or via a superoxide dismutase (SOD)-catalysed reaction H_2O_2 is more stable and therefore better suited for long-distance signalling, and transport across cell membranes to the vascular tissues is facilitated by aquaporins [12]. In plant tissues, H_2O_2 can react with $O_2^{\bullet-}$ via Fenton and Haber-Weiss reactions producing the highly reactive $HO^{\bullet}$, or can be converted to H_2O in reactions catalysed by POX and catalases (CAT) [13].

Several plant organelles and compartments are sources of ROS, especially those with high electron transport rates. Because of electron transfer from photosystem I (PSI) to O_2 during photosynthesis, chloroplasts are the main sources of $O_2^{\bullet-}$. Photorespiration on the other hand can also generate peroxisomal H_2O_2. In green tissues, excited chlorophyll and its tetrapyrrole derivatives near both photosystems additionally generate $^1O_2$, while mitochondrial contribution to plant ROS production is less significant, at least in photosynthetic tissues at moderate to high light intensities [14,15]. Finally, plasma membrane NADPHox and apoplastic enzymes such as POXs, oxalate- and amine-oxidases involved in cell wall cross-linking reactions, also greatly contribute to ROS generation in plants [7,15].

Enzymatic and non-enzymatic antioxidants balance ROS production in different organelles. The most important non-enzymatic antioxidants include glutathione (GSH), ascorbate (ASC), tocopherols and phenolic compounds, in addition to carotenoids and NAD(P)H [16]. The enzymatic ROS-scavenging system includes SOD, CAT, peroxidases (e.g. ascorbate and glutathione peroxidases, APX and GPX respectively), peroxiredoxins (PRX), thioredoxins (TRX), as well as enzymes of the glutathione-ascorbate redox cycle [17–19]. Excellent reviews regarding plant ROS and antioxidant systems can be found in [1,2,20].

The involvement of ROS production in redox biology is becoming a hot topic, as developmental or environmental conditions that alter redox homeostasis have been shown to modulate signalling events and to regulate cell metabolism and plant responses [21,22]. Redox switches, mostly based on Cys redox cycling, operate in plant transcription factors and enzymes, which undergo sulfhydryl to disulphide transitions (and vice versa) accompanied with conformational changes that modulate their biological function. These modifications can be triggered directly, for example by H_2O_2 reacting with the thiol group, or indirectly, changing the redox potential of different redox pairs, ultimately altering enzyme activity or gene expression [23–26]. Thus, elucidating how ROS and redox changes modulate signalling events leading to plant development adjustments and stress responses is of high importance. As these ROS regulated processes seem to be highly specific for each ROS type [27], it is essential to detect, accurately identify and localize the particular species produced in the cell in order to fully understand their involvement and regulatory role of distinct plant responses.

2. ROS and redox potential detection in plants: challenges and possibilities

2.1. Non-fluorescent probes

Traditionally, detection of plant ROS has been achieved by using non-fluorescent compounds that produce coloured precipitates upon reaction with (specific) ROS. Among these colorimetric probes, tetrazolium salts have been commonly used. Their reduction products, called formazans, are highly coloured and usually water-insoluble, which facilitates their detection by microscopy or absorption spectrometry when solubilised with appropriate solvents [28]. Tetrazolium salts have been extensively used as indicators of cell viability, mostly based on the functionality of reducing biological systems, e.g. to measure enzymatic redox respiratory activity during seed germination using 2,3,5-triphenyl tetrazolium chloride (TTC) [29], or *Helianthus tuberosus* mitochondrial dehydrogenases activity by 3-(4,5′-dimethyl thiozolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) [30]. Indeed, the selectivity of some tetrazoliums for specific ROS, e.g. nitroblue tetrazolium (NBT) for $O_2^{\bullet-}$, enabled the study of early steps in oxidative stress induction by different treatments and environmental conditions in plants [31]. Another popular colorimetric in situ ROS indicator is 3,3′-diaminobenzidine (DAB), which reacts with H_2O_2 in a peroxidase-catalysed reaction rendering a brown precipitate that can be microscopically imaged at tissue and cellular levels [32]. DAB has been used in studies spanning several plant species subjected to various treatments and conditions [33–35].

Chemiluminescence can be used to detect and measure ROS [36]. Lucigenin (bis-*N*-methylacridinium dinitrate, LC^{2+}) was reported as a selective superoxide chemiluminescent probe. However, it seems that it must first be oxidized to the radical $LC^{\bullet+}$ by enzymes or oxidized cofactors (e.g., flavoproteins) and then with $O_2^{\bullet-}$ to produce chemiluminescence. Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) is another commonly used chemiluminescent compound for ROS detection. Its selectivity for specific ROS is however very poor, reacting with $O_2^{\bullet-}$, $HO^{\bullet}$, $^{\bullet}NO$ and $^{\bullet}NO_2$, thiyl radicals ($GS^{\bullet}$) and a plethora of redox active intermediates. Both lucigenin and luminol can sensitize the production of ROS through their presence [36]. This low selectivity for distinct ROS is shared with many fluorescent redox probes and will be commented in the next sections. ROS-Glo™, a luciferin-based chemiluminescent assay now commercially available claims to be specific for H_2O_2 detection. Here, the luciferin precursor molecule is protected by a boronate moiety, which is liberated when exposed to H_2O_2. Once the boronate group is released, further chemical reactions generate luciferin chemiluminescence. Still, the specificity of this probe remains to be experimentally verified.

2.2. Fluorescence-based detection

Fluorescence detection is generally more sensitive than colorimetric methods. Its assessment is also more straightforward than chemiluminescent ones, and usually requires lower probe concentrations due to its very favourable signal-to-noise ratio compared to optical detection [37]. Also, as fluorescence imaging interferes less with biological processes in comparison to colorimetric and chemiluminescent reagents, it allows for simpler and more selective in vivo ROS detection in cells and intact tissues. Combined with appropriate fluorescent probes, subcellular monitoring and quantification of ROS dynamics can be highly informative [38]. However, several factors make ROS detection in plant tissues challenging: the restrained spatial and temporal dynamics of ROS, their short half-life (ranging from nanoseconds to seconds) and their cross-sensitivity to cellular antioxidants that compete with the probes for the ROS, reducing the signal measured and hampering their detection in plants [39].

High abundance of endogenous fluorescent compounds make in vivo ROS imaging challenging in plant cells and algae [40,41]. In green tissues, chlorophyll is the major contributor to autofluorescence, although significant interference also comes from cell wall components (e.g. cellulose and lignin) and other coloured molecules and pigments (e.g. carotenes, xanthophylls, flavonoids, anthocyanins, alkaloids, etc.) [42]. All these compounds exhibit distinct excitation and emission spectra that may overlap with exogenous fluorescent markers and hamper their detection, making reliable fluorophore quantification in plants more challenging than in other organisms (Fig. 1) [43]. The use of spectral imaging-based microscopy techniques, such as confocal and two-photon microscopy, can help to tackle the autofluorescence