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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.

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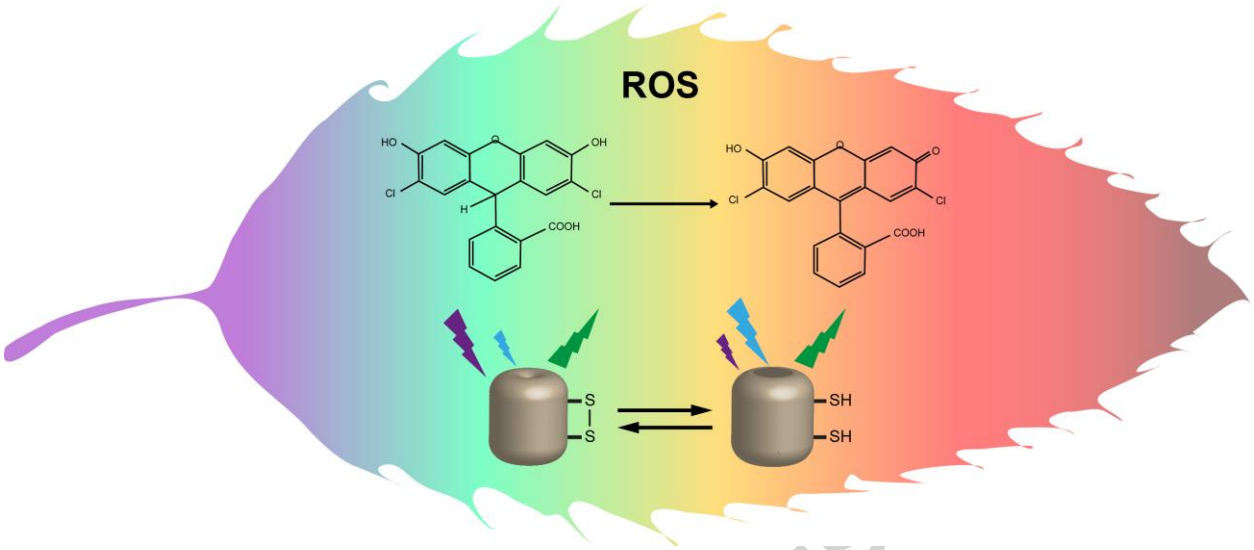
Graphical Abstract



Abbreviations

2-OH-E^+, 2-hydroxyethidium; $^{\bullet}NO$, nitric oxide; $^{\bullet}NO_2$, nitrogen dioxide radical; $^1O_2$, singlet oxygen; $^3O_2$, triplet oxygen; Al^{3+}, aluminium; APX, ascorbate peroxidase; ASC, ascorbate; CAT, catalase; Ca^{2+}, calcium; Cd^{2+}, cadmium; CFP, cyan fluorescent protein; CM-DCFH-DA, chloromethyl DCFH-DA; 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; 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; Hg^{2+}, mercury; $HO^{\bullet}$, hydroxyl radical ; $HO_2^{\bullet}$, hydroperoxyl radical; HRP, horseradish peroxidase; K^+, potassium; $LC^{2+,}$ lucigenin; $LC^{\bullet+}$, lucigenin radical; 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 bromide; NADPH, nicotinamide adenine dinucleotide phosphate; NADPHox, NADPH oxidase; NBT, nitroblue tetrazoluim; $O_2^{\bullet-}$, superoxide radical; $ONOO^{-,}$ peroxynitrite anion; POX, peroxidase; PRX, peroxiredoxin; PSI, photosystem I; PSII, photosystem II; RFP, red fluorescent protein; RNS, reactive nitrogen species; $RO^{\bullet}$, alkoxyl radical; $ROO^{\bullet}$, peroxyl radical; ROS, reactive oxygen species; SOD, superoxide dismutase;

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TRX, thioredoxin; TTC, triphenyl tetrazolium chloride; 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

Keywords

Reactive Oxygen Species (ROS); redox signalling; plant; *in vivo* detection; fluorescent probes; protein biosensors.

1.1 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 ($OH^{\bullet}$), 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 localisation and lifetime [4]. Thus, ROS production in plants is receiving increased 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

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[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 $OH^{\bullet}$, 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,

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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 regulation role of distinct plant responses.

1.2 ROS and redox potential detection in plants: challenges and possibilities

1.2.1 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

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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.

1.2.2 Fluorescence-based detection

Fluorescence detection is generally more sensitive than colorimetric methods. Its assessment is also more straightforward than chemiluminescent one, 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, and importantly because of its high signal-to-noise ratio, 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 **(Figure 1)** [43]. The use of spectral imaging-based microscopy techniques, such as confocal and two-photon microscopy can help to tackle the autofluorescence problem [44–46]. However, it is also important to note that alterations in (auto) fluorescence signals can result

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from different experimental treatments (e.g. NADPH or phenols increase) and therefore interfere with fluorescence quantification, especially when using short wavelength lasers (e.g. 405 nm). Nevertheless, autofluorescence background is still an issue to take into consideration when choosing the fluorescence probe, especially for epifluorescence microscopy users.

Thus, high fluorescence quantum yield probes or high expression of the fluorescent sensors can help surpass this difficulty. As always, background signal appraisal with proper controls and excitation regimes is required when quantitative measurements are performed [47].

1.3 Fluorescent dyes

Several chemically pure synthesised molecules, hereby termed probes or dyes, can be used for redox potential or ROS detection in plants. Ideally, such ROS probes should be non-toxic, display negligible photochemistry, show low background emission and be insensitive to other environmental parameters. Also, they should be membrane permeable to facilitate cell uptake when used for intracellular measurements (although some probes that require enzymatic catalysis for fluorescence production can be used for extracellular ROS monitoring by addition of the appropriate enzyme to the media). Fluorescent dyes should also be specific, highly reactive at low concentrations, produce stable and quantifiable non-diffusible products, have fast and reversible kinetics with high signal-to-noise ratio and not cross react with cellular antioxidants [48].

A common feature for most ROS probes is that they are usually non-fluorescent reduced membrane permeable precursors, which upon oxidation by (ideally a specific) ROS inside the cell, are converted to fluorescent derivatives with intensity being proportional to ROS accumulation. In the following section, we introduce the most popular ones used in plant systems.

1.3.1 Singlet Oxygen ($^1O_2$) detection

The most employed $^1O_2$ detection mechanism when using organic fluorophores is based on the formation of an endoperoxide on an aromatic molecule [38]. Usually an anthracene molecule is linked to a fluorophore (e.g. fluorescein derivative). Without $^1O_2$, the organic fluorophore molecule is excited, and before it has the possibility to emit a fluorescence photon, transfers energy or an electron to the linked anthracene. The excitation energy is then rapidly lost to the

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medium through internal molecular rearrangements. When $^1O_2$ is present however, the excited oxygen molecule reacts with the anthracene, producing a 1,4-cycloaddition on the central aromatic ring. This cycloaddition severely disrupts the former planar structure of the aromatic molecule and strongly decreases the kinetics of photochemistry from the fluorophore. With the probability for photochemistry greatly reduced, the fluorophore is free to emit light upon excitation, therefore signaling the presence of $^1O_2$. Two examples of $^1O_2$ sensors used in plants, dansyl-based probes and Singlet Oxygen Sensor Green, are introduced in the following sections.

1.3.1.1 Dansyl-based probes

DanePy (3-[N-(β-diethylaminoethyl)-N-dansyl] aminomethyl-2,2,5,5-tetramethyl-2,5- dihydro-1H-pyrrole) is used as a fluorescent, but also spin-trapping, probe for the estimation of $^1O_2$. Contrary to most other sensors that respond to ROS by increased fluorescence emission, the fluorescence of DanePy is quenched upon production of $^1O_2$. DanePy and other dansylated sterically hindered amines were designed to trap ROS [49]. DanePy showed best solubility in aqueous media and appeared selective to $^1O_2$, although $O_2^{\bullet -}$ or $HO^{\bullet}$, H_2O_2 and lipid peroxidation products also resulted in some fluorescence quenching. Importantly, presence of the $^1O_2$ scavenger histidine suppressed quenching of the fluorescence signal, suggesting competition between the scavenger and the probe for $^1O_2$. The probe was first used to detect $^1O_2$ in isolated spinach thylakoid membranes subjected to photoinhibition by high light in plants [49].

As the half-life of $^1O_2$ is very short due to its high reactivity and fast non-radiative deactivation [50], diffusion of $^1O_2$ within the cell is limited. It is therefore important that the sensor or probe is located at the site where the ROS is being generated. Later work with intact spinach leaves showed that DanePy located to chloroplasts [51]. This localization, together with the fluorescent properties of DanePy (emission at 500-600 nm, with a maximum at 545 nm, and little overlap with the autofluorescence of a plant leaf, see **Figure 1**), made it an ideal sensor for photosynthetic studies, e.g. stress resulting from photoinhibition. Local infiltration through a pinhole was shown efficient in order to deliver the sensor uniformly, and that this method caused less damage to the tissue and photosynthetic processes than e.g. vacuum infiltration. Using this probe *in vivo*, the authors could conclude that strong light produced mainly $^1O_2$ and

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only little $O_2^{\bullet-}$, while under UV-irradiation the situation was the opposite, where the main ROS produced consisted of $O_2^{\bullet-}$ [51].

Other researchers have used this probe to study generation of $^1O_2$ in illuminated leaves affected in chlorophyll breakdown [52]. In this study, dark-incubated leaves were exposed to light for various time periods. Leaf discs were subsequently infiltrated with DanePy in order to measure fluorescence quenching and $^1O_2$ production. It has also been successfully used to monitor $^1O_2$ production and stress responses upon dark-to-light shift in leaves of the *Arabidopsis flu* mutant [53]. The authors showed that generation of $^1O_2$ started within the first minute of illumination, and that it was confined to plastids. This study also confirmed the specificity of DanePy to $^1O_2$, as quenching of DanePy fluorescence only occurred upon illumination of pre-darkened leaves, and not because of the injury resulting from the leaves cutting and infiltration.

HO-1889NH is another dansyl-based sensor that differs only in one side-chain with DanePy. However, HO-1889NH reacted less with $^1O_2$ and showed cross-reactivity with $O_2^{\bullet-}$, which makes this probe less useful as a $^1O_2$-specific probe in plants [51].

1.3.1.2 Singlet Oxygen Sensor Green (SOSG)

Singlet Oxygen Sensor Green (SOSG) exhibits weak blue fluorescence that shifts to green upon reaction with $^1O_2$ [54]. This is the result of no longer exciting the anthracene moiety (feeble blue fluorescence) due to its reaction with $^1O_2$, thus opening the way for the fluorescein green emission (see 1.7.1 above). Valuable features as increased stability, high selectivity for $^1O_2$, and non-toxic effects on photosynthetic organisms makes SOSG a useful fluorescent probe to monitor $^1O_2$ generation in algae and plants [55]. However, recent studies comparing SOSG with other $^1O_2$ probes revealed that SOSG photosensitivity and unspecific interactions hampers its use in photosynthetic organisms [56]. Especially important is the high photosensitivity demonstrated by SOSG after low wavelength (< 600 nm) exposure which caused inhibition of photochemical yields of photosystem II (PSII) in tobacco leaves by 15% [56]. Additionally, UV radiation exposure leads to photobleaching of the probe mediated by radical species [57]. Thus, to counteract the effect of photosensitivity and photobleaching, appropriate experimental controls and data correction are critical when SOSG probe is used for $^1O_2$ monitoring in photosynthetic organisms.

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1.3.2 Superoxide radical ($O_2^{\bullet -}$) detection

1.3.2.1 Dihydroethidium (DHE)

Dihydroethidium (2,7-diamino-10-ethyl-9-phenyl-9,10-dihydrophenanthridine, DHE) is widely used as a fluorescent probe for $O_2^{\bullet -}$ detection [58,59]. The dye permeates cell membranes and specifically reacts with $O_2^{\bullet -}$, producing the red fluorescent 2-hydroxyethidium (2-OH-E^+) (see **Figure 2a**) that has spectral properties well suited for plant tissues (excitation and emission maxima around 500 nm and 600 nm, respectively, see **Figure 1**) [60]. However, DHE can also generate the fluorescent ethidium cation (E^+) upon oxidation by various catalysts inside the cell. E^+ is spectrally similar to 2-OH-E^+ and often accumulates at higher concentrations than 2-OH-E^+. Both oxidized DHE products bind DNA, resulting in an enhancement of the fluorescent signals [60,61]. It has thus been suggested that DHE should be considered a qualitative, and not a quantitative, readout of $O_2^{\bullet -}$ levels. For a detailed discussion about the chemistry of DHE in biological systems and their implications on $O_2^{\bullet -}$ detection, we recommend the excellent review by Zielonka and Kalyanaraman [62]. Importantly, Zielonka and Kalyanaraman highlight that precise determination of intracellular superoxide-specific 2-OH-E^+ levels requires HPLC analysis, and not only fluorescence-based microscopic assays, because of the spectral overlap between 2-OH-E^+ and E^+ [63]. Another disadvantage of DHE is the sensitivity to auto-oxidation and light [63]. Stability in culture medium and transport of DHE over multiple cellular compartments in tissues are other issues to consider [62]. In lupine embryos, an incubation time of 18 h was for example necessary to obtain a strong fluorescence signal [64].

Nevertheless, DHE derived probes are frequently used fluorescent dyes to study $O_2^{\bullet -}$ in plants, mostly in cultured cells [65,66] or smaller tissues such as root tips [67–70]. Exposure of *Arabidopsis* roots to rhizotoxic stressors showed that different stressors resulted in different location of $O_2^{\bullet -}$ production [67], although, as mentioned above, no quantitative analysis could be performed using DHE. In another study, where the localisation of ROS production was better characterized, abolishment of DHE fluorescence upon sodium azide (POX inhibitor) or diphenylene iodonium (DPI, NADPH oxidase inhibitor) treatment suggested the involvement of POX and NADPHox in $O_2^{\bullet -}$ generation in Cd-treated pea roots [69]. The same group later investigated the effect of Cd on antioxidants, ROS and $^{\bullet}NO$ metabolism of pea leaves [71]. Incubation of leaf sections with DHE indicated $O_2^{\bullet -}$ production in xylem vessels and adaxial sclerenchyma, epidermis, stomata and mesophyll cells. Supplementation of calcium (Ca^{2+})

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prevented accumulation of $O_2^{\bullet-}$ in mesophyll cells, but not in epidermis and vascular tissues where fluorescence was increased, suggesting a possible involvement of Ca^{2+} in ROS production. DHE has also been applied to isolated thylakoids samples [72], where PSII particles from plants deficient in PsbS, a PSII protein involved in non-photochemical quenching of chlorophyll fluorescence, were shown to generate more $O_2^{\bullet-}$ under high light stress.

Most importantly, specificity to $O_2^{\bullet-}$ of the detected fluorescent signal can be ensured by incubating control samples with the $O_2^{\bullet-}$-scavenger tetramethyl piperidinooxy before incubation with DHE [69,71,73,74]. Better imaging and preservation of signal over time [73], which may be required to characterize ROS distribution within plant tissues, can be achieved using different protocols for specimen embedding and sectioning, as reported in pea roots [69].

A positively charged mitochondria-targeted DHE variant called MitoSOX that accumulates in the mitochondrial matrix has also been developed. The reactivity of MitoSOX is similar to that of DHE, but due to its cationic nature it accumulates faster at the mitochondria, favoured by its electrochemical potential gradient. It has been used in plants to study $O_2^{\bullet-}$ kinetics and mitochondria-induced cell death resulting from aluminium (Al) stress in protoplasts and isolated mitochondria of *Arabidopsis* [75]. Using MitoSOX, the authors observed fluorescence emerging 10 min after Al treatment, which increased for another 20 min, indicating that $O_2^{\bullet-}$ and H_2O_2 formed at the mitochondria was involved in the oxidative burst induced by Al.

1.3.3 Hydrogen peroxide (H_2O_2) detection

Detection of H_2O_2 is particularly difficult for several reasons: i), H_2O_2 can be considered as a "secondary" ROS, as it is produced only after a "primary" ROS (e.g. superoxide) has been generated in the system being studied; ii) although its oxidation potential is relatively high (E^0= +1.78 V under standard conditions), it displays a certain "chemical inertness" due to kinetic factors, which reduce its chemical reactivity in a cellular environment [76]; iii) it tends to produce further ROS (e.g. $OH^{\bullet}$ radicals), which have a higher oxidation potential (E^0= +2.31 V under standard conditions) and faster reaction kinetics, thus altering the measurement due to the reaction of the H_2O_2 probe with these downstream ROS. Taken together, selective H_2O_2 detection can be a daunting task. The problem can be ameliorated by taking advantage of certain catalytic reactions driven by POXs in conjunction with the fluorescent probe (see Amplex Red® below). However, given the importance of the fluorescent 2,7-

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dichlorodihydrofluorescein (DCF)- and dihydrorhodamine 123 (DHR)-based dyes in the literature, we will introduce these probes in the following sections.

1.3.3.1 2,7-Dichlorodihydrofluorescein (DCF)-based probes

2'-7'- dichlorodihydrofluorescein diacetate (DCFH-DA) is perhaps the most frequently used dye when studying ROS and oxidative stress in plant cells and green algae. It belongs to the reduced fluorescein (dihydrofluorescein) dye family, in which a diacetate ester addition facilitates cell-permeability, allowing for better loading and trapping [54]. It is generally assumed that DCF-based probes are relatively specific detectors of H_2O_2. However, a critical review of the relevant literature that focuses on mechanistic issues shows that this is not the case, and is therefore deserved here a cautionary remark.

The established reaction mechanism for DCF-based probes is shown schematically in **Figure 2b.** The oxidation of the esterase-released form (DCFH) appear to be a two-step reaction: first the radical $DCF^{\bullet-}$ is produced after losing one electron, and then a second one-electron oxidation takes place, resulting in 2,7-dichlorofluorescein (DCF) [36] (see **Figure 2b**), which is retained in the cell, allowing for visualization by fluorescence microscopy and flow cytometry [75,77]. The same chemical behavior has been reported for dihydrorhodamine (DHR, see below). As a consequence, DCFH and DHR are rather unreactive to $O_2^{\bullet-}$ and H_2O_2, and requires production of the semiquinone radical for the reaction to proceed efficiently [36,38]. Also, certain POX catalyze the oxidation of these probes. To further complicate things, two semiquinone radicals can undergo a disproportionation reaction, whereby the reduced probe precursor and the fluorescent probe are generated. These intermediate semiquinone radicals can react directly with O_2, producing the fluorescent probe but also $O_2^{\bullet-}$ and subsequently H_2O_2 (**Figure 2b**). This is undesirable for two reasons, i) the fluorescent probe is being generated by reacting with a non-ROS compound (O_2); ii) an artificial increase in ROS occurs as superoxide is introduced in the system through an artefactual mechanism [36]. Therefore, it is more accurate to claim that these probes detect increased radical production, or as stated by Gomes and co-workers, they have "better use as a marker of the cellular oxidative stress than as indicator of the formation of H_2O_2 or other ROS and RNS" [38].

Photochemistry is another important aspect to consider when using these probes. Both DCF and rhodamine 123 (see 1.7.3.2 below) are particularly good fluorophores with high quantum

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yields of fluorescence [37]. But some photochemistry is unavoidable when illuminating these fluorophores. Once in an electronic excited state, the fluorophore often emits a fluorescent photon. However, there is a non-negligible risk that it undergoes a chemical reaction because of its energized state. Indeed, photoexcited DCF* reacts with GSH or NADH regenerating the probe radical and engaging available O_2 into redox chemistry (with DCF·/GS·/NAD·) producing $O_2^{\bullet -}$ [36]. Another photochemical reaction is the photosensitization of singlet oxygen ($^1O_2$) by the excited fluorophore, which is also a ROS. $^1O_2$ is a strong oxidizer and can cause false positive signals due to further probe oxidation [59]. All this taken together contributes to the relative non-selectivity of this dye [78,79].

In light of the presented facts on DCF-based probes, the following aspects should be taken into account when measuring ROS in living systems, including plants [36]: probe reactivity, catalysts role, probe intermediates and their presumable reactions, cellular distribution and interaction with antioxidants. Special attention should be given to the probe´s photochemistry once it has been produced in the system under study.

Additionally, as DCFH is soluble in both, the lipid and the aqueous phases, it is also important to remember that the reaction of DCFH with aqueous radicals is quickly prevented by soluble cellular antioxidants, but not in the lipophilic compartment. This feature makes DCFH a useful probe for detection of lipid hydroperoxides, especially DCFH derivatives that have been modified for enhanced lipophilic retention, e.g. carboxy-DCFH-DA [38].

To prevent passive leakage of DCFH-DA across the plasma membrane, which reduces the time-span for which this dye can be recorded, variants that aim to tackle this problem have been developed. The chloromethyl DCFH-DA derivate (CM-DCFH-DA), retention of the oxidised fluorescent product in living cells is increased because of a covalent bond formed between the chloromethyl group and intracellular thiol groups.

DCF-based probes have been extensively used to monitor ROS production in plants subjected to different stress stimuli and developmental cues. Some examples can be found in the following paragraphs.

Considering the multiple restraints described for DCFH-based probes, an appropriate experimental design is essential when using fluorescein-based dyes. In that regard, Potocky *et*

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al. studied the role of ROS generated during pollen tube development in *Nicotiana tabacum* [80]. General ROS production and distribution in pollen tubes was measured using cell permeable CM-DCFH-DA. To prevent signal limitations the authors established the optimal dye concentration for their experimental system where initial background was evaluated to avoid fluorescence signal overestimations. Moreover, utilization of the ROS scavenger MnTMPP (Mn-5,10,15,20-tetrakis(1-methyl-4-pyridyl)21H,23H-porphin), the NADPHox inhibitor DPI and antioxidants (GSH and ascorbate) allowed for the identification of NADPHox activity as the main ROS source during pollen tube growth. Similar studies of pollen tube growth in *Papaver* used CM-DCFH-DA in combination with DPI and external H_2O_2 supply to assess the distribution of ROS in pollen tubes along with their involvement in the self-incompatibility response [81].

As mentioned above, ROS and particularly H_2O_2 are generated in response to different stresses acting as signals throughout the plants. For instance, plant-pathogen elicitor proteins stimulate the production of H_2O_2, essentially through the activation of plasma membrane NADPHox, activating plant defense signaling and responses. Within this context, Sang *et al*. [82] generated transgenic lines of *Arabidopsis* that expressed a ROS-inducer harpin bacterial protein targeted to different subcellular compartments to study cytoplasmic and apoplastic H_2O_2 production. The authors compared ROS (DCFH-DA fluorescence) distribution with that of H_2O_2 using probes with better specificity: DAB for macroscopic detection and Amplex Red® and Amplex UltraRed® for cytoplasmic and apoplastic production site analysis respectively (see section 1.7.3.4) in *Arabidopsis* leaves. The NADPHox inhibitor DPI additionally helped to differentiate the subcellular localization of H_2O_2 or other ROS production. Based on their results, authors concluded that H_2O_2 might be translocated from the apoplastic space to the cytoplasm as a part of a plant pathogen defence mechanism [82].

Different plant genotypes and mutants are frequently used to study the physiological role of specific ROS during biological processes. In this respect, *Arabidopsis* represents a valuable tool for large-scale mutant screening. For example, double staining experiments in different *Arabidopsis* genotypes using two fluorescence probes (DCFH-DA and MitoSOX red) helped Martin and colleagues to determine that not only $O_2^{\bullet -}$ but also other mitochondrial ROS were involved in gametophyte development [83]. Likewise, *Arabidopsis* NADPHox defective mutants together with catalase (CAT) as peroxide scavenger were used by Wang *et al*. to elucidate the

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role of H_2O_2 in the heat shock signalling pathway that triggered *Arabidopsis* thermotolerance [84].

CM-DCFH-DA suitability as an indicator for *in vivo* monitoring of ROS using confocal laser scanning microscopy has been assessed in onion bulbs and *Arabidopsis* leaves, concluding that dye uptake might be limiting in some tissues, and protoplasts would be the best system to be used [85]. CM-DCFH-DA has also been used to characterize root responses to nutrient (potassium, K^+) starvation in *Arabidopsis* and maize [86]. The experimental strategy using a combination of *Arabidopsis* NADPHox mutant lines, the NADPHox inhibitor DPI, together with the use of H_2O_2 probe Amplex Red® and CM-DCFH-DA (in addition to transcriptional analysis) allowed the authors to conclude that K^+ starvation signalling and modulation of gene expression is mediated by ROS.

Despite acknowledged limitations of the DCFH-based probes, commercial availability in combination with their relative ease of use make them useful as tools for ROS generation and pro-oxidant status detection.

1.3.3.2 Dihydrorhodamine 123 (DHR)

In addition to DCFH-based probes, another dye frequently used as H_2O_2 indicator is dihydrorhodamine 123 (DHR) [87–89]. The ROS detection mechanism is essentially equal to that of DCF (see **Figure 2b**). DHR is a non-fluorescent membrane-permeable probe that is not reactive to H_2O_2 or $O_2^{\bullet-}$ in absence of a catalyst [36]. Instead it scavenges the $OH^{\bullet}$ generated from H_2O_2 in an iron-dependent Fenton reaction, generating the oxidized fluorescent product rhodamine 123. As for DCFH, DHR also reacts with $CO_3^{\bullet-}$, hypochlorous acid (HClO), $ONOO^-$ and other $^{\bullet}NO_2$ radicals. Therefore, and similarly to DCFH-DA, this lack of specificity for H_2O_2 makes it a marker of overall cellular redox environment or increased radical production instead of a hydrogen peroxide specific probe [90]. Also, as for $DCF^{\bullet}$ (see 1.7.3.1 above), the one-electron DHR radical ($DHR^{\bullet}$) intermediate reacts with O_2, leading to an artificial increase of the fluorescent signal. Still, several studies have used DHR to study ROS production in plants, e.g. in *Rubia cordifolia*, *Panax ginseng* and *A. thaliana* cells and callus [91]. In this work, the function of the *rolB* gene (for rooting locus *B* of *Agrobacterium rhizogenes*) in reducing ROS generation after exposure to different inducers was assessed in cultured cell lines with different *rolB* expression levels. ROS production under high light, in combination with Paraquat or menadione

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($O_2^{\bullet -}$ inducers at PSI and plasma membrane, respectively), was attenuated in cell lines overexpressing *rolB*, especially at the nucleus and plasma membrane. The authors concluded that *rolB* functions mainly at intracellular level, supressing ROS accumulation probably by enhancing antioxidant-enzyme gene expression. Notably, DCFH and DHR probes with similar photochemistry and ROS specificity were used. However, DHR showed an advantage over DCFH, as the former permitted the detection of ROS production not only in the cytosol, but also in mitochondria [91].

DHR was also used to measure ROS generation in *Arabidopsis* cell cultures in response to Cd exposure [88], and elicitor-triggered hypersensitive response and stomatal closure in epidermal strips of *N. benthamiana* [89]. In this work, the role of a vacuolar processing enzyme (VPE) as mediator of pathogen-induced ROS production and hypersensitive response was evaluated in different VPE-silenced *Nicotiana* lines. DHR allowed for ROS measurements in response to specific pathogens, showing reduced ROS generation and stomatal closure in the VPE-silenced guard cells. The authors concluded that VPE is involved in the regulation of defence-related gene expression and pathogen-induced stomatal closure [89].

Given the similarities in the chemical structures between DCF (hydroxyl substituents on the anthracene-like nucleus) and rhodamine 123 (amine substituents), it comes as no surprise that their chemical/photochemical behavior is similar. As such, the concerns highlighted in the previous section regarding DCF-based probes also apply to DHR-based probes.

1.3.3.3 CellROX®

Similar to DCFH-DA and DHR, CellROX® detects increased radical production. Upon oxidation by ROS, the otherwise non-fluorescent probe displays bright fluorescence. Lack of information about the exact chemical structures of these commercial products prevents a critical assessment of their molecular properties. Nevertheless, based on their absorption/emission spectra provided by the commercial supplier, it could be argued that CellROX® probably belongs to the DCF-like family of fluorophores (see 1.7.3.1 above). Members of the CellROX® family have overcome some of the limitations usually associated with dye probes, e.g. the limited number of probes with distinct colours (CellROX® variants display green, orange and red emission) required to perform dual or multiple labelling assays. Also, because of their intrinsic chemical properties, some CellROX® variants only emit fluorescence in a specific cell organelle, avoiding

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the compartment "targeting" limitation. For example, CellROX® Green only fluoresce after binding to DNA, restricting its signal to the nucleus, mitochondria or chloroplast. Though DNA binding seems to be essential to the CellROX® Green fluorescence, this fluorophore requires no further cellular processing and can be used for ROS extracellular measurements if DNA is present in the probe solution [92]. In contrast, CellROX® Orange and CellROX® Deep Red can be used in the cytosol as they do not require DNA binding to fluoresce [93]. Moreover, CellROX® Deep Red shows higher stability than traditional oxidative stress fluorescent probes (e.g. DCFH-DA) in addition to spectral properties better suited for plant imaging, with the possibility of multiplexing and simultaneous use with GFP or other probes. CellROX® Deep Red was used in *Matricaria chamomilla* to analyse the role of ROS and NO in Mn and Cd-induced stress [94,95]. In these works, application of several NO modulators showed that CellROX® Deep Red probe does not label a specific ROS, and complementary information from other dyes is needed to determine the ROS/RNS type generated after metal exposure.

1.3.3.4 N-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red®)

N-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red®) is a non-fluorescent substrate for POX that reacts with H_2O_2 to produce resorufin, a highly fluorescent product (**Figure 2c**). The POX-catalysed electron transfer is highly efficient, making Amplex Red® a specific and suitable probe to measure H_2O_2 formation and/or POX activity [59]. The excitation and emission maxima of this probe (568 and 581 nm, respectively) makes it suitable for use in plant tissue (see **Figure 1**). Further improved variants have been developed with increased sensitivity and contrast, e.g. Amplex UltraRed® that exhibits increased resistance to oxidation and works better in lower pH environments. Amplex Red® and its derivatives have shown capable of detecting H_2O_2 in plant tissues and cells, e.g. in the roots of seedlings of *Arabidopsis* following pre-incubation with H_2O_2 [96]. In another study, cultured tobacco BY-2 suspension cells were used to study oxidative burst defence responses to the pathogen elicitor cryptogein. Interestingly, H_2O_2-induction as a response to cryptogein treatment showed that Amplex Red®, but not Amplex UltraRed®, could penetrate the cells [97]. To ascertain that the recorded fluorescent signal was due to H_2O_2, CAT was added. Importantly, experiments where tobacco leaves were infiltrated with pre-oxidized Amplex Red® showed that resorufin could be detected also in leaf tissue, where it accumulated in the stomata and penetrated the guard cells, and localized mainly in the chloroplasts [35]. However, the study also showed that fluorescence of the oxidized probes started to decrease

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immediately after infiltration, also when leaves were kept in the dark. Fluorescence of resorufin was estimated to have a half-life of 6 to 8 min. In addition, both probes affected the leaf photochemistry, highlighting that concentrations should be kept at a minimum. Infiltration of Amplex Red® into the leaf resulted in fluorescence, which was reduced upon co-infiltration with CAT, indicating that the fluorescent signal was specific, mainly due to H_2O_2. Enhanced H_2O_2 specific fluorescence intensity was achieved by co-infiltration with HRP (avoiding the reaction-limiting step) upon high light treatment. In agreement with the results obtained in BY-2 cells [97], the authors concluded that Amplex Red® penetrates the leaf cells of tobacco more efficiently [35].

As Amplex Red® functions as an enzyme-dependent probe, it is susceptible to alterations in POX activity caused by various substances. For example, upon very high H_2O_2 concentrations resorufin can be used as substrate by POX, generating the non-fluorescent resazurin product that will lower the overall fluorescence (see **Figure 2c**) [98]. Moreover, intracellular reductants such as NADH or GSH can interact with HRP to produce $O_2^{\bullet -}$ and H_2O_2, resulting in oxidation of Amplex Red to resorufin without the requirement of exogenous H_2O_2 [99]. Also, broad wavelength irradiation (UV + VIS) of anaerobic NADH-resorufin dilutions results in generation of semiquinone products and reduced dihydroresorufin. If oxygen is admitted to the system after light exposure, these photoproducts react with O_2 producing $O_2^{\bullet -}$ and H_2O_2, which can lead to false positive results [100]. Therefore, perhaps the most valid conditions where the Amplex Red® probe can be used are measurement of extracellular H_2O_2 production (e.g. membrane NADPHox and SOD activity analysis) in the presence of externally added POX (such as HRP), or those involving plant extracts using appropriate controls [101]. Such studies have been performed in alfalfa roots upon Cd or mercury (Hg) exposure, where the kinetics of extracellular H_2O_2 production was followed up to 24 h after addition of the toxic metals [77,102]. Pre-incubation with the NADPHox inhibitor DPI affected the H_2O_2 response to Cd, but not Hg, suggesting different mechanisms of toxicity for the two metals. In other studies, Amplex Red® was used to detect both H_2O_2 production and POX activity in wheat and rice plants at the attack sites of larvae [103]. Further analysis of transcripts indicated that class III POX could play a role in ROS generation during these attacks.

1.3.3.5 Boronate-based probes: Peroxysensors

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Aromatic boronate-based fluorophores, known as the Peroxysensor family (Peroxy Green 1, PG1; Peroxy Yellow 1, PY1; Peroxy Orange 1, PO1; etc.), consist of a boronate moiety that masks a fluorophore (e.g. carboxy fluorescein). Upon oxidation by H_2O_2, the fluorophore becomes exposed and emits fluorescence [104,105]. The masking boronate substituents, chemical cycles including an O-B-O bridge are positioned at the periphery of the organic fluorophore. These cycles provide the increased specificity for H_2O_2. Probes can display one or two of such boronate groups. The cell-permeable boronate esters can be effectively modified in order to measure intracellular H_2O_2 in specific compartments [106]. However, the relatively low reaction kinetics for H_2O_2 results in competition with H_2O_2-scavenging enzymes, which reduces the overall signal. Additionally, aromatic boronate-based indicators can react with $ONOO^-$ faster than with H_2O_2, therefore reducing the specificity for ROS detection [107]. Nevertheless, the boronate sensor ContPY1 has been used to detect intracellular H_2O_2 formation in *Arabidopsis* cell cultures, protoplasts and leaves [108,109]. Higher fluorescence values obtained with the boronate-based probe ContPY1 compared to DCFH-DA in cell cultures and protoplast after elicitation of H_2O_2 production with COS-OGA (a complex of chitosan and oligogalacturonides that activate pathogen defense-signaling pathways and oxidative burst) were explained by the selective detection of H_2O_2 by the latter, and similar conclusions were obtained after wounding of leaves [108]. Complementary work using specific inhibitors of CAT, POX and NADPHox helped to elucidate the importance of cell wall and intracellular sources in H_2O_2 generation under normal and eliciting conditions [109]. However, the faster reaction kinetics of boronate probes for $ONOO^-$ than H_2O_2 could also interfere with the specific detection of H_2O_2 [110]. The specificity of these measurements should be confirmed by the use of $ONOO^-$ scavengers (e.g. uric acid) as additional experimental controls.

1.3.4 Other fluorescent dyes for ROS and redox detection

The development of reversible organochalcogen probes based on the incorporation of a selenium or tellurium atom into a fluorophore opens new possibilities for general redox status analysis. Organochalcogen-based probes show high reactivity with aromatic thiols, Cys and GSH [111], in addition to a wide range of ROS and RNS [112]. To date, we have however not found reports of its use in plants. The limited knowledge about the alterations ROS that and redox status cause to these molecules will require further investigation before they can be more widely used for redox biology studies.

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Another type of fluorescent probes is the HKGreen family of rhodol-based markers, which allowed to distinguish ROS from RNS in *Arabidopsis* leaves showing hypersensitive response [109,113]. Rhodol is a variation of the fluorescein family of fluorophores. Where fluorescein harbors two hydroxyl substituents, rhodol has one hydroxyl and one amine group. Therefore, rhodol can be considered as an intermediate compound between fluorescein and rhodamine.

In this review, we have focused on the most popular fluorescent dyes used in plants. For extended information about other fluorescent probes for ROS and RNS detection, we refer to Wardman and Chen [36,114].

1.4 The dyes chemistry in plant cells: assets and drawbacks

Fluorescent probes and dyes offer great possibilities to study ROS signalling *in vivo*, as discussed and exemplified above. Their chemistry and photochemistry are well known and rigorous controls can be undertaken in order to verify the obtained results. They are readily available from various suppliers and their chemical composition is reliably controlled. They have been the main approach to study redox chemistry and biology for many decades, not only in plants, but also in practically every known model for redox biology. Therefore, the results obtained can be directly compared with a very robust bibliographic database.

A redox probe´s cellular distribution can be predicted with relative accuracy thanks to the QSAR (Quantitative Structure-Activity Relationship) model [37], allowing for some information about the subcellular redox signaling involved, although not as conclusive as when using fluorescent protein sensors. It is also worth mentioning that experimental protocols employing redox dye probes are relatively easy to execute. Often, a simple incubation of the sample for a certain time period is the only required action to obtain the results. Of course, complementary controls must be included (e.g. to account for any foreseeable probe photochemistry), but in general the protocols are easier to implement compared to when using genetic probes.

However, the fluorescent probes and dyes are not without drawbacks. They are irreversibly oxidised, and do not recover their initial state making it difficult to study reaction kinetics and subtle shifts of redox potentials [59]. In addition, many of these probes react with various ROS and RNS or their derivatives, including the probes´ own intermediate states, and cellular antioxidants, which can jeopardize their specificity and cause artificial readouts that limit their

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use for quantitative analyses (**Figure 2d**) [36]. This further highlights the importance of the implementation of proper controls. Moreover, alterations in the cellular thiol/disulphide status or signal transduction may occur from ROS production/scavenging by the probe itself. Thus, only few studies have described simultaneous imaging of different ROS and RNS successfully in plants [73].

It is therefore important to understand the probe´s chemistry in detail, as well as the possible interactions with different ROS, RNS, antioxidants and other cellular molecules. Detailed knowledge of the probe´s specificity, sensitivity, photostability, solubility, permeability and intracellular distribution in biological systems must be obtained before an experiment using a particular fluorescent probe is designed. Hence, the implementation of carefully selected control reactions/conditions is mandatory to check probe photoactivation or photobleaching, pH-induced artefacts, false negatives due to catalysts limitation, artificial fluorescence amplification by intermediate radicals and possible interactions with antioxidants, to name some experimental parameters.

Researchers should consider the photochemical properties of the selected probe. Once the probe has been oxidized to its fluorescent state, the researcher must consider that other deactivation mechanisms can prevent further fluorescence, and redox chemistry of the excited probe can give rise to ROS in the system [115]. The excited probe can also transfer its excitation energy directly to O_2 in its ground state ($^3O_2$) producing $^1O_2$, which is another ROS [116]. These processes and chemical reactions are, to a greater or lesser extent, unavoidable. But certain courses of action can help minimizing their impact on the sample, e.g. keeping light exposure as low as possible, establishing the adequate probe precursor concentration, or reducing the sampling time. Although new and more specific dyes with improved characteristics have been developed, precise subcellular localization of fluorescent probes (except MitoSOX and similar probes) is still a limitation.

In summary, understanding each probe´s limitations, control of experimental conditions, adequate interpretation and cross-validation of the results with independent experimental methods is crucial for correct interpretation of the experiments [61]. For further information about the chemistry of dye-based probes, we refer to [38,117].

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1.5 Fluorescent sensor proteins for ROS and redox detection

The use of genetically encoded sensor proteins is becoming increasingly popular, as they fulfil most of the requirements for an ideal live imaging sensor [118]. Fluorescent genetically encoded biosensors are usually based on the green fluorescent protein (GFP), which was first used in plants in maize mesophyll protoplasts and Arabidopsis roots and shoots [119]. Perhaps the most important advantages offered by fluorescent protein (FP)-based probes (over fluorescent dyes) for *in vivo* imaging of living tissues are: i) permeation of a substrate is not required, making non-invasive techniques for *in vivo* monitoring of ROS/redox changes possible and avoiding leakage of the probe in long-term assays; ii) spatially and temporary control of the sensor expression in specific tissues and life cycle stages by inducible promoters; iii) possibility to target FPs to different organelles using sorting signals; iv) reversibility under changing cellular redox status, which enables dynamic changes monitoring and, v) specificity for distinct ROS species or redox couples, as many FP-based biosensors include naturally evolved protein sensor domains or fused enzymes for specific analytes (including ROS or redox couples), or sensitivity to redox changes that produce readouts (usually conformation changes or alterations in the chromophore milieu) reflected in the sensor fluorescent characteristics/properties.

Some of these sensors readout is intensiometric, meaning that single excitation and emission wavelengths are monitored. Such sensors are susceptible to factors not related to the experimental condition, e.g. expression level of the sensor or photostability. On the contrary, for ratiometric sensors a pair of emission or excitation wavelengths are recorded, where their ratio reflects the experimental condition or output. These sensors can be either excitation ratiometric sensors, where a single emission peak is monitored using two distinct excitation wavelengths, or emission ratiometric sensors, where a single excitation wavelength is used and two emission peaks are monitored. In these cases, artefacts from photobleaching and variations in protein expression level between different experiments are minimized and alteration of the spectral characteristics of the sensor is only related to the amount of ROS and/or oxidised/reduced redox pair exchange, thus making ratiometric sensors better suited for live imaging and quantitative measurements [120].

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Genetically encoded biosensors can be expressed transiently, using appropriate expression vectors, or stably integrated in the plant cell genome, making them flexible tools for co-visualization analyses. In that sense, less sample manipulation is required compared to cell uptake or loading of permeable fluorescent dyes. However, note that GFP biosynthesis involves H_2O_2 production [121]. Therefore, special care should be taken when studying cellular signalling processes using FP-based sensors, especially when fused to redox enzymatic systems, as their expression itself can generate/scavenge H_2O_2 or alter the cellular thiol system [121,122] that in turn may alter signalling processes and lead to acclimation or adaptation.

In addition to microscopy, other methods that take advantage of genetically encoded protein sensors for live cell imaging can be used. For example, laser scanning plate readers can be used to simultaneously measure fluorescence of a reasonable large number of plant tissue samples (e.g. leaf discs or seedlings) in a relatively short time and facilitating high-throughput screening assays [123]. On the other hand, flow cytometry and fluorescence-activated cell sorting, widely used to analyse mammalian cells in cultures, have so far been used to a limited extent in the plant field [124,125]. An important reason for this is that cell walls must be removed by enzymatic degradation to obtain protoplasts, which promotes stress and alters the physiology of plant cells. In addition, protoplasts are fragile due to the large volume constituted by vacuoles, making plant cells susceptible to breaking and hampering their manipulation.

One of the more important pitfalls of ROS dyes for live imaging of dynamic ROS or redox changes is the non-reversibility of the fluorogenic probe. Even though dye oxidation rate can be quantified and signal accumulation allows for high sensitivity, once oxidation occurs, most dyes cannot be reversed to their original form, limiting their use for live-cell imaging with dynamic quantification and measurement [38]. This can be beneficial for epifluorescence microscopy users, as signal accumulation can increase signal-to-noise ratio and help reduce background interferences during detection. On the contrary, at the expense of sensitivity, most protein-based sensors show reversible kinetics, which support ROS and redox pair's dynamic quantitative analyses. In addition, very few fluorescent dyes allow for ROS detection in particular subcellular compartments. The possibility of organelle-specific targeting is therefore a main advantage of genetically encoded biosensors, enabling live ROS monitoring in distinct

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cell compartments [126]. This property is necessary to unravel signalling events that are organelle and ROS-specific, as mentioned previously.

Better specificity for a certain ROS or redox pair is another key advantage of protein based sensors. In this regard, one of the best options to study ROS and redox regulated processes in plants is by using redox sensitive elements (e.g. transcription factors) fused to FPs. Changes in redox cellular balance alter the redox-sensing domain and subsequently the fluorescent properties of the protein. While most fluorescent sensors are nowadays based on ROS/redox-sensing elements of prokaryotic origin, large-scale transcriptome analyses of several plant species have given important knowledge about ROS/redox sensing networks that can be tested for generation of novel plant specific ROS and redox sensitive sensors [127–130].

Three main strategies have been employed for the development of protein-based fluorescent biosensors (**Figure 3**). Single FP-based sensors (**Figure 3a-d**) make use of the chromophore´s sensitivity to the environment, which can be modified through changes of specific amino acids (mutations) into the FP structure. Such engineering alters the three-dimensional structure of the FP and the interactions between the chromophore and its surroundings, facilitating the conversion between the protonated and anionic forms, which modify the sensor´s fluorescent properties. To engineer redox sensors, addition of redox sensitive residues such as Cys pairs at selected positions of the FP facilitates disulphide bond formation under fluctuating cellular redox conditions. This alters the FP´s chromophore environment, and changes the spectral properties of the sensor. Circular permutation of FPs (cpFPs) is a specific case in which the N- and the C-termini of the original protein are joined, and new N- and C-termini are created closer to the chromophore location where addition of ROS sensing domains affects the stabilization of the structure and alters the fluorescence of the sensor. Finally, FRET sensors (**Figure 3e**) are based on an energy transfer by resonance from a donor fluorophore to an acceptor fluorophore, which can happen if the emission spectrum of the donor overlaps with the excitation spectrum of the acceptor. Fusion of the two FPs into a single polypeptide chain, with additional sensing domain(s) that changes the conformation and distance between the donor and acceptor, can result in a FRET signal that reflects the status of the surrounding environment.

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1.5.1 Sensors based on single FPs

It is possible to exploit the fact that certain conditions affect the FPs in order to engineer a redox sensitive biosensor. For example, effective mutations added to the periphery of the chromophore can increase the sensitivity of the FP to a specific state/condition. Redox-sensitive YFP (rxYFP) and redox-sensitive GFP (roGFP) were created by introducing redox-reactive Cys residues at the surface of the FP. Alteration of the glutathione redox couple (2GSH/GSSG) potential, an accepted indicator of intracellular redox conditions [131], would change the oxidation status of these Cys and thus changing the fluorescent properties of the sensor. As they do not directly sense ROS, fluorescence is only altered when ROS accumulation in turn is able to shift dithiol/disulphide redox pairs in proteins and biothiols.

1.5.1.1 rxYFP

rxYFP was engineered by introducing pairs of cysteines into a yellow shifted GFP-derived protein (YFP), allowing disulphide bond formation in an oxidizing environment [132]. The three-dimensional structure of the wild type *Aequorea* derived FPs protect the chromophore from exposure to water. By substitution of two residues near the chromophore region to Cys, a reversible disulphide bond is formed under oxidising conditions that distorts the chromophore and decreases fluorescence intensity at 527 nm (**Figure 3a**). Upon reduction, chromophore function is restored by breakage of the disulphide bond and fluorescence is increased. Initially, Cys pairs were introduced at four specific locations of YFP, but only the N149C/S202C variant (named rxYFP) exhibited a substantial shift in the emission peak upon redox change (>2-fold). Changes in the 392/514 nm ratio (corresponding to the absorption peaks of the protonated non-fluorescent and anionic fluorescent forms of the chromophore, respectively) were reported. Exploiting such alterations of fluorescent properties it is possible to visualize *in vivo* cell redox status [132,133].

An important limitation of rxYFP is fluorescence quenching, which hampers ratiometric measurements. Thus, quantification relies on absolute values (fluorescence intensity readout), and both the oxidized and reduced conformations are estimated from the same excitation/emission peak [134,135]. In addition, rxYFP is pH sensitive, which further hinders accurate quantification.

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Initially, rxYFP was designed to be used for the determination of GSH/GSSG dynamic changes in yeast cytosol [136] and cultured human cells [135]. Such experiments showed the potential of FPs to monitor redox homeostasis at different subcellular compartments *in vivo*. However, rxYFP equilibrates very slowly with the glutathione pool, which depends on the GRX activity in the compartment where the GSH potential is measured (GRX catalyse thiol-disulphide exchange between the glutathione pool and the redox-sensitive protein). The improved rxYFP 3R version (for rxYFP200R/ 204R/227R) harbours three positively charged arginine residues close to the Cys pair involved in redox sensing, which stabilize the reactive groups in the Cys residues at physiological pH and increases its reactivity towards GSH/GSSG by 13-fold [137].

As previously mentioned, the thiol-disulphide exchange with the GSH pool is catalysed by glutaredoxins (GRXs), and therefore a rate-limiting factor in the rxYFP sensor equilibration with intracellular thiols. Thus, fusion with a recombinant GRX from yeast (rxYFP-GRX1p) significantly accelerated this reaction, and provided higher specificity for the 2GSH/GSSG redox pair [138]. This fusion-based relay has later been reproduced in other sensors (see below). On the contrary, rxYFP-TRX1 fusion with a human thioredoxin (TRX) did not improve 2GSH/GSSG specificity or kinetics [136].

1.5.1.2 roGFP

In a similar approach as for rxYFP, further development of redox sensitive FPs generated sensors capable of ratiometric measurements, e.g. the roGFPs [133,139]. These general thiol/disulphide status sensors are based on the *Aequorea* GFP, by insertion of Cys residues at positions S147 and Q204, which are very close to the N149/S202 residues modified in rxYFP. To maximize the effectivity of the disulphide reactions, a Cys residue at position 48 was substituted, resulting in roGFP1. The additional S65T mutation that generates a slight shift in the maximum excitation spectrum characteristic of EGFP, allowed for broader dynamic ratio between oxidized and reduced forms and gave rise to roGFP2 (**Figure 3b**) [133]. Although other combinations have been engineered and tested, roGFP1 and roGFP2 are the most frequently used versions.

Both roGFP1 and roGFP2 display two excitation peaks depending on redox state of the chromophore: about 390 nm for the protonated (neutral) form and about 480 nm for the non-protonated (anionic) form. Contrary to rxYFP, where the protonated form quenches the

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fluorescence of the protein, the protonated form of roGFPs is also fluorescent. Formation of the disulphide bridge produce structural rearrangements in the protein that modulate the equilibrium of the chromophore: oxidation increases excitation of the protonated form (390 nm) and decreases excitation of the anionic form (475 nm for roGFP1 and 490 nm for roGFP2). In other words, oxidation of the chromophore increases the 390/475 ratio in roGFP1 and the 390/490 ratio in roGFP2. These shifts in the excitation spectrum allow for ratiometric analyses, improving fluorescence quantification and making the readout less sensitive to protein expression levels or photobleaching [133,139]. Importantly, oxidation of the Cys residues is fully reversible.

Although formerly described as pH sensitive [139], the internal location of the chromophore in the FP barrel makes its protonation state only dependent on its nearby electrostatic milieu, presumably not being affected by the external pH. Indeed, when used as ratiometric indicators, roGFPs have been subsequently proven pH insensitive [140,141].

Measurement of redox potential in some subcellular compartments with very different redox potentials, e.g. oxidizing (such as endoplasmic reticulum, ER) or reducing environments (such as cytosol, chloroplasts and mitochondria) is difficult. For accurate measurements, the midpoint potential of the sensor should be as close as possible to the steady state redox potential of the compartment where the sensor is used. roGFP1 and roGFP2 have midpoint redox potentials between –280 mV and –291 mV [133], lower than for rxYFP (–261 mV), making them useful when monitoring small shifts in thiol/disulphide redox couple in reducing environments. Thus, they are especially suited for use in cytoplasm or even mitochondria where the redox potential is estimated to about –320 mV and –350mV respectively, but not at the approximately –208 mV of the ER [47,131]. As roGFP1 would mainly be reduced in the cytosol, it will properly detect oxidative changes in GSH potential while making reductive shifts more difficult to monitor (this applies even more for roGFP2, with a higher midpoint redox potential). roGFP variants with very negative GSH midpoint redox potential, such as roGFP3 (–299mV), can be further engineered to improve GSH measurements in reducing organelles such as the cytosol [139]. Limitations imposed by GSH midpoint redox potentials in the different organelles have been successfully faced by new roGFP1 and roGFP2 variants that expand the range of redox potential values. For example, roGFP1 was engineered in a similar way as rxYFP 3R (introducing three

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positively charged amino acids close to the reactive Cys) to increase its dynamic range, generating roGFP1-RX [142]. Also, the roGFP1-iX family was developed, where insertion of an amino acid (denoted by X) after C147 shifted the midpoint redox potential to between –229 mV and –246 mV (i.e. closer to that estimated at ER) [143]. Another variant, roGFP2-iL with midpoint redox potential about –238 mV, has been further modified and used in plants with severely depleted GSH synthesis [144]. Still, a high percentage of these new sensors are oxidised in the ER, making them better in measuring reductive shifts, compared to oxidative shifts, in GSH potential. Measurement of redox potential in specific organelles also requires the addition of targeting sequences to the roGFP probes. In this way, organelle specific roGFPs for use in different plant compartments have been generated, e.g. for cytosol [144–152] mitochondria [140,145,146,150–153], ER [131,140,149], plastids and peroxisomes [140,147,152,153]. Most of these studies have allowed for GSH redox potential analysis within different organelles related to different treatments or environmental conditions. For example, GSH redox potential showed a pro-oxidant shift in *Arabidopsis* mitochondria and peroxisomes, compared to chloroplasts and cytoplasm that was related to early events in dark-induced senescence [152]. Such involvement of mitochondria in the early signalling and redox alterations in *Arabidopsis* was also described for other stress-inducing events [146]. Additionally, long-term dark stress showed that younger leaves seem to have enhanced antioxidant capacity compared to older leaves [147]. It was also possible to determine the cytosolic GSH redox potential in mutants with altered GSH metabolism [144,149]; to study the importance of chloroplast ROS production and redox alterations that regulate intracellular transport through plasmodesmata [153]; to analyse ER GSH redox potential in plants [149] and its relationship to ER stress induction (in mammalian cells) [131]; or to establish the enhanced buffer capacity of *Arabidopsis* mitochondria over cytosol in pro-oxidant changes induced by water-stress and rehydration and the involvement of Asc in this response [148,151].

The exchange kinetics of roGFPs redox sensitive Cys with the GSH/GSSG is mainly dependent on the cellular GRXs [149]. Thus, when targeted to organelles with low or no GRX enzymatic activity, the sensor does no longer equilibrate with the 2GSH/GSSG redox couple. Knowing that GRXs reversibly transfer electrons from GSH to roGFP2, fusions of roGFP with human GRX1 have significantly improved the performance of the sensor when monitoring fast dynamic redox changes *in vivo* and increased its specificity for the 2GSH/GSSG couple [149,154,155].

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Moreover, they have allowed for upgraded calculations of GSH redox potentials in different organelles. Taken together, roGFP fusions to GRXs have additionally improved the sensitivity of this family of sensors for GSH redox potential from –320 mV (GRX1-roGFP2) to about –210 mV (GRX1-roGFP2-iL). This has been exploited in plants, e.g. in the cytosol of GSH deficient *rml1 Arabidopsis* mutants [144]. Since TRX may also oxidize roGFP2 by thiol/disulphide exchange, an alternative roGFP2 fusion with a human thioredoxin (TRX1-roGFP2) was created to assess the specificity of the sensor. This fusion protein further confirmed that on the contrary to GRX1, TRX1 does not confer dynamic responsiveness to changes in 2GSH/GSSG redox state [156].

Importantly, in physiological conditions, most Cys show low reactivity towards ROS and they are not oxidised directly by H_2O_2 (or other ROS) [157]. Several factors affect Cys reactivity towards different ROS, including the p*Ka* of the specific Cys residues and its surrounding environment within the protein [26,158–160]. Therefore, to create a H_2O_2 specific sensor highly reactive to this radical, a fusion between roGFP2 and a yeast oxidant receptor peroxidase (Orp1, a GPX-like enzyme known to oxidise the Yap1 transcription factor in the presence of H_2O_2) was generated (roGFP2-Orp1). In this sensor, the peroxidase uses the Cys of roGFP2 to reduce H_2O_2. As this reaction is nearly stoichiometric, reduction of nearly every H_2O_2 molecule results in a roGFP2 disulphide bond [161]. In *Saccharomyces cerevisiae*, Yap1 oxidation leads to conformational changes that allow nuclear import and specific gene transcription while TRX systems reduce back Orp1 (classified as a PRX due to its TRX-dependent thiol exchange) [23]. Thus, it seems that in plants roGFP2-Orp1 senses the balance between H_2O_2-induced disulphide formation and its dynamic reduction by cellular TRX. Other enzyme fusions have been engineered, e.g. using human GPX4 (roGFP2-GPX4) that also were successful for detecting H_2O_2 changes in yeast and human cell lines [161,162]. However, attempts to develop alternative H_2O_2 specific sensors based on PRX6 protein encountered great setbacks [161]. Recently, a new sensor consisting of roGFP2 fused to Tsa2, a typical 2-Cys PRX from *S. cerevisiae*, has been developed in yeast that resulted in improved affinity and therefore increased H_2O_2 sensitivity [163,164]. However, to the best of our knowledge, it has not yet been tested in plants.

1.5.1.3 Oba-Q: first blue redox sensors

Even though most FP reporters have emission peaks below 600 nm [165,166], efficient fluorescence imaging of plant green organs, as leaves, can be difficult [167]. The vast

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abundance of coloured pigments interferes with fluorescence detection of probes and sensors, a pitfall somewhat overcome by the development of Oba-Q (Oxidation balance sensed Quenching). Oba-Q was created from different FP variants (cyan, from mTurquose CFP, Oba-Qc; and blue, from Syrius, Oba-Qs; or from EBFP, Oba-Qb) and allowed for the development of the first blue-coloured protein-based redox sensors suitable for simultaneous imaging with other FP-based sensors. Similarly to roGFPs and rxYFPs, redox sensitive Cys residues account for the equilibration with the 2GSH/GSSG pool. Unfortunately, Oba-Q are not ratiometric: their fluorescence is quenched upon oxidation [168]. Oba-Qc fusion with human glutaredoxin-1 (Grx1-Oba-Qc) has also been developed and tested in mammalian cells, showing improved kinetics of the sensor [168]. However, to our knowledge Oba-Q has not yet been used to study ROS in plant cells.

1.5.2 Circular permutation-based sensors

Circular permutation engineering produce proteins whose amino acids (primary structure) have been changed, creating a protein with different N- and C-terminal that may alter some of the proteins properties. Modification of the FP structure makes the chromophore more exposed to the surrounding media, making the sensor pH-sensitive as changes in pH affects the ionic state of the chromophore. Thus, the circular permutated FPs (cpFPs) do not generate the fluorescence of the original protein, a property that can be exploited by the insertion of a functional domain into the cpFP that under specific conditions (e.g. redox potential changes) induces conformational variations, restoring or altering the fluorescence signal.

1.5.2.1 cpYFP

Development of specific sensors for individual ROS species was highly desirable, as most of the redox sensitive FP-based probes (e.g. rxYFP or roGFP) were not specific for a discrete ROS, but rather the thiol/disulphide system. cpYFP was generated by circular permutation and point mutation of the YFP variant EYFP (V68L/Q69K), where the original N- and C-termini were connected by a linker sequence [169]. cpYFP was initially described as a superoxide biosensor [48]. The authors observed brighter fluorescence of cpYFP under oxidizing conditions (thus being an intensiometric sensor), and it was described as an $O_2^{\bullet -}$ sensor not affected by H_2O_2 (**Figure 3c**) [48]. Addition of a mitochondrial targeting sequence allowed for the study of mitochondrial ROS accumulation in *Arabidopsis* roots, among other organisms [170,171].

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Transient "flashes" of fluorescence were reported, the so-called mitochondrial $O_2^{\bullet -}$ flashes or mitoflashes [48,61,172]. Although some authors suggest that cpYFP acts as an $O_2^{\bullet -}$ sensor, strong concerns regarding its specificity have been raised as the mechanism of $O_2^{\bullet -}$ interaction with the cpYFP remains unclear. Other authors have proposed that mitoflashes are caused by changes in pH (note that cpYFP fluorescence is quenched at pH below 6); for example, low pH increases chromophore protonation, alter its spectral excitation properties [126,173–175]. Although some authors that suggested cpYFP $O_2^{\bullet -}$ specificity do not exclude partial influence of pH in the sensor readout, they propose that the readout of the sensor can still depend on a ROS-related component, implying that the nature of these mitochondrial flashes still is a matter of debate [126]. However, recent work has showed that cpYFP is unresponsive to $O_2^{\bullet -}$ production [176].

1.5.2.2 HyPer

The H_2O_2 sensor HyPer is based on insertion of cpYFP into the redox-active regulatory domain of the *Escherichia coli* OxyR-H_2O_2 sensing protein [177]. OxyR is a transcription factor that regulates the expression of a set of redox-responsive genes in bacteria. It harbours several Cys residues, some of them critical for specific H_2O_2 sensing. Cys 199 has a low p*Ka* that enables its direct reaction with H_2O_2. Upon H_2O_2 exposure, a sulfenic acid is formed at the C199 residue, which condenses with C208 to form a (reversible) disulphide bond, producing conformational changes that promotes binding of the oxidised OxyR to DNA, activating transcription of antioxidant genes. OxyR sensing specificity was tested with different molecules in addition to H_2O_2. Since the sensing Cys pair resides in a hydrophobic pocket, accessibility of other oxidants such as superoxide anion seems to be restricted, showing high specificity for H_2O_2. Therefore, the insertion of cpYFP into OxyR domain greatly increases its H_2O_2 specificity, making Hyper a H_2O_2 specific sensor [158,177,178].

HyPer displays two excitation peaks (420 and 500 nm, reduced and oxidized forms respectively) with a single 516 nm emission maximum. Formation of the disulphide bond by H_2O_2 exposure alters OxyR structure and shifts the fluorescent properties of HyPer. Introduction of a Y203F mutation in the cpYFP allowed for additional visualization of the protonated form, enabling ratiometric measurement of H_2O_2 due to the shift in cpYFP excitation maximum (decreasing excitation of the protonated form at 420 nm while increasing the excitation of deprotonated

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form at 500 nm) (**Figure 3d**). Midpoint potential of the redox-responsive Cys couple of OxyR in HyPer is –185 mV [178].

As mentioned above, HyPer OxyR domain is highly selective for H_2O_2. Reduction of its oxidised Cys residues in plant cells is likely mediated by the 2GSH/GSSG pair, as for OxyR in *E. coli* [178], making HyPer a reversible sensor. Thus, HyPer senses the balance between H_2O_2-mediated disulphide formation and its reduction by GSH through GRX system [155]. In comparison to roGFP2-Orp1, HyPer displays a faster kinetics [161] which can be explained by its high sensitivity and direct reaction with H_2O_2, in contrast to POX-mediated oxidation for roGFP2-Orp1.

As for other cpYFP, HyPer fluorescence is sensitive to pH and care should be taken when using this sensor in organelles with different pH values. If possible, a ratiometric fluorescent pH-indicator such as SypHer, a H_2O_2 insensitive HyPer version with a C199S point mutation, can be used to monitor pH changes during the experiment [93,177]. Organelle targeted variants have been generated that might allow for simultaneous H_2O_2 monitoring in different cellular compartments [179]. However, special care should be taken when analysing such data, as pH dissimilarities and discrepancies in redox-sensitive Cys dynamic equilibration with thiol systems (known to vary between organelles) can give different fluorescence readouts in compartments with similar H_2O_2 production. Based on its asymmetrical equilibration with two different inputs (H_2O_2 and 2GSH/GSSG), that HyPer redox-sensitive Cys oxidation can either indicate high H_2O_2 generation in the ER compared to other organelles or slower efficiency of disulphide equilibration. The relative rates deciding this equilibrium and fluorescent readout will depend on the presence of enzymatic activities affecting the sensor exchange with the organelle thiol systems, also including enzymes as disulphide isomerases that use the thiol/disulphide exchange for protein folding in this compartment or improved kinetics of the sensor (see below).

Similarly, long-term experimental monitoring of cells expressing protein sensors that alter cellular thiol systems (e.g. redox relays and FP-fusions with GRX, TRX, PRX, etc.) can trigger acclimation/adaptation responses and therefore alter the fluorescence readouts dependent on the equilibration with these systems. To avoid such possible difficulties, transient expression or inducible promoters for FP-sensor expression should be considered.

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As for other protein-based sensors, organelle-specific localization sequences have been added to HyPer. A peroxisome targeted version capable of responding to addition of H_2O_2 has been tested in *Arabidopsis* epidermal cells, showing that the CAT-based H_2O_2 scavenging ability of the peroxisomes is mediated by Ca^{2+} [180]. HyPer was also key to describe the signalling role of plasma membrane aquaporins in guard cell H_2O_2-mediated signal transduction induced by plant hormones or biotic stress [181]. Although pH controls using a DCF-based probe were included in these experiments to monitor pH alterations that might affect HyPer readout, taking advantage of SypHer as pH sensor with same p*Ka* as HyPer would be advised when possible. Other studies have reported improved methods for the use of HyPer in different plant tissues [182]. The same authors monitored the oxidative burst induced by Al^{3+}-treatment in *Arabidopsis* roots, concluding that H_2O_2 and ROS mediated Al^{3+}-induced root elongation arrest. However, pH alterations were not monitored, hindering the interpretation of the data [183].

Recently, improved versions of HyPer were developed by mutating the OxyR domain. HyPer2 (A406V) showed an increased dynamic range [184], while HyPer3 (A406V/H34Y) even further improved dynamic range and kinetics [185]. HyPer2 has been used to study H_2O_2 dynamics in response to high light in *Nicotiana benthamiana* epidermal cells [186]. In this work, the authors constructed a similar pH control sensor (SypHer2), to ensure that the signal measured from Hyper2 were not due to pH alterations in the different compartments. Targeting of HyPer2 and SypHer2 to different organelles helped to study the photosynthetic control of nuclear gene expression responding to high light. Organelle targeting showed that a group of chloroplasts in close proximity to the nucleus were responsible for the H_2O_2 direct signalling between these two organelles, thus avoiding the cytosolic antioxidant systems that might inhibit such signalling.

Finally, red fluorescent HyPerRed, a circular permutated red FP (cpRed/cpmApple), was developed by replacing a Ca^{2+} sensing domain in a fluorescent Ca^{2+} sensor (R-GECO1) for the OxyR sensing domain, making HyPerRed specific for H_2O_2 monitoring [187]. Although non-ratiometric, the spectral characteristics of this variant allow for its simultaneous use with other green fluorescent sensors or probes used as volumetric controls, enabling quantitative calculations.

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Similarly to HyPer, these recent HyPer-derived sensors are pH-sensitive, requiring simultaneous use of appropriate pH controls (e.g. non-redox sensitive SypHer2 or HyPerRed-C199S).

1.5.2.3 rxRFP

A similar approach as used for HyPer was employed when generating rxRFP, the first red FP-based redox sensor. In this case, instead of the bacterial OxyR domain typical of HyPer proteins, a Cys pair was fused to a circular permutated red FP scaffold based on R-GECO1 (cpmApple/cpRed). The Cys pair formed a disulphide bond under oxidizing conditions, which altered the sensor´s fluorescence. Oxidation induced stabilization of the FP structure and caused increased fluorescence emission, while reduction resulted in loss of fluorescence. Thus, rxRFP is a red-shifted thiol/disulphide status probe [188].

rxRFP has also been fused to TRX (TrxRFP1) and targeted to different organelles to assess thioredoxin redox perturbations in mammalian cell lines [189]. Importantly, this sensor has been combined with spectrally different biosensor (green fluorescent GRX-roGFP2) to simultaneously monitor 2GSH/GSSG and thioredoxins dynamics upon treatments with enzyme inhibitors and oxidants, demonstrating specific cell responses to the distinct stimuli. This validates the parallel use of spectrally different FP-based biosensors and opens new possibilities for multiplexing experiments monitoring different signals/molecules.

1.5.3 FRET

In genetically encoded sensors using Förster-type fluorescence resonance energy transfer (FRET)-based technology, energy transfer from a donor fluorophore to an acceptor fluorophore can be measured to study cellular processes [190]. For FRET to happen both fluorophores need to be in very close proximity and the emission spectrum of the donor must overlap with the excitation spectrum of the acceptor. The excitation energy transfer happens without collision between the fluorophores [191]. Because the transfer from donor to acceptor takes place without a real photon being involved, just by electric interactions between electrons, distance is a crucial factor in FRET efficiency. This translates into a FRET distance dependence of ($\sim r^{-6}$) magnitude, which practically means FRET chance of taking place goes from 1 to 0 when distance changes from 1 to 10 nm. This feature provides FRET with exquisite sensibility to nanometer-scale distances, even when working in the optical range where light wavelengths are 100-fold larger [37]. Fusion of the two FRET FPs into a single polypeptide chain, linked by a

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functional domain undergoing conformational changes upon interaction with a substance or signal of interest, can result in a FRET signal that is finely modulated by the conditions of the surrounding environment, in our case the redox environment (**Figure 3e**). In the case of ROS and redox FRET sensors, the linker often contains Cys pairs, which can form intramolecular disulphide bonds under oxidative conditions, or specific ROS-sensing domains. Intramolecular disulphide bridge formation, or interaction between the sensing domains, induce conformational changes of the fusion proteins, altering the distance between FPs, which directly modifies FRET efficiency and therefore the fluorescent signal ratio between the donor and the acceptor FP. As the fluorescence signal recorded from the donor drops when its excitation energy is transferred to the acceptor FP (increasing the latter fluorescence signal), a ratio between the emission signals of both FPs can be measured, making FRET sensors ratiometric.

Nonetheless, a general drawback with FRET sensors is that they usually show a low dynamic range, and that FPs are intrinsically sensitive to pH. As FRET sensors contain two FPs, fluorescent interference caused by pH can affect both chromophores, making interpretation more difficult. However, considering p*Ka* values of the most usual FRET sensors and the average pH values in the cytosol, the possible artefacts found using FRET sensors expressed in this compartment are significantly reduced. In addition, selectivity of redox FRET biosensors is questioned due to the limited knowledge about the redox couples, or enzymes, that interact with the peptide linkers that regulate some of the sensors. Also, subcellular targeting of FRET-based sensors can be problematic due to the molecular size of the fusion proteins. Several examples of FRET sensors have been reported to measure different parameters, such as pH, Ca^{2+} and other ions, glucose, etc., in plant cells [192]. Although the development of ROS and redox FRET-based sensors in mammalian cells and yeasts has widened in the last years, its use in plants is still scarce.

1.5.3.1 First redox FRET-based sensors

The first FRET-based redox biosensors were developed and further improved by Kolossov *et al*. [193–195]. In these sensors, CFP and YFP were linked by redox sensitive polypeptides, containing Cys pairs that induced conformational changes upon oxidation, resulting in FRET (**Figure 3e**). The midpoint redox potential of an improved version (–143 mV, CY-RL7) [194]

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would be specially suited for extremely oxidising compartments (or mutants with exceptionally altered glutathione metabolism) with midpoint potentials above that of the ER. To date this sensor is best suited for detection of oxidative shifts in the 2GSH/GSSG exchange in the ER [144,195]. However, as for other FRET sensors, low dynamic range encourage further improvement of the sensor. In addition, ER targeted roGFP1-iL showed discrepancies compared to CY-RL7 readouts, suggesting that different characteristics of the sensors can affect the analysis and interpretation of the data requiring careful consideration [195].

1.5.3.2 HSP-FRET

Guzy and colleagues used a 69 amino acid Cys-containing redox-sensitive regulatory domain from the bacterial redox-regulated heat-shock protein HSP-33, placed between YFP and CFP [196]. Redox induced conformational changes in the linker caused CFP and YFP fluorophores to separate, resulting in decreased FRET signal. This FRET sensor was used to study cytosolic signalling in mammalian cell lines [197], although not yet in plant systems.

1.5.3.3 Redoxfluor

The FRET sensor Redoxfluor is also a CFP and YFP fusion, linked by tandem Cys-rich domains of Yap1 yeast transcription factor C-terminus that has been targeted to the peroxisomes. Redox alterations in the sensing linker induce FRET and spectral changes [198]. Redoxfluor has been successfully used in yeast and mammalian cells to study the redox state within peroxisomes. Although promising, to the best of our knowledge it has not yet been used in plants.

1.5.3.4 OxyFRET and PerFRET

Novel OxyFRET and PerFRET sensors are based on redox-sensitive linkers containing (tandem or single) Cys-rich domains, respectively. In an approach conceptually similar to Redoxfluor, OxyFRET is based on the H_2O_2 sensitive N- and C- terminal regions of Yap1 transcription factor, which is a component of the *S. cerevisiae* Orp1–Yap1 redox relay. However, H_2O_2 specificity requires the POX function of Orp1, a protein absent in many organisms. Thus, the cellular redox couples or enzymes responsible for the oxidation of this sensor in plants remain to be elucidated, questioning the specificity of this FRET biosensor. On the contrary, PerFRET harbours both a Yap1 Cys-rich domain and Orp1, flanked by the FPs pair, which increases H_2O_2 specificity [199].

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H_2O_2-induced increased (OxyFRET) and decreased (PerFRET) emission ratio was reported for cells expressing these FRET sensors. This could reflect distinct conformational changes induced at each sensor, reducing or increasing the distance of the FPs. These sensors were used to study H_2O_2 accumulation after NADPHox activation in mammalian cultured cells [199].

A summary of the most important FP-based sensors and their main characteristics for monitoring and measuring ROS and redox variations can be found in **Figure 3** and a recent review article [120].

1.5.4 Emerging photorreceptor-based sensors

Another class of alternative genetically encoded fluorescent proteins are based on natural bacterial and plant photoreceptor proteins containing flavin cofactors, such as the light-oxygen-voltage (LOV) sensing domain, instead of conventional FP proteins [200–204]. The limitations imposed by GFP-like proteins, e.g. their large size (~27 kDa) and the requirement of molecular oxygen to catalyse the chromophore formation that may become limiting during hypoxia, can be overcome using photoreceptors [202]. Under natural conditions, reception of UV/blue light by flavin chromophore (typically flavin mononucleotide, FMN) induces binding of the chromophore to a conserved Cys residue of the polypeptide that serves as a molecular photo-switch controlling signalling pathways. However, as the intrinsic green fluorescence of LOV photoreceptors (because the flavin also acts as a chromophore) is quenched by this bond (and subsequently recovered upon dark incubation), photoreceptors must be engineered (e.g. exchanging the conserved Cys for Ala) in order to create permanently fluorescent proteins [204].

These novel ROS and redox protein biosensors will likely bring new opportunities for live cell imaging, because of their small size, pH insensitivity and high photostability. Their fluorescent properties are independent of oxygen, which may be especially useful in the ROS studies and redox field, avoiding possible artefacts in the sensor readout.

For example, they have been used to analyse cell redox status in microaerobic and anaerobic conditions. In high-density bacterial cultures, photoreceptor-based reporters allowed for more accurate quantification of gene expression than oxygen-dependent traditional FPs [204,205]. Plant studies have also take advantage from the improved performance of photoreceptor

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variants to monitor systemic plant virus infections (e.g. better photochemistry, reduced gene expression silencing and better virus mobility due to the reduced protein size) [206]. Importantly, they can be combined with conventional FPs to generate novel FRET sensors [202]. For example, novel sensors based on FRET fusions with oxygen-sensitive YPF have been useful to study oxygen levels in the cytosol of *E. coli* [207].

1.6 Protein-based sensors: assets and drawbacks

Although genetically encoded ROS and redox sensors show clear advantages over fluorescent dyes, they are not devoid of inconveniences. For example, they require complex technical development and very fine adjustments for each single experimental condition. In addition, the size of some sensors (e.g. FRET constructs) may hinder their efficient expression in plant cells. Hence, protein expression levels and silencing can affect the fluorescence intensity readout, making comparisons between experiments difficult, mostly for non-ratiometric sensors. Similarly, changes in sensor photostability can create artefacts in the interpretation of the results for intensiometric sensors. In addition, these sensors require that the plant itself, or at least plant cell or plant tissue of interest, can be genetically modified. Therefore, genetically encoded sensors are only useful for species that can be stable (or transiently) transformed with foreign DNA.

On the other hand, some FRET sensors exhibit relatively low dynamic ranges that together with the fact that the different FPs may differ in photostability, chromophores maturation rate, pH sensitivity or other properties, can affect the interpretation of the data.

In addition, as indicated for HyPer and roGFP2-Orp1, H_2O_2-induced oxidation of these sensors needs to be reversed by cellular reductants as 2GSH/GSSG couple. This asymmetric equilibration of the probe complicates the analysis and interpretation of the data, especially when taken into account that it also competes with other cellular H_2O_2 scavengers present within the cells. Taken together, although protein-based sensors show great possibilities, all facts need to be carefully considered as the experimental requirements for these probes can be more demanding than for the corresponding dye-based sensors to avoid artefactual interpretation of the experimental results.

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1.7 Conclusions and perspectives

In this review, we have tried to summarize the present knowledge of *in vivo* ROS and redox fluorescent imaging, with a special focus on their use in plants. We wanted to cover both chemical dyes and probes, and sensors based on FP that can be expressed in the plant cell upon genetic incorporation of the sensor-encoding DNA. Although these two strategies are frequently used in mammalian and yeast systems, their use in plant systems is not as straightforward, mainly due to technical reasons (such as delivery of the probe inside plant cells) and the background signal coming from coloured and fluorescent molecules within plant tissues.

While fluorescent dyes are generally easier to use and signal accumulation allows for high sensitivity, FP-based sensors show high potential and important advantages over fluorescent dyes for monitoring and measuring dynamic ROS and redox variations. However, protein-based biosensors require careful calibration and validation when used quantitatively. Both sorts of indicators (dyes and FPs) can/should thus be used in parallel, as they can give complementary information.

FP sensors based on FRET are of great interest for future use in plant cells, and are also discussed in the text. However, their (to our knowledge) lack of implementation (until now) in plants is acknowledged. We also briefly refer to novel types of ROS sensors based on naturally occurring bacterial and plant photoreceptor proteins containing flavin cofactors. Such novel ROS and redox protein biosensors show exciting features such as small size, pH insensitivity and high photostability, and will likely be of importance in the near future.

While significant progress has been reached for the understanding of the physiological functions of ROS, their influence on cellular redox homeostasis, and their role as important intracellular signal transduction messengers, better and improved methods to measure ROS *in situ* and *in vivo* are desired. For that purpose, ROS and redox couples probes and sensors that offers better selectivity, accuracy, and spatiotemporal resolution will be essential for our future understanding of the remarkably complex plant ROS and redox signalling networks.

Accepted manuscript

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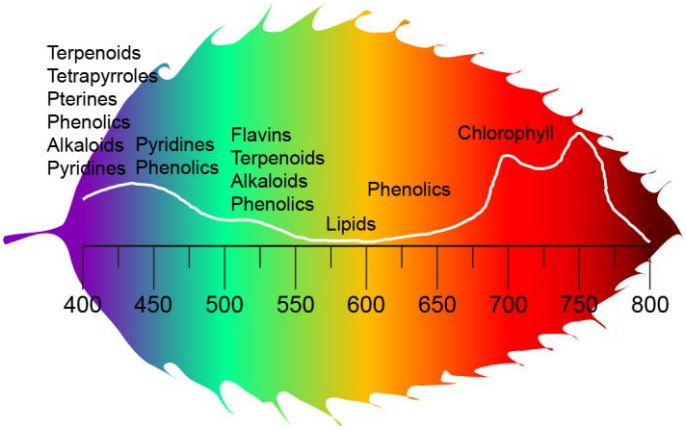

Figure 1. Autofluorescence spectrum of a typical green leaf, with the main plant compounds contributing to autofluorescence highlighted at their respective emission wavelength [43] (λ_{exc}=355 nm).

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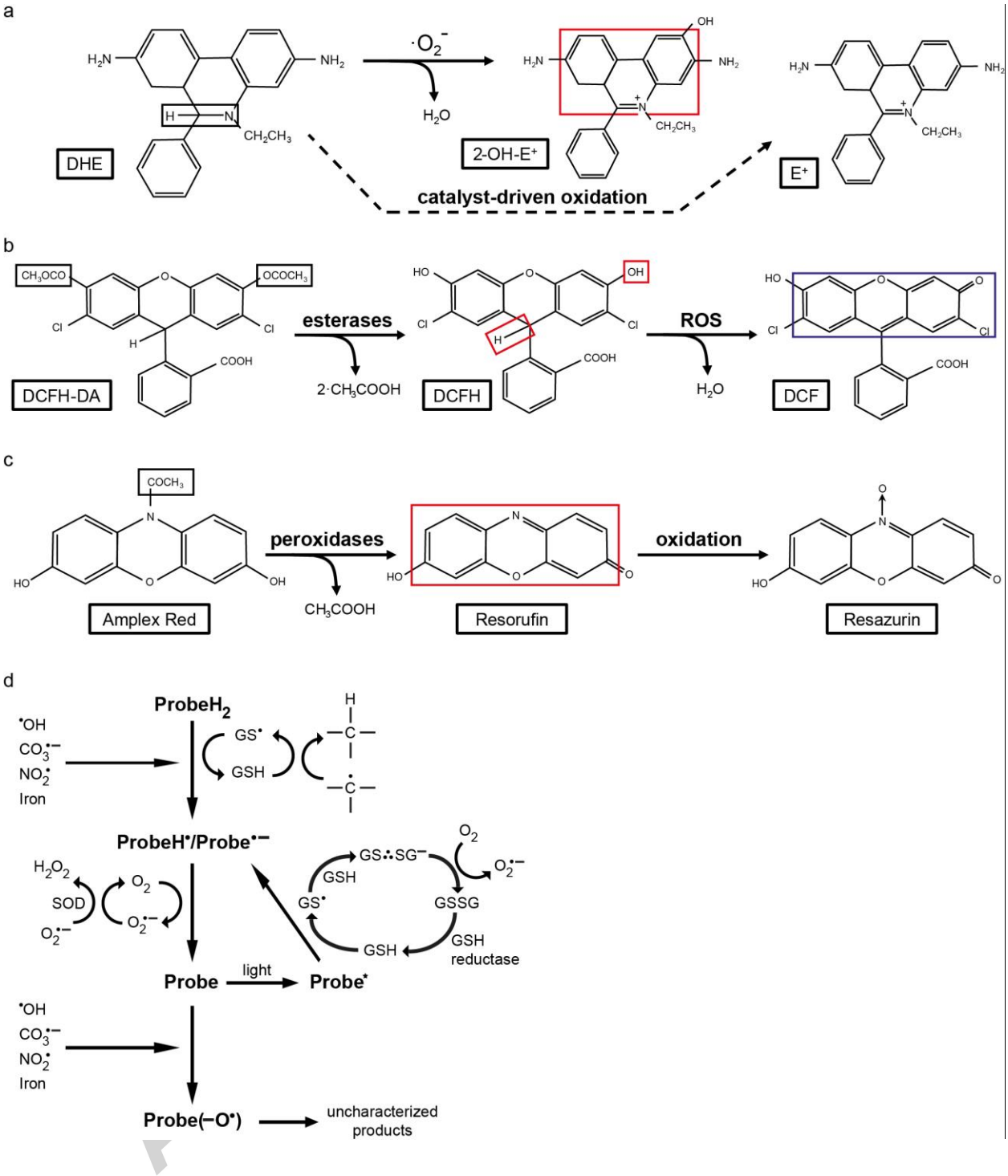

Figure 2. Schematics of the reaction mechanisms leading to ROS detection by dye-based probes. (**a**) In the presence of superoxide, DHE (left) is oxidized (black box) to 2-OH-E^+ (middle) and an electronic resonant structure is established in the phenanthrene-like rings (red box), which is fluorescent. Alternatively, DHE can be catalytically oxidized (dashed arrow) to E^+ (right), which features very similar fluorescence characteristics. (**b**) Probes based on DCFH-DA (left) cross the plasmatic membrane and esterases cleave the acetate groups (black boxes) to produce DCFH (middle). Then, DCFH is oxidized (red boxes) to DCF (right), and a resonant anthracene-like chemical structure is established (blue box)

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capable of fluorescence. The same scheme can be applied to DHR-based probes (not shown), only taking into account that the two hydroxyl substituents shown at the top of DCF are amino ($-NH_2$) groups in the case of rhodamine 123. (**c**) Amplex Red® (left) undergoes an oxidative deacetylation (black box) in the presence of cellular peroxidases, leading to the production of fluorescent resorufin (middle) (red box). Further oxidation is possible, which produces the non-fluorescent resazurin (right). (**d**) Simplified scheme demonstrating the complexity of possible interactions between fluorescent probes, oxidizing radicals and cellular antioxidants (adapted from [59]).

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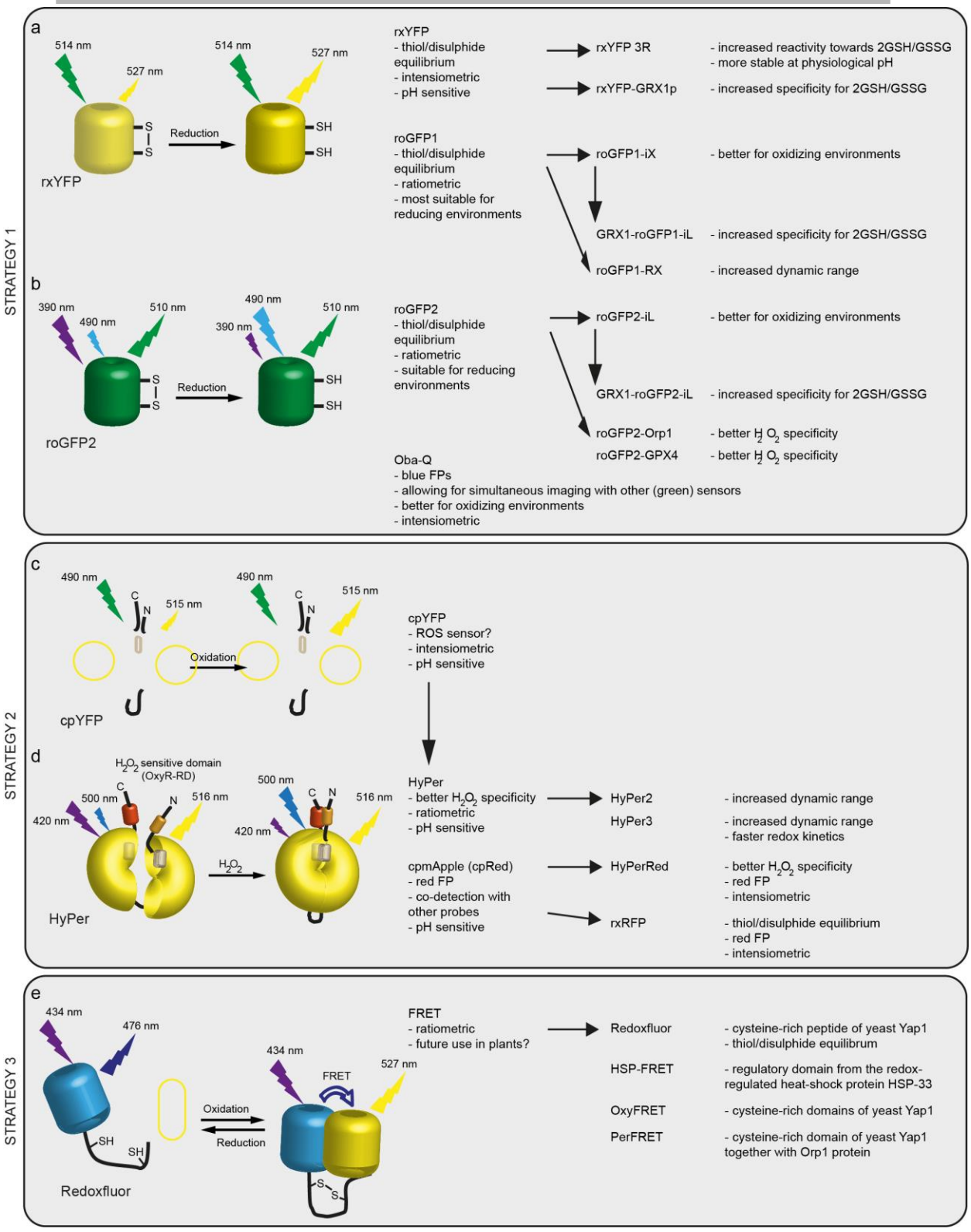

Figure 3. Three main strategies for development of FP-based ROS and redox sensors with examples. (**a, b**) Strategy 1 - single-FP based sensors, (**c**, **d**) Strategy 2 - circular permutated FP-based sensors, and (**e**) Strategy 3 - FRET-based sensors. In rxYFP (**a**) and roGFP2 (**b**), redox-reactive Cys introduced at the surface of the FPs alter the chromophore environment and change fluorescent properties. Circular

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permutation of YFP was initially described as a superoxide biosensor (cpYFP) (**c**), that was further engineered with a bacterial redox-active regulatory domain (OxyR-RD), generating H_2O_2 specific sensors (HyPer-family). (**e**) In FRET-based probes, alterations at redox-sensitive peptide linkers provoke conformational changes of the sensor that allow energy transfer from a donor to an acceptor fluorophore. The main characteristics of each sensor are highlighted.

Highlights

- Origin, homeostasis and function of reactive oxygen species (ROS) in plants
- Challenges and possibilities for fluorescent signal detection in plants
- Use and chemistry of fluorescent dyes for plant redox potential and ROS detection
- Fluorescent sensor proteins for plant ROS detection

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