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In vivo ROS and redox potential fluorescent detection in plants: present approaches and future perspectives

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Abstract

Reactive oxygen species (ROS) are metabolic by-products in aerobic organisms including plants. Endogenously produced ROS act as cellular messengers and redox regulators involved in several plant biological processes, but excessive accumulation of ROS cause oxidative stress and cell damage. Understanding ROS signalling and stress responses requires precise imaging and quantification of local, subcellular and global ROS dynamics with high selectivity, sensitivity, and spatiotemporal resolution. Several fluorescent vital dyes have been tested so far, which helped to provide relevant spatially resolved information of oxidative stress dynamics in plants subjected to harmful environmental conditions. However, certain plant characteristics, such as high background fluorescence of plant tissues *in vivo* and antioxidant mechanisms, can interfere with ROS detection. The development of improved small-molecule fluorescent dyes and protein-based ROS sensors targeted to subcellular compartments will enable *in vivo* monitoring of ROS and redox changes in photosynthetic organisms.

Keywords

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

Abbreviations

2-OH-E^+	2-hydroxyethidium
$^{\bullet}NO$	nitric oxide
$^{\bullet}NO_2$	nitrogen dioxide radical
$^1O_2$	singlet oxygen
$^3O_2$	triplet oxygen
ACO	acyl-CoA oxidase
ALO	aldehyde oxidase
APX	ascorbate peroxidase
AsA-GSH	ascorbate-glutathione cycle
AsA	ascorbate
BODIPY	4,4-difluoro--4-bora-3a,4a-diaza-s-indacene
CAT	catalase
CFP	cyan fluorescent protein
cpYFP	circular permutated YFP
CW	cell wall
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
E^+	ethidium
ETC	electron transport chain
FP	fluorescent protein
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
GOX	glycolate oxidase
GRX	glutarredoxin

GSH	reduced glutathione
GSSG	oxidised glutathione
H_2O_2	hydrogen peroxide
$HO^{\bullet}$	hydroxyl radical
$HO_2^{\bullet}$	hydroperoxyl radical
HRP	horseradish peroxidase
NADPH	nicotinamide adenine dinucleotide phosphate
NADPHox	NADPH oxidase
$O_2^{\bullet-}$	superoxide radical
$ONOO^-$	peroxynitrite anion
POX	peroxidase
RNS	reactive nitrogen species
$RO^{\bullet}$	alkoxyl radical
roGFP	reduction-oxidation sensitive GPF
$ROO^{\bullet}$	peroxyl radical
ROS	reactive oxygen species
rxYFP	redox sensitive YFP
SOD	superoxide dismutase
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl
XO	xanthine oxidase
YFP	yellow fluorescent protein

Content

1. ROS in plants: origin, homeostasis and detection

Reactive oxygen species (ROS) originate from incomplete reduction of molecular oxygen in aerobic organisms, generating reactive oxygen derivatives. Such derivatives can be free radicals possessing 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, such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), or ozone (O_3). In addition, reaction of $O_2^{\bullet -}$ and nitric oxide ($^{\bullet}NO$) generates peroxynitrite ($ONOO^-$) that acts both as ROS and reactive nitrogen species (RNS) [1]. Plant cells possess defence mechanisms such as antioxidants and ROS scavenging enzymes to prevent toxicity under normal growing conditions. When these barriers are overridden, ROS accumulation leads to oxidative stress causing protein denaturation, lipids peroxidation, and nucleotides degradation, which results in cellular damage and ultimately cell death [2–4]. However, ROS are also central signalling molecules to control the plants metabolism under stress conditions [5]. In fact, ROS production in plants have lately received increasing attention for its regulatory role in cell fate determination during development [6], organogenesis and differentiation [7], stress responses [8], plant immunity, wound response and acclimation processes [9–11].

In most cases, oxidative processes in plants (including oxidative stress) start with the activation of molecular oxygen in its ground state, triplet oxygen ($^3O_2$). Upon absorption of sufficient energy, it becomes more reactive and capable of accepting an electron from other cellular sources, such as the electron transfer chains (ETC) of mitochondria or chloroplasts, leading to formation of $O_2^{\bullet -}$ [12,13]. Enzymes such as NADPH-oxidases, peroxidases (POXs), lipo- and cyclo-oxygenases, cytochrome P450s and xanthine oxidases (XO) can also participate in electron transfer and ROS production. $O_2^{\bullet -}$ is subsequently converted into H_2O_2, spontaneously or *via* a superoxide dismutase (SOD)-catalysed reaction. H_2O_2, which can interact indirectly with $O_2^{\bullet -}$ *via* Fe^{2+}/Fe^{3+} in Fenton and Haber-Weiss reactions to produce the highly reactive $OH^{\bullet}$, is reduced to H_2O in reactions mediated by catalase (CAT) and ascorbate peroxidases (APX) [1,14–16].

Several plant organelles and compartments are sources of ROS, particularly those with high electron transport rates, such as chloroplasts, mitochondria and peroxisomes. Chloroplast $O_2^{\bullet -}$ production is mainly due to electron transfer from photosystem I to O_2 generated by photosystem II, while chlorophyll and its tetrapyrrole derivatives near both photosystems are main sources of $^1O_2$ in green tissues. Mitochondrial ETC contributes to plant ROS production, but in photosynthetic cells this source of ROS is less extensive than in mammalian cells. Matrix XO activity and membrane NAD(P)H-dependent ETC generate peroxisomal $O_2^{\bullet -}$ [19]. Peroxisomes also produce H_2O_2 through the photorespiratory glycolate oxidase reaction (GOX), fatty acid β-oxidation by acyl-CoA oxidase (ACO), enzymatic reactions of flavin oxidases, and the disproportionation of $O_2^{\bullet -}$ radicals by SOD. Endoplasmic reticulum $O_2^{\bullet -}$ originates from a NAD(P)H-dependent ETC involving a cytochrome p450. In addition, plasma membrane NADPH oxidases and apoplastic enzymes, such as POXs, oxalate- and amine-oxidases, also contribute to ROS generation in plants that is important for processes such as cell wall cross-linking, initiation of stress signalling, etc **(Figure 1)** [17]. An excellent overview on ROS production sites within the plant cell can be found in the review by Sharma *et al*. [18]**.**

Intracellular redox homeostasis controls a vast variety of biological processes, ranging from development to stress responses, and is balanced by ROS production and cellular antioxidants, both enzymatic and non-enzymatic [18]. Metabolites such as glutathione (GSH), ascorbate (AsA), tocopherols and phenolic compounds, in addition to carotenoids and NAD(P)H, are functionally the most important non-enzymatic antioxidants. The enzymatic ROS-scavenging system includes SOD, CAT, APX and other POX, peroxiredoxins and thioredoxins, as well as and the enzymes of the ascorbate-glutathione cycle (AsA-GSH) [19]. Some of these enzymes, e.g. glutathione peroxidase (GPX), are involved in the conversion of reduced and oxidized forms of different reductants [20].

Several signalling events are controlled by the redox cellular homeostasis, where pro-oxidant conditions induced by ROS accumulation have important effects on cell metabolism and plant responses. For example, H_2O_2 can reversibly modify thiol groups

of signalling transducer proteins, causing changes in redox-mediated processes in plants [8,21]. Thus, elucidation the cellular functions regulated by redox changes and the balance between ROS and antioxidants, and their effect on signalling, is receiving increasing attention. It is therefore essential to detect, localize and identify the specific radicals produced in the cell, to understand their role in regulating plant processes. However, ROS show complex spatial and temporal dynamics, having limited lifetime and being subjected to activity of the cellular antioxidant system. These complications make *in vivo* monitoring of ROS, and the subsequent events, a challenging task in plant cells [22].

While fluorescent probes offer thrilling possibilities to study ROS spatial-temporal signalling events *in vivo*, several problems may be encountered in complex biological systems such as intact plant cells and tissues. Thus, cellular antioxidants (e.g. AsA and thiols) can interfere with ROS detection, either by depleting the radicals generated or by reacting with the probes themselves. Therefore, the chemistry of the probe must be understood and its putative interaction with antioxidants, ROS-scavenging reaction intermediates and/or antioxidant enzymes [23]. In addition, fluorescence imaging in plant tissues is unusually challenging because of the high levels of intracellular fluorescent molecules [24]. Most autofluorescence in plant tissues originates from cell wall components (e.g. cellulose and lignin), chlorophylls, terpenoids (e.g. β-carotenes, xanthophylls), phenols (e.g. flavonoids, anthocyanins), and alkaloids [25]. These compounds display a wide array of excitation and emission spectra and can therefore interfere with detection of exogenous fluorescent markers, making reliable fluorophore quantification in plants more difficult than in other organisms **(Figure 2)** [26–28].

Fluorescence imaging is an excellent technique to visualize ROS due to the sensitivity, simplicity and selectivity offered by fluorescent ROS probes [29]. *In vivo* ROS detection in intact plant cells and tissues is therefore becoming more and more common, driven mainly by the development of novel small molecules and ROS fluorescent protein (FP)-based probes. This allows for specific tissue and/or subcellular analysis, in addition to spatiotemporal monitoring of ROS dynamics**.** However, to tackle the barriers

originating from plant fluorescent compounds it will be necessary to improve existing, or develop new, imaging methodologies for plant cells and tissues, so that high sensitivity and resolution, as well as specificity for distinct radicals, can be obtained.

In this review, we aim to summarize the current status of *in vivo* ROS imaging from a plant perspective, covering both small-molecule dyes and genetically encoded protein-based probes, and to highlight recent advances and future strategies for fluorescent ROS imaging in plants. A summary of ROS probes discussed in this review and their main characteristics (i.e. specificity, excitation/emission wavelengths, fluorescent product and examples of use in photosynthetic organisms), can be found in Supplementary Table 1.

2. ROS indicators: small molecules/fluorescent dyes frequently used in plants

ROS detection often results from oxidation of the ROS probe itself. In their reduced state, these dyes are usually stable molecules. Upon oxidation by ROS, they are converted into a molecule with fluorescent properties, due to the formation of resonant moieties. However, oxidation is unlikely selective and the major limitation of ROS detection and measurement, in addition to kinetics and quantitative calibration, is therefore specificity [23]. An ideal fluorescent ROS indicator should be non-toxic, specific and highly reactive at low concentrations, while not cross-react or outcompete cellular ROS antioxidants. In addition, the probe should display low background and high signal-to-noise ratio, present fast and reversible kinetics, be easy to load into cells or organelles with proper compartmentalization and without subsequent diffusion or metabolization. Resistance to photobleaching and low cost are also desirable features [30]. In the following section we will focus on the most frequently utilized dyes in plant systems, emphasizing their possibilities and limitations for use *in planta*.

2.1 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA)

Of the small-molecule fluorescent ROS dyes available, 2'-7'-dichlorodihydrofluorescein (DCFH) is perhaps the most frequently used when studying ROS signalling and

oxidative stress in plant cells and green algae. It belongs to the reduced fluorescein (dihydrofluorescein) group of leuco dyes, which are widely used for intracellular H_2O_2 and oxidative stress detection. The diacetate ester form (DCFH-DA) is cell-permeable, allowing easy loading and intracellular accumulation of non-fluorescent DCFH upon enzymatic dissociation by intracellular esterases [31]. The oxidation of DCFH by H_2O_2 or other oxidants produces the fluorescent product 2,7-dichlorofluorescein (DCF), which is retained within the cell and can be easily monitored by fluorescence microscopy and flow cytometry [32,33].

Some derivatives, such as carboxy-DCFH-DA show improved lipophilic affinity and retention [31]. DCFH-DA and its derivatives have been extensively used to visualize and measure intracellular, subcellular and extracellular production of H_2O_2 and other oxidants, or to monitor redox changes in plant cells and tissues subjected to different kinds of stress stimuli and developmental cues [34–43].

However, some issues should be considered when using DCFH-DA for ROS visualization and measurement. The main concern refers to the relative non-selectivity to distinct ROS species and oxidants, as DCFH does not react directly with $O_2^{\bullet -}$, H_2O_2 or $^{\bullet}NO$. Instead, metal ions- and POX-catalysed reactions generate potent oxidants capable of DCFH oxidation [44]. Therefore, DCFH oxidation can be produced by other one-electron transferring ROS, RNS, redox active metals (Fe^{2+} or Cu^{2+}) and metallo-enzymes (such as cytochrome C or heme-POXs), rather than H_2O_2 itself [45]. In addition, presence of cellular antioxidants (e.g. GSH, NADPH) and visible light irradiation can produce photoreduction of DCF, generating a semi-reduced DCF radical ($DFC^{\bullet}$) which in aerobic conditions can react with O_2 leading to $O_2^{\bullet -}$ formation and subsequently H_2O_2, resulting in an artefactual amplification of ROS [46]. Also, DCFH can suffer from photooxidation and photobleaching [47]. Furthermore, DCFH oxidation is irreversible, and the increase in DCF fluorescence, instead of fluorescence intensity *per se*, is used as readout of ROS level. Since DCFH is soluble in lipids, as well as in the aqueous phase, it is important to remember that the reaction of DCFH with aqueous radicals is suppressed by soluble cellular antioxidants, whereas not in lipophilic compartments,

making DCFH a suitable probe for detecting lipid hydroperoxides, especially its enhanced lipophilic derivatives [29].

In summary, DCFH-DA may be used as a general cellular oxidative stress redox marker rather than as a specific indicator for H_2O_2 or other ROS and RNS. In addition, complementary experimental controls must be performed to avoid misinterpretation of recorded fluorescence. However, DCFH-DA has been widely used to measure ROS production in multiple plant systems upon various treatments, e.g. *Arabidopsis thaliana* leaves after wounding [48] or pathogen attack [43]; to study the function of mitochondrial alternative oxidase in cultured tobacco cells [49]; characterization of the signalling cross-talk between plant phytohormones abscisic acid and auxins, analysis of self-incompatibility response, actin reorganization and programed cell death relationships in *Papaver sp.* [50]; rice autotoxicity response to ferulic acid exposure [51], etc. It is also widely used as a tool when studying green algae, e.g. ROS production in response to abscisic acid exposure [52], toxic metals [53], and osmotic stress [54].

2.2 N-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red)

Another probe frequently used to measure H_2O_2 is N-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red). It is a non-fluorescent substrate for POX that reacts with H_2O_2 in a 1:1 stoichiometric ratio to produce resorufin, a highly fluorescent product. Although also capable of being oxidized by other one-electron transferring oxidants, the electron transfer catalysed by POX is highly efficient, conferring Amplex Red H_2O_2 specificity. Furthermore, as only the end product resorufin presents fluorescence, this probe displays low background signal, making Amplex Red valuable when measuring H_2O_2 formation [47,55]. As for DCFH-DA, derivatives or Amplex Red showing improved sensitivity and contrast (e.g. Amplex UltraRed and other Amplex Red-based molecules) have been developed [31]. Amplex Red and its derivatives have been successfully used for *in vivo* detection of intracellular or extracellular H_2O_2 production in *Arabidopsis* [55] and tobacco leaves [56]; defence induction upon fungi attack in *Phaseolus vulgaris* suspension cultures [57]; and heat tolerance in the green algae *Chlamydomonas reinhardtii* [58]. Nevertheless, as with every other enzyme-dependent probe, this assay

is susceptible to interference with substances capable of altering POX enzymatic activity. Indeed, when H_2O_2 concentration is very high compared to that of Amplex Red, POX can use resorufin as a substrate, rendering resazurin (with little or no fluorescence) and affecting the total signal intensity [59]. Moreover, in biological systems, reducing metabolites (such as NADPH and GSH) may interfere with POX/Amplex Red assay by producing light-mediated photochemical oxidation of resorufin, yielding $O_2^{\bullet -}$ and H_2O_2 [60]. For this reason, *in vivo* experiments where Amplex Red is used to measure intracellular H_2O_2 can be challenging [61]. The most accurate conditions where this probe can be used may involve H_2O_2 release from the cell, e.g. cell wall NADPH oxidase activity analysis, and measurements of isolated organelle fractions where Amplex Red probe can be used to detect H_2O_2 in the presence of externally added POX (such as horse radish peroxidase). This has been done in alfalfa roots upon Cd or Hg exposure [32]; in wheat and rice studies of defence mechanisms induction by Gall Midge [62]; in tobacco BY2 suspension cultures characterization of oxidative burst defence response to pathogen elicitor cryptogein [63]; or cell wall ingrowth formation in *Vicia faba* cotyledon epidermal cells [64].

2.3 Dihydrorhodamine 123 (DHR)

Dihydrorhodamine 123 (DHR) is also frequently used as H_2O_2 indicator for general oxidative stress detection in plants [65–67]. DHR is a non-fluorescent lipophilic membrane-permeable probe that scavenges the $OH^{\bullet}$ generated from H_2O_2 in an iron-dependent Fenton reaction, rendering an oxidized fluorescent product, rhodamine 123. As for DCFH, DHR also reacts with $ONOO^-$ and other $^{\bullet}NO_2$ radicals, and therefore non-specific [68]. In addition, the one-electron intermediate DHR radical ($DHR^{\bullet}$) reacts with oxygen and causes an artefactual increase in fluorescence intensity. Thus, as for DCFH, interfering substances such as $^{\bullet}NO$, Fe^{2+} and $O_2^{\bullet -}$ should be carefully monitored and controlled using appropriate inhibitors. Nevertheless, several groups have successfully used this fluorophore to study plant-microbe interactions in *Rubia cordifolia*, *Panax ginseng*, and *A. thaliana* cells [69]; response to Cd exposure in *Arabidopsis* cell cultures [66]; elicitor-triggered hypersensitive response and stomatal closure in *N. benthamiana* [67]; or lipid peroxidation and oxidative changes induced by chlorophenols in wheat leaves [70]. A specially interesting application was the

evaluation of H_2O_2 production in green algae (*Chlamydomonas moewusii*) after herbicide (Paraquat) exposure, which was quantified by flow cytometry and cell sorting [71].

2.4 Dihydroethidium (DHE)

Dihydroethidium (DHE) is more reactive than DCFH or DHR toward superoxide radicals and therefore widely used as a specific fluorescent probe for $O_2^{\bullet-}$ [72,73]. The highly specific red fluorescent product generated, 2-hydroxyethidium (2-OH-E^+), shows shifted excitation and emission peaks, from 350 and 400 to 518 and 605 nm, respectively [40,43,47]. It freely permeates cell membranes, while in intact cells, DHE detection of $O_2^{\bullet-}$ is hindered by oxidants other than $O_2^{\bullet-}$, generating the two-electron oxidation product ethidium cation (E^+), another red fluorescent compound usually taken as equivalent to intracellular $O_2^{\bullet-}$ formation. E^+ often accumulates at higher concentrations and is capable of nuclear DNA binding, which strongly increases the fluorescence signal [74]. Because of these and other oxidative reactions, it has been suggested that measurement of 2-OH-E^+ is a qualitative, and not a quantitative, readout of intracellular and/or extracellular $O_2^{\bullet-}$ [47]. Another drawback is that these probes are light sensitive and prone to autooxidization. Nevertheless, DHE is a very popular fluorescent-dye to study $O_2^{\bullet-}$ in plants [75–78] and algae [71].

2.5 Emerging alternative dye-based probes

Aromatic boronate-based fluorophores, known as the peroxysensor family, can be oxidized by H_2O_2, giving rise to the corresponding fluorescent phenolic product [79,80]. The boronate esters are cell-permeable and can effectively be used for subcellular targeting, to measure intracellular H_2O_2 in distinct compartments [81]. In these probes, the boronate moiety masks a fluorophore (for example fluorescein). Upon reaction with H_2O_2, this moiety is released, exposing the fluorophore and emitting fluorescence. However, the reaction constant is relatively low, resulting in competition for H_2O_2 by faster H_2O_2-detoxifying enzymes and reduced signal. Nevertheless, ContPY1 boronate-based specific sensor of H_2O_2 has been used to detect intracellular H_2O_2 formation in *Arabidopsis* protoplasts and tissues [82,83]. However, aromatic boronate-based

indicators may also react with $ONOO^-$, in a stoichiometrically faster reaction than with H_2O_2, reducing the specificity of such probes for ROS detection.

A new generation of dyes allow the detection of several ROS and RNS with higher sensitivity. For example, HKGreen family of rhodol-based fluorescent indicators was more sensitivite to $ONOO^-$ than to H_2O_2, which permitted ROS to be distinguished from RNS in *A. thaliana* leaves showing hypersensitive response [84]. Other probes have also been used to detect products and derivatives from ROS reactions in various plants *in vivo*, such as BODIPY for hypochlorous acid in *Arabidopsis* protoplasts, tobacco BY2 cell cultures and *N. benthamiana* leaf tissue [85]; TEMPO and derivatives for singlet oxygen and general oxidative stress in *Arabidopsis* roots and tobacco leaves [86,87]; DanePy to monitor singlet oxygen production in isolated spinach thylakoids [88], *Arabidopsis* leaves [89,90]; and chloroplast-to-nucleus retrograde signalling in *Chlamydomonas* cells [91]. Recently, the development of a new group of stable and robustly reversible probes based on the incorporation of an organochalcogen atom, such as selenium and tellurium, into a chromophore or fluorophore molecule, has opened new possibilities for redox status monitoring. Organochalcogen-based probes show high selectivity for aromatic thiols, Cys and GSH [92] and a wide range of ROS and RNS [93]. However, the chemical and fluorescence transduction mechanisms of such probes in response to ROS and redox changes still needs to be elucidated. For further information about the chemistry of present and emerging dye-based probes the reader will find extensive information in the following references [29,44,94].

Despite the wide variety of small-molecule fluorescent dyes now available for ROS measurement, several concerns regarding these indicators must be taken into account in order to successfully design the experiments in which they are to be used. Some of these considerations are knowledge of the probe specificity and sensitivity; kinetics of the reaction under biological conditions; degree of cellular toxicity; (photo)stability and solubility in aqueous and lipid environments; permeability and intracellular distribution of the probe to properly set the loading conditions; interference from pH or biological compounds with their absorbance and/or emission; requirement of catalysts; production of probe intermediate radicals and their possible interactions with oxygen

and/or antioxidants; competition with cellular antioxidant system for the ROS; excitation and emission wavelengths of the fluorescent probe; illumination parameters to avoid photo-activation of the probe and/or photobleaching. When studying early events triggering ROS accumulation, alterations of signal transduction due to the scavenging of ROS by the indicators should also be kept in mind. In summary, it is important to understand the advantages and limitations of the indicators used, and to implement a strict control of the experimental conditions used and to adequately interpret the experimental data. Whenever possible, cross-validation using independent experimental methods and positive/negative controls with specific ROS scavengers is highly recommended [74].

3. Genetically encoded protein-based ROS probes in plant studies

The green fluorescent protein (GFP) is frequently used as a marker for gene expression and for protein tagging in prokaryotic and eukaryotic cells. The protein consists of 238 amino acids and forms an 11-stranded β-barrel surrounding a central helix, where the fluorophore, resulting from spontaneous cyclization and oxidation of the Ser65-Tyr66-Gly67 motif, is located [95]. GFP does not require supply of exogenous substrates or cofactors, and modified versions of the protein that offers unique excitation and emission characteristics have been generated, enabling co-expression and simultaneous detection of modified FP proteins with distinct colours [96,97].

FP-based ROS reporter molecules in plants may offer several advantages over dye-based fluorescent probes. Because FPs do not require permeation of substrates, non-invasive techniques can be used to monitor *in vivo* metabolic process upon ROS accumulation. Depending on the strategy chosen, ROS will either affect level or localization of the FP used, or alter its fluorescent properties, to generate a signal that correlates with the amount of ROS. Genes encoding FPs can be expressed in plant cells, either after stable integration into the plant cell genome, or transiently using expression vectors. With the help of organelle-specific targeting sequences included in the transgenic protein, ROS sensitive FP-derived probes that enable subcellular ROS

monitoring is feasible [98,99]. However, plant cell autofluorescence can severally hamper visualization of FPs expressed in specific cell types [26–28]. In green tissues, chlorophyll is the major contributor to autofluorescence with efficient absorbance in the blue spectrum up to 500 nm, and fluorescence from 600 to 800 nm (**Figure 2**). Although most fluorescent reporter proteins have emission peaks below 600 nm [96,97], the vast abundance of chlorophyll and other pigments in a green cells can substantially interfere with FP detection.

3.1 Redox sensitive probes: rxYFP and roGFP

Several GFP variants with shifted spectral properties are now available. Among them, a yellow shifted GFP protein (YFP) was point mutated to include two Cys in the nearby region of the chromophore. Upon oxidation, these sulfhydryl residues can reversely form a disulphide bond that causes a conformational change in the YFP molecule. This modification of the YFP structure results in altered fluorescent properties, making it a powerful tool as indicator of *in vivo* and real-time plant cell redox status [100,101]. Initially, four different redox sensitive YFPs (rxYFPs) were constructed by introducing Cys pairs at different locations of the 7 and 10 β-strands of YFP. Only the N149C/S202C variant exhibited a substantial fluorescent shift in the emission peak upon redox change (>2-fold) [100], confirming the importance of the location of the Cys pairs within the FP structure to obtain a chromophore with altered fluorescence properties. However, a major limitation of rxYFP is that quantification relies on absolute values, and both, the oxidized and reduced conformations are estimated from the same excitation/emission peaks (**Figure 3A**) [102,103]. In addition, rxYFP is highly sensitive to pH changes due to the chromophore *pKa* (close to pH 7), as occurs with other FPs. rxYFP was first used to determine the dynamic changes of cytosolic GSH/GSSG concentrations in yeast [104], highlighting the potential of FPs to detect *in vivo* changes in antioxidant metabolite levels and redox homeostasis at different subcellular compartments.

The second generation of redox sensitive FP probes belongs to the reduction-oxidation sensitive GPF (roGFP) class. These probes display two distinct maximum excitation peaks (405 nm for the neutral state and 488 nm for the anionic form), whose relative

amplitudes depend on the redox state of the fluorophore. This gradual shift in the excitation spectrum substantially improves fluorescence quantification as it allows for ratiometric analysis ($488_{exc}/405_{exc}$), enabling more accurate cellular redox status quantification compared to the rxYFP sensor [101,105]. While several roGFPs variants have been engineered and characterized, roGFP1 and roGFP2 are the most frequently used. Besides mutational changes performed in the roGFP1 variant (C48S, S147C and Q204C), roGFP2 contains an extra mutation (S65T) that generates a slight shift in the maximum excitation spectrum, allowing for larger dynamic ratio between oxidized and reduced forms (**Figure 3A**) [101]. Although roGFP1 is less pH-sensitive than roGFP2, the larger dynamic range of roGFP2 confers an advantage when used in confocal laser scanning microscopy.

Redox potential of specific organelles within the cell can also be studied by subcellular targeting of the roGFP probes using specific targeting sequences, e.g. for cytosol [106–111], plastids [108,112], peroxisomes [108,112], mitochondria [106,107,112,113] or endoplasmic reticulum [111,112]. As roGFP1 and roGFP2 have midpoint potentials between –280 mV and –290 mV [101,105], measurement of redox potential in some subcellular compartments is restricted, e.g. in the strong oxidizing environment on ER and the reducing conditions in other compartments (chloroplasts and mitochondria). This limitation has been faced by later roGFP variants that expand the range of redox potential values, such as the roGFP1-iX family with midpoint potential between –229 mV and –246 mV [114].

Experiments using roGFPs in plants to elucidate whether the fluorescent probes respond only to a specific redox pair, or the general cell redox buffer capacity, established that the oxidoreductases glutaredoxins (GRX) act reversibly by transferring electrons from GSH to the roGFP2 (**Figure 3B**) [111,115,116]. This finding explains the faster response of roGFP *in vivo* than when used in *in vitro* assays. Probes based on roGFP2 fusions have been developed, e.g. roGFP2 fused with GRX1 (GRX1::roGFP2), allowing the GSH/GSSG pool to be monitored. This probe extends the range available for cell GSH redox potential (E_{GSH}) measurement between –320 mV (GRX1::roGFP2) to about –210 mV (GRX1::roGFP-iL), as shown by Aller and colleagues who measured E_{GSH}

in the cytosol of GSH deficient *rml1 Arabidopsis* mutants [110]. GRX1::roGFP constructs facilitate faster redox equilibration with the GSH/GSSG redox couple than roGFPs alone. In addition, subcellular targeting of these probes allowed sensitive measurement of E_{GSH} in cellular compartments with low GRX enzymatic activity. Other examples of similar fusions are roGFP2-Orp1 and roGFP2-GPX4, with yeast POX Orp1 or human GPX4 fused to roGFP, that were successfully used for to detect changes in H_2O_2levels [117,118].

Despite recent advances, several concerns when using FP probes to measure redox status must be considered. For instance, a wide variety of coloured pigments synthesised by secondary metabolism are accumulating when plant cells are subjected to stress. This is particularly problematic as many plant pigments absorb light of similar wavelength as used for GFP excitation (usually 488 nm), resulting in decreased GFP fluorescence. Thus, pigment interference must be taken into account when measuring redox status in green tissues, and redox measurements based on roGFPs must be carefully calibrated and validated in order to avoid the influence resulting from autofluorescence. Another drawback when using roGFP redox sensors is pH sensitivity, the main general limitation for single-FP based probes. As fluorescence depends on the protonation state (neutral or anionic) of the GFP chromophore, care must be taken when FPs are targeted to cellular compartments with different pH conditions.

Similarly, special care should be taken if different GFP-based sensors are used simultaneously monitor distinct *in vivo* redox states in e.g. individual organelles, due to FP emission peak overlaps. This limitation has recently been partially resolved by the development of the Oba-Q (oxidation balance sensed quenching) protein, that is based on cyan FP (CFP) variants, and therefore better suited for simultaneous imaging with other FPs-based sensors, and whose fluorescence decreases dramatically under oxidative conditions [119]. In conclusion, despite recent progress in the development of roGFP based sensors, there is a need for development of more specific, stable and reliable plant redox sensitive FPs.

3.2 $O_2^{\bullet-}$ and H_2O_2 imaging

It soon became apparent that none of the FP-based probes developed could readily detect the cellular concentration of individual ROS, only their effect on the cellular redox balance or metabolic alterations. Thus, development of probes to study distinct ROS species was highly desirable. cpYFP was generated by circular permutation and point mutation of the YFP variant EYFP (V68L/Q69K), where the two original N- and C-termini were connected by the linker sequence VDGGSGGTG [120]. In that study, the authors found that the fluorescent properties of a chimeric protein consisting of M13 (a 26-residue peptide derived from the calmodulin-binding region of the skeletal muscle myosin light-chain kinase), cpYFP and calmodulin changed according to Ca^{2+} levels. In a subsequent study by Wang and colleagues, cpYFP itself was found to exhibit brighter fluorescence under oxidizing conditions, and was described as a $O_2^{\bullet-}$ sensor, not affected by H_2O_2 [30]. Using the cytochrome c oxidase subunit IV targeting sequence, mt-cpYFP was generated and successfully used to study mitochondrial ROS accumulation in *Arabidopsis* roots [121,122]. In addition, it was reported that mt-cpYFP displayed transient "flashes" of increased fluorescence, so-called mitochondrial $O_2^{\bullet-}$ flashes or mitoflashes [30,74,123]. The mechanism of how $O_2^{\bullet-}$ interacts with the cpYFP chromophore, and whether this interaction is specific, remains unclear. In addition, concerns about the nature and origin of these flashes has been raised [124–126]. Although available literature provides convincing evidences that cpYFP acts as a $O_2^{\bullet-}$ sensor, it cannot be ruled out that the reported "superoxide flashes", at least partially, are caused by changes in pH [98].

Another ROS sensitive probe is HyPer, which is composed of cpYFP inserted into the regulatory domain of *Escherichia coli* OxyR. In contrast to cpYFP alone, HyPer is sensitive to H_2O_2, but not $O_2^{\bullet-}$, oxidized glutathione (GSSG) or RNS [127]. HyPer displays a single emission spectrum peak at 516 nm, while two excitation spectrum peaks (420 and 500 nm, reduced and oxidized forms respectively). Upon H_2O_2 application, fluorescence intensity decreases at 420 nm excitation wavelength, while fluorescence increases at 500 nm, allowing for ratiometric measurement of H_2O_2. However, as for cpYFP and other GFP-based sensors, HyPer fluorescence is pH sensitive [127]. HyPer has successfully been used in plants [128], e.g. to monitor

oxidative stress in *Arabidopsis* roots subjected to aluminium treatment [129], and when studying responses to high light intensities in *Arabidopsis* seedlings [130]. HyPer was also shown to respond to H_2O_2 addition in guard cells of intact *Arabidopsis* epidermis [131]. Costa and colleagues additionally generated a peroxisome targeted version of HyPer by C-terminal fusion of the well-known peroxisomal targeting peptide sequence KSRM. Fluorescence was now confined to distinct vesicular structures corresponding to peroxisomes, and as for the cytoplasmic version, HyPer-KSRM in guard cells responded to H_2O_2 addition [131].

Recently, a red fluorescent sensor for H_2O_2 detection (HyPerRed) was described [132]. To generate this sensor, the cpYFP portion of HyPer was replaced with different circularly permuted red FPs, in combination with semi-random expression libraries where the length of the amino acid linkers between the cpFP and two flanking OxyR-RD parts of the sensor were varied. The best clone demonstrated an 80% increase in fluorescence upon H_2O_2 addition [132]. The performance of the red sensor was shown to be similar to its green analogues, and specific towards H_2O_2, as shown for HyPer. In addition, the authors created a mitochondrial targeted variant that allowed simultaneous use with cytoplasmic or mitochondrial redox biosensors detecting changes in H_2O_2, reduced-oxidized glutathione or pH.

4. Conclusions and future perspectives

The past decade has witnessed significant progress in understanding physiological functions of ROS, with their role as intracellular signal transduction messengers receiving increasing attention. Therefore, imaging ROS *in situ* and *in vivo*, with high selectivity, accuracy, and spatiotemporal resolution is essential for our understanding of the remarkably complex ROS signalling network. For that purpose, novel fluorescent protein-based ROS sensors and genetically engineered improved versions will further help deciphering these mechanisms at a molecular level.

In this regard, a novel class of fluorescent proteins and sensors have been recently developed. These probes are based on natural photoreceptor proteins containing

flavin cofactors, such as the light, oxygen and voltage receptors family [133,134], and have been successfully used to analyse cell redox status in microaerobic and anaerobic conditions [135,136], and upon plant virus infection [137].

While microscopy sensitivity and specificity is continuously improving, efficient fluorescence imaging of larger intact plant portions, especially of green tissues, still remains difficult. In this regard, Pasin and colleagues showed that laser scanning, combined with plate readers capable of detecting and quantitatively measure fluorescence, can be used to simultaneously study a reasonable large number of plant tissue samples (i.e. leaf discs or seedlings) in a relatively short time [138]. Flow cytometry and fluorescence-activated cell sorting, a widely used high throughput method for analysing individual cells of mammalian origin, is less frequently used with plant cells. The main reason is that individual protoplasts must be prepared by degrading the plant cell wall, using enzymes that often induce stresses and distort the physiology of these cells. Although fluorescence-activated cell sorting of protoplasts is more challenging than when used for mammalian cells, as the large volume of plant cell vacuoles make protoplasts prone to break, it has proved useful for the analysis of stably transformed plants and transiently transfected protoplasts [139–141].

4.1 FRET-based biosensors to study ROS and redox signalling in plants

The development of FPs variants with spectral shifts has enabled applications taking advantage of the possibility for fluorescence resonance energy transfer (FRET) to occur between adjacent fluorophores. FRET results in non-radiative energy transfer from an excited donor to a suitable acceptor (often CFP and YFP variants, respectively), when the two fluorophores are in close proximity (typically shorter than 10 nm). FRET can in this way be used to study molecular interactions, when two proteins of interest are fused to donor and acceptor FPs separately; a technique often called intermolecular or bimolecular FRET [142]. Another possibility offered by FRET is to engineer a single polypeptide that contains both donor and acceptor FPs, separated by a linker harbouring a domain capable of generating a conformational change upon induction of the process or stimuli to be studied, e.g. change in redox potential or phosphorylation

status. Conformational changes in these in intramolecular or unimolecular sensors, where the distance of the FPs is affected, will influence the FRET signal. These new generation of FRET biosensors are often used for simple ratiometric image analysis in cell biology, because of improved fluorescence dynamic range and high signal-to-noise ratio, and as they only require the expression of a single chimeric gene construct. In this sense, FRET can be a powerful tool for analysing both physiological changes and signalling events in response to ROS accumulation at a cellular level [142–144].

Although little information is known regarding the use of ROS-FRET biosensors in plants, glucose flux and Ca^{2+} signalling has successfully been monitored by FRET in leaves and roots [145–147]. However, for mammalian cells, several successful FRET-based ROS biosensors have been engineered. Kolossov *et al*. developed, and then further refined, intramolecular probes where CFP and YFP were linked by redox sensitive polypeptides, in which Cys embedded into the α-helical linker structures induced conformational changes resulting in FRET (**Figure 4A**) [148,149]. In a similar way, Yano and colleagues used tandem repeats from the C-terminal Cys-rich domain of the yeast transcriptional factor Yap1 to construct the FRET sensor Redoxfluor [150]. Using Redoxfluor in yeast and mammalian cells, the authors concluded that the redox state within peroxisomes was more reductive than in the cytosol, a requisite for ROS generation. In a different approach, Guzy and colleagues used a 69 amino acid Cys-containing regulatory domain from redox-regulated heat-shock *E. coli* protein HSP-33, cloned between YFP and CFP [151,152]. In response to oxidative stress, conformational changes in the redox-sensitive linker caused CFP and YFP to move apart, thus decreasing FRET emission and increasing the ratio of CFP/YFP fluorescence intensity. The resulting FRET sensor was used to assess cytosolic ROS signalling in mammalian cell lines of different origin, and enabled the authors to suggest that mitochondria function as O_2 sensors and signal hypoxic HIF-1α and HIF-2α stabilization by releasing ROS to the cytosol [152].

An alternative method to study signalling induced by ROS or redox changes by FRET is to create probes that respond to phosphorylation. If ROS or redox signalling affects the activity of specific kinases/phosphatases, activities of these enzymes can be monitored

by FRET probes that harbour phosphorylation sites for these kinases/phosphatases (**Figure 4B**). This has successfully been done for PKA and ERK (kinases of AGC and MAPK families), amongst others. In such intramolecular FRET sensors, the donor and acceptor FPs are linked by a domain that can bind phosphorylated amino acids, together with the substrate region or domain harbouring the phosphorylation site [153,154]. Upon phosphorylation of the substrate site, the binding domain will interact with the phosphorylated residue, resulting in a conformational change of the sensor and FRET signal (**Figure 4B**). In a similar way, FRET sensors to study ROS in plants could be generated, using substrate domains for ROS activated kinases, e.g. the AGC family kinase OXI1 or kinases from the MAPK family [155–159].

4.2 ROS regulated promoter-FP fusions

Another option to study ROS regulated processes in plants is to express FPs under the control of ROS-regulated promoters [160]. This approach is rather indirect, but if the promoter activity is tightly controlled by ROS it may enable ROS-mediated responses to be monitored. In this sense, Besseau *et al*. found that WRKY30 and WRKY53, which belong to the stress-induced WRKY transcription factor family in *Arabidopsis*, are upregulated in response to oxidative stress induced by ozone and H_2O_2 [161]. Large-scale transcriptome analyses of different plant species have given valuable information about ROS gene networks [162–166], which could be exploited to generate ROS sensitive marker genes and probes. However, extensive further research to understand the specific role of each factor in the network and to experimentally verify their function in profusely interconnected cellular processes is required. It must be emphasised that GFP and its variants are generally stable, producing long-lived reporter proteins that make dynamic processes difficult to study, such as transient ROS concentration changes, frequent under biotic and abiotic stresses. To overcome such limitations, labile GFP variants should be considered in order to more accurately follow altered gene expression [167–170].

In summary, protein-based probes are tools that when properly used present high potential for monitoring and quantification of ROS and redox variations, avoiding some

of the obstacles presented by small-molecule fluorescent dyes. Some of these dye-specific limitations are toxicity of the probe *per se* due to probe radical formation triggered by the excitation light, and requirement of endogenous enzymatic factors (e.g. esterases or POX), that might be more or less active depending on the treatments applied. On the other hand, protein-based probes often require more careful calibration and validation of the obtained data, mainly to exclude pH-induced artefacts. In this regard, depending on the specific experimental requirements, small-molecule fluorescent dyes can give important complementary information to that obtained from using protein-based probes.

In addition to development of novel protein-based sensors, increasing efforts in designing new small-molecule fluorescent ROS probes will help provide a more detailed picture of ROS mediated plant cellular responses to environmental changes and developmental cues *in vivo*.

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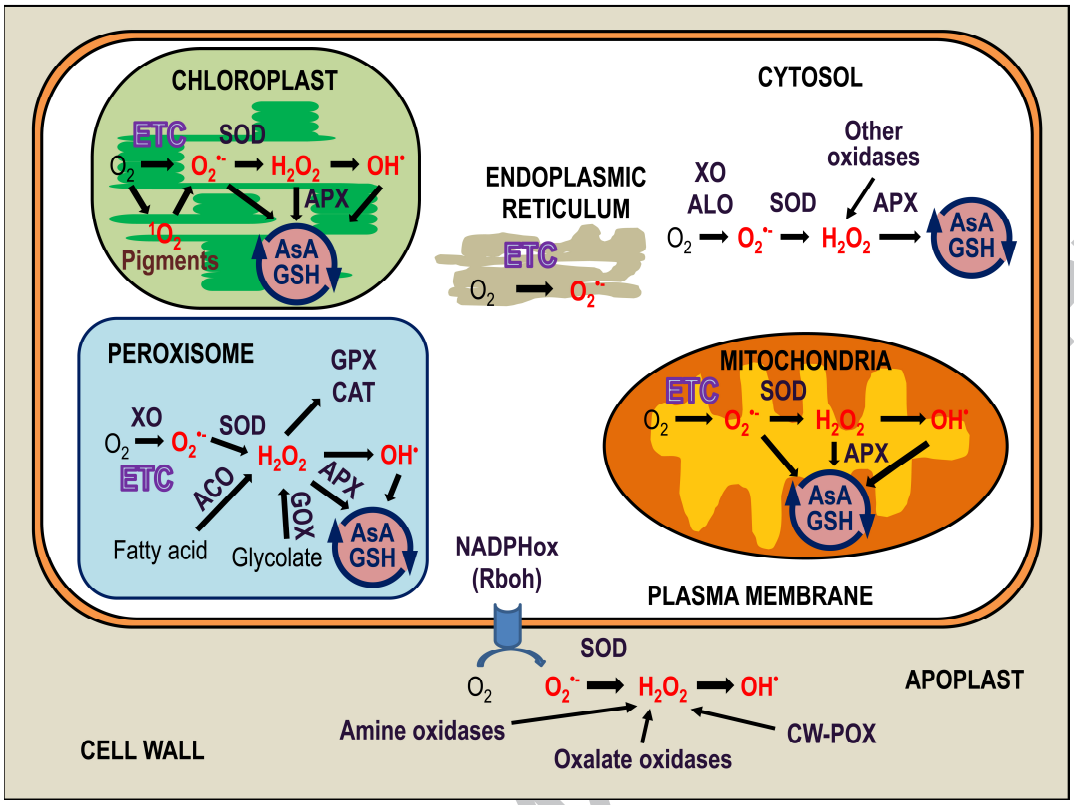
Figure legends

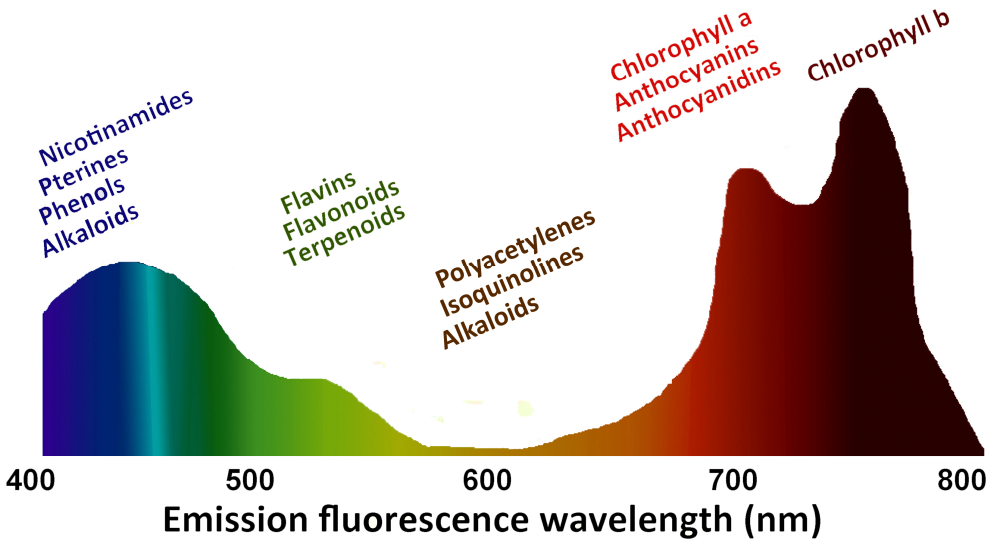
Figure 1. Sites of ROS production within the plant cell, together with important redox-related enzymes. AsA-GSH, ascorbate-glutathione cycle; AsA, ascorbate; ACO, acyl-CoA oxidase; ALO, aldehyde oxidase; APX, ascorbate peroxidase; CAT, catalase; CW-POX, cell wall peroxidases; ETC, electron transport chain; GOX, glycolate oxidase; GPX, glutathione peroxidase; GSH, reduced glutathione; H_2O_2, hydrogen peroxide; NADPHox, NADPH oxidase; POX, peroxidase; $O_2^{\bullet -}$ superoxide anion radical; $OH^{\bullet}$ hydroxyl radical; SOD, superoxide dismutase; XO, xanthine oxidase.

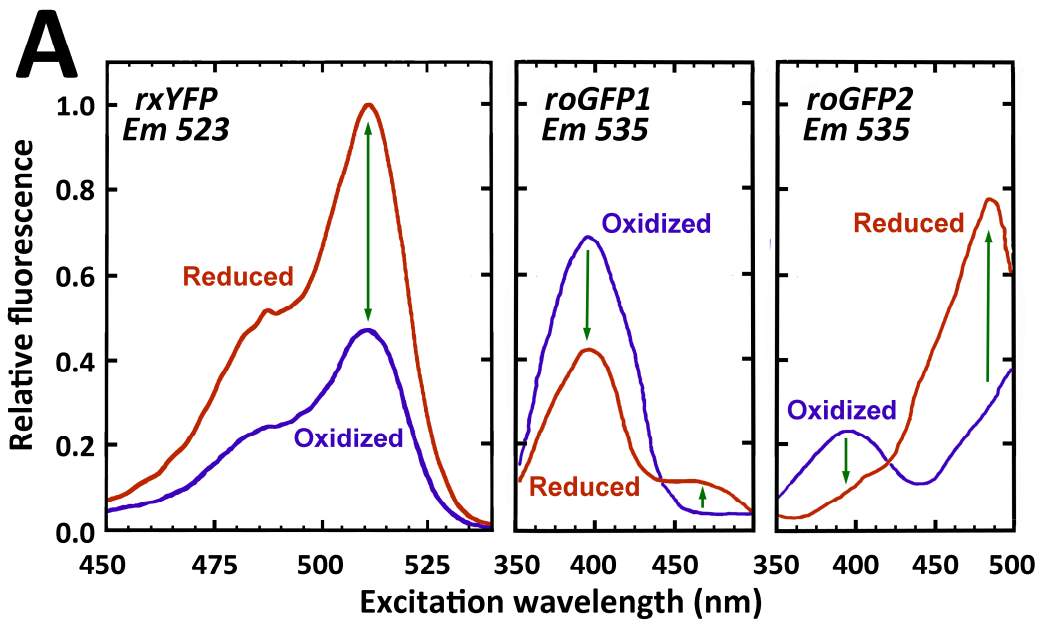
Figure 2. Typical ultraviolet autofluorescence spectra of a green leaf. Compounds that contribute to fluorescence emission are highlighted (λ_{exc}=355 nm, modified from Talamond *et al*. [24]).

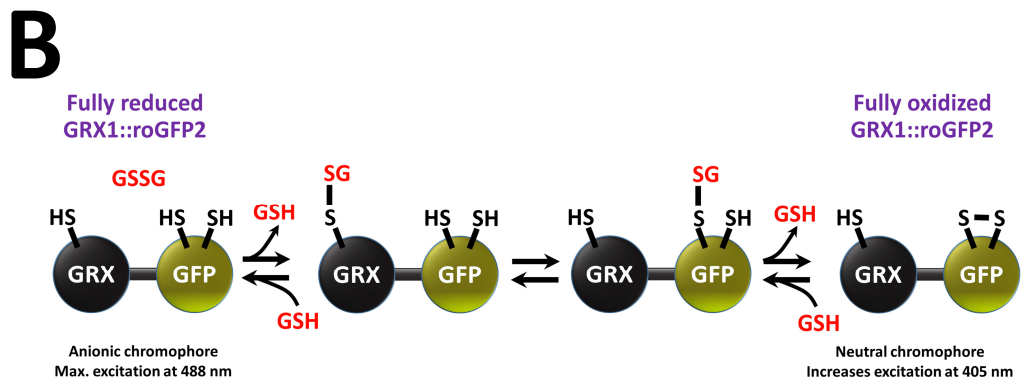
Figure 3. Single protein-based redox biosensors rxYFP and roGFP. (A) Excitation spectral response of redox sensitive rxYFP, roGFP1 and roGFP2 probes. Wavelengths typically used for quantitative (rxYFP) and ratiometric analysis based on spectral shifts (roGFPs) are marked with arrows. Em, emission wavelength (adapted from Østergaard *et al*. [100] for rxYFP, and Choi *et al*. [174] for roGFPs). (B) Schematic representation of the molecular mechanism for GRX1::roGFP2 biosensor (adapted from Meyer and Dick [116]). GSH, reduced glutathione; GSSG, oxidised glutathione; GRX, glutaredoxin; GFP, green fluorescent protein.

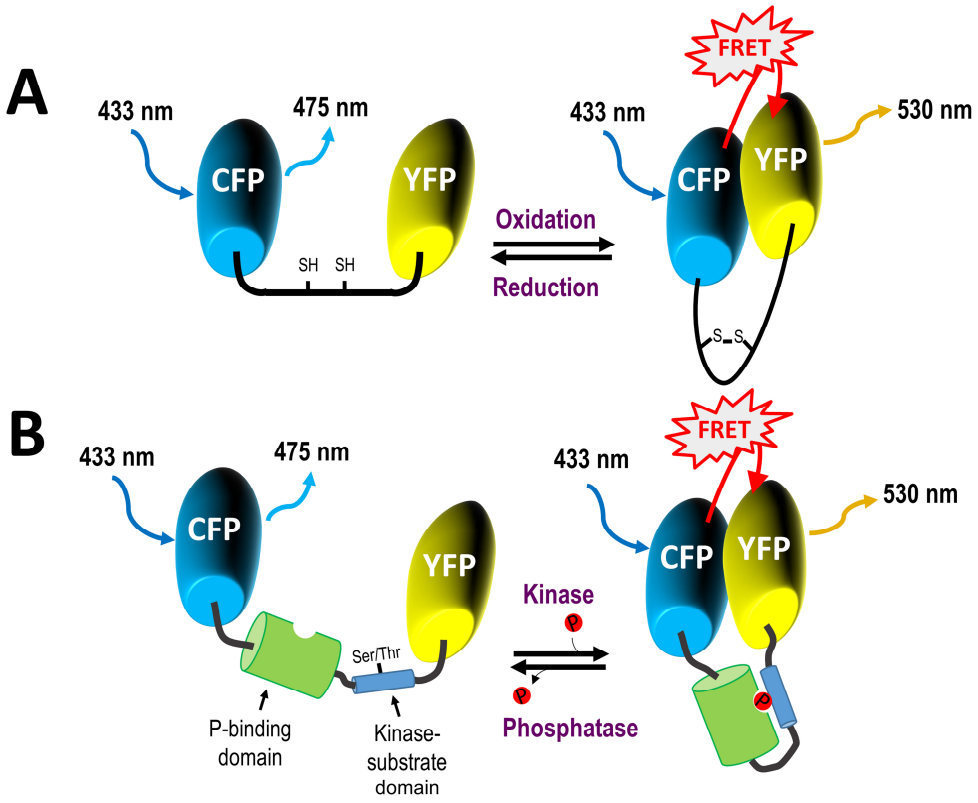
Figure 4. FRET-based ROS biosensors. (A) In the reduced state, excitation of the donor fluorophore (CFP) (dark blue arrow) mainly generates donor emission (light blue arrow), as donor and acceptor (YFP) are too distant from each other (due to extended linker conformation). Upon oxidation, disulphide bond formation causes the linker to fold, generating a conformational change that brings donor and acceptor fluorophores in close proximity, and induces FRET emission (yellow arrow) of the acceptor. Reduction of the disulphide bond restores initial conformation. (B) FRET-donor (CFP) is connected to the acceptor (YFP) via a linker domain containing a kinase/phosphatase substrate domain (Ser/Thr phosphorylation site), and a binding domain capable of binding the phosphorylated residue of the substrate domain. Upon phosphorylation by the specific kinase, the phospho-binding domain will interact with the phosphorylated residue and generate a conformational change that brings donor and acceptor fluorophores in close proximity. As for the redox sensitive probe, dephosphorylation by a phosphatase will reduce FRET. Using this approach, phosphorylation-dependent ROS signalling can be studied.











Highlights

- Description of ROS origin, homeostasis and *in vivo* detection in plants
- Small molecules and fluorescent dyes used for ROS and redox imaging in plants
- Protein-based fluorescent probes to study plant redox changes and ROS production
- Techniques and future strategies to study ROS signalling and redox changes