

The Fundamental Role of Reactive Oxygen Species in Plant Stress Response

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Abstract

Chemical, physical, and biotic factors continuously vary in the natural environment. Such parameters are considered as stressors if the magnitude of their change exceeds the current acclimation norm of the plant. Activation of genetic programs allows for conditional expansion of the acclimation norm and depends on specific sensing mechanisms, intracellular communication, and regulation. The redox and reactive oxygen species (ROS) network plays a fundamental role in directing the acclimation response. These highly reactive compounds like H_2O_2 are generated and scavenged under normal conditions and participate in realizing a basal acclimation level. Spatial and temporal changes in ROS levels and redox state provide valuable information for regulating epigenetic processes, transcription factors (TF), translation, protein turnover, metabolic pathways, and cross-feed, e.g., into hormone-, NO -, or Ca^{2+} -dependent signaling pathways. At elevated ROS levels uncontrolled oxidation reactions compromise cell functions, impair fitness and yield, and in extreme cases may cause plant death.

Key words Reactive oxygen species, Abiotic stress, Redox signaling, Redox regulation

1 Introduction

Plants are sessile organisms that have to cope with changing environmental conditions on time scales of minutes to years. On the one hand, plants exploit natural resources such as light and CO_2 in photosynthesis, mine minerals and tap water in the soil, and beneficially interact with bacteria and fungi. On the other hand, nutrient and water deficiencies, salinity, extreme temperatures, pathogens, and herbivores negatively interfere with plant fitness and yield. Regulatory circuitries allow for sensing the changing environmental parameters and control acclimation responses. If the intensity of such negative parameters increases, two effects reduce the fitness of plants: (1) The costs of activating and maintaining the acclimation and defense mechanisms compete for resource investments thereby decreasing growth, and (2) cumulative damage disturbs metabolism. Ultimately such disturbances

may cause cessation of growth and death. This stress concept is quite prevalent and has been extensively investigated in the last few decades, particularly with respect to the stress-specific signaling pathways, the involvement of redox- and ROS-related cues and the mechanisms mediating sensitivity and tolerance [1].

Most laboratory studies explore the effect of single stressors, while in nature organisms often encounter stress combination, e.g., high light, drought, and high temperature; or flood and salinity [2]. Abiotic stress can hardly be avoided and, from a global point of view, it impacts on plants stronger than biotic factors [3]. It is by now generally accepted that redox and ROS signaling decisively participate in perceiving stressful conditions, in regulating the acclimation response and in inducing damage progression and cell death. The qualitative and quantitative determination of relevant parameters often poses a challenge to the experimentalist. In the following the reader is introduced to the general stress concept and exemplarily to methods which have been used to assess the redox and ROS state of plant tissues under stress.

1.1 Generation of Reactive Oxygen Species

ROS are produced in discrete compartments of the plant cell.

1.1.1 Superoxide Anion Generation

In chloroplasts energy conversion is linked to the light-driven photosynthetic electron transport (PET). In mitochondria, the respiratory electron transport (RET) essentially converts reducing power generated from substrate oxidation to ATP. Within the transport chains electron carriers catalyze redox reactions and proton transfer to establish the electrochemical proton gradient for ATP synthesis. Both PET and RET can adopt a highly reduced state if energy input (light or NADH) is high and availability of intermediate carriers (plastoquinone, ubiquinone, cytochrome c) or terminal acceptors limited (NADP, O₂). Under such conditions superoxide anion ($\cdot\text{O}_2^-$) can be generated at photosystem I, mitochondrial complexes I and III and the quinones [4–6]. Furthermore, plasma membrane-localized NADPH oxidase (respiratory burst oxidase homologue: RBOH) releases $\cdot\text{O}_2^-$ into the apoplast which in particular participates in local and systemic signaling, plant immunity and stress responses [7, 8]. It should be noted that $\cdot\text{O}_2^-$ can react with nitric oxide to produce peroxynitrite (ONOO⁻) a highly reactive nitrogen species that cause protein nitration.

1.1.2 Singlet Oxygen

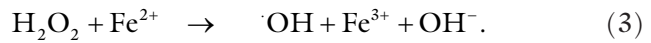
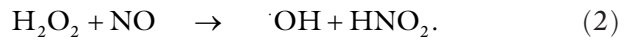
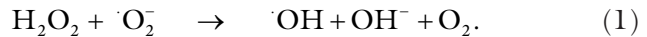
Photosensitizers such as excited chlorophylls in PS II are able to convert oxygen into its energized singlet state ($^1\text{O}_2$) being a highly reactive ROS that may damage the photosynthetic machinery. $^1\text{O}_2$ -overproducing mutants revealed signaling functions in combination with oxylipins like 12-oxo-phytodienoic acid (OPDA) for stress acclimation and cell death [9].

1.1.3 Hydrogen Peroxide

Superoxide dismutase converts $\cdot\text{O}_2^-$ to H_2O_2 , which is a less reactive since more stable and thus more abundant ROS. In peroxisomes H_2O_2 is produced as side product of oxidase reactions such as photorespiratory glycolate oxidase but also for example xanthine oxidase. At ambient CO_2 and O_2 concentrations, photorespiratory H_2O_2 is generated in stoichiometric amounts of 30–40% relative to CO_2 fixation rate. H_2O_2 is decomposed by several antioxidant systems, most prominently catalase in peroxisomes, type III peroxidases in the apoplast, and thiol peroxidases and ascorbate peroxidases in most other compartments including cytosol, chloroplast, and mitochondrion. In addition, H_2O_2 functions as an important cellular messenger in context of regular and stress signaling [10, 11].

1.1.4 Hydroxyl Radical

Some authors considered H_2O_2 as potentially being more toxic than based on its reaction constants with proteins and other cell constituents. Generally accepted is the ability of H_2O_2 to react with $\cdot\text{O}_2^-$, nitric oxide, some transition metals but also antioxidants such as ascorbate to produce $\cdot\text{OH}$ (Eqs. 1–4) [12, 13].



The Fenton reaction where H_2O_2 is reduced by redox active metal ions such as Fe^{2+} and Cu^+ , thereby generating $\cdot\text{OH}$, also occurs in metal-containing protein complexes [14]. $\cdot\text{OH}$ immediately reacts with proteins, nucleic acids, and lipids. Due to the high reactivity of $\cdot\text{OH}$ with multiple cell components efficient detoxification is not possible, instead it can be quenched in part by high concentrations of low molecular mass antioxidants such as proline, which often accumulate under stress [15]. $\cdot\text{O}_2^-$, H_2O_2 and $\cdot\text{OH}$ exhibit distinct reactivity toward certain amino acid residues in proteins, lipids, carotenoids, and nucleic acids. Lipid peroxidation can initiate radical chain reactions, which accelerate lipid oxidation. Protein oxidation may inhibit protein function due to fragmentation, modification of functional amino acid groups, or protein cross-linking [16].

1.2 Antagonists of ROS Accumulation

The balance between mechanisms to suppress ROS generation and efficient detoxification establishes low resting ROS concentrations in cells under normal growth conditions. Under such conditions, the thiol redox potential in the cytosol and stroma as measured by redox-sensitized green fluorescent protein (roGFP) coupled to glutaredoxins (Grx) is rather negative [17]. Inhibition of metabolism often leads to decelerated consumption of reducing power

and ATP. Consequently ADP is unavailable for mitochondrial and chloroplast ATP synthase and NADP⁺ is short as electron acceptor of photosystem I. Electron carriers of PET and RET get overreduced and the rate of electron transfer to O₂ increases. Thus, increasing stress intensity usually coincides with ROS accumulation and causes deviations from cell redox homeostasis. ROS detoxification takes place either by direct enzymatic reaction or by reductants that need to be regenerated.

The tripeptide glutathione (γ-Glu-Cys-Gly, GSH) is present at millimolar concentrations in plasmatic compartments of the plant cells and functions as thiol buffer, electron donor, substrate for conjugation and phytochelatin synthesis, and by S-glutathionylation as regulator of proteins [18]. Redox regulation of protein functions to a major extent depends on cysteine thiols, which can undergo multiple posttranslational modifications, namely disulfide formation, sulfenylation, sulfinylation, sulfonylation, S-nitrosylation, S-acylation, and S-glutathionylation. Such modifications have strong impact on protein function, but may also trigger redox-linked signaling [19]. Glutathione stabilizes the cellular thiol redox state. Oxidized glutathione GSSG is regenerated by glutathione reductase (GR) using NADPH as electron donor [20]. GSH serves as substrate in synthesis of phytochelatins, which participate in binding and transport of some heavy metal and metalloid ions, e.g., cadmium and arsenic [21, 22]. Hydrophobic antioxidants like tocopherols and carotenoids are localized in the thylakoid membrane and other membranes and quench ¹O₂ to protect lipid from peroxidation and to suppress radical chain reactions [23, 24].

Ascorbate is another important component of the plant redox system. In the Foyer-Halliwell-Asada cycle, ascorbate serves as reductant of H₂O₂ by ascorbate peroxidase (APX) [25, 26]. H₂O₂ originates from spontaneous breakdown or superoxide dismutase (SOD)-catalyzed conversion of [•]O₂⁻ [27]. The Foyer-Halliwell-Asada cycle functions in many subcellular compartments and has a particularly important role in chloroplasts. Electrons are extracted from water in photosystem II in the PET and are conditionally transferred to O₂ as alternative acceptor to produce [•]O₂⁻. This reaction allows for maintenance of electron flow through PET if other terminal acceptors are in short [28]. In addition to the thylakoid-bound ascorbate peroxidase (tAPX) and the soluble stromal sAPX in the chloroplast, APXs are present in the cytosol, the mitochondria and peroxisomes [29]. APX generates H₂O and monodehydroascorbate (MDHA), which is regenerated via dehydroascorbate (DHA), GSH and finally NADPH [30].

In parallel to the ascorbate-dependent water–water cycle, the ascorbate-independent thiol-based system detoxifies peroxides in many compartments. There are two main groups of high affinity thiol peroxidases, peroxiredoxins (PRX), and glutathione peroxidases (GPX) [31]. GPXs have a substrate preference for

lipid peroxides and the PRX group is able to react with a large spectrum of peroxides [32]. PRXs are classified according to their respective number of cysteines, binding interface and dimerization preference into groups of type A–D [33]. PRXs are characterized by a typical thioredoxin-like fold consisting of seven beta-sheets and five alpha-helices [34]. They reduce H_2O_2 to H_2O , alkylhydroperoxide to the corresponding alcohol and peroxynitrite to nitrite. Importantly, PRXs adopt different conformational states and physiological functions in dependence on their redox state such as peroxidase, chaperon, proximity-based oxidase, binding partner, and redox sensor in signaling [33].

Detoxification of generated ROS counteracts ROS accumulation, but cannot prevent eventual damage since the ROS molecules will diffuse from the site of generation through the cell until they are detoxified. Thus, the more efficient mechanism to decrease oxidative stress level is the suppression of ROS generation. In RET electrons are finally transferred to O_2 by complex IV, the cytochrome c oxidase (COX). Uncoupling protein and alternative oxidases (AOX) enable dissipation of excess reducing power and counteract ROS production in RET [35, 36]. In chloroplasts, the plastid terminal oxidase (PTOX) is part of chlororespiration and dissipates excess reducing power by transfer to O_2 [37, 38]. Thus, PTOX activation counteracts overreduction of the plastoquinone pool which otherwise could elicit oxidative stress [39, 40]. It should be noted that PTOX can produce $\cdot\text{O}_2^-$ as side product and thus might contribute to oxidative stress under certain conditions [41].

2 Regulation of Stress Acclimation

On the one hand, the discussed mechanisms of activating redox safety valves, strengthening ROS detoxification by antioxidant systems and maintenance of a strong redox buffer stabilize plants against redox- and ROS-incidences in a fluctuating environment. However, on the other hand, the homeostasis mechanisms must be leaky in order to enable redox-triggered acclimation responses. In addition to stress-specific signaling and responses, redox sensing and ROS-dependent regulation function as overarching principle in stress acclimation. The concept of redox regulatory networks in all cells consisting of redox input elements, redox transmitters, redox targets, redox sensors, and final electron acceptors has been advanced and provides a functional framework [42–44]. This network depends on ROS for reoxidation of redox-regulated targets. The balance between metabolic electron pressure into the network and drainage of electrons by final electron acceptors defines the state of the network. From this consideration it becomes clear that our understanding of the cellular

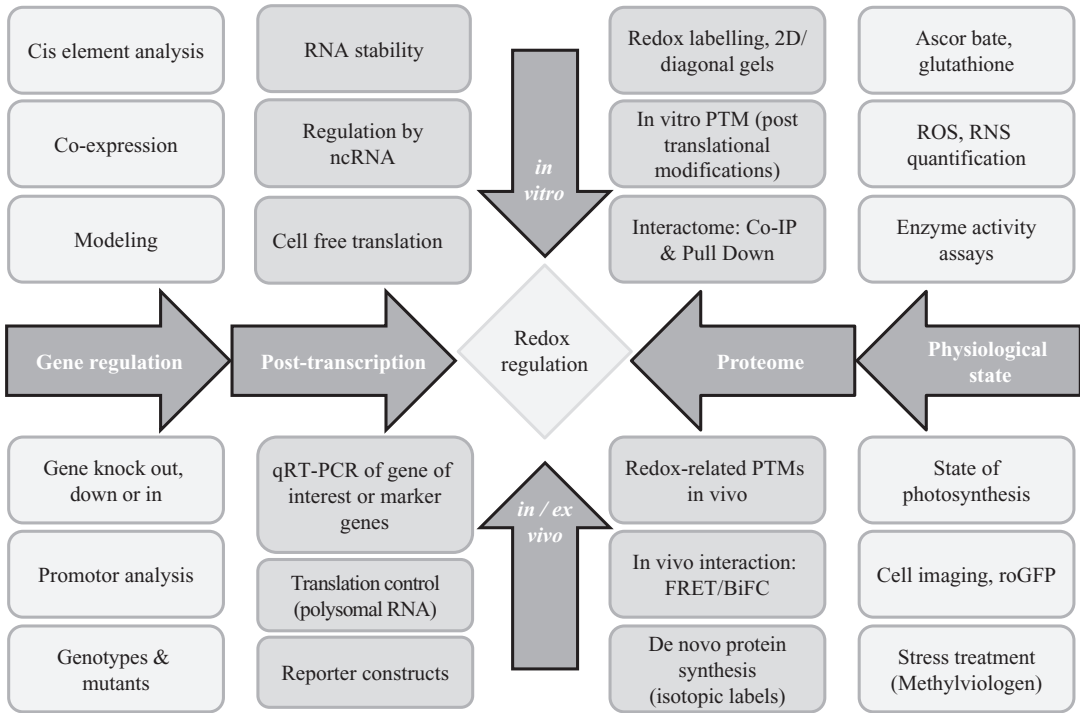


Fig. 1 Redox-focused overview of methods in redox and ROS research. The methods allow for various qualitative and quantitative assessments in the areas of transcriptome, proteome, and biochemical analyses

redox- and ROS-state must include sensitive analysis of the redox proteome and the involved regulatory mechanisms (*see* Fig. 1 for strategies).

Spatial and temporal specificity in ROS signatures comes from confined ROS generation, biological half-life time, substrate specificity and regulation of scavenging systems and compartmentation of sensors and targets [31, 45]. ROS and redox signaling pathways mutually interact with pathways controlled by Ca^{2+} -, reactive nitrogen species and phytohormones [46]. In the following paragraphs, three stressors, namely heat, high light, and salt, are exemplarily scrutinized to show the central role of ROS in plant stress responses.

2.1 ROS Response in Heat Stress

High temperatures have a huge impact on yield and productivity of plants [47, 48]. Rates of protein denaturation increase at higher temperatures. Therefore, mechanisms of protein stabilization by various types of chaperones and enhanced repair must counter protein inactivation and destabilization to maintain protein homeostasis and increase thermotolerance [49]. Responses to elevated temperatures include activation of ROS scavengers, heat shock transcription factors and proteins as well as increased osmolyte biosynthesis [50, 51]. Frank et al. [52] described significant correlations between ROS response and hormone signaling, sugar

accumulation and heat shock protein (HSP) translation. Transcript levels for stress markers like APX genes, RET and PET pathways, and heat shock factors are upregulated in maturing tomato microspores stressed for 2 h at 44 °C. The comparison between heat-tolerant and heat-sensitive cultivars indicates that oxidative stress functions as significant input signal for acquiring thermotolerance. This is especially reinforced by increased mRNA levels of ROS scavengers (tAPX, APX1, APX2, APX4-like, APX6-like, SIAPX3) in sensitive plants and basal levels of these markers before and after stress induction in tolerant plants. Tobacco bright-yellow 2 cells initiate the cell death program (PCD) after being subjected to a 55 °C heat-shock treatment. The PCD induction was associated with a twofold increased H_2O_2 accumulation and a tenfold increase of $\cdot\text{O}_2^-$ relative to the control. $V_{\max}$ of cAPX activity decreases up to fivefold during ROS-induced cell death. Pretreatment with the antioxidant ascorbate or SOD prior to heat stress stabilizes cAPX activity and protein amount to a level of unstressed control. In addition the reduction state of ascorbate decreased from 80% to 50% after 24 h together with a decrease in protein amount and mRNA of cAPX by 80% and 90%, respectively. Impaired kinetic properties and lower APX quantity are correlated, so it was suggested that protein modifications and inhibitory effects associated with elevated temperatures feed into this redox-regulatory network resulting in low $V_{\max}$ [53]. ROS scavenging systems like the ascorbate-dependent and the thiol peroxidase-dependent water–water cycles maintain redox and ROS homeostasis also in stress situations like heat [31, 54, 55].

Protein stabilization, renaturation, and degradation are crucial processes in acclimation to elevated temperature. Heat shock protein (HSP) amounts increase 10- to 200-fold during heat stress. HSPs assist in protein folding and prevent aggregation [56, 57]. HSP translation in Mammalia and *Drosophila* was reported to be stimulated in response to stress-induced ROS accumulation. The translational stimulation involves protein kinase pathways. HSPs constitute a functionally highly conserved and similarly regulated mechanism in all organisms [58, 59]. Heat shock factors (HSF) are transcription factors, which control expression of HSPs and other stress response genes in the heat stress response. HSFs bind to the heat shock-motif nAGAAAnnTTCTn which is contained not only in HSP promoters but also in many other stress-responsive genes with function in ROS signaling and detoxification, e.g., in cAPX1 [60–62]. The conserved mechanism in the heat stress response of humans and yeast involves H_2O_2 -dependent phosphorylation [63, 64]. This mechanism was also identified in plants where mitogen-activated protein kinases (MAPK) transduce the heat stress signal in tomato [65] and in *A. thaliana* [66]. Two H_2O_2 -sensitive MAPKs, namely MPK3 and MPK6 phosphorylate HSFA2 and HSFA4a and thereby connect the heat stress-induced signaling

with downstream activation of response genes [66]. A model for ROS-dependent oligomerization of AtHSFA8a and AtHSFA4a was suggested to trigger translocation to the nucleus and activation of target genes [67]. AtHSFA2 overexpressing Arabidopsis was subjected to short-term heat shock of 10 min. Upon heat shock HSFA2 could be detected in both the cytosol and nucleus. The *APX2* transcript accumulated 40-fold more in AtHSFA2 overexpressing plants than in wild type plants [68, 69]. Depending on the redox state of cysteine residues in HSFA8 in H₂O₂ treated protoplasts, a recent study indeed showed redox-dependent translocation to nucleus [70]. The Cys-Ser exchange in HSFA8 abolished the H₂O₂-induced translocation from the cytosol to the nucleus. This system should be tested in vivo.

Allakhverdiev et al. [71] reported that three processes in plant photosynthesis are especially vulnerable to elevated temperatures; (1) photosystem II (PSII) with its oxygen-evolving complex (OEC), (2) ATP-synthesis, and (3) carbon fixation in the Calvin-Benson cycle. Heat stress is associated with transient ROS accumulation. Accumulating H₂O₂ and ¹O₂ inhibit PSII repair by interfering with translation of *psbA* mRNA in cyanobacteria and plants [72–74]. High temperatures and high lipid fluidity negatively affect electron transport in photosynthesis. Especially mobility of thylakoid-bound plastoquinone as electron transmitter is a rate-limiting factor in photosynthesis under heat stress [75]. Unstacking of grana at high temperature favors the mobility of formerly densely packed PSII complexes to stroma-exposed sites and as a consequence improves accessibility to repair mechanisms for D1 subunits [76, 77].

2.2 ROS Response in High Light (HL) Stress

Absorption of excessive excitation energy by photosynthesis triggers acclimation responses on very fast to long-term time scales [78]. Enhanced ROS generation and accumulation occurs as an early response within minutes after increasing the light intensity. Singlet oxygen originates from excited chlorophylls. β -Carotene is regarded as highly important quenching component in photosystem II. Its oxidized derivatives β -cyclocitral and dihydroactinoidiolide appear to serve as retrograde signals from the chloroplast to the cytosol and nucleus [79, 80]. Transcript analyses on light-stressed *C. reinhardtii* indicate that these oxidized compounds activate signaling pathways that act on palindromic sequences in promoters called electrophile response elements for ¹O₂-related stress responses [81]. Reprogramming of gene expression in response to photosynthetic light stress involves kinase activity which links ROS-sensing and downstream activation of target genes. Light stressed ¹O₂-overproducing *flu* mutants overexpress *Oxidative Signal Inducible 1* (OXI1) which encodes an AGC kinase previously connected to cell death. Null mutants of *oxi1*

revealed less photo-induced oxidative damage and delayed cell death in high light [82]. Downstream activation of MAPKs through oxidative burst signaling was already reported as OXII function. Thus, there exists a tight coupling between ROS- and phosphorylation-dependent regulatory pathways and light stress-dependent gene expression [83].

The *flu* mutant is unable to suppress protochlorophyllide biosynthesis in darkness [84]. The accumulation of photosensitizers during darkness triggers $^1\text{O}_2$ release upon illumination and has allowed for studying the involved signaling pathways. Transcript profiling of *flu* plants showed upregulation of phytohormone-linked mRNAs previously reported for abscisic acid, ethylene, and jasmonate signaling, and also resembled patterns known from programmed cell death (PCD). The deduced mechanism assigns the decisive role to oxylipins, which control development of cell death or successful acclimation to $^1\text{O}_2$ stress [9].

A large scale microarray analysis in *flu* and the ROS-scavenging mutants *sod*, *cat2* and *apx1* exposed to various stresses including high light revealed overlapping and specific regulation of gene sets that illustrate the existence of complex redox signaling networks in plants. Cluster analysis of transcriptional responses pinpointed to nuclear-encoded signals characteristic for plastid $^1\text{O}_2$ production and provided evidence for patterns specific for the ROS type and subcellular localization. More than 500 annotated TFs in *A. thaliana* were differentially expressed showing the vast influence of changing ROS levels on plant cell transcription and redox regulation. Additionally these results identified unique transcriptional regulons based on specific bHLH, MYB, and NAM transcription factors found only in the high light stress sets [85]. A further transcript-targeted approach for ROS response focused on high light in catalase-deficient *A. thaliana* mutants with 65% or 20% residual catalase activity. This setup illustrated H_2O_2 -dependent defense responses. Transcriptional control of NAC, MYB, WRKY, and AP2-related TFs and phosphorylation of MAPKs increased after 3 h HL compared to wild type. In addition, H_2O_2 -dependent activation of photosynthetic proteins and ROS scavengers in plastids (e.g., peroxidases and PS I/II subunits) was delayed in catalase-deficient lines. The mutants were also interesting with regard to balanced resource investments into growth and defense. The energy-consuming translation machinery and protease activity were elevated in the catalase mutants indicative for increased protein turnover due to oxidation. Overall H_2O_2 is assumed to act together with master switches in the control of the light stress response [86, 87].

Chloroplast acclimation to high light is also linked to the redox state of the PQ pool [88–90]. The plastids have only a residual genome. Most genes for photosynthesis, respiration and also for

regulating redox imbalances like PQ redox state were transferred into nucleus genome. The communication between plastids and nucleus depends on retrograde signaling processes and includes information on ROS [91–95] in addition to metabolic, hormonal, and redox cues [78].

2.3 ROS Responses in Salt Stress

Salinity is a major threat for sustained crop production worldwide. Efficient salinity acclimation addresses osmolyte biosynthesis, ion homeostasis, transpiration control, and maintenance of ROS homeostasis [96, 97]. Stomatal closure decreases CO₂ availability and enhances processes leading to ROS accumulation such as photorespiration and Mehler reaction [98]. Guard cell contraction for gas control involves massive ion movement and often is mediated by ABA. The signaling also implicates MAPK and ROS synthesis [99, 100]. H₂O₂-treated *mpk9* and *mpk12* Arabidopsis were unable to close stomata like in WT controls [101]. The defect in H₂O₂ signaling was attributed to downstream elements such as nucleotide diphosphate kinase2 (AtNDK2), which is also needed for abiotic stress resistance and improved antioxidant capacity. AtNDK2 controls stress-dependent phosphorylation and signaling cascades by promoting AtMPK3/6 [102]. Rentel et al. [83] discovered additional components of signal transduction upstream of AtMPK3/6. As discussed above for light stress acclimation, OXI1 kinase activated AtMPK3/6 is suggested to act as a major player in oxidative burst mediated signaling. During ionic disturbances MPK6 acts as a regulatory hub in the control of Na⁺/K⁺ antiporters, supports membrane integrity and initiates RBOH-dependent signaling [103, 104]. MPK3 also promotes the expression of lipid transfer proteins and of the hybrid proline-rich protein (HyPRP) AZI1. These proteins improve seed germination and plant growth under salinity [105]. HyPRPs were reported to participate in cell wall cross-linking, thereby probably improving cell stability and membrane permeability against high salinity [106, 107].

Oxidative burst describes the sudden increase of ROS in the apoplastic space, which occurs as part of defense and signaling mechanisms. NADPH oxidases of the RBOH type transfer electrons from cytosolic NADPH to O₂ on the apoplastic site [108, 109]. Proline biosynthesis is enhanced by RBOH-dependent signaling and establishes safe osmotic relations in salt-stressed cultured tobacco cells [110, 111]. Salt stress activates expression of the RBOH isoforms C/D/F in Arabidopsis. This process depends on INT1 (increased tolerance to NaCl) since it is significantly decreased in *int1* mutants [112]. Increased H₂O₂ concentrations in the apoplastic space and endomembrane vesicles also foster ABA-dependent stomatal response to salt stress [113]. ROS originating from AtRBOHC stabilizes *SOS1* mRNA under NaCl stress.

Indeed *SOS1* strongly responds to salt stress but not to methyl viologen-induced oxidative stress. RBOHC links Na^+ homeostasis, ROS, and Ca^{2+} signaling [114]. The RBOH protein structure is characterized by the presence of EF-hand motifs for Ca^{2+} binding and constitutes a phosphorylation target under the control of MAPKs, ROS, and Ca^{2+} [115].

3 Conclusion

Maintenance of redox homeostasis is essential for sustained growth in a changing environment. Deviations from redox equilibrium by increased ROS generation shift the system to increased defense at the expense of growth. ROS signatures are linked to other signaling cues such as Ca^{2+} , protein kinases and phosphatases, hormones, metabolites, and nitric oxide. The question as to how a limited number of ROS namely $^1\text{O}_2$, $^{\bullet}\text{O}_2^-$, H_2O_2 , and $^{\bullet}\text{OH}$ can be involved in virtually all major stresses and how specificity of responses may be achieved is still unanswered. ROS signatures bear spatial and kinetic information depending on the type of abiotic stress [85]. Stress-dependent specificity of ROS signals might be encrypted in this type of poorly understood signature in time and space in combination with other signaling cues including activation of stress-specific sensors [45].

ROS action directly relates to redox homeostasis, cellular redox sensing, and the redox signaling network. Cysteine thiols play a unique role in this network. The thiol redox regulatory network consists of redox-switchable polypeptides and is composed of redox input elements, redox transmitters, redox targets, redox sensors, and ROS as final electron acceptors [42]. In addition to disulfide formation in and between polypeptides, Cys is subjected to S-glutathionylation, S-nitrosylation, sulfenylation, sulfinylation, sulfonylation, S-acetylation, and other modifications. Stress-induced disturbances are immediately recognized and responses elicited within seconds, e.g., upon high light exposure [116, 117]. Metabolic adjustment to new conditions is achieved by modulating the phosphoproteome, transcription factors, translation factors, and protein turnover. Often plants are not exposed to single stressors but face combinatorial stresses such as drought, heat, and high light. Understanding and dissecting the ROS pathways under such conditions pose a challenge to plant researchers. For this reason it is necessary to plan the projects along the proposed hypothesis, consider the available financial resources, and apply the most suitable methods. To this end, a selected overview of ROS-centered techniques is provided in Table 1.

Table 1
Exemplary overview of redox-targeted methods

Method	Advantage	Disadvantage	Scientific impact
ROS staining in tissues	Easy, visualization of ROS	Qualitative assessment of uncertain significance	*
Membrane integrity (lipid peroxidation, ion exchange)	Easy, ROS-related cell damage	Low sensitivity, late parameters of stress	*
Gel-based immune labeling by Western blotting	Easy assessment of PTMs	Requirement for specific antiserum, difficulty of blocking and labeling	**
Photosynthetic parameters	Established devices and theory		***
Metabolites (GSH, ASC, ...) and ROS quantification	Access to cellular redox features	Enzyme-coupled or cycling assays, difficulty of quenching	***
Thiol quantification	Quantitative analysis for protein thiols and free thiols	Low specificity	**
Microarray/RNA-Seq	Genome-wide RNA analysis, high sensitivity	Often expensive outsourcing, need for bioinformatics, transcript level	****
Transcript analysis, qPCR	Quantitative analysis RNA regulation	Specific targets, primer designs and adjustment	****
[³⁵ S] methionine or isotope labelling	Protein translation in vivo, time resolution	Institutional requirements	****
Cell imaging, fluorescent protein fusion	roGFP for redox balance, FRET and BiFC for interactions	Evaluation and controls require high experience, roGFP sensitivity range	****
Redox proteomics (2D redox gels, peptide identification)	Redox interaction on protein level, isolation and characterization of targets	Need for sophisticated proteomics facilities	*****
Enzyme activity assays	Quantitative kinetic parameters	In vitro, complex redox assays	***
Isothermal titration calorimetry (ITC)	Kinetic parameters (K, n, H, G, S) interactions and dynamics	High purity and amounts of proteins	****
Mass spectrometry	Detection of PTMs	Intensive purification and database requirements; high expert level	*****
Chromatographic analysis of proteins	Visualization of dynamics, modifications isolation and purification of targets	Protein-dependent adjustments, protein instability	****

The summary provides additional remarks from the authors' perspective as to "Advantages" and "Disadvantages" as well as a rating in context of "Scientific Impact." Values are given between (*) for least and (*****) for highest scientific potential

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