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REVIEW PAPER

Superoxide signalling and antioxidant processing in the plant nucleus

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Abstract

The superoxide anion radical ($O_2^{\cdot -}$) is a one-electron reduction product of molecular oxygen. Compared with other forms of reactive oxygen species (ROS), superoxide has limited reactivity. Nevertheless, superoxide reacts with nitric oxide, ascorbate, and the iron moieties of [Fe–S] cluster-containing proteins. Superoxide has largely been neglected as a signalling molecule in the plant literature in favour of the most stable ROS form, hydrogen peroxide. However, superoxide can accumulate in plant cells, particularly in meristems, where superoxide dismutase activity and ascorbate accumulation are limited (or absent), or when superoxide is generated within the lipid environment of membranes. Moreover, oxidation of the nucleus in response to environmental stresses is a widespread phenomenon. Superoxide is generated in many intracellular compartments including mitochondria, chloroplasts, and on the apoplastic/cell wall face of the plasma membrane. However, nuclear superoxide production and functions remain poorly documented in plants. Accumulating evidence suggests that the nuclear pools of antioxidants such as glutathione are discrete and separate from the cytosolic pools, allowing compartment-specific signalling in the nucleus. We consider the potential mechanisms of superoxide generation and targets in the nucleus, together with the importance of antioxidant processing in regulating superoxide signalling.

Keywords: Ascorbate, chloroplasts, glutathione, meristems, mitochondrial Complex 1, nitric oxide, RBOH-type NADPH oxidases, superoxide dismutases.

Introduction

Superoxide ($O_2^{\cdot -}$), which is an anion radical, is produced by the one-electron reduction of molecular oxygen. In aqueous media, protonation of superoxide can result in the formation of the uncharged hydroperoxyl radical (HOO·; pK_a of 4.8). Hence, the anion radical form is by far the predominant species at physiological pH ranges. A second reduction of superoxide would require the energetically unfavourable compression of two full negative charges, and hence superoxide is generally considered to be a better reducing agent than an oxidant. Superoxide does not readily cross lipid membranes and has a long lifetime within lipid environments. The same may also be true of certain membrane-less organelles (Fuentes-Lemus and Davies, 2023).

Superoxide is relatively unreactive towards most cellular molecules. However, the superoxide-dependent reduction of free Fe^{3+} releases Fe^{2+} from ferritin, and superoxide interacts

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with positively charged iron porphyrins and enzymes that contain iron–sulfur clusters. Such interactions can lead to the inactivation of enzymes that are important in energy production and amino acid metabolism, such as the tricarboxylic acid cycle enzyme aconitase (Scandroglio *et al.*, 2014). The superoxide-dependent inactivation of aconitase leads to enhanced glycolysis relative to oxidative phosphorylation in cellular energy generation. Moreover, nuclear [Fe–S] cluster-containing proteins are direct targets of superoxide. For example, superoxide-dependent oxidation of the [Fe–S] cluster in Repressor of Silencing 1 (ROS1) regulates meristem function and fate (Wang *et al.*, 2023, Preprint).

Reactive nitrogen species (RNS), such as nitric oxide (·NO), nitrogen dioxide (·NO_2), *S*-nitrosothiols (SNOs), and peroxynitrite ($ONOO^-$), often function together with reactive oxygen species (ROS) in plant responses to biotic and abiotic stresses. For example, NO and ROS play essential signalling roles in the orchestration of systemic acquired resistance (SAR) to pathogens (Wang *et al.*, 2014). The NO synthase (NOS) enzymes that are the primary producers of NO in animals (Zhang *et al.*, 2023) are absent from plants. Although NOS-like activity has been reported in plants (Astier *et al.*, 2018), nitrate reductase appears to be the main source of NO production, for example in plant immunity responses (Vitor *et al.*, 2013). Thus, although NO is able to regulate transcription factors and chromatin-modifying histone deacetylase functions, there is no known mechanism of NO production in the nucleus. NO-dependent regulation of epigenetic mechanisms and nuclear gene expression must therefore be achieved by NO import into the nucleus (Wurm and Lindermayr, 2021). When NO and superoxide are produced simultaneously, they react together at a diffusion-controlled rate to form peroxynitrite. Peroxynitrite is a primary agent responsible for post-translational protein modifications, particularly the nitrosylation of active site tyrosine residues in enzymes, as well as the nitrosylation of glutathione (GSH) and other cellular thiols. Peroxynitrite can cross cell membranes and thus enter the nucleus (Islam *et al.*, 2015). However, this highly reactive and powerful oxidant is unstable, decomposing to nitrate and dioxygen. We thus focus the following discussion on superoxide as a primary oxidant, whose abundance in different plant cell compartments is controlled by the expression of superoxide dismutase (SOD) and the abundance of low molecular weight antioxidants. In particular, we highlight recent evidence suggesting that $O_2{\cdot}^-$ functions as a physiological, local signalling molecule in the nucleus.

Superoxide as a signalling molecule

Superoxide, $O_2{\cdot}^-$, is highly soluble in water, where it is strongly solvated by four tightly hydrogen-bonded water molecules. The protonated form of superoxide, hydroperoxyl, HO_2, is a weak acid (pK_a of 4.8). Hence, the predominant species present in aqueous solutions at pH 7 is the small $O_2{\cdot}^-$ anion together with its strongly associated four water molecules. The $O_2{\cdot}^-$ anion is the substrate for the SOD enzymes.

Both $O_2{\cdot}^-$ and HO_2 are kinetically competent one-electron reductants. However, in most reactions only HO_2 (and not $O_2{\cdot}^-$) is the kinetically competent one-electron oxidant because of the need for either a proton or a coordinated metal ion to stabilize the peroxide dianion, O_2^{2-}, as it is formed (Sheng *et al.*, 2014). The disproportionation reaction is fastest at pH 4.8. In this situation, the concentrations of HO_2 and $O_2{\cdot}^-$ are equal, the former acting as an oxidant and the latter as a reductant. At high pH values, where the predominant species is $O_2{\cdot}^-$, where two superoxide anions repel each other and the unshielded O_2^{2-} is unstable, the disproportionation reaction cannot proceed and so $O_2{\cdot}^-$ is quite stable. While superoxide has the thermodynamic capacity to be a strong oxidant, it is generally not reactive with most cellular components. However, labile iron–sulfur clusters in the reduced state are rapidly and irreversibly oxidized by reaction with superoxide. Although it is likely that other superoxide targets remain to be discovered, labile iron–sulfur-containing species are considered to be major superoxide targets. Thus, superoxide is a selective oxidant, relatively unreactive with most components of cells, but highly reactive with some essential cellular components, particularly labile iron–sulfur-containing species. The lifetime of $O_2{\cdot}^-$ is considerably greater in aprotic media compared with aqueous solutions, and it thus has a greater stability when generated in lipid membranes. Moreover, although superoxide has a low membrane permeability, it can pass through anion channels (Andrés *et al.*, 2023).

In contrast to the rate of spontaneous non-enzymatic dismutation, which is relatively low at physiological pH values (2×10^5 M^{-1} s^{-1}), the SOD-catalysed dismutation reaction occurs at the almost diffusion-limited rate (~2×10^9 M^{-1} s^{-1}) (Sheng *et al.*, 2014). The lifetime of superoxide in biological systems is thus determined by the rate of chemical dismutation and the presence of SOD and low molecular weight antioxidants, particularly ascorbate. The rate constant for the reaction between ascorbic acid and superoxide (at pH 7.4) is estimated as 5.4×10 M^{-1} s^{-1} using the xanthine–xanthine oxidase system (Som *et al.*, 1983). In contrast, the rate constant of the bovine erythrocyte Cu,Zn-SOD was calculated to be 1000 times higher (4×10^9 M^{-1} s^{-1}; Gray and Carmichael, 1992). Hence, the lifetime of superoxide as a signalling molecule can be considered to depend on the presence of SODs and ascorbate, which essentially police this molecule.

Superoxide production and processing in plant cells

Oxygen delivery is essential for plant cell functions largely because of its role in aerobic respiration and ATP production through oxidative phosphorylation and the tricarboxylic acid

(TCA) cycle in the mitochondria. As a non-electrolyte, molecular oxygen can cross the lipid bilayer of membranes by passive diffusion, permeating directly through the bilayer. Oxygen diffusion in cells and across membranes is of vital importance, as it allows the mitochondria and organelles, including the nucleus, to receive the oxygen that they need to survive and function.

The electron transport carriers of the mitochondrial electron transport chain, particularly the NADH dehydrogenase complex (Complex 1), are a major source of superoxide in plant cells, as are also the chloroplast electron transport chain and the RBOH (respiratory burst oxidase homolog)-type NADPH oxidases (NOXs) of the plasma membrane (Moller, 2001; Dietz *et al.*, 2016; Foyer and Hanke, 2022; Lee *et al*, 2023; Miller and Mittler, 2023). Superoxide is also produced by other reactions, for example in ureide and nucleic acid catabolism by the enzymes xanthine oxidoreductase (XOR) and urate oxidase, and in sulfite oxidation by sulfite oxidase (Sandalio *et al.*, 2021).

Superoxide is generated by the photosynthetic electron transport chain, largely at PSI by the processes termed the 'Mehler reaction' or pseudocyclic electron flow (Foyer and Hanke, 2022). The extent of superoxide accumulation in chloroplasts is variable. For example, Arabidopsis and tobacco leaves have double the amount of superoxide under short-day growth conditions compared with long-day conditions (Michelet and Krieger-Liszkay, 2012). The reasons why superoxide accumulation is allowed to vary in such circumstances are unknown. Superoxide can also be produced in peroxisomes through the activities of enzymes such as peroxisomal XOR, which generates uric acid with concomitant superoxide generation, as part of purine base degradation in the nucleotide degradation pathway. In contrast, superoxide generation in the plant nucleus, particularly in response to environmental stress, has not been explored in the literature.

Superoxide levels are kept low in plant cells by the effective compartmentalization of oxygen reduction reactions and by the expression of SODs that have high affinities for superoxide. The primary function of SODs is to protect anaerobic organisms from ROS accumulation by scavenging $O_2^{\cdot -}$. Nevertheless, SODs generate another, less reactive ROS form, hydrogen peroxide (H_2O_2). The role of SODs is therefore to limit $O_2^{\cdot -}$ accumulation and diffusion distances. The SOD enzymes in plants are classified according to the catalytic metal ions they contain, namely MnSODs, FeSODs, NiSODs, and Cu,ZnSODs. Each type of SOD is localized in different intracellular compartments or extracellularly in the apoplastic space. The presence of SOD facilitates the tightly regulated and spatially oriented redox signalling through both $O_2^{\cdot -}$ and H_2O_2.

Current concepts consider that H_2O_2 is the predominant form of ROS used in redox signalling. However, increasing evidence suggests that superoxide also fulfils important signalling roles in plants and animals. For example, superoxide acts as a signal in young mutant animals to trigger changes of gene expression that prevent or attenuate the effects of subsequent ageing (Yang and Hekimi, 2010). Moreover, superoxide accumulation is important in the determination of stem cell and meristematic cell fate in plants and animals (Kim *et al.*, 2009; Soldner *et al.*, 2009; Tsukagoshi *et al.*, 2010; Zeng *et al.*, 2017). In plants, superoxide plays also an important role in xylem cell wall expansion, remodelling, lignification, and the induction of programmed cell death (PCD; Marzec-Schmidt *et al.*, 2020). Reduction of superoxide yields the non-radical ROS form, H_2O_2. In the presence of free transition metals, superoxide and H_2O_2 can give rise to the highly reactive hydroxyl radicals (Richards *et al.*, 2015), which are required for processes such as cell wall loosening and growth.

Apoplastic superoxide accumulation catalysed by enzymes such as amine oxidases plays an important role in cell wall loosening, allowing cell elongation during seed germination (Bailly, 2019). Superoxide generation accumulation by the plasma membrane-bound RBOH enzymes is triggered upon perception of chemical and/or physical cues (Yeung *et al.*, 2018). Such responses are relatively well characterized in relation to the plant immune system and the orchestration of local and systemic defence processes. Plants employ cell surface-resident pattern recognition receptors (PRRs) and intracellular nucleotide-binding domain leucine-rich repeat (NLR) receptors to detect the presence of pathogen-derived pathogen-associated molecular patterns (PAMPs) and effectors, activating PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI), respectively (Jones and Dangl, 2006). PTI and ETI can be triggered by a diverse range of PAMPs and effectors, respectively, each with a specific receptor (Barragan and Weigel, 2021). In addition, the release of host-derived damage-associated molecular patterns (DAMPs) that are sensed by various PRRs induces DAMP-triggered immunity (DTI; Tanaka and Heil, 2021). Some virulent pathogens use effectors or virulence factors to suppress PTI-associated ROS production. Microorganisms secrete effector proteins that can trigger a multitude of host defences depending on their target destination. Some effectors can pass through the nuclear envelope to reach targets in the nucleus, where they modify target gene expression either by subverting epigenetic modifications or by direct interference with the transcriptional machinery (Harris *et al.*, 2023). Several nuclear effectors modify phytohormone defence signalling and suppress immunity-associated host responses (Harris *et al.*, 2023). For example, the CRN63 and CRN115 effector proteins produced by *Phytophthora sojae* target a cytoplasm-localized catalase and re-localize with it to the host nucleus, thereby suppressing host cell death responses (Zhang *et al.*, 2015). Similarly, the HaRxL106 effector proteins from *Hyaloperonospora arabidopsidis* suppress host immunity by binding to the RADICAL-INDUCED CELL DEATH1 (RCD1) transcription factor and suppress the salicylic acid (SA)-induced transcriptional activation of defence genes (Wirthmueller *et al.*, 2018). Such studies illustrate the concept that ROS production and processing are key targets for manipulation by microorganisms. It remains to be demonstrated

whether symbiotic and pathogenic microorganisms are able to directly target superoxide signalling in the nucleus.

Of the Arabidopsis hypoxia-inducible *RBOH* genes in Arabidopsis, *RBOHD* and *RBOHF* are considered to be the most important in many stress responses (Chen *et al.*, 2015; Liu *et al.*, 2017; Yeung *et al.*, 2018). Moreover, anoxia-driven ethylene accumulation drives Ca^{2+} and calcium-dependent protein kinase (CDPK)5/13-mediated phosphorylation and activation of RBOH in the roots. The accumulation of superoxide but not hydrogen peroxide in this situation was found to be important in providing spatial cues for development of aerenchyma (Dunand *et al.*, 2007; Wany and Gupta, 2018).

Oxygen is a diffusible signal that controls many processes in plant and animal development, particularly the activity of stem cells. Mammalian stem cells reside in a hypoxic microenvironment that is considered to limit oxidative stress (Li *et al.*, 2021). However, ROS participate in activation of stem cells, stimulating them to enter the cell cycle upon exit from quiescence (Lyublinskaya *et al.*, 2015). A similar situation occurs in plant cells, such as those in the root apical meristem (RAM; de Simone *et al.*, 2017). The RBOH proteins play a pivotal role in this regulation, but the pathways involved remain poorly understood (Chapman *et al.*, 2019). Knocking out RBOH and mitochondrial Complex 1 subunits caused very similar phenotypes in stem cell maintenance (Zeng *et al.*, 2017), suggesting that both RBOH and NADH-dehydrogenase-derived superoxide molecules are important in the control of stem cell fate maintenance.

Oxidant and antioxidant functions in the plant cell nucleus

The nucleus is an organelle with a range of metabolic pathways and processes including DNA replication, transcription, and gene regulation. Accumulating evidence suggests that these metabolic pathways include enzymes that generate superoxide. Nuclear superoxide-producing enzymes are well characterized in human and animal cells. Of the enzyme families that use molecular oxygen, the 2-oxoglutarate-dependent dioxygenases are particularly important as they are involved in histone and DNA demethylation and hydroxylation reactions (Huang *et al.*, 2023). Interestingly, ascorbate is a specific cofactor for the Fe(II)- and 2-oxoglutarate-dependent dioxygenases that catalyse the addition of a hydroxyl group to various substrates (Wei *et al.*, 2021). Ascorbate is also a cofactor for the ten–eleven translocation (TET1−3) family of Fe(II)-dependent dioxygenases in mammalian cells, which are responsible for the removal of cytosine methylation in DNA (Zhithovich, 2020). The TET enzymes are Fe(II)-dependent dioxygenases that catalyse a series of consecutive oxidations of 5-methylcytosine. No TET-like enzymes have as yet been identified in plants, although 5-methylcytosine oxidation products, particularly 5-hydroxymethylcytosine (5hmC), have been reported (Mahmood and Dunwell, 2019). In addition, NOX4 generates superoxide specifically in the nucleus of specific human cell types including human vascular endothelial cells (Kuroda *et al.*, 2005). It is possible that NOX isoforms are also trafficked to the plant nucleus, not least because an increasing number of proteins display the ability to re-localize to the cell nucleus, for example by vesicle transport. Some transcription factors such as ERF74 translocate from the plasma membrane to the nucleus in response to hypoxia to regulate the transcription of *RBOHD* (Kosmacz *et al.*, 2015). All Arabidopsis RBOH proteins are generally targeted to the plasma membrane; RBOHD also had a potential nuclear localization signal (Table 1).

The animal and human cell SOD1 can rapidly re-localize to the nucleus in response to oxidative stress, where it acts as a nuclear transcription factor (Tsang *et al.*, 2014). SOD1 is localized in the nucleus under both normal and pathological conditions, where it functions as a regulatory protein in cell signalling, transcription, and ribosome biogenesis, contributing to oxidative stress responses and the control of growth (Xu *et al.*, 2022).

Table 1. Predicted nuclear localization of RBOH proteins in *Arabidopsis thaliana*

NADPH oxidase	Locus ID	TAIR database (GO cellular component)	LOCALIZER nuclei localization prediction	NLSP nuclear localization signal prediction	Probability (0–1)
RBOHA	At5g07390	PL, MT, NU	Yes	120–136	0.8
RBOHB	At1g09090	PL	–	–	–
RBOHC	At5g51060	PL, NU	Yes	51–60	0.966
RBOHD	At5g47910	PL, GL, NU	Yes	42–50	0.737
RBOHG	At4g25090	PL, VC	–	–	–
RBOHE	At1g19230	PL, NU	Yes	–	–
RBOHF	At1g64060	PL	–	–	–
RBOHI	At4g11230	PL	Yes	–	–
RBOHH	At5g60010	PL, NU	–	–	–
RBOHJ	At3g45810	PL, NU	Yes	–	–

Predictions were performed using LOCALIZER 1.0.4 and NLSP (Nuclear Localization Signal Prediction) tool. The LOCALIZER program is based on the collection of plant NLSP signals (https://localizer.csiro.au) and employs a Hidden Markov model.

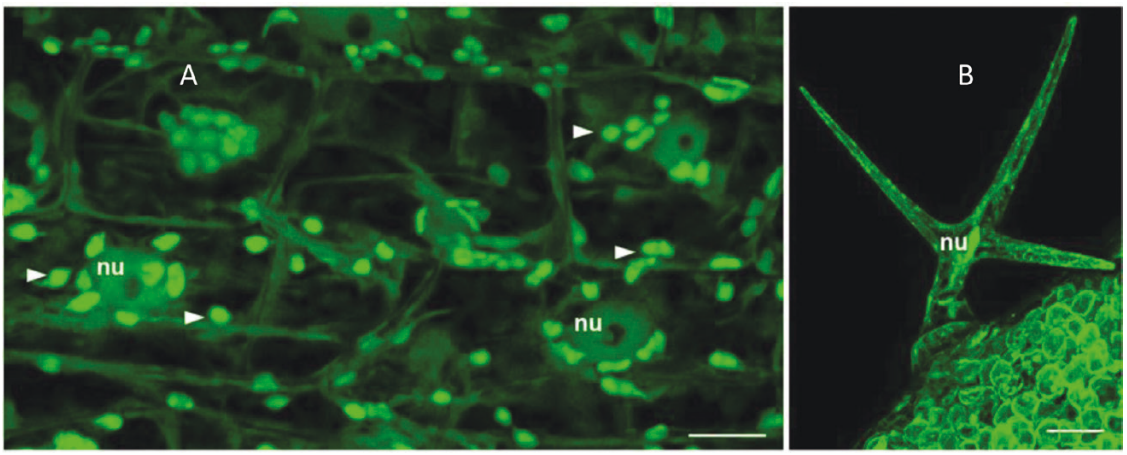


Fig. 1. Localization of iron superoxide dismutase 1 (FSD1) in the nucleus. Localization of FSD1–green fluorescent protein (GFP) fusions in the chloroplasts and the nucleus (arrows) of mesophyll tissue (A) and trichomes (B) see Dvořák *et al.* (2021). Nu, nucleus; chloroplasts are indicated by arrows.

The iron SOD (FSD1) is localized in the chloroplast stroma, the cytosol, and in nuclei (Fig. 1; Dvořák *et al.*, 2021; Melicher *et al.*, 2022). FSD1 was re-localized to the plasma membrane in response to salt stress (Dvořák *et al.*, 2021). A number of antioxidant enzymes are either localized in the nucleus, or can re-localize there in response to appropriate triggers (Foyer *et al.*, 2020).

The majority of the macromolecular traffic, including proteins, in and out of the nucleus, occurs through the nuclear pores. This transport is mediated by the Karyopherin-β (or Kap) family of nuclear transport receptors (Wing *et al.*, 2022). Nucleocytoplasmic traffic is mediated by 20 different Kaps in mammalian systems, that function either across the nuclear pore complex into the nucleus (importins), out of the nucleus (exportins), or in both directions (biportins). The presence of SOD in this compartment strongly suggests that superoxide is produced within the plant cell nucleus. Moreover, the presence of SODs in the nuclei of animal and plant cells demonstrates that H_2O_2 is generated directly in the nuclei.

The generation of both superoxide and hydrogen peroxide in the nucleus facilitates compartment-specific control of redox regulation and signalling. The plant cell nucleus is rich in glutaredoxins (GRXs), thioredoxins (TRXs), thiol reductases, as well as other redox-regulated proteins that exert redox control over nuclear processes and functions, such as gene expression, chromatin remodelling, and epigenetics (He *et al.*, 2018; Martins *et al.*, 2018). It was recently suggested that the redox modulation of transcription factors by the co-regulatory CC-type GRXs that are called ROXY, and other CC-type plant-specific GRXs are important in the evolution of plant developmental controls (Zachgo, 2023). The ancestral role for CC-type GRXs in modulating the activities of TGACG-binding (TGA) transcription factors is well established in the literature. For example, ROXY1 and the TGA transcription factor called PERIANTHIA (PAN) govern root meristem activities, acting together in the control of root development (Maß *et al.* 2020). Moreover, ROXY1 co-localizes with the active form of RNA polymerase II in the nucleus (Gutsche *et al.*, 2017).

Ascorbate peroxidases (APXs) and catalase have been localized in plant cell nuclei, along with other antioxidant enzymes (Al-Hajaya *et al.*, 2022; Table 2). Moreover, high concentrations of ascorbate (16.3 mM) and GSH (6.4 mM) were reported in the nuclei of *Arabidopsis thaliana* leaves (Zechmann, 2011, 2014, 2020; Zechmann *et al.*, 2011, Liu *et al.*, 2019; Chen *et al.*, 2021).

The concept that there is free diffusion of small molecules between the nucleus and cytosol through the nuclear pores is embedded in the literature. However, accumulating evidence suggests that the GSH pools of the nucleus are discrete. For example, the nuclear and cytosolic GSH pools are regulated independently during cell proliferation (Emmert *et al.*, 2023).

GSH co-localizes with nuclear DNA during the early stages of cells proliferation in plants and animals (Diaz Vivancos *et al.*, 2010). The recruitment and sequestration of GSH in the nucleus during the G_1 and S phases of the cell cycle had a profound impact on gene expression. GSH was suggested to act as a 'redox sensor' at the onset of DNA synthesis, maintaining the nuclear architecture by providing the appropriate redox environment for DNA replication, and safeguarding DNA integrity, epigenetic controls, and protein degradation by nuclear proteasome (García-Giménez *et al.*, 2013). Live-cell GSH imaging revealed that the GSH concentration of the nucleus is highest during S phase, and steadily decreases until mitosis (Emmert *et al.*, 2023). Such findings demonstrate that the nuclear GSH pool is regulated independently of that elsewhere in the cell. Moreover, the existence of different GSH concentrations in the nucleus and the cytosol strongly suggests that GSH does not diffuse freely across the nuclear envelope or through nuclear pores (Emmert *et al.*, 2023). The same may be true for

Table 2. Enzymes that process reactive oxygen species (ROS) in the nucleus

Constructs	Method	Localization	Reference
35S:FDS1–GFP, 35S:GFP–FDS1	*Arabidopsis thaliana*—stable transgenic lines	Nucleus, chloroplast	Dvořák *et al.* (2021)
UBQ:CAT2–sfGFP, UBQ:sfGFP–CAT2	*Arabidopsis thaliana*, mesophyll protoplasts transfection	Nucleus, peroxisome	Al-Hajaya *et al.* (2022)
35S:APX1–GFP	Arabidopsis thaliana, mesophyll protoplasts transfection	Nucleus, plasma membrane	Liu *et al.* (2019)
35S:APX1–GFP	*Nicotiana benthamiana*, leaf infiltration	Nucleus, cytoplasm	Chen *et al.* (2021)
35S:GPX8–GFP	Onion epidermal cells bombardment	Nucleus, cytoplasm	Gaber *et al.* (2012)

Compartmentation of catalase 2 (CAT2), ascorbate peroxidase 1 (APX1), iron superoxide dismutase 1 (FSD1), and glutathione peroxidase 8 (GPX8) in the nucleus, detected using green fluorescence protein (GFP) fusions.

ascorbate, with the ascorbate pools in the nucleus and the cytosol regulated independently. Infection of Arabidopsis plants with *Pseudomonas syringae* increased the ascorbate contents of the nuclei followed by a strong decrease that was accompanied by accumulation of ROS (Großkinsky *et al.*, 2012). Similarly, a large decrease in the ascorbate (58%) content of the nucleus was observed within 12 h after the infection of Arabidopsis wild-type plants with *Botrytis cinerea* (Simon *et al.*, 2013). The nuclear GSH pool increased by 300%, together with ROS accumulation in the nucleus (Simon *et al.*, 2013). Exposure to abiotic stress conditions also causes a decrease in the nuclear ascorbate contents relative to the cytosol (Koffler *et al.*, 2014a, b, 2015). In addition, nuclear ascorbate levels were either increased or remained unchanged following exposure to abiotic stress situations in the ascorbate-deficient *vtc2-1* mutants, whereas the cytosolic ascorbate contents decreased in the wild type (Zechmann, 2018).

The presence of a redox cycle within the plant cell cycle, with controlled oxidation at the early stages, suggests that the redox state of the nuclei is precisely controlled to facilitate cell cycle progression (de Simone *et al.*, 2017). Such regulation would suggest that superoxide is generated within the plant cell nuclei in response to the factors that trigger cell cycle progression. Exposure to abiotic stresses such as heat shock causes rapid oxidation of the nucleus (Babbar *et al.*, 2021). Similarly, inhibitors that are commonly used to modulate chloroplast and mitochondrial electron transport pathways in order to study organelle to nucleus signalling all increase oxidation in the nuclei as well as the cytosol (Karpinska *et al.*, 2017). Moreover, the chloroplast inhibitor lincomycin and the mitochondrial inhibitor antimycin caused a greater oxidation in the nuclei of stomatal guard cells than the cytosol (Karpinska *et al.*, 2017). Stress-induced oxidation of nuclei is crucially important for the regulation of gene expression and as associated signal transduction pathways, as well as the control of nuclear thiol–disulfide redox states by nucleoredoxins, GRXs, and TRX1 (Kneeshaw *et al.*, 2017). For example, the pathogen-inducible oxidoreductase Nucleoredoxin 1 (NRX1) targets H_2O_2-scavenging enzymes, including catalases. NRX1 forms a mixed disulfide intermediate with catalase *in vivo*, protecting H_2O_2-scavenging activity (Kneeshaw *et al.*, 2017).

Superoxide and cell to cell signalling

The ROS wave concept of cell to cell signalling that plays a pivotal role in local and systemic responses that underpin acclimation to stress (Waszczak *et al.*, 2018; Fichman *et al.*, 2022; Mittler *et al.*, 2022) requires an 'activated ROS production' state that is driven by RBOHs. The wave is propagated from cell to cell over long distances in order to activate gene expression that enhances the overall resilience of the plant to stress (Mittler *et al.*, 2022). The activation of RBOHs such as RBOHD and RBOHF was required to produce superoxide that is the essential driver of this process (Fichman *et al.*, 2022, 2023). The hydrogen peroxide receptor HPCA1 is required for the propagation of cell to cell ROS and calcium signals that underpin systemic signalling in response to different biotic and abiotic stresses (Fichman *et al.*, 2022). Thus, apoplastic H_2O_2 sensing and signalling are intrinsic to the systemic propagation of cell to cell ROS and calcium signals. However, such findings do not rule out a possible role for superoxide signalling and sensing in this process. Such pathways appear to have evolved as part of the quorum-sensing network in unicellular organisms. Evidence for the operation of the ROS wave pathway has now been presented in many organisms including unicellular algae and mammalian cells (Szechyńska-Hebda *et al.*, 2023; Fichman *et al.*, 2023).

Organelle to nucleus signalling pathways

Retrograde signalling pathways link the functional state of mitochondria and chloroplasts to the nuclear gene expression in order to facilitate acclimation (Wang *et al.*, 2020). Organellar retrograde signals are initiated by ROS including superoxide and hydrogen peroxide, and further mediated by oxidized compounds and proteins. Mitochondrial signals initiate cellular responses to hypoxia, including the regulation of heat-shock proteins and other molecular chaperones, transporters, the ALTERNATIVE OXIDASE1a (AOX1a), and other components of the alternative respiratory chain, such as NAD(P)H DEHYDROGENASE B2 (NDB2; Wagner *et al.*, 2018). The mitochondrial signalling cascade that triggers these responses is called the 'mitochondrial dysfunction stimulon'

(MDS; de Clercq *et al.*, 2013). The promotors of MDS genes are targeted by NAC (NO APICAL MERISTEM/ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR/CUP-SHAPED COTYLEDON) transcription factors (de Clercq *et al.*, 2013), particularly ANAC013 and ANAC017/REGULATORS OF ALTERNATIVE OXIDASE 1a (RAO2), WRKY-type transcription factors (WRKY15/WRKY40/WRKY63; Vanderauwera *et al.*, 2012), and ABI4 (ABSISIC ACID INSENSITIVE 4; Giraud *et al.*, 2009). These are downstream regulators of mitochondrial retrograde signalling pathways. CYCLIN-DEPENDENT KINASE E1 (CDKE1/RAO1), which is part of the mediator complex that bridges DNA-bound transcription factors to RNA polymerase II, links signalling input to transcription, in order to regulate AOX1a expression as part of the retrograde signalling hub from mitochondria as well as chloroplasts (Blanco *et al.*, 2014). Significant overlap between chloroplast and mitochondrial retrograde signalling pathways has been observed involving common or shared components (Wang *et al.*, 2023, Preprint). ROS are a key component of the intracellular signalling pathways that regulate the expression of nuclear genes in an ANAC017-dependent manner (Huang *et al.*, 2016; Jurdak *et al.*, 2021). Two key pathways that operate at the interface between chloroplast to nucleus and mitochondria to nucleus signalling have been characterized, namely the SAL1–PAP signalling pathway and the RADICAL-INDUCED CELL DEATH 1 (RCD1)-dependent pathway (Shapiguzov *et al.*, 2019). These organelle to nucleus retrograde signalling pathways overlap or converge in regulating nuclear gene expression. The SAL1 phosphatase, which is localized in chloroplasts and mitochondria, degrades 3'-phosphoadenosine-5'-phosphosulfate (PAP) to AMP and inorganic phosphate. In the absence of SAL1, PAP accumulates and inhibits the activity of the cytosolic 5'–3' exoribonuclease XRN4 and the nuclear 5'–3' exoribonucleases XRN2 and XRN3. The XRNs play significant roles related to non-coding RNAs, including small RNA metabolism, which results in the modulation of nuclear gene expression (Ratti *et al.*, 2020).

RCD1 is localized in the cytosol and nucleus. The abundance, nuclear localization, thiol redox state, and oligomerization are regulated by chloroplast ROS accumulation in order to coordinate plant stress responses, phytohormone signalling pathways, and growth and development (Tao *et al.*, 2023). RCD1 integrates retrograde signalling from the chloroplasts and mitochondria through ANAC013 and ANAC017. The *rcd1* mutation compromised responses to chloroplast ROS accumulation and changed AOX expression, as well as mitochondrial respiration and energy metabolism. The genes misregulated in the *rcd1* mutant had a significant overlap with the genes affected by the PAP signalling pathway and the MDS genes, including those for AOX1a and the sulfotransferase SOT12, an enzyme that generates PAP (Shapiguzov *et al.*, 2019).

Mutations in the gene encoding the rice chloroplast-localized pseudouridine synthase (OSPUS 1-1) result in albino seedlings under low temperatures because of aberrant chloroplast ribosome biogenesis (Wang *et al.*, 2022). Overexpression of mitochondrial MnSOD rescues the phenotype, as does the suppressor protein of *ospus 1-1*, which encodes a mitochondrial pentapeptide repeat (PPR) protein, suggesting that superoxide is involved in the co-ordination of mitochondria to nucleus and chloroplast to nucleus signalling pathways.

The role of superoxide in the regulation of plant development and stress responses

Superoxide generation is a pivotal driver of the cell cycle, pollen viability, microspore reprogramming towards sporophytic development, the regulation of female gametophyte patterning, and the maintenance of embryo sac polarity, as well as the prevention of self-pollination (de Simone *et al.*, 2017; Zur *et al.*, 2021; Sankaranarayanan *et al.*, 2020). ROS signals activate anaerobic core genes via the ERF-VII transcription factors (Sasidharan *et al.*, 2021). The RELATED TO AP-2.12 (RAP2.12) transcription factor regulates the expression of *HYPOXIA-RESPONSIVE UNIVERSAL STRESS PROTEIN 1* (*HRU1*) as well as *RBOHD* under hypoxia (Gonzali *et al.*, 2015). Oxygen levels can fall below 5% in the shoot apices and in the lateral root primordia (LRPs) of plants grown in air, leading to the expression of hypoxia-dependent genes (Shukla *et al.*, 2019). In contrast, hypoxia-responsive genes are not induced in the RAM under similar conditions (Eysholdt-Derzsó *et al.*, 2017; Shukla *et al.*, 2019). Oxygen gradients probably occur in the RAM, particularly because of the quiescent centre (QC) cells, which are deficient in antioxidants such as ascorbate and GSH (Jiang *et al.*, 2010). Flooding-induced hypoxic stress alters auxin flow and distribution in roots in a manner that can shift the redox state of the QC towards a more reduced environment, leading to QC activation and degradation of the meristem (Mira *et al.*, 2020).

The distinct spatiotemporal distribution of superoxide and hydrogen peroxide in shoot and root meristems is crucial for meristematic activities. Superoxide accumulates in meristems, where it serves to maintain cell divisions. In contrast, H_2O_2 is abundant in peripheral zones, promoting cell differentiation (Tsukagoshi *et al.* 2010; Zeng *et al.* 2017). Similarly, the spatial distribution of oxygen in plant organs is an important regulator of development, whose signalling functions connect developmental processes with metabolic activity (Weits *et al.*, 2023). Hypoxia is an established condition in the shoot apical meristem (SAM) that promotes leaf organogenesis which both increases and limits mitochondrial ROS production and also increases the activation of mitochondrial systems that remove ROS (Mailloux, 2020; Pucciariello and Perata, 2021). The oxygen signal is translated into transcriptional regulation through the N-degron pathway and the

regulated expression of ERF-VII (ETHYLENE RESPONSE FACTOR—group VII) transcription factors that link metabolic controls to the regulation of development in plants. Mitochondrial oxygen consumption is also linked to oxygen sensing through the inner mitochondrial membrane UNCOUPLING PROTEIN 1 (UCP1) (Barreto *et al.*, 2022). This regulates nuclear gene expression by inhibiting the cytoplasmic PLANT CYSTEINE OXIDASE (PCO) branch of the PROTEOLYSIS 6 (PRT6) N-degron pathway linking mitochondrial and nuclear functions during plant development. Moreover, the CC-type GRXs are particularly important in the control of plant developmental processes, in organs and tissues where low oxygen signalling contributes to meristem functions (Zachgo, 2023). Redox post-translational modifications of TGA transcription factors, which are targets of ROXYs, regulate their functions in plant development. For example, ROXY1 interacts with the TGA transcription factor PAN in the nucleus to regulate petal formation in *A. thaliana* (Li *et al.*, 2009). ROXY1 proteins also co-localize with all three different RNA polymerase II (RNAPII) isoforms in the nucleus and so regulate RNAPII-mediated transcription (Maß *et al.* 2020).

Mitochondrial metabolism resumes during seed imbibition in order to provide energy for the germinating embryo (Paszkiewicz *et al.*, 2017). Ethylene-generated mitochondrial superoxide production and ROS accumulation regulate seed dormancy alleviation, via a mechanism that involves MRR, nuclear ROS accumulation, the expression of *AOX1a* and *ANAC013*, and the activation of the ethylene canonical pathway (Jurdak *et al.*, 2021).

The concept that ROS signals regulate plant development and growth is well established in the literature (Tsukagoshi *et al*, 2010; Considine and Foyer, 2021; Schmidt and Schippers, 2015; Schippers *et al.*, 2016). Distinct spatial patterns of superoxide and H_2O_2 accumulation have been reported in the root (Zhou *et al.*, 2020). Similarly, stem cells maintain high levels of superoxide and low H_2O_2, while differentiating cells show opposite patterns. Superoxide accumulation is maintained by the regulation of RBOH activities and the expression of mitochondrial AOX, together with inhibition of SOD expression (Zeng *et al.*, 2017). AOX functions as a pre-oxidant defence system, limiting superoxide production, when electron flow via the cytochrome electron transport chain is restricted. Superoxide was shown to decrease the levels of H_2O_2 in the stem cell by activating the expression of peroxidases (Zeng *et al.*, 2017).

Superoxide accumulates in the SAM and RAM, preserving meristematic activity (Tsukagoshi *et al.*, 2010) and stem cell fate (Zeng *et al.*, 2017). Prevention of superoxide accumulation causes the termination of stem cell fate. Similarly, superoxide accumulation in the RAM defines the identity of undifferentiated meristematic cells (Tsukagoshi *et al.*, 2010). The preservation of superoxide signals in Arabidopsis is facilitated by repressed expression of the seven SODs in the central zone (Zeng *et al.*, 2017). Similarly, treatment with c-Myc and Oct44 represses the three human and mice SODs and prevents the induced pluripotent stem cell stage in mammalian cells (Kim *et al.*, 2009; Soldner *et al.*, 2009). The UPBEAT1 (UPB1) transcription factor mediates the balance between superoxide and H_2O_2 in the root in a manner that regulates the transition from cell proliferation to cell expansion and differentiation (Tsukagoshi *et al*, 2010). UPB1 regulates the H_2O_2 content in the root apex by inhibiting the expression of class III peroxidases which are able to produce superoxide in the elongation zone which establishes the ROS gradient distribution in the root meristem. The mutation of an ATP-dependent mitochondrial protease, AtFTSH4, causes oxidation in the cells in the SAM at 30 °C and affects the morphology of the mitochondria and functions of the SAM (Dolzblasz *et al.*, 2016).

Superoxide and hydrogen peroxide appear to fulfil antagonistic roles in plant stem cell regulation, which were established by distinct spatiotemporal patterns of ROS-metabolizing enzymes, particularly in roots (Dunard *et al.*, 2007; Eljebbawi *et al.*, 2020). Superoxide is markedly enriched in stem cells to activate WUSCHEL (WUS) and maintain stemness, whereas H_2O_2 is more abundant in the differentiating peripheral zone to promote stem cell differentiation. Moreover, H_2O_2 negatively regulates superoxide synthesis in stem cells, and increasing H_2O_2 levels or scavenging superoxide leads to the termination of stem cells.

ROS1, which encodes one of the key DNA demethylases, was recently identified as a target for superoxide in the nucleus of stem cells. ROS1, which directly excises 5-methylcytosine from DNA, is a repressor of transcriptional gene silencing that is responsible for demethylation of the promotor of the Type-B cytokinin response regulator ARABIDOPSIS RESPONSE REGULATOR 12 (ARR12). The Fe–S clusters of ROS1 are oxidized by superoxide and so activate the DNA glycosylase/lyase activity of the enzyme, increasing ARR12 expression and contributing to meristem cell maintenance (Wang *et al.*, 2023, Preprint). ARR12 thus acts downstream of ROS1-mediated superoxide signalling to maintain stem cell fate. The *ros1* mutants had lower levels of down-regulation of mRNAs encoding two stem cell regulators, WUS and CLV3, as well as reduced SAM sizes (Wang *et al.*, 2023, Preprint).

Conclusions and perspectives

Superoxide generation by RBOH enzymes is a pivotal driver of plant growth, development, and stress responses, and yet superoxide *per se* is often ignored as a signal in favour of hydrogen peroxide, which has well characterized roles in cell signalling. Accumulating evidence suggests, however, that superoxide is generated and processed in the nucleus, as illustrated in Fig. 2. Moreover, superoxide has key regulatory and signalling functions in the nucleus, particularly in meristem

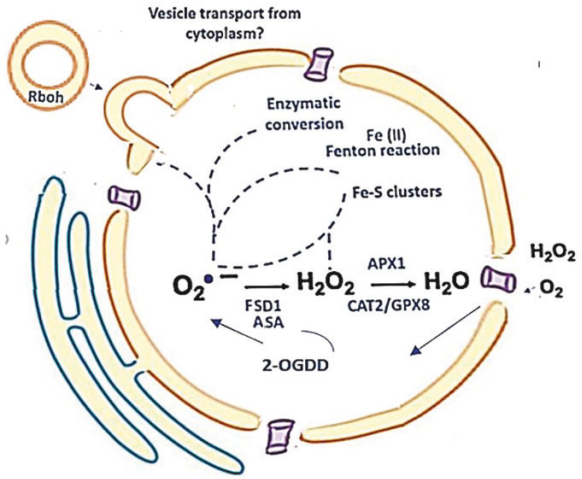


Fig. 2. Superoxide and antioxidant processing in the nucleus. Molecular oxygen and H_2O_2 may enter the nucleus through the nuclear pores. The Fe–S clusters of nuclear proteins are oxidized by superoxide ($O_2^{\bullet -}$). The oxidation of protein [4Fe–4S] clusters by molecular O_2 produces Fe (II) and $O_2^{\bullet -}$. Superoxide may also be generated by RBOH enzymes in the nucleus. These could be delivered to the nuclear membrane by vesicle transport from the plasma membrane. The nuclear antioxidant system includes ascorbate (AsA), GSH catalase 2 (CAT2), ascorbate peroxidase (APX1), iron superoxide dismutase 1 (FSD1), and glutathione peroxidase 8 (GPX8). 2-OGDD, Fe^{2+}/2-oxoglutarate-dependent dioxygenases.

maintenance and organ development, as well as other processes that incorporate multifaceted interplays between ROS and transcriptional regulators, phytohormones, and nutrients. Auxin-induced and RBOH-mediated superoxide production is important in the control of root and shoot architecture, as well as the establishment of the gravitropic curvature response in roots. Spatiotemporal regulation of the patterns of RBOH expression lead to superoxide accumulation in the apoplast in the region of the middle lamella regions of cells at pre-branch sites and LRPs during emergence, facilitating lateral root outgrowth by promoting cell wall remodelling of overlying parental tissues (Orman-Ligeza *et al.*, 2016). Superoxide participates in Fenton-type reactions in the apoplast/cell wall compartment to generate the hydroxyl radical formation required for cell wall remodelling. Moreover, the action of superoxide in the regulation of [Fe–S] cluster-containing proteins such as ROS1 is crucial to meristem maintenance and fate (Wang *et al.*, 2023, Preprint). It will be interesting to determine whether ROS1-mediated superoxide signalling is involved in the plant stress responses. The action of SODs and ascorbate in policing superoxide levels in the nucleus is thus central to superoxide signalling. However, the detection and quantitation of superoxide in the different compartments of the plant cell remain technically challenging. While there are a large number of established methods for superoxide detection, they are all problematic because of a lack of specificity. Data interpretation is therefore fraught with difficulty. The simplest are assays that measure superoxide accumulation in the apoplast/cell wall compartment of the cell, which is antioxidant poor, or when superoxide is released from cells into the surrounding solution. Most methods rely on superoxide scavengers, which react to produce a detectable product, such as the cell-permeable fluorescent probes that form the fluorescent product 2-hydroxyethidium, but none is wholly specific for superoxide (Akter *et al.*, 2021). A next generation of *in vivo* molecular probes for superoxide and hydrogen that can be targeted to specific intracellular compartments is urgently required, so that the mechanisms involved in superoxide production and metabolism in the nucleus can be fully explored.

While the movement of hydrogen peroxide across plant cell membranes through aquaporins is well documented, there is no known system for superoxide transport between different cellular compartments (Bienert and Chaumont, 2014). It is intriguing therefore that modulation of either the NADH dehydrogenase activity of respiratory chain Complex I (localized in mitochondria) or NADPH oxidase (localized in the plasma membrane) influences stem cell regulation (Zeng *et al.*, 2017). Moreover, the *atrbohD/F* double mutants had much lower levels of detectable superoxide (Zeng *et al.*, 2017). Such findings would suggest either that some of the RBOHD/F proteins are localized in the nucleus or that there are interactions between the different superoxide-generating systems in different cellular compartments. The positive relationship between nitrooleic acid, RBOH, and ROS suggests crosstalk between NO and ROS signalling (Gupta *et al.*, 2022). NO, which reacts with superoxide to form peroxynitrite ($ONOO^-$) is produced in high enough concentrations to outcompete SOD for superoxide. Moreover, peroxynitrite reacts relatively slowly with most biological molecules, making peroxynitrite a suitable transport metabolite and selective oxidant. Peroxynitrite can react with CO_2 and produce CO_3^- and $\cdot NO_2$, as well as catalysing post-translational modifications of target proteins. Taken together, these observations demonstrate that superoxide is a major driver of cell signalling that interacts with a wide range of other signalling molecules to regulate plant growth and development.

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Author contributions

BK and CF designed the review, discussed the data, and wrote the manuscript.

Conflict of interest

There are no conflicts of interest.

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Data availability

This manuscript does not contain original data

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