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Reactive oxygen species function as signaling molecules in controlling plant development and hormonal responses

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Abstract

Reactive oxygen species (ROS) serve as second messengers in plant signaling pathways to remodel plant growth and development. New insights into how enzymatic ROS-producing machinery is regulated by hormones or localized during development have provided a framework for understanding the mechanisms that control ROS accumulation patterns. Signaling-mediated increases in ROS can then modulate the activity of proteins through reversible oxidative modification of specific cysteine residues. Plants also control the synthesis of antioxidants, including plant-specialized metabolites, to further define when, where, and how much ROS accumulate. The availability of sophisticated imaging capabilities, combined with a growing tool kit of ROS detection technologies, particularly genetically encoded biosensors, sets the stage for improved understanding of ROS as signaling molecules.

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Introduction

The important role of reactive oxygen species (ROS) as signaling molecules that control plant form and function has received growing attention in recent years. Genetic and biochemical approaches have demonstrated that specific enzymes produce ROS under precise developmental or hormonal controls to initiate or propagate signaling pathways. There are multiple species of ROS, with hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$), and singlet oxygen ($^1O_2$) being most tightly linked to signaling [1]. H_2O_2, through the transfer of electrons, can oxidize reactive cysteine residues on proteins to form cysteine sulfenic acids (-SOH), which is a reversible modification. Sulfenic acids may be further oxidized to sulfinic acid ($-SO_2H$) and then to sulfonic acid ($-SO_3H$) under circumstances of excess ROS. Sulfenic acid may also react with free thiol groups (-SH) to form intra- or intermolecular disulfides [2].

Cysteine oxidation can change the structure, activity, and/or stability of proteins to either propagate or inhibit signals, much like the transfer of phosphates by kinases to specific hydroxyl groups on amino acids alters enzyme activity [3,4]. Oxidized target proteins can be identified by the presence of this cysteine sulfenic acid modification, the function of which can be tested by mutating the cysteine to a non-redox-sensitive amino acid [5]. Proteome-wide changes in cysteine oxidation in Arabidopsis proteins have been identified in response to treatment with H_2O_2, using specific probes that recognize sulfenic acids [5,6]. These redox proteomic datasets provide researchers with candidate proteins that may be the targets of ROS signaling pathways. Protein targets of oxidation include enzymes and transcription factors resulting in changes in their conformation, activity, and subcellular localization, with changes in transcription factor activity reprogramming the transcriptome [7]. Additionally, apoplastic ROS can oxidize cell wall polymers, which can either cause cell wall loosening or stiffening (depending on the ROS and the target polymer) to change cell growth [8,9].

This review focuses on the hormonal and developmental pathways in which ROS acts as signals to alter plant growth and development. We highlight plant tissues and subcellular locations in which ROS levels are developmentally or hormonally regulated and provide examples of how chemical probes and genetically encoded redox

Abbreviations

(ROS) Reactive Oxygen Species
(H_2O_2) Hydrogen Peroxide
($O_2^{\cdot-}$) Superoxide
($^1O_2$) Singlet Oxygen
(RBOH) Respiratory Burst Oxidase Homologs
(SOD) Superoxide Dismutase
(PRX) Class III peroxidase
(FER) FERONIA
(GEF) Guanine Nucleotide Exchange Factor
ROP (Rho of Plants) GTPases
(RH) Root hair
(ARF) Auxin Response Factor
(ACC) 1-aminocyclopropane-1-carboyxlic acid
(LR) Lateral Root
(LRP) Lateral Root Primordia
(CM H2DCF-DA) CM 2,7-dihydrodichlorofluorescein diacetate
(DAB) 3,3-diaminobenzidine
(DHE) Dihydroethidium
(PO1) Peroxy Orange1
(GSH) Glutathione
(ABA) Abscisic Acid
(roGFP) Redox-sensitive Green Fluorescent Protein
(DCF) Dichlorofluorescein
(NBT) Nitro blue tetrazolium

sensors have revealed how ROS accumulation patterns are regulated. Essential to these signaling responses are enzymes that produce and scavenge ROS. Once these enzymes are activated, the resulting changes in ROS can lead to oxidation of transcription factors and enzymes to remodel growth and development. These ROS-producing enzymes are summarized here in the context of how their functions are tied to efficient redox signaling. These studies build on the ability to detect accumulation of ROS, using a complement of chemical dyes whose fluorescence increases in response to oxidation or biosensors in which protein oxidation or reduction changes the properties of fluorescent proteins. Figure 1 illustrates a number of these patterns of ROS accumulation that are developmentally defined or hormonally regulated, detected with a range of methods for monitoring changes in ROS accumulation. Additionally, we highlight the development of genetically encoded biosensors [10,11] that have the potential to reveal changes in specific ROS types at subcellular levels and with temporal dynamics not previously possible.

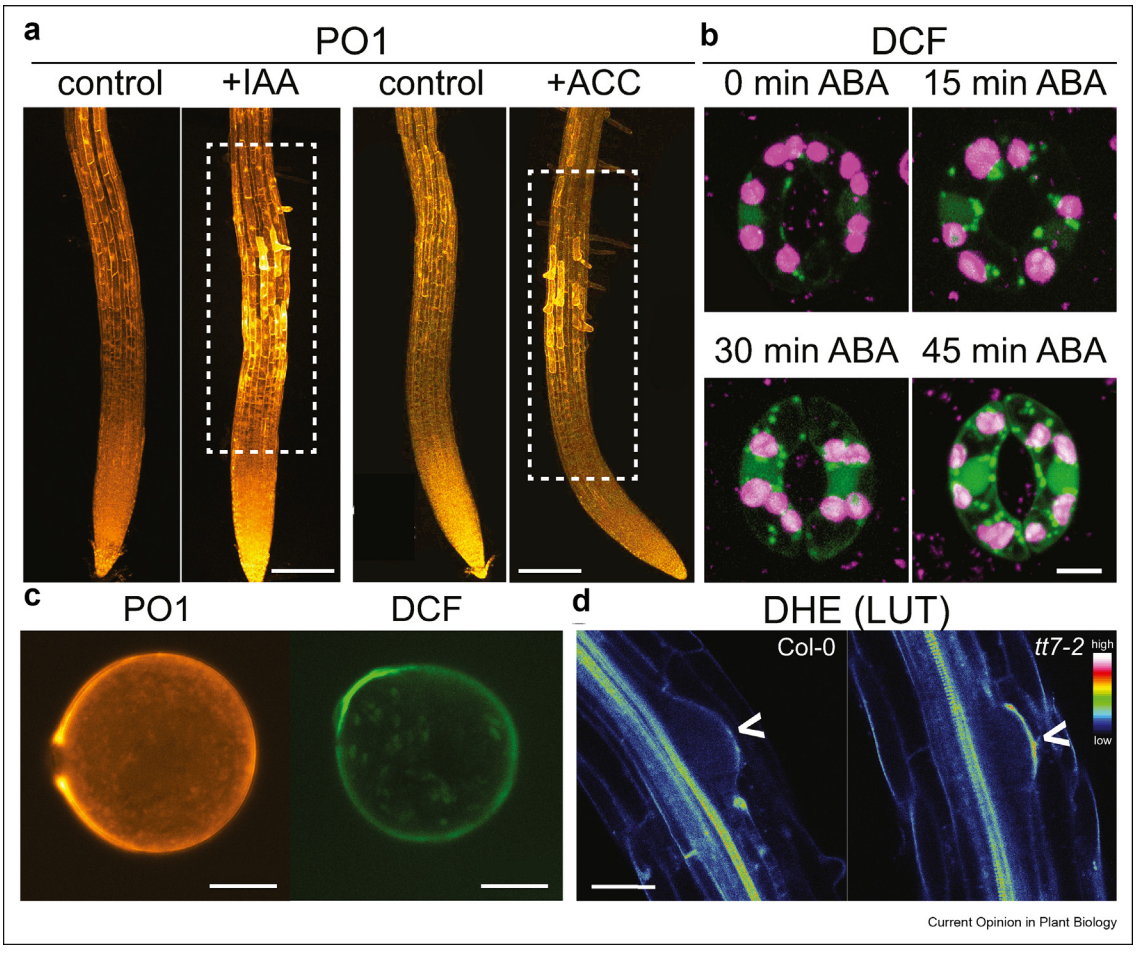
To maintain ROS homeostasis required for efficient signaling, numerous mechanisms are in place to ensure that ROS levels do not reach excess and damaging levels, which would lead to the irreversible oxidation of proteins, DNA, or lipids. To accomplish this, plants produce enzymes and specialized metabolites that function as antioxidants to restore ROS homeostasis [12]. This machinery can be turned on by the same signals that initiate ROS synthesis but with slower kinetics to allow transient increases in ROS. These antioxidant machineries are also critical for the response to high levels of ROS resulting from biotic and abiotic stresses [13–15]. This review does not focus on stress-induced ROS as others have reviewed both how these stressors affect localized ROS accumulation and long distance ROS signals [16–18]; however, we do highlight cases where ROS synthesis and antioxidant activity are balanced to allow for effective signaling. Although there are still many unanswered questions about the signaling pathways that plants employ to produce ROS, it is now clear that the balance of localized ROS synthesis and antioxidant systems that maintain ROS homeostasis is a powerful combination to control plant growth and development.

RBOH-dependent ROS production

Numerous ROS-producing enzymes participate in plant signaling pathways. The best characterized family are the respiratory burst oxidase homolog (RBOH) enzymes, which share sequence similarity with mammalian RBO/NADPH oxidase (NOX) enzymes [19]. RBOHs are located on the plasma membrane, where they transfer electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to molecular oxygen to produce extracellular superoxide, as shown in Figure 2 [19]. Superoxide is highly unstable and is rapidly dismutated to H_2O_2, either spontaneously or by superoxide dismutases (SODs) [20]. Aquaporins localized to the plasma membrane may then facilitate entry of extracellular H_2O_2 into plant cells [21–23]. The Arabidopsis genome encodes 10 RBOH family members (RBOHA-RBOHJ), each with distinct expression patterns across plant tissues (see Ref. [12], Figure 2) and with different functions, which have been reviewed previously [12], and are described below.

RBOHs may be regulated transcriptionally and at the level of enzyme activity [24–28] to control multiple growth and developmental responses. RBOH post-translational modifications can occur via multiple mechanisms, including through the binding of calcium and phosphatidic acid or transfer of a phosphate or nitrosyl moiety (i.e., phosphorylation or nitrosylation, respectively) [19,26,27]. During seed germination, *RBOHB* expression is regulated by abscisic acid (ABA) to control seed after-ripening and germination [28]. In Arabidopsis guard cells, the abundance of *RBOHD* and

Figure 1



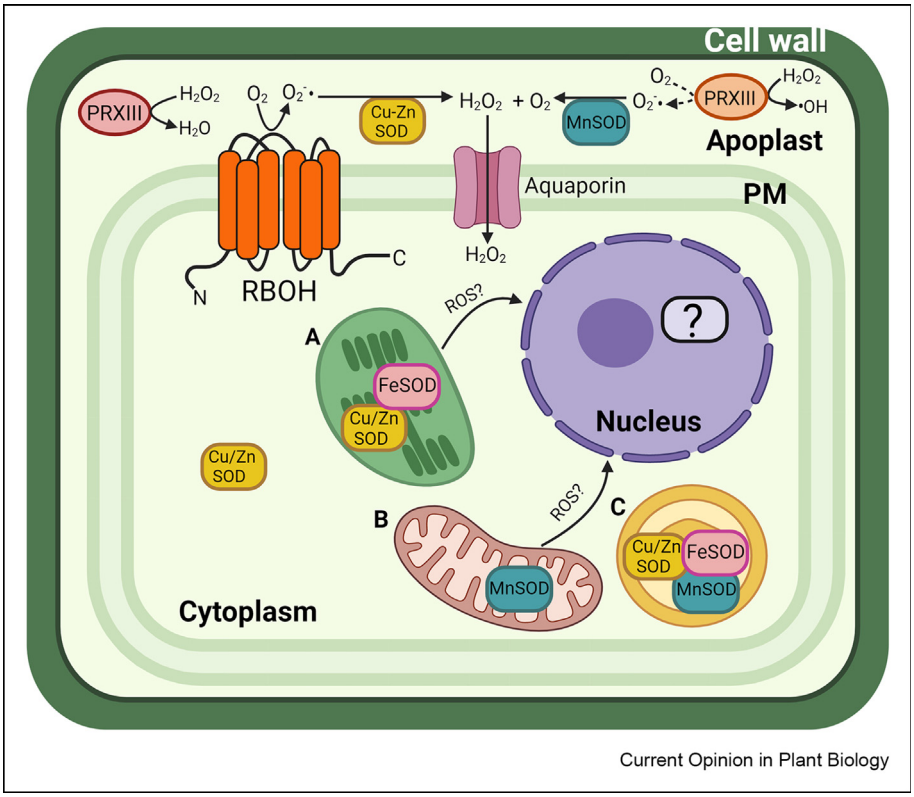
ROS are produced during development and in response to hormone perturbation. (**a**) Confocal images of wild-type (WT) Arabidopsis roots treated with IAA (adapted from Ref. [33]) or the ethylene precursor, ACC (adapted from Ref. [31], and stained with the hydrogen peroxide selective probe, peroxy orange 1 (PO1), revealing elevated ROS in root hair forming cells. Scale bars = 200 μm (**b**) Confocal images of WT tomato guard cells treated with ABA and stained with the general ROS sensor, dichlorofluorescein (DCF) (in green), visualized across a 45 min time course, with chlorophyll autofluorescence in magenta. ROS increases are observed in multiple subcellular locations with significant increases in the number and intensity of cytosolic puncta. Scale bar = 5 μm. Adapted from Ref. [82]. Copyright 2017 American Society of Plant Biologists. (**c**) Confocal images of WT tomato pollen grains stained with PO1 and the general ROS sensor DCF revealing differences in localized ROS accumulation around the pore from which pollen tubes emerge. Scale bars = 10 μm. Adapted from Ref. [51]. (**d**) Confocal images of Arabidopsis Col-0 and *tt7-2,* a mutant with a defect in quercetin synthesis, stained with the superoxide selective stain, dihydroethidium (DHE), which is visualized as a heat map. The *tt7* mutant has increased lateral root formation and elevated DHE signal over the lateral root primordia (indicated with caret symbol). Scale bar = 50 μm. Adapted from Ref. [85].

RBOHF transcripts is increased following ABA treatment and in response to abiotic stresses [29], and *rbohd/f* mutants have reduced ROS accumulation in guard cells and impaired stomatal closure [29]. In roots, transcript abundance of the root hair (RH)-localized *RBOHC* gene is increased by auxin treatment [30] and treatments that elevate ethylene [31], to increase RH initiation and/or elongation [31–33], *RBOHF* controls primary root elongation [34], and *RBOHD* controls lateral root (LR) initiation [35,36], which corresponds with cell type expression patterns of these genes (see Figure 1 in Ref. [12]).

Other ROS-producing enzymes activated by hormone signaling

Recent evidence has suggested that other ROS-generating enzymes play roles in hormone-induced and developmental ROS signaling; however, there is less information on the function of these enzymes [37]. Cellular organelles such as chloroplasts and

Figure 2



ROS-generating enzymes and their location within a cell. In the apoplastic space, ROS are produced by RBOHs [19], Cu/Zn superoxide dismutases (SOD) in cotton [41,128], the newly characterized Mn-SOD, MSD2 in Arabidopsis, as well as class III peroxidase (PRX) [37]. The best established signaling circuit is RBOH produced superoxide, (using NADPH as an electron donor), which is then converted to H_2O_2 by SOD. H_2O_2 then enters cells via aquaporins [21]. PRX enzymes have complex biochemistry but have been reported to scavenge hydrogen peroxide when in the resting state, but also the resting state enzyme can be reduced by superoxide and generate hydroxyl radicals when in the presence of hydrogen peroxide. PRX also have the ability to indirectly produce superoxide when oxidized, which can also be converted to H_2O_2 via SOD [37]. All three isoforms of SOD are also present within the cell found in the cytosol, chloroplasts (a), mitochondria, (b) and peroxisomes (c) [41,129]. ROS has been reported to travel from metabolic organelles to the nucleus and calcium treatment of isolated tobacco nuclei can generate ROS although the mechanism for this ROS synthesis is unknown [130]. Created with Biorender.com.

mitochondria also produce ROS as a by-product of electron transport to drive ATP synthesis [38], yet ROS in those organelles may also be produced by plant signaling pathways [39]. SOD is a ubiquitous enzyme that catalyzes the rapid dismutation of superoxide to hydrogen peroxide and molecular oxygen [40]. Plants have three isoforms of SODs with different metal co-factors: iron (Fe), manganese (Mn), or copper/zinc (Cu/Zn), and each is localized to distinct subcellular compartments, [40,41], as shown in Figure 2. The Arabidopsis genome has been predicted to encode three Cu/Zn SODs (CSD1, CSD2, and CSD3), three Fe-SODs (FSD1, FSD2, and FSD3), and was originally predicted to encode one highly conserved Mn-SOD (MSD1) [42,43]. However, a second cell wall-localized Mn-SOD (MSD2) has recently been identified and characterized [43]. Analysis of the *MSD2* promoter indicated the presence of *cis*-acting elements involved in ABA and light responses, and *MSD2* expression was reported to be elevated in etiolated seedlings [43]. Historically, SOD has been considered to have an antioxidant activity that is induced in response to stress with the sole purpose of reducing superoxide [44], yet these enzymes also produce H_2O_2 as a signaling molecule. Additionally, a study in yeast reported that the Cu/Zn superoxide dismutase (SOD1) plays a role in a redox signaling to regulate the stability of two plasma membrane kinases, which integrate nutrient sensing with metabolism [45]. SOD1 does this by physically associating with the kinase Yck1 and producing a local hydrogen peroxide flux that stabilizes this kinase.

Class III peroxidase (PRX) are another group of enzymes that participate in ROS signaling pathways that govern developmental processes. PRX are plant-specific, heme-containing enzymes that have been shown to be secreted into the apoplast [46]. In Arabidopsis, it has been reported that there are at least 75 genes encoding these enzymes [46]. PRX catalyze reactions that can produce ROS by oxidation of NADH to

an NAD radical to convert O_2 to superoxide, which can then be dismutated into H_2O_2, either nonenzymatically or via SODs. There is evidence for a secreted Cu/Zn-SOD in cotton [128], recent evidence suggests that a newly characterized Mn-SOD (MSD2) contains a secretory peptide and has been shown to localize to the cell wall [43] (Figure 2). PRX can also consume ROS as they can be reduced by superoxide, with the reduced PRX able to convert hydrogen peroxide to a hydroxyl radical [37,47]. The role of select PRX in producing ROS in response to hormonal signals to drive LR development [48] and RH initiation and elongation has been demonstrated using genetic approaches [30,31], which are detailed below. Other roles of PRX and their functions have been reviewed in detail by others [49,50].

ROS are signaling molecules controlling developmental and hormonal responses

Recent evidence supports the role of ROS as signaling molecules, which accumulate with precise spatial localization and whose levels are responsive to changes in hormonal and environmental signals. This section highlights examples of where ROS changes have been examined building on the examples shown in Figure 1. Localized ROS synthesis is an essential feature of flowering plant reproduction. ROS accumulate in pollen grains at the future site of pollen tube emergence (see Figure 1c) and at the tip of the pollen tube during polarized growth [51] to drive elongation [52]. Sperm cells are transported inside pollen tubes and are delivered to the female gametophyte, located inside the ovule, where a localized ROS burst is needed for fertilization to occur [53,54]. In Arabidopsis, RBOHH and RBOHJ localize to the pollen tube tip [55] and are regulated by locally elevated calcium [56]. Many calcium-dependent protein kinases are expressed in pollen [57], and calcium-dependent phosphorylation can regulate RBOH activity [26]. Calcium also directly binds to the EF hand domains of RBOHH and RBOHJ to induce ROS production [58]. These data suggest that a positive feedback loop occurs in which calcium activates RBOHs, leading to ROS production and activation of calcium channels that results in an increase in cytosolic calcium to drive tip growth [56].

Once the pollen tube elongates and reaches the ovule, ROS is required for tube rupture and subsequent sperm release [53]. The FERONIA (FER) gene encodes a plasma membrane-localized TOR-like kinase that interacts with guanine nucleotide exchange factors that activate Rho of Plants (ROP) GTPases that act as molecular switches in signaling pathways [59,60]. FER and RBOH activity are both required for localized ROS production at the entrance of the female gametophyte to facilitate pollen tube rupture and sperm release during fertilization [53]. Consistent with this biochemical function, the Arabidopsis *feronia* (*fer*) mutant has impaired fertility due to failed pollen tube rupture, decreasing fertilization rates [61,62].

Another source of tip-localized ROS in pollen tubes is polyamine oxidases, which use polyamines as a substrate to produce H_2O_2 [52,63]. Polyamine biosynthesis genes and polyamine oxidases are expressed in pollen tubes during pollen tube germination and elongation [63–65]. The polyamine, spermine, localizes to the pollen tube tip and when polyamine synthesis is chemically inhibited, pollen grains fail to germinate [66]. The ABC transporter, ABCG28, is also required for pollen tube elongation and ROS accumulation at the pollen tube tip. The *abcg28* null mutant is male sterile and has pollen tubes that have altered ROS accumulation, no tip-focused spermine localization, and fail to elongate [63]. Ectopic expression of ABCG28 restored polyamine and ROS accumulation and subsequent tip growth, supporting the hypothesis that ABCG28 facilitates vesicular trafficking of polyamines to the growing pollen tube tip [63].

Hormone signaling is a key driver of development that is necessary for plants to adequately respond to their changing environmental conditions. Hormones increase ROS in precise locations in roots to modify root development. RH are tip growing cells, like pollen tubes, but they differentiate from the root epidermis and ROS drives both their initiation (as shown in Figure 1a) [33] and elongation [67]. In Arabidopsis, RH form in an alternating pattern between hair cells, or trichoblasts, and nonhair cells, or atrichoblasts [68]. The levels of hydrogen peroxide are higher in the hair cells than nonhair cells (as shown in Figure 1a) [33], and accumulation at the tip of the growing hair cell is consistent with ROS driving RH formation and elongation [69]. Like pollen tubes, trichoblasts switch from cell elongation to polarized tip growth via localization of the small G-protein ROP to the site of RH initiation [70]. There is a sequential accumulation of specific ROPs and guanine nucleotide exchange factors at this site to drive RH initiation and elongation [71]. RBOH-induced ROS synthesis is required for polarized RH elongation, and RBOHC localization to the tip is dependent on ROP signaling [72]. The *root hair defective2* (*rhd2/rbohc*) mutant has impaired RH initiation [33] and forms short bulbous RH that fail to elongate [32] due to the decreased ROS localization at the tip of RH.

Both auxin and ethylene increase RH initiation and elongation with recent evidence suggesting PRX and RBOHs participate in hormone-induced RH initiation and elongation [30,31,33]. Elevated auxin leads to activation of several auxin response factors and the RH-specific transcription factor, RSL4. RSL4 directly regulates the expression of genes encoding PRX and RBOHs, which leads to ROS synthesis and polar RH

growth [30]. A mutant identified as *rhd2-6*, with a defect in the *RBOHC* gene, had decreased ROS localization at the tip of RH, and decreased H_2O_2 synthesis after treatments that elevate auxin, as shown in Figure 1 [32]. This mutant has both a defect in RH elongation [32] and RH initiation [33]. Ethylene signaling has also been shown to induce ROS accumulation and RH initiation [31], as shown in Figure 1a. Compared to Col-0, the null mutants *prx44-2* and *rhd2-6* had decreased RH initiation, elongation, and H_2O_2 accumulation when ethylene was elevated by treatment with the ethylene precursor, 1-aminocyclopropane-1-carboyxlic acid [31].

ROS is implicated in the differentiation of cells in the root apex, and the subsequent developmental progression of those cells to allow root elongation [7]. Superoxide and hydrogen peroxide gradients can be seen throughout the primary root, starting from the root tip [73]. Between the meristematic and elongation zone of the root, the transcription factor UPBEAT1 (UBP1) regulates ROS homeostasis by repressing PRX in the elongation zone [74]. RBOHD and RBOHF produce ROS in response to ABA signaling, which results in decreased auxin sensitivity and a shorter primary root [34]. A recent study also reported impaired primary root growth in *det2-9*, a mutant defective in brassinosteroid synthesis [75]. However, this mutant showed a significant increase in ethylene synthesis. Along with a hyper-accumulation of ethylene, this mutant had significantly higher superoxide accumulation. Superoxide levels in *det2-9/rbohD* and *det2-9/rbohD/F* mutants were comparable to *det2-9*, suggesting that RBOHs are not responsible for the increased ROS seen in this mutant. However, when broad spectrum PPX inhibitors were applied, *det9-2* root length was significantly increased and superoxide accumulation was reduced. These results suggest that brassinosteroid inhibits ethylene and superoxide synthesis via the peroxidase pathway, which leads to regulate primary root growth [75].

ROS signaling is also involved in cell wall lignification of endodermal cells that define the Casparian strip, which acts as a diffusion barrier [76]. Lignin is generated by the coupling of monolignols, which are oxidized by apoplastic PRX [77]. RBOHF is also required for the localized formation of lignin in the root [78]. Finally, recent evidence has indicated that the SGN1 kinase regulates Casparian strip formation via phosphorylation of RBOHs leading to a local increase in ROS [79].

The formation of LR is also defined by both ROS synthesis and scavenging. LR develop from pericycle cells within the primary root that form LR primordia (LRP), which then elongate, resulting in a branching root system that allows for enhanced plant stability and maximal water and nutrient uptake [80].Using genetic approaches one study reported that *RBOHD* and *RBOHF* were expressed in the vascular cylinder and LRP, and that *rbohd/f* null mutants had more emerged LR than wild type. These mutants displayed decreased signal from the general ROS sensors, CM 2,7-dihydrodichlorofluorescein diacetate (CM H2DCFDA), and 3,3-diaminobenzidine (DAB) staining. In contrast, the signal from dihydroethidium (DHE), a superoxide sensor, increased. This study also reported that in the *rbohd/f* null mutant treatment with auxin did not increase, LR formation as it did in wild-type plants. Together these results suggested that RBOHD and RBOHF negatively modulate LR development by inducing superoxide production in an auxin-independent manner [36]. However, another study reported a positive role of RBOHs in LR formation [35]. This second study reported that *RBOHA*, *RBOHC*, and *RBOHE* were strongly expressed in the endodermis, cortex, and epidermal cells overlying LRP [35] and that an overexpression construct containing RBOHD targeted to the LRP promoted LR emergence. The authors suggested that RBOH-induced ROS production facilitates LR formation by promoting cell wall remodeling [35].

Along with RBOHs, class III peroxidase (PRX, which are also abbreviated PER by some researchers) also play roles in hormone-induced LR development and have also been implicated in root branching. One study found that genes encoding PRX were highly represented in an LR-specific transcriptomic dataset [48]. Chemical approaches found that inhibition of PPX activity significantly reduced the number of emerged LR and found that null mutants, *per7-1* and *per57-1*, had significantly reduced numbers of emerged LR and lower LR density [48]. However, the role of PRX activity was found to be independent of auxin signaling, as treatment with the auxin indole-3-acetic acid (IAA) increased LR emergence in *per7-1* and *per57-1* and in *PER7* overexpression lines. Similarly, *PRX44* is increased in expression after 1-aminocyclopropane-1-carboyxlic acid treatment and *prx44* mutants have reduced ROS in RH and form fewer RH than Col-0 [31].

The ability of plants to close stomata in response to ABA is tightly regulated by ROS homeostasis within the guard cells that flank the stomatal pore [27]. In both tomato and Arabidopsis, ABA rapidly induces ROS production in guard cells to facilitate stomatal closure [81–83], with increases in H_2O_2 visualized by the hydrogen peroxide selective chemical probe peroxy orange 1 (PO1). ABA signaling [82], in response to drought or other stressors, results in increased RBOH phosphorylation followed by a transient ROS burst, then elevated calcium influx, leading to the subsequent loss of water and guard cell turgor pressure [84]. ABA-induced ROS production and stomatal closure were both impaired in *rbohf* and *rbohd/f* null mutants [29] as well as in plants treated with RBOH inhibitors [82]. However, treatment with exogenous H_2O_2 was able to close stomata in the *rbohd/f* double

mutant, suggesting RBOH-dependent ROS production is required for ABA-induced stomatal closure [29]. Guard cell ABA response has also been shown to be dependent on a tight balance between ROS synthesis and ROS scavenging by cellular antioxidant machinery [82,83], which will be discussed below.

Antioxidant systems and specialized metabolites keep ROS in check

For ROS to precisely regulate cell signaling pathways without resulting in deleterious side effects, cells are equipped with systems to prevent ROS from reaching levels sufficient to irreversibly oxidize macromolecules. There are many small molecule and enzymatic antioxidant systems that are conserved across the plant and animal kingdoms [1], but plants have also evolved a number of unique mechanisms to maintain ROS homeostasis. The most visible of these are specialized metabolites with potent antioxidant activity, with metabolites such as the colored anthocyanins and carotenoid pigments protecting plants from excess ROS [85]. What is particularly striking about these specialized metabolites is that the enzymes that synthesize them are often highly regulated so that their biosynthetic machinery is controlled by signaling pathways that may also produce ROS [86] and their localization can define the developmental positioning of ROS accumulation to drive developmental responses [33]. Consistent with these important roles, mutant plants deficient in these metabolites show altered development, and defects in signaling, as detailed below, as well as hypersensitivity to stress induced ROS [13,51,87].

The role of small molecule antioxidants and conserved enzymes in maintaining ROS balance has been extensively studied and reviewed [16]. In a few cases, these are tied to hormonal and developmental phenotypes. The small molecule glutathione (GSH) is a tripeptide that has a reactive sulfur group that can accept and donate electrons, which is present at very high levels (>1 mM) in most eukaryotic cells [14]. The oxidation of GSH is facilitated by several enzymes, including glutaredoxins, while it can be returned to a reduced state by glutathione reductase in order to keep the majority of the glutathione in cells reduced and available to buffer ROS increases [14]. There is evidence for the involvement of GSH in auxin-mediated primary and LR development [88]. Chemical and genetic approaches showed that plants deficient in GSH synthesis had altered ROS homeostasis and exhibited defects in primary root length, LR formation, and auxin transport [89,90]. GSH deficiency also increased auxin sensitivity by inhibiting ubiquitination and degradation of the Aux/IAA proteins and therefore preventing subsequent transcriptional activation of auxin-response genes. In contrast, a mutant in the gene encoding glutaredoxin (GRX27) had impaired auxin synthesis and signaling [89,91]. This work suggests that GSH-auxin crosstalk plays a role in the modulation of root architecture [88]. Other heme peroxidases that are present in the plant cell and are involved in hydrogen peroxide scavenging include ascorbate peroxidase and catalase [41]. These enzymes are involved in removing ROS produced by metabolism or environmental stimuli, such as pathogen attack [92,93], but their ties to signaling have received limited study.

Several classes of plant-specialized metabolites have been shown to act as antioxidants to prevent ROS from reaching damaging levels, with important roles documented for these molecules in managing stress-induced ROS [13], and more limited study of the function of these metabolites in modulating signaling induced ROS [94,95]. Specialized metabolites with antioxidants activity include carotenoids, ascorbate, tocochromanols, and flavonoids, with details on their structure and function and role in protecting plants from stress were summarized previously [12].

The role of flavonoids specialized metabolites in controlling signaling and developmental responses is the most studied. The flavonoid family contains multiple subgroups including flavonols, flavones, flavan-3-ols, flavanones, and anthocyanins [96]. The anthocyanin end products are purple, magenta, or blue allowing visual observation of their accumulation patterns, while a specific fluorescent dye can be used to monitor patterns of flavonol accumulation [33], allowing observation of changes during developmental, signaling, and stress responses. The biosynthetic pathway for these molecules is well characterized and in several species, mutants in genes encoding each biosynthetic enzyme have allowed dissection of the function of specific intermediates in signaling and development [95]. Additionally, multiple studies have demonstrated that the genes encoding the pathway enzymes are highly regulated in response to hormones and stresses to balance the amount of ROS produced by these signals [86].

The role of specific flavonols in regulating ROS and root development is well characterized in Arabidopsis. Null mutants in the first step of flavonol biosynthesis result in increases in both LR and RH formation [33,85]. As the flavonol biosynthetic pathway is encoded by single genes at each step and null mutations in these genes are available for all enzymes, the specific flavonols that control these developmental responses were identified. Quercetin is the flavonol that reduces RH initiation [33], while LR initiation is limited by synthesis of kaempferol [85]. When the accumulation of ROS is examined in these flavonol deficient mutants, there are increases in ROS in RH and LRP (as shown in Figure 1d) that are appropriately positioned to drive development [33,85]. Using dyes that monitor flavonol accumulation patterns and GFP fusions to pathway enzymes, the localization of flavonols indicates that they

are at low levels in epidermal tissues that give rise to RH, where ROS is at high levels, while flavonols accumulate at high levels in the cortical layer below the epidermis, where ROS is kept low [33]. This pattern is opposite to the levels of RBOHC, which is expressed at high levels in RH-forming cells of the epidermis to drive ROS synthesis and at low levels in more internal tissues [12]. Therefore, the balance of ROS synthesis and scavenging allows ROS-dependent tip growth of RH but limits ROS accumulation at internal tissues.

Flavonols accumulate at high levels in guard cells where they modulate ABA-dependent ROS accumulation but are at low levels in surrounding pavement cells [82,83]. Mutants in tomato and Arabidopsis that have impaired flavonol biosynthesis have higher ROS in guard cells and show enhanced ABA dependent stomatal closure [82,83]. Signals that increase flavonol synthesis, such as elevated levels of ethylene, reduce the ABA-dependent ROS synthesis, and reduce stomatal closure, demonstrating complex hormonal loops in which flavonols and ROS can fine tune stomatal opening and gas exchange on the surface of leaves [83].

In many species, flavonoids are essential for reproduction and modulate the levels of ROS in reproductive tissues, which may function to prevent ROS required for pollen tube tip growth and fertilization from reaching damaging levels. In maize, rice, and tomato, mutations or RNAi lines that are unable to produce flavonoids are infertile [97—99] although mutations that abolish flavonol synthesis in Arabidopsis have normal fertility [100,101]. Tomato mutants that have reduced flavonol synthesis set fewer seeds, have shorter pollen tubes, and increased ROS accumulation [51], with rice flaonol biosynthetic mutants exhibiting the same fertility and ROS alterations [102]. In tomato and rice mutants with impaired flavonols, there is also enhanced sensitivity to heat stress with pollen germination and pollen tube length impaired [51,102]. These data indicate that ROS scavenging by flavonols acts as a protective mechanism in pollen tubes with especially important roles during acute heat stress [51].

Approaches for monitoring hormone-induced ROS in plants

The ability to visualize changes in ROS in signaling pathways are constantly improving due to recent advances in both chemical and genetically encoded ROS sensors and increasing sophistication in the instrumentation for measuring fluorescence changes. Changes in ROS levels during signaling and development are most frequently detected via chemical probes. These probes are generally colorless or nonfluorescent, membrane permeable compounds entering cells in their reduced forms, with oxidation leading to changes in color or fluorescent properties [103]. The chemical properties and selectivity of these dyes are summarized in Table 1, which allow them to monitor the accumulation of different types of ROS [104,105]. CM H_2DCF-DA is the most commonly used probe to monitor increases in intracellular ROS accumulation, as it is oxidized by multiple species of ROS [106]. CM H_2DCF-DA diffuses across the plasma membrane, where it is trapped within the cell after cleavage by cellular esterases, with oxidation by intracellular ROS producing the highly fluorescent DCF molecule [107]. This probe has been used to report elevated levels of ROS induced by hormonal signals and during development including examples described above [29,51,62,69,81,83,108,109], as shown in Figure 1b and c. DCF's ease of use is balanced by limitations that include the absence of specificity for any single ROS type, its irreversibility, and its sensitivity to photo-oxidation during visualization [110].

The specific detection of H_2O_2 and O_2^- are of particular interest due to their crucial function in plant signal transduction cascades and imaging of their accumulation with fluorescent dyes and reporters visualized by confocal microscopy can review precise patterns of accumulation of ROS [41,111]. Colorimetric indicators such as DAB and nitroblue tetrazolium have been used to detect H_2O_2 and superoxide, respectively, in response to environmental stressors [112]. However, these histochemical techniques have multiple disadvantages including low sensitivity, limited specificity, and lengthy, destructive protocols. These limitations have led to the increasing use of more sensitive, less destructive fluorescence-based methods.

Hydrogen peroxide accumulation can be detected by PO1, which is a boronate-based probe, which becomes fluorescent upon irreversible H_2O_2-dependent oxidation of the boronate leaving group [113], as shown in Figure 1a and c. PO1 has a bright fluorescent signal, displays increased sensitivity compared to DAB and is cell permeable to detect intracellular H_2O_2. Amplex red is a noncell permeable chemical probe that reacts with H_2O_2, in the presence of horseradish peroxidase, to yield the fluorescent product resorufin [114]. This probe is sensitive to small changes in hydrogen peroxide and has been used to qualitatively monitor extracellular H_2O_2 in plant tissues such as roots and leaves [104,115]. Superoxide in plants has been detected using DHE and its mitochondrial-targeted version, MitoSOX, which are cell-permeable fluorescent probes that form the fluorescent product 2-hydroxyethidium (2-OH-E^+) following reaction with superoxide [85,109,116,117], as shown in Figure 1d. However, DHE is also able to react with other oxidants to form the ethidium cation, which is also highly fluorescent [118]. These two products can be differentiated through HPLC to quantify the superoxide-modified DHE molecule [118].

Although the use of chemical ROS probes has provided significant insight into ROS signaling in plants,

Table 1

Methodologies for ROS detection in plants.

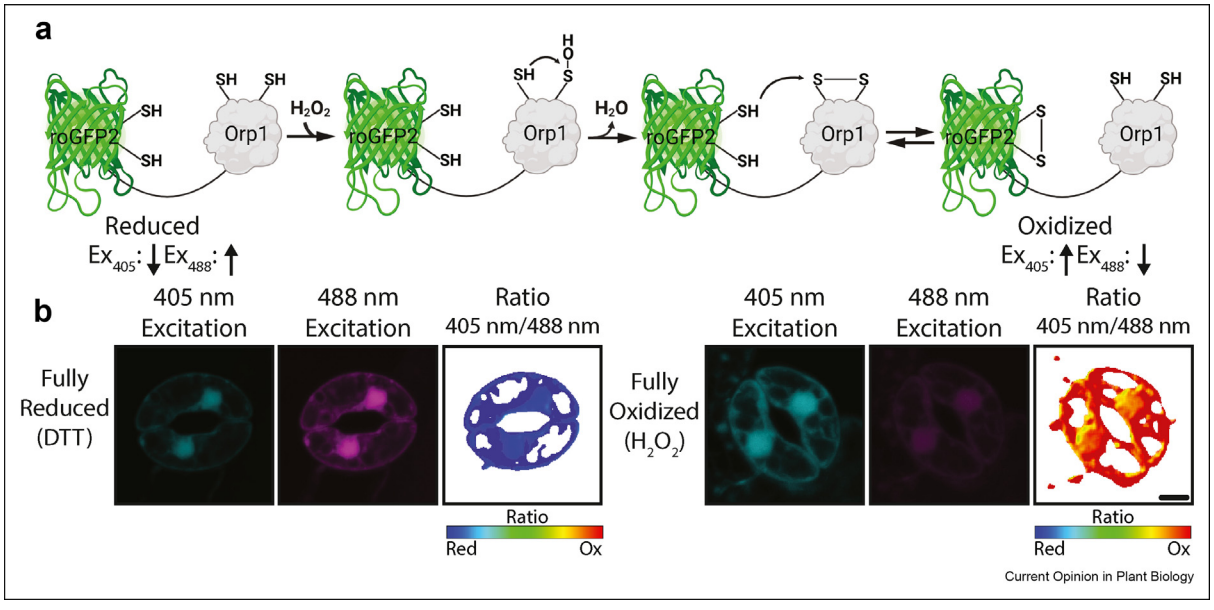
Type of detection method	Name	Detects	Limitations	Subcellular localization	Examples of use in plants
Fluorescent chemical probe	**CM H_2DCF-DA** Chloromethyl 2′,7′-dichlorodihydro-fluorescein diacetate	General increases in ROS accumulation	Irreversible, nonspecific, potential for photooxidation [110]	Intracellular Nontargeted	[51,83,85]
	OxyBurst Green H_2HFF-BSA Dihydro-2′,4,5,6,7,7′-hexafluoro-fluorescein	General increases in ROS accumulation	Irreversible, nonspecific, potential for photooxidation [69,110]	Extracellular	[69]
	BES H_2O_2-Ac 3′-O-Acetyl-6′-O-Pentafluoro-benzenesulfonyl 2′-7′-difluoro-fluorescein	H_2O_2	Irreversible, slow reactivity with H_2O_2 [104]	Intracellular Nontargeted	[132]
	Amplex red	H_2O_2	Irreversible, fluorescent product can be affected by pH, sufficient HRP required, potential for photooxidation [104]	Extracellular	[109,133]
	PO1 Peroxy orange1	H_2O_2	Irreversible, low efficiency of H_2O_2 detection [104]	Intracellular Nontargeted	[33,82]
	DHE Dihydroethidium	$O_2^{\cdot-}$	Irreversible, can react with multiple ROS types, may require more analytical methods for quantitation of $O_2^{\cdot-}$ [104,118]	Intracellular Nontargeted	[85]
	MitoSOX DHE covalently attached to triphenyl phosphonium	$O_2^{\cdot-}$	Irreversible, can react with multiple ROS types, reaction products may diffuse to nucleus [134]	Mitochondria	[135]
Genetically encoded biosensor	**Zat12p-ROS**	Expression of the ROS regulated transcription factor Zat12	Non-selective for a particular type of ROS [119]	Intracellular Nontargeted	[119]
	roGFP1/2	Glutathione redox potential	Not selective for a particular ROS type, measurements of reductive shifts in reducing environments or oxidative shifts in oxidizing environments may be unreliable [136]	Cytosolic-roGFP2 Mitochondrial-roGFP2 Plastid-roGFP2 Peroxisomal-roGFP1 ER-roGFP2	[121] [121] [137] [122] [138]
	roGFP2-Orp1	H_2O_2	Slower reaction time compared to HyPer probes [11]	Cytosolic-roGFP2-Orp1 (visible in nucleus) Mitochondrial- roGFP2-Orp1 Plastid- roGFP2-Orp1	[10] [10] [139]
	HyPer1/2	H_2O_2	Fluctuations in pH can skew interpretation [140]	Cytosolic-HyPer Peroxisomal-HyPer Nuclear-HyPer2 Chloroplast stroma-HyPer2	[23] [141] [142] [142]
	HyPer7	H_2O_2	May lack efficient cytosolic reduction mechanism [11]	Cytosolic-HyPer7	[11]
Colorimetric orobe	**DAB** 3, 3′-diamino benzidine	H_2O_2	Irreversible, long incubation time [143]	Non-targeted	[144]
	NBT Nitroblue tetrazolium	$O_2^{\cdot-}$	Irreversible, can react with O_2 to form further $O_2^{\cdot-}$ [145]	Non-targeted	[146]

genetically encoded biosensors that exhibit oxidation-dependent fluorescence changes in vivo have the potential for measuring dynamic responses with fewer technical limitations than chemical dyes. Genetically encoded sensors can employ different mechanisms to detect redox state, such as monitoring expression of a reporter gene fused to a redox-responsive gene [119], though this section will focus on two fluorescent protein-based biosensors. Redox-sensitive green fluorescent proteins (roGFP) were developed through the introduction of a pair of cysteines in adjacent β-strands proximate to the GFP chromophore [120]. Sulfenic acid formation at one of these residues results in disulfide formation between the two cysteines, causing a conformational change that alters the optical properties of the fluorophore. This provides a ratiometric readout that serves as an internal control and bypasses issues of differential dye uptake that may accompany the use of chemical dyes in certain contexts [107]. RoGFP has been used in a variety of plant tissues to monitor glutathione redox potential [121–123], but its limited selectivity for a particular ROS type and relatively slow response times compared to other sensors have led to improvements to this reporter. A new variant in which roGFP2 was fused to the yeast peroxidase Orp1 (also named GPX3) [124], has recently been transformed into Arabidopsis [10]. In the presence of H_2O_2, Orp1 is oxidized to rapidly form an intramolecular disulfide bond between two adjacent cysteine residues, which is then efficiently transferred to roGFP2 via thiol–disulfide exchange [124], which is shown in Figure 3.

RoGFP2-Orp1 is selective for H_2O_2, insensitive to changes in pH over a physiological range of pHs and can be reduced through the glutaredoxin/glutathione/glutathione reductase system, which allows for critical temporal measurement of changes in redox state in response to a stimulus [10]. Genetically encoded sensors are limited to reporting ROS accumulation only in the subcellular compartment in which they are targeted, but an expanded set of Arabidopsis lines with roGFP2-Orp1 targeted to the cytosol, mitochondria [10], and chloroplasts [11] are now available, reducing that limitation. Introduction of these sensors into genotypes with mutations in hormone signaling and redox-regulating machinery will provide new insight into regulation of ROS signaling in plants.

Another major class of ROS detecting genetically encoded sensors is the HyPer family. HyPer sensors consist of the H_2O_2-sensing domain from the bacterial transcription factor OxyR attached to a circularly permuted yellow fluorescent protein (cpYFP) [125]. Early HyPer variants have been used to monitor H_2O_2 in tissues including pollen [53], guard cells [23], and roots [126]. However, in contrast to roGFP2, cpYFP is highly

Figure 3



roGFP2-Orp1 is a genetically encoded ratiometric bioreporter for H_2O_2. (a) Schematic detailing the biochemical mechanism of roGFP2-Orp1 oxidation by H_2O_2 and resulting changes in fluorescence [10] Created with Biorender.com.(b) Representative micrographs of guard cells from individual excitation channels as well as ratiometric images following reduction with DTT or oxidation with H_2O_2 to illustrate the properties of the sensor. Fully reduced roGFP2 displays high signal intensity after excitation with the 488 nm laser line (magenta), while low-signal intensity excitation with the 405 nm laser lines yields low signal intensity (cyan). As roGFP2-Orp1 becomes fully oxidized, excitation at 405 nm results in increased emission intensity, while excitation with the 488 nm laser results in decreased emission intensity than when it is fully reduced. Ratiometric images were generated using the Redox Ratio Analysis software [131] to display fluorescence ratios calculated from the GFP fluorescence images excited at 488 nm and 405 nm.

sensitive to variations in pH [127]. The more recent HyPer7 variant displays increased sensitivity and dynamic range compared to roGFP2-Orp1 and exhibits a ratiometric readout that is largely unaltered by changes in pH although currently it is only targeted to the cytosol [11]. These genetically encoded biosensors have outstanding potential for monitoring the changes in ROS in living cells with temporal and spatial dynamics not previously possible.

Conclusions and future prospects

It is now evident that the concentrations of ROS change across development and in response to hormone and stress signals. Insight into the mechanisms that control the developmental localization and hormonal regulation of ROS-producing enzymes has enhanced our understanding of when ROS production can drive plant growth and development. Insight into how localized increases in hydrogen peroxide leads to reversible oxidation of specific cysteines in target proteins to change their activity is essential to understand the mechanisms of ROS signaling in plants. The improvement in genetically encoded ROS sensors that are able to monitor changes in H_2O_2 within distinct subcellular compartments will allow for a more precise understanding of when and where ROS changes are induced by hormonal and developmental signals. Equivalently important to productive ROS signaling are mechanisms to prevent ROS from reaching damaging levels, including insight into developmental and hormonal controls of synthesis of both small molecule, enzymatic, and specialized metabolites that are essential for maintaining ROS homeostasis. Together these new insights support a central role of ROS signals in plant development and hormonal responses.

Author contributions

REM, AEP, GKM conceptualized, drafted, and edited the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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