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REACTIVE OXYGEN SPECIES: Metabolism, Oxidative Stress, and Signal Transduction

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■ **Abstract** Several reactive oxygen species (ROS) are continuously produced in plants as byproducts of aerobic metabolism. Depending on the nature of the ROS species, some are highly toxic and rapidly detoxified by various cellular enzymatic and nonenzymatic mechanisms. Whereas plants are surfeited with mechanisms to combat increased ROS levels during abiotic stress conditions, in other circumstances plants appear to purposefully generate ROS as signaling molecules to control various processes including pathogen defense, programmed cell death, and stomatal behavior. This review describes the mechanisms of ROS generation and removal in plants during development and under biotic and abiotic stress conditions. New insights into the complexity and roles that ROS play in plants have come from genetic analyses of ROS detoxifying and signaling mutants. Considering recent ROS-induced genome-wide expression analyses, the possible functions and mechanisms for ROS sensing and signaling in plants are compared with those in animals and yeast.

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INTRODUCTION

The evolution of aerobic metabolic processes such as respiration and photosynthesis unavoidably led to the production of reactive oxyen species (ROS) in mitochondria, chloroplasts, and peroxisomes. A common feature among the different ROS types is their capacity to cause oxidative damage to proteins, DNA, and lipids. These cytotoxic properties of ROS explain the evolution of complex arrays of nonenzymatic and enzymatic detoxification mechanisms in plants. Increasing evidence indicates that ROS also function as signaling molecules in plants involved in regulating development and pathogen defense responses. In this review, we first describe the biological effects and functions of ROS in plants and then discuss open questions that need to be addressed in future research.

GENERATION OF ROS

Ground state triplet molecular oxygen is a bioradical with its two outermost valence electrons occupying separate orbitals with parallel spins. To oxidize a nonradical atom or molecule, triplet oxygen would need to react with a partner that provides a pair of electrons with parallel spins that fit into its free electron orbitals. However, pairs of electrons typically have opposite spins, and thus fortunately impose a restriction on the reaction of triplet molecular oxygen with most organic molecules (18, 51). However, ground state oxygen may be converted to the much more reactive ROS forms either by energy transfer or by electron transfer reactions. The former leads to the formation of singlet oxygen, whereas the latter results in the sequential reduction to superoxide, hydrogen peroxide, and hydroxyl radical (66) (Figure 1).

In plants ROS are continuously produced as byproducts of various metabolic pathways localized in different cellular compartments (37). Under physiological steady state conditions these molecules are scavenged by different antioxidative defense components that are often confined to particular compartments (3). The equilibrium between production and scavenging of ROS may be perturbed by a number of adverse environmental factors. As a result of these disturbances, intracellular levels of ROS may rapidly rise (36, 74, 102, 120). Plants also generate ROS by activating various oxidases and peroxidases that produce ROS in response to certain environmental changes (1, 13, 14, 35, 109). The rapid increase in ROS

Figure 1 Generation of different ROS by energy transfer or sequential univalent reduction of ground state triplet oxygen.

concentration is called “oxidative burst” (5). External conditions that adversely affect the plants can be biotic, imposed by other organisms, or abiotic, arising from an excess or deficit in the physical or chemical environment. Although plants’ responses to the various adverse environmental factors may show some commonalities, increases in ROS concentration triggered by either biotic or abiotic stresses are generally attributed to different mechanisms.

Biotic Strategies to Generate ROS

One of the most rapid defense reactions to pathogen attack is the so-called oxidative burst, which constitutes the production of ROS, primarily superoxide and H_2O_2, at the site of attempted invasion (5). Doke (1985) first reported the oxidative burst (35), demonstrating that potato tuber tissue generated superoxide that is rapidly transformed into hydrogen peroxide following inoculation with an avirulent race of *Phytopthera infestans*. A virulent race of the same pathogen failed to induce $O_2^{\bullet -}$ production. Subsequently, $O_2^{\bullet -}$ generation has been identified in a wide range of plant pathogen interactions involving avirulent bacteria, fungi, and viruses (71).

Several different enzymes have been implicated in the generation of ROS. The NADPH-dependent oxidase system, similar to that present in mammalian neutrophils, has received the most attention. In animals the NADPH-oxidase is found in phagocytes and B lymphocytes. It catalyzes the production of superoxide by the one-electron reduction of oxygen using NADPH as the electron donor. The $O_2^{\bullet -}$ generated by this enzyme serves as a starting material for the production of a large variety of reactive oxidants, including oxidized halogens, free radicals, and singlet oxygen. These oxidants are used by phagocytes to kill invading microorganisms, but at the same time they may also damage surrounding cells of the host. The core of the **PH**agocyte **OX**idase comprises five components: $p40^{PHOX}$, $p47^{PHOX}$, $p67^{PHOX}$, $p22^{PHOX}$, and $gp91^{PHOX}$. In the resting cell, three of these five components, $p40^{PHOX}$, $p47^{PHOX}$, and $p67^{PHOX}$, exist in the cytosol as a complex. The other two components, $p22^{PHOX}$ and $gp91^{PHOX}$, are localized in membranes of secretory vesicles. Separating these two groups of components ensures that the oxidase remains inactive in the resting cell. When the resting cells are stimulated, the cytosolic component $p47^{PHOX}$ becomes heavily phosporylated and the entire

cytosolic complex is recruited to the membrane, where it associates with the two membrane-bound components to assemble the active oxidase. Activation requires not only the assembly of the core components, but also the participation of two low molecular weight guanin-nucleotide-binding proteins (8).

In addition to the NADPH-oxidase of phagocytes, other NADPH-oxidases also associated with plasma membranes are found in a variety of cells (8). Thus, the NADPH-oxidase activity originally described by Doke (35) may not necessarily represent a homologue of the leukocyte-specific enzyme. However, subsequent studies have provided several lines of evidence strongly suggesting a common origin for both enzymes. Antibodies raised against human $p22^{PHOX}$, $p47^{PHOX}$, and $p67^{PHOX}$ cross-reacted with plant proteins of similar sizes (32, 116), and in several plant species *rboh* genes (*r*espiratory *b*urst *o*xidase *h*omologues) of $p91^{PHOX}$, the catalytic subunit of the NADPH-oxidase of phagocytes, have been found (64, 119). In rice, homologues of GTP-binding proteins, required for activating the animal enzyme, have been implicated in pathogen-induced cell death (63), and the putative plant plasma membrane NADPH-oxidase reportedly produces superoxide (108). Finally, knock-out mutations of two *Arabidopsis rboh* genes, *AtrbohD* and *AtrbohF*, largely eliminate ROS production during disease resistance reactions of *Arabidopsis* to avirulent pathogens (118), thus providing direct genetic evidence that two components of a plant NADPH-oxidase are required for ROS production during plant defense responses. Homologues of the $p47^{PHOX}$ and $p67^{PHOX}$ regulators of the mammalian NADPH-oxidase were not found in the *Arabidopsis* genome (26), which suggests that the plant NADPH-oxidase may be regulated differently than that in mammalian macrophages.

In addition to a plant-specific NADPH-oxidase, alternative mechanisms of ROS production have been proposed. Many peroxidases are localized in the apoplastic space and are ionically or covalently bound to cell wall polymers. Peroxidases can act in two different catalytic modes. In the presence of H_2O_2 and phenolic substrates they operate in the peroxidatic cycle and are engaged in the synthesis of lignin and other phenolic polymers. However, if the phenolic substrates are replaced by NADPH or related reduced compounds, a chain reaction starts that provides the basis for the H_2O_2-producing NADH-oxidase activity of peroxidases (22). Peroxidase H_2O_2 production is distinguished from that by the phagocyte-type NADPH-oxidase by different Km values for oxygen, different requirements for NADH and NADPH, and different sensitivities of the two enzymes to inhibitors such as cyanide, azide, and diphenyleneiodonium (DPI). Based on these differences rapid H_2O_2 production in some plant species triggered by pathogen attack and elicitor treatment has been attributed to the NAD(P)H-oxidase activity of apoplastic peroxidases (124).

In addition to its NAD(P)H-oxidase activity that gives rise to superoxide and hydrogen peroxide, in vitro studies of horseradish peroxidase suggest another activity of this enzyme: generating hydroxyl radicals (22). Similar to the $Fe^{2+/3+}$ catalyzed Haber-Weiss reaction, horseradish peroxidase can reduce hydrogen peroxide to hydroxyl radicals (22). Thus, one expects that whenever cell wall–bound peroxidases

come into contact with suitable concentrations of superoxide and H_2O_2, originating from the oxidative cycle of peroxidase or from other sources, hydroxyl radicals should form within the cell (110). This situation prevails, for instance, when $O_2^{\bullet -}$ and H_2O_2 levels are increased in plants in response to pathogen attack, followed by a hypersensitive reaction that leads to the death of host cells. However, producing hydroxyl radicals by cell wall–bound peroxidases may also be relevant for other physiological responses such as the controlled breakdown of structural polymers during rearrangement of cell walls in roots, hypocotyls, or coleoptiles (40, 94, 126).

In isolated tobacco epidermal cells two distinct ROS-producing mechanisms were activated after the addition of a fungal elicitor (1). One source of ROS production was identified as a NADPH-oxidase and/or a xanthine oxidase, whereas the second activity was attributed to a peroxidase and/or amine oxidase. It is not known whether these activities appear sequentially and thus are responsible for the two phases of ROS induction by fungal or bacterial elicitors that have been measured in plant cell cultures (9).

Chewing herbivorous insects mechanically wound plant tissue while feeding and induce an increase in hydrogen peroxide levels inside the plant reminiscent of the oxygen burst triggered by pathogens (12, 16). Based on the inhibition by DPI H_2O_2 production was linked to the NADPH-oxidase (96), thus invoking a common mechanism for the production of H_2O_2 during plant-pathogen interaction and the wound response. Hydrogen peroxide has been proposed to act as a second messenger for the induction of defense genes in response to wounding. However, these defense genes are different from those induced during plant-pathogen interactions. Thus, the specificity of the wound-induced defense response may not be derived from H_2O_2. Because the selectivity of the inhibitor used in the study (96) has been questioned (1, 39), a different source of H_2O_2 production cannot be ruled out.

Abiotic Strategies to Generate ROS

In plants, ROS are continuously produced predominantly in chloroplasts, mitochondria, and peroxisomes. Production and removal of ROS must be strictly controlled. However, the equilibrium between production and scavenging of ROS may be perturbed by a number of adverse abiotic stress factors such as high light, drought, low temperature, high temperature, and mechanical stress (36, 74, 102, 120).

CHLOROPLASTS ***HYDROGEN PEROXIDE/SUPEROXIDE*** Oxygen is continuously produced during light-driven photosynthetic electron transport and simultaneously removed from chloroplasts by reduction and assimilation. There are three types of oxygen-consuming processes closely associated with photosynthesis: (*a*) the oxygenase reaction of ribulose-1,5 bisphosphate carboxylase-oxygenase (Rubisco), (*b*) direct reduction of molecular oxygen by photosystem I (PSI) electron transport, and (*c*) chlororespiration (64).

Chlororespiration describes the reduction of oxygen resulting from the presence of a respiratory chain consisting of a NAD(P)H dehydrogenase and a terminal oxidase in chloroplasts that competes with the photosynthetic electron transport chain for reducing equivalents. This process has been largely studied in microalgae (11). Only recently have components of this respiratory chain also been found in higher plants (92). It is not known to what extent this process might contribute to ROS formation in chloroplasts of higher plants although the electron transport capacity of chlororespiration is only about $<1\%$ that of photosynthesis (e.g., 36a).

The two primary processes involved in formation of ROS during photosynthesis are the direct photoreduction of O_2 to the superoxide radical by reduced electron transport components associated with PSI and reactions linked to the photorespiratory cycle, including Rubisco in the chloroplast and glycolate-oxidase and CAT-peroxidase reactions in the peroxisome. When plants are exposed to light intensities that exceed the capacity of CO_2 assimilation, overreduction of the electron transport chain leads to inactivation of PSII and the inhibition of photosynthesis. Plants may use two strategies to protect the photosynthetic apparatus against photoinhibition: first, the thermal dissipation of excess excitation energy in the PSII antennae (nonphotochemical quenching), and second, the ability of PSII to transfer electrons to various acceptors within the chloroplast (photochemical quenching) (98). When plants are exposed to environmental stresses and the availability of CO_2 within the leaf is restricted, as may occur, for example, under drought or temperature stress, the reduction of oxygen by PSI (Mehler reaction) (79) and the photorespiratory pathway play a critical photoprotective role (Figure 2).

In leaves of C3 plants the photorespiratory oxygenation of ribulose 1,5-bisphosphate by Rubisco constitutes a major alternative sink for electrons, thereby sustaining partial oxidation of PSII acceptors and preventing photoinactivation of PSII when CO_2 availability is restricted. Rubisco catalyzes a competitive reaction in which oxygen is favored over CO_2 as a substrate as temperature increases or as the intracellular CO_2 concentration declines. This oxygenation reaction leads to the release of glycolate that is translocated from chloroplasts to peroxisomes. Its subsequent oxidation is catalyzed by the glycolate oxidase accounting for the major portion of H_2O_2 produced during photosynthesis (Figure 2). Oxygen reduction sustains significant levels of photosynthetic electron flux not only through its role in photorespiration but also by its direct reduction by PSI (7) (Figure 2). Superoxide radicals generated by the one-electron reduction of molecular oxygen by PSI are rapidly converted within the chloroplast to hydrogen peroxide by CuZn-superoxide dismutase (7). It has been suggested that photoreduction of O_2 to water by the Mehler ascorbate peroxidase pathway in intense light may involve up to 30% of the total electron transport (7). This would suggest that O_2 plays an important role as an alternative electron acceptor in photoprotection. Producing large amounts of ROS is an inevitable consequence of the photosynthetic reduction of oxygen, and plants would have to evolve efficient strategies to cope with the accumulation of these potentially toxic compounds that are integral components of oxygenic photosynthesis.

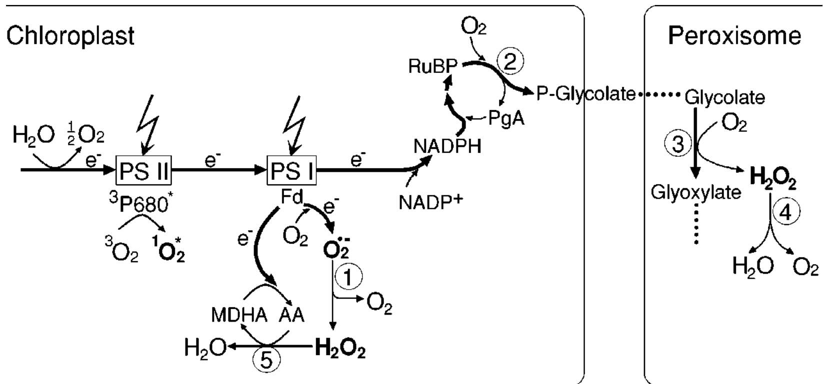

Figure 2 The principal features of photosynthetic electron transport under high light stress that lead to the production of ROS in chloroplasts and peroxisomes. Two electron sinks can be used to alleviate the negative consequences of overreduction of the photosynthetic electron chain: (*a*) the reduction of oxygen by PSI that generates superoxide and H_2O_2, and (*b*) the Rubisco oxygenase reaction and the photorespiratory pathway that lead to H_2O_2 generation within the peroxisome. Under light stress, increasing amounts of singlet oxygen are produced within PSII. Bold arrows show the main routes of electron transport. Key enzymes discussed in the text are shown in encircled numbers: 1) superoxide dismutase, 2) Rubisco, 3) glycolate oxidase, 4) catalase, and 5) ascorbate peroxidase.

Singlet Oxygen During photosynthesis singlet oxygen is continuously produced by PSII. The reaction center complex of PSII consists of cytochrome b_{559} and the heterodimer of the D1 and D2 proteins. The heterodimer binds the reaction center's functional prothestic groups including chlorophyll P680, pheophytin, and the quinone electron acceptors Q_A and Q_B. Excitation of the reaction center results in charge separation between P680 and pheophytin and the subsequent sequential reduction of Q_A and Q_B (10). When the redox state of the plastoquinone pool and Q_A and Q_B are overreduced because of excess light energy, charge separation cannot be completed and the oxidized P680 chlorophyll recombines with the reduced pheophytin. Under these conditions forming the triplet state of P680 is favored, leading to the generation of singlet oxygen by energy transfer. The release of singlet oxygen was first detected in preparations of isolated PSII particles (73), but was subsequently shown to occur in vivo (41, 54). During excess light stress that leads to photoinhibition of PSII, singlet oxygen production drastically increases (53).

In animals singlet oxygen may be produced metabolically during reduction of O_2 catalyzed by the phagocytic NADPH-oxidase (113). A homologue of this enzyme is activated during plant-pathogen interaction. Thus, it is conceivable that singlet oxygen also takes part in defense reactions of plants. However, there is presently no data to support this proposal.

MITOCHONDRIA In mammalian cells mitochondria are the major source of ROS (51). However, the relative contribution of mitochondria to ROS production in green tissues is very low (103). One reason that plant mitochondria do not produce more ROS could be the presence of the alternative oxidase (AOX) that catalyzes the tetravalent reduction of O_2 by ubiquinone. The AOX competes with the cytochrome bc_1 complex for electrons and thus may help to reduce ROS production in mitochondria. This suggestion is supported by findings that H_2O_2 induces the expression of AOX (128), and overproduction of AOX in transgenic cell lines reduces ROS production, whereas antisense cells with reduced levels of the AOX accumulate five times more ROS than control cells (77).

ROS DETOXIFICATION

In the presence of transition metal ions hydrogen peroxide may be reduced to hydroxyl radicals by superoxide. Superoxide and hydrogen peroxide are much less reactive than $OH^{\bullet}$. The main risk for a cell that produces the two former reactive oxygen intermediates may be posed by the intermediates' interaction, leading to the generation of highly reactive hydroxyl radicals. Because there are no known scavengers of hydroxyl radicals, the only way to avoid oxidative damage through this radical is to control the reactions that lead to its generation. Thus, cells had to evolve sophisticated strategies to keep the concentrations of superoxide, hydrogen peroxide, and transition metals such as Fe and Cu under tight control.

Nonenzymatic ROS Scavenging Mechanisms

Nonenzymatic antioxidants include the major cellular redox buffers ascorbate and glutathione (GSH), as well as tocopherol, flavonoids, alkaloids, and carotenoids. Mutants with decreased ascorbic acid levels (23) or altered GSH content (24) are hypersensitive to stress. Whereas GSH is oxidized by ROS forming oxidized glutathione (GSSG), ascorbate is oxidized to monodehydroascorbate (MDA) and dehydroascorbate (DHA). Through the ascorbate-glutathione cycle (Figure 3*c*), GSSG, MDA, and DHA can be reduced reforming GSH and ascorbate. In response to chilling, heat shock, pathogen attack, and drought stress, plants increase the activity of GSH biosynthetic enzymes (123, 125) and GSH levels (93).

A high ratio of reduced to oxidized ascorbate and GSH is essential for ROS scavenging in cells. Reduced states of the antioxidants are maintained by glutathione reductase (GR), monodehydroascorbate reductase (MDAR), and dehydroascorbate reductase (DHAR), using NADPH as reducing power (6, 120). In addition, the overall balance among different antioxidants must be tightly controlled. The importance of this balance is evident when cells with enhanced glutathione biosynthesis in chloroplasts show oxidative stress damage, possibly due to changes of the overall redox state of chloroplasts (24). Little is known about flavonoids and carotenoids in ROS detoxification in plants. However, overexpression of ß-carotene hydroxylase in *Arabidopsis* leads to increased amounts of xanthophyll in chloroplasts

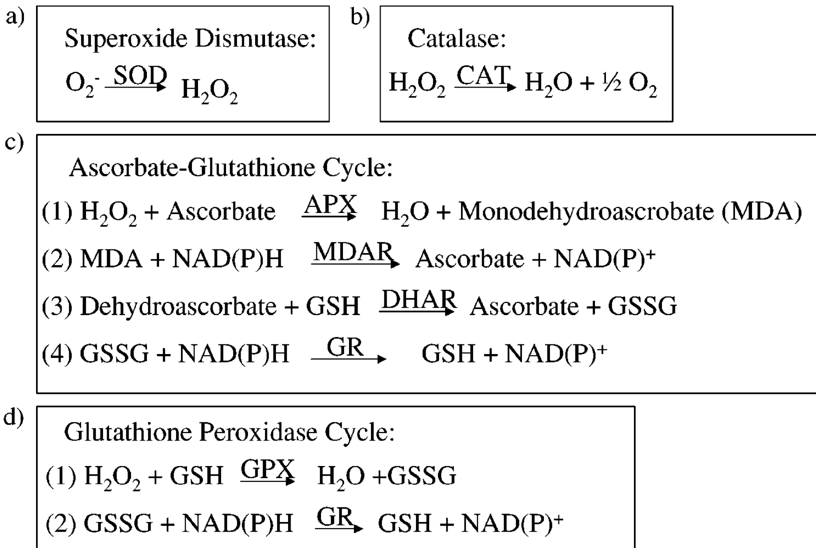

Figure 3 The principal modes of enzymatic ROS scavenging by superoxide dismutase (SOD), catalase (CAT), the ascorbate-glutathione cycle, and the glutathione peroxidase (GPX) cycle. SOD converts hydrogen superoxide into hydrogen peroxide. CAT converts hydrogen peroxide into water. Hydrogen peroxide is also converted into water by the ascorbate-glutathione cycle. The reducing agent in the first reaction catalyzed by ascorbate peroxidase (APX) is ascorbate, which oxidizes into monodehydroascorbate (MDA). MDA reductase (MDAR) reduces MDA into ascorbate with the help of NAD(P)H. Dehydroascorbate (DHA) is produced spontaneously by MDA and can be reduced to ascorbate by DHA reductase (DHAR) with the help of GSH that is oxidized to GSSG. The cycle closes with glutathione reductase (GR) converting GSSG back into GSH with the reducing agent NAD(P)H. The GPX cycle converts hydrogen peroxide into water using reducing equivalents from GSH. Oxidized GSSG is again converted into GSH by GR and the reducing agent NAD(P)H.

and results in enhanced tolerance towards oxidative stress induced in high light (27).

Enzymatic ROS Scavenging Mechanisms

Enzymatic ROS scavenging mechanisms in plants include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and CAT (Figure 3). SODs act as the first line of defense against ROS, dismutating superoxide to H_2O_2 (Figure 3*a*). APX, GPX, and CAT subsequently detoxify H_2O_2. In contrast to CAT (Figure 3*b*), APX requires an ascorbate and GSH regeneration system, the ascorbate-glutathione cycle (Figure 3*c*). Detoxifiying H_2O_2 to

H_2O by APX occurs by oxidation of ascorbate to MDA (equation 1 in Figure 3*c*), which can be regenerated by MDA reductase (MDAR) using NAD(P)H as reducing equivalents (equation 2 in Figure 3*c*). MDA can spontaneously dismutate into dehydroascorbate. Ascorbate regeneration is mediated by dehydroascorbate reducatase (DHAR) driven by the oxidation of GSH to GSSG (Equation 3 in Figure 3*c*). Finally, glutathione reductase (GR) can regenerate GSH from GSSG using NAD(P)H as a reducing agent. Like APX, GPX also detoxifies H_2O_2 to H_2O, but uses GSH directly as a reducing agent (equation 1 in Figure 3*d*). The GPX cycle is closed by regeneration of GSH from GSSG by GR (equation 2 in Figure 3*d*). Unlike most organisms, plants have multiple genes encoding SOD and APX. Different isoforms are specifically targeted to chloroplasts, mitochondria, peroxisomes, as well as to the cytosol and apoplast (6). Whereas GPX is cytoslic, CAT is located mainly in peroxisomes.

Specific roles for antioxidant enzymes have been explored via transgenic approaches. Overexpression of tobacco chloroplast SOD to chloroplasts did not alter tolerance toward oxidative stress, which suggests that other antioxidant mechanisms might be limiting (2). However, expression of a pea chloroplast SOD in tobacco increased resistance to methyl viologen–induced membrane damage (2). CAT is indispensable for oxidative stress tolerance because transgenic tobacco plants with suppressed CAT have enhanced ROS levels in response to both abiotic and biotic stresses (130).

The extent of oxidative stress in a cell is determined by the amounts of superoxide, H_2O_2, and hydroxyl radicals. Therefore, the balance of SOD, APX, and CAT activities will be crucial for suppressing toxic ROS levels in a cell. Changing the balance of scavenging enzymes will induce compensatory mechanisms. For example, when CAT activity was reduced in plants, scavenging enzymes such as APX and GPX were upregulated. Unexpected effects can also occur. When compared to plants with suppressed CAT, plants lacking both APX and CAT were less sensitive to oxidative stress (106). Because photosynthetic activity of these plants was decreased, reduction in APX and CAT might result in suppression of ROS production via chloroplasts.

THE ROLE OF ROS IN SIGNALING

ROS generation in cellular compartments such as the mitochondria or chloroplasts results in changes of the nuclear transcriptome, indicating that information must be transmitted from these organelles to the nucleus, but the identity of the transmitting signal remains unknown. Three principal modes of action indicate how ROS could affect gene expression (Figure 4). ROS sensors could be activated to induce signaling cascades that ultimately impinge on gene expression. Alternatively, components of signaling pathways could be directly oxidized by ROS. Finally, ROS might change gene expression by targeting and modifying the activity of transcription factors.

ROS Sensing by Histidine Kinases

In prokaryotes and fungi two-component signaling systems function as redox sensors (104, 129). In prokaryotes, two-component signaling systems usually consist of a histidine kinase that senses the signal and a response regulator that functions as a transcription factor. The transmembrane sensory kinase functions through its capacity to autophosphorylate a histidine residue in response to the presence or absence of an external stimulus. The phosphoryl group is subsequently transferred from the histidine to an aspartate residue in the response regulator. The induced conformational change in the response regulator alters its DNA binding affinity and thus promotes gene expression of certain promoters. Also in budding and fission yeast, histidine kinases of two-component signaling systems can function as sensors of oxidative stress (111). In contrast to animals, plants contain a range of two-component histidine kinases (56). Whether some of these proteins can function as ROS sensors is currently under investigation.

ROS Activation of Mitogen-Activated Protein Kinase (MAPK) Signaling Pathways

Although histidine kinases are part of two-component signal transduction systems in prokaryotes that act on their own, in fungi and plants these sensors are integrated into more complex pathways. The yeast Sln1 kinase transfers its phosphoryl group via the intermediary component Ypd1 to its final destination in the response regulator Ssk1. Stress inhibits autophosphorylation of Sln1 and hence the nonphosphorylated form of Ssk1 accumulates and activates the Hog1 mitogen-activated protein kinase (MAPK) cascade (50).

Sequence analyses of the rice and *Arabidopsis* genomes reveal an extraordinary complexity in MAPK signaling components that comprise more than 100 MAPK, MAPKK, and MAPKKK genes in these plants. MAPK signaling modules are involved in eliciting responses to various stresses, to hormones, and during cytokinesis. H_2O_2 activates several MAPKs (for review see 59). In *Arabidopsis*, H_2O_2 activates the MAPKs, MPK3, and MPK6 via MAPKKK ANP1 (67). Overexpression of ANP1 in transgenic plants resulted in increased tolerance to heat shock, freezing, and salt stress (67). H_2O_2 also increases expression of the *Arabidopsis* nucleotide diphosphate (NDP) kinase 2 (85). Overexpression of AtNDPK2 reduced accumulation of H_2O_2 and enhanced tolerance to multiple stresses including cold, salt, and oxidative stress. The effect of NDPK2 might be mediated by the MAPKs, MPK3, and MPK6 because NDPK2 can interact and activate the MAPKs. These data suggest a scenario in which various stresses induce ROS generation that in turn activate MAPK signaling cascades. Although neither the mechanism of activation nor the downstream targets of the MAPK pathways are yet known, ROS-induced activation of MAPKs appears to be central for mediating cellular responses to multiple stresses.

ROS Inhibition of Protein Phosphatases

Because H_2O_2 is a mild oxidant that can oxidize thiol residues, it has been speculated that H_2O_2 is sensed via modification of thiol groups in certain proteins. Recent work has identified human protein tyrosine phosphatase PTP1B to be modified by H_2O_2 at the active site cysteine (122). Inactivation of PTP1B by H_2O_2 is reversible and can be brought about by incubation with glutathione. A similar regulation likely occurs in plants because PTP1, an *Arabidopsis* PTP that can inactivate the *Arabidopsis* MPK6, can be inactivated by H_2O_2 (49). Also, phosphatases involved in abscisic acid (ABA) signaling within guard cells have been identified whose in vitro activity was modulated reversibly by H_2O_2 (80, 81).

ROS Activation of Transcription Factors

Comparing the mechanisms for ROS-induced gene expression in prokaryotes, fungi, and plants may reveal common mechanisms (44). In *E. coli*, the transcription factor OxyR is of paramount importance in oxidative stress signaling (133). In budding yeast, Yap1 plays a similar role. Budding yeast mutants deficient in Yap1 reveal that most ROS-induced genes depend on this transcription factor. OxyR and Yap1 are redox-sensitive transcription factors and modulate gene expression in response to oxidative stress. ROS regulates the activity of the transcription factors through covalent modification of cysteine thiol groups in OxyR and Yap1. Different types of ROS react with different cysteinyl residues and can give rise to differently modified products, possibly explaining how ROS species can induce different sets of genes via the same transcription factor (28, 29). One major difference between OxyR and Yap1 is that the yeast transcription factor is not sensing ROS directly but through the activity of Gpx3, which acts as hydroperoxidase and peroxiredoxin. The higher degree of complexity in yeast reflects the increased flexibility of eukaryote signaling systems. Accordingly, it is not surprising that additional regulation of redox-sensitive transcription factors was established in fission yeast (115).

Similar to yeast, plants have evolved a MAPK pathway and several protein phosphatases for ROS signaling. Although no redox-sensitive transcription factor has yet been identified in plants, it is likely that such transcription factors exist.

Gene expression in response to oxidative stress seems to be coordinated via the interaction of transcription factors with specific oxidative stress-sensitive *cis*-elements in the promoters of these genes. There is evidence that oxidative stress-responsive *cis*-elements exist in yeasts, animals, and plants. Work in budding and fission yeast has shown that homologs of the mammalian ATF and AP-1 transcription factors function as key mediators of diverse stress signals binding to conserved *cis*-regions of stress-inducible promoters (21, 43). Microarray analysis of H_2O_2-induced gene expression in *Arabidopsis* indicates potential H_2O_2-responsive *cis*-elements in genes regulated by H_2O_2 (31). One of these elements, the *as-1* promoter element, also has high homology with the redox-sensitive mammalian AP-1 *cis*-element (61). However, recent analysis of transgenic plants indicates that

ROS other than H_2O_2 activate this *as-1* element (42). Further analysis will reveal whether similarity exists among plant, animal, and fungal regulatory *cis*-elements of ROS-responsive genes.

ROS AS SIGNALS FOR GENE EXPRESSION

Transcriptome analysis with full genome chips has revolutionized our knowledge regarding gene expression. Oxidative stress affects approximately 10% of the yeast transcriptome (19, 21, 43). Exposure of yeast cells to various stresses including H_2O_2 defines a large set of genes denoted as common environmental stress response (CESR). CESR-induced genes play a role in carbohydrate metabolism, ROS detoxification, protein folding and degradation, organellar function, and metabolite transport. CESR-repressed genes are involved in energy consumption and growth, RNA processing, transcription, translation, and ribosome and nucleotide biosynthesis (19, 21, 43).

In plants, ROS-induced genes have been identified for receptor kinase (33), annexin (86), and peroxisome biogenesis (33). Recent approaches using cDNA profiling and DNA microarrays have analyzed large-scale gene expression in response to ROS. Following exposure of *Arabidopsis* cells to H_2O_2, a total of 175 genes (i.e., 1–2% of the 11,000 genes on the microarray) showed changes in expression levels (31). Of the 113 induced genes, several encoded for proteins with antioxidant functions or were associated with defense responses or other stresses. Still others coded for proteins with signaling functions.

Exposing a plant to sublethal doses of one stress that results in protection from lethal doses of the same stress at a later time is termed stress acclimation. Global changes in gene expression were analyzed in tobacco plants that were treated with superoxide-generating methyl viologen after pretreatment with a sublethal doses (127). Approximately 2% of the tobacco genes were altered in their expression in acclimated leaves. Genes with predicted protective or detoxifying functions and signal transduction were upregulated in acclimated leaves, implying a variety of cellular responses during acclimation tolerance.

The effects of oxidative stress on the *Arabidopsis* mitochondrial proteome have been analyzed (114). Whereas two classes of antioxidant defense proteins, peroxiredoxins, and protein disulphide isomerase accumulated in response to oxidative stress, proteins associated with the TCA cycle were less abundant.

By inhibiting H_2O_2 production, or facilitating its removal with scavengers such as CAT, genes encoding APX, pathogenesis-related (PR) proteins, glutathione S-transferase (GST), and phenylalanine ammonia-lyase (PAL) were identified (34, 62, 70). An alternative approach to study the effects of oxidative stress on the transcriptome is to induce oxidative stress by reducing antioxidant activity. CAT and ascorbate peroxidase antisense lines show elevated expression of SOD and GR (106). In contrast, MDAR, a key enzyme for the regeneration of ascorbate, was upregulated in plants with experimentally reduced CAT and ascorbate peroxidase

levels. An increase in expression of ROS detoxifying enzymes is compatible with compensatory mechanisms induced by oxidative stress. When tobacco plants deficient in CAT were grown in high-intensity light, they increased ROS production and PR protein levels, and showed enhanced disease resistance (20).

ROS AT THE INTERFACE BETWEEN BIOTIC AND ABIOTIC STRESSES

ROS and Plant Pathogen Defense

ROS play a central role in plant pathogen defense. During this response, ROS are produced by plant cells via the enhanced enzymatic activity of plasma-membrane-bound NADPH-oxidases, cell wall–bound peroxidases and amine oxidases in the apoplast (Figure 5*a*) (47, 52). Under these conditions, up to 15 μM H_2O_2 can be produced either directly or as a result of superoxide dismutation. In contrast to superoxide, H_2O_2 can diffuse into cells and activate many of the plant defenses, including PCD (programmed cell death) (26). During plant pathogen reactions, the activity and levels of the ROS detoxifying enzymes APX and CAT are suppressed by salicylic acid (SA) and nitrous oxide (NO) (65). Because during the plant pathogen defense reponse the plant simultaneously produces more ROS while decreasing its ROS scavenging capacities, accumulation of ROS and activation of PCD occurs. The suppression of ROS detoxifying mechanisms is crucial for the onset of PCD. ROS production at the apoplast alone without suppression of ROS detoxification does not result in the induction of PCD (30, 83). These data indicate an absolute requirement for the coordinated production of ROS and downregulation of ROS scavenging mechanisms.

Induction of PCD potentially limits the spread of disease from the infection point. During incompatible reactions, when a pathogen is detected as an enemy and defense responses including PCD are induced, H_2O_2 production occurs in a biphasic manner. The initial and very rapid accumulation of H_2O_2 is followed by a second and prolonged burst of H_2O_2 production. During compatible interactions, when a pathogen overcomes the defense lines and systemically infects the host plant, only the first peak of H_2O_2 accumulation occurs (9). It is not yet known whether these two distinct bursts arise from the same or different sources.

H_2O_2 generation occurs both locally and systemically in response to wounding (97). Recent work shows that H_2O_2 functions as a second messenger mediating the systemic expression of various defense-related genes in tomato plants (96). Previously, it was found that the oxidative burst in pathogen challenged *Arabidopsis* leaves activates a secondary systemic burst in distal parts of the plant, leading to systemic immunity via the expression of defense-related genes (4). It is possible that H_2O_2 is not the primary signal that is transmitted, and interactions with other signaling intermediates such as SA could also be involved.

Although the oxidative burst is a primary response to pathogen challenge that leads to PCD (15), and H_2O_2 induces PCD in various systems (34, 70, 112),

in some cases H_2O_2 is not required for PCD induction (46, 57). Studies show that a threshold exposure time of cells to H_2O_2 is required, during which period transcription and translation are necessary (34, 112). Pharmacological data indicate that removal of ROS during pathogen or elicitor challenge reduces PCD (34, 70). A recent genetic analysis confirms these data by showing that *Arabidopsis* knock-out lines lacking functional *rboh* genes (i.e., respiratory burst oxidase homolog genes) display reduced ROS generation and PCD following bacterial challenge (118). In line with this concept, tobacco plants with reduced CAT or ascorbate peroxidase expression show increased PCD to low doses of bacteria (83).

It is not clear yet to what extent PCD in plants and animals share similar mechanisms. Expression of the animal cell death suppressor genes Bcl-xl and Ced-9 in tobacco plants suppresses oxidative stress-induced cell death (82). In animals, mitochondria play a primary role in ROS production and in triggering programmed cell death. Mitochondrial ROS production has also been implicated in eliciting ROS-induced cell death in plants (76, 117). However, as mentioned earlier, in plants the majority of ROS is produced in chloroplasts and peroxisomes. Whether or not these differences necessitated the evolution of different mechanisms of PCD in plants and animals is not known yet.

ROS and Abiotic Stress

PCD occurs not only as a result of the oxidative burst following pathogen challenge, but also following exposure to abiotic stresses. For example, the ozone-induced oxidative burst results in a cell death process similar to the hypersensitivity response (HR) during plant-pathogen interactions (131). However, the role that ROS play during abiotic stresses appears to be opposite to the role that ROS play during pathogen defense. Upon abiotic stresses, ROS scavenging enzymes are induced to decrease the concentration of toxic intracellular ROS levels (Figure 5*b*). The differences in the function of ROS between biotic and abiotic stresses might arise from the action of hormones and cross-talk between different signaling pathways or from differences in the locations where ROS are produced and/or accumulate during different stresses. These considerations raise the question of how plants can regulate ROS production and scavenging mechansisms when they are exposed simultaneously to pathogen attack and abiotic stress. Evidence for the significance of such conflicting situations comes from experiments with tobacco plants showing reduced PCD after exposure to oxidative stress (83). The oxidative stress pretreatment resulted in increased levels of ROS scavenging enzymes, thereby abrogating the plants' ability to build up sufficient ROS for inducing PCD. In accordance with this model, CAT overproducing plants have decreased resistance to pathogen infection (101), wounding (97), and high light treatment (88). It was suggested that ROS act in conjunction with compound(s) that travel systemically and have the capacity to activate ROS production in distant parts of the plant (97). It is still debated whether ROS can travel long distances in the plant because most ROS are highly reactive and are detoxified immediately by the scavenging systems of the

apoplast. Future studies using plants with altered levels of ROS scavenging and/or ROS producing mechanisms might resolve this question.

ROS and Stomata

Recent work has shown that ROS are essential signals mediating ABA-induced stomatal closure. The phytohormone ABA accumulates in response to water stress and induces a range of stress adaptation responses including stomatal closure. Earlier work has shown that H_2O_2 induces stomatal closure (78) and that guard cells synthesize ROS in response to elicitor challenge (1, 68). H_2O_2 is an endogenous component of ABA signaling in *Arabidopsis* guard cells. ABA-stimulated ROS accumulation induced stomatal closure via activation of plasma membrane calcium channels (99). ABA-induced ROS synthesis also occurs in *Vicia faba* (132), but ROS production occurs at the plasma membrane and in the chloroplast. This study indicates the complexity of ROS signaling in this system. Various *Arabidopsis* mutants have been used to dissect ABA and ROS signaling in guard cells. In the *gca2* mutant, ABA increased ROS production, but H_2O_2-induced calcium channel activation and stomatal closure were absent in the mutant (99).

Protein phosphorylation is also involved in guard cell signaling, as shown by analysis of the ABA-insensitive *abi1* and *abi2* mutants. ABI1 and ABI2 encode protein phosphatase 2C enzymes that are both involved in stomatal closing. Using the *abi1* and *abi2* point mutants with strongly reduced phosphatase activities, it was shown that ABA is unable to generate ROS in abi1 mutants but ABA still induces ROS production in *abi2* mutants (89). These data indicate that ABI1 may act upstream and ABI2 downstream of ROS signaling.

A recently identified protein kinase functions between ABA perception and ROS signaling (90). *Ost1* was identified as an ABA insensitive mutant. OST1 kinase is activated by ABA in guard cell protoplasts of wild-type but not of *ost1* plants. ABA-induced ROS production was absent in *ost1* plants, although *ost1* stomata still closed in response to H_2O_2. The notion that OST1 regulates ROS production directly via the NADPH-oxidase is an attractive hypothesis that remains to be validated experimentally. As shown by the recent findings that NO can also mediate ABA-induced stomatal closure (91), guard cell behavior is probably not solely regulated by ROS.

ROS and Roots

A new role for H_2O_2 in auxin signaling and gravitropism in maize roots was revealed recently (60). Gravity and asymmetric auxin application induced ROS generation, and asymmetric application of H_2O_2 promoted gravitropism. An intracellular source of ROS was suggested because CAT application had no effect on gravitropism. The upregulation of oxidative stress-related genes during *Arabidopsis* gravitropism might indicate a wider role of ROS in this biological process (86).

CONCLUSIONS AND OPEN QUESTIONS

Although there has been rapid progress in recent years, there are still many uncertainties and gaps in our understanding of how ROS affect the stress response of plants. Here we focus on some of the more important questions that need to be addressed in the future.

Generally, ROS have been proposed to affect stress responses in two different ways. ROS react with a large variety of biomolecules, and may thus cause irreversible damage that can lead to tissue necrosis and may ultimately kill the plants (45, 105). On the other hand, ROS influence the expression of a number of genes and signal transduction pathways. These latter observations suggest that cells have evolved strategies to utilize ROS as environmental indicators and biological signals that activate and control various genetic stress response programs (25). This interpretation is based on the unstated assumption that a given ROS may interact selectively with a target molecule that perceives the increase of ROS concentration and translates this information into signals that direct the plant's responses to stress. ROS would be ideally suited to act as such signaling molecules. ROS are small and can diffuse short distances, and there are several mechanisms for ROS production, some of which are rapid and controllable, and there are numerous mechanisms for rapid removal of ROS.

There are at least three major possibilites of how ROS could act as biological signals in plants. (*a*) ROS could act as a second messenger and modulate the activity of a specific target molecule involved in signaling or transcription, as described above. (*b*) Many changes in gene expression that have been attributed to a signaling role of ROS could also result from their cytotoxicity. The toxicity of ROS has often been monitored by measuring lipid peroxidation. Polyunsaturated fatty acids within the lipids are a preferred target of ROS attack. Several of their oxygenation products are biologically active and may change the expression of specific genes (48). Thus, a given ROS may generate nonenzymatically a wide range of oxidation products, some of which may disseminate within the cell and act as a second messenger that triggers multiple stress responses. Whether or not these effects can be ascribed to a "signaling" role of ROS depends on how "signaling" is defined. (*c*) Finally, ROS may trigger stress responses in plants by modulating gene expression in a more indirect way. For instance, during detoxification of ROS in chloroplasts, large amounts of reductants such as ascorbic acid and glutathione are oxidized and shift the redox balance to a more oxidized state. For example, changes in the redox status of chloroplasts during a light-dark cycle are known to modulate the activity of many enzymes (38) and to influence the transcription of a variety of genes (38, 91). Redox changes in plants reverse the activity of the NPR1 protein, an essential regulator of plant systemic acquired resistance against pathogens (87). These few examples of how variable the effect of ROS on gene expression may be emphasize the importance of first identifying more precisely the targets and the modes of action of ROS during gene activation in plants under oxidative stress before a signaling role of a given ROS can be firmly established.

Depending on the character of the environmental stress, plants differentially enhance the release of ROS that are either chemically distinct or are generated within different cellular compartments (36, 91). For instance, during an incompatible plant-pathogen interaction, superoxide anions are produced enzymatically outside the cell and are rapidly converted to hydrogen peroxide that can cross the plasma membrane. The same ROS are also produced in chloroplasts exposed to high light stress, albeit by a different mechanism. The stress reactions of plants induced by pathogens differ from those induced by high light intensities (17, 52). If ROS act as signals that evoke these different stress responses, their biological activities should exhibit a high degree of selectivity and specificity that could be derived from their chemical identity and/or the intracellular locations where they were generated.

Most of what is known about oxidant-induced signaling was found in experiments using hydrogen peroxide as an oxidant. In some of these experiments hydrogen peroxide was added to cell cultures or plants either directly (31) or indirectly by using H_2O_2-generating enzymes (4). In both cases the intracellular distribution of H_2O_2 is difficult to control and the physiological significance of changes in gene expression that are induced under these conditions cannot easily be assessed. A more controlled release of H_2O_2 that is confined to specific compartments has become possible either by spraying plants with paraquat, a herbicide that under light generates H_2O_2 via superoxide predominantly within chloroplasts (79), or by modulating the expression of scavengers that are confined to different cellular compartments in transgenic plants (107, 121, 130).

If one accepts the notion that the chemical identity of a given ROS and the cellular topography of its release contribute to the specificity of a stress response, it is hard to imagine how H_2O_2 alone could act as a signal that triggers such specific responses unless differences in the intracellular distribution of H_2O_2-responsive targets mediate such specificities of stress responses. As mentioned above it is also possible that hydroxyl radicals derived from hydrogen peroxide are the primary triggers of some stress responses in plants. In contrast to hydrogen peroxide, hydroxyl radicals are very unstable and restricted to subcompartmental regions. They may give rise to biologically active oxygenation products that could preserve some of the topographical information that may be necessary to ensure specificity of stress responses.

Measuring global changes of gene expression in *Arabidopsis* cell cultures following treatment with hydrogen peroxide reveal major changes in gene expression (31). The surprisingly large number of genes that respond to an increase in hydrogen peroxide concentration would be in line with the proposed role of H_2O_2 as a ubiquitous signal for oxidative stress. However, the number of H_2O_2-responsive genes in cell culture was in marked contrast to that found in whole plants treated with low concentrations of paraquat (95). In the absence of visible necrotic lesions, very few genes were initially upregulated, some of which are intimately involved in the detoxification of H_2O_2, like ascorbate peroxidases I and II or ferritin I (62,

100). These genes were different from those activated by singlet oxygen, which suggests that chemical differences between the two ROS might have contributed to the selectivity of the induced stress responses (95). This apparent selectivity was lost when plants were exposed to higher paraquat concentrations that led to visible lesion formation. Under these stress conditions the primary cause for a given stress response may be difficult to separate from secondary effects that are superimposed.

This problem of discerning cause and effect has been highlighted in studies of tetrapyrrole biosynthesis and catabolism, in which several enzymatic steps were blocked experimentally (55, 72, 75, 84). A broad range of stress-related reactions were activated in these plants during illumination that have been attributed to the photodynamic activity of free tetrapyrrole intermediates that accumulated constitutively in these plants. However, many aspects of the responses are reminescent of those triggered by the release of superoxide and/or hydrogen peroxide during an incompatible plant-pathogen interaction (84). Either singlet oxygen, superoxide, and hydrogen peroxide may replace each other in triggering pathogen defense reactions, or the constitutive accumulation of photodynamically active tetrapyrrole intermediates and catabolites throughout the entire life cycle of these genetically modified plants may lead to photooxidative damage and injury that may promote a multifactorial induction of several overlapping secondary effects, some of which may mimic responses to pathogens. This latter interpretation agrees with in vivo measurements of ROS production in leaves under photooxidative stress, showing that singlet oxygen, superoxide, and hydrogen peroxide were produced simultaneously in the same leaf (41). If chemical specificity of a given ROS determines the specificity of stress responses, it would be difficult under these conditions to attribute a particular stress response to a well-defined ROS.

So far very few case studies suggest a selective signaling effect of a given ROS (58, 69, 95). This may be due partially to the fact that most of the previous studies have focused on analyzing the signaling role of hydrogen peroxide and—to a lesser extent—superoxide radicals, whereas other ROS such as hydroxyl radicals and singlet oxygen have been largely ignored. As described earlier, there are several lines of evidence suggesting that hydroxyl radicals may not only be a noxious side product of oxygen metabolism but may play a more significant role not only during oxidative stress but also during extension growth of roots, coleoptiles, and hypocotyls, or during seed germination. Singlet oxygen induces a specific set of stress responses (95). Its biological activity exhibits a high degree of selectivity that is derived from the chemical identity of this ROS and/or the intracellular location at which it is generated. These and other studies indicate that the biological activities of ROS may significantly differ from each other. Hence, "findings from experiments with hydrogen peroxide as a model oxidant should not be inappropriately generalized and taken as a fixed template for signaling events induced by all oxidants or by oxidative stress generated in a different way" (66).

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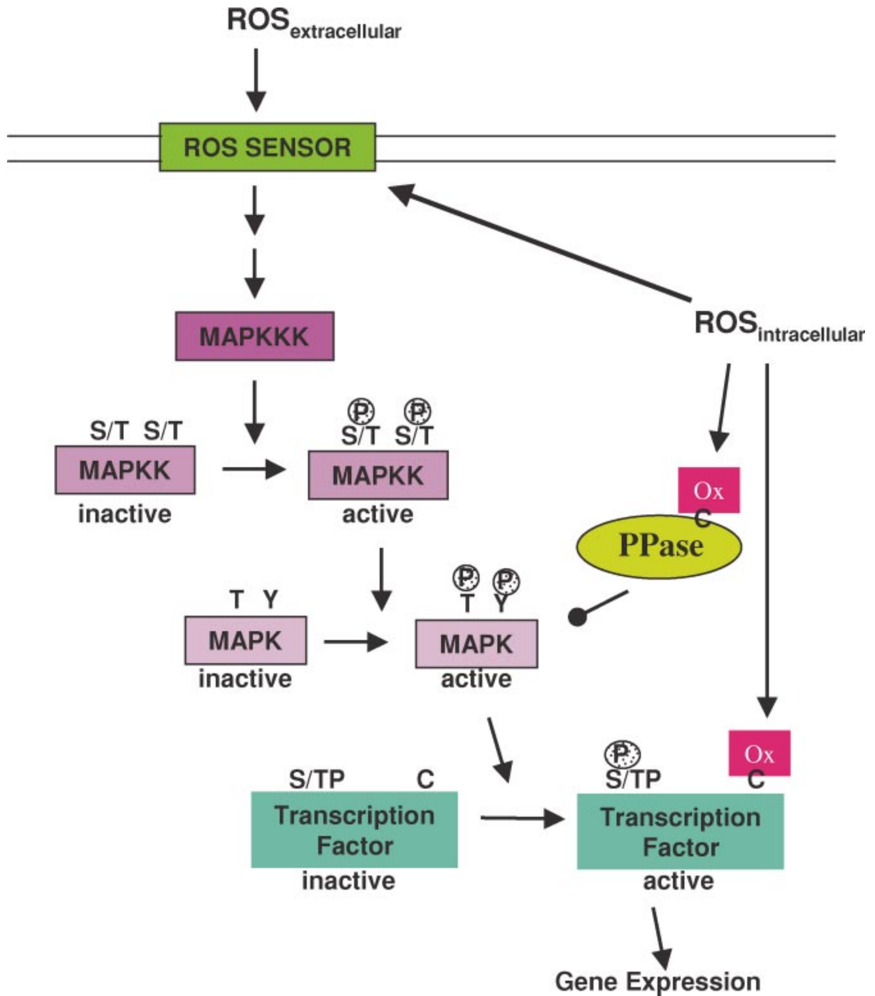

Figure 4 Schematic depiction of cellular ROS sensing and signaling mechanisms. ROS sensors such as membrane-localized histidine kinases can sense extracellular and intracellular ROS. Intracellular ROS can also influence the ROS-induced mitogen-activated protein kinase (MAPK) signaling pathway through inhibition of MAPK phosphatases (PPases) or downstream transcription factors. Whereas MAP kinases regulate gene expression by altering transcription factor activity through phosphorylation of serine and threonine residues, ROS regulation occurs by oxidation of cysteine residues.

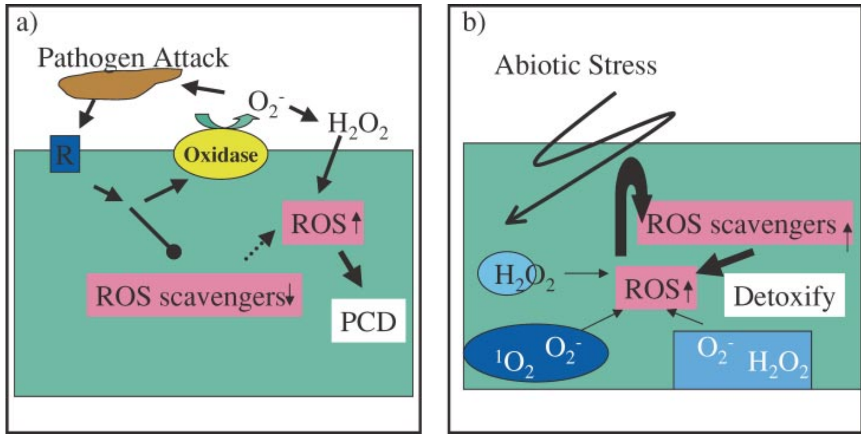

Figure 5 Different roles of ROS under conditions of (*a*) pathogen attack or (*b*) abiotic stress. Upon pathogen attack, receptor-induced signaling activates plasma membrane or apoplast-localized oxidases that produce superoxide radicals (O_2^-) that are highly toxic and can help to kill the invading pathogen. On the other hand, O_2^- is rapidly dismutated into hydrogen peroxide, which, in contrast to superoxide, can readily cross the plasma membrane. Intracellular ROS levels increase due not only to extracellular production of ROS but also by downregulation of ROS scavenging mechanisms. Overall, ROS amounts increase to critical levels and induce programmed cell death (PCD). During abiotic stress, ROS production occurs mainly in chloroplasts and mitochondria at the sites of electron transport, increasing intracellular ROS amounts to toxic levels. The cellular response encompasses upregulation of ROS scavenging mechanisms to detoxify increased amounts of ROS.