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Reactive oxygen gene network of plants

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Reactive oxygen species (ROS) control many different processes in plants. However, being toxic molecules, they are also capable of injuring cells. How this conflict is resolved in plants is largely unknown. Nonetheless, it is clear that the steady-state level of ROS in cells needs to be tightly regulated. In *Arabidopsis*, a network of at least 152 genes is involved in managing the level of ROS. This network is highly dynamic and redundant, and encodes ROS-scavenging and ROS-producing proteins. Although recent studies have unraveled some of the key players in the network, many questions related to its mode of regulation, its protective roles and its modulation of signaling networks that control growth, development and stress response remain unanswered.

Ever since the introduction of molecular oxygen (O_2) into our atmosphere by O_2-evolving photosynthetic organisms ~2.7 billion years ago, reactive oxygen species (ROS) have been the unwelcome companions of aerobic life [1]. In contrast to O_2, these partially reduced or activated derivatives of oxygen [singlet oxygen ($^1O_2$), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO\cdot$)] are highly reactive and toxic, and can lead to the oxidative destruction of cells [2]. Consequently, the evolution of all aerobic organisms has been dependent on the development of efficient ROS-scavenging mechanisms. In recent years, a new role for ROS has been identified: the control and regulation of biological processes, such as growth, cell cycle, programmed cell death, hormone signaling, biotic and abiotic stress responses and development [3–13]. These studies extend our understanding of ROS and suggest a dual role for ROS in plant biology as both toxic byproducts of aerobic metabolism and key regulators of growth, development and defense pathways.

The use of ROS as signaling molecules by plant cells suggests that, during the course of evolution, plants were able to achieve a high degree of control over ROS toxicity and are now using ROS as signaling molecules. Controlling ROS toxicity while enabling ROS such as H_2O_2 or O_2^- to act as signaling molecules appears to require a large gene network composed of at least 152 genes in *Arabidopsis*. This 'reactive oxygen gene network' of plants is described below.

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Modulation of ROS signaling by the reactive oxygen gene network of plants

Whereas Ca^{2+} signaling is predominantly controlled in plants by storage and release, ROS signaling is controlled by production and scavenging (Figure 1). Different developmental or environmental signals feed into the ROS signaling network and perturb ROS homeostasis in a compartment-specific or even cell-specific manner. Perturbed ROS levels are perceived by different proteins, enzymes or receptors and modulate different developmental, metabolic and defense pathways. ROS can be generated by various enzymatic activities, of which the best studied are NADPH oxidases, and removed by an array of ROS-scavenging enzymes (Table 1). The intensity, duration and localization of the different ROS signals are determined by interplay between the ROS-producing and ROS-scavenging pathways of the cell. This process requires a tight mode of regulation and might involve amplification and/or feedback inhibition loops. In addition to regulating the intensity and duration of the different ROS signals, the ROS-scavenging pathways are also responsible for maintaining a low steady-state baseline of ROS on which the different signals can be registered. The reactive oxygen gene network therefore modulates the steady-state level of ROS in the different cellular compartments for signaling purposes as well as for protection against oxidative damage.

It is possible that the use of ROS as versatile signaling molecules originated from their proposed use to sense stress. Most forms of biotic or abiotic stress disrupt the metabolic balance of cells, resulting in enhanced production of ROS. Simple organisms, such as bacteria or yeast, sense the enhanced production of ROS using redox-sensitive transcription factors and other molecular sensors, activate different ROS defense pathways, and regulate their metabolic pathways to lower the production rate of ROS [14,15]. This 'basic cycle' of ROS metabolism maintains a low steady-state level of ROS in cells. Variations on this pathway could have originated during evolution and contributed to the use of ROS as signaling molecules to control more specialized processes such as plant growth and defense, hormonal signaling, and development.

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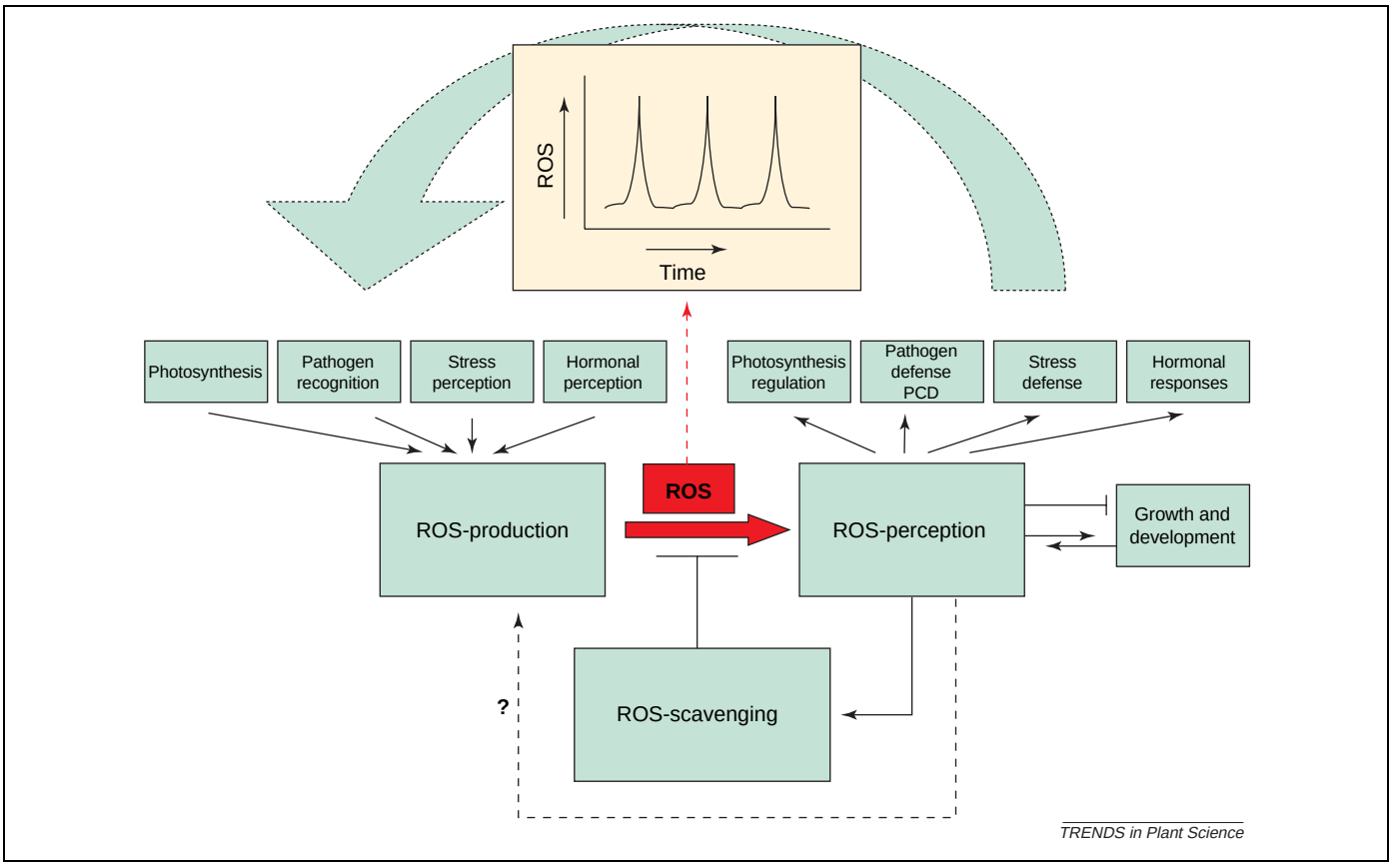


Figure 1. Modulation of reactive oxygen species (ROS) signaling by the reactive oxygen gene network of plants. Different cellular signals (e.g. pathogen recognition or stress perception) result in the enhanced production of ROS in cells by the ROS-producing pathways of the network. ROS are perceived by different ROS sensors and activate cellular responses (e.g. pathogen or stress defense). The intensity, duration and localization of the ROS signals are determined by interplay between the ROS-producing and the ROS-scavenging pathways. The ROS-scavenging pathways are also responsible for maintaining a low steady-state level of ROS on which the different signals can be registered. Modulation of ROS levels might also involve a positive feedback loop between ROS perception and ROS production (dashed line). In addition to activating or suppressing different cellular responses, ROS perception can affect growth and development (inhibition during stress or regulation during normal growth). Abbreviation: PCD, programmed cell death.

Production of ROS in plants

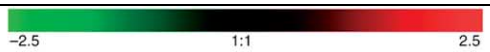
Organelles with a highly oxidizing metabolic activity or with an intense rate of electron flow, such as chloroplasts, mitochondria and microbodies, are a major source of ROS production in plant cells. Together with an extensive battery of oxidases, the plant cell is well armed for bountiful yet flexible ROS production. In chloroplasts, the primary sources of ROS production are the Mehler reaction and the antenna pigments [2]. Production of ROS by these sources is enhanced in plants by conditions limiting CO_2 fixation, such as drought, salt and temperature stress, as well as by the combination of these conditions with high-light stress. In C3 plants, limiting CO_2 conditions can also activate the photorespiratory pathway [16]. As part of this pathway, H_2O_2 is generated in peroxisomes by the enzymatic activity of glycolate oxidase. Production of H_2O_2 in microbodies can also occur during lipid catabolism as a side-product of fatty acid oxidation. In mitochondria, over-reduction of the electron transport chain is the main source of O_2^- production under specific stress conditions [17]. Additional sources of ROS in plant cells include the detoxifying reactions catalyzed by cytochromes in both the cytoplasm and the endoplasmic reticulum [18].

Plasma membrane NADPH-dependent oxidases, similar to the mammalian calcium-regulated NADPH oxidase (NOX5), have been the subject of intense investigation [19–23]. They are thought to play a key role in ROS signaling, and contain a multimeric flavocytochrome that forms an electron transport chain capable of reducing O_2 to O_2^-. Chemical inhibitors of mammalian NADPH oxidase (such as diphenylene iodinium) have been shown to block or impair ROS production during biotic or abiotic stress in plants [24–27]. Moreover, genes homologous to the mammalian subunit $gp91^{phox}$ have been identified in different plant genomes [28–31] (Table 1). In addition to NADPH oxidases, pH-dependent cell wall peroxidases, germin-like oxalate oxidases and amine oxidases have been proposed to generate ROS at the apoplast [32–34]. Although much attention has been given to NADPH oxidases and their possible role in cell signaling, other ROS-producing mechanisms in the mitochondria, apoplast and peroxisome are likely to play a role in ROS signaling in response to different stimuli or developmental signals.

Enzymatic components of the ROS-scavenging pathways of plants

Major ROS-scavenging enzymes of plants include superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX) and peroxiredoxin (PrxR) (Table 1). Together with the antioxidants ascorbic acid and glutathione [35], these enzymes provide

Table 1. Gene annotation and expression of the reactive oxygen species scavenging network of *Arabidopsis*[a]



Enzyme and reaction	Gene name	AGI code	Localization	AsCAT	KoAPX	KoSOD	Heat	Drought	Salt	Cold	HL
Superoxide dismutase (SOD)	FeSOD (FSD1)	At4g25100.3	**Chl**								
$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$	FeSOD (FSD2)	At5g51100.1	**Chl**								
	FeSOD (FSD3)	At5g23310.1	**Chl**								
	Cu/ZnSOD (CSD1)	At1g08830.1	**Cyt**								
	Cu/ZnSOD (CSD2)	At2g28190.1	**Chl**								
	Cu/ZnSOD (CSD3)	At5g18100.1	Per								
	MnSOD (MSD1)	At3g10920.1	**Mit**								
	MnSOD-like	At3g56350.1	Sec								
Ascorbate peroxidase (APX)	APX1	At1g07890.1	**Cyt**								
$2Asc + H_2O_2 \rightarrow 2MDA + 2H_2O$	APX2	At3g09640.1	**Cyt**				*				
	APX3	At4g35000.1	**Per**, chl								
	APX4	At4g09010.1	**Chl**								
	APX5	At4g35970.1	Per				*				*
	APX6	At4g32320.1	Cyt, chl, mit								
	APX7	At1g33660.1	Mit								*
	Stromal-APX	At4g08390.2	**Chl, mit**								
	Thylakoid-APX	At1g77490.1	**Chl**								
Monodehydroascorbate reductase (MDAR)	MDAR1	At1g63940.4	**Chl, mit**								
$MDA + NAD(P)H + H^+ \rightarrow Asc + NAD(P)^-$	MDAR2	At3g09940.1	Cyt				*	*			
	MDAR3	At3g27820.1	Cyt, mit								
	MDAR4	At3g52880.1	Cyt								
	MDAR5	At5g03630.1	Cyt								
Dehydroascorbate reductase (DHAR)	DHAR1	At5g16710.1	Chl, mit								
$DHA + 2GSH \rightarrow Asc + GSSG$	DHAR2	At5g36270.1	Cyt								*
	DHAR3	At1g75270.1	Cyt, chl								
	DHAR4	At1g19550.1	Cyt, chl								
	DHAR5	At1g19570.1	Cyt, chl								
Glutathione reductase (GR)	GR1	At3g24170.1	**Cyt**				*				
$GSSG + NAD(P)H \rightarrow 2GSH + NAD(P)^-$	GR2	At3g54660.1	**Chl, mit**								
Catalase (Cat)	Cat1	At1g20630.1	**Per**								
$2H_2O_2 \rightarrow 2H_2O + O_2$	Cat2	At4g35090.1	**Per**								
	Cat3	At1g20620.1	**Per**								
Glutathione peroxidase (GPX)	GPX1	At2g25080.1	Chl								
$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$	GPX2	At2g31570.1	Cyt, chl								
	GPX3	At2g43350.1	Mit								
	GPX4	At2g48150.1	Cyt								
	GPX5	At3g63080.1	ER								
	GPX7	At4g31870.1	Chl								
	GPX8	At1g63460.1	Cyt, chl								
	Phospholipid GPX6	At4g11600.1	Chl, mit								
Ferritin	Ferritin 1	At5g01600.1	**Chl**								
$Fe + P \rightarrow P\text{-}Fe$	Ferritin 2	At3g56090.1	Chl, mit								
	Ferritin 3	At2g40300.1	Chl, mit								
	Ferritin 4	At3g11050.1	Chl							*	*
NADPH oxidase	NADPH oxidase (RbohA)	At5g07390.1	Mem								
$NADPH + e^- + O_2 \rightarrow NADP^- + O_2^- + H^+$	NADPH oxidase (RbohB)	At1g09090.2	Mem								
ROS producer	NADPH oxidase (RbohC)	At5g51060.1	Mem								
	NADPH oxidase (RbohD)	At5g47910.1	Mem								
	NADPH oxidase (RbohE)	At1g19230.1	Mem								
	NADPH oxidase (RbohF)	At1g64060.1	Mem		*	*		*			
	NADPH oxidase (RbohG)	At4g25090.1	Mem								
	NADPH oxidase (RbohH)	At5g60010.1	Mem		*				*		*
	NADPH oxidase (RbohI)	At4g11230.1	Mem								
	NADPH oxidase (RbohJ)	At3g45810.1	Mem								
NADPH oxidase-like	See supplementary material (9 genes)										
Alternative oxidase (AOX)	AOX putative	At1g32350.1	**Mit**					*			
$2e^- + 2H^+ + O_2 \rightarrow H_2O$	AOX1A	At3g22370.1	**Mit**								
	AOX1B	At3g22360.1	**Mit**								
	AOX1C	At3g27620.1	**Mit**							*	
	AOX2	At5g64210.1	**Mit**								
	Immutans	At4g22260.1	**Chl**								
Peroxiredoxin (PrxR)	1-cys PrxR	At1g48130.1	Nuc								
$2P\text{-}SH + H_2O_2 \rightarrow P\text{-}S\text{-}S\text{-}P + 2H_2O$	2-cys PrxR A	At3g11630.1	**Chl**								
	2-cys PrxR B	At5g06290.1	Chl								
	2-cys PrxR F	At3g06050.1	**Mit**								
	PrxR Q	At3g26060.1	Chl								
	Type 2 PrxR -related	At3g03405.1	Cyt								
	Type 2 PrxR A	At1g65990.1	Mem, chl								
	Type 2 PrxR B	At1g65980.1	Cyt								
	Type 2 PrxR C	At1g65970.1	Cyt								
	Type 2 PrxR D	At1g60740.1	Cyt								
	Type 2 PrxR E	At3g52960.1	**Chl**, mit								
Thioredoxins (Trx)	See supplementary material (31 genes)										
$P\text{-}S\text{-}S\text{-}P + 2H^+ \rightarrow 2P\text{-}SH$											
Glutaredoxin (GLR)	See supplementary material (27 genes)										
$DHA + 2GSH \rightarrow Asc + GSSG$											

[a]Main reactive oxygen species (ROS) genes and their corresponding enzymatic activities are listed (a complete list can be viewed as supplementary material in the on-line version). With the exception of NADPH oxidase (a ROS producer), all genes included in the table encode ROS-scavenging enzymes. Arabidopsis genome initiative (AGI) codes were obtained from the TIGR annotation database. Predicted subcellular localization was determined with Ipsort, Predotar and Targetp. When in bold, localization was based on published data and on recent studies on the chloroplast and mitochondrial proteomes [78,79]. Relative expression data (mutant to wild-type or stress to control; right columns) visualized with Genesis software [80] was obtained from DNA chip analyses (Arabidopsis ATH1 Affymetrix chips). Comparative analysis of samples was performed using Affymetrix MAS5.0 software and the Silicon Genetics GeneSpring version 5.1. Each column represents the different mutants or stress conditions (AsCAT, antisense Cat2; KoAPX, knockout APX1; KoSOD, knockout CSD2). Red indicates an increase, green indicates a decrease. Positive and negative values indicate upregulation or downregulation compared with the control line or unstressed condition, respectively. Cells containing asterisks have one absent call (i.e. no detectable expression) in either control, mutant or stressed situation. Gray cells indicate absent calls in both samples. White cells indicate that the genes are not represented on the ATH1 GeneChip. Abbreviations: chl, chloroplast; cyt, cytosol; ER, endoplasmic reticulum; HL, high light; mem, membrane; mit, mitochondria; nuc, nuclei; per, peroxisomes.

cells with highly efficient machinery for detoxifying O_2^- and H_2O_2. The balance between SODs and the different H_2O_2-scavenging enzymes in cells is considered to be crucial in determining the steady-state level of O_2^- and H_2O_2. This balance, together with the sequestering of metal ions by ferritin and other metal-binding proteins, prevents the formation of the highly toxic HO· radical via the metal-dependent Haber–Weiss reaction or the Fenton reaction [2,36]. The cellular pools of the antioxidants ascorbic acid and glutathione are maintained in their reduced state by a set of enzymes capable of using NAD(P)H to regenerate oxidized glutathione or ascorbic acid (e.g. monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase; Table 1). Although dehydroascorbate reductases and glutaredoxins are indicated in Table 1 as capable of reducing dehydroascorbic acid, many other enzymes in plants can catalyze this reaction with different efficiencies [37]. In addition, monodehydroascorbate radicals can be reduced back into ascorbic acid via ferredoxin using electrons diverted from the photosynthetic apparatus in the water–water cycle in chloroplasts [38] (Figure 2). Scavenging of H_2O_2 can also be mediated in plants by 'classical' plant peroxidases (class III) using a range of reductants (not included in Table 1). These enzymes are encoded by a large gene family of at least 73 genes in *Arabidopsis* and are found in the cytosol, vacuole, apoplast or cell wall [39,40]. Although transcriptome analysis of knockout and antisense plants deficient in APX1 or CAT2 indicated that the steady-state level of transcripts that encode certain classical plant peroxidases is elevated in these plants, the significance of these findings is unknown at present. Additional research is needed to determine whether specific class III plant peroxidases contribute to the ROS-scavenging capacity of cells and should be included in the ROS gene network of plants.

Substrate affinity, reaction rate and enzyme concentration are important parameters when assessing the relative contribution of the different enzymes shown in Table 1 to ROS detoxification [6]. Under optimal conditions, a combination of these parameters allows enzymes such as the CuZnSOD to have a diffusion-limited reaction rate ($2\times10^9\ M^{-1}\ s^{-1}$) [2]. Membranes are highly susceptible to oxidative stress. In plant cells, they are protected by the activity of specific phospholipid glutathione peroxidases and by α-tocopherol (vitamin E), which is kept in its reduced state by the pool of reduced ascorbic acid [41–43]. Protection of cells against $^1O_2$ is generally believed to be mediated by carotenoids. Although it would be interesting to explore how these pathways, as well as those involved in the biosynthesis of ascorbate and glutathione, and those responsible for the repair of oxidative damage in cells, are linked to the genes described in Table 1, these mechanisms are not described here [20,35,44,45].

Cellular localization and coordination of the ROS-scavenging pathways of plants

The various scavenging enzymes encoded by the ROS network can be found in almost every subcellular compartment (Figure 2). In addition, usually more than one enzymatic scavenging activity per a particular ROS can be found in each of the different compartments (e.g. GPXs, PrxRs and APXs in the cytosol and chloroplast, and APXs and CATs in peroxisomes; Figure 2). When the relative function of the different enzymes in the different cellular compartments is considered, it is important to remember that ROS such as H_2O_2 can diffuse between different compartments [46]. Furthermore, transporters for the antioxidants ascorbic acid and glutathione are likely to be central in determining the specific concentrations of these compounds and the redox potential in the different cellular compartments [35,47]. An anonymous player in the ROS signaling network is the vacuole. Its ROS-scavenging and ROS-producing potentials are unknown. It is possible that this organelle, because of its relatively large cellular volume, plays an unanticipated essential role in the control of ROS metabolism in plants. Likewise, the antioxidant capacity and signaling role of the apoplast and peroxisomes have only recently begun to gain recognition [27,47,48]. However, the specific enzymes and genes involved in controlling ROS metabolism in these compartments are only partially known (Table 1; Figure 2).

Recent studies in *Arabidopsis* have suggested that the mode of coordination between different components of the ROS removal network of plants is complex [49,50]. For example, the application of light stress to *Arabidopsis* results in the induction of cytosolic and not chloroplastic defense enzymes [49,51,52], even though most ROS produced during light stress are thought to be generated in chloroplasts or peroxisomes [38]. In addition, at least three different enzymes of the ROS-scavenging pathways of plants have been found to be targeted to both the chloroplast and mitochondria, suggesting a high degree of coordination in defense responses between these different cellular compartments [37,53,54]. Future studies with knockout lines for the different ROS-scavenging or ROS-producing enzymes would reveal how the different branches of the ROS network co-operate during biotic and abiotic stresses.

Gene annotation and expression of the ROS network in *Arabidopsis*

Table 1 and the table in the supplementary material (available in the on-line version) summarize all known ROS-scavenging genes and NADPH oxidases in *Arabidopsis*. Expression data for the different genes in three different knockout or antisense lines (Apx1, CSD2 and Cat2) and in plants subjected to different abiotic stresses (e.g. drought, salt, cold or high light) are also included. Although data were assembled from different experiments and should only be considered from a qualitative point of view, Table 1 highlights two fundamental principles of a genetic network: redundancy and flexibility. Redundancy is evident from the transcript expression in the different knockouts. Thus, deficiency in Apx1 (KoAPX; Table 1) results in the enhanced expression of a type 2 PrxR (E) and a ferritin; deficiency in Cat2 (AsCAT; Table 1) results in the enhanced expression of a copper-binding protein, glutaredoxin and thioredoxin; and deficiency in CSD2 (KoSOD; Table 1) results in the enhanced expression of FeSODs, ferritin and CATs. Flexibility is evident from transcript expression in response to different stresses, as

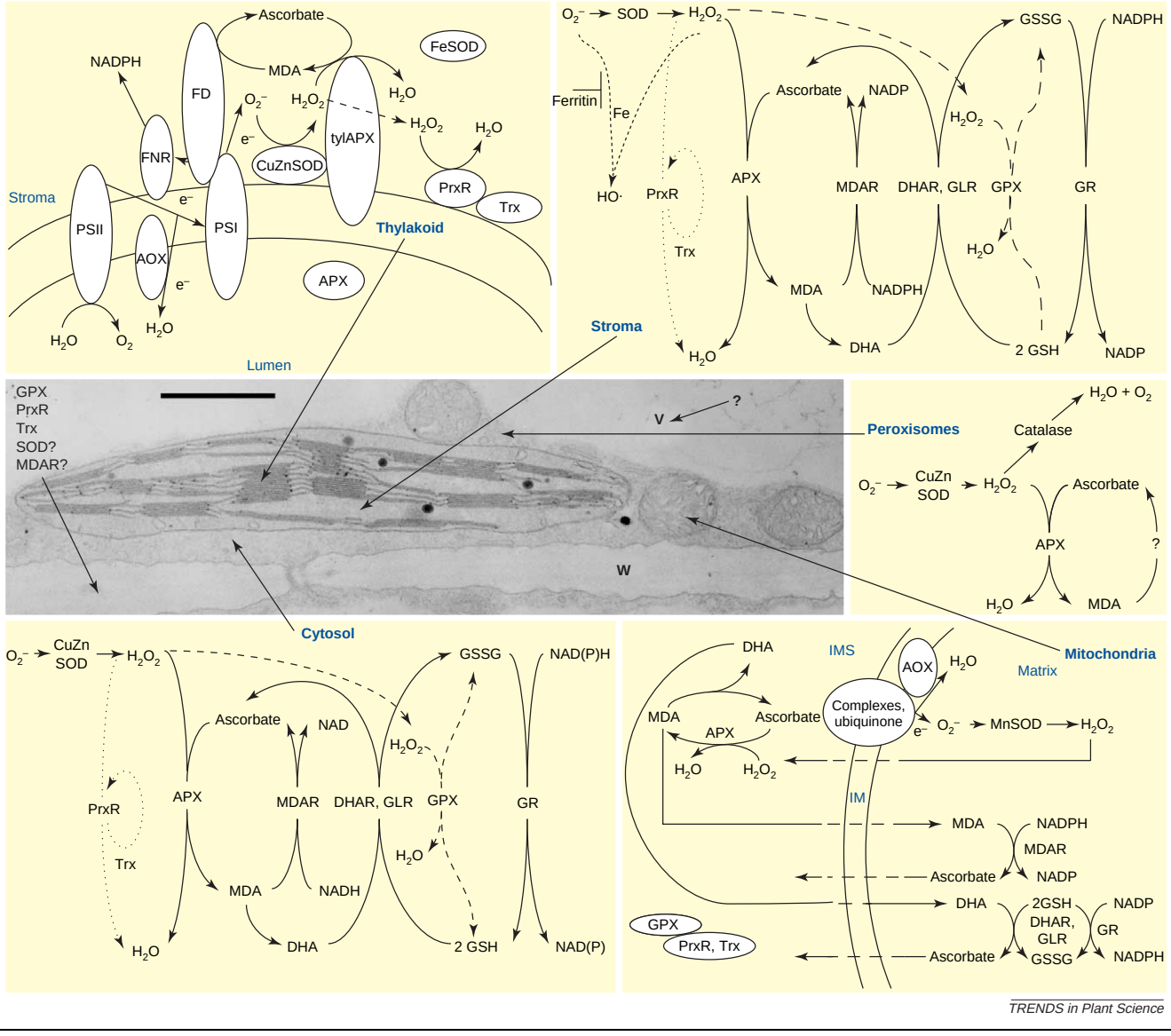


Figure 2. Localization of reactive oxygen species (ROS) scavenging pathways in plant cells. A transmission electron micrograph of a portion of a plant cell is used to demonstrate the relative volumes of the different cellular compartments and their physical separation (middle left). The enzymatic pathways responsible for ROS detoxification are shown. The water–water cycle detoxifies O_2^- and H_2O_2, and alternative oxidase (AOX; *Immutans*) reduces the production rate of O_2^- in thylakoids [top left; in some plants iron superoxide dismutase (FeSOD) might replace CuZnSOD in the chloroplast]. ROS that escape this cycle and/or are produced in the stroma undergo detoxification by SOD and the stromal ascorbate–glutathione cycle. Peroxiredoxin (PrxR) and glutathione peroxidase (GPX) are also involved in H_2O_2 removal in the stroma (top right). ROS produced in peroxisomes during photorespiration, fatty acid oxidation or other reactions are decomposed by SOD, catalase (CAT) and ascorbate peroxidase (APX) (middle right). SOD and other components of the ascorbate–glutathione cycle are also present in mitochondria. In addition, AOX prevents oxidative damage in mitochondria (bottom right). In principle, the cytosol contains the same set of enzymes found in the stroma (bottom left). However, these are encoded by a different set of genes and the major iron-chelating activity in the cytosol responsible for preventing the formation of HO· radicals is unknown. The enzymatic components responsible for ROS detoxification in the apoplast and cell wall (W) are only partially known, and the ROS-scavenging pathways at the vacuole (V) are unknown. Membrane-bound enzymes are depicted in white, GPX pathways are indicated by dashed lines and PrxR pathways are indicated by dotted lines in the stroma and cytosol. Although the pathways in the different compartments are mostly separated from each other, H_2O_2 can easily diffuse through membranes and antioxidants such as glutathione and ascorbic acid (reduced or oxidized) can be transported between the different compartments. Abbreviations: DHA, dehydroascrobate; DHAR, DHA reductase; FD, ferredoxin; FNR, ferredoxin NADPH reductase; GLR, glutaredoxin; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; IM, inner membrane; IMS, IM space; MDA, monodehydroascorbate; MDAR, MDA reductase; PSI, photosystem I; PSII, photosystem II; Trx, thioredoxin; tyl, thylakoid.

the ROS network responds in a highly specific manner to each of the different stresses included in Table 1.

Several studies have shown that biotic and abiotic stresses are accompanied by an oxidative burst mediated by NADPH oxidases [26,55]. Changes in the expression of NADPH oxidases provoked through perturbed ROS homeostasis by internal (knockouts) or external stresses suggest that transcriptional activation of certain NADPH oxidases is an essential intermediate step in the activation or amplification of defense responses [27,50,56,57].

When using changes in transcript expression as a measure for how important a specific gene is in protecting cells against ROS or other stresses, it is important to remember that, although many genes can play a key role in cell protection, their expression might not respond to stress. For example, transcript expression for *CSD2* or

chlAOX (*Immutans*) shows no response to the different stresses (Table 1). However, analysis of knockouts for these genes revealed that they play a pivotal role in chloroplast protection in the presence or absence of abiotic stress [50,58].

Key components of the reactive oxygen gene network identified by reverse genetics

Recent studies of knockout and antisense lines for Cat2, Apx1, chlAOX, mitAOX, CSD2, 2-cysteine PrxR and various NADPH oxidases have revealed a strong link between ROS and processes such as growth, development, stomatal responses and biotic and abiotic stress responses [7,8,50,52,57,59–62]. These findings demonstrate the complex nature of the ROS gene network in plants and its modulation of key biological processes. Although mutants for all the proteins listed above are viable, demonstrating the redundancy of the ROS network, a clear phenotype is associated with each of the different genes, suggesting that they play a key role in the ROS signaling network of plants.

Based on the analysis of the different mutants, Cat2, Apx1, ChlAOX, CSD2 and 2-cysteine PrxR are essential for the protection of chloroplasts against oxidative damage. Suppression of CSD2, for example, results in the induction of a high-light stress response in *Arabidopsis* plants grown under a low light intensity [50]. The absence of Apx1 results in reduced photosynthetic activity, augmented induction of heat shock proteins during light stress and altered stomatal responses [52]. Catalase deficiency triggers growth retardation and high sensitivity to ozone and high light stress [57]. By contrast, the absence of the NADPH oxidase genes *AtrbohD* and *AtrbohF* suppresses ROS production and the defense response of *Arabidopsis* against pathogen attack [7], and knockout of *AtrbohC* has an altered root phenotype [8]. *AtrbohD* and *AtrbohF* are also essential for abscisic acid signaling in guard cells [10]. In tomato, suppression of NADPH oxidase results in a highly branched phenotype and fasciated reproductive organs [62]. Characterization of additional mutants and double or triple knockouts for different genes in the network should reveal additional roles for the ROS signaling network of plants and unravel more links between ROS and different signaling pathways. These studies might also identify alternative or redundant components of the network that are unknown at present [63].

ROS signal transduction pathway of plants

Recent studies in *Arabidopsis* have uncovered some of the key components involved in the ROS signal transduction pathway of plants. Although the receptors for ROS are unknown at present, it has been suggested that plant cells sense ROS via at least three different mechanisms (Figure 3): (i) unidentified receptor proteins; (ii) redox-sensitive transcription factors, such as NPR1 or HSFs; and (iii) direct inhibition of phosphatases by ROS [6,13,20,64].

Downstream signaling events associated with ROS sensing involve Ca^{2+} and Ca^{2+}-binding proteins, such as

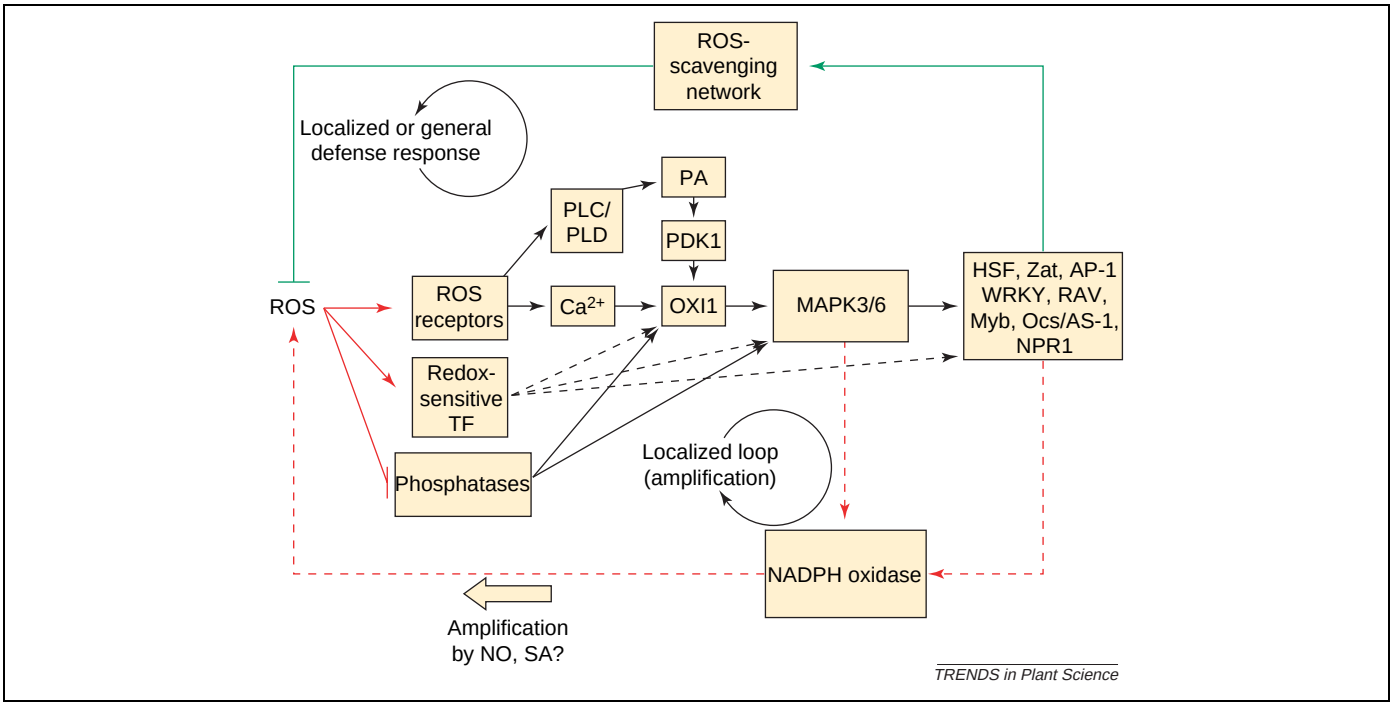


Figure 3. Generalized model of the reactive oxygen species (ROS) signal transduction pathway. ROS can be detected by at least three mechanisms (ROS receptors, redox-sensitive transcription factors and phosphatases). Detection of ROS by receptors results in the generation of Ca^{2+} signals and the activation of a phospholipase C/D (PLC/PLD) activity that generates phosphatidic acid (PA). PA and Ca^{2+} are thought to activate the protein kinase OXI1. Activation of OXI1 results in the activation of a mitogen-activated-protein kinase (MAPK) cascade (MAPK3/6) and the induction or activation of different transcription factors that regulate the ROS-scavenging and ROS-producing pathways. The activation or inhibition of redox-sensitive transcription factors by ROS might also affect the expression of OXI1 or other kinases and/or the induction of ROS-specific transcription factors. Inhibition of phosphatases by ROS might result in the activation of kinases such as OXI1 or MAPK3/6. Two different loops are shown to be involved in the ROS signal transduction pathway. A localized or general defense response (a negative feedback loop; solid green line) can be activated to suppress ROS, whereas a localized amplification loop (positive feedback loop; red dashed line) can be activated to enhance ROS signals via the activity of NADPH oxidases. Salicylic acid (SA) and nitric oxide (NO) might be involved in this amplification loop as enhancers. Abbreviations: HSF, heat shock factor; PDK, phosphoinositide-dependent kinase; TF, transcription factor.

calmodulin [65–67], the activation of G-proteins [68], and the activation of phospholipid signaling, which results in the accumulation of phosphatidic acid [69,70]. It is possible that the localization of ROS signals in specific cellular sites is similar to that of Ca^{2+} signals in response to stimuli [67]. The development of intercellular ROS sensors analogous to the fluorescence-based protein sensors for Ca^{2+} would help considerably in studying the spatial and temporal nature of ROS signaling in plants.

A recently identified serine/threonine protein kinase (OXI1) has been shown to play a central role in ROS sensing and the activation of mitogen-activated-protein kinases (MAPKs) 3 and 6 by Ca^{2+} [70]. This kinase is also activated by PDK1 through the phospholipase-C/D–phosphatidic-acid pathway [69]. A MAPK cascade involving MAPK3/6 acts downstream of OXI1 and controls the activation of different defense mechanisms in response to ROS stress [3,20]. The expression of different transcription factors is enhanced by ROS and includes members of the WRKY, Zat, RAV, GRAS and Myb families [50,52,56,57,59,64,71–73]. Recent studies using knockout plants have revealed that the zinc-finger protein Zat12 is required for *Apx1* expression and plant protection during oxidative stress [56], and that the highly conserved zinc-finger paralogs LOL1 and LSD1 have antagonistic effects on SOD and O_2^- accumulation [59].

The possible existence of positive amplification loops involving NADPH oxidases in ROS signaling has recently been suggested by pharmacological and genetic studies [27,56]. These loops might be activated by low levels of ROS and result in enhanced production and amplification of the ROS signals in specific cellular locations (Figure 3, localized amplification loop, red broken lines). The accumulation of ROS in cells might activate the ROS-scavenging pathways and result in the suppression of ROS in specific cellular locations or the entire cell (Figure 3, localized or general response, solid green lines). As indicated above, the interplay between the ROS-producing and ROS-scavenging pathways will determine the intensity, duration and localization of the ROS signals (Figures 1, 3). Although O_2^- and H_2O_2 have been considered to play key roles as ROS signal transduction molecules, recent studies have pointed to the existence of a $^1O_2$-specific signaling pathway [20,74].

Taking into account the complex nature of the ROS gene network (Figure 2, Table 1) and its integration into the web of plant signaling networks (Figure 1), we face a major challenge in dissecting the genetic network that controls ROS signaling in plants (Box 1). Large-scale transcriptome analyses coupled with proteomic and metabolomic analysis of plants perturbed at the levels of individual or multiple components of the ROS network will be essential for future studies. Integration of results obtained from these studies with the development of computer models [75] and bioinformatics tools [76,77] should enable us to gain a system-level understanding of ROS metabolism in plants.

Box 1. Questions and future challenges

- What are the sensors for reactive oxygen species (ROS) in plants?
- How are the different cellular signaling networks linked to the ROS gene network?
- How specific is the effect of different subcellular ROS signals and how are NADPH oxidases involved in generating these signals?
- What are the roles of the vacuole and the apoplast in ROS metabolism and signaling?
- How are transporters of ascorbic acid and glutathione (reduced or oxidized) involved in ROS metabolism and scavenging?
- How are salicylic acid and nitric oxide involved in ROS signaling during abiotic stress?

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Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tplants.2004.08.009

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