Oxidative stress, antioxidants and stress tolerance

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Traditionally, reactive oxygen intermediates (ROIs) were considered to be toxic by-products of aerobic metabolism, which were disposed of using antioxidants. However, in recent years, it has become apparent that plants actively produce ROIs as signaling molecules to control processes such as programmed cell death, abiotic stress responses, pathogen defense and systemic signaling. Recent advances including microarray studies and the development of mutants with altered ROI-scavenging mechanisms provide new insights into how the steady-state level of ROIs are controlled in cells. In addition, key steps of the signal transduction pathway that senses ROIs in plants have been identified. These raise several intriguing questions about the relationships between ROI signaling, ROI stress and the production and scavenging of ROIs in the different cellular compartments.

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Reactive oxygen intermediates (ROIs) are partially reduced forms of atmospheric oxygen (O_2). They typically result from the excitation of O_2 to form singlet oxygen (O_2^1) or from the transfer of one, two or three electrons to O_2 to form, respectively, a superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) or a hydroxyl radical (HO^-). In contrast to atmospheric oxygen, ROIs are capable of unrestricted oxidation of various cellular components and can lead to the oxidative destruction of the cell [1–4].

Production of ROIs in cells

There are many potential sources of ROIs in plants (Table 1). Some are reactions involved in normal metabolism, such as photosynthesis and respiration. These are in line with the traditional concept, considering ROIs as unavoidable byproducts of aerobic metabolism [1]. Other sources of ROIs belong to pathways enhanced during abiotic stresses, such as glycolate oxidase in peroxisomes during photorespiration. However, in recent years, new sources of ROIs have been identified in plants, including NADPH oxidases, amine oxidases and cell-wall-bound peroxidases. These are tightly regulated and participate in the production of ROIs during processes such as programmed cell death (PCD) and pathogen defense [2,4,5].

Whereas, under normal growth conditions, the production of ROIs in cells is low (240 $\mu M\ s^{-1}$ O_2^- and a steady-state level of 0.5 μM H_2O_2 in chloroplasts) [6], many stresses that disrupt the cellular homeostasis of cells enhance the production of ROIs (240–720 $\mu M\ s^{-1}$ O_2^- and a steady-state level of 5–15 μM H_2O_2) [6]. These include drought stress and desiccation, salt stress, chilling, heat shock, heavy metals, ultraviolet radiation, air pollutants such as ozone and SO_2, mechanical stress, nutrient deprivation, pathogen attack and high light stress [2,7–10]. The production of ROIs during these stresses results from pathways such as photorespiration, from the photosynthetic apparatus and from mitochondrial respiration. In addition, pathogens and wounding or environmental stresses (e.g. drought or osmotic stress) have been shown to trigger the active production of ROIs by NADPH oxidases [4,11–13]. The enhanced production of ROIs during stress can pose a threat to cells but it is also thought that ROIs act as signals for the activation of stress-response and defense pathways [9,14]. Thus, ROIs can be viewed as cellular indicators of stress and as secondary messengers involved in the stress-response signal transduction pathway.

Although the steady-state level of ROIs can be used by plants to monitor their intracellular level of stress, this level has to be kept under tight control because over-accumulation of ROIs can result in cell death [1–4]. ROI-induced cell death can result from oxidative processes such as membrane lipid peroxidation, protein oxidation, enzyme inhibition and DNA and RNA damage (the traditional concept). Alternatively, enhanced levels of ROIs can activate a PCD pathway, as was recently demonstrated by the inhibition of oxidative stress (paraquat)-induced cell death in tobacco by anti-apoptotic genes [15].

Because ROIs are toxic but also participate in signaling events, plant cells require at least two different mechanisms to regulate their intracellular ROI concentrations by scavenging of ROIs: one that will enable the fine modulation of low levels of ROIs for signaling purposes, and one that will enable the detoxification of excess ROIs, especially during stress. In addition, the types of ROIs produced and the balance between the steady-state levels of different ROIs can also be important. These are determined by the interplay between different ROI-producing and ROI-scavenging mechanisms, and can change drastically depending upon the physiological condition of the plant and the integration of different environmental, developmental and biochemical stimuli.

Scavenging of ROIs in cells

Major ROI-scavenging mechanisms of plants include superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) [1,7,16] (Table 1). The balance between SOD and APX or CAT activities in cells is crucial for determining the steady-state level of

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Table 1. Producing, scavenging and avoiding reactive oxygen intermediates in plants[a]

Mechanism	Localization	Primary ROI	Refs
Production			
Photosynthesis ET and PSI or II	Chl	O_2^-	[1,3]
Respiration ET	Mit	O_2^-	[2,25]
Glycolate oxidase	Per	H_2O_2	[31]
Excited chlorophyll	Chl	O_2^1	[1]
NADPH oxidase	PM	O_2^-	[4,5]
Fatty acid β-oxidation	Per	H_2O_2	[31]
Oxalate oxidase	Apo	H_2O_2	[2
Xanthine oxidase	Per	O_2^-	[31]
Peroxidases, Mn^{2+} and NADH	CW	H_2O_2, O_2^-	[4,5]
Amine oxidase	Apo	H_2O_2	[46]
Scavenging			
Superoxide dismutase	Chl, Cyt, Mit, Per, Apo	O_2^-	[7]
Ascorbate peroxidase	Chl, Cyt, Mit, Per, Apo	H_2O_2	[1,3]
Catalase	Per	H_2O_2	[16]
Glutathione peroxidase	Cyt,	H_2O_2, ROOH	[47]
Peroxidases	CW, Cyt, Vac	H_2O_2	[1]
Thioredoxin peroxidase	Chl, Cyt, Mit	H_2O_2	[48]
Ascorbic acid	Chl, Cyt, Mit, Per, Apo	H_2O_2, O_2^-	[3,8]
Glutathione	Chl, Cyt, Mit, Per, Apo	H_2O_2	[3,8]
α-Tocopherol	Membranes	ROOH, O_2^1	[1]
Caretenoids	Chl	O_2^1	[1]
Avoidance			
Anatomical adaptations	Leaf structure, epidermis	O_2^-, H_2O_2, O_2^1	[44,49]
C_4 or CAM metabolism	Chl, Cyt, Vac	O_2^-, H_2O_2	[49]
Chl movement	Cyt	O_2^-, H_2O_2, O_2^1	[44]
Suppression of photosynthesis	Chl	O_2^-, H_2O_2	[49]
PS and antenna modulations	Chl	O_2^-, O_2^1	[44]
Alternative oxidases	Chl, Mit	O_2^-	[25,50]

[a]Abbreviations: Apo, apoplast; Chl, chloroplast; CW, cell wall; Cyt, cytosol; ET, electron transport; Mit, mitochondria; O_2^1, singlet oxygen; Per, peroxisome; PM, plasma membrane; PS, photosystem; ROI, reactive oxygen intermediate; Vac, vacuole.

superoxide radicals and hydrogen peroxide [17]. This balance, together with sequestering of metal ions, is thought to be important to prevent the formation of the highly toxic hydroxyl radical via the metal-dependent Haber–Weiss or the Fenton reactions [1]. The different affinities of APX (μM range) and CAT (mM range) for H_2O_2 suggest that they belong to two different classes of H_2O_2-scavenging enzymes: APX might be responsible for the fine modulation of ROIs for signaling, whereas CAT might be responsible for or the removal of excess ROIs during stress.

The major ROI-scavenging pathways of plants (Fig. 1) include SOD, found in almost all cellular compartments, the water–water cycle in chloroplasts (Fig. 1a), the ascorbate–glutathione cycle in chloroplasts, cytosol, mitochondria, apoplast and peroxisomes (Fig. 1b), glutathione peroxidase (GPX; Fig. 1c), and CAT in peroxisomes (Fig. 1d). The finding of the ascorbate–glutathione cycle in almost all cellular compartments tested to date, as well as the high affinity of APX for H_2O_2, suggests that this cycle plays a crucial role in controlling the level of ROIs in these compartments. By contrast, CAT is only present in peroxisomes, but it is indispensable for ROI detoxification during stress, when high levels of ROIs are produced [16]. In addition, oxidative stress causes the proliferation of peroxisomes [18]. Drawing upon the model for bacteria [19], a dense population of peroxisomes might be highly efficient in scavenging of ROIs, especially H_2O_2, which diffuses into peroxisomes from the cytosol.

The water–water cycle (Fig. 1a) draws its reducing energy directly from the photosynthetic apparatus [3]. Thus, this cycle appears to be autonomous with respect to its energy supply. However, the source of reducing energy for ROI scavenging by the ascorbate–glutathione cycle (Fig. 1b) during normal metabolism and particularly during stress, when the photosynthetic apparatus might be suppressed or damaged, is not entirely clear. In animals and yeast, the pentose-phosphate pathway is the main source of NADPH for ROI removal [20,21]. Because CAT does not require a supply of reducing equivalents for its function, it might be insensitive to the redox status of cells and its function might not be affected during stress, unlike the other mechanisms (Fig. 1).

Antioxidants such as ascorbic acid and glutathione, which are found at high concentrations in chloroplasts and other cellular compartments (5–20 mM ascorbic acid and 1–5 mM glutathione) are crucial for plant defense against oxidative stress [8]. Consequently, both mutants with suppressed ascorbic acid levels [22] and transgenic plants with altered content of glutathione [23] are hypersensitive to stress conditions. It is generally believed that maintaining a high reduced per oxidized ratio of ascorbic acid and glutathione is essential for the proper scavenging of ROIs in cells. This ratio is maintained by glutathione reductase (GR), monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR) using NADPH as reducing power (Fig. 1) [3,8]. In addition, the overall balance between different antioxidants has to be tightly controlled. Enhanced glutathione biosynthesis in chloroplasts can result in oxidative damage to cells rather than their protection, possibly by altering the overall redox state of chloroplasts [23]. It has also been suggested that the oxidized:reduced ratio of the different antioxidants can serve as a signal for the modulation of ROI-scavenging mechanisms [24].

Avoiding ROI production

Avoiding ROI production might be as important as active scavenging of ROIs. Because many abiotic stress conditions are accompanied by an enhanced rate of ROI production, avoiding or alleviating the effects of stresses such as drought or high light on plant metabolism will reduce the risk of ROI production. Mechanisms that might reduce ROI production during stress (Table 1) include: (1) anatomical adaptations such as leaf movement and curling, development of a refracting epidermis and hiding of stomata in specialized structures; (2) physiological adaptations such as C_4 and CAM metabolism; and (3) molecular mechanisms that rearrange the photosynthetic apparatus and its antennae in accordance with light quality and

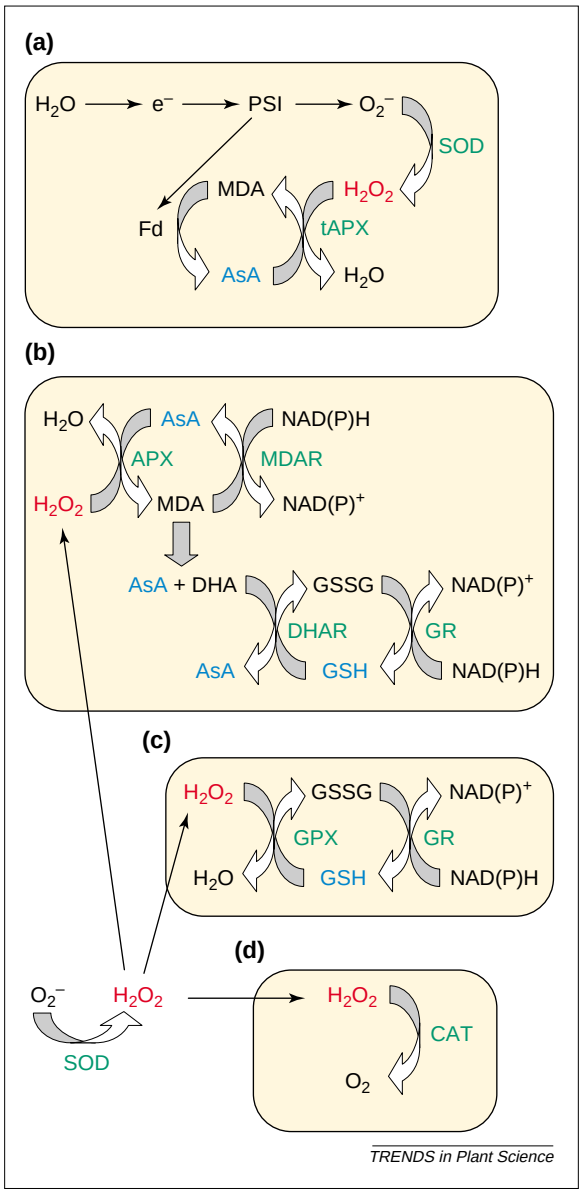

Fig. 1. Pathways for reactive oxygen intermediate (ROI) scavenging in plants. (a) The water–water cycle. (b) The ascorbate–glutathione cycle. (c) The glutathione peroxidase (GPX) cycle. (d) Catalase (CAT). Superoxide dismutase (SOD) acts as the first line of defense converting O_2^- into H_2O_2. Ascorbate peroxidases (APX), GPX and CAT then detoxify H_2O_2. In contrast to CAT (d), APX and GPX require an ascorbate (AsA) and/or a glutathione (GSH) regenerating cycle (a–c). This cycle uses electrons directly from the photosynthetic apparatus (a) or NAD(P)H (b,c) as reducing power. ROIs are indicated in red, antioxidants in blue and ROI-scavenging enzymes in green. Abbreviations: DHA, dehydroascorbate; DHAR, DHA reductase; Fd, ferredoxin; GR, glutathione reductase; GSSG, oxidized glutathione; MDA, monodehydroascorbate; MDAR, MDA reductase; PSI, photosystem I; tAPX, thylakoid-bound APX.

intensity or completely suppress photosynthesis. By balancing the amount of light energy absorbed by the plant with the availability of CO_2, these mechanisms might represent an attempt to avoid the over-reduction of the photosynthetic apparatus and the transfer of electrons to O_2 rather than for CO_2 fixation.

ROI production can also be decreased by the alternative channeling of electrons in the electron-transport chains of the chloroplasts and mitochondria by a group of enzymes called alternative oxidases (AOXs). AOXs can divert electrons flowing through electron-transport chains and use them to reduce O_2 to water (Fig. 2). Thus, they decrease ROI production by two mechanisms: they prevent electrons from reducing O_2 into O_2^- and they reduce the overall level of O_2, the substrate for ROI production, in the organelle. Decreasing the amount of mitochondrial AOX increases the sensitivity of plants to oxidative stress [25]. In addition, chloroplast AOX is induced in transgenic plants that lack APX and/or CAT, and in normal plants in response to high light [50].

Production and scavenging of ROIs in different cellular compartments

Recent manipulations of ROI-scavenging pathways in different cellular compartments suggest some intriguing possibilities. For years, the chloroplast was considered to be the main source of ROI production in cells and consequently one of the main targets for ROI damage during stress. However, it has recently been suggested that the chloroplast is not as sensitive to ROI damage as previously thought [26]. The mitochondrion is another cellular site of ROI production. However, recent studies suggest that the mitochondrion is also a key regulator of PCD in plants and that enhanced ROIs levels at the mitochondria can trigger PCD [27].

Both the mitochondrion and the chloroplast contain ROI-scavenging mechanisms. By contrast, little is known about the ROI-scavenging properties of the nucleus, which might contain redox-sensitive transcription factors [28]. Because H_2O_2 can diffuse through aquaporins [29], ROIs produced at a specific cellular site (e.g. the chloroplast during stress or the apoplast during pathogen attack) can affect other cellular compartments, overwhelm their ROI-scavenging capabilities and alter the pattern of gene expression during stress, pathogen infection or PCD. In support of this assumption, stresses that result in the enhanced production of ROIs at the chloroplast induce cytosolic and not chloroplastic ROI-scavenging mechanisms [24,30], and ROI production at the apoplast induces the production of pathogenesis-response proteins [4]. Because the plant mitochondria and nuclei are involved in the activation of PCD [27], the level of ROIs that reaches these compartments during stress or pathogen challenge needs to be tightly controlled to prevent abnormal PCD activation. The cytosol, with its ascorbate–glutathione cycle, and the peroxisomes, with CAT, might therefore act as a buffer zone to control the overall level of ROIs that reaches different cellular compartments during stress and normal metabolism.

The importance of peroxisomes in ROI metabolism is beginning to gain recognition [31]. Peroxisomes are not only the site of ROI detoxification by CAT but also the site of ROI production by glycolate oxidase and fatty acid β-oxidation. In addition, peroxisomes might be one of the cellular sites for nitric oxide (NO) biosynthesis [31]. In animal cells, NO activates fatty acid β-oxidation and enhances the production of ROIs in cells. However, although NO has been shown to be involved in ROI-induced cell death in plants [32] and NO is known to be a key regulator of pathogen responses [5], little is known about how NO is involved in the response of plants to abiotic stresses.

Redundancy in ROI-scavenging mechanisms

Some of the complex relationships between the different ROI-scavenging and ROI-producing mechanisms have been revealed in transgenic plants with suppressed production of ROI-detoxifying mechanisms. Thus, plants with suppressed APX

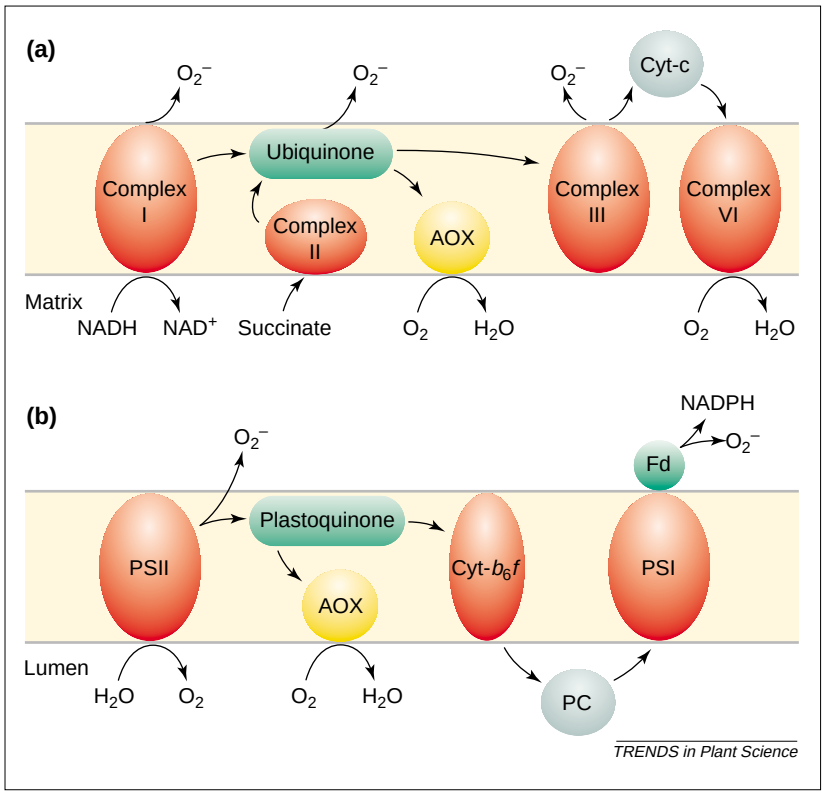

Fig. 2. Involvement of alternative oxidase (AOX) in reactive oxygen intermediate (ROI) avoidance. In both the mitochondrial electron-transport chain (a) and the chloroplast electron-transport chain (b), AOX diverts electrons that can be used to reduce O_2 into O_2^- and uses these electrons to reduce O_2 to H_2O. In addition, AOX reduces the overall level of O_2, the substrate for ROI production, in the organelle. AOX is indicated in yellow and the different components of the electron-transport chain are indicated in red, green or gray. Abbreviations: Cyt-b_6f, cytochrome b_6f; Cyt-c, cytochrome *c*; Fd, ferredoxin; PC, plastocyanin; PSI, PSII, photosystems I and II.

production induce SOD, CAT and GR to compensate for the loss of APX, whereas plants with suppressed CAT production induce APX, GPX and mitochondrial AOX [16,50]. CAT and APX are not completely redundant because they do not compensate for the lack of each other, as shown by the sensitivity of plants

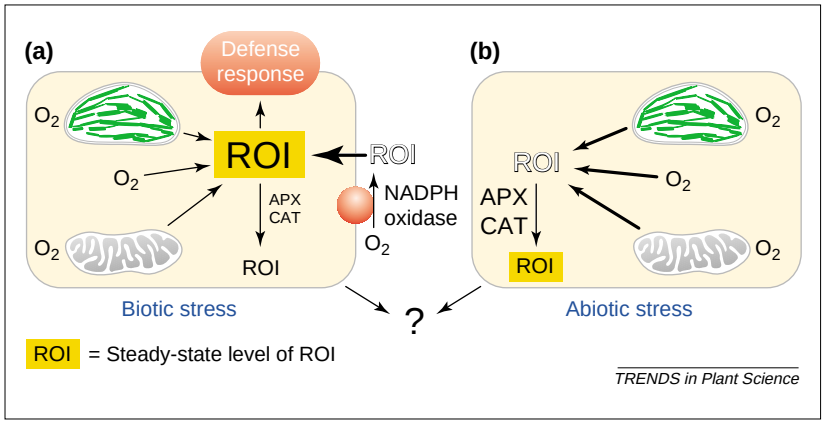

Fig. 3. Differences in the steady-state levels of reactive oxygen intermediates (ROI) during biotic stress and abiotic stress. Biotic stress (a) results in the activation of NADPH oxidase and the suppression of ascorbate peroxidase (APX) and catalase (CAT). This leads to the over-accumulation of ROI and the activation of defense mechanisms. Abiotic stress (b) enhances ROI production by chloroplasts and mitochondria. However, by inducing ROI-scavenging enzymes such as APX and CAT, it reduces ROI levels. The question mark indicates that little is known about the regulation of ROI metabolism during a combination of biotic and abiotic stresses. Chloroplasts are indicated in green, mitochondria in gray and the steady-state levels of ROI in yellow.

with reduced APX or CAT levels to environmental stresses and pathogen attack [33]. Interestingly, plants with suppressed APX and CAT appeared, at least under a defined set of environmental conditions, to be less sensitive to oxidative stress than plants with lowered APX or CAT levels. These plants had reduced photosynthetic activity, enhanced chloroplastic AOX production and enhanced expression of genes of the oxidative and reductive pentose-phosphate pathway and MDAR, possibly to avoid ROI production as well as to enhance the non-enzymatic detoxification of H_2O_2 by ascorbic acid [50].

ROIs at the interface between biotic and abiotic stresses

ROIs play a central role in the defense of plants against pathogen attack. During this response, ROIs 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 [4,5]. H_2O_2 produced during this response (up to 15 μM; directly or as a result of superoxide dismutation) is thought to diffuse into cells and, together with salicylic acid (SA) and NO [34], to activate many of the plant defenses, including PCD [35]. The activity of APX and CAT is suppressed during this response by the plant hormones SA and NO [34], the production of APX is post-transcriptionally suppressed [36] and the production of CAT is downregulated at the level of steady-state mRNA [37]. Thus, the plant simultaneously produces more ROIs and at the same time diminishes its own capacity to scavenge H_2O_2, resulting in the over-accumulation of ROIs and the activation of PCD. The suppression of ROI-scavenging mechanisms together with the synthesis of NO appears to be crucial for the activation of PCD because, in their absence, increased ROI production at the apoplast does not result in the induction of PCD [32,33].

The role ROIs play during PCD appears, therefore, to be opposite to the role they play during abiotic stresses, during which ROIs induce ROI-scavenging mechanisms such as APX and CAT that decrease the steady-state level of ROIs in cells (Fig. 3). The differences in the function of ROIs between biotic and abiotic stresses might result from the action of hormones such as SA and NO, from cross-talk between different signaling pathways (Fig. 4) or from differences in the steady-state level of ROIs produced during the different stresses. The apparent conflict in ROI metabolism between biotic and abiotic stresses (Fig. 3) raises the question of how the plant manipulates its rate of ROI production and ROI scavenging when it comes under biotic attack during an abiotic stress. In support of the possible existence of such a conflict, tobacco plants that were previously subjected to oxidative stress (and consequently had a higher level of antioxidative enzymes) had a reduced rate of PCD compared with unstressed control plants [33]. In addition, plants that overproduce CAT have a decreased resistance to pathogen infection [38].

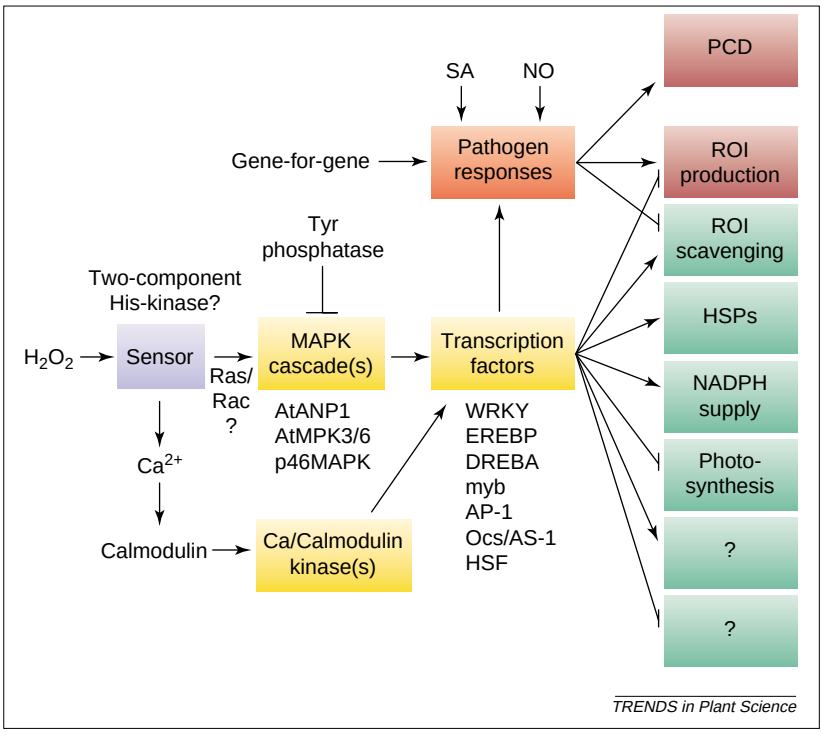

Fig. 4. A suggested model for the activation of signal transduction events during oxidative stress. H_2O_2 is detected by a cellular receptor or sensor. Its detection results in the activation of a mitogen-activated-protein kinase (MAPK) cascade and a group of transcription factors that control different cellular pathways. H_2O_2 sensing is also linked to changes in the levels of Ca^{2+} and calmodulin, and to the activation or induction of a Ca^{2+}–calmodulin kinase that can also activate or suppress the activity of transcription factors. The regulation of gene expression by the different transcription factors results in the induction of various defense pathways, such as reactive oxygen intermediate (ROI) scavenging and heat-shock proteins (HSPs), and in the suppression of some ROI-producing mechanisms and photosynthesis. There is also cross-talk with the plant–pathogen signal transduction pathway, which might depend on pathogen recognition by the gene-for-gene mechanism and can result in an inverse effect on the regulation of ROI-production and ROI-scavenging mechanisms, as well as on the activation of programmed cell death (PCD). The plant hormones nitric oxide (NO) and salicylic acid (SA) are key regulators of this response.

ROI signal transduction pathway

Recent studies have identified several components involved in the signal transduction pathway of plants that senses ROIs. These include the mitogen-activated protein (MAP) kinase kinase kinases AtANP1 and NtNPK1, and the MAP kinases AtMPK3/6 and Ntp46MAPK [39,40]. In addition, calmodulin has been implicated in ROI signaling [9,41]. A hypothetical model depicting some of the players involved in this pathway is shown in Fig. 4. H_2O_2 is sensed by a sensor that might be a two-component histidine kinase, as in yeast [9]. Calmodulin and a MAP-kinase cascade are then activated, resulting in the activation or suppression of several transcription factors. These regulate the response of plants to oxidative stress [9,42]. Cross-talk with the pathogen-response signal transduction pathway also occurs and might involve interactions between different MAP-kinase pathways, feedback loops and the action of NO and SA as key hormonal regulators. This model (Fig. 4) is simplified and is likely to change as research advances our understanding of this pathway.

ROIs act as signals that mediate the systemic activation of gene expression in response to pathogen attack [43], wounding [11] and high light [44]. They were suggested to act in conjunction with a compound that travels systemically and activates their production in distal parts of the plant, where they mediate the induction of gene expression [11]. The involvement of ROIs in the regulation of stomatal closure [13] and in other cellular responses involving auxin [39,45] might suggest that more signaling pathways involving ROIs as inducers of systemic signals await discovery. It is unlikely that ROIs can travel systemically because they are highly reactive and would be scavenged along the way by the many antioxidative mechanisms and antioxidants present in the apoplast. However, it is possible that a wave of activity similar to the 'oxidative burst' is activated in cells along the systemic path and in distal tissues, resulting in the accumulation of ROIs. Future studies using plants with altered levels of ROI-scavenging and/or ROI-producing mechanisms might resolve this question.

Future challenges and questions

The cause of cell death induced in plants by oxidative stress is not known. Is it simply the toxicity of ROIs that damages cells or is it the activation of a PCD pathway by ROIs? It is possible that the level of H_2O_2 that is currently thought to kill cells by direct cellular damage actually induces PCD [15,27], and it might require a higher level of ROIs to kill cells by direct oxidation. Perhaps future studies applying oxidative stress to mutants deficient in different PCD pathways will answer this question.

Many questions related to ROI metabolism remain unanswered (Box 1). We are currently at an exciting time, when most of the technologies required to answer these questions are in place. Thus, a comprehensive analysis of gene expression using microarrays and chips, coupled with proteomics and

Box 1. Questions and future challenges

- How toxic are reactive oxygen intermediates (ROIs) to plant cells?
- What are the sensors for ROIs in plants?
- What regulatory networks control the production and scavenging of ROI in plants? How are they coordinated? Is nitric oxide involved in abiotic stress signaling?
- How is ROI metabolism regulated during a combination of abiotic and biotic stresses, and during multiple abiotic stresses?
- Are there undiscovered or neglected ROI-producing and/or ROI-scavenging mechanisms [a]?
- What is the chloroplast to nucleus signal involved in sensing ROI stress?
- What is the source of reducing energy [i.e. NAD(P)H] for ROI removal during normal metabolism and stress?
- Is the diffusion of H_2O_2 through aquaporins regulated?
- Can we develop imaging tools to study the subcellular location of ROIs in plants?

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metabolomics to follow different antioxidants and related compounds during oxidative stress, should answer many of these questions. This analysis can be performed on plants responding to abiotic stresses, biotic insults or combinations of both, and can be complemented by using mutants with altered ability to produce or scavenge ROIs. In addition, the development of cellular markers that enable the non-destructive quantification of different ROIs in the different cellular compartments, like the markers used for Ca^{2+} imaging, will considerably advance our understanding of ROI metabolism.

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