Chapter 6

ROS Regulation of Antioxidant Genes

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ABSTRACT

Over the last decade our understanding of the role of ROS has progressed from the classical view of adverse toxic metabolic by-products inadvertently associated with aerobic life to include the newly emerging role of signaling molecules regulating growth, development and coordinating responses to abiotic and biotic stress. A recent series of discoveries have given scientists new insights into ROS-dependent gene activation and the molecular mechanisms involved. The majority of information of the regulatory role of ROS on gene expression derived from experiments using: i) transgenic plants overexpressing or suppressing antioxidant genes in order to reduce or increase the intracellular ROS levels, respectively; ii) mutants impaired in ROS generation or scavenging; iii) direct application of ROS; iv) application of ROS generating compounds. Results of these experiments provided significant information on ROS-dependent signaling pathways and ROS-responsive genes. A number of genes involved in defense, signal transduction, transcription, metabolism as well as cell structure have been identified revealing a highly dynamic and redundant network of ROS-producing and ROS-scavenging genes. Antioxidant genes are central players in this network and their function has profound effect in controlling ROS levels and cellular redox balance. ROS, on the other

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hand can remarkably regulate the level of antioxidant gene expression providing a feedback regulation mechanism of ROS levels, which is a critical component in modulation of signaling networks. This chapter focuses on recent developments on the role of ROS in activation of gene expression with an emphasis on ROS-scavenging enzyme systems.

Introduction

Oxygen supports aerobic life of land plants granting them with great energetic benefits but on the other hand challenges them through an endless formation of reactive oxygen species (ROS). ROS, namely singlet oxygen ($^1O_2$), superoxide radical (O^-_2), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^\cdot$), are by-products of the energy-generating processes of photosynthetic and respiratory electron transport chains (ETC). Consequently, chloroplasts, mitochondria and peroxisomes are the main organelles of ROS producers in plant cells. ROS are highly reactive and toxic based on their ability to react indiscriminately with almost all biomolecules provoking destructive protein modifications, DNA strand breaks, purine oxidations, protein-DNA crosslinks and β-oxidation of lipids (Van Breusegem and Dat 2006). Thus, evolution of all aerobic organisms has been dependent on the development of efficient enzymatic and non-enzymatic ROS-scavenging mechanisms, referred as antioxidant machinery.

Under physiological conditions the antioxidant machinery is sufficient to maintain equilibrium between production and scavenging of ROS, commonly known as redox homeostasis. However, due to their static lifestyle, plants are interminably exposed to unfavorable environmental conditions such as temperature extremes, high light intensities, drought, salinity, air pollution and pathogen attack, all known to increase the rate of ROS generation. When ROS production overwhelms the cellular scavenging capacity suspending cellular redox homeostasis, the results is a rapid and transient excess of ROS, known as oxidative stress (Scandalios et al. 1997). Under such circumstances reactivity of ROS is discerned as necrotic lesions on plant tissues due to a heaved production of lipid-derived radicals that lead to lipid peroxidation of organellar and cellular membranes, affecting cellular functioning and resulting ultimately in membrane leakage and cell lysis. As increased production of ROS is the rule than the exception, plant evolution has necessitated a tight regulation of ROS equilibrium attained through a complex gene network that operates in all subcellular compartments. Major ROS-scavenging enzymes include the superoxide dismutase (SOD) that dismutates O^-_2 to H_2O_2 followed by the coordinated action of a set of five enzymes namely catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and peroxiredoxins (Prx) that reduce H_2O_2 (Mittler et al. 2004). All ROS-detoxifying enzymes known to date

are encoded by nuclear genes that are processed properly to function in various subcellular compartments (Table 1).

Along with non-enzymatic antioxidants, such as ascorbic acid (vitamin C), tocopherols (vitamin E), and glutathione (GSH), antioxidant enzymes work in concert to sustain an intracellular steady-state level of ROS that promotes plant growth, development, cell cycle, hormone signaling, and reinforces responses to abiotic and biotic environmental stressors (Foyer and Noctor 2005, Mittler et al. 2004, Van Breusegem and Dat 2006).

SODs are the front line of defense as they rapidly dismutate O^{-}_{2} to H_2O_2. SODs comprise a multigene family of nuclear encoded enzymes present in every subcellular compartment including chloroplasts, mitochondria, peroxisomes, glyoxysomes, cytosol, and apoplast. The number of SOD genes varies among plant species (Alscher et al. 2002). According to their metal cofactor at their active site, plant SODs are classified into three groups: the copper zinc SOD (Cu/ZnSOD), the manganese SOD (MnSOD), and the iron SOD (FeSOD). The Cu/ZnSODs are found in the cytosol, apoplast, peroxisomes and chloroplasts. FeSODs are localized

Table 1 ROS-scavenging enzymes

Antioxidant enzymes	**Function**	**Subcellular location**
Superoxide dismutase (SOD)	$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow O_2 + H_2O_2$	(chloro) plastids, mitochondria, cytosol
Ascorbate peroxidase (APX)	H_2O_2 + 2AsA → $2H_2O$ + 2MDA	(chloro) plastids, mitochondria, peroxisomes, cytosol
Catalase (CAT)	$2H_2O_2 \rightarrow 2H_2O + O_2$	peroxisomes
Glutathione peroxidase (GPX)	H_2O_2 + 2GSH → H_2O + GSSG	(chloro) plastids, mitochondria, cytosol
Peroxiredoxin (Prx)	2P-SH + H_2O_2 → P-S-S-P + $2H_2O$	(chloro) plastids, mitochondria, peroxisomes, cytosol
Peroxidase (POD)	H_2O_2 + $(ROH)_2$ → $2H_2O$ + $R(O)_2$	Cytosol, cell wall bound
Alternative oxidase (AOX)	$2e^- + 2H^+ + O_2 \rightarrow H_2O$	Mitochondria, (chloro) plastids
Glutathione S-transferase (GST)	ROO^- + 2GSH → GSSG-ROOH	Cytosol, nucleus
Glutathione reductase (GR)	GSSG + NAD(P)H → 2GSH + $NAD(P)^-$	(chloro) plastids, mitochondria, peroxisomes, cytosol
Monodehydroascorbate reductase (MDAR)	MDA + NAD(P)H + H^+ → AsA + $NAD(P)^-$	(chloro) plastids, mitochondria, peroxisomes, cytosol
Dehydroascorbate reductase (DHAR)	DHA + 2GSH → Asc + GSSG	(chloro) plastids, mitochondria, peroxisomes, cytosol

in the chloroplasts, and are resistant to KCN inactivation. MnSODs are found in mitochondria and are resistant to H_2O_2 inhibition. Noteworthy, that both Cu/ZnSOD and FeSOD are inhibited by H_2O_2, thus they co-exist with robust H_2O_2-scavenging systems such as enzymes of the ascorbate-glutathione cycle (Foyer et al. 1997, Scandalios 1997). The intracellular balance of SODs and the different H_2O_2-scavenging enzymes is apparently crucial in determining the steady-state level of O^{-}_2 and H_2O_2. This balance, along with the sequestering of metal ions by ferritin and other metal-binding proteins, prevents the formation of the highly toxic $HO^{\cdot}$ via the metal-dependent Haber-Weiss reaction or the Fenton reaction (Halliwell and Gutteridge 1999). Plants do not possess enzymatic systems to scavenge $HO^{\cdot}$ thus preventing its generation, keeping low the steady-state level of O^{-}_2 and H_2O_2 to avoid their interaction is the only way of defense.

Removal of H_2O_2 is achieved by a complex network of antioxidant enzymes and non-enzymatic antioxidants such as tocopherols, ascorbic acid (AsA) and glutathione that work in concert to detoxify H_2O_2. In the plant cell, elimination of H_2O_2 is undertaken by a set of antioxidant enzymes, encoded by nuclear genes, including CAT, APX, GPX and Prx. Of them, catalases are unique in decomposing H_2O_2 without additional reductant, thus providing the cell with an energy efficient mechanism. CATs are indispensable, responsible for the gross removal of intracellular H_2O_2 generated in peroxisomes during photorespiration (Scandalios et al. 1997). CATs are distinguishable of alternative H_2O_2-scavengers by very high turnover rate but rather low affinity towards H_2O_2. Consequently, they are responsible for the gross removal of H_2O_2 generated in peroxisomes of photosynthetic plant tissues. CATs are predominantly localized in peroxisomes although their presence in mitochondria is still unclear.

On the other hand, APXs catalyze the reduction of H_2O_2 with concomitant consumption of ascorbate as the reducing agent. Thus APX activity depends solely on the availability of reduced ascorbate, while reduced glutathione can be used in some instances. Under normal conditions the cellular pool of ascorbate is kept at the reduced state by a set of enzymes, namely mono-dehydroascrobate reductase (MDAR) and dehydroascorbate reductase (DHAR) capable of using NAD(P)H to regenerate oxidized ascorbate (Mittler et al. 2004). APXs exhibit a very high affinity to H_2O_2 thus acting at the micromolar and submicromolar range. Localized in all subcellular compartments including peroxisomes, chloroplasts, mitochondria and cytosol, APXs are ideally suited for a fine tuning of sensitive redox balances with low H_2O_2 concentrations that are important for regulatory mechanisms.

Alternative enzymes involved in removal of H_2O_2 are GPXs and Prxs. The major function of GPXs is the reduction of phospholipid hydroxyperoxides to form corresponding alcohols using thioredoxins

(Trx) as preferred electron donors (Navrot et al. 2006). They also scavenge H_2O_2 in the ascorbate-glutathione cycle (Foyer et al. 1997). Thereby, GPXs protect cell membranes from peroxidative damage, maintaining cellular integrity. GPXs are present in most subcellular compartments and are involved in the response to both abiotic and biotic stresses by acting as general peroxide scavengers (Navrot et al. 2006). Recent evidence has shown that some GPXs may also be involved in redox transduction under stress conditions (Miao et al. 2006). Our view of plant Prxs was profoundly modified by the recent sequencing programs which revealed a number of genes encoding not only the previously identified but also numerous new ones. In plants Prxs are classified into five subgroups one of them being the incorrectly named GPXs. Prxs are mainly localized in organelles such as chloroplasts and mitochondria, while counterparts are also found in the cytosol. Prxs are thiol-dependent peroxidases capable of eliminating H_2O_2 and a variety of peroxides through conserved reactive catalytic cysteines, which are regenerated by reducing systems (Horling et al. 2002). Therefore, regeneration of Prxs after a peroxidatic reaction constrains them to compete for electron donors with other target proteins. This characteristic is considered as redox sensor; therefore it is perceived that Prxs along with GPXs act as intracellular redox sensors that transmit information of the cellular levels of ROS to the redox network. Increasing evidence has accumulated that Prxs maintain a central function beyond peroxide detoxification (Foyer and Noctor 2005). They control and initiate cell signaling affecting photosynthesis, mitochondrion-dependent and chloroplast-dependent nuclear gene expression and activation of enzymes of the Calvin cycle (Dietz 2008). These are all thought to be linked and interfere with the redox regulatory network.

In addition to the aforementioned ROS scavenging enzymes, a number of enzymes found in various subcellular compartments are involved in maintaining redox homeostasis either by scavenging directly particular ROS and ROS-byproducts or by replenishing antioxidants. In that respect these enzymes could be also considered antioxidants. Such enzymes include alternative oxidases (AOXs), peroxidases (PODs), glutathione S-transferases (GSTs), MDAR and DHAR. Plant mitochondria are thought to be a major site of H_2O_2 production under normal metabolism. Under stress conditions such as high light intensities, the mitochondrial electron transport chain is overwhelmed enhancing production of ROS. Mitochondria contain an alternative respiratory pathway sustained by AOX, which is a ubiquinol oxidase that transfers electrons from reduced ubiquinone to molecular oxygen, producing water as the reduced product (Siedow and Umbach 1995). Accumulating evidence suggests that AOX, which is encoded in plants by a small multigene family, play significant role under adverse environmental conditions in two ways: modulating

plant growth and development, and protecting cells from oxidative stress (Polidoros et al. 2009). Apart from mitochondria, AOX-like enzymes are also found in the thylakoids of chloroplasts. The *Arabidopsis immutans* mutant is due to a recessive AOX homologue targeted to the chloroplasts (Aluru et al. 2009). PODs comprise a group of non-donor specific plant peroxidases for which guaiacol is a common donor, thus named guaiacol peroxidases. PODs catalyze reduction of H_2O_2 utilizing guaiacol as reductant, however other functions of PODs are still unclear. Recently, two distinct PODs have been separated from the plasma membrane (Mika and Luthje 2003). MDAR and DHAR are members of the glutathione-ascorbate cycle that operates in chloroplasts and cytosol. MDAR and DHAR are responsible for the reduction of ascorbate that is utilized as reductant by APX (Mittler et al. 2004). GSTs comprise a family of enzymes localized in various subcellular compartments. The primary function of many GSTs is conjugation of GSH (glutathione) to a variety of molecules such as xenobiotics or intermediates of secondary metabolites (Mylona et al. 1998). GSTs detoxify breakdown products of lipid peroxides. They are induced *inter alia* by ROS and pathogen challenge (Mylona et al. 2007; Polidoros et al. 2005). Certain GSTs play roles as peroxidases or in regenerating ascorbate from dehydroascorbate (Foyer and Noctor 2005, and references therein).

In this chapter we examine recent data revealing that deviation from the cellular redox balance acts as a signal that affects regulation of antioxidant genes. In recent years ROS have been implicated in the control and regulation of biological functions, such as growth, cell cycle, programmed cell death, hormone signaling, biotic and abiotic stress responses and development. Emerging evidence indicates that production of ROS and activation of redox-dependent signaling cascades are involved in the regulation of the antioxidant genes, which in turn affect the intracellular level of ROS and may provide a feedback control of the ROS-dependent biological processes.

Experimental Systems Utilized for Modulation of ROS Levels

Under environmental stress conditions, several chemically distinct ROS are generated simultaneously in various subcellular compartments hence a causal link between accumulation of a specific ROS and its signaling or damaging effects has always been difficult to establish. Clearly, it is desirable to assay ROS specifically, ideally within cells and in specific sub-cellular locations. Thus various histochemical assays have been developed including dansyl-2,2,5,5-tetramethyl-2,5-dehydro-*1H*-pyrrole (known as DanePy) for $^1O_2$ (Hideg et al. 1998), nitroblue tetrazolium for O^-_2 (Berridge and Tan 1998), 3-3′-diaminobenzidine (DAB) a classic

photometric assay for H_2O_2 in leaf tissues and lately fluorescent probes AR (Ampex Red, 10-acetyl–3,7-dihydroxyphenoxazine) and AUR (Amplex Ultra Red) that image H_2O_2 are gaining popularity (Thordal-Christensen et al. 1997, Groten et al. 2005, Šnyrychova et al. 2009). However, histochemical assays imprint the excess accumulation of ROS whereas the modulating role of ROS is thought to be attained by minimal ROS increases that trigger signaling cascades. Along with histochemical assays a set of experimental *in vivo* systems, both pervasive and non-pervasive, are employed to address regulation and signal transduction issues of ROS.

The primary experimental system utilized in assessing the effects of ROS accumulation is that of exogenous application of either a particular ROS or a compound that pervade the cells to induce intracellular production of ROS. Pervasive-systems utilize cell suspension cultures, seedlings and specific parts of plants such as *Zea mays*, *Nicotiana tabacum*, *N. plumbaginifolia*, *Arabidopsis thaliana*, *Oryza sativa, Poplar* and a set of exogenously applied stressors including, menadione, paraquat, toxins, herbicides, H_2O_2, ozone, plant hormones, extreme temperatures and light intensities (Feierabend 2005, Polidoros and Scandalios 1997). Menadione and paraquat are known redox cycling compounds that interrupt ETC of chloroplasts and mitochondria leading to generation of O^{-}_{2}. Herbicides such as 3-aminotriazole (AT) are known to inhibit catalases, therefore uptake of AT results in intracellular accumulation of H_2O_2 (Scandalios et al. 1997). Toxins derived from fungi such as cercosporin and *Alternaria alternata* toxin (AAL) are known to interact with biomolecules inducing generation of ROS. H_2O_2 is a product as well as a substrate of antioxidant enzymes with an inhibitory action for antioxidant enzymes in high concentrations. Its application was coined to detect primarily changes in antioxidant enzyme systems and in genes expression. Ozone, an air pollutant is used as a pervasive compound that upon entrance is converted to O^{-}_{2} and H_2O_2 in the apolplast. Use of plant hormones such as abscisic acid (ABA), salicylic acid (SA), methyl jasmonate (JA) and auxin is based on evidence indicating that plant hormones stimulate ROS generation and that antioxidant enzymes are under hormonal regulation during plant development (Scandalios et al. 1997). Temperature extremes, and light intensities were shown to induce production of ROS. Simultaneous studies have shown that some catalases are photoinactivated while others are under light regulation, known as circadian rhythm (Feierabend 2005). Conclusively pervasive systems allow a thorough investigation of antioxidant enzyme activation, gene expression, detection of stress-induced ROS production and observation of changes in physiological parameters.

However, pervasive systems certainly constrain a thorough elucidation of ROS-generating systems and of identifying which specific ROS is

active within a subcellular compartment to exert its effects on signal transduction pathway. Solution to this problem is granted by transgenic and mutant plants in which only one of the various forms of ROS reaches high levels and ideally gives rise to stress responses that is easily scored and its specificity delineated. Transgenics of model-plants overexpressing a particular antioxidant enzyme provided the first evidence to increase stress tolerance of plants and crops. Conversely, transgenic plants with suppressed level, deficiency in a particular antioxidant enzyme consolidate the role of ROS-scavenging enzymes and provide the basis to decipher ROS-mediated signaling. Significant advances in our understanding of ROS-signaling was recently gained utilizing *Arabidopsis* mutants such as the lesion stimulating disease (*lsd*), the fluorescent (*flu*) and the *immutans* (*im*) that due to a deficiency, accumulation of a particular ROS in specific subcellular compartment is induced upon exogenous application of stressors. Subsequently delineation of particular ROS-induced gene expression is deciphered and in combination with specific deficiencies in antioxidant enzymes the ROS-mediated signal transduction pathways are starting to emerge.

Overall, pervasive- and non-pervasive systems were used to assess changes in antioxidant enzyme activities and isoforms composition by spectrophotometric assays, zymogram analyses and immunochemical studies (Feierabend 2005, Mittler et al. 2004, Polidoros and Scandalios 1997, and references therein). In the 90s with the advent of molecular biology tools and the acquired knowledge of cDNAs for a number of antioxidant genes, *in vitro* antioxidant gene expression studies were predominantly attained by northern hybridization analyses followed by RT-PCR (real time polymerase chain reaction) and nowadays by transcriptome profiling utilizing cDNA- and oligo DNA-microarray technology (Vij and Tyagi 2007).

Undoubtedly the wide genome sequences have paved the way to the –omics era that embrace various parallel dynamic approaches including transcriptomics, proteomics coupled with metabolomics along with the use of mutants and transgenics enable gene expression, function and protein interactions profiling in a high throughput mode.

Over the last decade our perception of ROS has been revolutionized. ROS primarily believed to be inevitable harmful by-products of aerobic metabolism that had to be eliminated, to date are accepted to act as signaling molecules in plants. Compelling evidence indicate that tightly regulated production of ROS is beneficial to the plant as they modulate a broad range of physiological processes including, senescence (Peng et al. 2005), photorespiration and photosynthesis (Noctor and Foyer 1998), stomatal movement (Bright et al. 2006) cell cycle (Mittler et al. 2004), growth and development (Foreman et al. 2003) and programmed cell death (Bethke and Jones 2001, Fath et al. 2002). Moreover, ROS are known

to mediate signal transduction pathways and stress responses, however, the molecular mechanisms by which these processes occur have not yet been fully clarified (Hancock et al. 2006, Neill et al. 2002).

The development of sensitive and selective techniques for *in vivo* detecting and measuring ROS including cellular localization and concentration, provided the tools to explore their role in stress response and signal transduction pathways. On the other hand, the specific effects such as accumulation, and signaling of metabolically generated ROS within a particular subcellular compartment were assessed using transgenic plants with overexpressed or suppressed ROS-scavenging enzymes as well as mutant lines harboring particular deficiencies. Notably the genome projects have allowed broad analyses of gene expression profiles by microarray methodology of plants under physiological and stress conditions. Consequently, identification of numerous genes including transcription factors, protein kinases, ROS-scavenging enzymes, ion channels and others were revealed furthering our understanding of the components of molecular mechanisms involved in optimal and stress conditions.

Antioxidant Genes Regulation by ROS

Singlet oxygen

Until recently, the significance of singlet oxygen ($^1O_2$) was obscure due to lack of methods enabling its generation and detection (Hideg et al. 2002). Under physiological conditions, singlet oxygen is generated in illuminated chloroplasts during photosynthesis as insufficient energy dissipation leads to formation of chlorophyll in triplet state that can transfer its excitation energy to molecular O_2 producing $^1O_2$ (Asada 2006). The plant counteracts this by the use of carotenoids that can quench directly $^1O_2$, a role shared with tocopherols in a phenomenon known as thermal dissipation (Holt et al. 2005). However, in stress conditions such as high light intensities when the absorption energy exceeds the capacity of CO_2 assimilation, excited triplet chlorophyll molecules (namely P_{680}) in photosystem II interact with molecular O_2 to endorse generation of high levels of $^1O_2$ and cause photooxidative damage to plants (Asada 2006). Under high light intensities, superoxide radicals (O_2^{-}) are also generated in the chloroplast, at PSII through the Mehler reaction (Hideg et al. 1998). Gene expression studies have shown that high light intensities induced expression of nuclear genes. Given the fact that $^1O_2$ is a short-lived molecule, unable to cross the chloroplast membranes, it is likely that communication signaling of organelle to the nucleus (also referred as retrograde signaling) is mediated through activation of second messengers, such as H_2O_2. In photoinhibition O^{-}_2 and H_2O_2 are also generated; however O^{-}_2 is rapidly converted via SOD to H_2O_2 that is scavenged by chloroplastic APX (Asada 2006).

Since several distinct ROS are generated simultaneously during stress in chloroplasts, it is rather impossible in wild-type plants to attribute stress-induced changes of nuclear gene expression to elevated levels of a particular ROS. Solution to this problem became available with use of the *Arabidopsis thaliana* conditional *flu* mutant. *flu* mutants overaccumulate the free protochlorophyllide, that acts as a potent photosensitizer generating $^1O_2$ during illumination (Op Den Camp et al. 2003). A major consequence of this $^1O_2$ generation is a rapid change in nuclear gene expression, that affects 5% of the total genome, probably through the transfer of a $^1O_2$-derived signal from the plastid to the nucleus (Op Den Camp et al. 2003). Immediately after the release of $^1O_2$ the growth rate of mature plants decreases whereas seedlings bleach and die. These two stress responses are caused by $^1O_2$-dependent activation of genetically determined stress response programs. Many of the $^1O_2$-induced genes are different form those activated by O^{-}_2 or H_2O_2 suggesting that $^1O_2$ and O^{-}_2/H_2O_2 signaling occurs via distinct pathways, (Laloi et al. 2006). Antioxidant genes activated in *flu* mutant after a dark/light shift included glutaredoxin (a member of the *Prx* family), *POD*s, NADPH oxidase, *Aox1a*, *MDAR*, *DHAR*, ascorbate oxidase (*AO*), as well as signal transduction genes such as MAP kinase kinase 4 (*MAPKK4*), the protein phosphatase *ABI1* and several protein kinases (Op Den Camp et al. 2003). Conversely, genes that were not affected after dark/light shift included *APX1*, *ferritin1*, *SOD* and *CAT*. These genes are known to be induced by O^{-}_2/H_2O_2, however, the apparent lack of activation of these genes in the *flu* mutant after a dark/light shift suggested that the concentration of O^{-}_2/H_2O_2 in these plants is too low to affect the mutant's early stress responses (Op Den Camp et al. 2003). It is worth noting that for transcriptome analysis only genes that exhibit a change of ≥ 2.5 fold are usually selected (Aluru et al. 2009, Gadjev et al. 2006, Gechev et al. 2006, Op Den Camp et al. 2003).

Overexpression of the thylakoid bound *APX* (*tAPX*) that reduces the chloroplastic level of H_2O_2 (Murgia et al. 2004) in *Arabidopsis flu* mutant background enhanced the $^1O_2$-induced nuclear gene expression after a dark/light shift and increased intensity of $^1O_2$-mediated cell death and growth inhibition compared with the *flu* parental line (Laloi et al. 2007). These results suggested that H_2O_2 antagonizes the $^1O_2$-mediated signaling of stress responses as seen in the *flu* mutant.

Compelling evidence of antioxidant gene regulation by ROS was obtained in *Arabidopsis immutans* (*im*) mutant that lack colored carotenoids in chloroplasts developing green-white sectors in leaves. Growth under low light intensity results in nearly all-green plants while high light intensity causes enhanced white sector formation resulting in nearly all-white plants (Wetzel et al. 1994). Early biochemical studies have shown that white sectors of *im* plants accumulate phytoene due to a

blocked step in carotenogenesis. It is now established that IMMUTANS (IM), a homologous to mitochondrial AOX, servers as a terminal oxidase in thylakoid membranes transferring electrons to molecular O_2 to yield H_2O (Aluru et al. 2006). Given that carotenoids are the main scavengers of $^1O_2$, white sectors of *im* plants operate as sinks of $^1O_2$ that result in photooxidative damage. Subsequently, gene expression analysis of white sectors under high light intensities provides information of $^1O_2$-induced responses. Transcriptome analysis of *im* white sectors revealed that many of the induced genes are involved in oxidative stress responses. Oxidative stress response genes that are largely induced include *Cu/ZnSODs* (*CDS1*, *CDS2* and *CDS3*), *FeSOD* (*FSD3*), catalase1 (*CAT1*), ferritin1, heat shock protein 70 (*HSP70*), peroxidases (*POD*), a group of Prxs commonly known as glutathione peroxidases (*GPX2, GPX7*), stromal ascorbate peroxidases (*sAPX*), glutathione reductase (*GR1*) and alternative oxidases (*AOX1a and AOX1d*). Conversely, antioxidant genes that were repressed include FeSOD1 (*FSD1*) and catalase3 (*CAT3*), thylakoid ascorbate peroxidases (*tAPX*) and *DHAR* (Aluru et al. 2009). Repression of *tAPX* could be due to lack of proper thylakoid membranes, since *im* white sectors contain abnormal chloroplasts, while repression of *CAT3* and *FSD1* could be due to light, since expression of these genes is under circadian regulation (Kliebenstein et al. 1998, Polidoros and Scandalios 1997).

Transcriptome analysis of *im* green sectors revealed activation of several genes previously shown to be induced under highlight conditions, such as genes of anthocyanin biosynthesis, as well as genes uniquely induced in *im* green sectors. Induction of anthocyanin biosynthesis genes has previously been reported in response to photooxidative stress (Gadjev et al. 2006, Rizhsky et al. 2003). The unique induction of a large number of ROS scavengers and several other defence-responsive genes involved in heat, ABA, cold, dehydration and salt stress, suggests activation of different signal transduction pathways between *im* white and green sectors in response to high light.

It is now established that retrograde signaling (communication of chloroplast to the nucleus) is mediated through activation of second messengers such as EXECUTER proteins (EX1, EX2) thylakoid proteins encoded by nuclear genes (Lee et al. 2007). Recently gained new insights of the mode of action of EX1 and EX2 show that both act in concert to transfer ROS-related signals from the plastid to the nucleus. EX2 is a modulator attenuating and controlling activity of EX1 which depends upon lipid peroxidation events of the plastid (Przybyla et al. 2008). Another component of this retrograde signaling is the recently identified *Arabidopsis* GUN1 (GENOMES UNCOUPLED) a nuclear encoded plastid protein, that mediates ROS and/or redox responses (Koussevitzky et al. 2007). Whereas, the primary identified LSD1 (*lsd1*, lesion simulating

disease resistance) acts as a cellular hub for the basic Leu zipper (bZIP) transcription factor, outside the nucleus under oxidative stress conditions in wild-type *Arabidopsis* plants (Kaminaka et al. 2006). Conclusively, operational control of retrograde signaling is attained by a combination of factors including redox poise, different ROS, photosynthetic electron transport and possibly many others awaiting to become forward.

Superoxide radical

Major sources of superoxide radical (O^{-}_{2}) are the electron transport chains (ETC) of the energy producing organelles, mitochondria and chloroplasts. O^{-}_{2} is also produced in peroxisomes, cytosol as well as in the apoplastic space (Agrawal et al. 2003). O^{-}_{2} is a short lived, 2–4 μs, ROS unable to transverse the phospholipid bilayer because it is charged, and therefore its action is restricted to the close proximity of its generation site (Bhattacharjee 2005 also see the chapter of Bhattacharjee in this volume). O^{-}_{2} is rapidly dismutated to H_2O_2 by SOD enzymes that are present in all cellular compartments. However, a number of environmental conditions including drought, salinity, temperature extremes, excessive light, and exposure to herbicides, xenobiotics and air pollutants accelerate generation of O^{-}_{2} exceeding the scavenging capacity. It is conceivable that O^{-}_{2} increase, due to its short half-life and limited diffusion, is probably communicated to the nucleus through second messengers. An excellent example of this is the *Arabidopsis* runaway cell death mutant *lsd1* produces uncontrolled levels of superoxide, leading to changes in defense gene expression and cell death lesions (Jabs et al. 1996). Although O^{-}_{2} is the ROS implicated here, it seems rather surprising that H_2O_2 did not have a similar effect, given that O^{-}_{2}-mediated signaling is achieved by EX1 and EX2, as previously referred.

Antioxidant gene/enzyme regulation by O^{-}_{2} is mainly obtained by exogenous application of bipyridyl compounds. Bipyridyl compounds such as paraquat, also known as methyl viologen, and benzyl viologen are redox-active molecules that are taken up by the cell, undergo univalent reduction and subsequently transfer their electrons to oxygen, forming O^{-}_{2} and regenerating oxidized paraquat or benzyl viologen that may engage in successive rounds of redox cycling (Halliwell and Gutteridge 1999). Even though paraquat can be reduced by a number of enzymes and electron transfer systems of the plant cell, photoreduction in chloroplasts represents the most efficient pathway followed by that of mitochondria that operates in light or dark. Therefore, exposure to paraquat during illumination results in O^{-}_{2} formation mainly in chloroplasts, while in dark generation of O^{-}_{2} is favored in mitochondria and microsomes (Halliwell and Gutteridge 1999).

The effects of paraquat-derived O^{-}_{2} on antioxidant genes in *Nicotiana plumbaginifolia* showed an increase in transcript levels for mitochondrial *MnSOD*, chloroplastic *FeSOD* and cytosolic *Cu/ZnSOD* in presence of light. Whereas treatment of plants with paraquat in dark induced expression of cytosolic *Cu/ZnSOD* only (Tsang et al. 1991). Exposure of pea plants to paraquat or salt stress (which also generates O^{-}_{2}) induced increases activities of the antioxidant enzymes SOD, APX and GR (Donahue et al. 1997, Gomez et al. 1999). However, it was reported that the observed increase in antioxidant enzyme activities did not correlate with mRNA levels. It is worth noting that, changes in transcript expression as a measure for how important a specific antioxidant gene is in protecting plant cells against ROS or other stresses is not always the case. Further evidence substantiating induction of antioxidant enzyme activities indicated up-regulation of the antioxidant enzymes CAT, APX, SOD and GR activities in leaves and roots of wild-type salt tolerant tomato plants in response to salt-derived O^{-}_{2} stress (Mittova et al. 2004). Recent observations in cotton leaves and callus tissue to salt and paraquat generated O^{-}_{2} stress showed increases of activities of the antioxidant enzymes SOD, CAT, APX, GR and total POX (Vital et al. 2008). Pretreatment of cotton tissues with N-acetyl-L-cysteine (NAC) an O^{-}_{2} scavenger, completely removed the salt- or paraquat-derived O^{-}_{2} and inhibited up-regulation of antioxidant enzyme activities. These results suggest that O^{-}_{2} mediates regulation of antioxidant enzyme activities; however the mechanism for this regulation remains still unknown. Compelling evidence for induction of both, genes and enzyme activities to paraquat- and benzyl viologen-produced O^{-}_{2} were shown in maize embryos. O^{-}_{2} up-regulated activities of SOD and CAT enzymes and induced the expression of mitochondrial *MnSOD*, cytosolic *Cu/ZnSODs*, *CAT1*, *CAT2* and *GST1* genes (Mylona et al. 2007). Further evidence for O^{-}_{2}-accumulating gene induction is obtained from ozone treatments. Ozone (O_3) is an atmospheric pollutant that breaks down in the apoplast forming mainly O^{-}_{2} and H_2O_2. Acute (single) or chronic (3, 6 and 10 consecutive days) exposure to O_3 of maize seedling exhibited increases in transcript levels of catalases *CAT1*, *CAT3*, glutathione S-transferase (*GST1)*, mitochondrial *MnSOD* and cytosolic *Cu/ZnSODs* in leaves (Ruzsa et al. 1999). However, transcript levels of *CAT2* and chloroplastic *Cu/ZnSOD* were down-regulated.

Substantial evidence of O^{-}_{2} regulation of antioxidant gene expression became available by studies on transgenic plants with suppressed SOD. Transcriptome analysis of knockdown *Arabidopsis* plants with suppressed expression of chloroplastic Cu/ZnSOD (*CSD2*), accumulating-O^{-}_{2} under optimal conditions exhibited induction of chloroplast and nuclear encoded genes (Rizhsky et al. 2003). Induction of chloroplastic transcripts although, photosynthesis is down-regulated indicates operation of a highly specific

redox sensor mechanism in the thylakoids. Induced nuclear genes include *FeSOD*, catalases (*CAT1, CAT3),* chloroplastic ferritin (*ferritin1*), *POD* and genes of the anthocyanin biosynthesis. Whereas down-regulated genes include *CAT2*, chloroplastic *APX*, cytosolic and chloroplastic *GR*, *GPX*s, chloroplastic *Prxs* and the plasma membrane NADPH oxidase. Intriguingly, chloroplastic *APX* was suppressed, while chloroplastic *FeSOD* was induced and cytosolic *APX1* and *APX2* were unaffected, suggesting that different components of the antioxidant water-water cycle are regulated by the O^{-}_{2}-mediating signal transduction pathway.

In recent comparative transcriptome analyses among wild type *Arabidopsis* plants exposed to paraquat or ozone and transgenic plants in which the activity of an individual antioxidant enzyme was suppressed including *CAT1-*, *APX1-* and *Cu/ZnSOD*-deficiency under optimal conditions exhibited induction of a number of chloroplast and nuclear encoded genes (Gadjev et al. 2006). The comparative analysis of O^{-}_{2}-induced nuclear genes included those with a 5-fold increase such as *HSPs* (heat shock proteins), *GST* (glutathione S-transferase), anthocyanin biosynthesis genes and a number of transcription factors. *GSTs* (glutathione S-transferases) constitute a complex family of proteins known to be responsive to O^{-}_{2} and H_2O_2 (Mylona et al. 1998, 2007, Polidoros and Scandalios 1999) and to a variety of abiotic stresses. Accumulation of anthocyanins in response to O^{-}_{2}-induced stress as previously reported (Rizhsky et al. 2003) possibly indicates a photooxidative stress in chloroplasts that could cause a retrograde signaling pathway. It is crucial to note that organellar derived signaling may be modulated by interaction with components of other signaling pathways, particularly those involved in responses to abiotic stresses, as well with signaling from other organelles.

Given that O^{-}_{2} is produced in response to various abiotic stresses, overexpression of SOD in model plants would render them stress-tolerant. However, in some transgenics overexpression of cytosolic or chloroplastic SOD provided moderate or minimal tolerance, attributed to the type of overexpressed *SOD* and its subcellular localization (Allen et al. 1997). Light in that direction was shed recently with the discovery that microRNA (miRNA) molecules regulate antioxidant gene expression. Studies in Arabidopsis have identified miR398, a repressor of cytosolic (*CSD1*) and chloroplastic (*CSD2*) *Cu/ZnSOD* expression (Sunkar et al. 2006). Transgenic *Arabidopsis* plants exhibiting down-regulation of miR398 and overexpression of the *CSD2* showed increased tolerance to paraquat- and salt-induced oxidative stress, revealing a direct connection between miRNA pathway and *CSD1* and *CSD2* post-transcriptional regulation. Following, sequence data analysis revealed that miR398 and its target sites on cytosolic and chloroplastic Cu/ZnSOD mRNA are conserved in dicotyledonous and monocotyledonous plants. Further studies showed

that posttranscriptional regulation of SOD genes by miR398 in response to ABA or salt stress in plants is twofold: a dynamic regulation within a plant species and a differential regulation between different plant species (Jia et al. 2009). Conclusively, these data suggest that miR398 exerts its role through distinct regulatory mechanism in response to abiotic stress. Whether this regulation is mediated by ROS or other signaling molecules and which signal transduction pathways are involved remain to be elucidated.

Hydrogen peroxide

Under physiological conditions H_2O_2 is mainly produced in peroxisomes during photorespiration. Peroxisomal H_2O_2 production is by far the biggest producer of H_2O_2 in photosynthetic cells (Foyer and Noctor 2005). In mitochondria and chloroplasts, H_2O_2 is generated from SODs that dismutate O^{-}_2 generated by electron leakage from the ETCs. H_2O_2 levels of cytosol, a result of leakage from subcellular compartments could be further elevated through the function of cytosolic SODs. H_2O_2 is also produced during β-oxidation of fatty acids in glyoxysomes of seeds (Del Rio et al. 2006). Other potential sources of H_2O_2 include NADPH-oxidase, referred as rboh, located at the plasma membrane and a number of enzymes of the extacellular matrix, which participate in regulation and synthesis of cell wall components as well as in apoplastic oxidative burst (Agrawal et al. 2003). Recently, H_2O_2 has been shown to act as a key regulator in a broad range of physiological processes including, senescence (Peng et al. 2005), photorespiration and photosynthesis (Noctor and Foyer 1998), stomatal movement (Bright et al. 2006) cell cycle (Mittler et al. 2004), growth and development (Foreman et al. 2003) and programmed cell death (Bethke and Jones 2001, Fath et al. 2002). Under stress conditions, such as high light intensities, temperature extremes, drought, salinity, UV irradiation, air pollutants, exposure to xenobiotics, metals and pathogen attack, generation of H_2O_2 is further enhanced in various cellular compartments. It is well documented that stress produced H_2O_2 is connected with changes in nuclear gene expression; hence its availability has to be precisely regulated maintaining cellular redox homeostasis and mediating signal transduction pathways. Subsequently, removal of H_2O_2 has to be monitored in its site(s) of generation as well as in the whole cell due to its diffusion ability.

Modulation of gene expression by H_2O_2 has received much attention as it is generated in response to a variety of stress stimuli and it is likely to mediate cross-talk between different signaling pathways (Bowler and Fluhr 2000). A number of studies have shown that manipulation of plant antioxidant levels result in cross-tolerance to subsequent exposure of plant to oxidative stress situations (Neill et al. 2002). Although the field of plant

pathogenesis has certainly led the way in oxidative stress signaling for many years (Lamb and Dixon 1997), accumulating evidence supporting the signaling role of H_2O_2 in defense responses to abiotic stresses are now available (Desikan et al. 2008, Hancock et al. 2006, Neill et al. 2002). The first demonstration of H_2O_2 inducible gene expression in plants was that of *GPX* and *GST* genes in soybean cell suspension cultures (Levine et al. 1994). This was also the first evidence of cross-membrane trafficking of H_2O_2 involved in redox signal transduction pathway in plants. In recent years, specific induction of defense responses has been obtained with direct H_2O_2 treatments or by stressors that induce its generation. Exogenously applied H_2O_2 induced expression of cytosolic SODs in maize embryos (Scandalios 1997). Stress by H_2O_2 of transgenic tobacco plants with 10% of wild-type catalase activity showed that catalase was crucial for maintaining the redox balance during oxidative stress (Willekens et al. 1997). Substantial evidence supporting this showed that high levels of exogenous applied H_2O_2 induce expression of catalase *CAT1, CAT2* and *CAT3* genes in leaves of maize seedlings (Polidoros and Scandalios 1999). Induction of *CAT3* gene superimposed its circadian regulation, demonstrating a direct signaling action in the regulation of the major H_2O_2-scavenging enzymes. It is worth noting that the effects of exogenous H_2O_2 depends on the rate at which it is degraded, which presumably determines its concentration at its site of action. Maize *CAT* genes are also induced in response to wounding and pathogen attack, which also generate H_2O_2 (Guan and Scandalios 2000).

Transgenic tobacco (*Nicotiana tabacum*) plants with antisense suppression of *CAT1* showed increased *APX* and *GPX* levels and a 4-fold decrease in ascorbate pool in response to oxidative stress (Willekens et al. 1997). On the other hand, transgenic tobacco with suppressed *CAT* and *APX* activities were less sensitive to oxidative stress compared to single antisense plants suppressing either peroxisomal *CAT* or cytosolic *APX*, suggesting that lack of H_2O_2-scavenging mechanisms might have turned on an alternative mechanism for cellular protection (Rizhsky et al. 2003). The pivotal role of peroxisomal catalase in decomposing photorespiratory H_2O_2 and modulating the signaling role of this ROS was recently shown in *Arabidopsis CAT2* deficient plants (Queval et al. 2007, Vandenabeele et al. 2004). Specifically, photorespiratory generated H_2O_2 modulates nuclear transcriptional programs influencing expression of cytosolic, chloroplastic and mitochondrial proteins, providing additional evidence for the importance of intraorganellar communication within the plant's defense response. Specifically *CAT2* gene expression plays an indispensable role in preventing redox perturbation under ambient air conditions preventing photooxidation thus its expression is necessary for optimal growth and redox homeostasis in photorespiration. *CAT2* deficiency results in distinct photoperiod-dependent redox signaling that

modulates responses to oxidative stress in the acclimation versus the cell death decision in stress conditions.

Given the regulatory role of catalases in redox homeostasis in presence of photorespiration, clearly peroxisomes are significant regulators of cellular redox state. Circumstantial evidence remarking this exhibited that exogenous H_2O_2, wounding and pathogen attack induced expression of genes encoding proteins required for peroxisome biogenesis (Lopez-Huertas et al. 2000). Leaf peroxisomes from plants treated with cadmium showed an enhancement of H_2O_2 concentration, an increase of the activity of antioxidant enzymes involved in the ascorbate-glutathione cycle and slight peroxisomal proliferation (Del Rio et al. 2006). Up-regulation of the antioxidative systems was also observed in peroxisomes of leaves and roots in wild-type salt tolerant tomato plants in response to salt-induced oxidative stress (Mittova et al. 2004).

In *Arabidopsis* mRNA of peroxisome targeted *APX* increased in response to cold, UV light, treatment with H_2O_2 and paraquat (Zhang et al. 1997). H_2O_2 induces expression of cytosolic ascorbate peroxidase (*APX1*) gene of *Arabidopsis* plants during paraquat treatment and under high-light conditions (Karpinski et al. 1997, Storozhenko et al. 1998). Transcript levels of cytosolic *APX1* were significantly increased by H_2O_2 or paraquat treatment in rice cell suspension cultures (Morita et al. 1999). Addition of diethyldithio-carbamate (a SOD inhibitor resulting in lower H_2O_2 levels) reduced the induction of *APX*, whereas inhibition of CAT or APX activity (resulting in H_2O_2 accumulation) increased *APX* mRNA levels (Morita et al. 1999). Similar observations of cytosolic *APX* gene induction by paraquat and high light stress were reported in pea, maize, Arabidopsis and spinach revealing that cytosolic APX isozyme is the most stress-responsive among different members of the APX gene family (Davletova et al. 2005, Yoshimura et al. 2000). Transgenic tobacco plants expressing antisense RNA for the cytosolic *APX* showed increased susceptibility to ozone (Orvar and Ellis 1997), whereas overexpression of cytosolic *APX* provided increased resistance to paraquat treatment in tobacco plants (Allen et al. 1997). Similarly, in *Arabidopsis* knockout mutant of cytosolic *APX1* exhibited accumulation of H_2O_2 under optimal conditions, whereas exposure to light stress resulted in induction of *CAT, GPX* and a number of heat shock protein (*HSP*) genes (Pnueli et al. 2003). Furthermore, knockout mutant of cytosolic *APX1* exhibited altered stomatal responses and suppressed growth and development suggesting its important regulatory roles. Compelling evidence for the role of cytosolic *APX1* revealed that it is essential for the proper function of chloroplastic APXs and in its absence both thylakoid *APX* (*tylAPX*) and *s/mAPX* (stromal/mitochondrial *APX*) are degraded under high light stress (Davletova et al. 2005). Overexpression

of *tAPX* in *Arabidopsis* plants exhibited increased resistance to paraquat-induced photooxidative stress (Murgia et al. 2004).

AOX is a nuclear encoded enzyme responsive to a variety of abiotic and biotic stresses. Exogenously applied H_2O_2, SA or nitric oxide (NO) on maize seedlings induced expression of *AOX1a* (Polidoros et al. 2005). *Arabidopsis* knockout mutant of mitochondrial *AOX1a* altered the stress-response networks of the cell, making them more susceptible to stress. Deficiency of AOX1a led to remarkable changes in transcriptome of *Arabidopsis* plants even under normal conditions. These changes include several genes encoding components involved in ROS defense, signaling, transcription factors, and proteins located in mitochondria and chloroplasts indicating that retrograde signaling are altered (Giraud et al. 2008).

In *Arabidopsis* suspension cultures, H_2O_2 induced expression of *GST* (Desikan et al. 1998). Exogenous H_2O_2 induced expression of *GST1* leaves of maize seedlings (Polidoros et al. 2005). GST comprises of a family of nuclear encoded enzymes involved in cellular detoxification processes following various abiotic stresses, including exposure to xenobiotics and metals (Mylona et al. 2007). GSTs appear also as binding proteins, for example in the anthocyanin biosynthesis, as well as of tetrapyrroles and porphyrins, therefore there is considerable potential for cell signaling role (Foyer and Noctor 2005).

GPXs comprise a family of isoenzymes that use thioredoxin to reduce H_2O_2 and organic and lipid peroxides, thereby protecting cells against oxidative damage (Gama et al. 2008). In plants GPXs are nuclear encoded enzymes localized in the cytosol, chloroplasts and most subcellular compartments are involved in response to both abiotic and biotic stress. To date, GPXs comprise a group of the Prxs (peroxiredoxins) family (Navrot et al. 2006). Recent data have shown that expression of *GPXs* is enhanced in response to abiotic and biotic stresses including salinity, heavy metal toxicity, bacterial and viral pathogen infections (Avsian-Kretchmer et al. 2004, Ramos et al. 2009) via H_2O_2-mediating signal transduction pathway. *GPX1* of citrus is induced in response to salt-stress and exogenous H_2O_2 and ABA (Avsian-Kretchmer et al. 2004). *GPX1* promoter analysis has shown that salt induction is mediated via ROS predominantly formed as H_2O_2 in an intracellular process, whereas induction by exogenous H_2O_2 involves a different signaling pathway that NADPH oxidase is involved. Surprisingly the promoter of *GPX1* did not respond to exogenous ABA although *GPX1* transcripts increased in response to ABA in citrus. In *Arabidopsis*, it has been demonstrated that GPX specifically relays the H_2O_2 signal to other signaling molecules such as abscisic acid (Dietz 2008). Overexpression of GPX either in the cytosol or chloroplast of tobacco plants resulted in suppressed stress-induced production of lipid peroxides (Foyer and Noctor 2005 and references therein). Using a proteomic approach it

was demonstrated that expression of various mitochondrial antioxidant defense proteins including Prxs are up-regulated following exposure to H_2O_2 (Sweetlove et al. 2002).

ABA is known to induce generation of H_2O_2. Studies in maize seedlings have shown that ABA induces expression of catalase genes (*CAT1, CAT2, CAT3*) and of cytosolic Cu/ZnSOD (*SOD4* and *SOD4a*) (Scandalios 2005). Analysis of the maize *CAT1* promoter revealed the presence of two ABA responsive elements ABRE1 and ABRE2 (Guan and Scandalios 2000). Recent evidence demonstrated that ABA-induced *CAT1* expression in *Arabidopsis* is mediated by a MAPK cascade and that activation of *CAT1* may be part of the feedback regulation of H_2O_2 signaling (Xing et al. 2008). ABA also induces stomatal closure mediated via H_2O_2-signaling that activates calcium permeable channels on the plasma membrane (Desikan et al. 2008).

It is now well accepted that H_2O_2 mediates signal transduction pathways. H_2O_2 is generated in chloroplasts, mitochondria, peroxisomes, cytosol, apoplastic space and cell walls in response to various abiotic stresses. With a comparatively longer half-life of 1ms, lower toxicity compared to other ROS and diffusion capacity, it is favored as an intra- and intercellular messenger (Bhattacharjee 2005, Desikan et al. 1998). However, to function as a signaling molecule, H_2O_2 needs to cross the inner and outer membranes of the chloroplast and peroxisomes, but its polar nature might limit its capacity to diffuse through hydrophobic membranes unassisted. Recent evidence proposes that H_2O_2 transport might be mediated by aquaporin channels (Bienert et al. 2007). However, the necessity of aquaporins for H_2O_2 movement *in vivo* is yet to be determined. Accumulation of H_2O_2 induces Ca^{2+} channels, generation of Ca^{2+}/calmodulin (CaM) complexes and activation of mitogen-activated kinase (MAPK) cascade. Transcription factors (TFs) such as ZAT10, ZAT12 and ABI4 are known to be induced in response to various abiotic and biotic stresses. Transcriptome analysis has shown that nuclear encoded TFs could be induced specifically to accumulation of a particular ROS while others are induced by all types of ROS (Scarpeci et al. 2008). Thus so far a number of TFs have been shown to modulate antioxidant gene responses.

Hydroxyl radical

Hydroxyl radical ($HO^{\cdot}$) is generated by H_2O_2 and O^{-}_2 in presence of iron or copper ions via the Haber-Weiss or Fenton reaction (Halliwell and Gutteridge 1999). Due to its charge, $HO^{\cdot}$ is a strongly oxidizing ROS that can potentially react with all biological molecules (Bhattacharjee 2005). Plant cells have no enzymatic mechanism to eliminate this highly reactive ROS, thus its formation is restricted through the combined action of SOD,

CAT, APX, Prx as well as ferritin that sequesters Fe. Cause of $HO^{\cdot}$ toxicity and its short half life about 2–4 μs, there are no data of antioxidant gene induction by $HO^{\cdot}$. However, evidence indicates that $HO^{\cdot}$ plays a regulatory role in cell wall loosening, root elongation, growth, leaf extension and in oxidative burst in response to fungi challenge (Vreeburg and Fry 2004).

Conclusions and Future Prospects

Over the last decade our view of ROS has changed to realize their dual role: the already known adverse role of toxic metabolic by-products requiring antioxidant defense mechanisms to protect cells from their detrimental effects, and the newly emerging role of signaling molecules regulating growth, development and coordinating responses to abiotic and biotic stress. Several parameters of the ROS generation, scavenging and signaling have been uncovered, but how their dual role is controlled is largely unknown. To date the majority of information of ROS regulation of genes are from global gene expression studies in wild-type and transgenic plants that overexpress or suppress a particular antioxidant enzyme in response to application of specific ROS and ROS generators.

Considerable progress towards that direction is made by use of mutants specifically impaired in ROS generation or response that delineate ROS specificity and provide significant data of ROS-responsive genes. Deficiencies in particular antioxidant gene/enzymes has shown that they are indispensable not only as ROS scavengers but as regulators of stress-response, growth and development. A number of genes involved in defense, signal transduction, transcription, metabolism as well as cell structure have been identified. Studies in *Arabidopsis* and other plant species have revealed a network of ROS-producing and ROS-scavenging genes that is highly dynamic and redundant. Regulation of this network and the fine tuning between ROS-production and ROS-scavenging that is required to enable the regulatory role of ROS in modulation of signaling networks that control growth, development and stress responses, are central questions that remain unanswered. Antioxidant genes are central players in this network and their function has profound effects in controlling ROS levels and the redox balance of the cell. More importantly, recent evidence suggest that ROS can regulate the level of antioxidant gene expression and thus provide a feedback loop in regulation of ROS levels, that is critical component of the role ROS perform.

A schematic representation of ROS signaling networks that regulate antioxidant gene expression and lead to fine tuning of ROS levels in the cell is depicted in Fig. 1. Environmental insult (abiotic and biotic stress) induces enhanced generation of ROS in organelles and cytosol, which in turn orchestrates signal transduction pathways that activate gene expression.

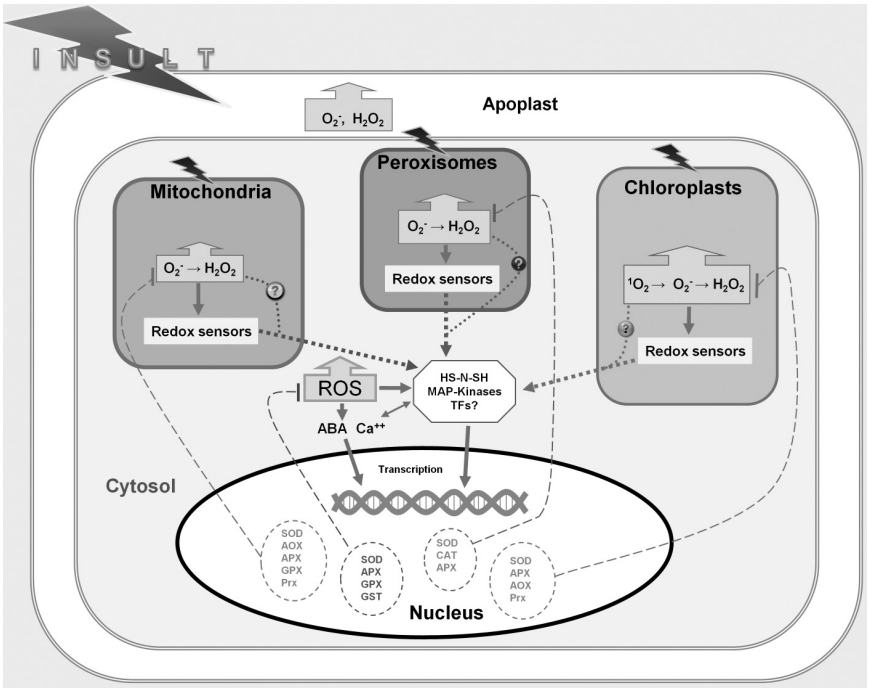

Fig. 1 Simplified working model of known and predicted ROS networks involved in operational control of nuclear gene expression. Dotted lines represent retrograde signaling and dashed lines represent feedback regulation of ROS levels. Solid lines indicate transcription activation. Abbreviations: HS-N-SH, cytosolic thiol protein sensors; MAP-kinases, mitogen activated kinase cascade; SOD, superoxide dismutase; APX, ascorbate peroxidases; AOX, alternative oxidase; CAT, catalase; GST, glutathione S-transferase; GR, glutathione reductase, Prx, preoxiredoxins.

Ubiquitous redox sensors containing thiol groups are thought to play a central role in perceiving perturbations in redox balance. Retrograde signaling (communication of organelles to nucleus) is mediated by well characterized mediators, for example mitogen activated protein kinase (MAPK) cascades and other known or yet unknown transcription factors (TFs). Simultaneously, abiotic stress induces ABA accumulation that leads to accumulation of ROS and intracellular Ca^{++} affecting the activation of MAPKs and leading to antioxidant gene responses. Possible involvement of other components in this signal transduction is not clear. Consequently, ROS-dependent signaling activate expression of antioxidant genes that decrease ROS levels in a feedback loop regulation resulting in a fine tuning of the redox balance in the cell that controls growth, development and abiotic and biotic stress responses.

Yet, many questions regarding antioxidant genes related to the mode of regulation of particular isoforms, the protective roles and the modulation of signaling networks that control growth, development and stress responses remain unanswered. The challenge is to define the specific role each antioxidant gene has undertaken in the ROS-gene network of the cell and link the regulation of the gene to the regulation of the network as a whole under various conditions. Such knowledge may ultimately be exploited to modulate ROS-related plant processes and enable breeding of better performing crop plants.

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