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REVIEW ARTICLE

Reactive Oxygen Species, Antioxidants and Signaling in Plants

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Several reactive oxygen species (ROS) are continuously produced in plants as byproducts of many metabolic reactions, such as photosynthesis, photorespiration and respiration. Depending on the nature of the ROS species, some are highly toxic and rapidly detoxified by various cellular enzymatic and nonenzymatic mechanisms. Oxidative stress occurs when there is a serious imbalance between the production of ROS and antioxidative defence. ROS participate in signal transduction, but also modify cellular components and cause damage. ROS is highly reactive molecules and can oxidize all types of cellular components. Various enzymes involved in ROS-scavenging have been manipulated and over expressed or down regulated. An overview of the literature is presented in terms of primary antioxidant free radical scavenging and redox signaling in plant cells. Special attention is given to ROS and ROS-anioxidant interaction as a metabolic interface for different types of signals derived from metabolisms and from the changing environment.

Keywords: antioxidants, gene expression, MAPK signaling, ROS

When plants are subjected to environmental stress conditions such as high light intensity, temperature extremes, drought, high salinity, herbicide treatment, or mineral deficiencies, the balance between the production of reactive oxygen species and the quenching activity of the antioxidants is upset, often resulting in oxidative damage (Spychalla and Desborough, 1990). The main sites of ROS are mitochondria, chloroplast, peroxisomes, plasmamembrane and apoplast. Limited CO_2 fixation due to stress conditions leads to a decrease in carbon reduction by the Calvin cycle and to a decrease in oxidized $NADP^+$ to serve as electron acceptor in photosynthesis. When ferrodoxin is overreduced during photosynthetic electron transfer, electrons may be transferred from PS-I to oxygen to form superoxide radicals ($O_2^{-\bullet}$) by the process called Mehler reaction (Hsu and Kao, 2003). This triggers chain reaction that generates more aggressive oxygen radicals. Photorespiration has evolved to prevent overreduction of ETC by regeneration of $NADP^+$ (Kozaki and Takeba, 1996). As part of photorespiratory, H_2O_2 is formed in the peroxisomes. H_2O_2 is also produced from β-oxidation of fatty acids as a byproduct. Xanthine oxidase is the other source of ROS in the peroxisomes, which generates $O_2^{-\bullet}$ during the catabolism of purines. In NADH-induced production of $O_2^{-\bullet}$ has been demonstrated in the peroxisomal membranes of castor bean endosperm and pea leaves (del Rio et al., 1998). NADPH induced production of $O_2^{-\bullet}$ has also been characterized in the peroxisomal membranes of pea leaves (Lopez-Huertas et al., 1999).

The intracellular generation of ROS occurs at the mitochondrion due to leakage of the electrons at the ubiquinone: cytochrome b region and at the matrix side of complex 1 (NADH dehydrogenase) (Moller, 2001). H_2O_2 generation and regulation by uncoupling of ETC and oxidative phosphorylation have also been demonstrated.

At endoplasmic reticulum, $O_2^{-\bullet}$ is formed as a result of detoxification reactions catalysed by the cytochromes particularly cytochrome P_{450}. ROS is also generated by NADPH-dependent oxidases at the plasma membrane level or extracellularly in the apoplast. In the plasma membranes, NADPH oxidases generate ROS during both biotic and abiotic stresses (Mittler, 2002). In the apoplast, pH- dependent cell wall peroxidases, germin like oxalate oxidase and amine oxidases are sources of H_2O_2. The H_2O_2 formed may be utilized by wall bound peroxidases in lignification and cell wall strengthening both during normal growth as well as in response to external stimuli such as wounding and pathogenesis (Srivalli et al., 2003). Under abiotic stresses $O_2^{-\bullet}$ production enhances from 240 to 720 μM S^{-1} and that of H_2O_2 in chloroplast from 5 to 15 μM (Polle, 2001; Mittler, 2002).

ROS causes damage to lipids, proteins and DNA (McCord, 2000). Peroxidation of membrane lipids occurs when ROS reacts with unsaturated fatty acids leading to leakage of cellular contents, rapid desiccation and hence cell death (Fig. 1). The harmful effect of ROS is due primarily to their ability to initiate a variety of autoxidative chain reactions on unsaturated fatty acids (Smirnoff, 2000). Oxidative attack on proteins results in site specific amino acid modifications, fragmentation of the peptide chain, aggregation of cross linked reaction products and increased susceptibility to proteolysis. ROS can also induce numerous lesions in DNA that cause deletions, mutations and other lethal genetic effects (Srivalli et al., 2003). Plants with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to this oxidative damage (Table 1) (Serres and Mittler, 2006). The ability of plant tissues to mobilize enzymatic defense against uncontrolled lipid peroxidation may be an important facet of their tolerance (Srivalli et al., 2003). Primary intracellular plant antioxidant expression are closely related to their metabolic state and is responding to constantly fluctuating environment (Stohr and Stremlau, 2006; Mullineaux et al., 2006). Discoveries made over the past few decades have demonstrated that ROS are not only destructive, but can also be important signals. At sublethal levels, ROS have been shown to activate defence responses

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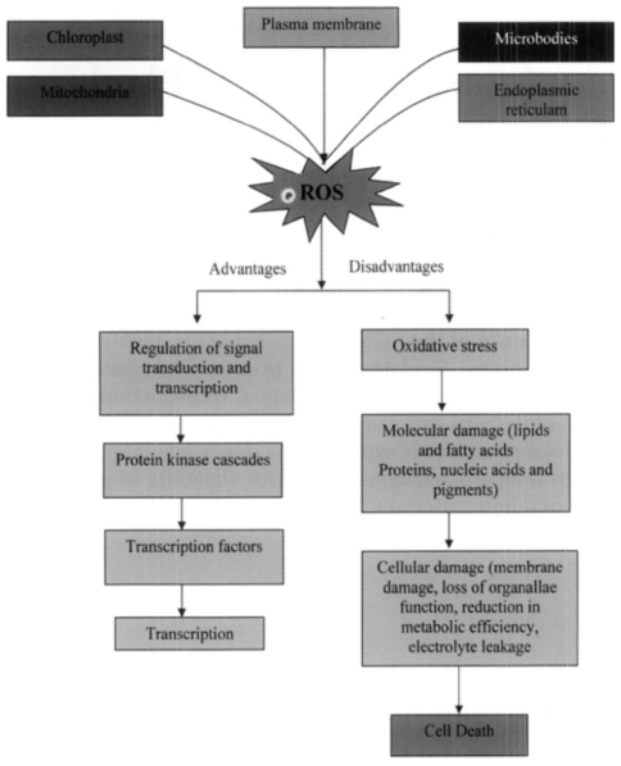

Figure 1. Sites of reactive oxygen species (ROS) and the biological consequences leading to a variety of physiological dysfunctions that can lead to cell death.

in model organisms such as *S. cerevisiae* and *Escherichia coli*. Activating these defences enhances survival during subsequent oxidative stress from the same ROS, but how these signals are converted into changes in gene expression is not yet clear. This review throws light on enzymatic and non-enzymatic antioxidants and signaling during the ROS production.

ANTIOXIDANTS IN PLANTS

Abiotic stress results in the formation of ROS in plants which creates a condition called oxidative stress that can damage cellular components (Apel and Hirt, 2004). Plants have developed efficient antioxidant system that can protect plants from this disaster (Mittler et al., 2004). The toxic effects of ROS are counteracted by enzymatic as well as non- enzymatic antioxidative system such as: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), Ascorbic acid (AsA), Tocopherol, Glutathione and phenolic compounds etc (Fig. 2). Normally, each cellular compartment contains more than one enzymatic activity that detoxifies a particular ROS. For example, the cytosol contains at least three different enzymatic activities that scavenge H_2O_2: APX, GPX, and PrxR (Nobuhiro and Mittler, 2006). Anaerobic bacteria possess rubredoxin and neelaredoxin, the reduced form of iron-sulphur proteins, which helps them to convert $O_2^{-\bullet}$ radicals to H_2O_2 (Abreu et al., 2001). Thioredoxin-peroxyredoxin system, though, localized in plant chloroplasts, have also been found in cyanobaceria where they are involved with the detoxification of H_2O_2. Presence of these enzymes in almost all cellular compartments clears their crucial role of ROS detoxification for the survival of the plant (Mittler et al., 2002) (Fig. 3). Activities of antioxidant enzymes have been directly correlated with the stress tolerance in plants and augmented ability to scavenge ROS has been observed in the plants that grow in sublethal levels of stress (Fecht-Christoffers et al., 2003).

In plants over 150 genes encode for different ROS-detoxifying or ROS-producing enzymes forming well organised ROS gene web (Mittler et al., 2004). During the past few years, considerable progress has been made in understanding how plants protect themselves against oxidative stresses and pathogens. Several genes encoding for plant antioxidant enzymes have been cloned, characterized, and used in the construction of transgenic lines (Sarowar et al., 2005). Genetic analysis of the paraquet tolerant *Conyza bonariensis* indicated that all the three enzymes of Halliwell-Asada pathway, i.e. SOD, APX and catalase, co-segregate (Shaaltiel et al., 1988). Manipulation of genes that protect and maintain cellular functions or those maintain structure of cellular components has been the major target of attempts to produce plants that have enhanced stress tolerance. Qualitative and molecular analysis of the transgenic plants overexpressing different enzymes of Halliwell-Asada cycle can provide us greater insights into the oxidative stress tolerance mechanism which in turn leads to the increased abiotic stress tolerance. Several recent studies have been aimed at enhancing ROS protection by the constitutive overexpres-

Table 1. ROS scavenging and detoxifying enzymes.

Enzyme	EC number	Reaction catalysed
Superoxide dismutase	1.15.1.1	$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ 2H_2O_2 + O_2$
Catalase	1.11.1.6	$2H_2O_2\ O_2 + 2H_2O$
Glutathione peroxidase	1.11.1.12	2GSH + PUFA–OOH GSSG + PUFA + $2H_2O$
Glutathione S-transferases	2.5.1.18	RX + GSH HX + R-S-GSH*
Phospholipid-hydroperoxide glutathione peroxidase	1.11.1.9	2GSH + PUFA-OOH (H_2O_2) GSSG + $2H_2O$
Ascorbate peroxidase	1.11.1.11	AA + H_2O_2 DHA + $2H_2O$
Guaiacol type peroxidase	1.11.1.7	Donor + H_2O_2 oxidized donor + $2H_2O$
Monodehydroascorbate reductase	1.6.5.4	NADH + 2MDHA NAD^+ + 2AA
Dehydroascorbate reductase	1.8.5.1	2GSH + DHA GSSG + AA
Glutathione reductase	1.6.4.2	NADPH + GSSG $NADP^+$ + 2GSH

*R may be an aliphatic, aromatic or heterocyclic group; X may be a sulfate, nitrite or halide group.

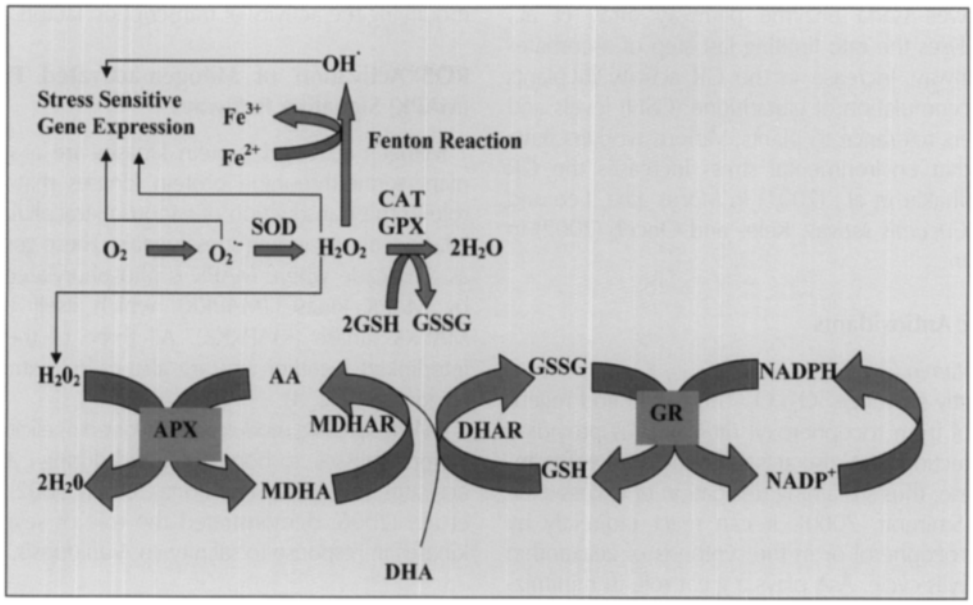

Figure 2. ROS generation, antioxidant Pathway and activation of stress-sensitive gene expression. SOD (Superoxide dismutase), CAT (Catalase), GPX (Glutathione peroxidase), GSH (Glutathione), GSSG (Oxidized glutathione), APX (Ascorbate peroxidase), AA (Ascorbate), MDHA (Manodehydroascorbate), MDHAR (Manodehydroascorbate reductase), DHA (Dehydroascorbate), DHAR (Dehydroascorbate reductase).

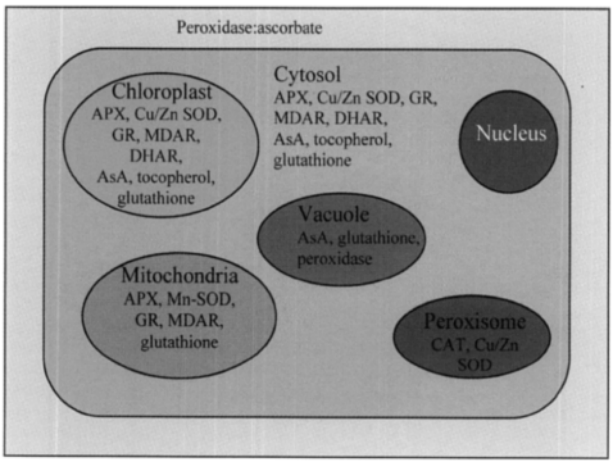

Figure 3. Antioxidant resources in plant cells.

sion of antioxidant defence enzymes in transgenic plants (Lee et al., 2007).

Enzymatic Antioxidants

Superoxide dismutase (SOD; EC 1.15.1.1)

The metalloenzyme superoxide dismutase converts $O_2^{-\bullet}$ to H_2O_2 (Table 1) and was first demonstrated in maize which contained six genetically and biochemically distinct isozymes (Scandalios, 1993). The upregulation of SODs is implicated in combating oxidative stress caused due to abiotic stress and have a critical role in the survival of plants. Coinciding increase in SOD activity under salt stress have been observed in various plants viz. barley (Liang, 1999), tomato (Shalata et al. 2001), mulberry (Harinasut et al. 2003).

Catalases (EC 1.11.1.6)

Catalases are the principal scavenging enzymes which can directly dismutate H_2O_2 and is indispensable for ROS detoxification during stress (Van Breusegem et al., 2001). Sudhakar et al., (2001) have shown that CAT activity increases by more in salt tolerant mulberry cultivars S1 than salt susceptible cultivar ATP at higher concentration of NaCl. The *Escherichia coli* catalase encoded by the *katE* gene overexpressed under CaMV35S promoter in Japonica rice (*Oryza sativa*) conferred transgenic rice plants tolerance to the salt and catalase activity being about 1-5 to 2.5 times more than non-transgenic plants (Nagamiya et al., 2007).

Glutathione Peroxidases (GPX, EC 1.11.1.9)

Besides scavenging of H_2O_2, GPxs also serve to detoxify products of lipid peroxidation formed due to activity of ROS (Table 1). GPx decomposes peroxides to water (or alcohal) while simultaneously oxidizing GSH. GPx competes with catalase for H_2O_2 as a substrate and is the major source of protection against low levels of oxidative stress.

Ascorbate peroxidase (APX, EC 1.11.1.1)

APX is involved in scavenging of H_2O_2 in water-water and ascorbate-glutathione cycles and utilizes AsA as the electron donor. The APX family consists of at least five different isoforms including thylakoid and microsomal membrane bound forms, as well as soluble stromal, cytosolic and apoplastic enzymes (Noctor and Foyer, 1998). APX has a higher affinity for H_2O_2 (μM range) than CAT and POD (mM range) and it may have a more crucial role in the management of ROS during stress or it may be responsible for the fine modulation of ROS for signaling.

Glutathione reductase (GR, EC 1.6.4.2)

GR catalyses the NADPH dependent reaction of disulphide bond of GSSG and is thus important for maintaining the reduced pool of glutathione (Table 1). The role of Glutathione and GR in H_2O_2 scavenging has been well estab-

lished in Halliwell-Asada enzyme pathway (Bray et al., 2000). GR catalyses the rate limiting last step of ascorbate-glutathione pathway. Increase in the GR activity in plants results in the accumulation of glutathione (GSH) levels and ultimately confers tolerance to plants. Various workers have demonstrated that environmental stress increases the GR activity e.g, Sudhakar et al., (2001) in *Morus alba*, Lee and Lee, (2000) in *Cucumis sativas*, Keles and Oncel, (2002) in *Triticum aestivum*.

Non-enzymatic Antioxidants

Ascorbic acid (Vitamin C)

AsA can directly scavenge $^1O_2$, $O_2^{-\bullet}$ and $^\bullet OH$ and regenerate tocopherol from tocopheroxyl radical, thus providing membrane protection. AsA also acts as co-factor of violaxanthin de-epoxidase, thus sustaining dissipation of excess excitation energy (Smirnoff, 2000). It can react indirectly by regenerating α-tocopherol or in the synthesis of zeaxanthin in the xanthophylls cycle. AsA plays a great role in minimizing the damage caused by oxidative process. This is performed by its synergitic action with other antioxidants (Foyer and Noctor, 2005).

Tocopherols

α-tocopherols prevent the chain propagation step in lipid autooxidation and this makes it an effective free radical trap. The increase in tocopherols in conjunction with AsA has been implicated as one of the primary responses of water deficit conditions in rice (Boo and Jung, 1999). Oxidative stress activates the expression of genes responsible for the synthesis of tocopherols in higher plants (Wu et al., 2007). Antioxidants including α-tocopherol and ascorbic acid have been reported to increase following triazole treatment in tomato and these may have a role in protecting membranes from oxidative damage, thus contributing to chilling tolerance (Shao et al., 2007). Increase in tocopherol during water stress in plants have been demonstrated by many workers (Wu et al., 2007; Shao et al., 2007). Pourcel et al., (2007) have shown that drought stress led to an increase of 1 to 3-fold of α-tocopherol concentration in some grass species.

ROLE OF ROS IN SIGNALING

Signal transduction is a process enabling information to be transmitted from the outside of the cell to various functional elements inside the cell, i.e. the outside signal results in changes in nucleur transcriptome. ROS sensors could be activated to induce signaling cascades that ultimately impinge on gene expression. Alternatively, components of signaling pathways could be directly oxidized by ROS. Signaling mediated by ROS involves hetero-trimeric G-proteins (Pfannschmidt et al., 2003) and protein phosphorylation regulated by specific MAP kinases and protein Tyr phosphatases (Kiffin et al., 2006; Foyer and Noctor, 2005). The biochemical and structural basis of kinase pathway activation by ROS remains to be established in higher plants, but thiol oxidation possibly plays a key role (Gapper and Dolan, 2006). Finally, ROS might change gene expression by targeting and modifying the activity of transcription factors.

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

Mitogen-activated protein kinases are a specific class of plant serine/threonine protein kinases that play a central role in the transduction of various extracellular and intracellular signals, including stress signals. These generally function as a cascade where MAPK is phosphorylated and activated by MAPK kinase (MAPKK), which itself is activated by MAPKK kinase (MAPKKK). All three of these kinases are interlinked together and are also called extracellular receptor kinases (Fig. 4).

MAPK signaling modules are involved in eliciting responses to various stresses, to hormones, and during cytokinesis. H_2O_2 activates several MAPKs (Jonak et al., 2002). Sanan-Mishra et al., (2006) demonstrated the role of several plant MAP kinases in response to salinity. In *Arabidopsis*, H_2O_2 activates

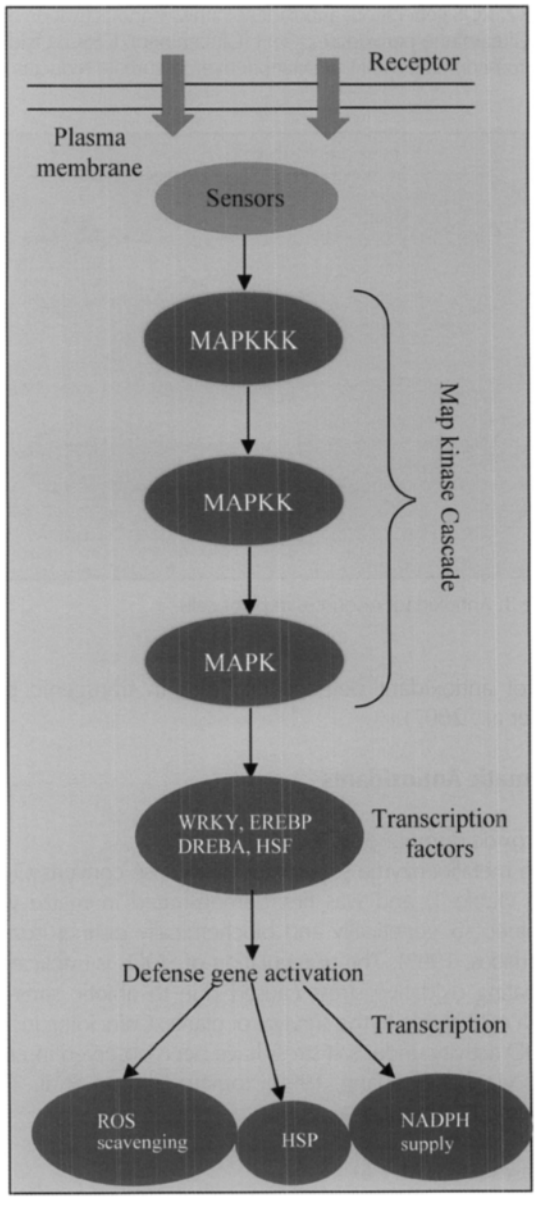

Figure 4. The MAP kinase signal transduction pathway.

the MAPKs, MPK3, and MPK6 via MAPKKK ANP1, the overexpression of ANP1 in transgenic plants resulted in increased tolerance to heat shock, freezing, and salt stress (Kovtum et al., 2000). H_2O_2 also increases expression of the *Arabidopsis* nucleotide diphosphate (NDP) kinase 2 (Moon et al., 2003). Accumulation of H_2O_2 was reduced to a greater extent by overexpression of AtNDPK2 which enhanced tolerance to cold, salt and oxidative stress. The effect of NDPK2 might be mediated by the MAPKs, MPK3, and MPK6 because NDPK2 can interact and activate the MAPKs. Zhang et al., (2006) isolated a new MAP kinase gene (CbMAPK3) from *Chorispora bungeana* plant and its transcript level was upregulated in response to salinity and cold stresses.

The reports suggest that stress are responsible for the generation of ROS. ROS activates MAPK signaling cascades which suggest that a MAPK kinase signaling cascades helps in mediating stress tolerance in plants.

ROS as Signals for Gene Expression

The microarray expression analysis has revolutionized our knowledge regarding gene expression on a genomic scale, rendering thousands of genes assayable in a single experiment (Schena et sl., 1995). DNA microarrays can comprehensively examine gene expression networks during oxidative stress. There is now significant progress being made in surveying gene expression in response to H2O2 in *Escherichia coli* (Zheng et al., 2001), yeast (Gasch et al., 2000; Causton et al., 2001), animals (Finkel and Holbrook, 2000), and higher plants (Desikan et al., 2000).

A genome-wide transcription profile of *E. coli* cells exposed to H_2O_2 was examined with a DNA microarray composed of 4169 *E. coli* open reading frames (Zheng et al., 2001). Gene expression was measured in isogenic wild type and *oxyR* deletion mutants (Δ*oxyR*) to confirm that the H_2O_2-response regulator OxyR activates most of the H_2O_2-inducible genes. Several new H_2O_2 inducible genes were also identified: some were members of the OxyR regulon and some induced by an OxyR-independent mechanism suggestive of other H_2O_2 sensors and regulators in *E. coli* (Christman et al., 1985). The results from the *E. coli* microarrays clearly indicate that the activities of transcription factors in addition to OxyR and SoxRS are likely modulated by oxidative stress.

In plants, ROS-induced genes have been identified for receptor kinase (Desikan et al., 2000), annexin (Moseyko et al., 2002), and peroxisome biogenesis (Desikan et al., 2000). Recent approaches using cDNA profiling and DNA microarrays have analyzed large scale gene expression in response to ROS. Following exposure of *Arabidopsis* cells to H_2O_2, a total of 175 genes (i.e., 1-2% of the 11,000 genes on the microarray) showed changes in expression levels (Desikan et al., 2001). Out of 175 genes 62 are repressed and 113 are induced. Of the 113 induced genes, several encoded for proteins with antioxidant functions or were associated with defense responses or other stresses. Still others coded for proteins with signaling functions. A portion of these genes have functions in defense response, cell rescue and signaling, and transcription, underscoring the pleiotropic effects of H_2O_2 in the response of plants to stress. Most of the genes out of 175 have no direct role in oxidative stress but may be linked to oxidative stress indirectly, as a consequence of other biotic and abiotic stresses, explaining their sensitivity to H_2O_2. H_2O_2 induced genes encoding transcription factors, suggesting that they may mediate downstream H_2O_2 responses. As in other organisms, expression of the MAPKs in *Arabidopsis* is induced by oxidative stress, which in turn can mediate the induction of oxidative stress-responsive genes (Kovtum et al., 2000).

Vranova et al., (2002) analysed the gene expression of tobacco plants treated with methyl viologen (MV) which generates superoxides. Approximately 2% of the tobacco genes were altered in their expression in acclimated leaves. Genes with predicted protective or detoxifying functions and signal transduction were upregulated in acclimated leaves, implying a variety of cellular responses during acclimation tolerance.

Oxidative stress affects approximately 10% of the yeast transcriptome (Causton et al., 2001; Chen et al., 2003; Gasch et al., 2000). Exposure of yeast cells to various stresses including H_2O_2 defines a large set of genes denoted as common environmental stress response (CESR). Whole genome expression patterns in *Saccharomyces cerevisiae* cells exposed to H_2O_2, in addition to other stresses, indicated that ~2/3 of the genome is involved in the response to environmental changes, and the global set of genes induced/repressed by each environmental signal were identified (Gasch et al., 2000; Causton et al., 2001). The response to oxidative stress involves ~1/3 of the yeast genome and the maximal effects on gene expression occur slightly later relative to other stresses examined during similar time-courses, with most of the transcriptome returning to prestress levels within 2 h following exposure to H_2O_2 (Gasch et al., 2000). CESR-induced genes play a role in carbohydrate metabolism, ROS detoxification, protein folding and degradation, organellar function, and metabolite transport. CESR-repressed genes are involved in energy consumption and growth, RNA processing, transcription, translation, and ribosome and nucleotide biosynthesis (Causton et al., 2001; Chen et al., 2003; Gasch et al., 2000).

By inhibiting H_2O_2 production, or facilitating its removal with scavengers such as CAT, genes encoding APX, glutathione Stransferase (GST) were identified (Desikan et al., 1998; Karpinski et al., 1999). CAT and ascorbate peroxidase antisense lines show elevated expression of SOD and GR (Rizhsky et al., 2002). In contrast, MDAR, a key enzyme for the regeneration of ascorbate, was upregulated in plants with experimentally reduced CAT and ascorbate peroxidase levels. An increase in expression of ROS detoxifying enzymes is compatible with compensatory mechanisms induced by oxidative stress.

CONCLUSION AND FUTURE PERSPECTIVE

The generation of reactive oxygen species (ROS) represent a constant source of assaults upon our genetic material which can be either enhanced or partly reduced by nutritional, hormonal and environmental influences. ROS pose a threat to crop production because they are unable to detox-

ify effectively by the ROS scavenging machinery. The unquenched ROS react spontaneously with organic molecules and cause membrane lipid peroxidation, protein oxidation, enzyme inhibition and DNA and RNA damage.

Analysis can be performed on plants responding to abiotic or biotic stresses or combinations of both, and can be complemented by using mutants with altered ability to produce or scavenge ROS. Since the scavenging pathway is quite complex, the use of transgenic plants to probe the role of the antioxidant system still continues to be an important approach. The harmful effect of oxidative stress is counteracted by the antioxidant action of both enzymatic and non-enzymatic antioxidants. Plants overexpressing antioxidant enzymes have been engineered with the aim of increasing stress tolerance by directly modifying the expression of these ROS scavenging enzymes. Positive effects of SOD and APX overexpression and other antioxidants have been reported by various workers. However, the protective effects were marginal and were observed under strictly controlled conditions. Elevated GSH biosynthetic capacity in the chloroplasts of transgenic tobacco plants paradoxically caused increased oxidative stress because of the failure of the redox sensing process in the chloroplast (Creissen et al., 1999). Besides exacerbating cellular damage, ROS can act as ubiquitous signal molecules in higher plants. Higher plant cells can be considered as a series of interconnecting compartments with different antioxidant buffering capacities determined by differences in synthesis, transport and/or degradation.

Redox regulation of gene expression by oxidants and antioxidants is emerging as a vital mechanism in the growth and development of the plant. ROS serve as a common factor in regulating various signaling pathways. Similar stresses also activate MAPKs with kinetics that either precede or parallel H_2O_2 production, indicating that MAPKs may be one of several converging points in the defense-signaling network. Genomic tools are accelerating the discovery of ROS-responsive genes on a global scale and are expanding our understanding of the oxidative stress response and the pleiotropic roles of ROS in signaling and gene expression.

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