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Reactive Oxygen Species Metabolism in Plants: Production, Detoxification and Signaling in the Stress Response

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ABSTRACT

The production of reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2), in plants is a common event in metabolic and physiological processes. ROS are normally formed in photosynthesis and respiration by the chloroplast and mitochondrial electron transfer chains, respectively, and in metabolic reactions taking place in the peroxisomes. As these active oxygen species are destructive to cellular components such as lipids, nucleic acid and proteins, plant cells are equipped with non-enzymatic and enzymatic antioxidant defense systems comprising ascorbate, glutathione, phenols, catalases, superoxide dismutases and peroxidases. Biotic and abiotic stress, such as salinity stress, excess of heavy metals, mechanical shock, UV light, exposure to ozone, water deficiency and pathogen attack, also increase ROS production. In the latter case the release of ROS, referred to as the "oxidative burst", is one of the earliest responses activated following pathogen recognition and has been suggested to play a pivotal role in the integration and the coordination of the plant defense responses. In this review we summarize the current knowledge about ROS production and oxidative defense in plants. The role of ROS will be discussed in the frame of stress responses, with emphasis on the plant-pathogen interaction.

Keywords: abiotic stress, oxidative burst, oxygen radicals, plant-pathogen interaction, ROS, ROS signaling
Abbreviations: AsA, ascorbic acid; **CAT**, catalase; **ET**, ethylene; **GSH**, glutathione; **JA**, jasmonic acid; **HR**, hypersensitive response; **MAPK**, mitogen-activated protein kinase; **NADPH**, nicotinamide adenine dinucleotide phosphate; **NO**, nitric oxide; **PCD**, programmed cell death; **ROS**, reactive oxygen species; **SA**, salicylic acid; **SAR**, systemic acquired resistance; **SOD**, superoxide dismutase

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INTRODUCTION

The production of reactive oxygen species (ROS), also known as reactive oxygen intermediates (ROI) or active oxygen species (AOS), as byproducts of metabolic processes such as respiration and photosynthesis is the price to pay for the advantages of aerobic life. The derivatives of molecular oxygen, including superoxide anion $O_2^{\bullet-}$, singlet oxygen $^1O_2$, hydroxyl radical $OH^{\bullet}$ and hydrogen peroxide H_2O_2 (**Table 1**), are in fact toxic and can damage cellular constituents leading to cell death. The ability to survive these cellular toxins depends on enzymatic and non-enzymatic detoxification mechanisms coordinately acting to reduce cellular damage under oxidative conditions. In this frame, Foyer and Noctor (2005) aptly described the plant cell as a network of compartments whose varying antioxidative buffering capacities are determined by differences in synthesis, transport and/or degradation of antioxidants.

Upon exposure to various stress conditions, such as temperature extremes, excess of light, air pollutants, anoxia, heavy metals, water deficit and pathogen attack (Smirnoff 1993; Lamb and Dixon 1997; Dat *et al*. 2000; Blokhina *et al.* 2003; Sgherri *et al.* 2003; Kangasjärvi *et al*. 2005), the equilibrium between ROS generation and removal is often shifted towards the former, leading to an increased production and accumulation of oxidative species. It is now clear that a common theme in the response to both biotic and abiotic stress is the generation of ROS that can be by themselves the primary cause of adverse effects and/or be involved in signal transduction pathways and changes in gene expression.

ROS GENERATION AND SCAVENGING

In plant cells ROS are continuously produced as a consequence of normal metabolism in virtually all the intracellular organelles, in particular in the chloroplasts, mitochondria and peroxisomes (Elstner 1982; Smirnoff 1993; Apel

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Invited Review

Table 1 Mechanisms for the generation of Reactive Oxygen Species in plant cells. ^{3}Chl*, chlorophyll triplet state; Fd_{red}, reduced ferrexoxin; Fd_{ox}, oxidized ferredoxin; Fe-S_{red}, reduced Rieske FeS center; Fe-S_{ox}, oxidized Rieske FeS center; M, trace metals (Fe^{2+}, Cu^{+}); $Q_{A\ red}$, reduced quinone A; $Q_{A\ ox}$, oxidized quinone A; RH, lipid.

Oxidative species	Production mechanisms
Superoxide radical ($O_2^{\bullet-}$)	$Fd_{red} + O_2 \rightarrow Fd_{ox} + O_2^{\bullet-}$
	$Fe\text{-}S_{red} + O_2 \rightarrow Fe\text{-}S_{ox} + O_2^{\bullet-}$
	$Q_{A\ red} + O_2 \rightarrow Q_{A\ ox} + O_2^{\bullet-}$
	$Cu^{+} + O_2 \leftrightarrow Cu^{2+} + O_2^{\bullet-}$
Hydrogen peroxide (H_2O_2)	$O_2^{\bullet-} + O_2^{\bullet-} + 2H^{+} \leftrightarrow H_2O_2 + O_2$
Hydroxyl Radical ($OH^{\bullet}$)	Haber-Weiss cycle:
	$O_2^{\bullet-} + M^{n+} \rightarrow O_2 + M^{(n-1)+}$
	$M^{(n-1)+} + H_2O_2 \rightarrow OH^{-} + OH^{\bullet} + M^{(n+1)}$
	overall:
	$H_2O_2 + O_2^{\bullet-} \rightarrow OH^{-} + OH^{\bullet} + O_2$
	Fenton reaction:
	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + OH^{\bullet}$
Singlet oxygen ($^1O_2$)	$^3Chl^* + O_2 \rightarrow {}^1O_2$
Hydroperoxyl radical ($HO_2^{\bullet}$)	$O_2^{\bullet-} + H^{+} \rightarrow HO_2^{\bullet}$
Lipid radical ($R^{\bullet}$)	$RH + OH^{\bullet} \rightarrow R^{\bullet} + H_2O$
Lipid peroxyl radical ($ROO^{\bullet}$)	$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$
	$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$
Lipid hydroperoxide (ROOH)	$RH + {}^1O_2 \rightarrow ROOH$

and Hirt 2004) (**Fig. 1**). In the chloroplast ROS are generated in the photoreduction of oxygen to H_2O_2 by photosystem I electron transport (Mehler reaction), whose primary product is superoxide anion, and in photodynamic reactions occurring under conditions that limit electron transfer through the photosystems, such as high light intensity and low CO_2 concentration (Elstner 1982; Apel and Hirt 2004; Mittler *et al.* 2004). Superoxide anion can also be produced by the leaking of electrons to molecular oxygen from electron transport chains in photosystems I and II (Sgherri *et al.* 1996). Finally, insufficient energy dissipation during photosynthesis leads to the formation of chlorophyll triplet state that can generate singlet oxygen by energy transfer to ground-state oxygen, and its production is increased during excess light stress (Apel and Hirt 2004; Asada 2006; Halliwell 2006). H_2O_2 and superoxide radical, but also nitric oxide (NO), are produced in peroxisomes and glyoxysomes (del Río *et al.* 2002). The generation of superoxide involves both the xanthine oxidase reaction in the organelle matrix and a small electron transport chain at the peroxisomal membrane level. H_2O_2 on the other hand derives from the glycolate oxidase reaction, the β-oxidation of fatty acids in the catabolism of lipids, the enzymatic reaction of flavin oxidases and the disproportionation of superoxide radicals (del Río *et al.* 2002; Halliwell 2006). At variance with mammalian cells, where mitochondria are the main source of ROS, the contribution of the mitochondrial electron transfer chain to ROS production in plant cells is low (Apel and Hirt 2004). This has been ascribed to the presence of the enzyme alternative oxidase (AOX) that limits the production of ROS by oxidizing ubiquinol in an O_2-dependent reaction. However, the contribution of mitochondria to ROS generation in the dark and in non-green tissues must not be underestimated (Rhoads *et al*. 2006) and mitochondrial ROS evolution can increase as a consequence of oxidative stress conditions that lead to ROS accumulation in other cellular compartments thereby altering the normal mitochondrial function (Overmyer *et al*. 2003). Another possible source of $O_2^{\bullet-}$ in plants is the electron leakage to oxygen during the monooxygenase reaction catalyzed by the cytochromes, in particular cytochrome P_{450}, in the cytoplasm and the endoplasmic reticulum (Urban *et al*. 1997). Finally, as will be discussed in more detail later, the NADPH-dependent oxidase complex of plant plasma membranes, pH-dependent cell wall peroxidases, germin-like oxalate oxidases, and amine oxidases have also been identified as sources of ROS during biotic stress (Lamb and Dixon 1997; Grant and Loake 2000; Mittler *et al*. 2004).

In order to control the accumulation of these toxic oxygen derivatives, in particular hydroxyl radicals and singlet oxygen, plant cells are equipped with a battery of enzymatic and non-enzymatic antioxidant systems to preserve the integrity of proteins and other cellular components (Dat *et al.* 2000; Apel and Hirt 2004). The main ROS-scavenging enzymes and the reactions they catalyze are reported in **Table 2**. Plant cells possess several classes of superoxide dismutase (SOD) enzymes, considered the first line of defence against ROS (De Gara *et al*. 2003; Apel and Hirt 2004; Asada 2006). SODs are characterized by the presence of diverse metal ions in their active site i.e. Cu-Zn, Mn and Fe and are located mainly in chloroplasts, mitochondria and peroxisomes as well as in the cytosol (del Río *et al.* 2002). The presence of SOD and of other enzymatic and non-enzymatic antioxidants has also been detected in the apoplastic space (Vanacker *et al.* 1998). Catalases (CAT), tetrameric enzymes containing a heme prosthetic group, are predominantly localized in the peroxisomes for scavenging H_2O_2 before it can diffuse to other cellular compartments and react with metal ions (Fenton reaction) to form the highly reactive hydroxyl radical (Dat *et al.* 2000; del Río *et al.* 2002). Three main isoforms of catalases are known, CAT1, CAT2 and CAT3, present in different plant species and responding to various stress conditions (Dat *et al.* 2000). H_2O_2 can also be removed very efficiently by peroxidases, that can be either cytosolic or cell wall-bound. Ascorbate peroxidase, glutathione peroxidase and guaiacol-type peroxidase belong to this class (Blokhina *et al*. 2003).

Glutathione (GSH), L-ascorbic acid (AsA), phenolic compounds, tocopherols, carotenoids and violaxanthin, antheraxanthin and zeaxanthin cooperating in the xanthophyll cycle are the most important non-enzymatic antioxidants (Smirnoff 1993; Gruszecki 1995; Rice-Evans *et al*. 1997; Noctor and Foyer 1998; Zancani and Nagy 1999; Grace and Logan 2000; Maeda and DellaPenna 2007). In addition to its role in the detoxification of xenobiotics, the tripeptide GSH (γ-Glu-Cys-Gly) is involved in the protection against oxidative stress by reacting chemically with ROS, such as H_2O_2, and taking part in the enzymatic ascorbate-glutathione cycle (Halliwell-Asada-Foyer pathway) (Noctor and Foyer 1998; Noctor *et al*. 2002). GSH is oxidized to glutathione disulphide (GSSG) and regenerated by glutathione reductase that reduces GSSG to 2GSH with NADPH as co-

Table 2 Enzymatic antioxidant systems functioning in defence against oxidative injury during plant stress responses. Ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase take part in the ascorbate-glutathione cycle (Halliwell-Asada-Foyer cycle). AsA, ascorbic acid; DHA, dehydroascorbate; GSH, glutathione; GSSG, glutathione disulphide; MDHA, monodehydroascorbate; R, aliphatic, aromatic or heterocyclic group; X, sulfate, nitrite or halide group (modified from Blokhina *et al*. 2003).

Enzymatic antioxidant	EC number	Reaction catalysed
Superoxide dismutase	1.15.1.1	$O_2^{\bullet-} + O_2^{\bullet-} + 2H^{+} \rightarrow H_2O_2 + O_2$
Catalase	1.11.1.6	$2H_2O_2 \rightarrow O_2 + 2H_2O$
Ascorbate peroxidase	1.11.1.11	$2AsA + H_2O_2 \rightarrow 2MDHA + 2H_2O$
Monodehydroascorbate reductase	1.6.5.4	$2MDHA + NAD(P)H \rightarrow 2AsA + NAD(P)^{+}$
Dehydroascorbate reductase	1.8.5.1	$DHA + 2GSH \rightarrow GSSG + AsA$
Glutathione reductase	1.6.4.2	$NAD(P)H + GSSG \rightarrow NAD(P)^{+} + 2GSH$
Peroxidases	1.11.1.7	$H_2O_2 + R(OH)_2 \rightarrow 2H_2O + R(O)_2$
Violaxanthin de-epoxidase	1.10.99.3	Violaxanthin + $AsA \rightarrow$ Zeaxanthin + $DHA + H_2O$
Glutathione *S*-transferase	2.5.1.18	$RX + GSH \rightarrow HX + R\text{-}S\text{-}GSH$

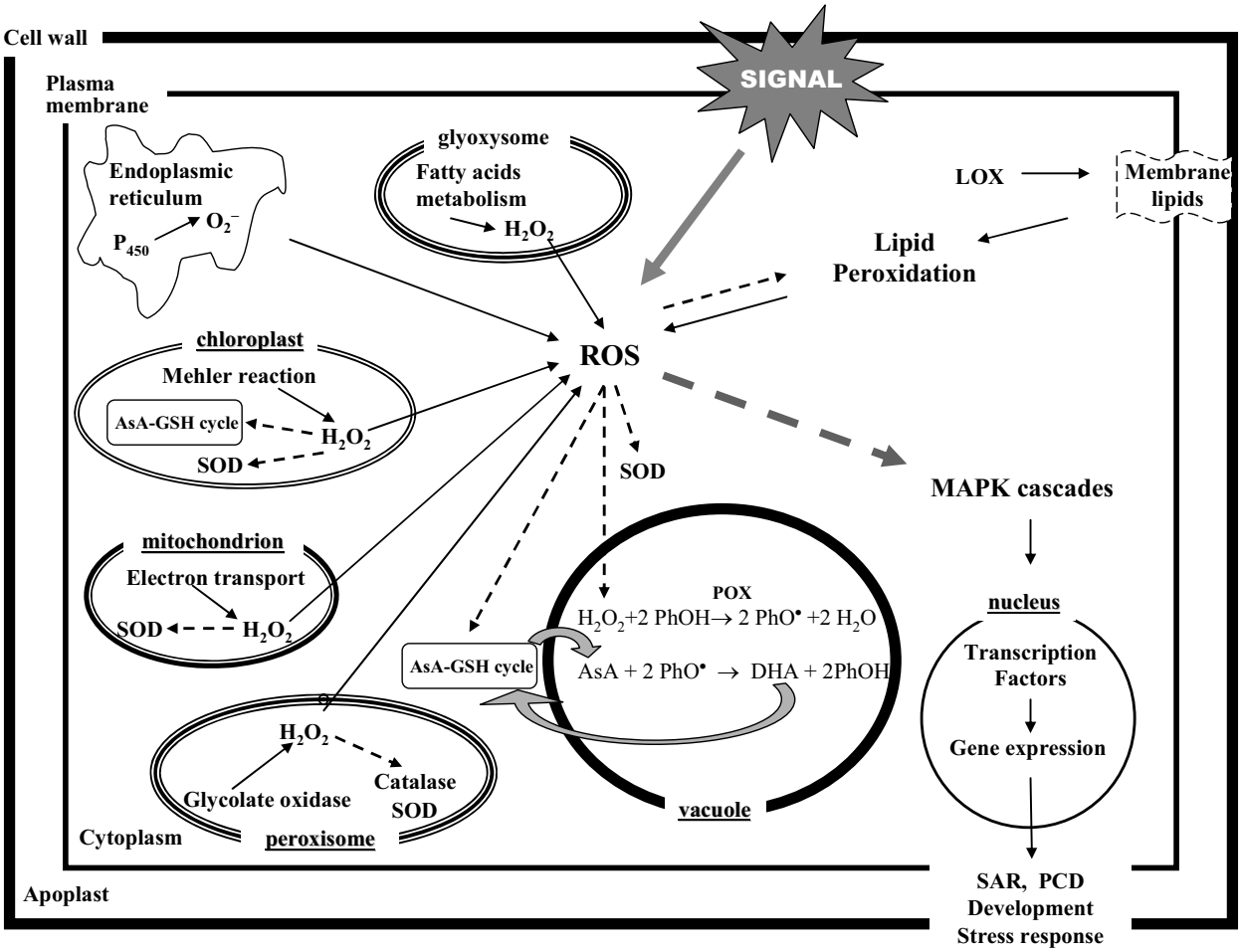

Fig. 1 Main sites of reactive oxygen species production in plant cells. AsA, ascorbic acid; DHA, dihydroascorbate; GSH, glutathione; LOX, lipoxygenase; MAPK, mitogen-activated protein kinase; P_{450}, cytochrome P_{450}; PCD, programmed cell death; PhOH, phenolic compounds; PhO˙, phenoxyl radicals; POX, peroxidase; ROS, reactive oxygen species; SAR, systemic acquired resistance; SOD, superoxide dismutase.

factor (**Table 2**). There are several examples of the positive correlation between GSH biosynthesis and/or accumulation and enhanced stress tolerance following treatment with heavy metals, herbicide safener or in heavy metal hyperaccumulating species (Freeman *et al*. 2004; Mullineaux and Rausch 2005; Sun *et al.* 2005). AsA on the other hand is the most important antioxidant in plant cells, where it exerts ROS scavenging function both directly and by the ascorbate-peroxidase reaction that reduces H_2O_2 to H_2O (Smirnoff 1993). The oxidation of ascorbate forms the radical monodehydroascorbate (MDHA) that can be either reduced to ascorbate by ferredoxin, cytochrome *b*561 or reductases, or dismutated to dehydroascorbate (DHA). Dehydroascorbate in turn can be reduced by specific reductases (DHARs), glutathione-*S*-transferases, GSH, thioredoxins and glutaredoxins (Noctor 2006). For instance, sensitivity to the air pollutant ozone is correlated with the ascorbate status of the leaf tissue (Kangasjärvi *et al*. 2005) and AsA deficiency in the *Arabidopsis thaliana* mutant *vitamin c-1* (*vtc1*) determines enhanced sensitivity to oxidative stress caused by exposure to ozone, sulphur dioxide, low temperatures and UV-B light (Conklin *et al*. 1996).

Photosynthetic organisms, i.e. plants, algae and most cyanobacteria, also synthesize the lipophilic antioxidants α, β, γ and δ-tocopherols (vitamin E) (Krieger-Liszkay and Trebst 2006; Maeda and DellaPenna 2007). In particular, α-tocopherol has the capacity of quenching singlet oxygen (Munné-Bosch 2005), while γ-tocopherol can react with NO forming 5-nitro-γ-tocopherol, therefore modulating NO endogenous level in plant tissues (Desel *et al.* 2007). The oxidized tocopherol radicals and hydroperoxides thus formed are recycled by reductants such as ascorbate or GSH (Krieger-Liszkay and Trebst 2006). GSSG and DHA are then regenerated in a non-enzymatic pathway involving other molecules with antioxidant activity such as lipoic and dihydrolipoic acid (Navari-Izzo *et al.* 2002). The hydroperoxides formed in the reaction of tocopherols with $^1O_2$ can also be cleaved to tocopherylquinones in the chloroplast lumen, rendering irreversible this type of oxidative reaction (Munné-Bosch 2005; Krieger-Liszkay and Trebst 2006). As tocopherols are located in the membranes their primary role is considered to be to protect polyunsaturated fatty acid acyl chains from oxidative damage interrupting the peroxidative chain reaction (Munné-Bosch and Alegre 2002; Maeda and DellaPenna 2007). However, recent data seem to sustain a reshaping of the role of tocopherols in the protection against oxidative stress imposed by high light by virtue of the surprisingly poor phenotypic effects observed in tocopherol-deficient mutants and leading to propose that they are but one of numerous components taking part in this function (Maeda and DellaPenna 2007). Another class of nonenzymatic scavengers is represented by phenolic compounds, the most abundant class of plant secondary metabolites derived from the phenylpropanoid biosynthetic pathway, including anthocyanins, flavonoids and isoflavonoids, tannins, coumarins, stilbenes and structural polymers such as lignin and suberin (Dixon and Paiva 1995; Grace and Logan 2000). The antioxidant capacity of polyphenols derives from their properties as reducing agents as well as from their ability for metal chelation that inhibits the formation of hydroxyl radical by the Fenton reaction (Rice-Evans *et al.* 1997; Grace and Logan 2000). The reduced state of phenolics, necessary for their effective function as antioxidants, is maintained by the ascorbate present in the vacuole that can also function as a chemical reductant of both quinones and semiquinones (Yamasaki and Grace 1998). The biosynthesis of phenols is induced upon exposure to environmental stresses such as high light, UV-B, metals, wounding/herbivory or

pathogen attack (Dixon and Paiva 1995; Grace and Logan 2000; Winkel-Shirley 2002). Sgherri *et al*. (2003) by studying the effect of copper excess on *Raphanus sativus* showed that the content of phenolic acids, such as chlorogenic, caffeic and p-coumaric acid as well as of total and reduced ascorbate, increased with the intensification of Cu treatment and proposed an interrelation between the cytoplasmic ascorbate-gluthatione cycle and the phenols stored in the vacuole, according to the results of Takahama and Oniki (1997) and Zancani and Nagy (1999).

The regulation of the intracellular concentration of ROS is based on the equilibrium between their production and removal, the so-called called "redox homeostasis" (Mittler 2002; Foyer and Noctor 2005). The antioxidant system has therefore a dual function, (i) to modulate the concentration of ROS and set the threshold for the induction of the appropriate responses to different stress conditions and (ii) to scavenge excess ROS before they can damage cellular constituents. The efficient removal of ROS at their generation sites depends on the sub-cellular localization of the antioxidant mechanisms, which is particularly true for the photosynthetic apparatus as the main site for ROS formation in plant cells and the target of abiotic stressors such as high light, sulphur dioxide and photodynamic herbicides (Alscher *et al.* 1997). Furthermore enzymatic and non-enzymatic scavengers, in addition to their individual capacity for ROS detoxification, take part in complex cycles of redox reactions (the Halliwell-Asada-Foyer cycle, the ascorbate peroxidase cycle, the water-water cycle) (Elstner 1982; Mittler 2002; Asada 2006) where they cooperate in scavenging ROS and regenerating the active forms of antioxidants to keep the pool fully functional. The importance of the antioxidant network can be further inferred from its redundancy whose aim is to counterbalance possible failures in one or more elements. Knockout and antisense lines for key enzymes in the pathway such as CAT, ascorbate peroxidase, peroxiredoxin and SOD are viable and show compensatory increases in the expression of other genes with similar function (Mittler *et al*. 2004); in mutants with low AsA concentration, the tocopherol pool is increased; finally, high light sensitivity in *Arabidopsis* can be obtained only in double mutants defective in both GSH and tocopherol (Krieger-Liszkay and Trebst 2006).

ROS IN THE PLANT STRESS RESPONSE

Plants are sessile organisms and as such are particularly susceptible to environmental fluctuations. The key for their survival is the ability to sense these changes and for this reason they have evolved highly effective and coordinated mechanisms to perceive and respond to stress. To the same end, plants are endowed with a remarkable amount of phenotypic plasticity, i.e. the capacity to modify their development, physiology and life history according to the environmental conditions (Sultan 2000). Adaptive plastic responses include the constraints imposed on growth and development by environmental conditions and adaptive changes that guarantee that a given organism will maintain its function and reproduce in multiple environments. They affect not only the contingent success of organisms in their natural context, but also their ability for colonization of different ecological niches and for evolutionary diversification.

As previously mentioned, the production and accumulation of reactive oxygen species is a common feature in plant stress (Dat *et al.* 2000; Baier *et al.* 2005; Torres *et al.* 2006). Besides their role in the onset and modulation of the biotic and abiotic stress response, ROS participate in a range of biological processes such as senescence, phytohormone signaling, stomatal closure, cell expansion and plant development (Laloi *et al*. 2004; Bhattacharjee 2005; Torres and Dangl 2005) (**Fig. 1**).

ROS in plant-pathogen interactions

When plant defenses to pathogens are taken into account non-specific and specific responses must be distinguished. The non-specific responses are displayed by every plant towards most potentially pathogenic microbes and are a major component of the so called non-host resistance (Heath 2000). The specific responses on the other hand are triggered by the interaction between plant genotypes possessing specific resistance (*R*) genes and pathogen genotypes possessing the corresponding avirulence (*Avr*) genes (incompatible interaction). This gene-for-gene mechanism (Flor 1947) leads to the induction of a rapid defense response, the hypersensitive response (HR), characterized by localized cell death at the site of infection (Dangl and Jones 2001), and to the acquirement of long-lasting, broad-spectrum Systemic Acquired Resistance (SAR) to subsequent infections induced in plants surviving pathogen challenge (Ryals *et al*. 1994). When the plant is susceptible to the infection by a virulent pathogen the interaction is called compatible and disease ensues. The earliest events in the resistance response are calcium influx, alkalinization of the extracellular space, production of ROS (the "oxidative burst") and NO, and protein kinase activation (Dangl and Jones 2001). The onset of the HR is also accompanied by a general "transcriptional reprogramming" induced by pathogen challenge that involves the coordinated expression of numerous genes coding for transcription factors, pathogenesis-related or PR-proteins, enzymes involved in biochemical pathways from primary and secondary metabolism and leading to the production of phytoalexins, lignin and salicylic acid (SA) (Hahlbrock *et al*. 2003).

The first evidence for an oxidative burst in the plant response to pathogens was reported by N. Doke (1983a) in potato tuber tissue infected with the late blight oomycete *Phytophthora infestans* or treated with cell wall components (CWC) from the same pathogen. The production of superoxide anion was observed only in response to an incompatible, but not to a compatible, race of the pathogen and was inhibited by the addition of SOD. In potato protoplasts treated with the same elicitor superoxide generation could be detected as fast as 2 min after the addition of CWC to the reaction mixture, making this the earliest pathogen response known (Doke 1983b). Using a fluorescence transition assay as detection method, Apostol *et al.* (1989) showed in soybean cell suspension cultures that the treatment with an elicitor from the fungus *Verticillium dahliae* stimulated an oxidative burst detectable less than 1 min after elicitor addition. This burst was mainly derived from the rapid formation of H_2O_2 and its use by extracellular peroxidases to oxidize susceptible substrates. Since these pioneering studies the involvement of ROS in the interaction between plants and a large number of viral, bacterial and fungal pathogens as well as upon treatment with various elicitors of the defense response has been described (Keppler *et al.* 1989; Vera-Estrella *et al*. 1992; May *et al*. 1996; Wojtaszek 1997 and references therein). Further experimental evidence of ROS involvement in the defense response came from experiments aimed at obtaining pathogen-resistant transgenic plants through the manipulation of ROS metabolism. Increased H_2O_2 production in potato due to the insertion of a glucose oxidase enzyme from *Aspergillus niger* conferred to plants a broad-spectrum disease resistance resulting from induction of specific genes, SA accumulation and cell wall lignification (Wu *et al*. 1995).

The production of ROS follows a different time course in the incompatible with respect to the compatible interaction. In the former the burst is biphasic, characterized by a transient first phase with low amplitude, considered to be a non specific reaction, followed by a secondary, more sustained phase of higher magnitude (Lamb and Dixon 1997). In the presence of virulent pathogens only the first phase is induced. A biphasic oxidative burst is also observed in the response to ozone (Kangasjärvi *et al.* 2005) and in the presence of excess of metals such as copper (Raeymaekers *et al.* 2003).

One of the most striking features of the oxidative burst observed in plant cells undergoing pathogen attack is its

similarity to the innate immune response in higher animals, where the production of superoxide by granulocytes and its subsequent dismutation to hydrogen peroxide have been shown to have a role in the phagocytosis process. ROS seem not to have a direct function in killing the invading microorganisms, as was originally thought, but rather to determine a pH change in the phagocytic vacuole in order to create an optimal environment for the action of enzymes such as neutral proteases (Segal 2005). In mammalian cells a NADPH oxidase called the respiratory burst NADPH oxidase (RBO) is responsible for ROS generation (Babior *et al.* 2002). The mammalian enzyme is constituted by five subunits, the two plasma membrane proteins $gp91^{phox}$ and $p22^{phox}$, that occur as a heterodimeric flavohemoprotein known as cytochrome b_{558}, and a cytosolic complex of three regulatory proteins $p40^{phox}$, $p47^{phox}$, $p67^{phox}$. Upon stimulation the regulatory complex moves to the plasma membrane and associates with the cytochrome b_{558} to form the active oxidase. For the activation of the complex the low molecular weight GTPase(s) Rac1 or Rac2 are also required. As the production of ROS in plants can be blocked by specific inhibitors of the mammalian NADPH oxidase such as diphenylene iodonium (DPI), plant homologues of the animal genes have been searched for leading to the isolation of several homologues of $gp91^{phox}$ (*rboh* genes) in *A. thaliana*, rice, tomato and potato (Torres and Dangl 2005). The plant Rboh proteins are larger than their mammalian counterparts. The C-terminal domain shows significant homology and a conserved membrane topology to the human $gp91^{phox}$ while the extended hydrophilic N-terminal domain has two EF hand motifs that bind Ca^{2+} ions and a significant similarity to the human RanGTPase-activating protein 1 (RanGAP1), a key regulator of the Ras-related nuclear small GTP-binding protein Ran (Keller *et al.* 1998). This finding also suggests a different regulatory mechanism for the activation of the oxidative burst in plants. The N-terminal domain could project into the cytoplasm and respond to the increase in Ca^{2+} induced by pathogen avirulence factors, leading to the rapid induction of the oxidative burst to initiate the HR in infected cells near to the pathogen penetration site (Keller *et al*. 1998). The phosphorylation of specific serine residues in the N-terminal region of the NADPH oxidase by a calcium-dependent protein kinase (CDPK) has recently been proposed by Kobayashi *et al*. (2007) to take part in the regulation of Rboh in potato. Furthermore in rice, as in animal cells, a homologue of the human Rac regulatory subunit of the NADPH oxidase complex (OsRac1) has been shown to regulate ROS production by NADPH oxidase and cell death (Kawasaki *et al.* 1999), and to be involved in disease resistance acting downstream of the *R* genes (Oono *et al.* 2001). In *A. thaliana* the *rboh* genes constitute a family of ten members (*AtrbohA* to *J*) (Torres and Dangl 2005). The isolation of *Atrboh* mutants allowed the identification of two genes, *AtrbohD* and *F*, specifically involved in the accumulation of ROS during the resistance response to bacterial and fungal pathogens (Torres *et al*. 2002). In particular, *AtrbohD*, and its tobacco counterpart *NtrbohD*, are responsible for ROS production during the interaction with avirulent pathogens or after elicitor treatment, while the contribution of *AtrbohF* is more limited and seems to be important in the regulation of HR. These genes probably encode components of a plant NADPH oxidase that produce superoxide.

Besides the plasma membrane-bound NADPH oxidase complex, the accumulation of ROS can also be due to apoplastic enzymes such as peroxidases, amine, diamine and polyamine oxidases, and oxalate oxidases (Grant and Loake 2000; Mittler *et al*. 2004). For instance, a germin-like oxalate oxidase protein that can produce H_2O_2 from oxalic acid has been identified in the incompatible interaction between barley and powdery mildew (*Blumeria graminis* f. sp. *hordei*) (Zhang *et al*. 1995), and the involvement of an apoplastic peroxidase in the oxidative burst has recently been reported in *A. thaliana* (Bindschedler *et al*. 2006). Finally, H_2O_2 produced in tomato leaves infected with the fungus *Botrytis cinerea* is derived from the activity of peroxidase and superoxide dismutase in the apoplast (Patykowski and Urbanek 2003).

It is therefore clear that different enzymes can be the source of ROS for the oxidative burst. However, understanding the contribution and the interactions of the diverse ROS-generating systems in the plant defense response is not an easy task. In order to explain the role of peroxidases and NADPH oxidases in the oxidative burst and in defense, Bindschedler *et al*. (2006) have proposed a model where apoplastic peroxidases generate a first burst of ROS upon recognition of pathogen-associated molecular patterns (PAMPs) associated with basal resistance. Reactive oxygen species thus produced could subsequently activate NADPH oxidases for the generation of the plasma membrane-associated oxidative burst in incompatible interactions.

The generation and accumulation of ROS in the course of the plant defense response is accompanied by changes in the cellular antioxidative systems. The activities of ROS-scavenging enzymes and antioxidants have been found to decrease or increase depending on the plant-pathogen system studied making it difficult to define a clear and univocal role for these systems. On one hand it is clear that ROS accumulation can be achieved either by an enhanced production and/or a lower removal, so a decrease in antioxidants or scavenging enzymes activity could be necessary to ROS build up during the defense response for pathogen killing and signaling purposes. Antioxidant enzymes activity was found to decrease in plant-virus interactions, where the resulting oxidative stress could contribute to virus replication and spreading (Clarke *et al.* 2002; Hernández *et al.* 2004), as well as in tomato roots reacting hypersensitively to the infection by the nematode *Meloidogyne incognita*, concomitant with an increase in ROS generation leading to accumulation of ROS and cell death (Zacheo and Bleve-Zacheo 1988). A diminished activity of class I and II CAT and of ascorbate peroxidase has also been linked to the onset of hypersensitive cell death in the interaction between tobacco and tobacco mosaic virus (TMV) (Dorey *et al*. 1998; Mittler *et al*. 1998). On the other hand, in incompatible interactions an increase in ROS scavengers has been observed as well and has been related to the need to control ROS production before they can damage extensively the tissue. An increase in the activity of class II CAT, glutathione reductase and peroxidase was detected following virus infection or elicitor treatment in tobacco (Montalbini and Buonaurio 1995; Dorey *et al*. 1998) and cowpea (Moshati *et al.* 1993), where it was involved in the cross-linking of cell wall components. By comparing different species of the genus *Lactuca* resistant or susceptible to the pathogen *Bremia lactucae* Sedlarova *et al.* (2007) found that only the resistant genotypes were characterized by accumulation of H_2O_2 in infected cells together with enhanced activity of the corresponding scavenging enzymes. Similar results have been obtained in the wheat (*Triticum aestivum*)-*Fusarium graminearum* interaction, where an up-regulation of antioxidants was detected only in the resistant cultivar 'Ning 7840' (Zhou *et al.* 2005). However, Montalbini and Buonaurio (1986) and Buonaurio *et al*. (1987) observed that the increased SOD activity in both the *Nicotiana* - TMV and *Phaseolus vulgaris* - *Uromyces phaseoli* interactions was not sufficient to protect tissues from oxygen toxicity in the course of the HR. An up-regulation of ROS-scavenging enzymes in the course of the interaction between plants and pathogens has also been detected in transcriptome analyses by means of the DNA microarray technology. In a comprehensive study of *A. thaliana* transcriptome changes during SAR, Maleck *et al.* (2000) found that the expression of genes encoding proteins involved in redox regulation such as peroxidase C and glutathione-*S*-transferase (GST) was upregulated. The expression levels of genes involved in free radical scavenging were also increased in *A. thaliana* after inoculation with an incompatible strain of the fungus *Alternaria brassicicola* (Schenk *et al*. 2000) and in a resistant rice cultivar upon infection with the leaf blight pathogen

Xanthomonas oryzae pv. *oryzae* (Kottapalli *et al*. 2007).

It must also be mentioned that, in the course of the host-pathogen coevolution race, some pathogens have acquired the ability to manipulate the ROS-scavenging system and exploit ROS production to their own advantage. This behaviour is typically observed in the infections by necrotrophic pathogens, such as *B. cinerea*, where a decreased level of antioxidants facilitates infection by promoting the spreading of cell death (de Gara *et al.* 2003; Torres *et al.* 2006).

Functions of ROS in the plant defense response

ROS produced in the oxidative burst following pathogen attack or elicitor treatment have multiple roles in the biotic stress response of plants that are summarized in **Fig. 2**.

The peroxidation of membrane lipids is a commonly observed event in the HR, where the resulting oxidative membrane damage may lead to cell death (Croft *et al.* 1990; May *et al.* 1996). Lipid peroxidation can occur both enzymatically and non-enzymatically, by the action of lipoxygenases (LOX) or of reactive oxygen species, respectively. LOX enzymes catalyze the hydroperoxidation of fatty acids and lead to the production of active oxygen species such as singlet oxygen, superoxide radicals and hydroxyl radicals derived from the latter by the action of SOD (Slusarenko *et al.* 1991). The resulting fatty acid hydroperoxides degenerate autocatalytically producing radicals that can propagate lipid peroxidation as a chain reaction (**Fig. 3**). Peroxidized fatty acids metabolites have been implicated as signal molecules in the activation of plant defenses as the production of the phytoalexin glyceollin in soybean (Degousée *et al*. 1994), or in cell death (Knight *et al*. 2001; Montillet *et al*. 2005). In the tocopherol-deficient *Arabidopsis* mutant *vitamin e2* (*vte2*) (Sattler *et al*. 2006) and following infection with *B. cinerea* in tomato or induction of peroxide stress in tobacco suspension cells (Thoma *et al*. 2003), the increase in general oxidation and non-enzymatic lipid peroxidation products (malondialdehyde and phytoprostanes, respectively) induced the accumulation of phytoalexins and the expression of genes involved in signal transduction, primary and secondary metabolism. Once more, a common theme in the defensive responses of plants and animals can be detected, as phytoprostanes belong to the chemical class of isoprostanes, mediators of oxidative stress in animals produced from arachidonic acid by a free-radical catalyzed mechanism (Roberts and Morrow 1997).

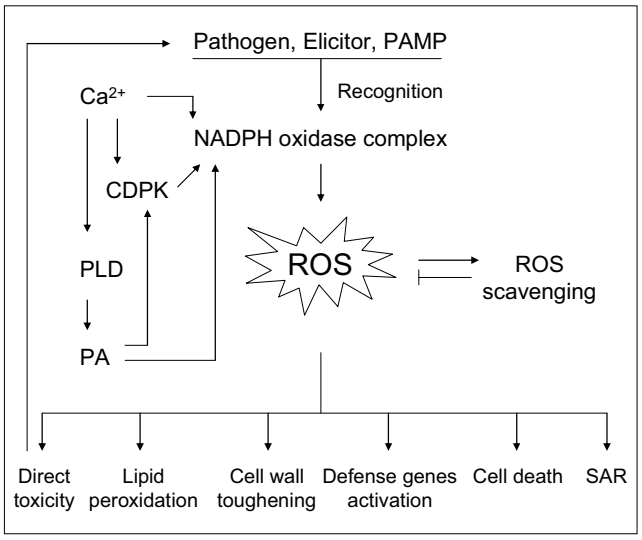

Fig. 2 Production and functions of reactive oxygen species in the plant defense response. CDPK, calcium-dependent protein kinase; PA, phosphatidic acid; PLD, phospholipase D; PAMP, Pathogen Associated Molecular Pattern; ROS, reactive oxygen species; SAR, Systemic Acquired Resistance.

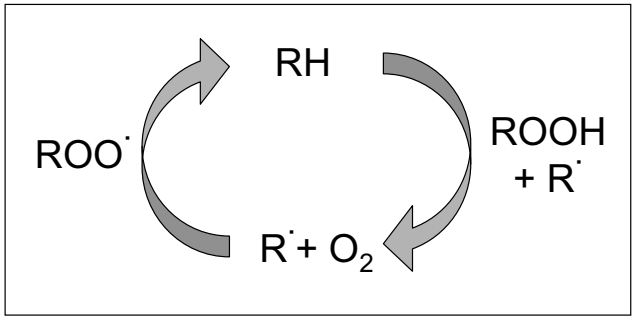

Fig. 3 Chain reaction for the propagation of lipid peroxidation among the plasmamembrane fatty acids. R·, lipid radical; RH, lipid; ROO·, lipid peroxy radical; ROOH, lipid hydroperoxide.

During the plant defense response H_2O_2 accumulation from the oxidative burst at the cell surface can drive the oxidative cross-linking of cell wall structural proteins, i.e. Hydroxyproline-Rich Glycoproteins (HRGPs) and tyrosine-rich proteins, after pathogen attack or elicitation contributing to the toughening of the cell wall barrier to pathogen ingress (Lamb and Dixon 1997; Wojtaszek 1997; Dat *et al.* 2000). Both the insolubilization of HRGPs in the cell wall (Bradley *et al*. 1992) and the inhibition of protoplast release by cell-wall degrading enzymes (Brisson *et al*. 1994) have been observed after treatment of bean or soybean cells with glucan elicitor from the oomycete *Phytophthora megasperma* f. sp. *glycinea* or H_2O_2. The response showed the same time course as the oxidative burst, and was inhibited by the addition of antioxidants such as catalase or AsA.

ROS are also involved in cell death, a prominent feature of the HR in incompatible plant-pathogen interactions where it is linked to disease resistance and has been proposed to block the spreading of the pathogen (Greenberg and Yao 2004; Van Breusegem and Dat 2006). The death of hypersensitively reacting cells, however, is not a passive necrotic process, but an active form of programmed cell death (PCD). Furthermore, in order to avoid that the spreading of cell death would irreversibly damage the whole tissue, the PCD programme is strictly regulated. The genes involved in PCD pathways have been identified through the isolation of lesion mimic mutants, such as *A. thaliana lsd1* (*lesion-simulating disease resistance response 1*) where the HR is not properly controlled and cell death spreads throughout the infected leaf (Lorrain *et al.* 2003).

In cells undergoing hypersensitive death the main events are vacuole collapse, ion fluxes (K^+, Cl^- efflux, Ca^{2+} influx), changes in pH and depolarization of the plasma membrane together with chromatin condensation, DNA laddering and cytochrome *c* (Cyt *c*) release (Jones 2001; Hofius *et al.* 2007). The latter features correspond to well-known markers of apoptosis in animal cells, where several stimuli activate a self-destruction programme requiring Cyt *c* release for the induction of a cysteine protease (caspase) cascade (Hengartner 2000). The proteolytic attack of caspases on different substrates causes the typical features of PCD such as nuclear shrinking and budding, loss of cell shape, DNA laddering and ultimately leads to cell death. Support to this similarity came from the finding that a caspase-like proteinase activity is involved in hypersensitive cell death in the interaction between tobacco and an incompatible strain of *Pseudomonas syringae* pv. *phaseolicola* (Del Pozo and Lam 1998). However, no homologues of animal caspases have been identified in plant genomes to date, but only putative caspase-like families called metacaspases (Watanabe and Lam 2004; Hofius *et al.* 2007).

The involvement of ROS in hypersensitive cell death has been demonstrated both pharmacologically, by showing that its typical features in plant cells could be blocked by treatment with antioxidants, inhibitors of transcription and translation (indicating the requirement for *de novo* protein synthesis) or of components of the signal transduction pathways, and genetically by the isolation of lesion mimic mutants and by the characterization of transgenic plants with altered antioxidant levels (Lorrain *et al.* 2003; Van Breusegem and Dat 2006). Being very reactive, some ROS like

hydroxyl radicals or singlet oxygen have obviously the potential to cause cell death by directly damaging cellular constituents. However, the link between ROS and cell death is not as straightforward as it could be thought at first sight. The oxidative burst *per se* is not sufficient to trigger cell death and its interaction with NO and phytohormones is necessary to this end. Concerning NO, a signal molecule in the vertebrate vascular, nervous and immune systems, Delledonne *et al*. (2001) have shown that in plant, as in animal, cells its cooperation with ROS is necessary for the induction of the hypersensitive reaction and that ROS and NO production must be fine-tuned to efficiently induce the HR. In animal macrophages NO and $O_2^{\bullet-}$ react to form peroxynitrite ($ONOO^-$), which is toxic to pathogen and tumor cells. On the contrary in plant cells $ONOO^-$ is not an essential intermediate for hypersensitive cell death and there is no evidence for a direct interaction between NO and $O_2^{\bullet-}$. Induction of cell death and killing of the pathogen are achieved instead by the synergistic interaction between NO and H_2O_2 produced from superoxide by SOD during the HR. However, NO can have a dual role in the modulation of plant cell death: it cooperates with ROS in its induction and can also protect plant cells from ROS damage by increasing the levels of scavenging enzymes such as CAT, SOD, GST and AOX (Wendehenne *et al*. 2004).

The phytohormones SA, ethylene (ET) and jasmonic acid (JA) are also implicated in the control of cell death induced by ROS (Hofius *et al.* 2007). The effect of SA seems to depend on a concentration gradient at the infection site. Where SA concentration is high, i. e. at the very site of pathogen penetration, cell death is promoted to block pathogen growth and diffusion, while low levels of SA in the adjacent cells promote cell survival and impede the spreading of the lesions. The spreading of cell death from HR sites is controlled also by ROS produced by the respiratory burst NADPH oxidase homologues AtrbohD and AtrbohF, which negatively regulate cell death induced by SA or its analog benzothiadiazol (Torres *et al*. 2005). McDowell and Dangl (2000) have proposed that SA, NO and ROS could act together in the modulation of cell death. The production of ROS and NO upon recognition of an avirulent pathogen stimulates the biosynthesis of SA as both NO and ROS can up-regulate the transcription of Phe ammonia lyase, a key enzyme in phenylpropanoid biosynthesis (Hancock *et al.* 2002). SA in turn enhances the responses induced by ROS and NO creating a cycle of positive feedback regulation that amplifies the initial signal. Salicylic acid interacts also with ET in the regulation of PCD, creating a feedback cycle that positively influences the ROS-dependent cell death. However, in some cases a PCD pathway independent of ET has been identified (Van Breusegem and Dat 2006; Hofius *et al.* 2007). Finally, JA seems to control lesion formation, even if this event can be JA-independent as well (Lorrain *et al*. 2003; Greenberg and Yao 2004). The effect of JA in the modulation of the cell death response depends on the chemical nature of the oxygen radical signal (superoxide/H_2O_2 versus singlet oxygen) and on its concentration with respect to other phytohormones such as SA (Van Breusegem and Dat 2006; Hofius *et al.* 2007).

ROS signal transduction

Both superoxide anion and H_2O_2 are involved in signal transduction pathways leading to the plant defense response at the local and the systemic level. There is evidence that calcium ions and calcium-binding proteins, lipid-based signals, protein kinase cascades and G proteins are involved in these pathways (McDowell and Dangl 2000; Nakagami *et al.* 2005; Lecourieux *et al*. 2006; Torres *et al.* 2006; Laxalt and Munnik 2002; Ma and Berkowitz 2007) (**Figs. 1, 2**).

A modification of ion fluxes, and in particular an increase in cytosolic Ca^{2+} concentration, is one of the earliest events induced by pathogen attack or elicitor treatment. An influx of Ca^{2+} ions through the plasma membrane ion channels is considered an essential component in the signal transduction chain leading to the HR and to the activation of the defense response, and in fact its inhibition blocks ROS production (Jabs *et al*. 1997 and references therein). The activity of calcium-permeable ion channels is modulated by cyclic nucleotides and by heterotrimeric G protein-dependent phosphorylation (Lecourieux *et al*. 2006; Ma and Berkowitz 2007). Furthermore, calcium can exert a direct control on ROS generation at the level of the NADPH oxidase complex by binding to the EF hands in the N-terminal domain of plant $gp91^{phox}$ homologues (Keller *et al.* 1998; Sagi and Fluhr 2001) and *via* the phosphorylation of ser residues in the same domain by a calcium-dependent protein kinase (Kobayashi *et al*. 2007). As the transduction of Ca^{2+} signals is based on calmodulin, a small acidic protein present in all eukaryotes that binds Ca^{2+} and activates downstream targets, its involvement in the plant oxidative burst has been investigated. In this frame, the enzymes NAD kinase and catalase have been identified as targets for calmodulin action (Harding *et al*. 1997; Yang and Poovaiah 2002), pointing to a dual role of calcium in the regulation of H_2O_2 homeostasis: a positive regulation that could act directly through the activation of NADPH oxidase or indirectly on NAD kinase, and a negative regulation through the stimulation of catalase. Recent data have demonstrated that signal transduction downstream from Ca^{2+} influx and leading to the HR also requires NO, whose biosynthetic enzyme nitric oxide synthase can be activated through calmodulin by an increase of Ca^{2+} in the cytoplasm (Ma and Berkowitz 2007). NADPH oxidase activity and ROS generation are modulated also by lipid-based signal transduction systems i.e. phospholipase D (PLD) and its product phosphatidic acid (PA) (Sang *et al.* 2001). PLD gene expression and PA production have been observed following exposure to H_2O_2, elicitors and pathogens, and induce the expression of elicitor-responsive genes and the biosynthesis of phytoalexins (Wang *et al.* 2006). Furthermore, PA produced by phospholipases D and C can function as a second messenger activating protein kinase cascades, CDPK and affecting ion channels activity (Laxalt and Munnik 2002).

The signaling pathway downstream from ROS includes mitogen-activated protein kinases (MAPK) cascades typically represented by three protein kinases, MAPKKK, MAPKK and MAPK (Asai *et al*. 2002; Nakagami *et al*. 2005). Mitogen-activated protein kinases responding to pathogens and elicitor molecules have been identified in a number of plants, including *A. thaliana*, tobacco, parsley, alfalfa, rice and tomato (Nakagami *et al*. 2005). The tobacco MEK2, salicylate-induced (SIPK) and wound-induced protein kinases (WIPK) and their *Arabidopsis* orthologues MPK3 and MPK6, are also induced by H_2O_2. In particular, the activation of MEK3 and MEK6 in response to H_2O_2 is mediated by the MAPKKK ANP1 and by a nucleoside diphosphate kinase 2 (AtNDPK2) (Nakagami *et al*. 2005; Fujita *et al*. 2006). The *A. thaliana* OXI1 (OXIDATIVE SIGNAL INDUCIBLE1) putative serine/threonine kinase has been recently identified as a further signaling element upstream from the MAPK cascade (Rentel *et al*. 2004). The final targets of these MAPK cascades are transcription factors that modulate the expression of specific genes in a stimulus-dependent manner, and in fact ROS increase the expression of members of several families of transcription factors such as WRKY and Myb (Laloi *et al*. 2004; Mittler *et al*. 2004). The activation of gene expression by transcription factors can also be controlled by the redox balance of the cell as has been shown for NPR1, an essential mediator of SAR (Dong 2004). Upon the SA-induced onset of SAR, NPR1 is localized to the nucleus (Kinkema *et al*. 2000) where it interacts with basic leucine zipper type (bZIP) transcription factors belonging to the TGA family for the activation of the expression of genes encoding PR-proteins (Zhang *et al*. 1999). In non-induced conditions NPR1 is present as a large, inactive oligomeric complex whose monomers are held together by disulphide bonds. The oxidative burst that occurs upon SAR induction establishes a transient increase in the cellular reduction potential

leading to a conformational change in the NPR1 complex and to the appearance of active monomers that localize in the nucleus and activate PR gene expression (Mou *et al.* 2003). By examining in detail the interaction between NPR1 and the transcription factors TGA1 and TGA4, Després *et al*. (2003) have shown that in non-induced conditions both transcription factors are kept in an inactive state through the formation of an intramolecular disulphide bond between two oxidized cysteine residues. The induction of SAR causes the reduction of the disulphide bridges and the breakage of the bonds thus allowing the interaction NPR1/ TGA and the subsequent activation of PR gene expression. How the reduced cellular state is established as a consequence of the SA-triggered initial oxidative burst is not clear. According to Dong (2004) it could be obtained by either activating the expression of genes coding for detoxifying enzymes such as GST induced by SA or by increasing the reducing power of the cell via the induction of the pentose phosphate pathway through the activation of the key enzyme glucose-6-phosphate 1-dehydrogenase induced by pathogen infection. Fobert and Després (2005) have also proposed that the redox changes of NPR1 and the TGA transcription factors could be modulated by oxidoreductases such as thioredoxins and glutaredoxins.

Finally, ROS have been proposed to have a direct role in the establishment of SAR. Alvarez *et al*. (1998) have shown that the systemic signal for the induction of SAR in tissues distant from the infection site could be the production of ROS. They have observed that in *A. thaliana* plants inoculated with an avirulent strain of the bacterium *P. syringae* the production of ROS and H_2O_2 is not restricted to the inoculation site where the HR occurs, but can be detected also at distant sites as systemic micro-oxidative bursts that initiate SAR development. The systemic generation of ROS by the micro-oxidative bursts could function in the establishment of SAR activating the defense response throughout the plant, albeit at low levels, through the production of less reactive second messengers, such as H_2O_2 and JA. However, the role of H_2O_2 as a potential second messenger has been questioned by other studies (Dorey *et al*. 1998; Torres *et al*. 2006).

ROS and abiotic stress: the response to ozone

A detailed description of the role of ROS in the plant's response to abiotic stress is beyond the scope of this review, as our main focus is the involvement of ROS in biotic stress. However we will take into account oxidative stress caused by ozone (O_3) being particularly relevant *per se* as an example of abiotic stress and in the present context because the acute, high-level exposure to this air pollutant induces a series of events that are typical of the pathogen defense response (Sandermann *et al.* 1998). Ozone is in fact considered a sort of abiotic "elicitor" of the defense response.

The effect of ozone on plants depends on a series of variables i.e. the plant species or cultivar, the ozone concentration and the duration of the exposure (Sharma and Davis 1994; Baier *et al.* 2005). Chronic exposure to low ozone levels reduces plant growth and crop yield without visible foliar damage, an effect generally attributed to enhanced respiration, reduced transpiration and photosynthesis, and premature senescence, while high levels of ozone lead to the appearance of necrotic lesions and/or chlorotic symptoms on leaves. A biphasic oxidative burst involving a NADPH-dependent oxidase (Rao and Davis 1999) and resulting in the generation and accumulation of H_2O_2 and superoxide anion has been proposed to be the main mechanism for ozone-induced cell death (Langebartels *et al*. 2002). A clear spatial and quantitative correlation between the accumulation of ROS and ozone damage has in fact been found in several plant species, including *Arabidopsis*, tobacco, tomato and birch (Wohlgemuth *et al*. 2002; Overmyer e*t al.* 2003). The study of ozone-induced gene expression has revealed that the ozone-dependent gene induction overlaps to a certain extent with that observed during the HR, is correlated with the rapid accumulation of SA and requires a SA-dependent signaling pathway (Sharma and Davis 1994; Sharma *et al*. 1996; Sandermann *et al*. 1998). Genes whose expression is increased upon exposure to ozone include those involved in the flavonoid and phenylpropanoid pathways, SA-signaling, ethylene and JA biosynthesis as well as encoding PR proteins and antioxidant enzymes aimed at decreasing the level of toxic intracellular ROS (Sharma and Davis 1994; Li *et al.* 2006; Tosti *et al.* 2006). Consistent with the known reduction in photosynthesis and induction of senescence, ribulose-1,5-bisphosphate carboxylase/oxygenase large and small subunit and binding protein genes were down-regulated, while senescence-associated genes were induced (Tosti *et al*. 2006).

The complex chain of events involved in O_3 perception and downstream signal transduction has been clearly described by Kangasjärvi *et al*. (2005). Ozone enters plant tissues through the stomata and gains access to the apoplastic fluid surrounding the leaf cells where its degradation forms ROS that are attacked by antioxidants such as ascorbate. When the level of apoplastic ROS exceeds the detoxifying capacity of scavengers, two independent signal transduction chains are activated, one depending on heterotrimeric G-proteins and the other on a MAPK cascade. The former induces ROS production in the chloroplast and subsequently activates a plasma membrane respiratory burst NAPDH oxidase, enhancing the production of ROS in cells adjacent to the lesion. The MAPK cascade on the other hand could be involved in the up-regulation of the biosynthesis of ET, which cooperates with SA in the perception and transduction of the ROS signal to nearby cells (Overmyer *et al*. 2003; Kangasjärvi *et al*. 2005). Lesion containment depends on the synthesis of JA, based on lipid peroxidation products released as a consequence of cell death, which blocks ethylene signaling most likely at the level of hormone perception. A role in limiting lesion growth has been proposed also for SA that inhibits the ET-biosynthetic enzyme ACC oxidase. Both activation of MAPK and NADPH oxidase are dependent on intracellular calcium increase that takes place in the early stages of signal transduction, probably via the oxidative activation of calcium channels and changes in the protein phosphorylation pattern with MAPK cascades again playing a key role (Baier *et al*. 2005). Finally, abscisic acid (ABA) has a role in the regulation of the stomatal state and therefore of O_3 influx to leaves (Torres and Dangl 2005; Kangasjärvi *et al*. 2005). The occurrence of stomatal closure upon exposure to ozone is a well known phenomenon and the involvement of ABA in this process has been demonstrated, probably with ROS functioning as intermediates.

The common features of the response to ozone and pathogens underscore a general motif in the stress response in that the signal transduction pathways for different stimuli often converge on common effectors and activate overlapping sets of genes (Wan *et al*. 2002 and references therein). ROS, including the ROS-generating NADPH oxidase, MAPK cascades and lipid-derived signal compounds have been identified as major "points of convergence" for different plant responses (Mithöfer *et al.* 2004; Fujita *et al.* 2006). Besides being involved in ROS production in hypersensitively reacting cells, the *Arabidopsis AthbohD* and *AthbohF* genes are also expressed in stomatal guard cells where H_2O_2 induces an ABA-mediated increase of Ca^{2+} in the cytoplasm leading to stomatal closure (Torres and Dangl 2005). As a further confirmation of the interplay between these two pathways, fungal elicitors have been shown to induce both ROS and stomatal closure by the same mechanism (Klusener *et al*. 2002). These same genes seem also to be responsible for the generation of ROS signals in the response to ozone stress (Joo *et al*. 2005). Finally, signal transduction systems based on PLD and PA take part as well in the response to several abiotic stress conditions such as metal excess, drought, cold or wounding (Navari-Izzo *et al.* 2006 and references therein).

CONCLUSIONS

Reactive Oxygen Species are clearly emerging as a leitmotif in plant life, being involved in most physiological responses to stress as well as in developmental processes such as stomatal closure, root elongation and cell expansion (Lamb and Dixon 1997; Grant and Loake 2000; Overmyer *et al*. 2003; Apel and Hirt 2004; Laloi *et al*. 2004). It is therefore not surprising that at least 152 genes are comprised in the so-called "reactive oxygen gene network" in the *Arabidopsis* genome, involving multiple enzymes for ROS production and scavenging (Mittler *et al*. 2004).

One of the main points is that the generation of active oxygen species is a key process shared between the response to both biotic and abiotic stress where ROS can either seriously damage cells or be interpreted as a signal for the activation of downstream responses. The outcome of the oxidative stress depends probably on the existence of a threshold in the level of ROS accumulated as a result of stress conditions. Only if the amount of ROS remains below this threshold they can be used in cell signaling processes, otherwise if severe oxidative stress occurs, with a very high ROS production and a drastic decrease in the level of antioxidants, irreversible cell damage occurs eventually leading to cell death. From an evolutionary point of view, a signaling strategy based on ROS is easy to set up and has several advantages, ROS in fact (i) are normally generated during basal metabolic processes such as photosynthesis and respiration and therefore the components necessary for their production and scavenging are already present in the cell, (ii) are produced in both biotic and abiotic stress conditions and (iii) as their generation occurs very rapidly upon stress, linking a response to an early event ensures that it will be rapid and effective as well.

A further point concerns the extreme complexity of the whole system centered on ROS in terms of signal perception, transduction and regulation that has not yet been elucidated in its entirety. Firstly, the components of the signaling system upstream and downstream from ROS such as modifications of ion fluxes, activation of MAPK cascades and G-proteins, are shared between most physiological and developmental events and, secondly, ROS are intertwined with all the main mediators of the defense response (SA, JA, ET). McDowell and Dangl (2000) have proposed that the complexity of the network could stem from the need to carefully control an important, costly and potentially harmful response, as is the defense response to pathogens, and as a consequence of the perpetual plant-pathogen coevolution race.

However, more general considerations can be drawn in this context. Multi-step and/or branched networks allow for greater versatility and flexibility in regulation, as every step can be subjected to positive or negative control and linked to different inputs and/or outputs. One of the major themes in the evolution of complex organisms is the strategy of using a relatively small number of highly conserved and unconstrained signal transduction and transcription core processes that can interact with a variety of other processes for different purposes, in different environments and cell types, conferring an extraordinary capacity for physiological and evolutionary adaptability (Gerhart and Kirschner 1997). A typical example of this strategy is represented by regulatory modules ("contingency units") based on MAPK cascades, that are widespread in eukaryotes and used to link inputs to outputs in as diverse situations as cell growth, differentiation and death, embryogenesis, responses to biotic and abiotic stress, light and hormones (Pearson *et al*. 2001; Nakagami *et al*. 2005). The conservation of versatile circuits underlies a fundamental principle in that during evolution innovation is mostly achieved not by the appearance of novelty but rather by using what is already available to obtain a new outcome, a behaviour embodied in the concept of "molecular tinkering" as proposed by Jacob (1977).

The responses of plants to various stress conditions can therefore be described as "variations on a common theme", an aspect even more pronounced in plant-pathogen interactions where a continuous process of coevolution takes place. If the widespread adoption of a few model species has allowed to obtain an increasingly clear picture of the common features underlying these responses, only the study of diverse plant systems will help in understanding the multiple facets of a fascinating phenomenon.

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