Update on Signal Transduction

Active Oxygen Species in Plant Defense against Pathogens[1]

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Plant disease resistance to pathogens such as fungi, bacteria, and viruses often depends on whether the plant is able to recognize the pathogen early in the infection process. The recognition event leads to a rapid tissue necrosis at the site of infection, which is called the HR. The HR deprives the pathogen of nutrients and/or releases toxic molecules, thereby confining pathogen growth to a small region of the plant. This response provides resistance to the great majority of potential pathogens (nonhost or species resistance). For a given plant species, a much more limited number of true pathogens exhibit the ability to evade the host recognition system and grow extensively within the plant without evoking host necrosis at all or only after considerable delay. In this case, the plant exhibits susceptibility and the extensive growth of the successful pathogen can cause varying degrees of damage. However, certain races within pathogenic bacterial or fungal species are recognized by certain cultivars or genotypes of the host plant species and the HR is triggered. These observations indicate that there is an ongoing evolution of the host plant's ability to recognize pathogen races that were previously unrecognized while the pathogen evolves to avoid recognition by a previously resistant host.

Recognition of pathogens triggers a large range of inducible defense mechanisms that are believed to contribute to overall resistance in the plant. The mechanisms induced at the site of infection and associated with the HR include synthesis of antimicrobial compounds called phytoalexins, synthesis of hydrolytic enzymes that attack fungi and bacteria, and alterations in the synthesis of cell-wall structural proteins (for review, see Lamb et al., 1989). Many of these responses are due to transcriptional activation of specific genes that are collectively known as plant defense or defense-related genes. Defense gene regulation has been extensively studied both in intact plant-pathogen interactions and in model systems in which plant cell suspensions are treated with pathogen-derived molecules termed elicitors.

Several rapid processes characteristic of the HR appear to involve primarily activation of preexisting components rather than changes in gene expression. One of these rapid processes is the striking release of AOS, which is termed the oxidative burst. This response to pathogens or elicitors has been observed in diverse monocotyledonous and dicotyledonous species including rice, tobacco, soybean, and spruce. The

AOS are toxic intermediates that result from successive one-electron steps in the reduction of molecular O_2. The predominant species detected in plant-pathogen interactions are superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-). The oxidative burst is correlated with the HR in a number of plant-pathogen interactions and therefore may be an important element contributing to disease resistance.

This review first describes the occurrence of the oxidative burst in several plant-pathogen interactions. Second, recent progress toward understanding the regulation and properties of the molecular components involved in the generation of AOS is highlighted. Third, possible functions of AOS that may contribute to disease resistance are discussed. The reader is also referred to an excellent past review on this subject (Sutherland, 1991).

CHEMISTRY OF AOS DURING THE OXIDATIVE BURST

AOS are routinely generated at low levels by plant cells due to electron transport in chloroplasts, mitochondria, and enzymes in other cell compartments involved in reduction-oxidation processes. The first reaction during the pathogen-induced oxidative burst is believed to be the one-electron reduction of molecular oxygen to form superoxide anion (O_2^-). In aqueous solutions, the superoxide anion undergoes spontaneous dismutation in an overall reaction written as

$$2\ O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2 \tag{1}$$
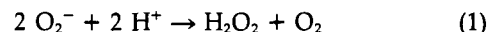
This reaction occurs at a higher rate at acidic pH, such as is found in the cell wall (O_2^- half-life < 1 s [Sutherland, 1991]). This reaction is also catalyzed by superoxide dismutase enzymes that occur in the cytosol, chloroplasts, and mitochondria (Scandalios, 1993).

O_2^- can also act as a reducing agent for transition metals such as Fe^{3+} and Cu^{2+}. These metals may be reduced even if they are complexed with proteins or low mol wt chelators. One important consequence of metal reduction is that it can lead to the H_2O_2-dependent formation of hydroxyl radicals (OH^-). For example,
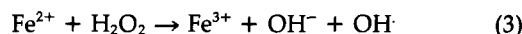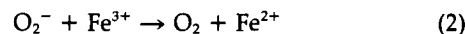
$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+} \tag{2}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^- \tag{3}$$

Abbreviations: AOS, active oxygen species; HR, hypersensitive response; SAR, systemic acquired resistance.

The reaction shown in Equation 3 is called the Fenton reaction. Because of its extreme reactivity and its documented formation in cells producing O_2^- and H_2O_2, OH· is believed to be a major AOS responsible for modifications of macromolecules and cellular damage. The hydroxyl radical initiates radical chain reactions including lipid peroxidation, enzyme inactivation, and degradation of nucleic acids. By comparison, O_2^- and H_2O_2 are weaker oxidizing agents. However, O_2^- has been shown to react with proteins containing Fe-S$_4$ clusters or heme groups, and H_2O_2 can attack thiol groups of proteins or GSH (Thompson et al., 1987).

OCCURRENCE OF AOS DURING PLANT DEFENSE RESPONSES

Fungal Pathogens

The first observations of AOS generation during plant-pathogen interactions were made in studies of potato tubers infected with races of the potato blight fungus *Phytophthora infestans* (Doke, 1983a). Doke observed that inoculation of aged potato discs with a race that results in an HR increased the reduction of both Cyt c and the dye nitroblue tetrazolium in the bathing solution. Inclusion of superoxide dismutase in the bathing solution inhibited these reactions. These tests are typically used to indicate the presence of O_2^-. In contrast, another fungal race that infects tubers without eliciting the HR did not increase Cyt c reducing activity. These findings suggest that O_2^- generation may be involved in host cell hypersensitivity.

Similarly, AOS generation was associated with the HR in the race-specific interaction between tomato and the fungus *Cladosporium fulvum* (Vera-Estrella et al., 1992). It was found that apoplastic (extracellular) fluid from infected leaves exhibiting HR stimulated AOS generation within 2 min in cell-suspension cultures of the same cultivar. However, apoplastic fluid from uninfected plants or plants infected with a race that does not elicit HR had little effect on AOS levels. In these studies, AOS levels were measured in the culture medium by a luminol-dependent chemiluminescence assay. In this assay, luminol is oxidized in the presence of H_2O_2 by peroxidases present in the culture medium and an unstable derivative emits light upon return to its ground state (Keppler et al., 1989).

Nonspecific fungal elicitors that lack cultivar specificity also have been shown to trigger the oxidative burst. Soybean cell suspensions treated with two different nonspecific elicitors produced AOS within 1 to 2 min (Apostol et al., 1989). AOS in the culture medium were detected by the peroxidase-mediated oxidation of a membrane-impermeant fluorescent dye, pyranine, to a nonfluorescent compound. Bean cell suspensions treated with nonspecific elicitors also rapidly produced AOS as measured by the chemiluminescence method (Anderson et al., 1991).

Bacterial Pathogens

AOS have also been reported in race-specific interactions of plant cells with pathogenic bacteria. Tobacco plants infected with *Pseudomonas syringae* pv *syringae* exhibited an HR that was accompanied by increased O_2^- generation and

lipid peroxidation (Adam et al., 1989). In contrast, a transposon mutant of *P. syringae* pv *phasiollicola* that had lost the ability to induce an HR failed to induce these changes. In tobacco cell suspensions treated with *P. syringae*, all bacterial treatments resulted in an initial, rapid oxidative burst (0–1 h) as detected by the chemiluminescence assay (Keppler et al., 1989). However, a second, prolonged increase in AOS levels (3–6 h) was observed in cells treated with HR-causing bacteria but not in cells treated with non-HR-causing bacteria. Recently, a protein termed harpin has been identified as an elicitor of the HR in tobacco caused by *Erwinia amylovora*. Harpin-producing *E. amylovora* induced AOS production in tobacco cell suspensions after a lag of 2 h, but AOS were not induced by *E. amylovora* transposon mutants that do not produce harpin (Baker et al., 1993). Moreover, addition of cell-free extracts of *Escherichia coli* expressing the harpin gene increased AOS levels within 5 to 10 min.

Viruses

In studies of the interaction of tobacco and tobacco mosaic virus, O_2^- was detected in leaf discs undergoing an HR using the Cyt c reduction assay (Doke and Ohashi, 1988). In contrast, little O_2^- was detected in uninfected or systemically infected leaf discs. The reduction of nitroblue tetrazolium by AOS produces a blue compound that serves as a useful histochemical assay of AOS production. This assay showed that the O_2^--generating activity appeared to occur at sites of subsequent necrotic lesion development.

MOLECULAR MECHANISMS FOR PRODUCTION AND TURNOVER OF AOS DURING THE DEFENSE RESPONSE

Identification of the enzyme(s) responsible for the oxidative burst and the molecular components involved in activation of the enzyme(s) is a major current challenge. Figure 1 presents a working model that depicts possible signaling pathways leading to the production of extracellular AOS during the HR. This model is based on the available evidence. However, it must be emphasized that the actual identities of the components and their location in the signal-transduction pathway remain to be clarified.

Several plant receptors that bind plant or fungal cell wall-derived elicitors have been localized to the plasma membrane. In soybean, plant cell wall-derived polygalacturonic acid and fungal cell wall-derived carbohydrate/(glyco)protein preparations are known to stimulate the oxidative burst (Apostol et al., 1989), and their receptors were found on the plasma membrane (Horn et al., 1989). It is likely that other fungal or plant cell wall carbohydrate or (glyco)protein elicitors that promote the oxidative burst also bind to plasma membrane-bound receptors. Elicitor receptors may be associated with heterotrimeric GTP-binding or G proteins, as has been shown for a wide variety of animal transmembrane receptors. Agents known to interact with heterotrimeric G proteins were shown to promote AOS generation in soybean cell cultures either in the presence or absence of elicitor (Legendré et al., 1992).

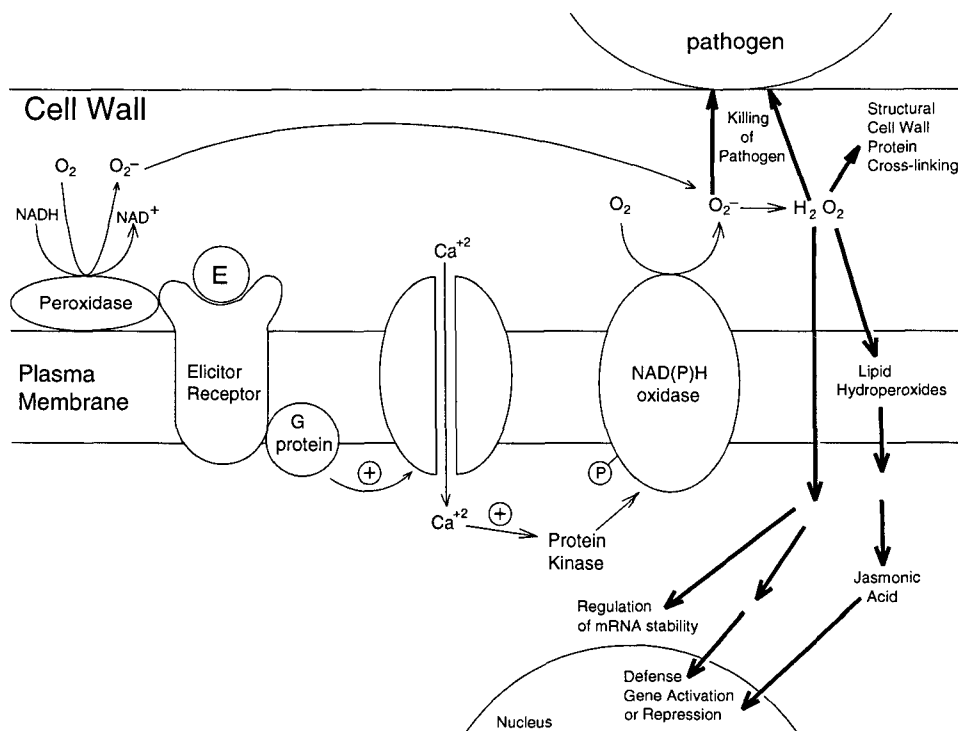AOS generation in several species appears to depend on

Figure 1. A speculative model showing possible components involved in AOS generation and effects of AOS. Assignment of actual components and sequence of components requires additional data. Elicitor receptors may be coupled to active oxygen synthesis via G proteins, increased intracellular Ca due to Ca channel opening, and activation of a protein kinase that activates a membrane-bound NAD(P)H oxidase by phosphorylation. Alternatively, occupation of elicitor receptors may stimulate a membrane-associated peroxidase by unknown mechanisms, which results in O_2^- synthesis. O_2^- spontaneously dismutates to H_2O_2, which is membrane permeable. O_2^- and H_2O_2 contribute to killing of the pathogen, whereas H_2O_2 also participates in oxidative cross-linking of cell wall proteins and regulation of host gene expression.

increased intracellular Ca and protein kinase activation. Depletion of Ca in the medium reduced the fungal elicitor-induced active oxygen formation, whereas a Ca ionophore, A23187, induced active oxygen formation in spruce cell suspensions (Schwacke and Hager, 1992). La^{3+}, a Ca^{2+} channel blocker, inhibited bacterial elicitor-induced AOS production in tobacco (Baker et al., 1993). The protein kinase inhibitors staurosporine and K252a inhibited elicitor-induced increases in active oxygen in spruce and tobacco cultures, respectively (Schwacke and Hager, 1992; Baker et al., 1993). One reasonable pathway consistent with the data is that elicitor receptor coupled with a G protein leads to Ca influx, which then activates a Ca^{2+}-dependent protein kinase and ultimately the O_2^--generating oxidase (Fig. 1).

The oxidase(s) that catalyzes the formation of O_2^- has to date been characterized only in cells or crude membrane preparations. A NADPH-dependent oxidase evolved O_2^- in microsome preparations from sliced aged potato tubers. This activity increased in microsomes from tubers undergoing an HR to *P. infestans* (Doke, 1983b) and in elicitor-treated protoplasts (Doke, 1985). Although the specific membrane system containing the NADPH oxidase was not defined, it is likely that the oxidase resides in the plasma membrane or is associated with its external surface because the release of O_2^- was extracellular (O_2^- is poorly diffusible across membranes).

The NADPH oxidase in microsomes from tobacco mosaic virus-infected leaves was Ca^{2+} dependent (Doke and Ohashi, 1988). The NADPH oxidase associated with the oxidative burst in plants may be structurally related to the multisubunit NADPH oxidase in mammalian phagocytes responsible for O_2^- generation (Morel et al., 1991). In mammals, several cytosolic proteins, including the phosphorylated protein p47, must assemble with a membrane-bound Cyt to form the active NADPH oxidase. The essential features of the model shown in Figure 1 indicating the involvement of heterotrimeric G-protein activity, increased Ca^{2+}, protein kinase activity, and phosphorylation of the NADPH oxidase have been generally established in the oxidative burst pathway of phagocytes (Morel et al., 1991).

Another oxidase that could be the source of the oxidative burst is a NADH-dependent peroxidase that is associated with the external surface of the plasma membrane (Sutherland, 1991; Vera-Estrella, 1992). Peroxidase generation of O_2^- occurs in healthy tissues to provide the H_2O_2 for lignification. Involvement of peroxidases in elicitor-induced AOS generation is suggested by the reduced formation of lipid peroxides and phenolic compounds in tomato cells in the presence of cyanide and salicylhydroxamic acid, two known inhibitors of peroxidases (Vera-Estrella et al., 1992). However, these inhibitors can act on other cellular targets. There

is no information on the signaling pathway coupling elicitor receptors and NADH-dependent peroxidases.

Numerous mechanisms are available to limit the duration of the oxidative burst and its toxic consequences in plant cells. First, the increased rate of AOS generation induced by pathogen or elicitor exposure is transient in essentially all interactions examined (Schwacke and Hager, 1992; Baker et al., 1993; Legrendre et al., 1993). In soybean cells, the AOS-generating activity was down-regulated or desensitized to subsequent exposures to the elicitor (Legrendre et al., 1993). Second, endogenous reducing agents and antioxidant enzymes are abundant in plant cells that are capable of neutralizing the AOS. These include superoxide dismutase, cytosolic and peroxisomal forms of catalase, ascorbate and ascorbate peroxidase, and glutathione and glutathione peroxidase (Thompson et al., 1987). Oxidative stress is known to induce several of these antioxidant systems (Scandalios, 1993), and elicitor treatment of alfalfa and bean cells increases the levels of glutathione (Edwards et al., 1991). Despite these defenses, the oxidative burst may significantly increase intracellular AOS levels.

ROLES OF AOS IN PLANT DEFENSE

A major question in studies of the oxidative burst during plant-pathogen interactions is whether the oxidative burst plays a causal role in pathogen growth restriction or whether AOS are merely produced as a consequence of other metabolic changes during the HR. The current evidence is limited but several studies indicate that AOS directly reduce pathogen viability. In studies of the bacteria-induced HR in tobacco cell suspensions, O_2^- scavengers added with *P. syringae* pv *tabaci* inhibited the O_2^- production, lipid peroxidation, and increased recovery of bacteria (Keppler and Baker, 1989). Spore germination for a number of fungal pathogens has been shown to be inhibited by micromolar concentrations of H_2O_2 (Peng and Kuc, 1992). It has been estimated that cells responding to polygalacturonic acid elicitor produce roughly 1.2 mM H_2O_2, averaged for a mass of cells (Legendré et al., 1993). This concentration of H_2O_2 is roughly equivalent to that present in activated mammalian phagocytes as part of their chemical arsenal (Morel et al., 1991).

The toxicity of AOS or AOS-derived compounds may contribute to host cell death during the HR as well. Lipid peroxidation and generation of lipid free radicals after elicitor or pathogen exposure has been extensively documented (Adam et al., 1989; Keppler and Baker, 1989). The hypothesis that host cell death is due at least in part to the elevated AOS levels is supported by studies showing that addition of antioxidants reduces death of hypersensitively responding cells (Keppler and Novacky, 1987). In addition, lower levels of antioxidant enzymes in different bean cultivars were correlated with greater resistance to pathogen infection (Buonaurio et al., 1987).

The oxidative burst appears to play a novel role in strengthening the plant cell wall to pathogen attack. Elicitor treatment of bean or soybean cells was shown to result in H_2O_2-mediated oxidative cross-linking of specific structural proteins (Bradley et al., 1992). This response was rapid (initiated in 2 min), appeared to depend on de novo-synthesized H_2O_2,

and increased wall resistance to the action of fungal wall-degrading enzymes.

The possibility that AOS serve as signal intermediates that induce phytoalexin biosynthesis has received considerable attention. Doke (1983a) initially observed that exogenous superoxide dismutase inhibited both O_2^- generation and phytoalexin accumulation in aged potato discs infected with an HR-inducing fungal pathogen. Similar inhibitory effects of antioxidant enzymes or free radical scavengers on phytoalexin production have been reported for a number of plant species, including pea, soybean, and cotton (Epperlein et al., 1986; Apostol et al., 1989). Depending on the elicitor and plant used, the degree of inhibition of phytoalexin accumulation by antioxidant mechanisms can vary, suggesting that pathways independent of the oxidative burst contribute to the regulation of phytoalexin accumulation. In white clover cell suspensions treated with bacteria, a variety of antioxidant treatments failed to inhibit phytoalexin accumulation (Devlin and Gustine, 1992). However, a lack of effects obtained with exogenous antioxidant treatments does not exclude AOS involvement, since the reactivity and accessibility of the AOS to these agents may vary in different experimental systems. In addition, exogenous H_2O_2 alone or exposure to an AOS-generating system such as xanthine:xanthine oxidase has been shown to stimulate phytoalexin accumulation in the absence of elicitor (Rogers et al., 1988; Apostol et al., 1989).

The mechanisms of AOS regulation of phytoalexin accumulation and other intracellular defense-related changes are unknown. Since the accumulation of phytoalexins has been shown to be due primarily to transcriptional activation, it is likely that AOS ultimately affect gene expression (Fig. 1). In addition, AOS may regulate the stability of defense-related mRNAs (Zhang et al., 1993). Treatment of bean cell suspensions with H_2O_2 induced the accumulation of mRNAs encoding Phe ammonia-lyase, chalcone synthase, and chalcone isomerase (enzymes required for phytoalexin biosynthesis) and a basic endochitinase, whereas a mRNA encoding a Pro-rich protein was degraded (Y. Sharma, K. Sathasivan, N. Bays, and M.C. Mehdy, unpublished data). Inhibition of the elicitor-induced oxidative burst was correlated with reduced regulation of these mRNAs. These data support a role for AOS as signaling intermediates in pathways leading to defense-related gene expression but do not exclude the occurrence of AOS-independent pathways.

Potential regulatory molecules modulating gene expression, which are affected by increased AOS levels, are GSH and GSSG or their derivatives. Exogenous application of GSH increased Phe ammonia-lyase and chalcone synthase transcripts in bean cell suspensions (Wingate et al., 1988). A later study reported that both GSH and GSSG elicit Phe ammonia-lyase enzyme activity and phytoalexin accumulation (Edwards et al., 1991). Whereas elicitor treatment was shown to increase GSH and other thiols, experiments using an artificial precursor for glutathione suggested that an increase in intracellular GSH alone was insufficient to cause phytoalexin accumulation in bean and alfalfa cells (Edwards et al., 1991). In carrot, inhibition of glutathione synthesis induced phytoalexin accumulation (Guo et al., 1993). The cellular ratio of GSH to GSSG may be more important in the regulation of defense-related gene expression than the absolute amounts

of either form. Sulfhydryl compounds have been shown to induce transcription of a cytosolic copper/zinc superoxide dismutase in tobacco (Hérouart et al., 1993). In mammals, sulfhydryl compounds and/or perturbants of glutathione metabolism regulate the activities of proteins such as the NF-κB transcription factor (Schreck et al., 1991), protein kinases (Nose et al., 1991), and the Bcl-2 protein, which inhibits most types of apoptotic cell death (Hockenbery et al., 1993).

Lipid peroxides generated by nonenzymic reaction of AOS with lipids may serve as precursors in the synthesis of jasmonic acid, a known regulator of several defense-related genes expressed during the HR (Farmer and Ryan, 1992). This AOS-dependent source of lipid peroxides may augment an enzymic pathway for their production involving phospholipases and lipoxygenase (Farmer and Ryan, 1992). Clearly, more work is needed to identify cellular targets modified by AOS action and their roles in signal transduction.

The above discussion has focused on the roles of AOS during the HR. Another response of plants to necrotizing pathogens is to develop enhanced resistance to the same or unrelated pathogens throughout the plant over a period of days (referred to as SAR). A recent study indicates that salicylic acid, which is widely believed to be the inducer of specific plant defense genes during the development of SAR, acts by inhibiting catalase activity (Chen et al., 1993). The somewhat increased H_2O_2 concentrations detected in salicylic acid-treated leaves, presumably resulting from catalase inhibition, was suggested to serve as a second messenger in the transcriptional activation of pathogenesis-related protein genes. These findings should stimulate research to more precisely define the role of AOS in systemically induced defense-related genes.

CONCLUSIONS AND FUTURE PROSPECTS

The oxidative burst associated with pathogen recognition has now been well established for many plant-pathogen interactions. Several exciting developments have focused attention on this redox perturbation as a possible direct effector of resistance and as a signal modulator of later defense responses. The AOS may function extracellularly as agents for pathogen killing, both at the level of the cell wall for oxidative cross-linking of proteins and intracellularly as signal intermediates triggering changes in gene expression and possibly as toxic molecules contributing to host cell death. The postulated dual nature of AOS in plant-pathogen interactions is reminiscent of its destructive and gene-regulatory roles during the immune response (Schreck et al., 1991). In plants, the molecular machinery for generating AOS, the nature of the coupling to elicitor receptors, and AOS regulation of diverse cellular processes related to defense are only beginning to be elucidated. Rapid future progress in understanding the role of the increased AOS levels during the HR and systemic acquired resistance will benefit from consideration of the large body of information on oxidative stress in plants and animals, application of precise tools such as specific inhibitors to studies of the oxidative burst, chemical and physical methods for measuring AOS levels, and molecular and genetic approaches.

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