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MINI-REVIEW

Generation of Superoxide Anion and Hydrogen Peroxide at the Surface of Plant Cells

Angelo Vianello[1] and Francesco Macrì[1]

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Abstract

In addition to well-known cell wall peroxidases, there is now evidence for the presence of this enzyme at the plasma membrane of the plant cells (surface peroxidase). Both are able to catalyze, through a chain of reactions involving the superoxide anion, the oxidation of NADH to generate hydrogen peroxide. The latter is oxidized by other wall-bound peroxidases to convert cinnamoyl alcohols into radical forms, which, then polymerize to generate lignin. However, there are other enzymes at the surface of plasma membranes capable of generating hydrogen peroxide (cell wall polyamine oxidase), superoxide anion (plasma membrane Turbo reductase), or both (plasma membrane flavoprotein?). These enzymes utilize NAD(P)H as a substrate. The Turbo reductase and the flavoprotein catalyze the univalent reduction of Fe^{3+} and then of O_2 to produce Fe^{2+} and O_2^-, respectively. The superoxide anion, in the acidic environment of the cell wall, may then dismutate to H_2O_2. These superoxide anion- and hydrogen peroxide-generating systems are discussed in relation to their possible involvement in physiological and pathological processes in the apoplast of plant cells.

Key Words: Anion superoxide; cell wall; hydrogen peroxide; NADH oxidase; plant cell; plasma membrane; surface peroxidase.

Introduction

NAD(P)H oxidoreductases have been identified in nearly all plant membranes, although the most extensively studied are those associated with the inner and the outer mitochondrial, and endoplasmic reticulum membrane (Møller and

[1]Section of Plant Physiology and Biochemistry, Institute of Plant Protection, University of Udine, via Cotonificio 108, I-33100 Udine, Italy.

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Lin, 1986). In recent years, following the pioneering work of Craig and Crane (1981) and Ivankina and Novak (1981), redox activities have been shown in isolated plant plasma membranes (De Luca *et al.*, 1984; Barr *et al.*, 1985a; Blein *et al.*, 1986; Buckhout and Hrubec, 1986; Sandelius *et al.*, 1986; Asard *et al.*, 1987; Brightman *et al.*, 1988; Luster and Buckhout, 1988; Buckhout *et al.*, 1989; Valenti *et al.*, 1990), or at the surface of cells (Chalmers *et al.*, 1984; Lin, 1984; Misra *et al.*, 1984; Barr *et al.*, 1985b; Thom and Maretzki, 1985; Neufeld and Bown, 1987) and organs (Rubinstein *et al.*, 1984; Sijmons *et al.*, 1984; Böttger *et al.*, 1985; Qiu *et al.*, 1985; Böttger and Lüthen, 1986; Bown and Crawford, 1988; Ivankina and Novak, 1988; Lüthen and Böttger, 1988). Endogenous NAD(P)H, in the case of protoplasts and organs, and exogenously supplied NAD(P)H, with isolated membranes, are mainly utilized as source for reducing several physiological (O_2 and Fe^{3+}) or artificial electron acceptors.

In general, the physiological significance of these oxidoreductases is still unclear, but increasing evidence suggests that they are related to some essential physiological functions (Bienfait, 1985; Lüttge and Clarkson, 1985; Crane *et al.*, 1985; Morré *et al.*, 1988; Crane and Barr, 1989).

Peroxidases are hemoproteins widely distributed in plant cells, which play an active metabolic role. They are isoenzymes with specific participation in physiological processes. Many peroxidases are relatively unspecific for the hydrogen donor (phenolic substances, ascorbate, amines, indolacetic acid, etc.), but they have an almost absolute requirement for peroxide as oxidant (Butt, 1980; van Huystee, 1987). Peroxidases are also able to catalyze oxidation of NADH, in a reaction involving the superoxide anion, to produce hydrogen peroxide (Akazawa and Conn, 1958; Yamazaki and Yokota, 1973). Two major cationic and anionic peroxidase isoenzymes have been recognized. They are distributed inside and outside the plant cell. The only organelles that do not exhibit peroxidase activity are mitochondria and peroxisomes. Extracellular peroxidases are usually classified as free, ionically bound, or covalently attached to the cell wall and account for 36% of the total peroxidase activity (Li *et al.*, 1989). In addition to the cell wall peroxidases, in recent years this activity has also been demonstrated in plasma membrane preparations (Møller and Bérczi, 1986; Askerlund *et al.*, 1987), at the surface of protoplasts (Coleman and Chalmers, 1988; Pantoja and Willmer, 1988) and organs (Spreen Brouwer *et al.*, 1986).

This paper deals with these surface peroxidases and NAD(P)H oxidoreductases, describing their possible roles in some physiological and pathological processes, involving hydrogen peroxide and superoxide anion, and occurring at the level of plasma membrane and cell wall intended as a continuum.

Surface Peroxidases

Some recent studies have demonstrated that the NADH oxidase activity in isolated plasma membrane vesicles or at the surface of protoplasts and organs may be ascribed to peroxidase-like enzymes (Møller and Bérczi, 1986; Spreen Brouwer *et al.*, 1986; Askerlund *et al.*, 1987; Coleman and Chalmers, 1988; Pantoja and Willmer, 1988) (Table I).

NADH oxidase in the plasma membrane may be distinguished from peroxidase activity according to a number of properties. In fact, peroxidase activity is cyanide or catalase sensitive and is also favored by high concentrations of NADH (1 mM). This type of activity is low without phenols or manganese and is very active at acidic pH. To the contrary, NADH oxidase activity is assayed with low concentrations of NADH (< 0.2 mM) at neutral pH and is insensitive to cyanide, catalase, and superoxide dismutase (Crane and Barr, 1989).

Plasma membrane vesicles isolated from wheat roots exhibit an O_2 consumption, after the addition of approximately 1 mM NADH. This activity is strongly stimulated by salicylhydroxamic acid (SHAM, an inhibitor of the alternative oxidase of plant mitochondria), while it is inhibited by KCN, is insensitive to antimycin A, and is abolished by catalase. During oxidation, H_2O_2 is formed (Møller and Bérczi, 1985). In the absence of SHAM the rate of O_2 uptake increases with increasing acidity from pH 8.5 to 4.5, without a pH optimum. Conversely, in the presence of SHAM, the pH optimum is at approximately 6.5, and 0.8 mol of H_2O_2 are produced for every 1 mol of oxygen consumed. This NADH oxidation activity appears to be located on the external face of the plasma membrane because in this work vesicles predominantly right side-out were used and, in addition, the activity is not

Table I. NADH Oxidases Related to Peroxidase-like Activity of Plant Plasma Membranes or at the Surface of Protoplasts and Organs

Plant source		Specific activity	References
		(nmol O_2 mg^{-1} min^{-1})	
Plasma membranes	Wheat	12–29	Møller and Bérczi, 1986
	Cauliflower	6	Askerlund *et al.*, 1987
	Radish	1.0–1.5	Vianello *et al.*, 1990
	Barley	20–24	Brüggemann and Moog, 1989
		(nmol O_2 10^6 prot · min^{-1})	
Protoplasts	Carrot	75	Coleman and Chalmers, 1988
	C. communis	9	Pantoja and Willmer, 1988
		(μmol O_2 g dry wt^{-1} h^{-1})	
Roots	Pea	182	Spreen Brouwer *et al.*, 1986
	Maize	311	Spreen Brouwer *et al.*, 1986

enhanced by Triton X100 (Møller and Bérczi, 1986). It has been suggested that this external NADH oxidation activity is identical to the cytochrome $P_{420/450}$ present in plasma membrane, which, as is known, may oxidize NADH to produce H_2O_2 in the absence of substrate (Murray *et al.*, 1985). However, as suggested by Spreen Brouwer *et al.* (1986), the inhibition caused by catalase and KCN on this activity seems to point to the involvement of a peroxidase in SHAM-stimulated root respiration. The latter conclusion is confirmed by the results obtained on plasma membranes from cauliflower inflorescences. These membranes and intracellular membranes show an NAD(P)H oxidase activity (2 mM NAD(P)H) stimulated by phenolic compounds. Besides SHAM, ferulic acid, coniferyl alcohol, *n*-propyl gallate, naringenin, kaempherol, and caffeic acid strongly stimulate the activity, which is inhibited by catalase, superoxide dismutase, and KCN. Most of the activity is soluble. However, the membrane-bound activity is highly enriched in the plasma membranes compared to intracellular membranes and appears to be mediated by a peroxidase or peroxidase-like enzyme (Askerlund *et al.*, 1987).

In agreement with the above results, a peroxidase activity associated with the plasma membrane of *Commelina communis* guard-cell protoplasts was described. These cells, in the dark, exhibit a stimulated O_2 consumption upon 1.5 mM NADH addition. The oxygen uptake, monitored at pH 6.1, is dependent on Mn^{2+} and is stimulated 10- to 15-fold by SHAM. Catalase, KCN, and ascorbate, the latter a superoxide scavenger, inhibit the SHAM-stimulated O_2 uptake. The activity increases with time in the incubation medium of the protoplasts, indicating that the enzyme is excreted (Pantoja and Willmer, 1988). Oxidation of exogenous NAD(P)H (1 mM) by isolated carrot protoplasts was also demonstrated. NAD(P)H oxidation is absent in freshly prepared protoplasts and increases after 1.5–2 h. The characteristics of this activity are: stimulation by Mn^{2+} and monophenols, inhibition by superoxide dismutase and catalase, and pH optimum at 6.5 (Coleman and Chalmers, 1988). As shown for guard-cell protoplasts of *C. communis*, the activity may be released from the protoplasts by washing. These observations suggest that in this case peroxidases are involved, and this is confirmed by the cytochemical localization of the enzyme (Chalmers *et al.*, 1986).

SHAM, at low concentrations (below 15 mM), stimulates oxygen consumption in pea and *Plantago* sp., whereas it is inhibitory at higher concentrations (De Visser and Blacquèrie, 1984). These results were subsequently confirmed and extended by intact roots of pea and maize. The O_2 uptake requires 1 mM NAD(P)H as substrate. It is stimulated by low concentrations of SHAM and by 2-methoxyphenol, and inhibited by KCN, gentisic acid, and catechol; the latter is a superoxide free radical scavenger. The stimulation is due to activation of a peroxidase located inside the cell or in the cell wall (Spreen Brouwer *et al.*, 1986). However, in the light of the results presented

above, it is more probable that this peroxidase is located at the surface of the cell (cell wall and/or plasma membrane).

Superoxide-Generating Oxidoreductase Activity

Radish plasma membrane vesicles show an NAD(P)H oxidation activity (4 mM NAD(P)H) at acidic pH (4.5–5.0), which is inhibited by KCN, ascorbic acid, catalase, and SOD, the latter added either before or during NADH oxidation. SHAM, Mn^{2+}, and H_2O_2 do not affect this activity. Ferrous and, to a lesser extent, ferric ions strongly stimulate NADH oxidation. This activity elicits anion superoxide formation (Vianello and Macrì, 1989). Salicylate and benzoate, two known hydroxyl radical scavengers, inhibit both basal and iron-stimulated NADH oxidation. The iron chelators EDTA (ethylenediaminetetraacetic acid) and DFA (deferoxamine mesylate), at high concentrations (2 mM), inhibit NADH oxidation, whereas they are ineffective at lower concentrations (80 μM). Hydrogen peroxide is not detected during NADH oxidation but, in the presence of salicylate, it is formed in significant amounts (116 nmol mg^{-1} protein). NADH oxidation is also associated with Fe^{2+} oxidation which is partially inhibited by catalase and prevented by superoxide dismutase, while ferric ion reduction is abolished by catalase and unaffected by superoxide dismutase (Vianello *et al.*, 1990). A similar NADH oxidation activity is present in *Acer pseudoplatanus* protoplasts. Both β-NADH and α-NADH may be used as substrates (Macrì and Vianello, unpublished results). Therefore, this oxidation activity appears to be similar to that described for the endoplasmic reticulum and differs from the dehydrogenases of mitochondria which specifically use β-NADH (Møller and Lin, 1986).

An oxidoreductase activity dependent upon 0.6 mM NADH addition is also present in plasma membrane vesicles from barley roots. This activity is stimulated by SHAM, quinhydron, Fe^{2+}-EDTA, and Fe^{3+}-EDTA, but not by $K_3[Fe(CN)_6]$ or $K_4[Fe(CN)_6]$. The stimulating effect of iron chelates on oxygen consumption is due to Fe^{2+} and can be inhibited by bathophenanthroline disulfonate, SOD and PCMS (*p*-chloromercurophenylsulfonic acid) (Brüggemann and Moog, 1989). The activity has a pH optimum at approximately 7.0, and no free hydrogen peroxide is detected by addition of catalase, suggesting that the Fe^{2+}-induced oxygen consumption is different from the peroxidase activity described by others (Møller and Bérczi, 1986; Askerlund *et al.*, 1987; Coleman and Chalmers, 1988; Pantoja and Willmer, 1988). The inhibition caused by SOD indicates the involvement of a superoxide anion during NADH oxidation activity. However, the lack of subsequent formation of hydrogen peroxide can be explained by considering that dismutation of the superoxide anion is rapid only at acidic pH (Fridovich, 1978).

Components of Redox Systems

Although the components of electron transfer systems of different complexity have been described in plasma membrane of mammalian cells (Crane *et al.*, 1985), very little is known on the nature of redox components of plant plasma membranes. The best characterized electron carriers in plant membranes are flavins and cytochromes of the *b* type (Møller *et al.*, 1988; Crane and Barr, 1989). Among the latter, cytochromes of the *b* type are present in a broad range of plant species. In addition, a light-reducible cytochrome *b* has been reported in corn coleoptiles, cauliflower inflorescences, and spinach leaves, while a cytochrome of the $P_{420/450}$ type is present in cauliflower inflorescences. Flavins have been detected only in a few cases.

Potential Redox Reactions

It is well known that, as first suggested by Yamazaki and Yokota (1973), peroxidase (PX) may cause the oxidation of NAD(P)H through the following reactions (Crane and Barr, 1989):

(1) $NADH + O_2 \longrightarrow O_2^{\cdot-} + NAD^{\cdot} + H^+$

(2) $NAD^{\cdot} + O_2 \longrightarrow O_2^{\cdot-} + NAD^+$

(3) $2O_2^{\cdot-} + 2H^+ \longrightarrow O_2 + H_2O_2$

(4) $O_2^{\cdot-} + NADH + H^+ \longrightarrow NAD^{\cdot} + H_2O_2$

(5) $2NADH + H_2O_2 \xrightarrow{PX} 2NAD^{\cdot} + 2H_2O$

(6) $PX + O_2^{\cdot-} \longrightarrow$ Compound III

(7) Compound III $\longrightarrow$ Peroxidase $+ O_2$

In this scheme of reactions the superoxide anion is produced by a very low nonenzymatic NADH reduction which is inhibited by SOD (1). Reactions (2), (3), and (4) are also nonenzymatic and are stimulated by SOD (3) or Mn^{2+} (4). Peroxidase (PX) catalyzes the subsequent reactions (5) which is inhibited by cyanide and catalase. Such enzyme may then react with the superoxide anion to produce compound III (oxyperoxidase) (6) which is converted back to active peroxidase. Phenols inhibit reaction (6), while they stimulate reaction (7). This sequence of reactions is favored by a high concentration of NADH and by acidic pH (Crane and Barr, 1989).

As suggested, NADH oxidase activity of cauliflower inflorescence and wheat root plasma membranes, *C. communis* guard-cell, and carrot protoplasts satisfy these requirements and point to the involvement of surface peroxidases in the generation of hydrogen peroxide (Møller and Bérczi, 1986; Askerlund *et al.*, 1987; Coleman and Chalmers, 1988; Pantoja and Willmer, 1988). The results on NADH oxidation of radish plasma membrane vesicles, although in agreement with the above postulated mechanism of peroxidase

activity (e.g., inhibition by KCN, catalase, and SOD, added before NADH), differ for the lack of stimulation induced by SHAM, H_2O_2 and Mn^{2+}, and for the inhibition by SOD (Vianello and Macrì, 1989; Vianello *et al.*, 1990), even if added during NADH oxidation (Halliwell, 1978). In addition, the presence of contaminating iron ions appears to be crucial for this activity (Vianello and Macrì, 1989).

In plasma membranes there are some redox systems in which flavoproteins play a major role (Møller *et al.*, 1988; Crane and Barr, 1989) and which utilize NADH as a reducing agent.

(1) $FpH_2 + Fe^{3+} \longrightarrow FpH^{\cdot} + Fe^{2+} + H^+$

(2) $FpH^{\cdot} + O_2 \longrightarrow Fp + O_2^{\cdot-} + H^+$

(3) $2H^+ + 2O_2^{\cdot-} \longrightarrow H_2O_2 + O_2$

(4) $H_2O_2 + Fe^{2+} \longrightarrow OH^{\cdot} + OH^- + Fe^{3+}$

(5) $H_2O_2 + Fe^{3+} \longrightarrow Fe^{2+} + O_2^{\cdot-} + 2H^+$

It is possible that, as suggested by Cakmak *et al.* (1987) for the reduction of ferric ions before they are taken up by cells, a reduced flavoprotein (FpH_2) may react with Fe^{3+}, through a univalent reaction, to produce a radical flavoprotein ($FpH^{\cdot}$) (1). The latter, reacting with oxygen, generates a superoxide anion (2) which, at acidic pH, dismutates to H_2O_2 (3). Hydrogen peroxide, through a Fenton's reaction, may react with Fe^{2+} to produce hydroxyl radicals (4), or with Fe^{3+} to generate the superoxide anion (5).

Relation to Other Oxidoreductases

Trans-plasma membrane electron transport linked to ferricyanide or oxygen reduction has been demonstrated in isolated plasma membranes, protoplasts, and organs (Craig and Crane, 1981; Lin, 1984; Misra *et al.*, 1984; Rubinstein *et al.*, 1984; Barr *et al.*, 1985b; Böttger *et al.*, 1985; Qiu *et al.*, 1985; Blein *et al.*, 1986; Böttger and Lüthen, 1986; Neufeld and Bown, 1987). This electron transport has been associated to proton extrusion (Craig and Crane, 1981; Ivankina and Novak, 1981; Lin, 1984; Böttger *et al.*, 1985; Blein *et al.*, 1986; Böttger and Lüthen, 1986; Neufeld and Bown, 1987; Bown and Crawford, 1988; Ivankina and Novak, 1988). The activity appears to be a system subsidiary to the proton pumping ATPases for energizing the plasma membrane (Marrè and Ballarin-Denti, 1985; Sze, 1985). This conclusion is consistent with the observation of the generation of a membrane potential by electron transport in plasma membrane vesicles (Hassidim *et al.*, 1987). However, the reduction of ferricyanide in corn roots causes inhibition of K^+ uptake (Kochian and Lucas, 1985) and depolarizes the trans-plasma membrane electrical potential in *Elodea densa* and *Lemna gibba* leaves (Lass *et al.*, 1986; Marré *et al.*, 1988). In addition, in isolated membranes (Macrì

and Vianello, 1986; Giannini and Briskin, 1988), cells of sugarcane (Komor *et al.*, 1987), and organs (Guern and Ullrich-Eberius, 1988), the oxidation of NADH, in the presence of ferricyanide, only leads to a scalar acidification of the medium. Donor and acceptor sites for the NADH-ferricyanide reductase appear to be located on the cytoplasmic surface of plasma membranes (Askerlund *et al.*, 1988), although Böttger (1989) shows evidence that internalized NADH reduces external ferricyanide in right side-out vesicles. The effects of exogenous NADH on ion movements (inhibition of K^+ uptake and H^+ extrusion) in roots (Kochian and Lucas, 1985) and sugarcane cells (Komor *et al.*, 1987), however, may be due to the overlapping of peroxidase activity (Crane and Barr, 1989). In any case, it is clear that the NADH oxidase, with low affinity sites for oxygen, is coupled to proton extrusion (Böttger and Lüthen, 1986) and is controlled by hormones (Brightman *et al.*, 1988; Böttger and Hilgendorf, 1988; Morré *et al.*, 1988). NAD(P)H is the probable physiological electron donor in protoplasts and organs for the reduction of ferricyanide (Chalmers *et al.*, 1984; Craig and Crane, 1981; Böttger and Lüthen, 1986) or oxygen (Chalmers and Coleman, 1983).

In plants, iron is taken up as a ferric chelate or, after reduction, as ferrous ion (Römheld, 1987). Ferric reduction occurs at the plama membrane of root epidermic cells and is mediated by an inducible trans-membrane redox system (Turbo-reductase) which is present only in dicotyledonous and monocotyledonous (not grasses) plants. This system appears to be separate from the redox chain carrying electrons from NADH to ferricyanide (Standard reductase) which is ubiquitous in plants (Bienfait, 1985).

On the basis of results obtained with maize roots and *Elodea densa* leaves, redox activity does not provide an additional and independent pathway for H^+ transport. The activity may be an important factor in regulating the proton pumping ATPase of plasma membrane (Rubinstein and Stern, 1986; Marrè *et al.*, 1988; Trockner and Marrè, 1988).

Redox activities also appear to be involved in other physiological processes, such as phenomena related to blue light-inducible absorbance changes (LIAC) (Widell, 1987) and reactivation of primary (H^+-ATPase) and secondary active transport (H^+ cotransport) by maintaining, at the reduced state, functional SH groups of the transport proteins (Bienfait and Lüttge, 1988; Elzenga *et al.*, 1989).

Involvement of Oxygen Free Radicals and Hydrogen Peroxide in Functions of Plasma Membrane and Cell Wall of Plant Cells

Anion superoxide and hydrogen peroxide are involved in a variety of physiological and pathological phenomena of plants and this subject has been reviewed (Elstner, 1982; Thompson *et al.*, 1987).

Hydrogen peroxide, formed through reactions catalyzed by peroxidases which involve peroxide anion (Elstner and Heupel, 1976; Mäder and Amberg-Fisher, 1982), is implicated in the peroxidative polymerization of cinnamoyl alcohols, leading to lignin biosynthesis (Harkin and Obst, 1973; Stafford, 1974; Mäder and Füssl, 1982).

The degradation of lipids in senescing membranes and the ensuing release of free fatty acids seem to be mainly triggered by the activity of membrane peroxidases and lipoxygenases, although the activity of the latter enzymes takes place only after the fatty acid substrates, linoleic acid and linolenic acid, become available. Similarly, the wounding of certain plant tissues is also associated to a series of reactions leading to peroxidation that involves the generation of oxygen free radicals (Thompson *et al.*, 1987).

Activation of lipoxygenase and oxygen free radical generation leading to lipid peroxidation are also fundamental events during the early stages of the response of plant to infection (Keppler and Novacky, 1986, 1987; Rogers *et al.*, 1988; Ullrich-Eberius *et al.*, 1988; Peever and Higgins, 1989). In particular, anion superoxide may play a role in the synthesis of phytoalexins in response to infection of potato tubers by *Phytophtora infestans* (Doke, 1983, 1985; Chai and Doke, 1987), in bacteria-induced hypersensitive reaction (Keppler *et al.*, 1988), and in rice tissue treated with elicitors from *Pyricularia oryzae* (Haga *et al.*, 1986). The rapid production of H_2O_2 and its use by extracellular peroxidases has been recently described in plant cell cultures treated with elicitors of defense reactions, suggesting that hydrogen peroxide plays a crucial role in defense response to pathogens (Apostol *et al.*, 1989). Increase in NADH-duroquinone reductase in tobacco leaves treated with a bacterial protein-lypopolysaccharide complex was also reported. This response is interpreted as a mechanism to protect plasma membrane from oxygen free radical-induced damage (Valenti *et al.*, 1989). In other cases, hydroxyl radicals are involved in the abiotic elicitation of phytoalexins in legumes (Epperlein *et al.*, 1986). Conversely, singlet oxygen does not appear to be involved in bacterial-induced hypersensitive reaction in tobacco cells (Salzwedel *et al.*, 1989).

The superoxide anion is generated in the plasma membrane of root rhizodermal cells of dicotyledonous plant species stressed by iron deficiency (Cakmak *et al.*, 1987). In zinc deficiency, an enhanced superoxide anion generation and impaired detoxification of superoxide anion and hydrogen peroxide can lead to high levels of these oxygen species which, in turn, may increase the peroxidation of membrane lipids (Cakmak and Marschner, 1988a). The effects on the integrity of plasma membranes are, at least in part, due to the generation of $O_2^{\cdot-}$ by a membrane-bound NAD(P)H oxidase (Cakmak and Marschner, 1988b).

Membrane systems are one of the primary sites of dissecation injury in plants (Senaratna and McKersie, 1983). Membrane disruption, following dissecation stress, is mediated by a free radical mechanism with the consequent de-esterification of membrane phospholipids and accumulation of saturated free fatty acids which alter the physical properties of the membranes (Senaratna *et al.*, 1987). Membrane disassembly is also related to freezing injury (Kendall and McKersie, 1989) and accelerated seed aging (Gidrol *et al.*, 1989). In both cases lipid peroxidation, which is triggered by oxygen free radicals, accounts for at least a part of the damage.

Role of Surface Oxidases in the Apoplast of Plant Cells

Plant plasma membranes have some NADH oxidation activities capable of generating superoxide anion and hydrogen peroxide (Fig. 1). Surface peroxidases are enzymes that are bound to the outer surface of the membrane. As reported above, they generate hydrogen peroxide, through a reaction chain involving superoxide anion, and can utilize NADH as reducing substrate (Møller and Bérczi, 1986; Spreen Bouwer *et al.*, 1986; Askerlund *et al.*, 1987; Coleman and Chalmers, 1988; Pantoja and Willmer, 1988). The function of this peroxidase appears to be very similar to that of the well-known cell wall peroxidases associated with H_2O_2 generation (Elstner and Heupel, 1976; Mäder and Amberg-Fisher, 1982). The physical contiguity and the same catalytic activity exhibited by plasma membrane and cell wall peroxidases suggest a synergistic physiological role for these enzymes.

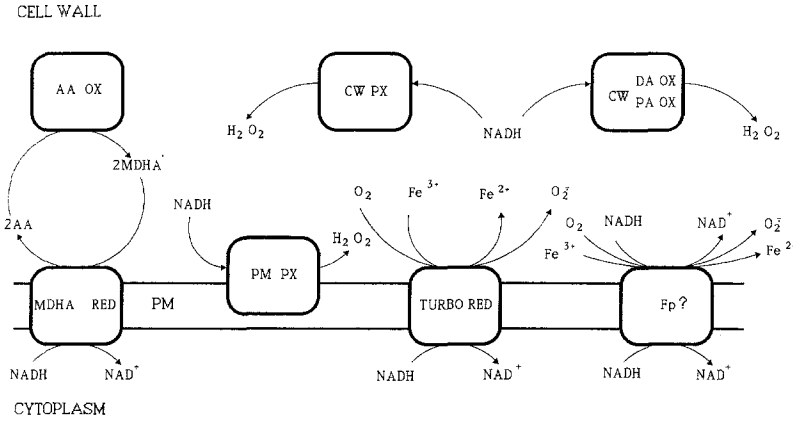

Fig. 1. NADH oxidases involved in the generation of free radicals and hydrogen peroxide at the surface of plant cells. PM, plasma membrane; PX, peroxidase; Fp, flavoprotein; AA, ascorbic acid; MDHA, monodehydroascorbate; DA OX, diamine oxidase; PA OX, polyamine oxidase; CW, cell wall.

Other results point to the involvement of plasma membrane NADH-reduced flavoproteins that, through an univalent reaction requiring Fe^{3+}, produce superoxide anion and hydrogen peroxide. The hypothetical flavoprotein seems to have the catalytic site on the external face of the plasma membrane (Vianello and Macrì, 1989; Vianello *et al.*, 1990). Both peroxidase and flavoprotein utilize, therefore, extracellular NADH. This substrate may be generated by the activity of a cell wall-bound malate dehydrogenase (Stephens and Wood, 1974; Gross, 1977; Gross and Janse, 1977; Mäder and Schloss, 1979; Tipton and Thowsen, 1985; Li *et al.*, 1989). The malate for this activity may be exported from the cell by a malate/oxalacetate shuttle (Gross, 1977). Gross *et al.* (1977), in their model of lignin biosynthesis, hypothesized that H_2O_2, besides being formed by peroxidase, may be generated from NADH via a nonenzymic reaction or through a flavoprotein catalyzing this reaction. This function may be accomplished by the NADH oxidase of radish plasma membrane (Vianello and Macrì, 1989; Vianello *et al.*, 1990). This activity can be important in young tissues, such as those used to isolate these membranes (24 h old seedlings), since peroxidase activity increases with age (Crane and Barr, 1989).

The inducible NADH oxidase (Turbo reductase) related to reduction of ferric chelate may be a third source of superoxide anion in the apoplastic site of the cell. However, in this case, cytoplasmic NAD(P)H is consumed (Sijmons *et al.*, 1984). In addition to peroxidase, β-glucosidase, malate dehydrogenase, arylesterase (Li *et al.*, 1989), and amine oxidases in the apoplast of some *Leguminosae* and *Gramineae* were also described. The latter enzymes may oxidize diamines and polyamines to produce hydrogen peroxide (Angelini and Federico, 1989). An NADH oxidase (semidehydroascorbate reductase) localized in the plasma membrane is able to reduce semidehydroascorbate produced by a cell wall ascorbate oxidase (Morré *et al.*, 1986). However, generation of anion superoxide or hydrogen peroxide are not involved in these reactions.

It is well known that phagocytic cells (neutrophils, monocytes, monocyte-derived macrophages, and eosinophils) have a marked capacity of generating anion superoxide through single electron transfers from NADPH to molecular oxygen. Subsequently, superoxide anion dismutates, in acid pH, to H_2O_2. Neutrophils (but not macrophages) and eosinophils contain a myeloperoxidase and an eosinophil peroxidase, respectively. The interaction of these peroxidases with a suitable halide allows the formation of OCl^- and OBr^-. These are particularly active oxygen species which may account for the antimicrobial activity of these cells (Segal, 1989).

The mechanism of resistance to pathogens in plants is less understood. Frequently, as a consequence of an infection, a rapid and localized "hypersensitive" response occurs near to the region parasitized, which leads to death of cells surrounding the site of pathogen invasion. This reaction is mediated

by multiple biochemical components which act together: production of phytoalexins, secretion of enzymes, and deposition of extracellular molecular barriers. In general, an increase of respiration (Uritani and Asahi, 1980) and peroxidase activity (Kawashima and Uritani, 1963; Yu and Hampton, 1964; Sako and Stahmann, 1972; Vance *et al.*, 1976; Svalheim and Robertsen, 1990) is frequently associated with infections of plants by pathogens. As reported above, superoxide anion formation has been established in several host–parasite interactions (Doke, 1983, 1985; Haga *et al.*, 1986; Chai and Doke, 1987; Keppler *et al.*, 1988), although hydroxyl radicals seem to be the major factor in the response of legumes to abiotic agents (Epperlein *et al.*, 1986).

Although circumstantial, these results suggest the involvement of oxygen free radical generating systems in defense mechanisms of plants (Ullrich-Eberius *et al.*, 1988). It is clear that in plasma membrane and cell wall there are enzymes capable of generating oxygen free radicals and hydrogen peroxide. In particular, peroxidase is an enzyme which is present with diverse isoenzymatic forms. Treatment with elicitors (pectic fragments of the fungal cell wall) may induce substantial changes in the activity of some of these which results in the appearance of new peroxidase isoenzymes and in a rapid formation of H_2O_2 (Apostol *et al.*, 1989). Therefore, the response of plants to elicitors suggests an increase in lignin biosynthesis caused by disease (Bruce and West, 1989). Even though there is no evidence, it is possible that some of these isoenzymes, in particular the isoforms unrelated to lignin biosynthesis, may also be associated with the generation of other toxic oxygen species, as demonstrated for animal phagocytic cells (Segal, 1989). NADH oxidoreductases generating superoxide anion might be an initial defense mechanism. Iron ions present in the cell wall can produce hydroxyl radicals by the Haber–Weiss reaction:

$$O_2^{\cdot-} + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

$$Fe^{2+} + H_2O_2 \longrightarrow OH^{\cdot} + OH^{-} + Fe^{3+}$$

This may be an additional mechanism of defense and can account for previously reported results (Epperlein *et al.*, 1986). In addition, ferric ion, which is the common state of iron in the apoplast, may be reduced directly by H_2O_2 through a relatively slow reaction:

$$Fe^{3+} + H_2O_2 \longrightarrow O_2^{\cdot-} + Fe^{2+} + 2H^{+}$$

to produce superoxide anion and ferrous ions, supporting the view that reduction of Fe^{3+} may also occur before uptake in the cell-wall space (Tipton and Thowsen, 1985).

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