Plant Cell Physiol. 38(10): 1118-1126 (1997)

Generation of Superoxide Anion and Localization of CuZn-Superoxide Dismutase in the Vascular Tissue of Spinach Hypocotyls: Their Association with Lignification

Ken'ichi Ogawa [1,4], Sumio Kanematsu [2] and Kozi Asada [3,5]

[1] *Department of Botany, Faculty of Science, Kyoto University, Kyoto, 606-01 Japan*
[2] *Department of Food Science and Technology, Minami-Kyushu University, Takanabe, Miyazaki, 884 Japan*
[3] *The Research Institute for Food Science, Kyoto University, Uji, Kyoto, 611 Japan*

The sites of generations of superoxide anions and hydrogen peroxide in cross sections of hypocotyls from spinach seedlings were located by staining with nitroblue tetrazolium (NBT) and with starch-iodide, respectively. Formazan, produced upon the reduction of NBT by superoxide, was observed mainly in the vascular tissue only in the presence of inhibitors of CuZn-superoxide dismutase (CuZn-SOD), and its formation was suppressed under anaerobic conditions. Thus, NBT was reduced to formazan specifically by the superoxide anions generated in vascular tissue. The reduction of NBT was suppressed by inhibitors of NAD(P)H oxidase, but neither by cyanide nor azide, indicating the involvement of NAD(P)H oxidase in the generation of superoxide anions in the vascular tissue. Starch-I_2 complex also was formed in the vascular tissue, but not in the presence of either the CuZn-SOD inhibitor or the NAD(P)H oxidase inhibitor, indicating that the hydrogen peroxide is produced via the catalytic disproportionation with CuZn-SOD of the superoxide generated by NAD(P)H oxidase. Generations of superoxide anions and hydrogen peroxide in the vascular tissue were particularly apparent in the xylem and associated with the sites of distribution of CuZn-SOD as determined by an immunohistochemical method, and also with the location of lignin as determined by the phloroglucin-HCl reaction.

Key words: Apoplast — Hydrogen peroxide — Lignin synthesis — Spinach (*Spinacia oleracea*) — Superoxide — Superoxide dismutase (EC 1.15.1.1).

Hydrogen peroxide is a substrate for peroxidase-dependent reactions, such as the biosynthesis of lignin and suberin, and the decomposition of IAA during plant development, and for defenses against infection by pathogens. Peroxygenases also require hydrogen peroxide for the oxygenation of substrates such as indole, phenols and linoleic acid (Ishimaru and Yamazaki 1977, Hamberg and Hamberg 1996). Hydrogen peroxide is produced either by the two-electron reduction of dioxygen in reactions catalyzed by divalent oxidases, such as glycolate oxidase, or by the disproportionation of the superoxide anion. Superoxide is produced via the univalent reduction of dioxygen by electron donors in reactions catalyzed by univalent oxidases, such as xanthine oxidase, NAD(P)H oxidase and aldehyde oxidase, or via the autooxidation of electron carriers, such as the primary electron acceptor of PSI (Asada and Takahashi 1987). Spontaneous disproportionation of superoxide radicals proceeds at an appreciable rate to produce hydrogen peroxide and dioxygen ($10^5\ M^{-1}\ s^{-1}$ at pH 7), but the generation of hydrogen peroxide in apoplastic regions of elicitor-treated cells is suppressed by an inhibitor specific for CuZn-superoxide dismutase (CuZn-SOD) (Auh and Murphy 1995) which catalyzes the disproportionation of superoxide anions at a diffusion-controlled rate ($2 \times 10^9\ M^{-1}\ s^{-1}$). These observations indicate that the superoxide radicals that are generated in vivo react with cellular components prior to their spontaneous disproportionation when SOD is inactivated. Thus, SOD appears to be required for the generation of hydrogen peroxide from the superoxide via the enzyme-catalyzed disproportionation.

In spinach leaves, up to 40% of the so-called "cytosolic" (as distinct from chloroplastic) isoforms of CuZn-SOD are localized in the apoplast, and the sites of localization of the apoplastic CuZn-SOD are associated with the lignifying cells in the leaf and hypocotyl tissues (Ogawa et al. 1995b, 1996). Furthermore, we have demonstrated a preliminary evidence for an association of the sites of distribution of CuZn-SOD with the sites of the generation of superoxide and of lignifying tissues in spinach hypocotyls (Ogawa et al. 1996). However, the cells in which superoxide is generated for the supply of hydrogen peroxide for lignification and the enzymes participating in the generation of superoxide remain to be determined.

Abbreviations: BSA, bovine serum albumin; DDC, *N,N*-diethyldithiocarbamate; DPI, diphenyleneiodonium; NBT, nitroblue tetrazolium; PBS, phosphate buffered saline (10 mM sodium phosphate, pH 7.4, and 150 mM NaCl); SOD, superoxide dismutase.

[4] Present address: Chemical Research Laboratories, Toray Industries Inc., 9-1 Oe-cho, Minato-ku, Ngagoya, 455 Japan.
[5] To whom reprint requests should be addressed to his present address: Department of Biotechnology, Faculty of Engineering, Fukuyama University, Gakuen-cho-1, Fukuyama, 729-02 Japan.

In the present work, we showed the generating enzyme of superoxide anions in the vascular tissue of spinach hypocotyls and assessed the contribution of CuZn-SOD and NAD(P)H oxidase to the formation of hydrogen peroxide using inhibitors of CuZn-SOD, guaiacol peroxidase and NAD(P)H oxidase. Further, detailed distribution of CuZn-SOD, lignin and the generation sites of superoxide and hydrogen peroxide in spinach hypocotyls is demonstrated, indicating the involvement of CuZn-SOD in the supply of hydrogen peroxide for lignification of the cell walls.

Materials and Methods

Plant material—Spinach seeds were germinated on moist vermiculite in a growth chamber (20°C; daily illumination for 12 h of 1,500 lux; humidity, 100%), and cross sections (100–300 μm thick) of hypocotyls from 13-d-old seedlings, cut 1 mm from the top, were prepared with a razor blade for histochemical analysis. For immunohistochemical analysis, hypocotyls from 13-d-old spinach seedlings were prefixed in 4% paraformaldehyde in 10 mM sodium phosphate, pH 7.4, and 150 mM NaCl (PBS) at 4°C overnight, and then were rinsed six times with PBS for 20 min each. Subsequently, cross sections, cut 1 mm below the top, or longitudinal sections (100–300 μm thick) including the region 1 mm from the top were prepared as described above.

Antibody against cytosolic CuZn-SOD—The antibody against "cytosolic" CuZn-SOD from spinach leaves was prepared as described previously (Kanematsu and Asada 1990). This antibody cross-reacted with "cytosolic" isoforms of CuZn-SOD that included the apoplastic isoform, but not with the isoform of CuZn-SOD from chloroplasts (Kanematsu and Asada 1990, Ogawa et al. 1995a, b, 1996).

Detection of lignin in hypocotyls—Sections mounted on glass slides were incubated with 1% phloroglucin in ethanol and, just before the ethanol was evaporated, 10% HCl (w/v) was added. Subsequently, the sites of lignin were identified as regions of dark red-brown staining under a light microscope (BH and PM-20, Olympus, Tokyo, Japan).

Detection of superoxide in hypocotyls—Generation of superoxide was detected under the light microscope by the formation of blue formazan after incubation of the sections for 30 to 120 min in 0.25 mM NBT in 10 mM sodium phosphate, pH 7.8. To examine the effects of inhibitors on the superoxide-dependent reduction of nitroblue tetrazolium (NBT), inhibitors of CuZn-SOD (1 mM *N,N*-diethyldithiocabamate (DDC), 10 mM KCN or 1 mM NaN_3; Asada et al. 1975) were added to the NBT-containing solution. When the effects of inhibitors of NAD(P)H-oxidase were examined, the sections were preincubated with either 10 mM imidazole or 50 μM diphenyleneiodonium (DPI) (Murphy and Auh 1996) for 30 min prior to the incubation with NBT. For the depletion of dioxygen, the sections in a petri dish were subjected to a reduction in pressure with an aspirator just after the sections had been incubated with NBT, and then nitrogen gas was introduced. Before the observation of the sections, the formazan deposited on the periphery of the section was washed out with ethanol, by which the formazan formed by the wounding was removed (Ogawa et al. 1996).

Detection of hydrogen peroxide in hypocotyls—Generation of hydrogen peroxide was located as a dark blue starch-I_2 complex on the cut surface of the sections as described by Olson and Varner (1993). Potato starch (4%, w/v) in 100 mM potassium iodide was boiled and then cooled to room temperature. The sections were incubated with the starch-iodide for 3 min and observed under the light microscope. When the effects of inhibitors on the generation of hydrogen peroxide was tested, the sections were preincubated with 2 mM DDC or 100 μM DPI.

Immunohistochemical detection of CuZn-SOD in hypocotyls—Prefixed sections were incubated with 2% (w/v) glycine for 1 h to quench any remaining paraformaldehyde. Then, the sections were immunostained on glass slides that had been coated with 1% (w/v) gelatin as described by Ogawa et al. (1996). The sections on the gelatin-coated glass slides were incubated for 1 h with a blocking solution that consisted of 0.8% (w/v) bovine serum albumin (BSA), 0.1% (v/v) IGSS quality gelatin (Amersham Japan, Tokyo), 5% (w/v) goat serum albumin and 2 mM NaN_3 in PBS, and then they were rinsed with a washing medium, which consisted of 0.8% (w/v) BSA, 0.1% (v/v) IGSS quality gelatin and 2 mM NaN_3 in PBS. Thereafter, the sections were allowed to cross-react with the antibody against "cytosolic" CuZn-SOD at 200-fold dilution in an incubation mixture that consisted of 0.8% (w/v) BSA, 0.1% (v/v) IGSS quality gelatin, 1% (w/v) goat serum albumin and 2 mM NaN_3 in PBS for 4 h at room temperature. The sections were rinsed three times with the washing medium for 15 min each and then incubated with the antibody raised in goat against rabbit IgG that had been conjugated with gold particles (diameter, 1 nm; Amersham Japan, Tokyo) at 50-fold dilution in the incubation mixture. After reaction with the second antibody for 4 h at room temperature, the sections were washed three times with the washing medium for 15 min each and then similarly with PBS. Finally, they were washed three times with water for 5 min each. The images of the immunogold particles were enhanced with a silver enhancement kit (Amersham Japan, Tokyo), and the immunolabeled sections were observed under the light microscope.

Results and Discussion

Effects of inhibitors on the production of superoxide anion in the vascular tissue of spinach hypocotyls—When sections of hypocotyls were incubated with NBT for 120 min, little of the blue precipitate of formazan that is produced as a result of the reduction of NBT was found in the vascular tissue (Fig. 1A). However, the blue precipitate was found in the vascular tissue after incubation in the presence of DDC, an inhibitor of CuZn-SOD (Fig. 1B), as observed previously (Ogawa et al. 1996). Incubation of the sections with either KCN or NaN_3, which inhibits both CuZn-SOD and peroxidases, induced a high intensity of staining with formazan as that in the presence of DDC (Fig. 1C, D, respectively). Only a little formazan was formed under anaerobic conditions (Fig. 1E). The production of formazan was suppressed by preincubation of the sections with inhibitors of NAD(P)H oxidase, either DPI or imidazole, for 30 min, prior to the incubation with NBT even in the presence of DDC (Fig. 1F, G).

Enhanced reduction of NBT in the presence of DDC, an inhibitor of CuZn-SOD, can be inferred from the increase in the steady-state concentration of superoxide anions to allow the formazan formation by the superoxide when the CuZn-SOD-catalyzed disproportionation of superoxide was blocked. This increase was suppressed under

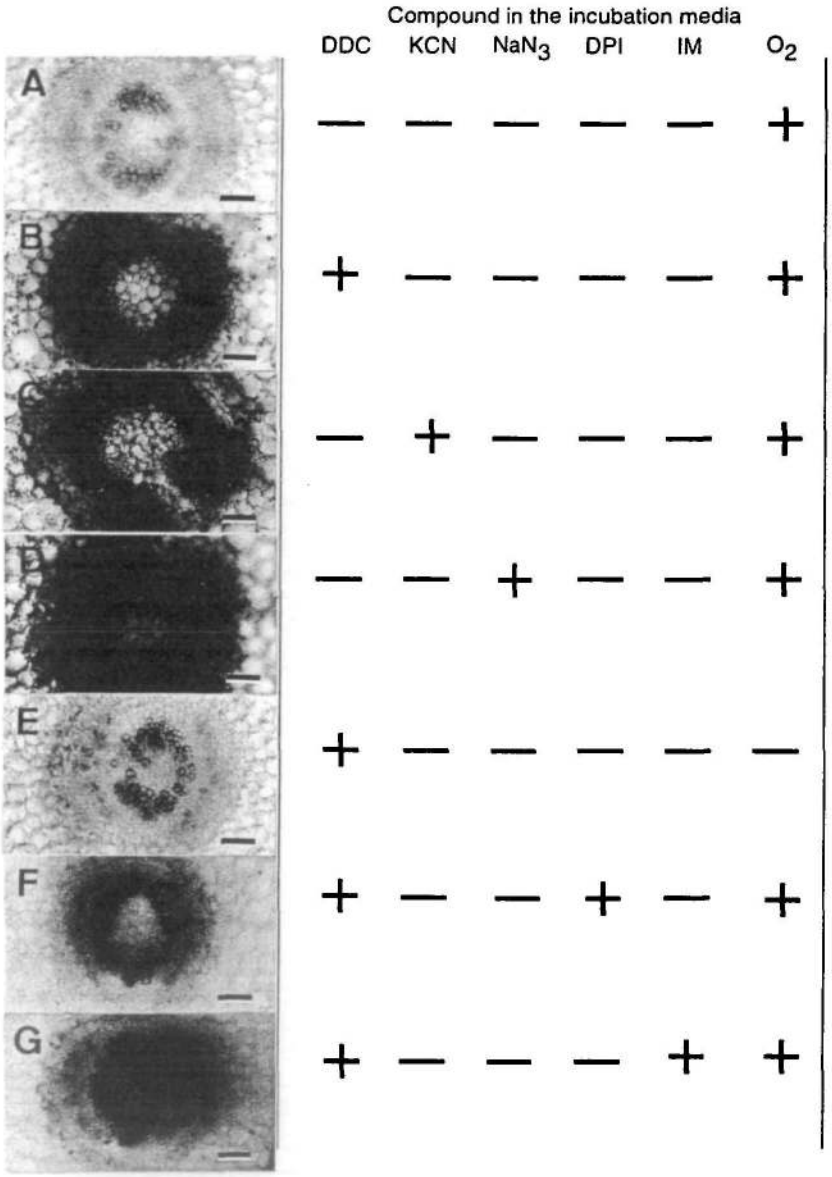

Fig. 1

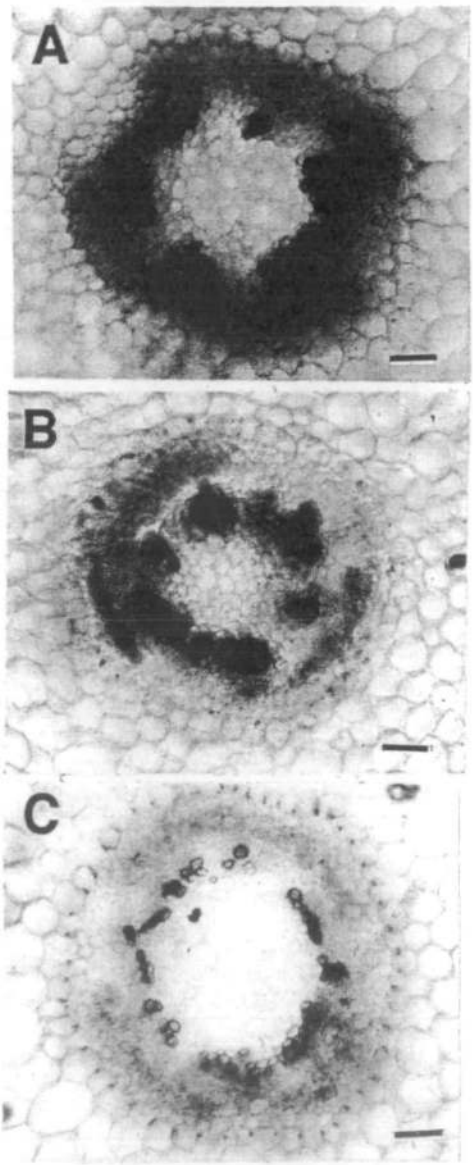

Fig. 2

Fig. 1 Effects of inhibitors and dioxygen on the formation of formazan in vascular tissues of spinach hypocotyls. Cross sections of hypocotyls of 13-d-old seedlings, cut 1 mm from the top, were used, and generation of superoxide was detected as a blue precipitate of formazan that was formed as a result of the reduction of 0.25 mM NBT in 10 mM sodium phosphate, pH 7.8, as described in Materials and Methods. The duration of incubation with NBT was 120 min. Each micrograph was obtained after incubation of a section with NBT in a medium that included the compounds [1 mM *N,N*-diethyldithiocarbamate (DDC), 10 mM KCN, 1 mM NaN_3, 10 mM imidazole (IM), 50 μM diphenyleneiodonium (DPI)] indicated in the same row, where + and − represent the presence and absence of the compound at the top of the column. Scale bars, 100 μm. DPI and IM were applied to the sections prior to the incubation with NBT. A control cross section (untreated) is shown in Fig. 3B.

Fig. 2 Effects of inhibitors on the formation of starch-I_2 complex in the vascular tissue of spinach hypocotyls. The cross sections of the hypocotyl from a 13-d-old seedling were prepared as in Fig. 1, and generation of hydrogen peroxide was located as dark blue starch-I_2 complex, as described in Materials and Methods. A, Formation of the starch-I_2 complex in the control section; B, in the presence of DDC; C, in the presence of DPI. DDC (2 mM) and DPI (100 μM) were applied to the sections prior to the incubation with the starch-iodide solution. Scale bars, 100 μm.

anaerobic conditions, a result that suggests that the formazan in vascular tissue represents the generation of superoxide. In the presence of an inhibitor of NAD(P)H oxidase, either DPI or imidazole, such generation of superoxide was suppressed in spite of the inhibition of CuZn-SOD by DDC, an indication that the generation of superoxide observed in vascular tissue was catalyzed by an enzyme similar to the NADPH oxidase of mammalian neutrophiles.

Effects of inhibitors on the production of hydrogen peroxide in the vascular tissue of spinach hypocotyls—When the sections of hypocotyls were incubated with the starch-iodide solution, a dark blue starch-I_2 complex was found in the vascular tissue and its location pattern was similar to that of the formazan (Fig. 2A), indicating that the generation site of hydrogen peroxide was the same as that of superoxide in the vascular tissue. Such distribution of generation site of hydrogen peroxide is consistent with that in the hypocotyl tissue of *Zinnia elegans* (Olson and Varner 1993). The production of hydrogen peroxide as detected by starch-I_2 complex was suppressed by the CuZn-SOD inhibitor DDC and the NAD(P)H oxidase inhibitor DPI (Fig. 2B, C), although DDC did not completely suppress its generation compared with DPI. The difference between their effects on the generation of hydrogen peroxide may reflect that, to some degree, the superoxide is spontaneously disproportionated to hydrogen peroxide and dioxygen because DDC has no effect on NAD(P)H oxidase and superoxide is intensively generated in the xylem. The present results indicate that hydrogen peroxide formed in the vascular tissue is derived from the superoxide anion radicals generated by NAD(P)H oxidase, and the superoxide would disappear without the formation of hydrogen peroxide on account of its interaction with cellular components if CuZn-SOD could not function. Thus, we propose that in the vascular tissue of spinach hypocotyl, the hydrogen peroxide is generated via the following pathway;

$$2O_2 + NAD(P)H \rightarrow 2O_2^- + NAD(P)^+ \quad \text{(NAD(P)H oxidase)}$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad \text{(CuZn-SOD)}.$$

When cultured cells of broadbean, French bean, cow pea, rice, rose, soybean, tomato, tobacco and so on, are treated with elicitors, hydrogen peroxide and superoxide anion radicals have been shown to be produced transiently by the cells (Doke et al. 1991, Elstner 1991, Kuchitsu and Shibuya 1994, Mehdy 1994, Auh and Murphy 1995, Robertson et al. 1995, Murphy and Auh 1996). The elicitor-induced generation of superoxide has been shown to be suppressed by an inhibitor of NAD(P)H oxidase (Auh and Murphy 1995). The ratio of NADH to NAD^+ decreases with elicitor-induced increases in the uptake of oxygen (Robertson et al. 1995). All these reports together suggest the presence of NAD(P)H oxidase in plant cells. The present observations indicate the involvement of NAD(P)H oxidase in the generation of superoxide not only in the elicitor-treated tissues, but also in normal or sound tissues.

A catalytic cycle consisting of NADH, phenols, Mn^{2+} ions and guaiacol peroxidase has been proposed as the superoxide-generating system for the supply of hydrogen peroxide for lignification (Elstner and Heuple 1976, Gross et al. 1977, Halliwell 1978). Guaiacol peroxidase is a family of peroxidase, as represented by horseradish peroxidase, for the metabolism of cellular components, and distinguished from a family of the hydrogen peroxide-scavenging peroxidase, ascorbate peroxidase (Asada 1992). Addition of either KCN or NaN_3, which inhibits both peroxidases and CuZn-SOD (Asada et al. 1975), did not suppress the generation of superoxide, but tended, rather, to enhance it (Fig. 1C, D). Furthermore, DPI and imidazole, which did not inhibit either peroxidase or CuZn-SOD, suppressed almost completely the generation of superoxide (Fig. 1F, G) and hydrogen peroxide (Fig. 2C). Thus, the participation of guaiacol peroxidase in the generation of superoxide is unlikely, at least, in spinach hypocotyls.

The site of generation of superoxide anions in spinach hypocotyls—In cross sections of spinach hypocotyls that had been incubated with NBT in the presence of DDC for 90 min, blue formazan was distributed mainly in the vascular tissue, with a little in the cortex and the epidermal cells (Fig. 3A). The untreated sections showed only the pale-brown color in the vessels (Fig. 3B). In the vascular tissue, when the incubation with NBT was short (30 min), formazan was found only on the thickened cell walls in the xylem elements with secondary thickenings (Fig. 3C, arrow), but not on all the thickened cell walls (Fig. 3C, arrowhead). After a 90-min incubation, formazan was found not only in the xylem but also in the phloem (Fig. 3D, arrow). Thus, the superoxide anions were generated mainly in xylem and to a much lesser extent in phloem.

Four stages during the differentiation of tracheary elements in situ are likely to occur: (1) cell elongation, (2) development of the patterned secondary thickenings of the cell walls that contain β-1,3-glucan and β-1,4-glucan, (3) accumulation of lignin in the secondary thickenings of the cell walls, and (4) cell death for conversion to vessels (for review, see Fukuda 1996). Hydrogen peroxide is required at stage (3) for the oxidation of phenyl propanoid catalyzed by guaiacol peroxidase, with the resultant polymerization of phenyl propanoid radicals. Furthermore, in maize, stage (1) has been shown to be inhibited by hydrogen peroxide because hydrogen peroxide impairs the extensibility of the cell wall (Schopfer 1996). Stages (1) through (4) should also be discernible in the vascular tissue, where the cells of each stage are located in close proximity to each other. Since the cells at stage (1) cannot elongate if hydrogen peroxide diffuses within the vascular tissue, the generation time of hydrogen peroxide should be limited to stage (3). There-

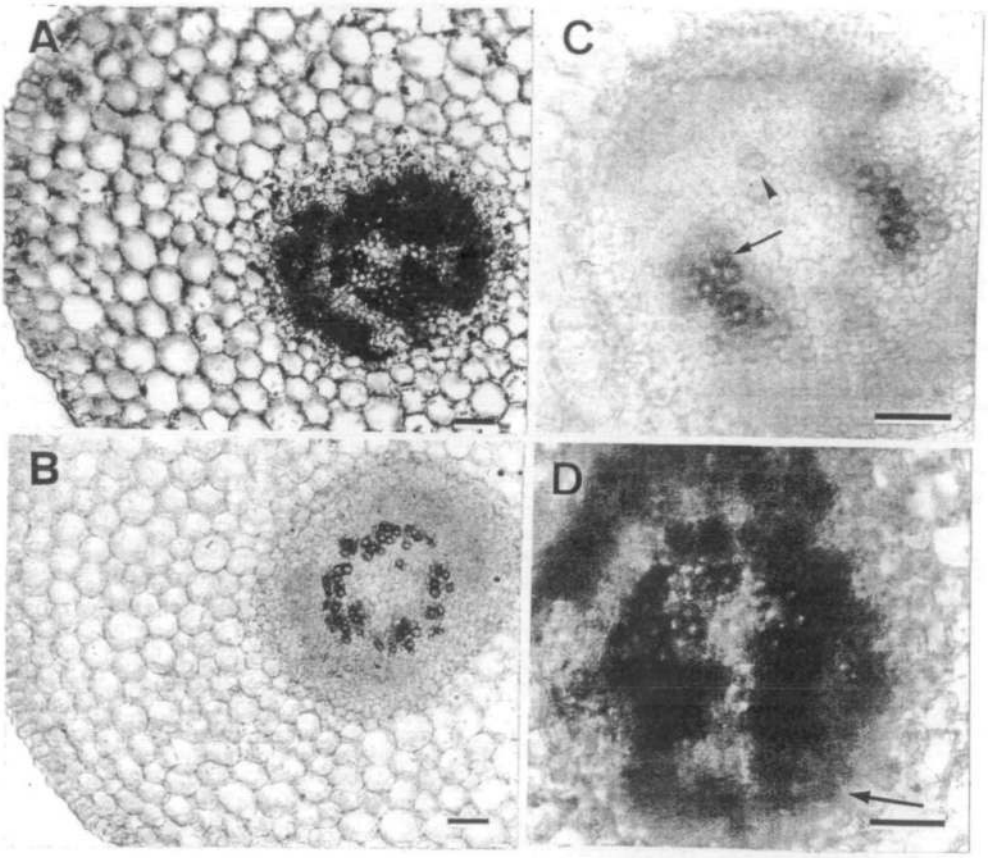

Fig. 3 Distribution of sites of generation of superoxide in hypocotyls of spinach, as indicated by blue precipitates of formazan. A, Precipitation of formazan in a cross section of the hypocotyl from a 13-d-old seedling that had been incubated with NBT and DDC as described in Fig. 1B for 90 min. B, an untreated cross section of A; C, high-magnification view of vascular tissue after a 30-min incubation under the conditions described for Fig. 1B; D, precipitation of formazan after a 90-min incubation as in C. Scale bars, 100 μm.

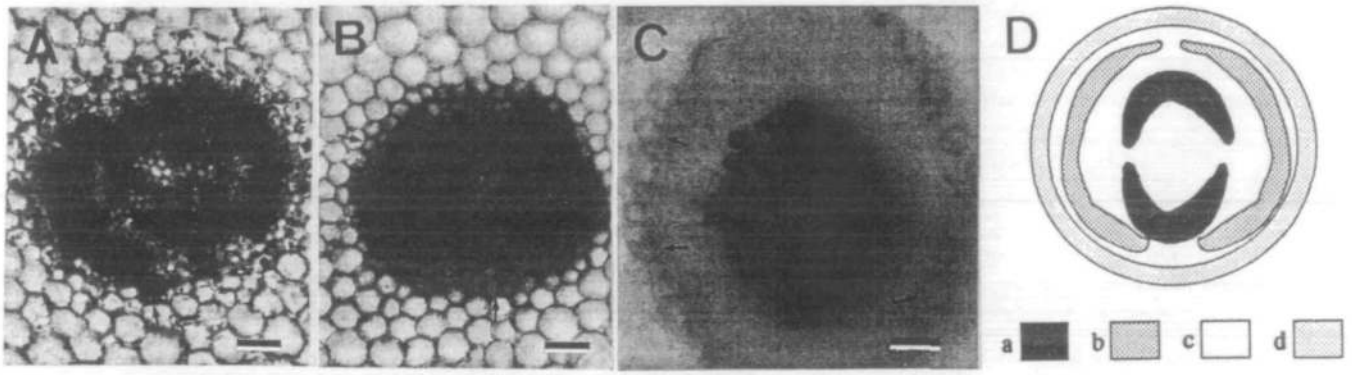

Fig. 5 Comparison of the distribution of lignin and the so-called "cytosolic" CuZn-SOD with that of sites of generation of superoxide in spinach hypocotyl. A, Formation of formazan at sites of generation of superoxide under the same conditions as described for Fig. 3A; B, immunolabeling of "cytosolic" CuZn-SOD; C, staining of lignin by phloroglucin-HCl. Scale bars, 100 μm; D, Schematic distribution of sites of generation of superoxide, hydrogen peroxide, CuZn-SOD and lignin in the vascular tissue of a spinach hypocotyl. The area (a), corresponding to the xylem, generates considerable superoxide and hydrogen peroxide and contains both CuZn-SOD and lignin. The area (b), corresponding to the phloem, generates a little less superoxide and hydrogen peroxide and contains lower amounts of CuZn-SOD than those in A but does not contain much lignin. The area (c), including fiber elements, pith and the cambial zone, generates relatively little superoxide and hydrogen peroxide and contains lower amounts of CuZn-SOD than those in the areas (a) and (b) and no lignin. The area (d), corresponding to the endodermis, generates relatively a little superoxide and hydrogen peroxide and contains both CuZn-SOD and suberin.

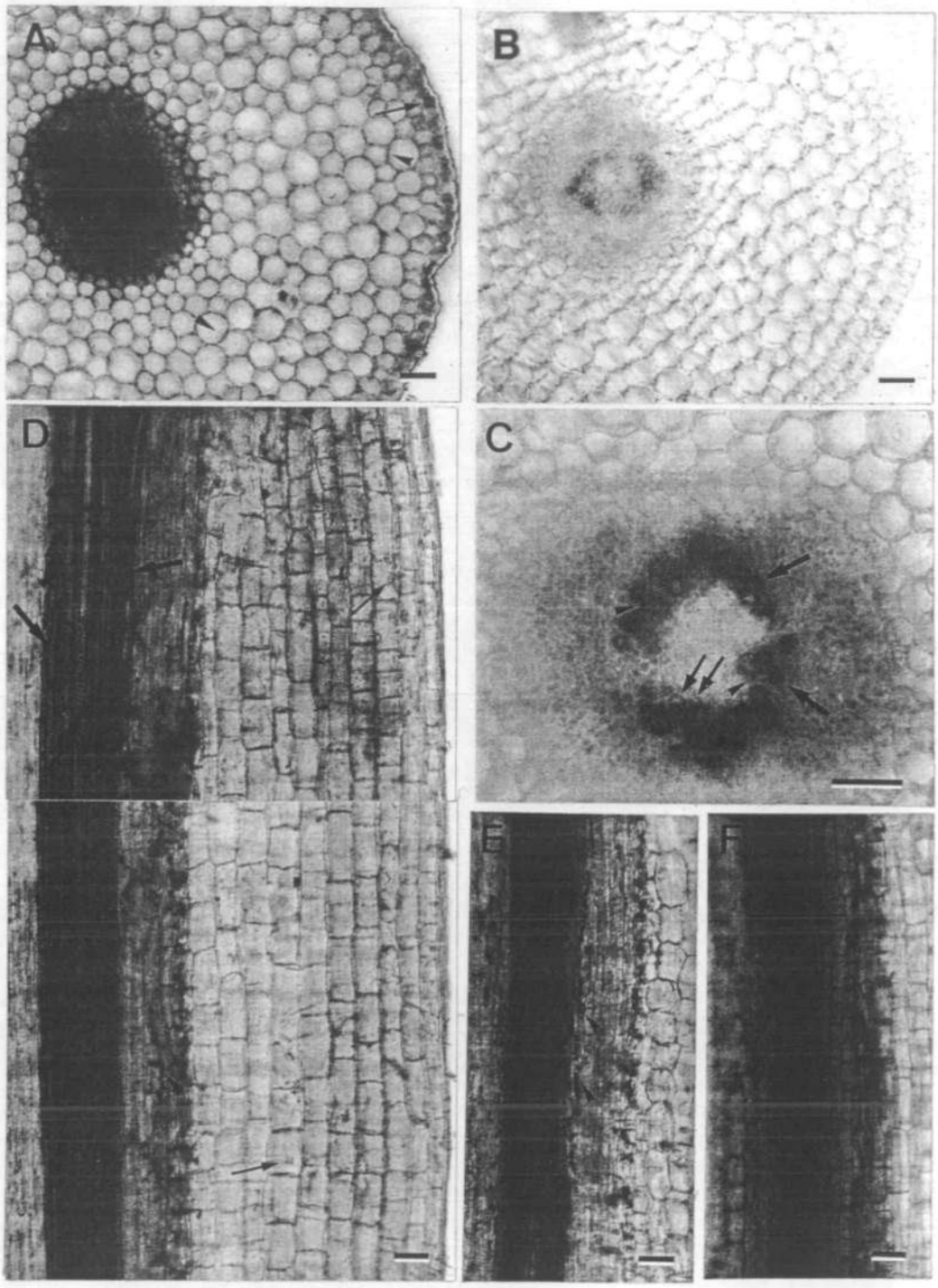

Fig. 4 Immunohistochemical distribution of the "cytosolic" CuZn-SOD in spinach hypocotyls. Immunohistochemical staining of "cytosolic" CuZn-SOD in hypocotyls from 13-d-old seedlings was performed as described in Materials and Methods. Immunostaining of "cytosolic" CuZn-SOD is visible as brown to dark-brown region. A, a cross section of a hypocotyl immunostained with the antibody against "cytosolic" CuZn-SOD; B, control section of A (without the primary antibody); C, high-magnification view of A; D, E and F, longitudinal sections of hypocotyl cut at different sites. Scale bars, 100 μm. Another control section of A (untreated) is shown in Fig. 3B.

fore, the cells with secondary thickenings which precipitated formazan (Fig. 3C, arrow) appear to be at stage (3), and those which precipitated little formazan (Fig. 3C, arrowhead) at stage (2). Superoxide is not generated in the completely lignified tissues of spinach hypocotyls and is generated only in the cells adjacent to the lignified tissues (Ogawa et al. 1996). The completely lignified cells correspond to cells after stage (4) and, therefore, the cells with a formazan precipitate can be assumed to be at stage (3) and to be adjacent to lignified cells. The various levels of superoxide detected in the xylem elements might reflect stages of the differentiation of the vessels.

Lignification also occurs in phloem fiber, and so the precipitation of formazan in the phloem is likely to indicate that the cells are in the process of accumulation of lignin. Hydrogen peroxide and guaiacol peroxidase have been detected not only in the xylem but also in the phloem (Harkin and Obst 1973, Olson and Varner 1993, Fukuda 1996). Therefore, the generation of superoxide in the phloem appears to be correlated with the supply of hydrogen peroxide for lignification.

Distribution of CuZn-SOD in spinach hypocotyls When cross sections of hypocotyls were immunohistochemically labeled with antibodies against "cytosolic" CuZn-SOD, the product of immunoreaction with the antigen was found mainly in the vascular tissue and epidermal cells as brown to dark-brown silver staining (Fig. 4A). In contrast, control cross sections without the primary antibody showed only a light brown color on the cell walls of the vessels (Fig. 4B). In the vascular tissue, the staining in xylem was so deep that it was almost black, but that in the phloem was less (Fig. 4A, C). Most CuZn-SOD was located on thickened cell walls of the cells in the xylem elements (Fig 4C, large arrows), although all of the xylem cells did not always contain CuZn-SOD in the thickened cell wall (Fig. 4C, small arrows). Thus, CuZn-SOD in vascular tissue was distributed (Fig. 4A, C) at the sites of generation of superoxide (Fig. 1, 3). In addition to that in the vessels, CuZn-SOD was distributed in the symplast of xylem parenchyma cells (Fig. 4C, arrowhead). CuZn-SOD was located also on the symplast in the epidermal cells (Fig. 4A, arrow), with a little on that in cortex (Fig. 4A, arrowheads), where superoxide was generated as detected by formazan (Fig. 3A). We have demonstrated the location of "cytosolic" CuZn-SOD in nuclei and tonoplasts of spinach mesophyll cells by immunogold electron microscopy. (Ogawa et al. 1996). Epidermal cells have wax-rich cell walls where cutin is produced from fatty acids (Brett and Waldron 1996, Heldt 1996). The fatty acids moiety of cutin are oxygenated (Post-Beittenmiller 1996), probably catalyzed by peroxygenase.

Similarly to the location of CuZn-SOD in the cross sections, the immunostaining of longitudinal sections of the top 1 mm of hypocotyl revealed that CuZn-SOD was distributed mainly in the vascular tissue (Fig. 4D). The xylem was most deeply immunostained to nearly black in color and the location of CuZn-SOD in the secondary thickenings of vessel elements was observed as vertical stripes of immunostaining (Fig. 4D, large arrows). Faint immunolabeling of CuZn-SOD was found on the cytoplasm in the cortex (Fig. 4D, small arrows), resembling that in the cross section in Fig. 4A. The insides of the parenchyma cells adjacent to the xylem were also found to contain CuZn-SOD (Fig. 4E, arrowheads). The parenchyma cells and their cell walls also were immunolabeled by the antibody against CuZn-SOD (Fig. 4D, F, arrowheads).

Thus, in spinach hypocotyls, the sites with strongest immunolabeling of "cytosolic" CuZn-SOD were the vascular tissue and the epidermis. The CuZn-SOD-containing vascular tissue corresponds to the sites of the expression of the promoter of the gene for a "cytosolic" CuZn-SOD in tobacco, as determined with a reporter gene for β-glucuronidase (Hérouart et al. 1994). The present work confirmed the previous results obtained by the immunogold labeling and electron microscopy (Ogawa et al. 1995b, 1996). Strong immunolabeling of CuZn-SOD was consistently found on the cell walls of lignifying cells, future vessels, and especially on the secondary thickenings of the cell walls. Previous works have shown that, in the vascular tissue, CuZn-SOD is distributed in the cells where superoxide is generated, and not in completely lignified cells (Ogawa et al. 1996).

Comparison of the location sites of lignin, suberin and CuZn-SOD and the generation site of superoxide in vascular tissue—The xylem, in which lignin was stained by the phloroglucin-HCl reaction (Fig. 5C, p. 1122), corresponded to the sites of distribution of CuZn-SOD (Fig. 5B) and to the generation site of superoxide (Fig. 5A). Location of CuZn-SOD and the generation of superoxide were observed also in the phloem, but little lignin was detected in the phloem, because of its low levels to detect by the phloroglucin-HCl reaction. Phloem fiber cells are to be lignified (Olson and Varner 1993) and, therefore, location of CuZn-SOD and generation of superoxide in the phloem might be correlated with the biosynthesis of lignin.

The endodermal cells generally contain suberin, which is synthesized from lignin precursors and fatty acids in higher plants. Its biosynthesis requires hydrogen peroxide for the oxidation of monophenols as does the synthesis of lignin (Brett and Waldron 1996, Heldt 1996). Faintly staining in the cell walls of endodermal cells by the phloroglucin-HCl reaction may indicate the location of suberin in them (Fig. 5C, arrows). The endodermal cells also contained CuZn-SOD (Fig 5B, arrows) and generated superoxide radicals (Fig. 5A, arrows), which may participate in the generation of hydrogen peroxide for the biosynthesis of suberin.

As schematically shown in Fig. 5D, in the vascular tissue of spinach hypocotyls, the sites of distribution of

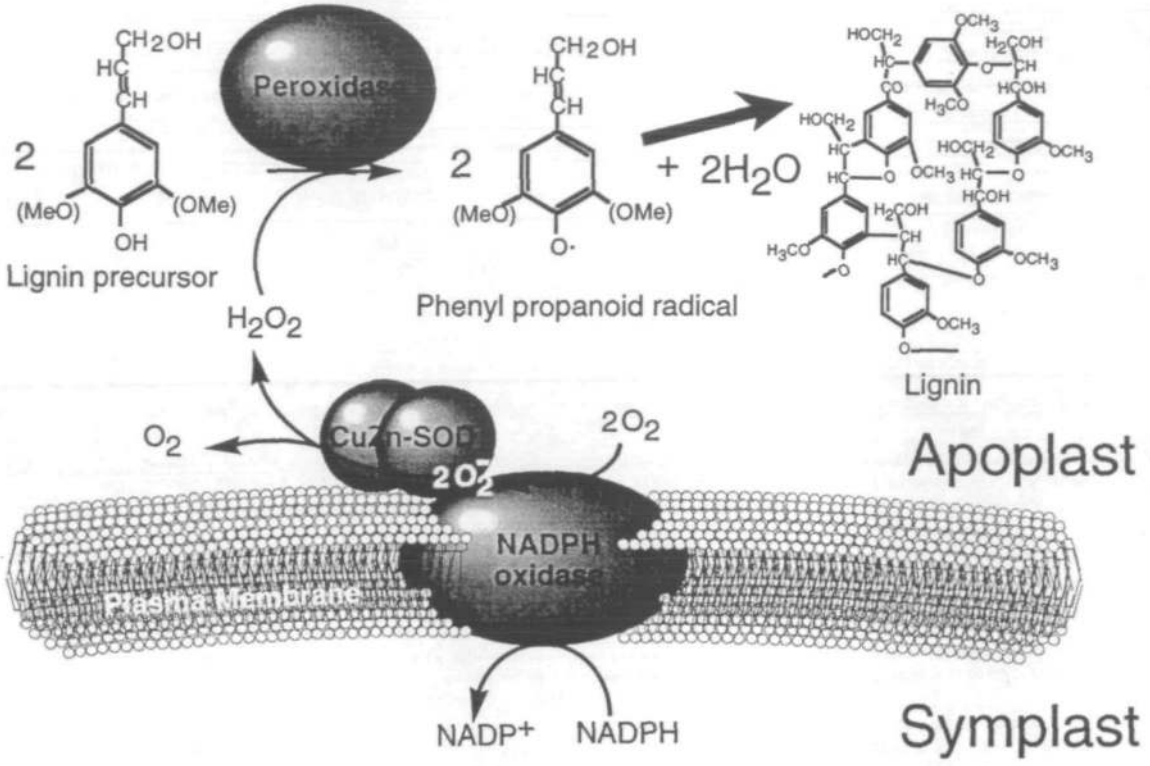

Fig. 6 A proposed scheme for supplying hydrogen peroxide to lignin biosynthesis.

CuZn-SOD and of generation of superoxide (Fig. 1, 3 and Fig. 5A) and hydrogen peroxide (Fig. 2A) were apparently associated with each other. The association of the generation of superoxide and hydrogen peroxide with CuZn-SOD in the xylem allows the sites of generation of hydrogen peroxide to be restricted to those of the generation of superoxide, at which where the lignification occurs. In contrast to the present results, when tissue printing method is applied to hypocotyls of bean and soybean, little starch-I_2 complex is found in the xylem tissue (Schopfer 1994). This might be due to a rapid consumption of the hydrogen peroxide generated in the xylem prior to its diffusion to tissue printing membrane.

Concluding remarks—The present study not only confirmed the association of the generations of superoxide and hydrogen peroxide with CuZn-SOD, but also revealed the detailed sites of generation of superoxide in vascular tissue, especially in the xylem, of spinach hypocotyls. Further, the production of superoxide is catalyzed by NAD(P)H oxidase, but not by peroxidase in the vascular tissue. We here showed a possibility that generation of superoxide and the expression of CuZn-SOD are synchronously regulated, depending on the stage of differentiation of xylem cells. In this context, it should be noted that the xylem parenchyma cells that surrounded the vessels contained CuZn-SOD in the cytoplasm (Fig. 4C, E). These cells may correspond to the sites of expression of promoters of the gene for the cinnamyl alcohol dehydrogenase and phenylalanine ammonia-lyase (Feuillet et al. 1995) and to sites of the accumulation of the lignin precursor, coniferin, as anticipated from the localization of a coniferin-specific β-glucosidase in the xylem fiber elements (Dharmawardhana et al. 1995). Furthermore, we have shown that either the CuZn-SOD-inhibitor DDC or the NAD(P)H oxidase inhibitor DPI inhibits the accumulation of lignin in the secondary cell wall of the cultured cells of *Zinnia elegans* (Ogawa et al. 1997). These results lend further support to our hypothesis that CuZn-SOD is essential for lignification (Ogawa et al. 1996) and to the participation of NAD(P)H oxidase in the generation of superoxide for the hydrogen peroxide in the biosynthesis of lignin.

We propose the possible system for supplying hydrogen peroxide to the biosynthesis of lignin as schematically shown in Fig. 6. In the presence of the inhibitors of CuZn-SOD, little hydrogen peroxide was detected in spite of the generation of superoxide (Fig. 2). Thus, superoxide anions disappear through their interactions with cellular components if CuZn-SOD fails to catalyze the rapid disproportionation of superoxide to hydrogen peroxide and dioxygen. Furthermore, interaction of superoxide with guaiacol peroxidase results in the formation of Compound III, which is an inactive form in the catalytic cycle of peroxi-

dase (Metodiewa et al. 1992). Thus, CuZn-SOD is essential to lignification since it supplies hydrogen peroxide and protects the peroxidase from inactivation by superoxide.

The present work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (no. 04273101) from the Ministry of Education, Science and Culture of Japan, and by a grant from the Human Frontier Science Program. K.O. was the recipient of a fellowship from the Japan Society for the Promotion of Science for Japanese Junior Scientists.

References

Asada, K. (1992) Ascorbate peroxidase—a hydrogen peroxide-scavenging enzyme in plants. *Physiol. Plant.* 85: 235–241.

Asada, K. and Takahashi, M. (1987) Production and scavenging of active oxygen in photosynthesis. *In* Photoinhibition. Edited by Kyle, D.J., Osmond, C.B. and Arntzen, C.J. pp. 227–287. Elsevier, Amsterdam.

Asada, K., Takahashi, M. and Nagate, M. (1975) Assay and inhibitors of spinach superoxide dismutase. *Agric. Biol. Chem.* 38: 471–473.

Auh, C.-K. and Murphy, T.M. (1995) Plasma membrane redox enzyme is involved in the synthesis of O_2^- and H_2O_2 by *Phytophthora* elicitor-stimulated rose cells. *Plant Physiol.* 107: 1241–1247.

Brett, C. and Waldron, K. (1996) The cell wall and intracellular transport. *In* Physiology and Biochemistry of Plant Cell Walls. pp. 114–136. Unvin Hyman, London.

Dharmawardhana, D.P., Ellis, B.E. and Carlson, J.E. (1995) A β-glucosidase from lodgepole pine xylem specific for the lignin precursor coniferin. *Plant Physiol.* 107: 331–339.

Doke, N., Miura, Y., Chai, H.-B. and Kawakita, K. (1991) Involvement of active oxygen in induction of plant defense response against infection and injury. *In* Active Oxygen/Oxidative Stress and Plant Metabolism. Edited by Pell, E. and Steffen, K. pp. 84–96. American Society of Plant Physiologists.

Elstner, E.F. (1991) Metabolisms of oxygen activation in different compartments of plant cells. *In* Active Oxygen/Oxidative Stress and Plant Metabolism. Edited by Pell, E. and Steffen, K. pp. 13–25. American Society of Plant Physiologists.

Elstner, E.F. and Heuple, A. (1976) Formation of hydrogen peroxide by isolated cell walls from horseradish (*Armoracia lapathifolia* Gilib.). *Planta* 130: 175–180.

Feuillet, G., Lauvergeat, V., Deswarte, C., Pilate, G., Boudet, A. and Grima-Pettenati, J. (1995). Tissue- and cell-specific expression of a cinnamyl alcohol dehydrogenase promoter in transgenic poplar plants. *Plant Mol. Biol.* 27: 651–667.

Fukuda, H. (1996) Xylogenesis: initiation, progression, and cell death. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47: 299–325.

Gross, G.G., Janse, C. and Elstner, E.F. (1977) Involvement of malate, monophenols, and superoxide radical in hydrogen peroxide formation by isolated cell walls from horseradish (*Armoracea lapathifolia* Gilib.). *Planta* 136: 271–276.

Halliwell, B. (1978) Lignin synthesis: the generation of hydrogen peroxide and superoxide by horseradish peroxidase and its stimulation by manganese (II) and phenolics. *Planta* 140: 81–88.

Harkin, J.M. and Obst, J.M. (1973) Lignification in trees: indication of exclusive peroxidase participation. *Science* 180: 296–298.

Hamberg, M. and Hamberg, G. (1996) Peroxygenase-catalyzed fatty acid epoxidation in cereal seeds. *Plant Physiol.* 110: 807–815.

Heldt, H.W. (1996) Die Phenylpropanoide umfassen eine Vielfalt pflanzlicher Sekundärmetabolite und Zellwandbestandteile. *In* Pflanzenbiochemie. pp. 421–438. Spectrum Akademischer Verlag, Heidelberg.

Hérouart, D., Montagu, M.V. and Inzé, D. (1994) Developmental and environmental regulation of the *Nicotiana plumbaginifolia* cytosolic Cu/Zn-superoxide dismutase promoter in transgenic tobacco. *Plant Physiol.* 104: 873–880.

Ishimaru, A. and Yamazaki, I. (1977) Hydroperoxide-dependent hydroxylation involving "H_2O_2-reducible hemoprotein" in microsomes of pea seeds. *J. Biol. Chem.* 252: 6118–6124.

Kanematsu, S. and Asada, K. (1990) Characteristic amino acid sequences of chloroplast and cytosol CuZn-superoxide dismutases in spinach, rice and horsetail. *Plant Cell Physiol.* 31: 99–112.

Kuchitsu, K. and Shibuya, N. (1994) *N*-Acetylchitooligosaccharides, specific fungal elicitor for defense responses, induce transient generation of active oxygen species in suspension-cultured rice cells. *In* Frontiers of Reactive Oxygen Species in Biology and Medicine. Edited by Asada, K. and Yoshikawa, T. pp. 255–256. Elsevier, Amsterdam.

Mehdy, M.C. (1994) Active oxygen species in plant defense against pathogens. *Plant Physiol.* 105: 467–462.

Metodiewa, D., de Melo M.P. and Escobar, J.A. (1992) Horseradish peroxidase-catalyzed aerobic oxidation and peroxidation of indole-3-acetic acid. *Arch. Biochem. Biophys.* 296: 27–33.

Murphy, T.M. and Auh, C-K. (1996) The superoxide synthases of plasma membrane preparations from cultured rose cells. *Plant Physiol.* 110: 621–629.

Ogawa, K., Kanematsu, S., Takabe, K. and Asada, K. (1995a) Attachment of CuZn-superoxide dismutase to thylakoid membranes at the site of superoxide generation (PSI) in spinach chloroplasts: detection by immunogold labeling after rapid freezing and substitution method. *Plant Cell Physiol.* 36: 565–573.

Ogawa, K., Kanematsu, S. and Asada, K. (1995b) Spinach chloroplastic and cytosolic CuZn-SODs are localized at the site of superoxide generation. *In* Photosynthesis: from Light to Biosphere. Edited by Mathis, P. Vol. IV. pp. 339–342. Kluwer Acad. Publishers, Dordrecht.

Ogawa, K., Kanematsu, S. and Asada, K. (1996) Intra- and extra-cellular localization of "cytosolic" CuZn-superoxide dismutase in spinach leaf and hypocotyl. *Plant Cell Physiol.* 37: 790–799.

Ogawa, K., Nakashima, J., Takabe, K., Okada, K. and Asada, K. (1997) Generation of superoxide is accompanied with biosynthesis of lignin in tracheary element differentiation of cultured *Zinnia elegans* cells. *Plant Cell Physiol.* 38: s65.

Olson, P.D. and Varner, J.E. (1993) Hydrogen peroxide and lignification. *Plant J.* 4: 887–892.

Post-Beittenmiller, D. (1996) Biochemistry and molecular biology of wax production in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47: 405–430.

Robertson, D., Davies, D.R., Gerrish, C., Jupe, S.C. and Bolwell, G.P. (1995) Rapid changes in oxidative metabolism as a consequence of elicitor treatment of suspension-cultured cells of French bean (*Phaseolus vulgaris* L.). *Plant Mol. Biol.* 27: 59–67.

Schopfer, P. (1994) Histochemical demonstration and localization of H_2O_2 in organs of higher plants by tissue printing on nitrocellulose paper. *Plant Physiol.* 104: 1269–1275.

Schopfer, P. (1996) Hydrogen peroxide-mediated cell-wall stiffening in vitro in maize coleoptiles. *Planta* 199: 43–49.

(Received January 16, 1997; Accepted July 11, 1997)