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Intra- and Extra-Cellular Localization of "Cytosolic" CuZn-Superoxide Dismutase in Spinach Leaf and Hypocotyl

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Immunogold-electron microscopic analysis of spinach leaves done with the antibody specific for "cytosolic" CuZn-superoxide dismutase (SOD) indicates that SOD is localized in the apoplast, in the nucleus and in, or near, the tonoplast. The association of CuZn-SOD with the nucleus indicates it has a role in preventing fatal mutation caused by reactive species of oxygen. The localizing site of CuZn-SOD in the apoplastic region of spinach leaf tissues corresponds to that of the accumulation of lignin. In spinach hypocotyl "cytosolic" CuZn-SOD is localized in vascular tissues where lignification and the generation of superoxide respectively were shown by the phloroglucin-HCl reaction and formation of formazane from nitroblue tetrazolium. Because hydrogen peroxide is required for lignification via the peroxidase-catalyzed reaction, the CuZn-SOD in the apoplast appears to function in the biosynthesis of lignin by causing rapid disproportionation of the superoxide anion radical prior to its interaction with cellular components and peroxidase.

Key words: Apoplast — Immuno-gold labeling — Lignin synthesis — Rapid freezing — Spinach (*Spinacia oleracea*) — Superoxide dismutase (EC 1.15.1.1).

Superoxide anion radical is generated unavoidably in cells via the univalent reduction of dioxygen, and hydrogen peroxide is produced through its disproportionation. The superoxide anion radical and the hydrogen peroxide produce the hydroxyl radical via the transition metal ion-catalyzed Haber-Weiss reaction, and the hydroxyl radical is highly reactive with cellular components. Superoxide anions, therefore, should be immediately scavenged at the site of generation to suppress formation of hydroxyl radical. The superoxide-scavenging enzyme superoxide dismutase (SOD) has been shown to be localized in such organelles as the chloroplasts and mitochondria where superoxide radicals are generated. Further, we have shown the attachment of CuZn-SOD to the stromal faces of the thylakoids of chloroplasts where the photogeneration site of superoxide (PSI) is located (Ogawa et al. 1995a, b).

"Cytosolic" and chloroplastic isozymes of CuZn-SOD have been found in plants (Kanematsu and Asada 1990, Bowler et al. 1994). The chloroplastic CuZn-SOD is exclusively located in chloroplasts, and its amino acid sequence differs from the sequences of "cytosolic" CuZn-SODs (Kanematsu and Asada 1990). Chloroplastic CuZn-SOD is characterized by a transit sequence for targeting chloroplasts, whereas no "cytosolic" CuZn-SOD has a transit sequence (Bowler et al. 1994). Although several isoforms of "cytosolic" CuZn-SOD have been found in various plant species, only one isoform of the chloroplastic enzyme has been detected. Each isoform of "cytosolic" CuZn-SOD is thought to be localized in different compartments of plant cells. In addition to cytoplasm- and chloroplast-localized enzymes, CuZn-SOD activity has been found within the intermembrane spaces of mitochondria (Jackson et al. 1978), but no cDNA for CuZn-SOD with a targeting sequence to mitochondria has yet been isolated. CuZn-SOD also has been found in the glyoxysomes of watermelon (Sandalio and del Río 1987, Bueno and del Río 1992, Bueno et al. 1995). Four isoforms of extracellular CuZn-SOD have been reported in Scotch pine, but the NH_2-terminal amino acid sequences and isoelectric points of these extracellular CuZn-SODs are different markedly from those of symplastic CuZn-SODs (Streller and Wingsle 1994).

To find the in situ localization site(s) of "cytosolic" CuZn-SOD in spinach leaf cells, we made immunohistochemical and immuno-electron microscopic studies using the antibody against "cytosolic" CuZn-SOD after the rapid freezing and substitution that was done as described by Ogawa et al. (1995a). We found that "cytosolic" CuZn-SOD is localized in the cytosol near the vacuole, the nucleus and the apoplastic region. Subsequently, locations of CuZn-SOD, lignin and superoxide generation in spinach hypocotyl were simultaneously determined, and we proposed that CuZn-SOD in the apoplast functions in lignification. Part of this work has been presented in a preliminary form (Ogawa et al. 1995b).

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

Materials and Methods

Antibodies against chloroplastic and "cytosolic" CuZn-SODs

—CuZn-SOD I ("cytosolic") and CuZn-SOD II (chloroplastic) were purified from spinach leaves, and the respective antibodies against them were raised in rabbits, as described by Kanematsu and Asada (1990). The isolated CuZn-SODs I and II used for antigens showed a high purity, which allows to determine the amino terminal sequences of over 50 amino acid residues (Kanematsu and Asada 1990). After precipitation of the respective antisera with ammonium sulfate at 50% saturation, the IgG fraction was purified by passage through a column of DEAE Affi-Gel Blue. The purified antibody against chloroplastic CuZn-SOD is specific for chloroplastic CuZn-SOD, and the antibody against "cytosolic" CuZn-SOD is specific for "cytosolic" CuZn-SOD; it does not cross-react with chloroplastic CuZn-SOD. The antibody against "cytosolic" CuZn-SOD, however, cross-reacted with two isoforms of "cytosolic" CuZn-SOD and does not distinguish CuZn-SOD I from other forms of "cytosolic" CuZn-SOD from spinach (Kanematsu and Asada 1990). Western blotting analyses of the crude extracts from spinach leaves showed that each antibody specifically cross-reacted with the respective CuZn-SODs corresponding to those of activity staining of SOD and no other cross-reacted bands with the antibodies was found (data not shown).

Plant materials—Spinach seeds were germinated in moistened vermiculite in a growth chamber (23°C; 12 h of 1,500 lux; humidity, 60%), and the primary leaf and hypocotyl of 13-d-old seedlings were used. The seedlings of 20-d-old spinach were transplanted in the field, and leaves and hypocotyl of two-month-old plants also were used in the experiments.

Immunogold labeling of CuZn-SOD—Rapid freezing and substitution of spinach leaf and hypocotyl tissues for the immunogold labeling were done by the procedure reported previously (Ogawa et al. 1995a), except that the substitution solution was replaced with 0.35% glutaraldehyde and 0.5% tannic acid in acetone. Then the tissues embedded in LR White resin (London Resin Co. Ltd., U.K.) were cut into thin sections, and then the immunogold labeling was done as described by Ogawa et al. (1995a). The diameter of the gold particles conjugated with the secondary antibody was 15 nm. Samples were observed in a Hitachi transmission electron microscope (H-700).

Extracellular washing fluid for the spinach leaves—Mature spinach leaves with petioles (100 g FW) were washed with water three times for 5 min each, then dipped in the washing solution (water or 500 mM NaCl in 50 mM sodium acetate, pH 5.5), then subjected to infiltration at 2,660 Pa. The infiltrated solution, which represented the washing fluid of the apoplastic region, was then collected by centrifugation of the leaves at $500 \times g$ for 2 min, and designated the extracellular washing fluid. This fluid was concentrated in an Amicon ultracentrifuge (Amicon Inc., Beverly, U.S.A.).

Immunoblot assay of CuZn-SOD in the extracellular washing fluid—Mature spinach leaves were homogenized with 50 mM potassium phosphate, pH 7.6, containing 0.6 mM EDTA, after which the homogenate was centrifuged at $10,000 \times g$ for 15 min. The supernatant was used in the immunoblotting analysis of CuZn-SOD in whole leaves. The supernatant and the extracellular washing fluid were dot-blotted on polyvinylidene difluoride membranes (Bio-Rad, Tokyo, Japan), after which the membranes were immunostained with the antibodies against "cytosolic" and chloroplastic CuZn-SODs using Western blotting. The contents of the "cytosolic" and chloroplastic CuZn-SODs in the extracellular washing fluid relative to those in the whole leaves were determined.

Assay for SOD—One unit of SOD activity was defined as the amount of enzyme required to inhibit the reduction of Cyt *c* by 50% with superoxide anion radicals generated by the xanthine-xanthine oxidase system as described by Kanematsu and Asada (1990). A reaction volume of 1 ml was used in our assay, as compared with the 3 ml used in the original method of McCord and Fridovich. The observed enzymatic activity, therefore, was divided by three for the unit defined by McCord and Fridovich (1969). CuZn-SOD activity was estimated as the amount of activity inhibited by 1 mM KCN.

Immunohistochemical labeling of CuZn-SOD—Spinach hypocotyls were fixed overnight with 4% paraformaldehyde in PBS at 4°C, after which the sections were rinsed six times with PBS for 15 min. Cross sections (100–300 μm thick) of hypocotyl cut 1 mm below the top were prepared manually with a razor blade and quenched with 2% glycine (w/v) for 1 h. The sections then were immunostained on a slide glass coated with 1% gelatin. The immunostaining procedure was the same as immunogold labeling, except that the diameter of the gold particles conjugated with secondary antibody was 1 nm and the images of the particles were enhanced by the use of a silver enhancement kit (Amersham Japan, Tokyo). The immunolabeled sections were observed under a microscope (X-Tr and PM-20, Olympus Tokyo, Japan).

Detection of lignin in the hypocotyl—Cross sections (100–300 μm thick) of hypocotyl were cut 1 mm below the top with a design knife. The sections were mounted on the slide glasses and incubated with 1% phloroglucin (w/v) in ethanol. Just before the ethanol vaporized 3.2 M HCl was added, and the location of lignin was seen as red staining under the microscope.

Detection of superoxide in hypocotyl—The site of the production of superoxide in the tissue sections was located, on the basis of the superoxide-dependent formation of formazane from NBT in the presence of an inhibitor of CuZn-SOD. Cross sections (100–300 μm thick) of hypocotyl cut 1 mm from the top were prepared as described above. The sections were incubated in a mixture of 0.25 mM NBT, 2 mM sodium *N,N*-diethyldithiocarbamate in 50 mM potassium phosphate, pH 7.8, for 20 min, then observed under a microscope. The production of O_2^- could be pinpointed by the formation of formazane under the conditions in which CuZn-SOD is inhibited by *N,N*-diethyldithiocarbamate (Asada et al. 1975)

Results and Discussion

Localization of "cytosolic" CuZn-SOD within mesophyll cells of spinach leaves—Three isozymes of CuZn-SOD have been isolated from spinach. One, found in Percoll-purified intact chloroplasts (Kanematsu and Asada 1990), is designated the chloroplastic isozyme (CuZn-SOD II). Actually the chloroplastic CuZn-SOD is exclusively localized in chloroplasts as determined by immunogold-electron microscopic analyses (Ogawa et al. 1995a). The other two isozymes are considered "cytosolic" isozymes (CuZn-SODs I and III) because they could not be found in intact chloroplasts but were major isoforms in nonphotosynthetic tissues (Kanematsu and Asada 1990). The in situ distributions of "cytosolic" CuZn-SOD in spinach mesophyll cells from two-month-old seedlings were studied by the immuno-electron microscopic method after rapid freezing and substitution as described by Ogawa et al. (1995a). Immunogold particles were found nowhere if the preimmune serum was used, as described by Ogawa et al. (1995a). No immu-

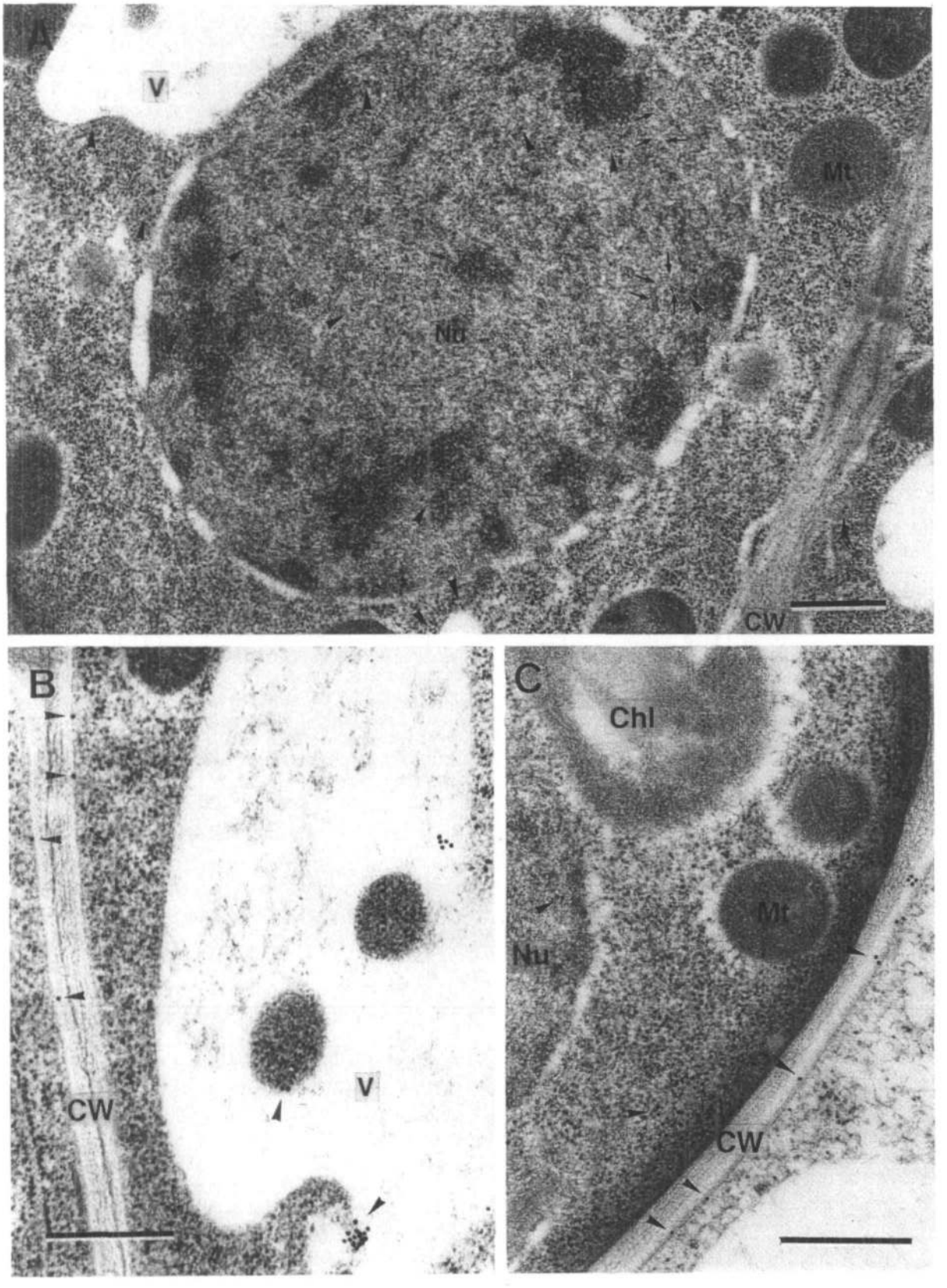


Table 1 Distribution of immunogold particles specific for "cytosolic" CuZn-SOD in the cellular compartments of spinach mesophyll cells (%)

Cytosol	Nucleus	Mitochondrion	Chloroplast	Vacuole	Golgi	Apoplast
3.8	24.4	0.2	2.1	23.3 (18.6)[a]	1.5	44.2

Values are percentages of a total of 581 immunogold particles for various mesophyll cell compartments from 15 electron micrographs.
[a] This value is the percentage of immunogold particles on the "tonoplast". "Vacuole" and "tonoplast" include the 15-nm layer of the cytosol on the surface of the tonoplast.

nogold particles that label "cytosolic" CuZn-SOD could be found on the chloroplasts or the mitochondria of the mesophyll cells. The absence of "cytosolic" CuZn-SOD in the chloroplasts was expected because of its absence in isolated chloroplasts (Kanematsu and Asada 1990) and because chloroplasts are immunogold stained only by the antibody against the chloroplastic isozyme (Ogawa et al. 1995a, b). Immunogold particles of "cytosolic" CuZn-SOD were found on the nuclei (Fig. 1A), on or near the tonoplast (Fig. 1B) and on the apoplast (Fig. 1C). The distribution of the immunogold particles labeling "cytosolic" CuZn-SOD in mesophyll cells of spinach was determined (Table 1). More than 40% of the immunogold particles of "cytosolic" CuZn-SOD were present on the apoplast and about 25% each in the nuclei and vacuole, including the 15-nm layer on the cytosolic faces of the tonoplast.

Distribution of CuZn-SOD in the nuclei—The location of CuZn-SOD in the nuclei (Fig. 1A) indicates possible production of superoxide anions in the nuclei. This is the first indication of the localization of SOD in plant nuclei. CuZn-SOD would be transported to the nuclei through the nucleic pores because it is large enough to pass through. In fact, proteins of small molecular mass have been shown to be translocated through the nucleic pore (Peters 1986). In mammalian cells, NAD(P)H-dependent production of O_2^- in the nucleus has been shown (Bartoli et al. 1977, Peskin and Shlyahova 1986, Kukielka et al. 1989, Puntarulo and Cederbaum 1992), and localization of CuZn-SOD in the nuclei of human cells (Crapo et al. 1992) and rat hepatocytes (Chang et al. 1988) has been reported.

Immunogold particles specific for "cytosolic" CuZn-SOD appear to be attached to the DNA observed as filaments (Fig. 1A, arrows). Counting of the immunogold particles in the nuclei (142) from 6 micrographs indicates the association of 80.3% of the particles with the DNA filaments. The CuZn-SOD in spinach nuclei therefore may protect the DNA against oxidative damage by this association. In *Escherichia coli* cells Mn-SOD is associated with DNA (Steinman et al. 1994), and it seems to have a defensive role in protecting the DNA against modifications by active species of oxygen.

Distribution of CuZn-SOD in the vacuole—Nearly 80% of the immunogold labels on the vacuole are near or on the tonoplast (within 15-nm from the membranes, Fig. 1B). This distribution of "cytosolic" CuZn-SOD near or on the tonoplast is a strong indication that superoxide anions are generated near the tonoplast, but to our knowledge there is little information about this.

Distribution of CuZn-SOD in the apoplast—Immunogold particles specific for "cytosolic" CuZn-SOD were distributed on the apoplast of cells in the spongy tissues. The particles mainly were near or on the plasma membranes (Fig. 1B, C). The interspace between the plasma membrane and the cell wall, which is expected to develop into the secondary thickenings, contained CuZn-SOD (Fig. 2A, B). Immunogold particles also are present on a vesicle-like structure surrounded by plasma membranes (arrowheads, Fig. 2C), which would represent cross section of secondary thickenings of cell wall. Secondary thickenings that are rich in lignin are often seen in vessels. When the immunogold-electron microscopic observations of differentiating treachery elements that are the to-be vessels were made, immunogold particles were found on the secondary thickenings of the cell wall (Fig. 3A), and the labeling density was higher when compared with that on the apoplast of the mesophyll. Notably, immunogold particles were present not only near or on the plasma membranes but deep within the secondary thickenings. No immunogold particle, however, was found on the secondary thickenings when the preimmune serum was used (Fig. 3B). The precursors of lignin

Fig. 1 Immunogold labeling of "cytosolic" CuZn-SOD in a spinach mesophyll cell from two-month-old seedlings. Substitution and fixation were done with 0.35% glutaraldehyde and 0.5% tannic acid in acetone. The immuno-gold labeling specific for "cytosolic" CuZn-SOD was performed as described in Materials and Methods. Scale bars, 500 nm. Chl, chloroplast; CW, cell wall; Nu, nucleus; Mt, mitochondrion; V, vacuole. No gold particles were detected on the cell when the preimmune serum was used. Arrowheads show gold particles indicative of "cytosolic" CuZn-SOD. Arrows in nucleus (A) show the attachment of "cytosolic" CuZn-SOD to the DNA. Gold particles are present on the nucleus (A) and on the cytosol near the vacuoles (A and B). Gold particles also are present on the cell wall (A, B and C).

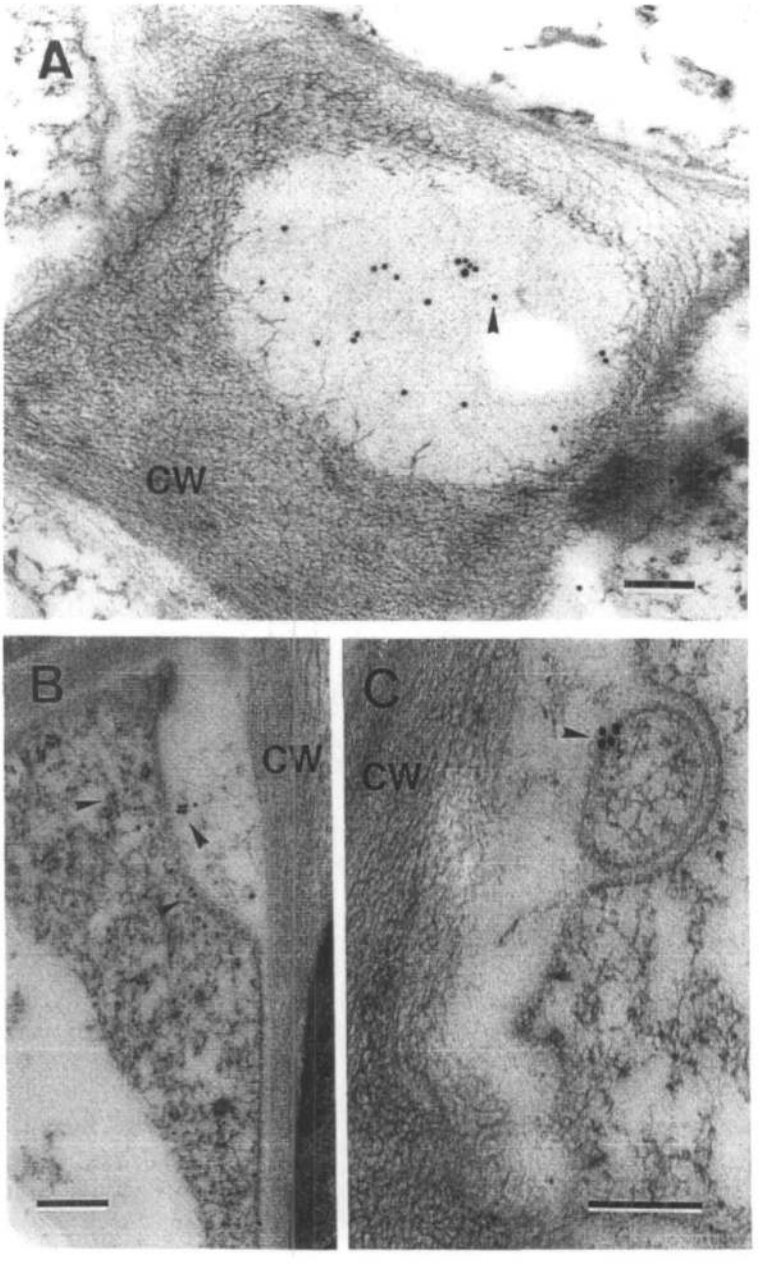


Fig. 2 Distributions of immunogold labels specific for "cytosolic" CuZn-SOD in a spinach mesophyll cell from spongy tissue of two-month-old seedlings. Substitution and fixation were done with 0.35% glutaraldehyde and 0.5% tannic acid in acetone. Scale bars, 200 nm. CW, cell wall. Arrowheads show gold particles specific for "cytosolic" CuZn-SOD. The intraspace contains the immunolabels specific for "cytosolic" CuZn-SOD (A). From another angle of the cell corner, immunogold particles are present on the cytoplasm and in the interspace between the plasma membrane and cell wall (B). Some immunolabels are present on the vesicle-like structure in the intraspace (C), which is also found in Fig. 1A.

and the enzymes involved in lignification accumulate in the secondary thickenings of the cell wall which contain a large amount of lignin (Smith et al. 1994). The localization of the CuZn-SOD in the secondary thickenings therefore seems to indicate its association with lignification.

CuZn-SOD in the extracellular washing fluid—To confirm the localization of CuZn-SOD in the apoplastic compartments, we collected the extracellular washing fluids of mature spinach leaf tissue (two-month old plants) and separately determined the "cytosolic" and chloroplastic CuZn-SOD contents using the antibodies specific for each isozyme (Table 2). The extracellular washing fluid obtained with the high salt buffer contained nearly 10% of the total "cytosolic" CuZn-SOD of the leaf tissue, whereas the con-

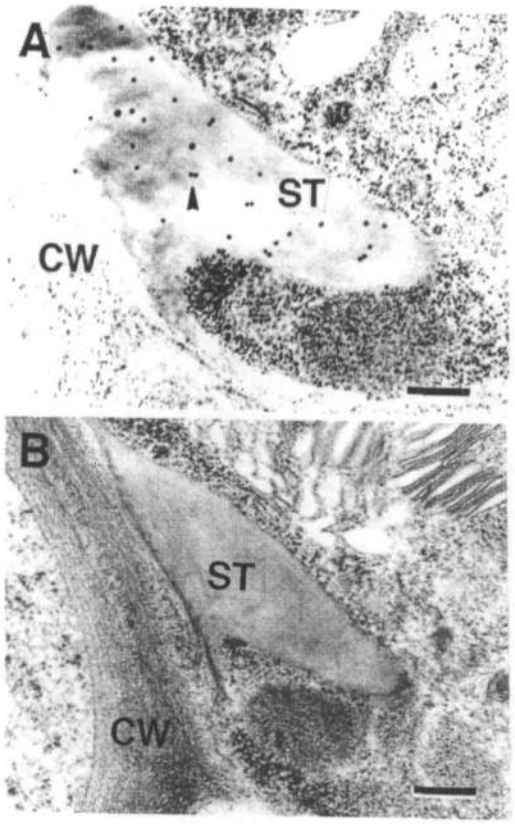


Fig. 3 Immunogold labeling of "cytosolic" CuZn-SOD in the secondary thickening of cell wall of a spinach vascular cell from two-month-old seedlings. Substitution and fixation were done with 0.35% glutaraldehyde and 0.5% tannic acid in acetone. Immunogold labeling was performed as described in Materials and Methods. Scale bars, 200 nm. CW, cell wall; ST, secondary thickening of the cell wall. A, immunostained with the antibody against "cytosolic" CuZn-SOD; B, immunostained with the preimmune serum. Immunogold particles specific for "cytosolic" CuZn-SOD are present on the secondary thickening of cell wall.

tent for water was less than 1%. In contrast, the chloroplastic CuZn-SOD content of the extracellular washing fluid was very low as compared with that of "cytosolic" CuZn-SOD, and was not affected by the washing medium. Further, when the extracellular washing fluid was applied to native PAGE, the coomassie brilliant blue staining image showed that the components of protein in the extracellular washing fluid were quite different from those in the whole leaf extracts (data not shown). Thus, there was little disruption of the cell membranes, which would cause leakage of symplastic SOD, during the collection of the extracellular washing fluid. These findings confirm that "cytosolic" CuZn-SOD is located in the apoplastic compartments and is bound to the cell wall or the plasma membranes via ionic interactions. The amount extracted, 10%, did not, however, correspond to the findings for the immunogold-electron microscopic observation, more than 40%

Table 2 Ratios of "cytosolic" and chloroplastic CuZu-SODs in the extracellular washing fluid to those in the buffer extract of spinach leaves (%)

Isozyme	Water	500 mM NaCl
Cytosolic CuZu-SOD	0.75	9.5
Chloroplastic CuZn-SOD	0.15	0.062

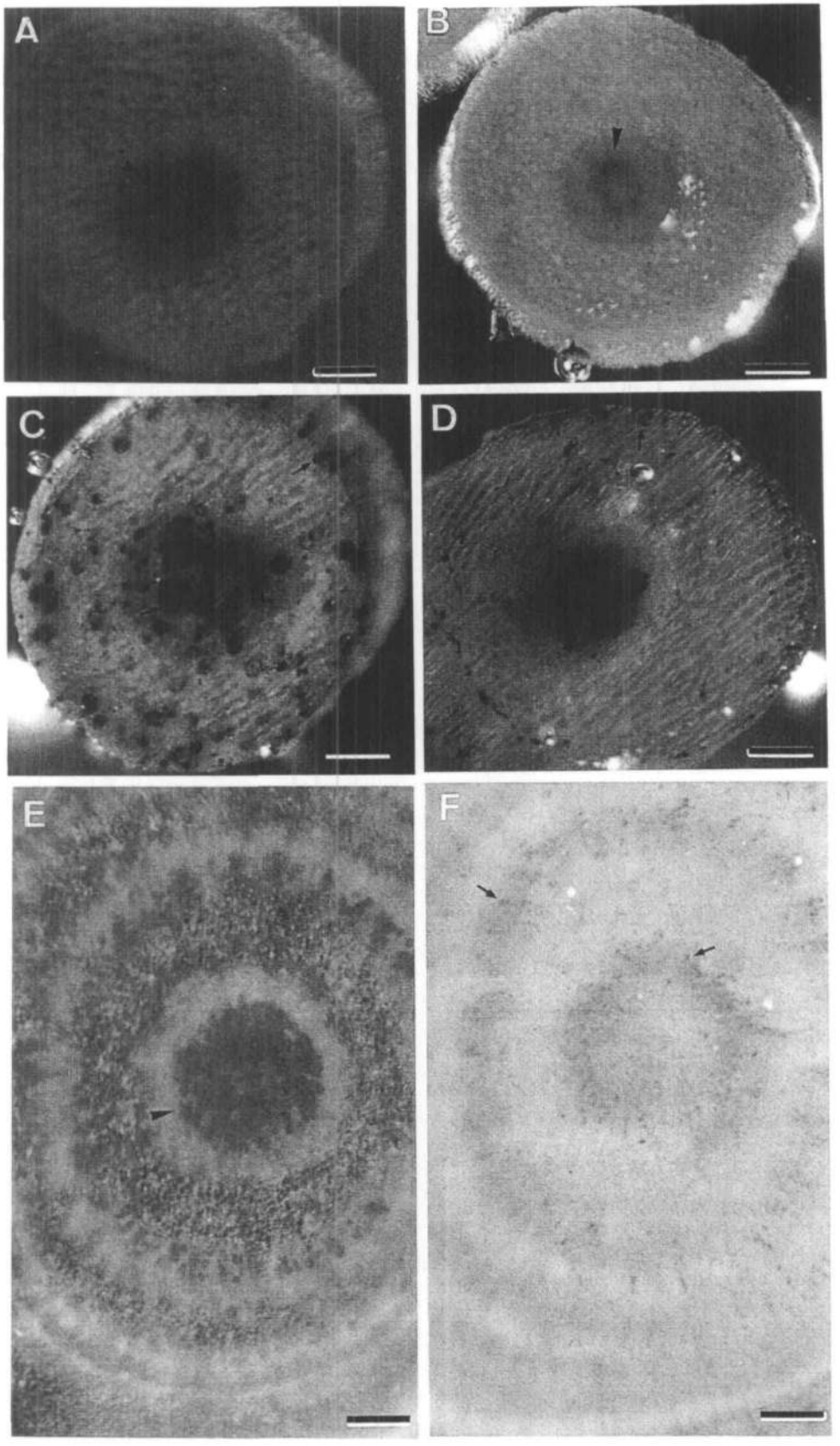
Extracellular washing fluids were obtained with water or 500 mM NaCl in 50 mM sodium acetate, pH 5.5 (500 mM NaCl). The respective total CuZn-SOD activities in the buffer extract, and in the extracellular washing fluids obtained with water and with 500 mM NaCl in 50 mM sodium acetate were 2.31, 0.04, and 0.30 unit g FW^{-1}.

(Table 1), evidence that this "cytosolic" CuZn-SOD was localized also in the apoplastic compartment away from the easily washable sites.

The "cytosolic" CuZn-SOD from spinach has no transit peptide, as deduced from its cDNA (Sakamoto et al. 1990), and the isolated "cytosolic" and chloroplastic CuZn-SODs do not contain carbohydrates. How the CuZn-SOD synthesized in the symplast is transported to the apoplastic compartments through the plasma membranes therefore has yet to be shown, but apoplastic exocytosis is a plausible mechanism.

Distribution of CuZn-SOD in hypocotyl—Immunogold-electron microscopic analyses of spinach hypocotyl using the antibody against "cytosolic" CuZn-SOD showed that the distribution of CuZn-SOD in the hypocotyl is similar to that in the leaves. In particular, immunogold particles were consistently localized in the apoplastic compartment (data not shown). The distribution of "cytosolic" CuZn-SOD at microscopic level in the hypocotyl therefore was investigated to determine the relation among "cytosolic" CuZn-SOD, generation of superoxide and lignification.

The distribution of CuZn-SOD in spinach hypocotyl was determined immunohistochemically with antibody against "cytosolic" CuZn-SOD. To clarify whether CuZn-SOD participates in lignification, we simultaneously determined the sites of lignin accumulation, superoxide generation, and the localization of CuZn-SOD in hypocotyl cross sections cut 1 mm below the top of hypocotyls from 13-d-old spinach seedlings. Intensive immunolabeling of "cytosolic" CuZn-SOD on the vascular tissues were seen as brown to dark brown immunogold-silver staining, but there was little staining on the pith or cortex (Fig. 4A). When the preimmune serum was used, no immunolabeling was observed (data not shown). Cells containing lignin were stained red in vascular tissues (arrowhead, Fig. 4B) by the phloroglucin-HCl reaction. Superoxide generation



mainly was found in the vascular tissues with some in the cortex and epidermis as indicated by the precipitates of formazane (arrows, Fig. 4C) produced via O_2^--dependent reduction of NBT in the presence of an inhibitor of CuZn-SOD, *N,N*-diethyldithiocarbamate. No formazane was formed without treatment of the section with the inhibitor of CuZn-SOD, confirming that the formazane is produced by O_2^-.

When sections were stained by the phloroglucin-HCl reaction for lignin just after treatment with NBT for superoxide generation, formazane precipitates present in the cortex and epidermal cells disappeared (Fig. 4D). In this case, most of the formazane formed in the cortex and epidermis was removed by the ethanol solution of phloroglucin during the second staining. Formazane precipitates probably are deposited on the periphery of the cross section, because of the superoxide generated by the wounding of the cells. The remaining formazane, that formed inside the tissues, was found mainly in the vascular tissues, a little being present in the epidermis. The site of superoxide generation in the vascular tissues (arrow, Fig. 4D) was adjacent to cells containing lignin in their vascular tissues (arrowhead, Fig. 4D) and corresponds to the site of the accumulation of the lignin precursor (Dharmawardhana et al. 1995) and the expression of lignification-related enzymes (Feuillet et al. 1995). The site of superoxide generation adjacent to the lignin-containing cells (arrow, Fig. 4D) appeared to be associated with that of the most intensive immunolabeling of "cytosolic" CuZn-SOD (arrowhead, Fig. 4A).

Superoxide generation and the immunogold labels of CuZn-SOD were weak in the hypocotyl cross sections from the same seedlings cut 1 mm above the base end, although lignin was intensively stained (data not shown). This indicates that the basal part of hypocotyl is older than the top, and reflects the stage of development of each part of the hypocotyl. These observations suggest that superoxide generation and the expression of CuZn-SOD are regulated in accordance with the stage of cell differentiation because they were not present in completely lignified tissues that represent the last stage of cell differentiation.

To determine how lignification and superoxide generation in the hypocotyl are affected by plant development, we tested the top parts of the hypocotyls of two-month-old plants, and compared the findings with those for 13-d-old plants (Fig. 4A–D). The region of the lignin-containing tissues in hypocotyls of two-month-old seedlings (Fig. 4E) was expanded in comparison with that of the 13-d-old seedlings (Fig. 4B). The tissues of the hypocotyls of two-month-old seedlings that corresponded to the tissues within the endodermis of hypocotyls from the 13-d-old seedlings (primary vascular tissue) were lignified, and the secondary vascular tissues, which also contained lignin, appeared circularly concentric. This shows that primary vascular tissue is lignified in mature hypocotyl. In contrast, in mature hypocotyl, superoxide generation was found only outside the lignified primary vascular tissue (primary xylem), not in the primary xylem. The formazane precipitates were associated consistently with the lignifying tissues (arrows, Fig. 4F) located around the primary and secondary xylems. In the mature hypocotyl, however, the intensity of the formazane precipitate was low in comparison to the intensity in the 13-d-old seedlings (Fig. 4C, D), indicative of decreased production of superoxide in the vascular tissues with aging.

Concluding remarks—"Cytosolic" CuZn-SOD from plants has been characterized at the molecular and genomic levels, but little evidence of its cellular localization is available. We have shown its compartmentation in the nuclei, the tonoplast, and the apoplastic regions of spinach leaf tissues. "Cytosolic" CuZn-SOD localized in the spinach symplast seems to protect the nucleus and tonoplast from oxidative damages by reactive species of oxygen. To the contrary, the site of "cytosolic" CuZn-SOD in the apoplast of the leaf and hypocotyl is associated with the sites of the accumulation of lignin and the generation of superoxide anion radicals. CuZn-SOD in the vascular tissues therefore is very probably associated with lignin biosynthesis.

Hydrogen peroxide, which is necessary for the biosynthesis of lignin via the guaiacol peroxidase-catalyzed reaction and for protection against fungal and bacterial infections, is thought to be produced via superoxide anion generated by the univalent reduction of dioxygen catalyzed by NAD(P)H oxidase (Mehdy 1994, Murphy and Auh 1996) or Mn^{2+} and phenol-mediated oxidase activity of guaiacol peroxidase (Elstner and Heuple 1976, Gross et al. 1977, Halliwell 1978).

$$2O_2 + \text{NAD(P)H (or } RH_2) \rightarrow 2O_2^- + \text{NAD(P)}^+ \text{ (or R)} + H^+ \text{ (or } 2H^+) \quad (1)$$

in which RH_2 represents an electron donor for peroxidase. The actual generation of superoxide anion radicals has been reported in isolated plant cell wall (Elstner and Heuple 1976, Gross et al. 1977, Halliwell 1978, Gaspar et al. 1991). Moreover, infection with pathogens or their elicitors induces the transient generation of superoxide radicals in

Fig. 4 Distribution of "cytosolic" CuZn-SOD, lignin and superoxide generation in spinach hypocotyl. Immunostaining of "cytosolic" CuZn-SOD (A, arrowhead) and detections of lignin (B, arrowhead) and superoxide (C, arrows) in the hypocotyls of 13-d-old spinach seedlings were done in cross sections cut 1 mm from the top of the hypocotyl, as described in Materials and Methods. Simultaneous detection of lignin (D, arrowhead) and superoxide (D, arrows) was made when lignin was stained just after incubation of the section with NBT for 30 min under the conditions described in C. Lignin (E, arrowheads) and superoxide (F, arrows) in hypocotyls of two-month-old plants were stained by the method described for B and C. Scale bars, 50 μm.

cultured cells (Doke et al. 1991, Elstner 1991, Kuchitsu and Shibuya 1994, Mehdy 1994). Our findings presented here show that superoxide is generated in vascular tissues, depending on the differentiation stages in sound plants.

The superoxide anion radical generated would be disproportionated spontaneously to hydrogen peroxide and dioxygen at an appreciable rate ($\sim 10^5\ M^{-1}\ s^{-1}$ at pH 7.0) if no reactant for the radical is available in the cell. In the presence of SOD, the radical is catalytically disproportionated at a diffusion-controlled rate ($2 \times 10^9\ M^{-1}\ s^{-1}$ at pH 7.0) (reaction (2)).

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (2).$$

Superoxide anion radical, however, may interact with cellular components (AH and A) unless it is immediately disproportionated by SOD. Possible reactions of the superoxide anion radical generated are

$$O_2^- + AH + H^+ \rightarrow H_2O_2 + A \quad (3)$$

$$O_2^- + A + H^+ \rightarrow O_2 + AH \quad (4).$$

Reaction (3) represents the oxidation of AH, e.g. ascorbate, phenols and thiols, by superoxide anion, and reaction (4) the reduction of A, e.g. Cyt *c* and quinones, by superoxide. In fact, only when CuZn-SOD is inhibited by *N,N*-diethyldithiocarbamate (Asada et al. 1975), the generation of superoxide anion in vascular plant tissue has now been shown here. In the cultured rose cells, no accumulation of hydrogen peroxide has been detected when *N,N*-diethyldithiocarbamate was added (Auh and Murphy 1995), indicating superoxide disappeared mainly via reaction (4) in these cells. If reaction (4) is the major reaction of superoxide in vascular tissues, no hydrogen peroxide is available to the peroxidase-catalyzed lignin formation. Further, if either AH or A is the substrates for the lignin biosynthesis such as coniferyl and sinapyl alcohols or aldehydes, the rapid disproportionation of superoxide by CuZn-SOD would be indispensable to the production of lignin.

In addition, guaiacol peroxidase, which participates in the biosynthesis of lignin, interacts with superoxide anion to form Compound III (reaction (5)) thereby losing activity, because Compound III is not a catalytic intermediate (Metodiewa et al. 1992),

$$O_2^- + \text{Peroxidase} \rightarrow \text{Compound III} \quad (5).$$

The localization of CuZn-SOD in apoplastic regions such as the secondary thickenings of the cell wall and the cell corners is associated with the localization of guaiacol peroxidases (Smith et al. 1994, Zimmerlin et al. 1994).

Our findings suggest that the rapid, catalytic disproportionation of the superoxide is necessary for the biosynthesis of lignin prior to the radical's interacting with cellular compounds and guaiacol peroxidase. We propose that one physiological function of the CuZn-SOD in the apoplastic compartment is the rapid disproportionation of superoxide which facilitates the biosynthesis of lignin by supplying the substrate hydrogen peroxide and suppressing inactivation of the participating enzyme, guaiacol peroxidase.

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