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**Function of CuZn-Superoxide Dismutase in Protection from
and Utilization of Active Species of Oxygen in Plants**

(植物の活性酸素に対する防御と利用におけるCuZn-スーパーオキシドジスムターゼの機能)

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Chapter 1 General Introduction

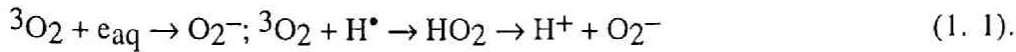
Plants are grown under the stress conditions. In midsummer daytime, plants are inevitably exposed to high intensities of light over $2,000 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$. In addition to it, chilling and heating, nutritional limitation, submergence and fumigation to air pollutants, and UV-irradiation affect their growth and development. These stresses cause generation of active species of oxygen, in which superoxide is primarily generated via the univalent reduction of dioxygen. On the other hand, plants spontaneously produce the active species of oxygen in such a physiological response and developmental metabolism as resistance to pathogen infection, lignification, suberization and IAA decomposition. Plants regulate cellular levels of the active species of oxygen; if the generation is negative to plants, the active species of oxygen are lowered by the scavenging systems and, if the generation of active species of oxygen is required, plants promote it. In the present thesis, I demonstrated mechanisms concerning scavenging and utilizing of the active species of oxygen in plants. In this chapter, I describe an overview of fields related to the thesis and then the purpose of the present thesis. First, I describe the generation and action of active species of oxygen, and their scavenging, especially, in chloroplasts. Secondly, as examples for utilization of active species of oxygen, biosynthesis of lignin and defence mechanism against pathogen infection are described. Finally, superoxide dismutase which is a key enzyme in the present thesis will be mentioned.

1. 1 Generation and action of active species of oxygen and protection from them in chloroplasts

Dioxygen in the atmosphere is ground state oxygen ($^3\text{O}_2$), characterized by its triplet state with two unpaired electrons having parallel spins in two antibonding molecular orbitals, $2P\pi_x^*$ and $2P\pi_y^*$. Therefore, ground state oxygen is a biradical ($\bullet\text{O}_2\bullet$).

Superoxide (O_2^-) is produced by the univalent reduction of triplet dioxygen via (a) spontaneous oxidation of radicals and compounds with a low redox potential, (b) one-electron oxidases such as xanthine oxidase, aldehyde oxidase, dihydroorotate dehydrogenase and NAD(P)H oxidase, (c) superoxide-released oxygenases such as indolamine-2,3-dioxygenase and 2-nitropropane oxygenase, and (d) release of superoxide from oxygen-carrying proteins such as oxyhemoglobin and from oxygenated enzyme intermediates such as peroxidase Compound III.

The production of superoxide through the aerobic irradiation of water by ionizing radiation and UV light are included in (a). In such case, superoxide is produced via the univalent reduction of triplet dioxygen by the reduced species of radiolytic products of water, aquo electron (e_{aq}^-) and hydrogen radical (H^\bullet) (reaction 1. 1)



Hydrogen peroxide (H_2O_2) is produced through either the disproportionation of superoxide (1. 2) or the reduction of O_2^- by ascorbate, thiols, ferredoxin and manganous ions (reaction 1. 3).



Beside the above reactions, hydrogen peroxide is directly produced by the two electron oxidase such as glycolate, glucose, amino acid and sulfite oxidases via the oxidation of respective substrates as follows,



Recombination of hydroxyl radical (HO^\bullet) possibly produces hydrogen peroxide as follows,



Once hydroxyl radical is produced, it rapidly oxidizes cell components because of its high reactivity with cell components and of extremely higher concentrations of the cell components as compared with that of HO^\bullet . Therefore, the production of hydrogen peroxide through reaction (1. 5) is unlikely to occur in chloroplasts.

1. 1. 1 Generation of active species of oxygen in chloroplasts

Overreduction of the electron carriers in the intersystem between PSI and PSII leads to a quite dangerous situation in chloroplasts. Under the conditions where supply of CO_2 to chloroplasts is limited due to unfavorable environmental conditions, the electron acceptor NADP^+ is so deficient that the electron carriers in the intersystem are kept at the reduced state and that the reaction center excited Chl (${}^1\text{Chl}^*$) does not proceed to charge separation to the Chl^+ and the reduced primary electron acceptor. Under such conditions, ${}^1\text{Chl}^*$ decays to its semistable triplet excited state (${}^3\text{Chl}^*$), which rapidly transfers its excitation energy to the triplet ground state of dioxygen (${}^3\text{O}_2$), producing the singlet excited state of dioxygen (${}^1\text{O}_2$);



${}^1\text{O}_2$ is highly reactive with thylakoid lipids, proteins and pigments, and ${}^1\text{O}_2$ inactivates PSII reaction center under some conditions.

Dioxygen can be an alternative electron acceptor at PSI in illuminated chloroplasts. The photoreduction rate of O_2 is 10-20 $\mu\text{mol mg Chl}^{-1} \text{h}^{-1}$ (Asada and Takahashi 1987) even under favorable conditions for chloroplasts to facilitate its maximum rate of CO_2 fixation;



In isolated thylakoid membranes, dioxygen is mainly photoreduced in the aprotic membrane interior region, probably near the FeS center X or center A/B of PSI. The superoxide generated in the thylakoid membrane lives for relatively long times and diffuses to the both sides of the thylakoid membrane. Possibly, electrons are transferred from the reducing side to the oxidizing side of PSI via the superoxide anion radicals, leading to a superoxide-mediated cyclic electron transport. If protons are supplied into the membranes by either low pH medium or the proton gradient formation across the thylakoid membranes, superoxide is disproportionated in the aprotic interior region of the membrane to hydrogen peroxide, causing enhancement of the photoreduction of dioxygen.

1. 1. 2 Target molecules for active species of oxygen and their protection from oxidative stresses in chloroplasts

Under the photostressed conditions, generation of reactive species of oxygen and radicals is suppressed by relaxation mechanisms and, furthermore, almost all of the reactive species of oxygen are scavenged by the scavenging system in the chloroplasts. However, if photooxidative stresses exceed the capacity for protection against them, the components of cells in the leaves are oxidized by the reactive species of oxygen. Visible damages such as chlorolysis appear only after the generation of reactive species of oxygen are increased by the oxidation of target molecules due to photostress, therefore, chlorophyll is not a primary target molecules for the reactive species of oxygen in chloroplasts. The primary target molecules characterized so far are several enzymes of Calvin cycle, D1 protein of PSII and reaction center of PSI. Inactivation of such target molecules lowers the capacities for photo-utilization, promoting the generation of reactive species of oxygen, and the photodamages are amplified and visualized.

1. 1. 2. 1 Reaction center of photosystem II

Under photon-excess, bright light conditions, PSII is primarily inactivated (Aro et al. 1993). In most cases, the electron transfer from the reaction center chlorophyll (P680) to QA and QB via pheophytin (Phe) is suppressed due to overreduction of the acceptors from QA by either strong light or unavailability of the electron acceptors for the linear electron flow. This inhibition of PSII by the suppression of electron transfer from P680 to the acceptor has been referred to as the acceptor-side photoinhibition. Under such conditions, recombination between $^3\text{P680}^*$ and Phe^- occurs (Vass et al. 1992, 1993),



In vitro dioxygen is not necessary for initiation of the inactivation. At the initial stage for which dioxygen is not required, $^3\text{P680}^*$ inactivates the intra-electron transport in the reaction center of PSII, probably through the Type I process in which $^3\text{P680}^*$ directly oxidizes the target molecules (TH₂) via hydrogen abstraction,



At this stage no breakdown of the D1 protein of the reaction center of PSII has been observed. Under aerobic conditions, ${}^3\text{P680}^*$ transfers its excitation energy to ${}^3\text{O}_2$ producing ${}^1\text{O}_2$ (Hidég et al. 1994a, b, Macpherson et al. 1993, Mishra et al. 1994, Miyao 1994),



The singlet oxygen participates in triggering the proteolytic degradation of D1 protein via the oxidation of amino acid residues. ${}^1\text{O}_2$ may oxidize not only D1 protein, but also chlorophyll and other PSII components, which enhances further the photoinhibition of the acceptor side (Mishra et al. 1994).

In addition to acceptor side photoinhibition, the situation occurs that the rate of electron donation from water in the oxidizing side in PSII is slower than that of excitation of the reaction center. This may occur when the water oxidase activity in PSII is lowered by a release of Ca^{2+} from the oxygen-evolving complex into the lumen by ΔpH formation in the thylakoids under photon-excess conditions (Krieger et al. 1992), or by a release of the extrinsic proteins required for the oxidation of water in PSII resulting from exposure to freezing temperatures (Wang et al. 1992). Under such conditions, life times of P680^+ and Tyr Z^+ in PSII are prolonged, and these strong oxidative radicals have a chance to oxidize PSII components. Until this primary step dioxygen is not required, but under aerobic conditions O_2^- (Chen et al. 1992, Ananyev et al. 1994) and HO^\bullet (Hidég et al. 1994b) are photoproduced, which promotes further the photoinactivation. The interaction of superoxide (Chen et al. 1992), the hydroxyurea radicals (Kawamoto et al. 1994) or the azidyl radicals (Kawamoto et al. 1995) with Tyr Z^* modifies the Tyr Z residue and inhibits the electron transport to P680 from the water oxidase complex. In the case of the donor-side photoinhibition also, the proteolytic degradation of D1 protein is triggered by these oxidants (Miyao 1994).

In the case of either acceptor-side or donor-side photoinhibition, D1 protein is the target molecule and is oxidatively damaged by photogenerated oxidants including reactive species of oxygen. Following this oxidative damage, the D1 protein is then degraded by a thylakoid-bound protease. It is not understood a reason why the D1 protein is the target molecule but not the D2 protein, which is another subunit of the reaction center of PSII. However, chloroplasts have a high capacity for the de novo synthesis of D1 protein which is encoded in the chloroplast DNA, and its biosynthesis is regulated by its translation, not by transcription. The de novo synthesized precursor of D1 protein is inserted into the damaged PSII reaction center complex and is processed at the carboxy terminal (Takahashi et al. 1988), and the PSII activity is then recovered. Because of this rapid repair mechanism of the PSII reaction center, the turnover rate of D1 protein is the highest among chloroplast proteins (Aro et al. 1993).

1. 1. 2. 2 Reaction center of photosystem I

In addition to PSII, PSI also is inactivated when isolated thylakoids are illuminated under strong light (Satoh 1970, Inoue et al. 1986, 1989). The photoinhibition is remarkable when the primary electron acceptors of PSI are reduced, as in the case of PSII. Under such conditions the electron flow from A_0 (chlorophyll) and A_1 (phylloquinone) to F_x and F_A/F_B is suppressed, and the probability of recombination of $P700^+$ with either A_0^- or A_1^- increases producing $^3P700^*$. Unlike the situation for 3P680 in PSII, 3O_2 cannot quench $^3P700^*$ in PSI to produce 1O_2 (Sétif et al. 1981; Takahashi and Katoh 1984). However, dioxygen is required for the initiation of photoinhibition in PSI, which is not the case for photoinhibition of PSII except for Bryopsis chloroplasts (Satoh and Fork 1982). Therefore, even if $^3P700^*$ participates in the inactivation of PSI components via the type I process without the mediation of oxygen, it would not be the primary event.

Recently, it has been shown that when cucumber (Terashima et al. 1994, Sonoike and Terashima 1994) and potato (Havaux and Davand 1994) leaves are illuminated at low temperature, PSI is photoinactivated before PSII is. Furthermore, PSI of spinach thylakoids is also photoinactivated under weak light before PSII is (Sonoike 1995). The primary photoinactivation site is assigned to be either F_x , F_A , F_B and A_1 (Sonoike and Terashima 1994, Sonoike et al. 1995), and dioxygen is required, at least for the initiation. Thus, it is very likely that superoxide and hydrogen peroxide, photogenerated by autooxidation of F_x , F_A/F_B , participate in the inactivation of the reducing side of the reaction center of PSI. The participation of reduced oxygens in the photoinhibition of PSI is supported also by the observation that synthesis of SOD, APX and MDA reductase is induced during cold acclimation of poplar twigs (Nakagawara and Sagisaka 1984) and spinach leaves (Schöner and Krause 1990). The induction of catalase also has been shown in the cold-acclimated maize (Prasad et al. 1994).

1. 1. 2. 3 CO_2 -fixation cycle enzymes

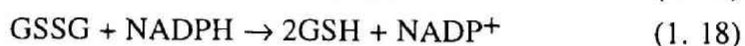
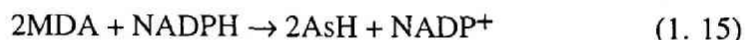
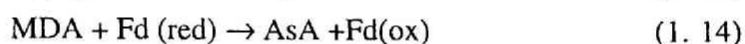
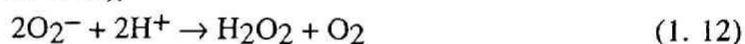
Kaiser (1976) found that CO_2 fixation is inhibited by hydrogen peroxide and subsequently this is turned out to be due to the inhibition of the H_2O_2 -sensitive enzymes, fructose 1,6-bisphosphatase, ribulose 5-phosphate kinase, sedoheptulose 1,7-bisphosphatase (Kaiser 1979) and $NADP^+$ -glyceraldehyde-3-phosphate dehydrogenase (Tanaka et al. 1982). These enzymes have thiol groups participating in the catalytic reactions, and their oxidation to the disulfide form by hydrogen peroxide converts the enzymes to an inactive form. The Fd-thioredoxin system reduces the disulfide-forms of the enzymes to recover their activities.

The operation of the CO_2 -fixation cycle is suppressed if even one of the enzymes participating in the cycle is inactivated, consequently, CO_2 fixation is lost at low

concentrations of hydrogen peroxide with a 50% inhibition at only 10 μM (Kaiser 1976). In illuminated chloroplasts hydrogen peroxide can accumulate to this concentration in 0.5 s if its scavenging system is not functioning. The thiol enzymes are generally sensitive to superoxide and hydroxyl radical, therefore, it is very likely that these enzymes are susceptible to reactive species of oxygens other than hydrogen peroxide. Inactivation of the enzymes of the CO_2 -fixation cycle by singlet oxygen has also been demonstrated (Jung and Kim 1991). In addition to the above enzymes for CO_2 fixation, it has been shown that the glutamine synthetase of the chloroplasts participating in photorespiration is sensitive to the hydroxyl radical generated in the vicinity of the targeting His residue (Fucci et al 1983).

1. 1. 3 Scavenging of active species of oxygen in chloroplasts (see reviews of Asada 1994, 1996, Asada et al. 1998)

Superoxide generated in chloroplasts is disproportionated to dioxygen and hydrogen peroxide by SOD (reaction 1. 12), and the hydrogen peroxide generated is reduced to water by ascorbate peroxidase (APX) producing monodehydroascorbate radical (MDA) (reaction 1. 13),



where AsH is ascorbic acid. MDA is regenerated by either reduced ferredoxin (reaction 1. 14) or MDA reductase (reaction 1. 15) in the stroma. If MDA is completely regenerated, dehydroascorbate (DHA) reductase seems dispensable. In addition to AsH, chloroplasts, however, contain GSH which can be the electron donor of DHA reductase for regeneration of AsH (reaction 1. 17). Actually, DHA reductase-deficient mutant (Yamasaki et al. 1995) is very sensitive to photostress, indicating that relatively small amount of MDA is spontaneously disproportionated to DHA and AsH (1. 16). In spite of high content of GSH in the stroma, little or no activity of GSH peroxidase is found in land plants. Glutathione reductase is present in the stroma, implying also that spontaneous disproportionation of MDA cannot be neglected. The above pathway is schematically shown in Figure 1. 1.

In the present thesis, I revealed microcompartmentation of CuZn-SOD at the site of generation of superoxide in spinach chloroplasts and demonstrated its effects on the photoresistance in Chapter 2.

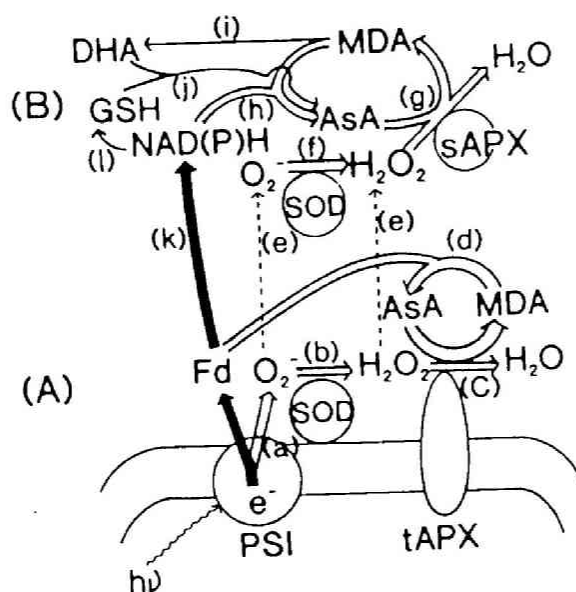


Fig. 1. 1. Thylakoid and stromal scavenging systems of superoxide and hydrogen peroxide in chloroplasts from Asada (1994). (A) Thylakoid scavenging system: (a) Diffusion to the stroma of superoxide radicals generated within the membrane, reduction of hydrogen peroxide by AsA catalyzed with thylakoid-bound or peripheral SOD, (c) reduction of hydrogen peroxide by AsA catalyzed with thylakoid-bound APX (tAPX) localized on the stroma thylakoids, (d) reduction of monodehydroascorbate radical (MDA) by reduced ferredoxin (Fd) in PSI. (B) Stromal scavenging system: (e) Diffusion of superoxide and hydrogen peroxide to the stroma from the thylakoid periphery, (f) disproportionation of superoxide catalyzed by stromal SOD, (h) reduction of MDA by NAD(P)H catalyzed by MDA reductase, (i) spontaneous disproportionation of MDA to dehydroascorbate (DHA) and AsA, (j) reduction of DHA to AsA by GSH catalyzed with DHA reductase, (k) reduction of NADP⁺ by the reduced ferredoxin (Fd) catalyzed with Fd-NADP reductase and production of NADH catalyzed by transhydrogenase, (l) reduction of GSSG by NADPH catalyzed by glutathione reductase.

1. 2 Utilization of active species of oxygen in plants

1. 2. 1 Biosynthesis of Lignin

Dried cells of land plants consist mainly of cellulose, lignin and hemicellulose, which are synthesised during their development. Lignin is a heterogeneous polymer of phenyl propanoid, so called monolignol, via the radical polymerization. Biosynthesis of monolignol has been intensively studied so far (Fig. 1. 2, Campbell and Sederoff 1996) and properties and gene regulation of many key enzymes involved in them have been investigated (Higuchi 1990, Lewis and Yamamoto 1990, Fukuda 1996, Campbell and Sederoff 1996). Precursors of lignin, monolignols, which constitute the main backbone(s) of lignin, differ depending on species of plants; coniferyl alcohol is found in lignin from all species, sinapyl alcohol in that from angiosperms, and *p*-coumaryl alcohol in that from only *Poaceae* (Lewis and Yamamoto 1990).

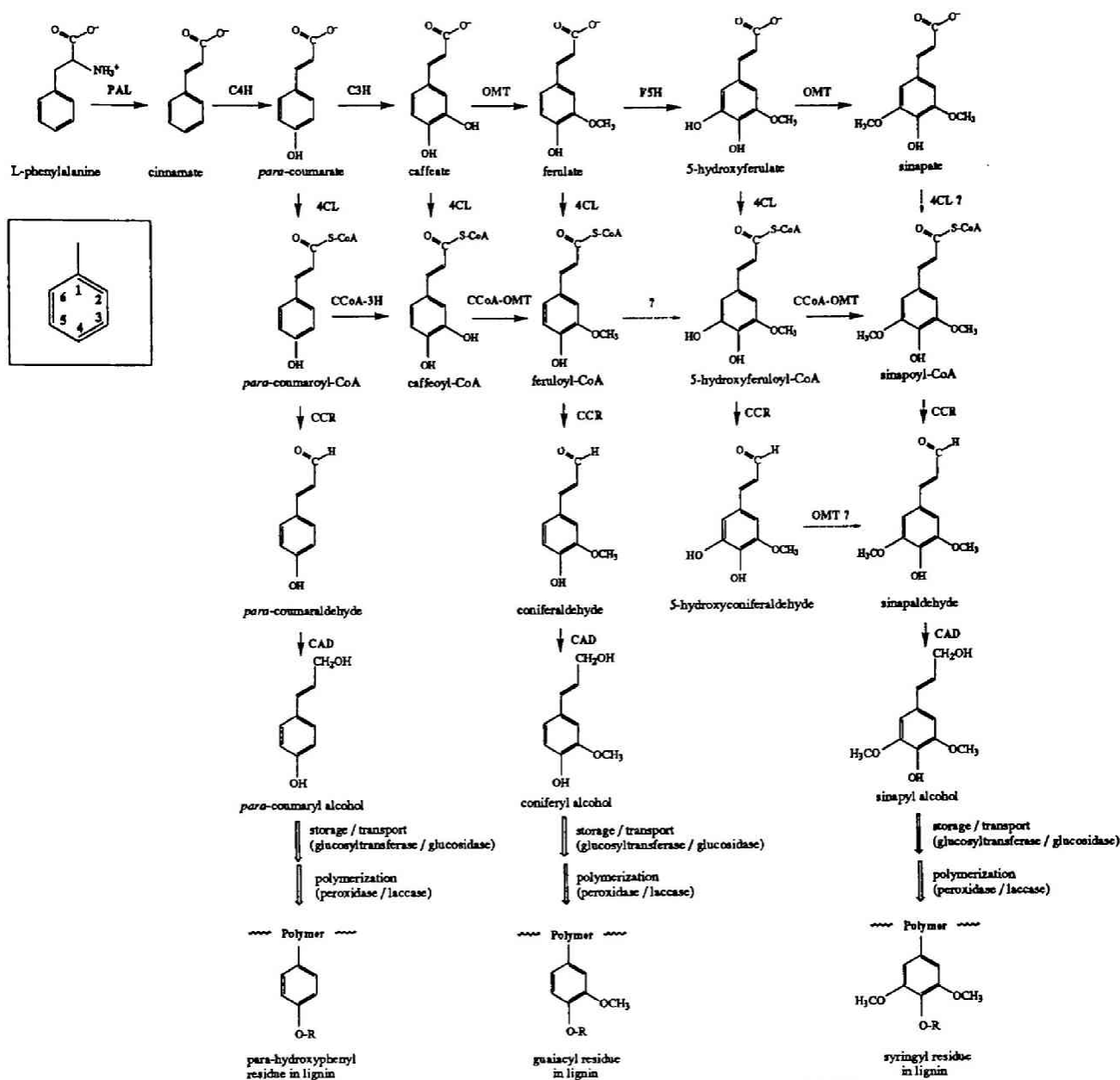


Fig. 1. 2. The lignin biosynthetic pathway from Capbell and Sedroff (1996). This general pathway for lignin biosynthesis has been inferred from studies of specific steps in several diverse species.

Biosynthesis of lignin requires not only monolignols, but also oxidants of the monolignols for their radical formation at the last step of biosynthesis of lignin. Oxidation of them are supposed to be catalyzed by either peroxidases (donor: H_2O_2 oxidoreductase) or laccases (*p*-diphenol: O_2 oxidoreductase). Laccases as well as peroxidases mediate the polymerization of monolignols to produce lignin-like polymer in vitro (Freudenberg 1968). Furthermore, laccase has been purified and characterized (O'Malley et al. 1993), and is revealed to be associated with deposition of lignin in pine (Udagama-Randeniya 1992, Bao et al 1993). However, its specific activity are 100-fold lower than that of peroxidase (0.4 vs $40 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$; Sterjiades et al. 1993)

and is not necessarily distributed in all plants (Lewis and Yamamoto 1990). Thus, it remains suspectful that laccase is universal mediator of the last step of biosynthesis of lignin. On the other hand, although there are many isoforms of peroxidase in all plants, it is difficult to assign the association of one isoform with lignification due to the variation of isoforms and to the wide range of substrates. Nevertheless, acidic peroxidase predominantly contained in tobacco shows a high affinity for coniferyl alcohol (Mäder 1992) and overexpression of this enzyme promotes the accumulation of lignin-like compound in the pith tissue of the stem (Lagrimini 1991). Taking universal distribution of peroxidase in plants into an consideration, it is likely to suggest that lignification is primarily mediated by peroxidase. Deposition of lignin in cell walls are not even, and so investigation of relation between depositon of lignin and the localization and expression of each isozyme of peroxidase would provide more precise information on association of peroxidases with lignification.

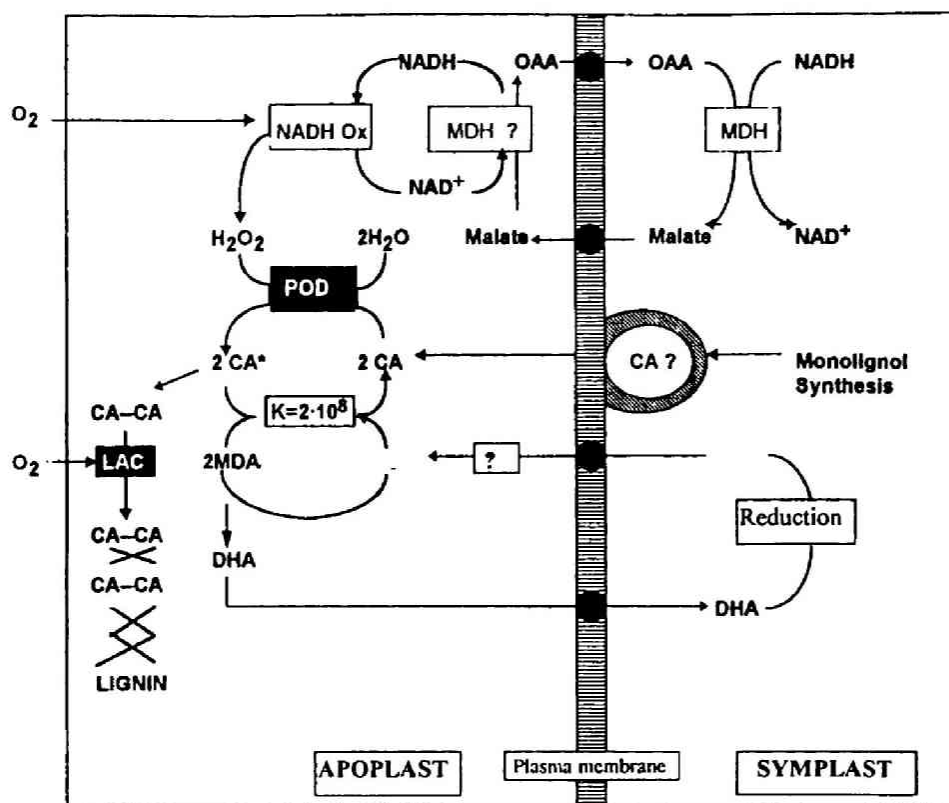


Fig. 1. 3. Hypothetic view of lignification in the cell wall under control of ascorbate from Polle et al. (1997). OAA, oxalacetate; CA, coniferyl alcohol; CA*, phenoxo radical; AsA, ascorbate; MDA, monodehydroascorbate radical; DHA, dehydroascorbate radical; MDH, malate dehydrogenase; POD, peroxidase; NADH ox., NADH oxidase; LAC, laccase; K, apparent reaction constant.

Lignification catalyzed by peroxidases requires hydrogen peroxide. So far the generation of hydrogen peroxide has been studied since the early stage of lignin research (Elstner and Heupel 1976, Gross et al. 1977, Halliwell 1978, Mäder et al. 1980, Mäder and Amberg-Fischer 1982). An idea that NADH oxidase activity of peroxidase themselves supplies hydrogen peroxide from NADH in apoplastic compartment (Fig. 1. 3) has been believed for about two decades. Oxidation of NADH by peroxidase is, however, not enough to account for the amount of deposited lignin (Polle et al. 1997). In Chapter IV-III, I demonstrated that hydrogen peroxide is produced not by peroxidase, but by the putative NADP(H) oxidase. In this respect, CuZn-SOD is turned out to function as both supplier of hydrogen peroxide and protector of peroxidase.

1. 2. 2 Hypersensitive response against pathogen

Plant resistance against pathogens such as fungi, bacteria and viruses depends on whether the plant is able to recognize the pathogen at an early stage in the infection process. When plants recognize it, the rapid tissue necrosis at the site of infection, which is called the hypersensitive response, trigger a large range of inducible defense mechanisms participating in overall resistance in the plant. The mechanisms, which are associated with the hypersensitive response, include synthesis of antimicrobial compounds called phytoalexins and synthesis of hydrolytic enzymes. Many of these responses are induced by transcriptional activation of specific genes.

Several events occurring at the early stage of the hypersensitive response involve primarily activation of preexisting components rather than changes in gene expression. One of these events is the striking release of superoxide and hydrogen peroxide, which is called oxidative burst. Hydrogen peroxide is the disproportionation product of superoxide which is transiently generated by the putative NAD(P)H oxidase. This resisting response is induced by pathogen derived molecules termed elicitor. In model system, plant cell wall-derived molecules also are used as an elicitor. This signal transduction has been well studied and thought to be as described in Figure 1. 4 on the basis of the works so far. Several plant receptors that bind plant or fungal cell wall derived-elicitors are localized on the plasma membrane (Horn et al. 1989). Elicitor receptors are associated with heterotrimeric GTP-binding or G proteins, deduced from the fact that G protein interacting agents promote oxidative burst independently of elicitor (Legendré et al. 1992). Oxidative burst occurs depending on increased intracellular Ca and protein kinase activation (Schwacke and Hager 1992, Baker et al. 1993).

Oxidative burst induced by the above mechanism play three main roles in resistance against pathogens: Reduction of pathogen viability, increase of wall resistance to the action of fungal wall-degrading enzymes such as cross-linking of extensin and lignin deposition, and signal for induction of successive events such as phytoalexin accumulation

Studies of the bacteria-induced hypersensitive response in tobacco cell suspensions indicate that scavengers of superoxide suppressed peroxidation of lipid, but increased infection by bacteria (Keppler and Bader 1989). In addition to its hydrogen peroxide functions as not fungicide but also substrate of biosynthesis of lignin, composition of which is different from that of normal lignin (high content of *p*-coumaryl alcohol unit). Deposition of lignin around the infected area limits spread of pathogen. Exogenous SOD inhibited generation of superoxide in aged potato disks, infected with an hypersensitive response inducing pathogen (Doke 1983) and, in *Arabidopsis*, hypersensitive responses are induced by superoxide and inhibited by SOD, indicating that superoxide is a signal for transduction mechanism (Jabs et al. 1996).

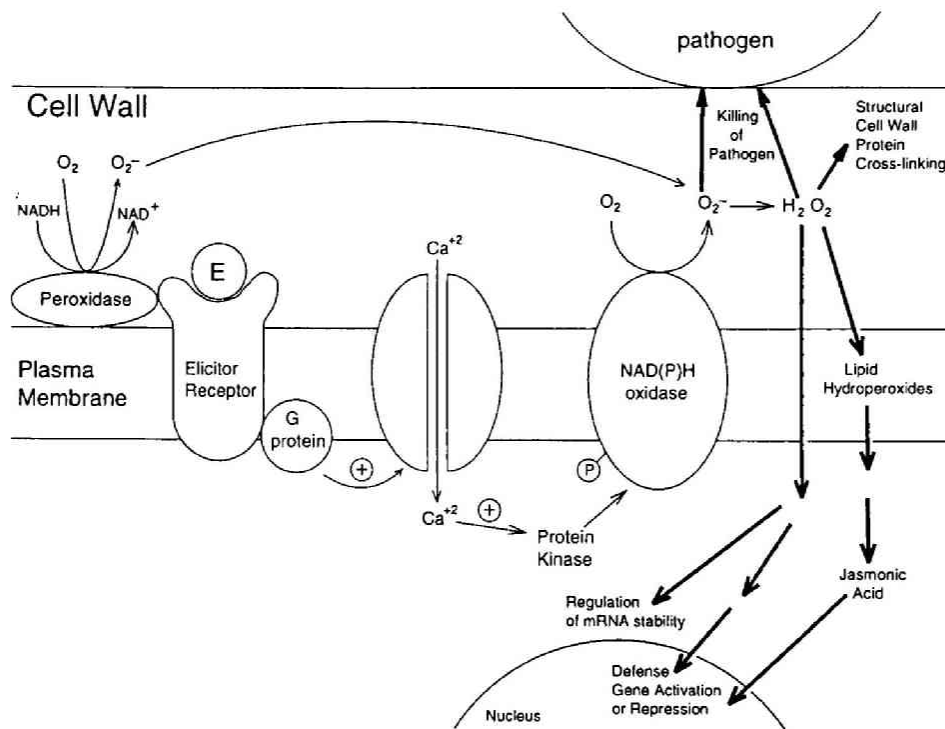


Fig. 1. 4. A speculative model showing possible components involved in generation of active species of oxygen from Mehdy (1994). Assinment of actual components and sequence of components requires additional data. Elicitor receptors may be coupled to production of active species of oxygen via G proteins, increased intracellular Ca due to Ca channel opening, and activation of a protein kinase that activates membrane-bound NAD(P) oxidase by phosphorylation. Alternatively, occupation of elicitor receptors may stimulate a membrane-associated peroxidase by unknown mechanisms, which results in production of superoxide. Superoxide anion radcals spontaneously disproportionated to hydrogen peroxide and dioxygen.

1. 3 Key enzyme: Superoxide dismutase

1. 3. 1 Isoforms of SODs and their molecular properties

SODs catalyzed the disproportionation of O_2^- at a diffusion-controlled rate and its prosthetic metal is either Mn (Mn-SOD), Fe (Fe-SOD) or Cu and Zn (CuZn-SOD). The molecular and enzymatic properties of the three isoforms of SOD are summarized in Table 1. 1. Fe-SOD and Mn-SOD have a high level of amino acid sequence similarity (Parker and Blake 1988), which is true for their three-dimensional structures as determined by X-ray analysis (Stallings et al. 1985). Fe-SOD and Mn-SOD are either a homo-dimer or -tetramer. Although their own metal cannot be replaced mutually, *E. coli* has a hybrid SOD whose subunits are Fe-SOD and Mn-SOD (Clare et al. 1984).

Amino acid sequences of CuZn-SOD are quite different from those of Fe-SOD and Mn-SOD, and its three-dimensional structure also is different from those of other SODs. Cu is the functional metal for the disproportionation of O_2^- but Zn, which is replaceable with Cu (Valentine et al. 1979, Free and Briggs 1975) and Co (Rotilio et al. 1974), supports the three-dimensional structure of CuZn-SOD. CuZn-SOD is a homo-dimer in which intradistance between the copper atoms in two subunits is 3.4 nm (Kitagawa et al. 1991) and the disproportionation of O_2^- is catalyzed independently by the two subunits. Beside them, extracellular SOD has been found in mammalian cells (Hyalmarsson et al. 1987, Marklund 1982). Metal contents of this SOD is the same as the intracellular CuZn-SOD, but the extracellular CuZn-SOD (EC-SOD) is a homo-tetramer and has a glycosylated domain for attaching to heparin. In extracellular space of Scot pine, CuZn-SOD has been found, but it is not a glycoprotein (Streller and Wingsle 1994).

Velocity of disproportionation of superoxide catalyzed by SOD is as high ($2 \times 10^9 M^{-1} s^{-1}$) as a diffusion-controlled rate. The electron transfer reaction of superoxide with the prosthetic metal ion of SOD is immediately finished when the superoxide anion radical encounters SOD, and so the whole rate of disproportionation of superoxide with SOD is controlled by the encounter rate of superoxide with SOD rather than by the electron transfer. The prosthetic metal ion at the reaction center occupies only 0.1 % of surface area of the SOD molecule and, assuming that reaction of superoxide with SOD was regulated by the encounter, the rate constant of reaction of superoxide with SOD should be $10^7 M^{-1} s^{-1}$. However, this rate constant is lower two orders of magnitude than the actual rate constant of reaction of superoxide with SOD. In respect of CuZn-SOD, the positively charged arginine residue is located near the reaction center and conservative in CuZn-SODs found in all organisms, and is demonstrated to attract electrostatically superoxide to the reaction center Cu ion (Getzoff et al. 1983, 1992).

In vivo rate of SOD-catalyzed disproportionation of O_2^- would be lowered due to high viscosity by high concentrations of protein in cells. In fact, the rate constant of SOD with O_2^- is reduced as the viscosity of reaction medium is increased by addition of

glycerol (Rotilio et al. 1972). Detailed estimation of the rates of the CuZn-SOD-catalyzing and spontaneous disproportionations of superoxide under in vivo conditions is discussed in Chapter 2

Table 1. 1. Isozymes of SOD and their molecular properties.

Property	CuZn-SOD	Mn-SOD	Fe-SOD
Molecular Weight	32,000	42,000, 85,000	42,000, 85,000
Subunit structure (molecular weight)	α_2 (16,000)	α_2 or α_4 (21,000)	α_2 or α_4 (21,000)
Amino acid residue number	151~156	198~211	190~248
Metal contents (atoms/subunit)	1 Cu and 1 Zn	1 Mn	1 Fe
Intradistance between metals located at reaction center	34 Å (Cu-Cu)	18 Å (Mn-Mn)	18 Å (Fe-Fe)
λ_{\max} (nm)	258, 680	280, 470	280, 350
Molar absorbance coefficient ($M^{-1} s^{-1}$) (nm)	200~300 (680)	900 (470)	3000~4000 (350)
Characteristic amino acid composition	Null or low content of Try, Trp	—————	—————
Specific activity (unit mg protein ⁻¹)	3000	3000	3000
Rate constant of reaction of O_2^- with SOD ($M^{-1} cm^{-1}$)	2×10^9	2×10^9	2×10^9
Optimum pH	4.8~9.7	6~8	6~8
K_m (O_2^- , mM)	0.35	0.2	0.08
Molecular activity(molecules/s)	10^6	10^6	10^6
$E_{m,7}$ (V)	0.28	0.26~0.31	0.23~0.27
Inhibitors	Inactivation by H_2O_2 KCN NaN ₃ Diethyldithiocarbamate Phosphate Phenylglyoxal	Noninactivation by H_2O_2 Noninhibition by KCN NaN ₃ CH ₃ Cl Ethanol	Inactivation by H_2O_2 Noninhibition by KCN NaN ₃ NaF Dithizone Toluene 3,4-dithizone

1. 3. 2 Distribution of SODs in organisms

Distribution of Fe-SOD, Mn-SOD and CuZn-SOD (including EC-SOD) in organisms at various stages at evolution is summarized in Fig. 1. 5 (Ogawa and Asada 1995). SOD is widespread in almost all the aerobic organisms with exception of such bacteria as *Pediococcus*, *Leuconostock* and *Lactobacillus*. Such SOD-null bacteria contain Mn in the form of Mn-polyphosphate at higher concentrations than the SOD in bacteria do, and such Mn-chelates catalyze the disproportionation of superoxide instead of SOD (Archibald and Fridovich 1981).

Not only aerobic organisms, but also many anaerobic organisms contain SOD. Even taxonomically ancient fermentative bacteria have Fe-SOD in many cases, and SOD-null anaerobic bacteria are more apt to die by air than SOD-containing anaerobic bacteria are (Gregory et al. 1978). Anaerobic methane-producing bacteria (Kirby et al. 1981), sulfate-reducing bacteria (Hatchikian and Henry 1977) and (photosynthetic) green sulfur bacteria (Kanematsu and Asada 1978a, b) have Fe-SOD at the similar level as aerobic ones do. Facultative aerobic non-sulfur purple bacteria possess Mn-SOD. Thus, SOD had been already acquired by "anaerobic" bacteria prior to the accumulation of dioxygen in the atmosphere on account of the appearance of cyanobacteria. These results indicate that, 3×10^9 years ago, before the supply of dioxygen evolved by cyanobacteria, dioxygen even at quite low concentrations (below 10^{-4} -fold relative to the present concentration) suffered organisms. Acquisition of SOD at such an early stage of evolution of organisms might be contributed to the evolution of organisms resistant to oxidative stresses accompanying with an increase of the concentration of dioxygen in the atmosphere.

In most cases, aerobic bacteria contain Fe-SOD and Mn-SOD. *E. coli* contains only Fe-SOD under anaerobic conditions, whereas the biosynthesis of Mn-SOD is induced only under aerobic conditions (Hassan and Fridovich 1977). This observation corresponds to the fact that anaerobes contain only Fe-SOD. Taking it into an consideration that Mn-SOD has a high levels of sequence simlality with Fe-SOD, Fe-SOD appeared to be the first SOD acquired by the organisms, and Mn-SOD stems from Fe-SOD during the subsequent evolution of organisms.

Land plants (mosses, ferns, gymnosperms and angiosperms) have CuZn-SOD as the major SOD, and Mn-SOD is expressed in chloroplasts, mitochondria and glyoxysomes. In several plants such as *Gingko* (Duke and Salin 1985) and water lilly (Salin and Lyon 1983), Fe-SOD is a major SOD. Tobacco changes the expression of Fe-SOD and CuZn-SOD depending on its age (Bowler et al. 1991), and even spinach has Fe-SOD at a trace level (Kanematsu and Asada 1991). However, no Fe-SOD so far have been found in *Poaceae* plants (Bowler et al. 1994). On the other hand, Metazoa and Eucaryomycota in which CuZn-SOD is major SOD have Mn-SOD but not Fe-SOD. Now, there are interesting questions to be solved; Why expression of Fe-SOD depends

on the species of plants; why Fe-SOD is not expressed in *Poaceae* plants, and why Fe-SOD disappeared in animals.

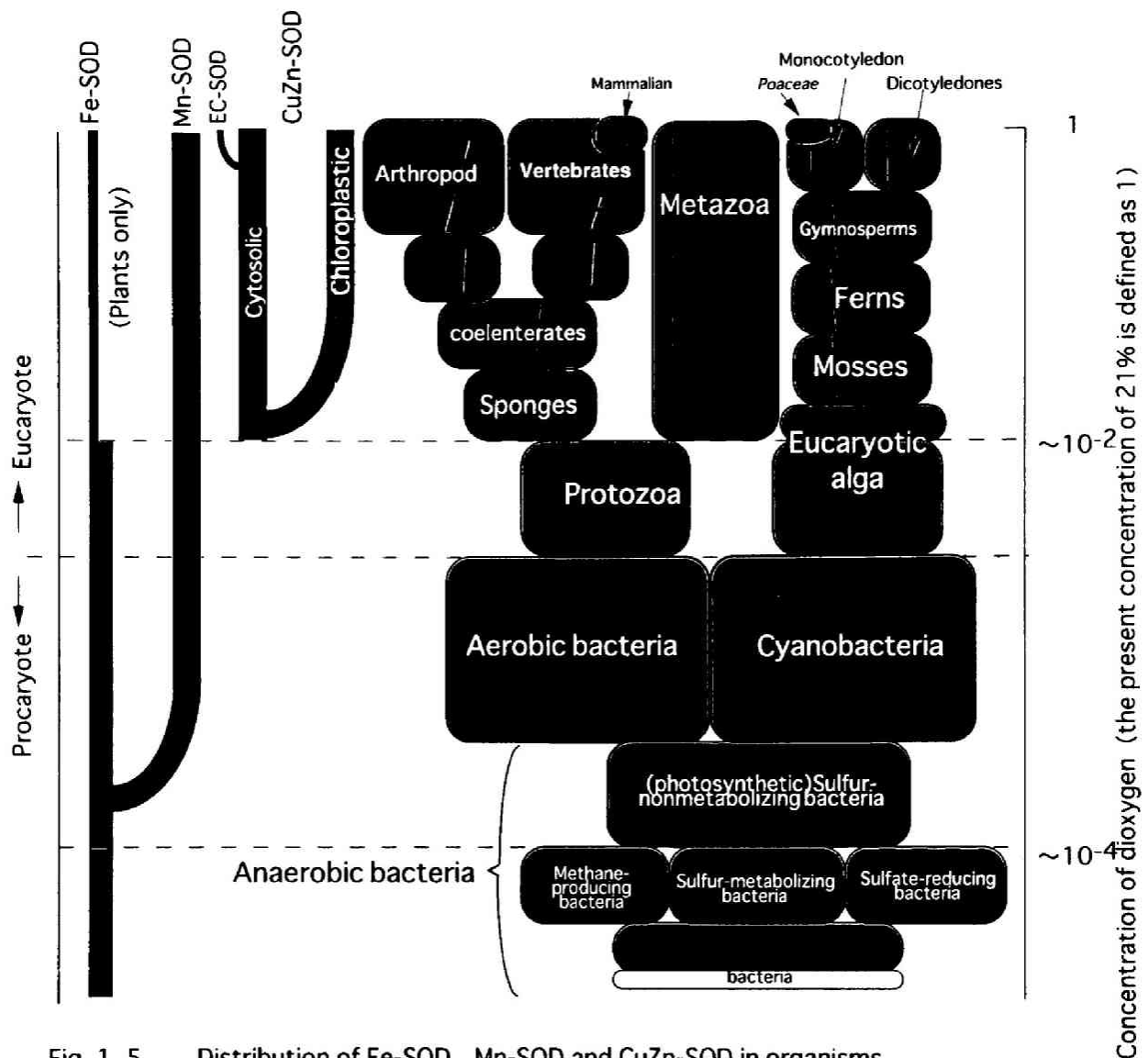


Fig. 1. 5. Distribution of Fe-SOD, Mn-SOD and CuZn-SOD in organisms

Y axis in right represents atmospheric dioxygen concentrations at the period of appearance of each organism (10^{-4} and 10^{-2} of the present dioxygen concentration correspond to 3×10^9 and 6×10^8 years ago, respectively). Colors in the figure indicate respective isoform of SOD. \square , non; \bullet , Fe-SOD; \circ , Mn-SOD; \blacktriangle , cytosolic CuZn-SOD; \blacklozenge , chloroplastic CuZn-SOD; \blacklozenge , EC-SOD. Chloroplastic and cytosolic CuZn-SOD are only in plants and their amino acid sequences are different from each other. In bacteria, hybrid SOD consisting of Fe-SOD and Mn-SOD are contained in bacteria. EC-SOD has been only in mammals. Branches in left indicate molecular evolution of SOD on the basis of similarities of amino acid sequences.

Considering that CuZn-SOD is not found in Protozoa, but it is mainly contained in Metazoa and Eucaryomycota, and, among eucaryotic algae, CuZn-SOD is found only in the most evolved algae, *Chara*, *Nitella* and *Spirogyra* (Kanematsu and Asada 1989), CuZn-SOD is thought to be have acquired prior to the evolution of land plants. The time of acquirement of CuZn-SOD in eucaryotic algae corresponds to that in Metazoa and Eucaryomycota, which is deduced to be 6×10^7 years ago in the Cambrian era. The concentration of dioxygen in the atmosphere at this era had been supposed to raise to the 10^{-2} -fold level of the present. Amino acid sequence of CuZn-SOD is different from those of Fe-SOD and Mn-SOD, then CuZn-SOD seems to have been acquired at this era as a new SOD from different ancestor from those of Fe-SOD and Mn-SOD. Now another question is coming up: Why organisms select three SOD even though little difference has been found in their enzymatic properties. This question is fundamentally significant and its significance is emphasized by the issued topics of genetic disease due to the mutation of SOD. In this respect, my thesis of Chapter 2 will help for solving the question.

1. 3. 3 Localization of SODs in cells and the primary purpose of the present thesis

As described above, in land plants, Fe-SOD is found in chloroplast, Mn-SOD is found in chloroplasts, mitochondria and peroxisomes, and CuZn-SOD has been shown to be located in chloroplasts and other compartments (see detailed introduction in Chapter 3. 1). However, considering short life time of active species of oxygen, these SOD are localized at the site of superoxide generation prior to the interaction of superoxide with the target molecules. In *E. coli*, Mn-SOD binds to genomic DNA whereas Fe-SOD is not (Steinman et al. 1994), though both isoforms have a high level of similarity with each other. No more information about microcompartmentation of SOD in cells were available at the start of this thesis. Thus, I made up mind to investigate microlocalization of isozymes of CuZn-SOD in organellar using immuno-electron microscopy, which was enabled to apply to spinach leaves in this thesis (Chapter 2. 1 and 3. 1). Then, I intended to reveal the physiological role of the investigated CuZn-SODs on the basis of their localization in spinach leaf tissue. As a result, CuZn-SODs play both protective role in environmental stresses (2. 2) and biosynthetic in development of plants (3. 2 and 3. 3).

Chapter 2 Protection from Active Oxygens in Chloroplasts

2. 1 Attachment of CuZn-Superoxide Dismutase to Thylakoid Membranes at the Site of Superoxide Generation (PS I) in Spinach Chloroplasts: Detection by Immuno-Gold Labeling After Rapid Freezing and Substitution Method

Introduction

In chloroplasts, dioxygen is univalently photoreduced to the superoxide anion radical (O_2^-) in PS I (Asada et al. 1974). It seems likely that O_2^- is photogenerated within the thylakoid membranes via the autooxidation of the Fe-S centers X and A/B in the PS I reaction center complex, as indicated by the suppression of the release of superoxide anions from the membrane upon trapping by a hydrophobic catechol derivative but not by hydrophilic one (Takahashi and Asada 1988). Diffusion of the superoxide anions generated within the thylakoid membrane to the surface of the membrane is enhanced when protons are available to the superoxide anion, as the result of an increased rate of spontaneous disproportionation of the anions (Takahashi and Asada 1988; Hormann et al. 1993), and the superoxide anion-mediated cyclic flow of electrons around PS I within the membrane has been proposed (Asada 1994). In this way, a superoxide anion generated within the membrane donates an electron to either Cyt *f* or plastocyanin, and diffusion of the superoxide anion to the surface of membrane is suppressed. The observed rate of photoreduction of dioxygen is about $20 \mu\text{mol mg Chl}^{-1} \text{h}^{-1}$ (Asada and Takahashi 1987) and represents the rate of release of superoxide anions at the surface of the thylakoid membranes.

The superoxide anions released to the stroma of chloroplasts are disproportionated to dioxygen and hydrogen peroxide in a reaction catalyzed by the CuZn-SOD that is present in the stroma in most angiosperms. Chloroplastic and cytosolic isozymes of CuZn-SOD have been found in plants, and the respective isozymes have characteristic amino acid sequences (Kanematsu and Asada 1990). Chloroplastic CuZn-SOD is localized in the stroma in a soluble form (Asada et al. 1973) and is also found in the lumen (Hayakawa et al. 1984). Mn-SOD has been found in a membrane-bound form in spinach thylakoids (Hayakawa et al. 1985) and in cyanobacteria (Okada et al. 1979).

The hydrogen peroxide produced from the superoxide radicals in chloroplasts is reduced to water by ascorbate in a reaction catalyzed by ascorbate-specific peroxidase (Asada 1992). In spinach chloroplasts, ascorbate peroxidase is present in the stroma in a soluble form and in the thylakoid in a membrane-bound form (Miyake and Asada 1992, Miyake et al. 1993). The thylakoid-bound ascorbate peroxidase has been found mainly in the stromal thylakoid where the superoxide-photogenerating site, namely,

the PS I complex, is also located (Miyake and Asada 1992). Furthermore, the thylakoids can photoregenerate ascorbate from the monodehydroascorbate radical, which is the primary product of oxidation in a reaction catalyzed by ascorbate peroxidase, and this photoreduction of the ascorbate radical is mediated by the reduced ferredoxin produced in PS I (Miyake and Asada 1992, 1994). Thus, the thylakoid-bound ascorbate peroxidase and the ferredoxin-dependent photoreduction of the monodehydroascorbate radical function as the primary system for scavenging hydrogen peroxide on thylakoid membranes in the vicinity of the PS I complex. In addition to this thylakoidal scavenging system, the stromal system, consisting of soluble ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase, is also involved in the scavenging of hydrogen peroxide in chloroplasts (Asada and Takahashi 1987, Asada 1994).

In the thylakoidal system for scavenging hydrogen peroxide, the thylakoid-bound ascorbate peroxidase is localized at or near the site of generation of superoxide radicals, namely, the PS I complex. Therefore, the superoxide-disproportionating enzyme CuZn-SOD seems likely to be localized in the vicinity of the PS I reaction center complex to supply hydrogen peroxide to the thylakoid-bound peroxidase. In the present communication, I report that soluble chloroplastic CuZn-SOD is not uniformly distributed in the stroma but is localized mainly on the stromal-face of thylakoid membranes in spinach chloroplasts, as revealed by immuno-gold labeling with an antibody against the chloroplastic CuZn-SOD. The sections for immuno-labeling were prepared by a rapid freezing and substitution method that allows preservation of cell structure (Nishizawa and Mori 1989, Inomata et al. 1992, Nicolas and Bassot 1993) and retains cells in a more authentic state than those subjected to chemical fixation. The observed localization of CuZn-SOD on the stromal-face of thylakoid membranes indicates that SOD scavenges superoxide radicals at the sites where they are generated and that the local concentration of SOD in chloroplasts is 36-fold higher than that previously estimated on the basis of its uniform putatively distribution in the stroma.

Materials and Methods

Plant materials — Spinach was grown hydroponically in a greenhouse or obtained from a local market. Mature leaves were used for the present experiments.

Antibodies against chloroplastic and cytosolic CuZn-SODs — Antibodies against chloroplastic CuZn-SOD II and cytosolic CuZn-SOD I from spinach were raised in rabbits, as described by Kanematsu and Asada (1990). The respective antibodies were purified by passage through a column of DEAE Affi-Gel Blue after precipitation with ammonium sulfate at 50% saturation. The purified antibody against the chloroplastic CuZn-SOD cross-reacted only with the corresponding antigen, but not with the

cytosolic isozymes from spinach. On the other hand, the antibody against the cytosolic CuZn-SOD cross-reacted with two isozymes of the cytosolic CuZn-SOD from spinach but not with the chloroplastic CuZn-SOD (Kanematsu and Asada 1990).

Rapid freezing and substitution — For rapid freezing of samples, the lower epidermis of spinach leaves was peeled off by hand, and then the peeled segments of leaves were immediately plunged into liquid propane or Freon-12 that had been cooled with liquid nitrogen. As a result, the mesophyll cells attached to the lower epidermis froze rapidly without formation of ice crystals within the cells. Such crystals were frequently encountered when the intact sections of leaves were directly frozen by the same procedure. Subsequently, the segments were rapidly transferred to liquid nitrogen and were then substituted with either 0.35% glutaraldehyde and 0.5% tannic acid in acetone or 0.5% tannic acid in acetone by incubation at -80°C for 2 days. The temperature of the substituted samples was gradually raised to room temperature, with the bottles of samples held at -20°C for 2 h, at 4°C for 1 h and then a room temperature for 5 min.

Embedding in LR White resin — After temperature equilibration of room temperature, the sample was rinsed three times with acetone for 15 min each and then similarly with ethanol. The sample was infiltrated with 33% (v/v) LR White resin (The London Resin Co. Ltd., U. K.) in ethanol, with 66% (v/v) resin in ethanol and then with 100% resin for 1 h each, and thereafter with fresh resin overnight. The sample was cured in a gelatin capsule at 50°C for 24 h and then stored with silica gel at 4 °C.

On-grid immunostaining — The cured blocks were cut into ultra-thin sections on an ultramicrotome (Ultra Cut E; Reichert). The ultra-thin sections, mounted on nickel grids, were quenched by 2% glycine in PBS, if they had been fixed with glutaraldehyde. Then the ultra-thin sections on the nickel grids were floated on distilled water for 10 min, and blocked by incubation in a mixture of 0.8% BSA, 0.1% IGSS quality gelatin, 5% goat albumin and 2 mM NaN₃ in PBS for 30 min. Thereafter the sections were rinsed with the washing solution, which consisted of 0.8% BSA, 0.15 IGSS quality gelatin and 2 mM NaN₃ in PBS for 5 min, and then they were allowed to cross-react with the antibodies against CuZn-SOD at an appropriate dilution in an incubation mixture that consisted of 0.8% BSA, 0.1% IGSS quality gelatin, 1% goat albumin and 2 mM NaN₃ in PBS for 1-4 h at room temperature, for 1 h at 37°C or overnight at 4°C. Then, the specimens were rinsed three times with the washing solution for 10 min each, and incubated with the antibody against rabbit IgG that had been conjugated with gold particles (diameter 15 nm; Amersham Japan, Tokyo) at an appropriate dilution in the incubation mixture. After the reaction with the second antibody for 1- 4 h at room temperature, the sections were washed three times with the washing solution for 15 min each and then

similarly with PBS. Thereafter, the samples were postfixed with 2% glutaraldehyde in PBS and washed three times with distilled water for 5 min each.

II. I. II. VI *Observations by transmission electron microscopy* - The sections, mounted on the nickel grids, were observed with a transmission electron microscope at 100 kV (JEM-100C or JEM- 2000ES, JEOL, Tokyo, Japan) after double staining with 2% uranyl acetate and 1% lead citrate.

Results

Distribution of chloroplastic CuZn-SOD in mesophyll cells — Three isozymes of CuZn-SOD have been identified in spinach. One has been found in Percoll-purified intact chloroplasts and is referred to as the chloroplastic isozyme, and the other two are referred to as cytosolic isozymes. The cytosolic isozymes are not found in Percoll-purified intact chloroplasts, but they are present at high levels in nonphotosynthetic tissues (Kanematsu and Asada 1990). The antibodies against the chloroplastic and cytosolic CuZn-SODs cross-reacted only with the respective antigens. Using these isozyme-specific antibodies, I performed an immuno-electron microscopic analysis of CuZn-SOD in mesophyll cells of spinach.

When the mesophyll cells were immuno labeled with the gold particles and the antibody specific for the chloroplastic CuZn-SOD (Fig. 2. 1. 1), most of the gold particles were found on the chloroplasts, with some on the Golgi vesicles. No gold particles were found on the mitochondria or the cytosol. Thus, the chloroplastic CuZn-SOD is specific to the plastids. The occurrence of the chloroplastic CuZn-SOD in the Golgi vesicles suggests its processing in the Golgi for targeting to chloroplasts since CuZn-SOD is encoded in the nuclear DNA and not in chloroplast DNA. However, the occurrence of the chloroplastic CuZn-SOD in the Golgi vesicles and the function of the vesicles in this regard remains to be determined because CuZn-SOD is not a glycoprotein. When the antibody specific for the cytosolic CuZn-SOD were applied to the mesophyll cells, gold particles were not found on chloroplasts and they were localized mainly over or beside cell walls (results not shown). The localization of cytosolic CuZn-SOD in spinach mesophyll cells will be discussed in detail elsewhere. The specific localization of the two isozymes of CuZn-SOD in the mesophyll cells, as determined by immuno-gold labeling, reflects the previously reported cellular distribution of two isozymes of CuZn-SOD, determined by fractionation of cells (Kanematsu and Asada 1990).

Distribution of chloroplastic CuZn-SOD within chloroplasts — Fractionation of intact chloroplasts into thylakoids and stroma by osmotic shock and centrifugation showed that almost all the CuZn-SOD occurs in the stroma in a soluble form, with a small amount of the enzyme being present in the lumen space. Little CuZn-SOD activity has been found in thylakoid membranes after washing with buffer (Hayakawa

et al. 1984). From these results, it has been assumed that chloroplastic CuZn-SOD is localized in the stroma. Immunocytochemical localization of the soluble, chloroplastic isozyme of CuZn-SOD within chloroplasts indicates, however, that its distribution is not uniform in the chloroplasts. Typical immuno-gold labeling, as shown in Figure 2. 1. 2, indicated that the immuno-gold particles specific for the chloroplastic CuZn-SOD were predominantly localized on the thylakoid membranes, and only a few particles were localized in the lumen, as judged from the location of the gold particles on or inside the membranes. A small number of gold particles was observed in the stroma and the envelope. Furthermore, I observed immuno-gold particles on the stromal thylakoids and also on the stroma-facing ends and margins of the grana thylakoids, but there were very few gold particles on the stacked regions of grana thylakoids.

Quantitative analysis of the distribution of the immuno-gold particles that were specific for the chloroplastic CuZn-SOD within chloroplasts was performed using 25 electron micrographs of chloroplasts, with a total of 733 gold particles in all (Table 2. 1. 1). The immuno-gold particles on the thylakoid membranes were counted as "on thylakoid" and those that were not on thylakoid membranes, even if they were quite close to the membranes, were counted as "in stroma". Over 70% of the gold particles were "on thylakoid", and nearly 60% of the gold particles "on thylakoid" were found on the stromal thylakoids, with the remaining ones being on the grana thylakoids. The distribution of gold particles on the grana thylakoids was limited to the stromal-facing membranes, including the ends and margins of the stacked thylakoids, and few gold particles were detected on the stacked regions of the grana thylakoids.

It has been demonstrated that the PS I complex is concentrated on the stromal thylakoids and also on the stromal faces of thylakoid membranes of the grana, while the PS II complex is found in the stacked region of the grana thylakoids (Anderson and Melis 1983, Andreasson and Albertsson 1993). Thus, at least, 70% of the chloroplastic CuZn-SOD was localized in thylakoid regions in which the PS I complex and the thylakoid-bound ascorbate peroxidase (Miyake and Asada 1992) are also localized. The photoreduction of the monodehydroascorbate radical that is produced in the peroxidase-catalyzed reaction is mediated by ferredoxin in the PS I complex (Miyake and Asada 1994). Therefore, the scavenging of superoxide and of the hydrogen peroxide that is produced by the SOD-catalyzed disproportionation of the superoxide seems to occur in the vicinity of the site of photogeneration of superoxide prior to the diffusion of these molecules into the stroma where the active oxygen-sensitive enzymes are localized. The local concentrations of SOD in chloroplasts and the effects of compartmentalization of SOD on the stroma-facing thylakoid membranes on the diffusion of superoxide radicals to the stroma are assessed in Discussion.

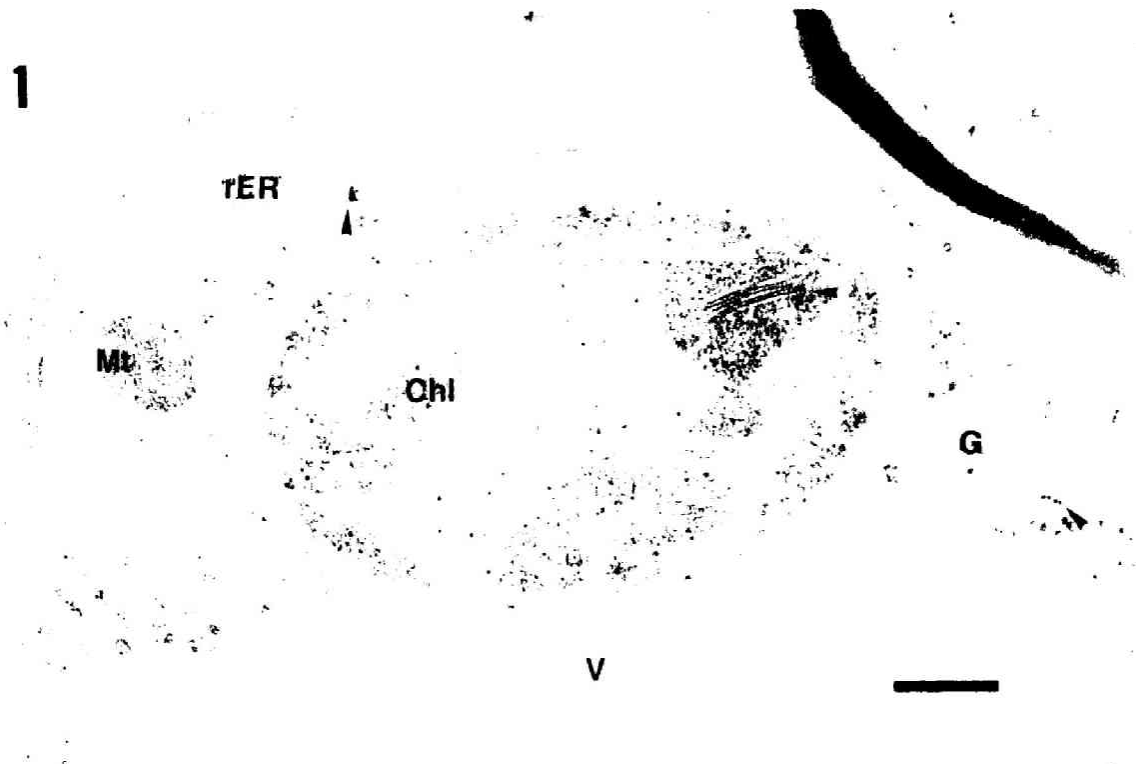


Fig. 2. 1. 1. Immuno-gold labeling of the chloroplastic CuZn-SOD in a spinach mesophyll cell. Substitution and fixation were performed using 0.35% glutaraldehyde and 0.5% tannic acid in acetone and the immuno-gold labeling was performed as described in Materials and Methods. Scale bar, 500 nm. Chl, Chloroplast; G, Golgi apparatus; rER, rough endoplasmic reticulum; Mt, mitochondrion; V, vacuole. No gold particles were found on the cell when control serum was used. Arrowheads represent gold particles indicating the chloroplastic CuZn-SOD in chloroplasts and Golgi vesicles.

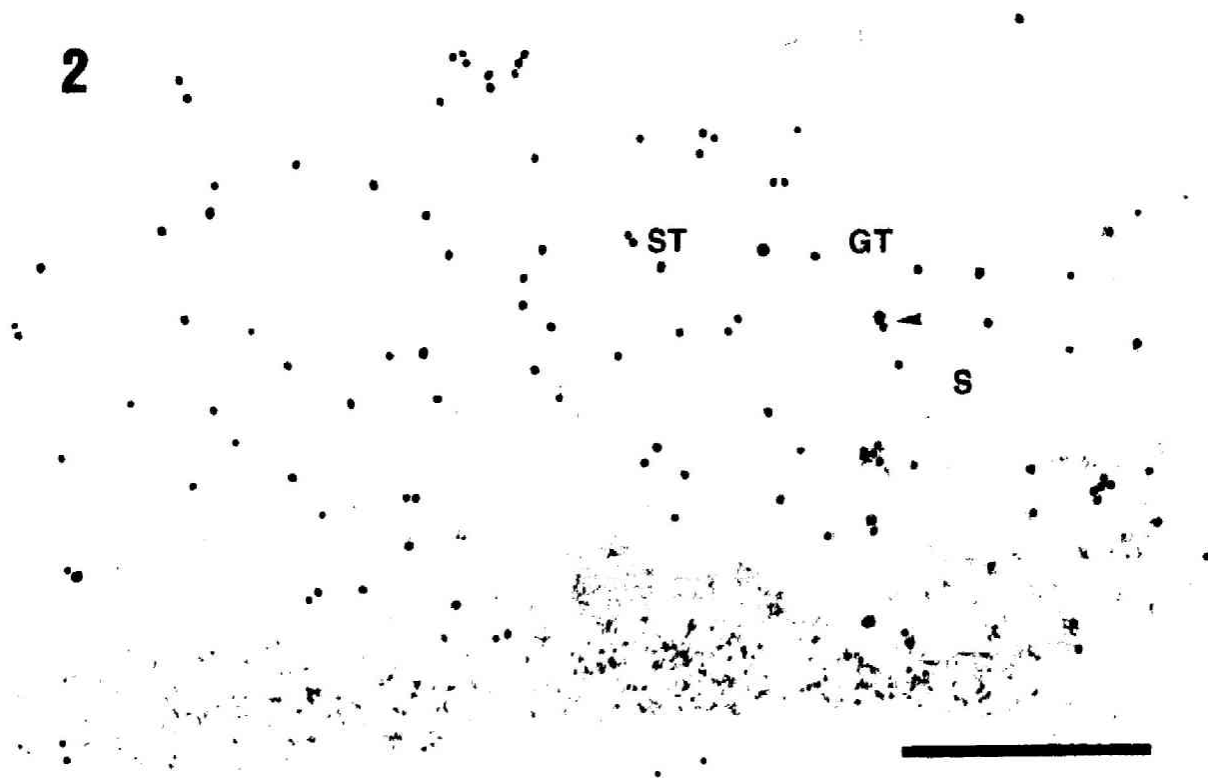
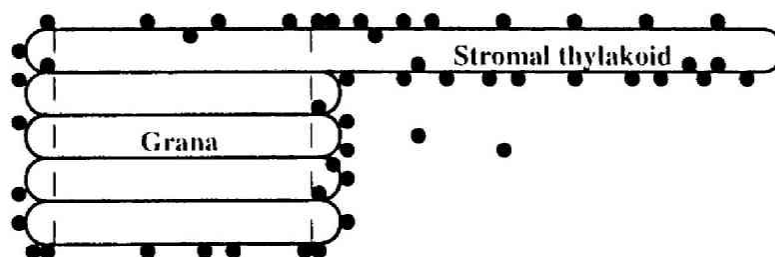


Fig. 2. 1. 2. Immuno-gold labeling of chloroplastic CuZn-SOD in a spinach chloroplast. Gold particles (arrowheads) indicating the chloroplastic CuZn-SOD are located on the thylakoid and the envelope. The section of the chloroplast was substituted and fixed with 0.5% tannic acid in acetone. Scale bar, 500 nm. S, Stroma; ST, stromal thylakoid; GT, grana thylakoid

Table 1. Distribution of the gold particles specific for chloroplastic CuZn-SOD within spinach chloroplasts.

Compartment	Distribution (%)
"In stroma"	27.1
"On thylakoid"	72.9
Total	72.9
{ Stroma thylakoids	41.9
{ Grana thylakoids	31.0
{ Margins exposed to stroma	8.6
{ Ends exposed to stroma	19.6
{ Not exposed to stroma	2.7



Discussion

Local concentrations of CuZn-SOD on thylakoid membranes and in the remaining stroma — The present electron micrographs indicate the thicknesses of the thylakoid membrane and the lumen were 6 nm and 14 nm, respectively (total thickness of thylakoid, 26 nm). The average diameter of the gold particles was 15 nm (Fig. 2. 1. 2).. The relative areas, as percentages, of the stroma, the stromal thylakoids and the grana thylakoids including the lumen were estimated to be 72.0, 5.3 and 22.7%, respectively, from three electron micrographs of a chloroplast. Because these areas are expected to be proportional to the volumes of thylakoid membranes including the lumen, and of the stroma in chloroplasts, the volumes of the stroma, the stromal thylakoids and the grana thylakoids are estimated to be 18.7, 1.4 and 5.9 $\mu\text{l mg Chl}^{-1}$, respectively) if we assumed that the volume of spinach chloroplasts is 26 $\mu\text{l mg Chl}^{-1}$ (Heldt et al. 1973). Furthermore, we can see in the electron micrographs that the average number of thylakoids in a stack of grana thylakoids is ten, of which three protrude as the stromal thylakoids.

The thylakoid is so flat that we can estimate the approximate volume occupied by stroma-facing thylakoid membranes of x nm in thickness (V , liter mg Chl^{-1}) from the following equation:

$$V = \left(\frac{0.053 \times 2x}{26} + \frac{0.227 \times 2x}{26 \times 10} \right) \times 26 \times 10^{-6} = 1.51 \times 10^{-7} x \quad (1).$$

The first term represents the volume occupied by the x -nm layer on the both sides of the stromal thylakoids and the ends of the grana thylakoids, and the second term that on the stroma-facing margin of the grana thylakoids.

When I used immuno-gold particles of 10 nm in diameter, a similar distribution of gold particles specific for the chloroplastic CuZn-SOD was observed on the stroma-facing thylakoid membranes as when I used the 15 nm-gold particles (data not shown). Therefore, the maximum distance of CuZn-SOD from the surface of the thylakoid is 5 nm (radius of the gold particle), since only gold particles over the thylakoid membranes were counted as "on thylakoid". The molecular dimensions of dimeric CuZn-SOD from spinach have been determined to be $3.3 \times 6.2 \times 3.6$ nm (Kitagawa et al. 1991) and are similar to the dimensions occupied by CuZn-SOD that was observed by immunogold labeling on the thylakoid membranes. Therefore, almost all the CuZn-SOD seems to have been attached to the stroma-facing thylakoid membranes, and the volume in which CuZn-SOD is localized (V) was estimated to be 7.55×10^{-7} liter mg Chl^{-1} , if x is 5 nm, from equation (1).

The activities of CuZn-SOD in the stroma and the lumen of spinach chloroplasts have been determined to be 113 and 5.3 units mg Chl^{-1} , respectively. In addition, Mn-SOD has been found in the thylakoids at 2.7 units mg Chl^{-1} in a membrane-bound form (Hayakawa et al. 1984, 1985). From the specific activity (3,100 units mg

protein⁻¹) and molecular weight (32, 000) of CuZn-SOD, the amount of CuZn-SOD in the entire stroma was estimated to be 1.14×10^{-9} mol mg Chl⁻¹. Since 72% of the CuZn-SOD in the stroma was attached to the surface of the thylakoid membranes (Table 2. 1. 1), the local concentration of CuZn-SOD on the stroma-facing thylakoid membranes ([SOD]) is given as follows:

$$[\text{SOD}] = \frac{1.14 \times 10^{-9} \times 0.72}{V} = 1.08 \times 10^{-3} \text{ M} \quad (2).$$

The concentration of CuZn-SOD in the remaining stroma is estimated to be 17 μM from the volume of the stroma apart from the 5-nm layer on the surface of the stroma-facing thylakoids and the relative level of CuZn-SOD in the stroma (Table 2. 1. 1). Thus, as compared with the local concentration of CuZn-SOD on the surface of the stroma-facing thylakoids (1.08 mM), that in the bulk of the stroma is very low. If the chloroplastic CuZn-SOD were uniformly distributed in the stroma, its concentration would be 60 μM .

The molar ratio of chloroplastic CuZn-SOD to the reaction center of PS I (P700) can be estimated to be 0.98 from the level of the SOD in spinach chloroplasts (Hayakawa et al. 1984, 1985)- if P700 is present at a level of one molecule per 430 molecules of Chl (Boardman 1973). Therefore, about 0.7 molecule of CuZn-SOD can be assumed to be attached to the thylakoid membrane in the vicinity of each PS I complex.

Diffusion coefficient of O₂⁻ and the rate constants of its disproportionation in the stroma — The diffusion coefficient of O₂⁻ (D) and the rate constants of its SOD-catalyzed disproportionation (k_{SOD}) and of its spontaneous disproportionation (k_{spo}) are expected to be decreased by an increase in the viscosity of the solution. The viscosity of the stroma should be quite high because of the high concentration of proteins in the stroma (about 40%, w/v) as estimated from the level of soluble chloroplast proteins, 7.7 mg protein mg Chl⁻¹ (Kirk 1978), and the volume of the stroma, 18.7 μl mg Chl⁻¹. I determined the viscosity of 40% bovine serum albumin (w/v) relative to that of water (η_{rel}), using an Ostwald viscometer at 20 °C and found it to be 69. Because the diffusion coefficient is inversely proportional to viscosity, D of O₂⁻ in the stroma should be about $1.4 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ if that in water is $10^{-5} \text{ cm}^2 \text{ s}^{-1}$ at 20 °C.

The second-order rate constants for the spontaneous disproportionation of the superoxide radical (k_{spo}) and for the disproportionation of the superoxide radical catalyzed by CuZn-SOD (k_{SOD}) have been determined to be $5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (Bielski 1978) and $1.8 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ (Rotilio 1972) in water. The rate of the spontaneous disproportionation in the stroma would be decreased to $7 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ because of the decreased diffusion of the superoxide radical. When a superoxide anion radical encounters CuZn-SOD, the positively charged amino acid residues around the Cu atom

in the reaction center play an important role in the ionic guidance of the substrate (Getzoff et al. 1992), which allows the diffusion-controlled reaction between superoxide anions and the catalytic Cu atom of CuZn-SOD. In facts, the k_{SOD} in glycerol at various concentrations is inversely proportional to the root of η_{rel} (Rotilio 1972). Therefore, k_{SOD} in the stroma is expected to be $2.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$. Employing these estimated values, we can estimate the concentration gradient of the superoxide from the site of its photoproduction on the surface of the thylakoid membranes to the stroma.

Effects of the compartmentalization of CuZn-SOD on the concentration gradient of superoxide from the site of its photoproduction and on its half-life — In accordance with Fick's law, the diffusion of O_2^- is given as follows:

$$\frac{\partial [O_2^-]_d}{\partial t} = D \frac{\partial^2 [O_2^-]_d}{\partial d^2} \quad (3)$$

where t , d and $[O_2^-]_d$ represent time, distance from the site of photogeneration of O_2^- and the concentration of O_2^- at distance d , respectively. For the estimation of $[O_2^-]_d$, the contributions of the spontaneous and SOD-catalyzed disproportionation of superoxide anions should be included. Thus, equation (3) is modified as follows:

$$\frac{\partial [O_2^-]_d}{\partial t} = D \frac{\partial^2 [O_2^-]_d}{\partial d^2} - k_{SOD}[SOD][O_2^-]_d - k_{SPO}[O_2^-]_d^2 \quad (4)$$

where $[SOD]$ represents the molar concentration of CuZn-SOD.

Under steady-state conditions, the left side of equation (4) is equal to zero and the third term can be disregarded because it is negligibly small as compared to the second term, if CuZn-SOD is present. Therefore,

$$D \frac{\partial^2 [O_2^-]_d}{\partial d^2} = k_{SOD}[SOD][O_2^-]_d \quad (5).$$

Integration of equation (5) gives equation (6),

$$D \left(\frac{\partial [O_2^-]_d}{\partial d} \right)^2 - D \left(\frac{\partial [O_2^-]_0}{\partial d} \right)^2 = k_{SOD}[SOD] \{ [O_2^-]_d^2 - [O_2^-]_0^2 \} \quad (6).$$

Because equation (6) is an identical equation, variable terms and constant terms on the left and right sides are equal, respectively, and equation (6) can be simplified as follows:

$$\frac{\partial [O_2^-]_d}{\partial d} = -\sqrt{\frac{k_{SOD}[SOD]}{D}} [O_2^-]_d \quad (6').$$

Fick's law gives also equation (7) for the flux of O_2^- ,

$$J_d = \frac{1}{A} \frac{\partial m}{\partial t} = -D \frac{\partial [O_2^-]_d}{\partial d} \quad (7)$$

where J_d represents the flux of the superoxide anion through the plane at a distance of d nm from the surface of the thylakoid membranes, A represents the area of the plane that is equivalent to the stroma-facing surface of the thylakoid membranes, and m

represents the amount of the superoxide. From equations (6') and (7), $[O_2^-]_0$ is given as follows:

$$[O_2^-]_0 = \frac{1}{\sqrt{k_{SOD}D[SOD]}} J_0 \quad (8).$$

The flux of superoxide ejected from the thylakoid membranes, J_0 , is estimated to be $1.3 \times 10^{-12} \text{ mol cm}^{-2} \text{ s}^{-1}$ from the following parameters: the observed rate of photoproduction of superoxide from the thylakoid membranes, which is $2.0 \times 10^{-5} \text{ mol mg Chl}^{-1} \text{ h}^{-1}$ (Asada and Takahashi 1987); the density of the PS I complex on the stroma-facing surface of the thylakoid membranes, which is $3.5 \times 10^3 \mu\text{m}^{-2}$ (Staehelin 1976); molar ratio of P 700 to 100 Chl, which is 0.3 (Boardman 1970, Jacobi 1977); and the average molecular weight of Chl, which is 897.2 (ratio of Chl *a* to Chl *b* = 2.8).

By integration of equation (6'), $[O_2^-]_d$ at the steady state can be calculated from equations (9) and (9').

$$[O_2^-]_d = [O_2^-]_0 \exp\left(-\sqrt{\frac{k_{SOD}[SOD]_1}{D}} d\right) \quad (0 \text{ nm} \leq d \leq 5 \text{ nm}) \quad (9)$$

$$[O_2^-]_d = [O_2^-]_0 \exp\left(-\sqrt{\frac{k_{SOD}}{D}} \{\sqrt{[SOD]_2} d - 5(\sqrt{[SOD]_1} - \sqrt{[SOD]_2})\}\right) \quad (5 \text{ nm} \leq d) \quad (9')$$

where $[SOD]_1$ and $[SOD]_2$ are the local concentrations of CuZn-SOD in the compartmentalized layer of 5 nm in thickness on the stroma-facing thylakoid membranes and in the rest of the stroma, respectively. Thus, equation (9) represents the concentration gradient of superoxide in the compartmentalized region of CuZn-SOD on the stroma-facing thylakoid membranes, and equation (9') represents that in the remaining stroma.

Figure 2. 1. 3 shows the dependence on the distance from the surface of the thylakoid membranes (d) of the steady-state concentration of O_2^- ($[O_2^-]_d$) and of the flux of O_2^- (J_d). By compartmentalization of CuZn-SOD on the membranes (solid lines), as compared with the uniform distribution of CuZn-SOD throughout the stroma (broken lines), the steady-state concentration of O_2^- on the surface of the membrane ($[O_2^-]_0$) decreases to $7.2 \times 10^{-9} \text{ M}$ from $3.0 \times 10^{-8} \text{ M}$. Furthermore, with the attachment of CuZn-SOD to the membrane, O_2^- is very effectively disproportionated prior to diffusion into the stroma, and the steady-state concentration of the O_2^- at a distance of 5 nm from the surface of the membrane is the same as it would be at 55 nm if SOD were uniformly distributed in the stroma. The same is true for the flux of O_2^- : the attachment of SOD reduces the flux within a short distance. Thus, the attachment of CuZn-SOD to the surface of the membrane at 0.7 molecules per molecule of P700 allows scavenging of the O_2^- photoproduced in the PS I complex within 5 nm, prior to any diffusion into the stroma where the active species of oxygen-labile enzymes are localized.

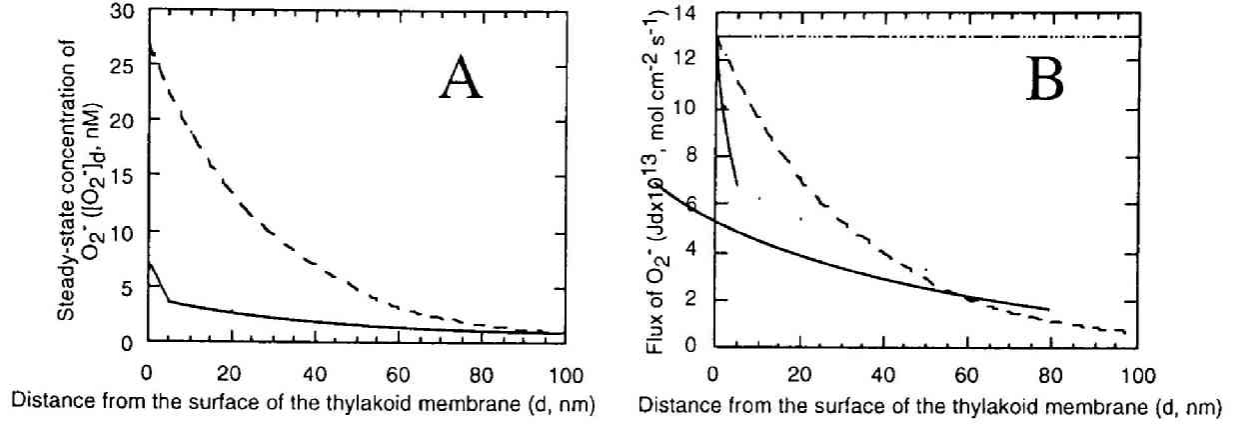


Fig. 2. 1. 3. Gradients in the steady-state concentration ($[O_2^-]_d$) and the flux of O_2^- (J_d) from the surface of thylakoid membranes to the stroma; effects of microcompartmentalization of CuZn-SOD on $[O_2^-]_d$ (panel A) and J_d (panel B). The effects of the compartmentalization of CuZn-SOD on the steady-state concentration ($[O_2^-]_d$) and the flux rate of O_2^- (J_d) were estimated from equations (9) and (9'), and (7) in the text, respectively (solid lines). For this estimation, we employed k_{SOD} ($2.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$) and D ($1.4 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$) in the stroma, $[SOD]_1$ ($1.08 \times 10^{-3} \text{ M}$ in the 5-nm layer on the thylakoid surface), and $[SOD]_2$ ($1.7 \times 10^{-5} \text{ M}$ in the rest of the stroma). The broken lines show $[O_2^-]_d$ and J_d if CuZn-SOD were to be uniformly distributed over the stroma. Estimations were made using equations (7) and (9) and assuming that $[SOD]_1$ is $6.0 \times 10^{-5} \text{ M}$. The dotted line in panel B indicates the flux of O_2^- in the absence of SOD. In the absence of SOD, $[O_2^-]_0$ is $1.4 \times 10^{-5} \text{ M}$, and $[O_2^-]$ at 100 nm from the surface of the membrane is calculated to be $1.4 \times 10^{-5} \text{ M}$, using the following equations,

$$J_d = \sqrt{\frac{2k_{spo} D [O_2^-]_d^3}{3}} \quad \text{and} \quad \frac{1}{\sqrt{[O_2^-]_d}} - \frac{1}{\sqrt{[O_2^-]_0}} = \sqrt{\frac{k_{spo}}{6D}} d.$$

The half-life of O_2^- is estimated from the pseudo first-order constant for the disproportionation of O_2^- which is $2.2 \times 10^5 \text{ s}^{-1}$, if we assume that k_{SOD} in the stroma is $2.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ and $[\text{SOD}]_1$ is 1.08×10^{-3} . The estimated value is $3.2 \times 10^{-6} \text{ s}$, which is shorter than the half-time of the linear electron flow in the thylakoid by four orders of magnitude. These results indicate that the O_2^- that is photoproduced in the PS I complex is immediately disproportionated by the attached CuZn-SOD prior to the subsequent photogeneration of O_2^- , and no accumulation of O_2^- occurs in the vicinity of the PS I complex.

Concluding remarks — The present electron-microscopic observations, made after immunogold labeling specific for the chloroplastic CuZn-SOD, indicate the attachment of the enzyme to the stroma-facing thylakoid membranes at nearly one molecule per PS I complex at about 1 mM. The compartmentalization of CuZn-SOD on the stroma-facing thylakoid suggests the prompt scavenging of the O_2^- that is ejected from the membrane prior to any diffusion of O_2^- into the stroma. The attachment of CuZn-SOD to the thylakoid membranes lowers the steady-state concentration of O_2^- on the surface of the membranes (Fig. 2. 1. 3), and the product of SOD-catalyzed disproportionation, H_2O_2 , is reduced to water immediately in a reaction catalyzed by the thylakoid-bound ascorbate peroxidase, which has been shown also to be located in the vicinity of the PS I complex (Miyake and Asada 1992, Miyake et al. 1993). The thylakoid-bound ascorbate peroxidase is present at about one molecule for each two PS I complexes, and the rate of the reaction catalyzed by the peroxidase is high ($k_1 = 1.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and $k_3 = 2.6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$; Miyake et al. 1993), also indicating rapid scavenging of H_2O_2 prior to its diffusion into the stroma. The monodehydroascorbate radical generated in the peroxidase-catalyzed reaction is photoreduced to ascorbate by photoreduced ferredoxin at a rate of $10^7 \text{ M}^{-1} \text{ s}^{-1}$ in the thylakoids (Miyake and Asada 1994). Thus, the enzymes for scavenging O_2^- and H_2O_2 and the system for regeneration of ascorbate from its radical are all compartmentalized in the vicinity of the O_2^- -generating PS I complex, protecting the active oxygen-sensitive enzymes of the CO_2 -fixation cycle by effectively scavenging O_2^- and H_2O_2 prior to any possible interactions.

Several of the enzymes of the CO_2 -fixation cycle in the stroma, such as fructose-1,6-bisphosphatase, glyceraldehyde-3-phosphate dehydrogenase, ribulose-5-phosphate kinase and sedoheptulose-1,7-bisphosphatase are very sensitive to H_2O_2 (Kaiser 1979). It has been shown that ribulose-1,5-bisphosphate carboxylase, ferredoxin-NADP reductase and the above-listed enzymes, except for fructose-1,6-bisphosphatase, form a multienzyme complex, and the complex is bound to the thylakoid membranes (Süss et al. 1993). The compartmentalization of the CO_2 -fixation enzymes on the thylakoid membranes would be very effective for driving of

the cycle by the photoproduced NADPH and ATP, but the complex confronts the risk of inactivation by the active species of oxygen photogenerated at the same site. The microcompartmentalization of the scavenging enzymes on the surface of thylakoid membranes might be essential to reduce such a risk.

At present it is not known how the soluble CuZn-SOD is bound or attached to the stroma-facing thylakoid membranes. Weak interactions with the membrane and also with the complex of CO₂-fixation enzyme might play a role in the "targeting" of the chloroplastic CuZn-SOD to the vicinity of the PS I complex. In this respect, it is of interest to note that, in humans, a point mutation in CuZn-SOD causes amyotrophic lateral sclerosis (Deng et al. 1993). The mutation is not in the active-site domain and the mutant SOD retains activity. It seems likely that the mutation in domains other than the reaction center disturbs proper "targeting" of soluble CuZn-SOD in human cells.

2. 2 Tobacco Chloroplastic CuZn-superoxide Dismutase Cannot Function Without Its Localization at the Site of Superoxide Generation (PS I)

Introduction

Three isozymes of superoxide dismutase (SOD) have been found in plants: Fe-SOD, Mn-SOD and CuZn-SOD, although they share almost the same enzymatic properties. CuZn-SOD, which is a major SOD in plants, has several isozymes, which can be classified to, at least, two types on the basis of primary structures and cellular location; cytosolic and chloroplastic isozymes (Kanematsu and Asada 1990). In contrast to plants, mammalian have usually only one isoform of CuZn-SOD. Cellular and suborganellar distributions of CuZn-SOD have been studied in detail in spinach leaves (Ogawa et al. 1995b). Distribution of Fe-SOD is restricted to the certain kinds of plant species and is found in chloroplasts, and Mn-SOD are in mitochondria, glyoxysomes and chloroplasts (Bowler et al. 1994). Chloroplastic CuZn-SOD is distributed only in chloroplasts (Ogawa et al. 1995a, b) and glyoxysomes (Bueno et al. 1995), and cytosolic CuZn-SOD has been found in apoplast, tonoplast and nuclei (Ogawa et al. 1996b). Recently apoplastic SOD is demonstrated to be associated with lignification for supply of hydrogen peroxide (Ogawa et al. 1997a, b). Physiological functions of SODs in other subcellular compartments have not been clearly assessed.

I have already shown that spinach chloroplastic CuZn-SOD attaches to the stroma-facing thylakoid membrane to which the PSI complex is localized (Ogawa et al. 1995a, b, Chapter 2. 1). Such association of CuZn-SOD is mediated by magnesium ions (Ogawa et al. 1996a). However, we do not know to what degree the chloroplastic CuZn-SOD actually contributes to the scavenging of reactive species of oxygen in chloroplasts.

In chloroplasts, superoxide is produced via the univalent reduction of oxygen in PSI complex (Asada et al. 1974). The reduction of dioxygen mediated by the flavoenzyme monodehydroascorbate reductase at PSI has been revealed to lead to the relaxation of the intersystem between PSII and PSI rather than the disadvantaged situations (Asada et al. 1998). Under photon-excess environments excess electrons in the intersystem reduce dioxygen to superoxide, and microcompartmented CuZn-SOD and ascorbate peroxidase immediately reduce it to water (Asada 1996). Reduction of dioxygen catalyzed by monodehydroascorbate reductase occurs only when the NADP⁺ is not available. Even in the absence of monodehydroascorbate reductase, superoxide is generated in the aprotic region of the thylakoid membranes (Takahashi and Asada 1988) and its diffusion to either stroma or lumen is accelerated if protons are available on the surface of the thylakoid membranes. Thus, the diffusion of superoxide to the lumen side is enhanced by the formation of proton gradient across the membranes (Takahashi and Asada 1988, Hormann et al. 1993). Besides it,

superoxide-dependent cyclic flows around PSI has been proposed (Asada 1994, 1996). Transgenic tobacco overexpressing chloroplastic Mn-SOD in chloroplasts exhibits phototolerance of PSI and that Fe-SOD PSII (Slooten et al. 1996). However, no significant change is observed in transgenic tobacco overproducing chloroplastic CuZn-SOD in the chloroplasts (Van Camp et al. 1994). To elucidate the effects of attachment of CuZn-SOD to the thylakoid membranes on the protection from photostress, transgenic plants having reduced activity of CuZn-SOD in chloroplasts by means of the antisense method were constructed, and the phototolerance of the tobaccos were investigated among the transgenic plants. To address the function of chloroplastic CuZn-SOD more, I transformed a transgenic plant overexpressing mitochondrial Mn-SOD in the chloroplasts (Bowler et al. 1991) by insertion of CuZn-SOD in the antisense orientation using another selection marker.

I report herein that chloroplastic CuZn-SOD is indispensable even when the Mn-SOD and Fe-SOD are expressed in the tobacco chloroplasts and that SOD cannot function without its localization at the site of superoxide generation.

Materials and Methods

Plant materials and culture conditions — Wild-type tobacco used for the genetic transformations is *Nicotiana tabacum* var Petit Havana SRI. SRI transgenic plant overexpressing mitochondrial Mn-SOD (ov-Mn) were obtained as described by (Van Camp et al. 1995). Briefly, the coding sequence of the mature, mitochondrial Mn-SOD of *Nicotiana plumbaginifolia* was coupled in frame behind the chloroplast transit sequence of the pea Rubisco (for details, see Bowler et al 1991). For overexpression of antisense gene of chloroplastic CuZn-SOD in SRI and ov-Mn, PCR products (primers used were synthesized on the basis of tomato chloroplastic CuZn-SOD gene and additive sequences were Sac I and Hin III sites as shown in Fig. 2. 2. 1, right) as template of cDNA of wild type of the plants were inserted to binary vector, pMAT137 in the antisense orientation as shown in Figure 2. 2. 1 (left). Primary transformants were selected on basal Murashige and Skoog (1962) medium (MS medium) containing 200 mg / liter kanamycin and 200 mg / liter hygromycin, were grown up to the stage which the seeds were obtained. The seeds were germinated for a week on the moisten filter paper containing 200 mg/ liter kanamycin and 200 mg / liter hygromycin for further selection. The germinated seedlings were transferred and grown on MS medium for two weeks at 22 °C under the light intensity of 50 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$. Thereafter, the transgenic plants were transferred and grown in a pot under light intensities of 50, 100 and 1000 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$.

Fluorescence measurements — Chlorophyll fluorescence yield was measured with a Mini-PAM fluorometer (Walz, Effeltrich, Germany). A PAM chlorophyll fluorometer was used for measurement of P700⁺. The accessory modules PAM-102

and PAM-103 were used for actinic light and saturation pulse control, respectively. Far-red illumination was provided by an array of 7 LEDs peaking at 735 nm (Shinko KL571). Saturating single turnover flashes were obtained from a miniature Xe-discharge lamp (XMT-103, Walz) under the control of the PAM-103 Trigger Control Unit.

Measurement of activities of SOD isozymes in chloroplasts — Tobacco leaves (30 g) were homogenated by a Waring blender with 200 ml of the homogenizing medium (10 mM ascorbate, 1 g / liter polyvinylidene, 0.33 M sorbitol, 5 mM MgCl₂, 2 mM EDTA, 10 mM sodium dihydrogen pyrophosphate, with adjustment of pH to 6.4 by NaOH). The homogenate was filtered through four layers of cheesecloth and a layer of nylon mesh, and then were centrifuged at 2,000 × g for 30 s. The sediments were suspended in 4 ml of the suspension medium consisting of 0.33 M sorbitol, 5 mM MgCl₂, 10 mM NaCl, 2 mM EDTA, 0.5 mM sodium pyrophosphate, 50 mM HEPES-KOH, pH 7.8. The suspended chloroplasts were fractionated by centrifugation at 4,000 × g using a Percoll-density gradient (5 ml of 40 % and 3 ml of 70% Percoll (Sigma, St. Luis, USA) in the suspension medium). The chloroplasts layered between 40% and 70% Percoll were collected and washed three times with the suspension medium by centrifugation at 4,000 × g for 1 min. The sedimented chloroplasts were suspended in 4 ml of the suspension medium.

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, Fe-SOD, and Mn-SOD activities were separately determined using KCN and hydrogen peroxide, as the inhibitor of CuZn-SOD and inactivation treatment of CuZn-SOD and Fe-SOD, respectively.

Results and Discussion

Construction of transgenic tobaccos and their SOD contents in chloroplasts — SOD activities in the chloroplasts of transgenic and wild-type tobaccos were determined using the third leaves from the top of two month-old plants (Table 2. 2. 1). Overexpression of antisense gene in ov-Mn (ov-Mn,an-CuZn) and SRI (an-CuZn) suppressed CuZn-SOD activity in chloroplasts but not completely. In the chloroplasts of an-CuZn, Fe-SOD activity was also suppressed in addition to CuZn-SOD activity. Overexpression of mitochondrial Mn-SOD results in suppression of both Fe-SOD and CuZn-SOD activities in chloroplasts. In activity-staining of SOD, where native Mn-SOD and introduced mitochondrial Mn-SOD are recognized (Bowler et al. 1993),

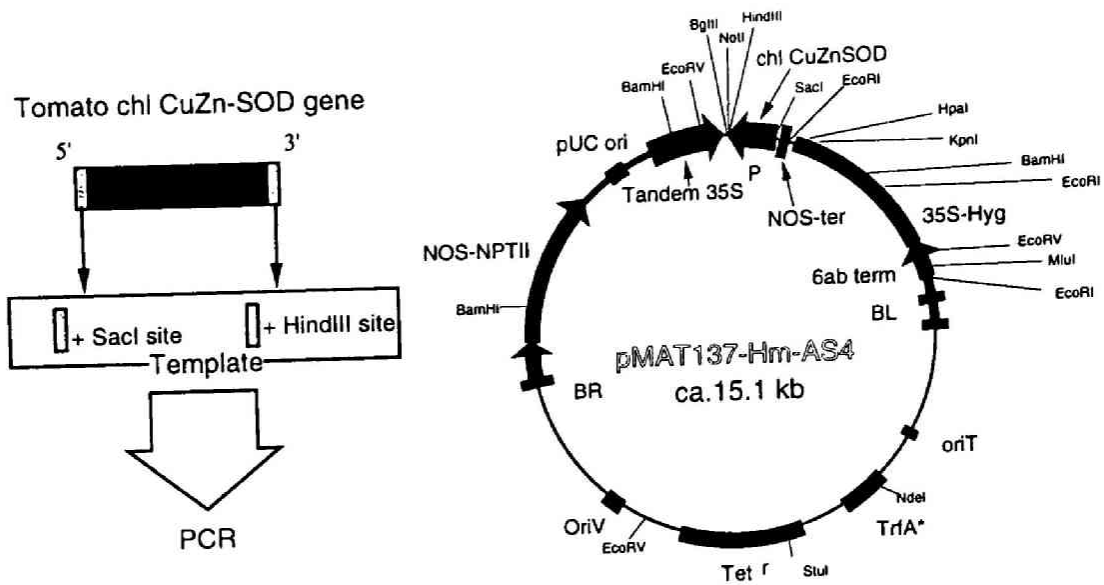


Fig. 2. 2. 1. Procedure and construction of antisense transformant of CuZn-SOD.

Table 2. 2. 1. SOD activities in chloroplasts of wild-type (WT) and transgenic tobaccos. Values are activity of the SOD relative to that of CuZn-SOD in wild-type tobacco.

Transformant	Mn-SOD	Fe-SOD	CuZn-SOD
SRI (WT)	ND ^a	98	100
ov-Mn	747	32	32
ov-Mn, an-CuZn	1000	50	18
an-CuZn	ND ^a	60	17

^aNot detected

native Mn-SOD activity was also suppressed similarly to Fe-SOD and CuZn-SOD activity by the overexpression of mitochondrial Mn-SOD (data not shown). The above observations indicate that total SOD activity is regulated to the same level in the chloroplasts by an unknown mechanism.

Phenotypic differences among the transgenic tobaccos — The phenotypic differences between transgenic and wild-type tobaccos were not observed when they were grown under the conditions of a low intensity of light ($50 \mu\text{mol photon m}^{-2} \text{s}^{-1}$). Under a high intensity of light ($1,000 \mu\text{mol photon m}^{-2} \text{s}^{-1}$), however, transgenic plants harboring CuZn-SOD (an-CuZn and ov-Mn,an-CuZn), whether mitochondrial Mn-SOD is present or absent, exhibit bleaching of leaves. Besides it, such transgenic tobaccos were easily photodamaged by exposure to high intensity of light ($2,000 \mu\text{mol photon m}^{-2} \text{s}^{-1}$) for 360 min (Fig. 2. 2. 2). Bleaching of leaves in an-CuZn and ov-

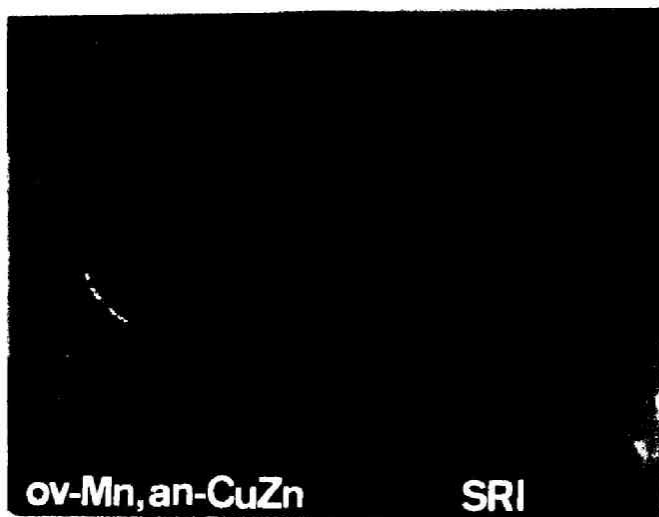


Fig. 2. 2. 2. Bleaching of transgenic tobacco harboring chloroplastic CuZn-SOD by their exposure to high intensity of light. Transgenic and wild-type tobaccos were exposed to continuous light ($2,000 \mu\text{mol photon m}^{-2} \text{s}^{-1}$) for 6 h in such a way as shown in figure (upside). Transgenic tobacco harboring chloroplastic CuZn-SOD and overproducing mitochondrial Mt-SOD in the chloroplasts (ov-Mn,an-CuZn) exhibited its photo-damage (left, downside figure as indicated below the figure), whereas SRI was apparently little affected by this treatment. As for an-CuZn, the result was similar to that of ov-Mn,an-CuZn. ov-Mn exhibited almost consistent results with SRI but several plants were photodamaged by this treatment. Treatment was repeated individually more than 20 times for 360 min each transgenic line.

Mn,an-CuZn was initiated at the center of the area surrounded by veins. Transgenic tobacco harboring cytosolic isoform of CuZn-SOD (another control plant), however, exhibited bleaching of leaves along with vascular bundles and did not show the bleaching at the corresponding areas of the an-CuZn and ov-Mn,an-CuZn (data not shown).

Electron transports of transgenic tobaccos — Effects of preillumination at gradually increasing intensity of light ($50-1,500 \mu\text{mol photon m}^{-2} \text{s}^{-1}$, each for 5 min, total photons were $0.7 \text{ mol photon m}^{-2}$) on the electron transports in the transgenic and wild type tobaccos were studied (Fig. 2. 2. 3) by means of Fv/Fm measurement. Electron transports of an-CuZn and ov-Mn,an-CuZn were reduced by the illumination. Electron transport in ov-Mn, however, was not significantly reduced as compared with SRI. To investigate the degrees of photoinhibition of PSII in the transgenic plants as compared with SRI, the plants were exposed to continuous light for various times (3 min to 6 h) and PSII activity was determined by Chl fluorescence parameter (F_m) (Fig. 2. 2. 4). Observed difference among transgenic and wild-type plants was significant (Fig. 2. 2. 4) but did not appear to account for the apparent differences as for the photodamage.

Inactivation of PSI in transgenic tobaccos harboring CuZn-SOD — Exposure of an-Cu and ov-Mn,an-CuZn to a high light ($2,000 \mu\text{mol photon m}^{-2} \text{s}^{-1}$) affected little PSII activity in spite of photobleaching of the leaves. I investigated photoinhibition of PSI by following the redox reaction of P700 after exposure to a strong light ($2,000 \mu\text{mol photon m}^{-2} \text{s}^{-1}$) in the transgenic and wild-type tobaccos. Reoxidation of P700 by far-red light after the reduction by multiple turnover flash light (50 ms) was delayed with illumination time in an-CuZn and ov-Mn,an-CuZn and such delay was associated with the reduction of $P700^+$ levels, indicating photodecomposition of reaction center of PSI. The levels of $P700^+$ at the beginning of exposure to a strong light were of little difference, and thereafter the levels of an-CuZn and ov-Mn,an-CuZn were more rapidly decreased than SRI was (Fig. 2. 2. 5), indicating that the decomposition of PSI in an-CuZn and ov-Mn,an-CuZn occurs earlier than that in SRI. Differences in degree of photodamage in PSII between the CuZn-SOD-harbored transgenic tobacco and SRI (Fig 4) were smaller than that in PSI (Fig. 2. 2. 5), suggesting that CuZn-SOD protects mainly PSI from photodamage. As for ov-Mn and ov-Fe, results were similar to SRI but, in ov-Mn, several plants exhibited the similar results to those of the transgenic plants harboring CuZn-SOD (data not shown), which may be attributed to decreased activities of native CuZn-SOD and Fe-SOD in the chloroplasts (Table 2. 2. 1).

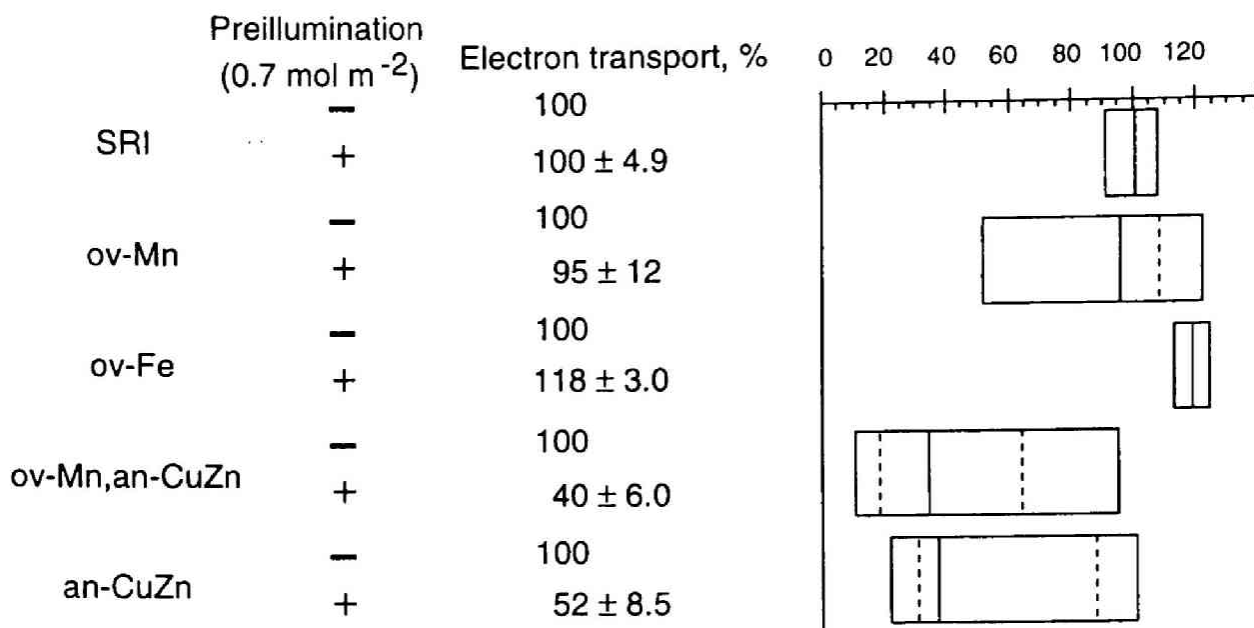


Fig. 2. 2. 3. Effects of preillumination with gradually increasing intensity of light on the electron transport of the transgenic and wild-type tobaccos. Plants were preilluminated by the gradually increasing intensity of light ($50\text{-}1,500 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$) for 40 min, and total photon dose was $0.7 \text{ mol photon m}^{-2}$. In the column of preillumination, + and - represent whether the plant was preilluminated or not. Electron transport of each plant was measured from F_v/F_m and shown as the percentage of the plant to that of SRI (center). Left figure shows the distribution of observed values of each line. Values represent mean \pm SE in 25 individual plants.

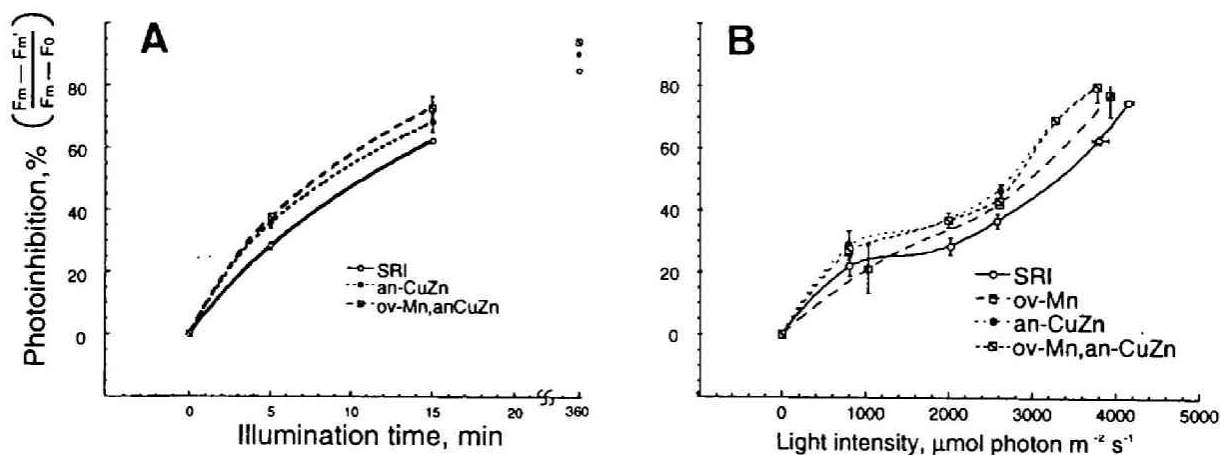


Fig. 2. 2. 4. Photoinhibition of PSII in transgenic and wild-type tobaccos by high light intensity of light. Panel A shows values of $100 \times (F_m - F_m') / (F_m - F_0)$ in transgenic and wild-type tobaccos after five-min illumination at indicated intensities. Panel B shows those for the plants after preillumination for indicated times at a light intensity of $2,000 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$. F_m and F_0 were measured prior to the illumination and F_m' and F_0' were measured 10 min after the illumination. Each point was measured as for seven plants of each transgenic line. Bars represent SE for each point.

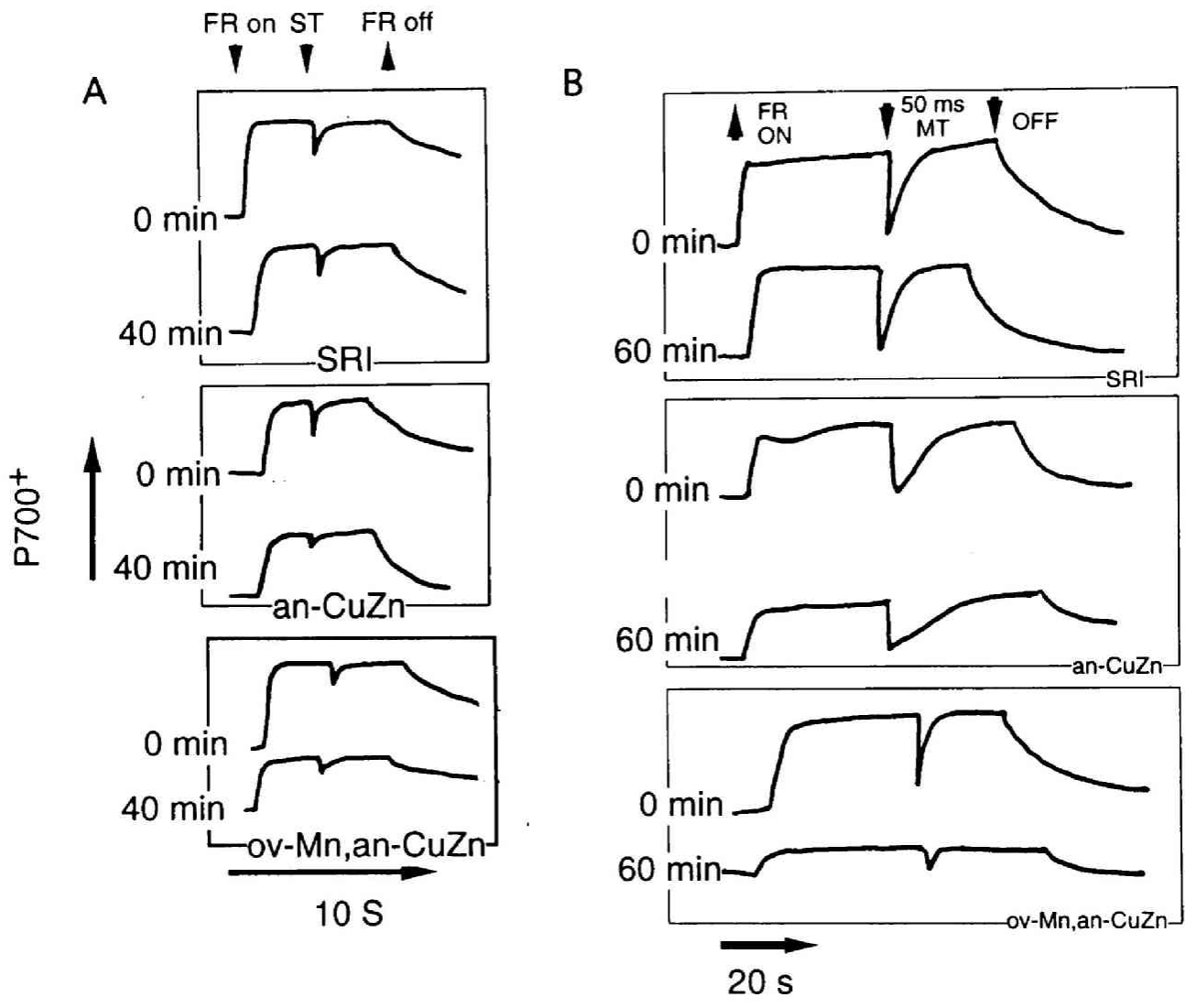


Fig. 2. 2. 5. Photodamage of PS I by a high intensity of light in transgenic and wild-type tobaccos. P700 were oxidized by far-red light (ON, arrows) as described in Materials and Methods, and then single turnover pulse light (14 μ s) (A) or multi turnover pulse light (50 ms) (B) was given (ST or MT, arrows). Subsequently, turning off the far-red light (OFF, arrows). The leaves were preilluminated by a light of 2,000 μ mol $m^{-2} s^{-1}$ for the indicated times.

Concluding remarks — The three isozymes of SOD have the similar enzymatic properties, but the results obtained here demonstrated that physiological function of each SOD isozyme is different each other. Differences in their functions in chloroplasts are very likely to be attributed to their microcompartmentalization within chloroplasts. Actually, immunoelectron microscopy has shown the location of chloroplastic CuZn-SOD at the site of PSI complex (Ogawa et al 1995a), and Fe-SOD specifically prevent photodamage of PSII rather than PSI (Van Camp et al. 1996). Present results demonstrate that chloroplastic CuZn-SOD actually protect PSI from photodamage by means of microcompartmentation at the site of superoxide generation, and, furthermore, give the possibility that mutation of CuZn-SOD cannot easily be replaced by other SODs. When we intend to recover lost function of a cell due to mutation of CuZn-SOD, introduction of the native CuZn-SOD or the SOD with similar features to the cell is thought to be the most effective. With this respect, design of microtargeting of the enzymes are expected for cure of genetic diseases due to mutation of SODs.

Now the environmental problem is issued, the construction of transgenic plants for the enhancement of stress tolerance will be intended more and more. In such a case, not only the targeting of SOD to organelles but also its microtargeting to the generation site of O_2^- should be considered.

Chapter 3. Utilization of Active Oxygens in Lignin Biosynthesis

3. 1 Intra- and Extra-cellular Localization of "Cytosolic" CuZn-Superoxide Dismutase in Spinach Leaf and Hypocotyl

Introduction

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, I have shown the attachment of CuZn-SOD to the stromal faces of the thylakoids of chloroplasts where the photogeneration site of superoxide (PS I) is located (Ogawa et al. 1995a, b).

"Cytosolic" and chloroplastic isoforms 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 (Sandalo 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 NH₂-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, I 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). I found that "cytosolic" CuZn-

SOD is localized in the cytosol near the vacuole, the nucleus and the apoplasmic region. Subsequently, locations of CuZn-SOD, lignin and superoxide generation in spinach hypocotyl were simultaneously determined, and I proposed that CuZn-SOD in the apoplast functions in lignification.

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 2660 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, USA).

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 PVDF 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 defined 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 dark red-brown 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 immunogold 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 chloroplastic isozyme (Ogawa et al. 1995a, b). Immunogold particles of "cytosolic" CuZn-SOD were found on the nuclei (Fig. 3. 1. 1A), on or near the tonoplast (Fig. 3. 1. 1B) and on the apoplast (Fig. 3. 1. 1C). The distribution of the immunogold particles labeling "cytosolic" CuZn-SOD in mesophyll cells of spinach was determined (Table 3. 1. 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. 3. 1. 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

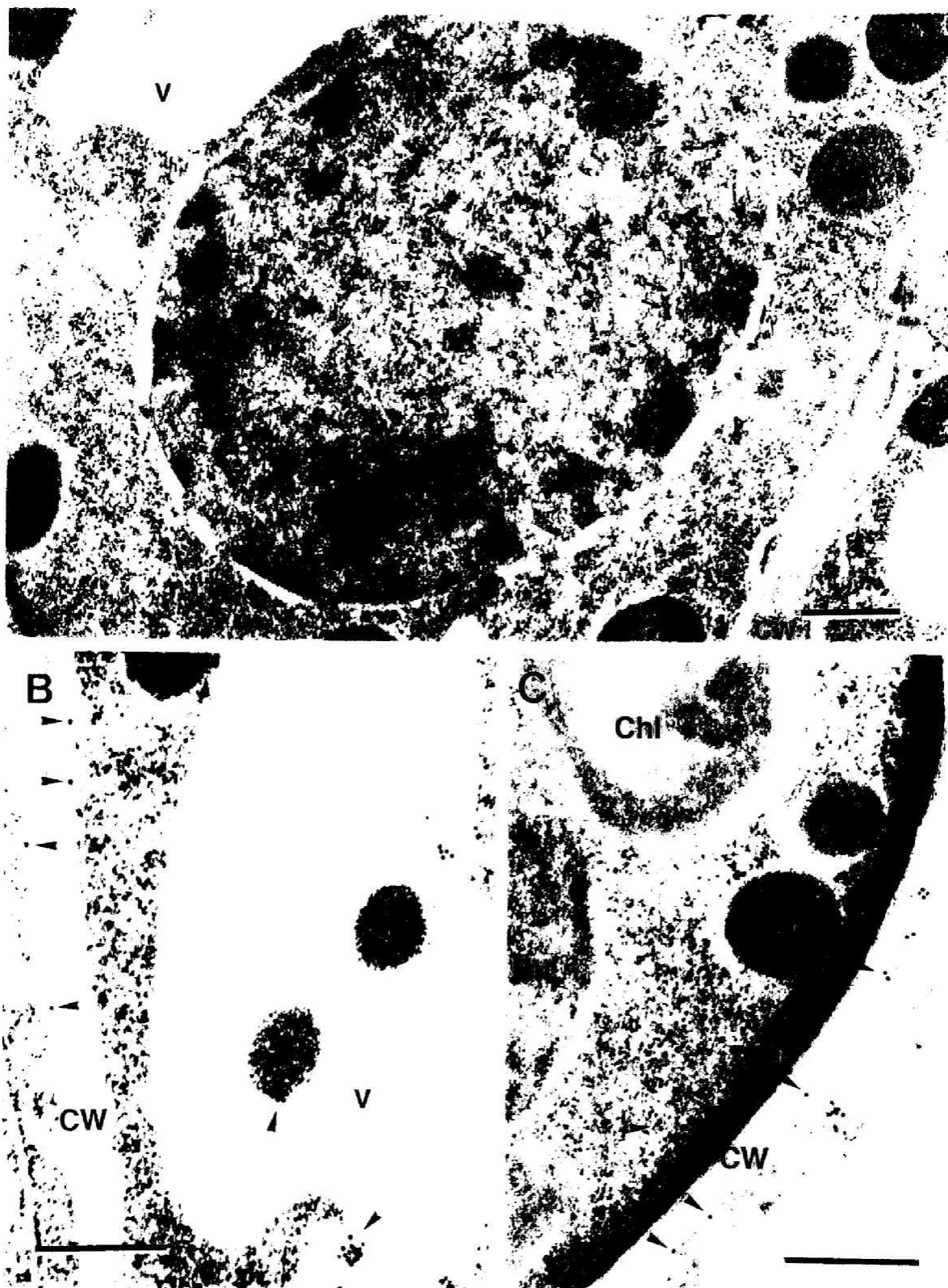


Fig. 3. 1. 1. Immunogold labeling of "cytosolic" CuZn-SOD in a spinach mesophyll cell from two-month-old seedlings. Substitution and fixation were done with 0.35% glutaraldehyde and 0.5% tannic acid in acetone. The immuno-gold labeling specific for "cytosolic" CuZn-SOD was performed as described in Materials 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. 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).

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.

Table 3. 1. 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 immuogold particles on the "tonoplast". "Vacuole" and "tonoplast" constitute the 15-nm layer of the cytosol on the surface of the tonoplast.

Immunogold particles specific for "cytosolic" CuZn-SOD appear to be attached to the DNA observed as filaments (Fig. 3. 1. 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. 3. 1. 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. 3. 1. 1B and C). The interspace between the plasma membrane and the cell wall, which is expected to develop into the secondary thickenings, contained CuZn-SOD (Fig. 3. 1. 2A and B). Immunogold particles also are present on a vesicle-like structure surrounded by plasma membranes (arrowheads, Fig. 3. 1. 2C), which would represent cross section of



Fig. 3. 1. 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 intraspaces contain 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 intraspaces (C), which is also found in Fig. 3. 1. 1A.

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 tracheary elements that are the to-be vessels were made, immunogold particles were found on the secondary thickenings of the cell wall (Fig. 3. 1. 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. 3. 1. 3B). The precursors of lignin 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.

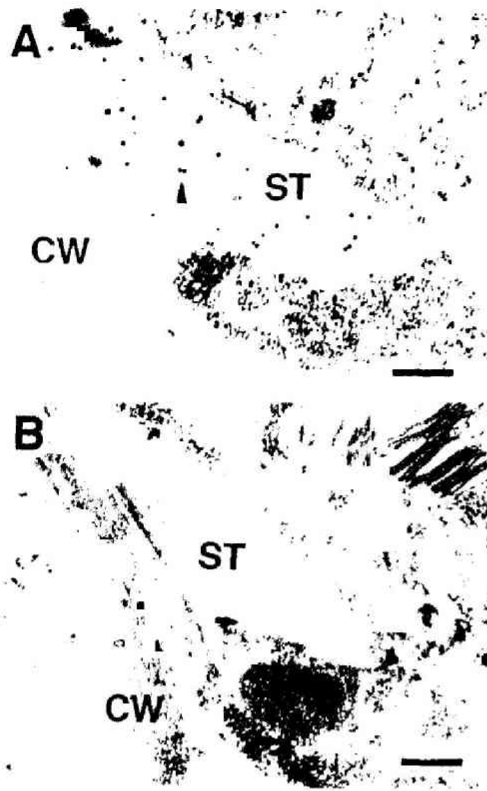


Fig. 3. 1. 3. Immunogold labeling of "cytosolic" CuZn-SOD in the secondary thickening of cell wall of a spinach vascular cell from two-month-old seedlings. Substitution and fixation were done with 0.35% glutaraldehyde and 0.5% tannic acid in acetone. Immunogold labeling was performed as described in 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 (B).

CuZn-SOD in the extracellular washing fluid — To confirm the localization of CuZn-SOD in the apoplastic compartments, I 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 3. 1. 2). The extracellular washing fluid obtained with the high

Table 3. 1. 2. Ratios of "cytosolic" and chloroplastic CuZn-SODs in the extracellular washing fluid to those in the buffer extract of spinach leaves (%).

Isozyme	Water	500 mM NaCl
Cytosolic CuZn-SOD	0.72	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

salt buffer contained nearly 10% of the total "cytosolic" CuZn-SOD of the leaf tissue, whereas the content 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 3. 1. 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, I simultaneously determined the sites of lignification, 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. 3. 1. 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. 3. 1. 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. 3.

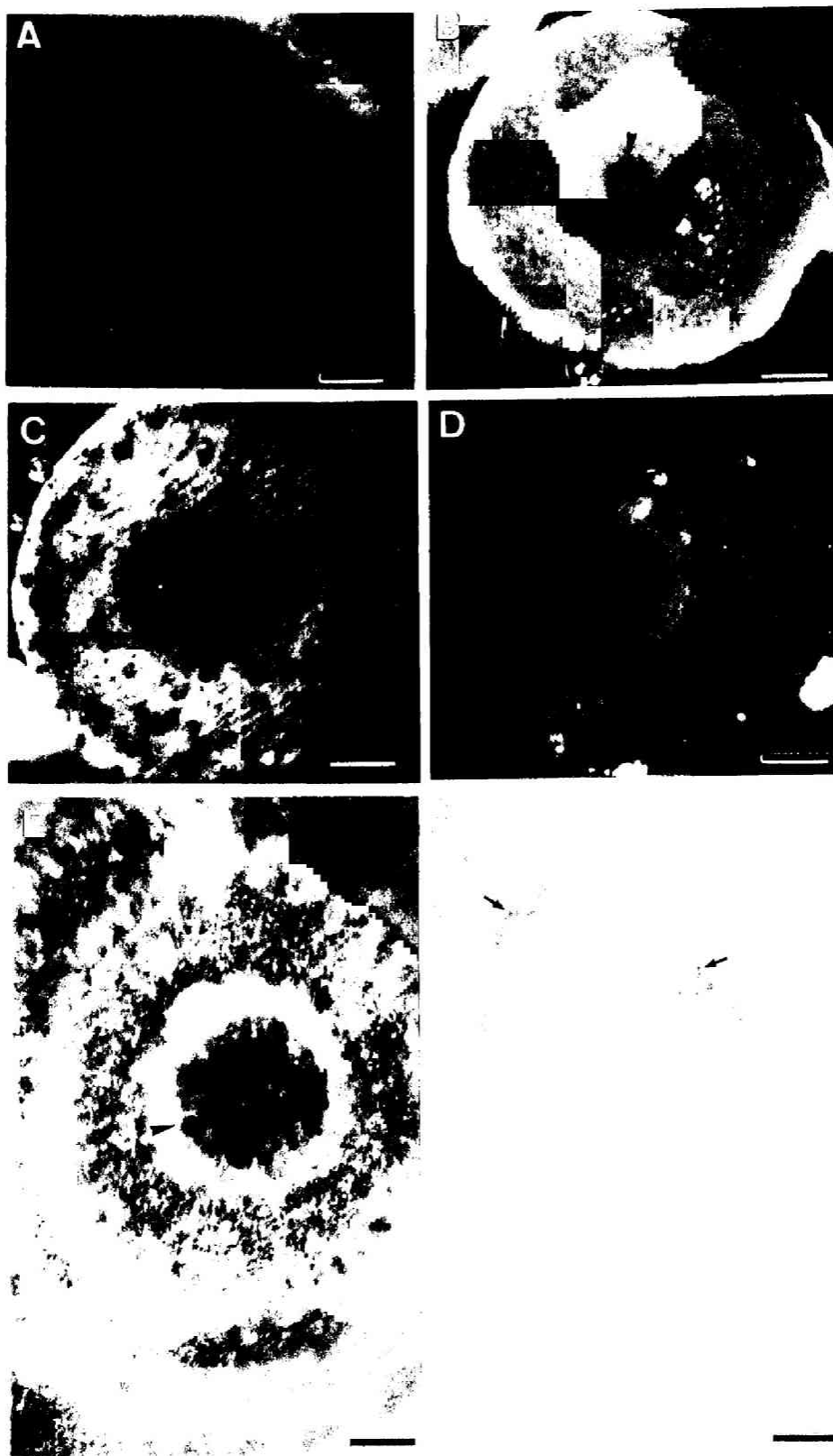


Fig. 3. 1. 4. Distribution of "cytosolic" CuZn-SOD, lignin and superoxide generation in spinach hypocotyl. Immunostaining of "cytosolic" CuZn-SOD (A, arrowhead) and detections of lignin (B, arrowhead) and superoxide (C, arrows) in the hypocotyls of 13-d-old spinach seedlings were done in cross sections 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 μ m.

1. 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. 3. 1. 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 peripher 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. 3. 1. 4D) was adjacent to cells containing lignin in their vascular tissues (arrowhead, Fig. 3. 1. 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. 3. 1. 4D) appeared to be associated with that of the most intensive immunolabeling of "cytosolic" CuZn-SOD (arrowhead, Fig. 3. 1. 4A).

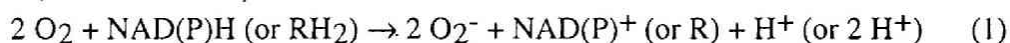
Superoxide generation and the immunogold labels of CuZn-SOD were weak in the hypocotyl cross sections from the same seedlings cut 1 mm above the base end, although lignin was intensively stained (data not shown). This indicates that the basal part of hypocotyl is older than the top, and reflects the 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, I tested the top parts of the hypocotyls of two-month-old plants, and compared the findings with those for 13-d-old plants (Fig. 3. 1. 4A-D). The region of the lignin-containing tissues in hypocotyls of two-month-old seedlings (Fig. 3. 1. 4E) was expanded in comparison with that of the 13-d-old seedlings (Fig. 3. 1. 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. 3. 1. 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. 3. 1. 4C and 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. I 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 seem 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 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).



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 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)).



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



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*-diethylthiocarbamate (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*-diethylthiocarbamate 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),



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. I 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.

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

Introduction

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 PS I (Asada and Takahashi 1987). Spontaneous disproportionation of superoxide radicals proceeds at an appreciable rate to produce hydrogen peroxide and dioxygen ($10^5 \text{ M}^{-1} \text{ s}^{-1}$ 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 ($2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$). 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, 1996b). Furthermore, I 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. 1996b). 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.

In the present work, I 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, 1996b).

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*-diethyldithiocarbamate (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. 1996b).

Detection of hydrogen peroxide in hypocotyls — Generation of hydrogen peroxide was located as a dark blue starch-I₂ 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. (1996b). 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 NaN₃ 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 NaN₃ 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 NaN₃ 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. 3. 2. 1A). However, the blue precipitate was found in the vascular tissue after incubation in the presence of DDC, an inhibitor of CuZn-SOD (Fig. 3. 2. 1B), as observed previously (Ogawa et al. 1996b). Incubation of the sections with either KCN or NaN₃, which inhibits both

CuZn-SOD and peroxidases, induced a high intensity of staining with formazan as that in the presence of DDC (Fig. 3. 2. 1C and D, respectively). Only a little formazan was formed under anaerobic conditions (Fig. 3. 2. 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. 3. 2. 1F and G).

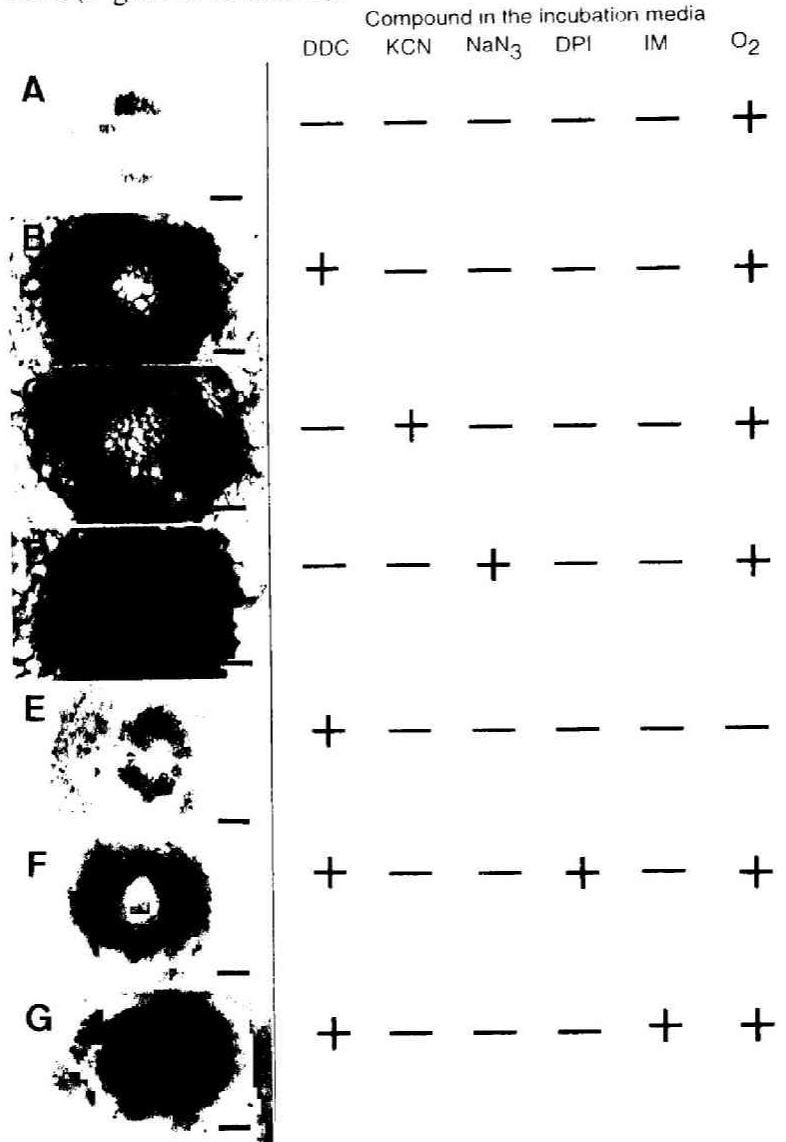


Fig. 3. 2. 1. Effects of inhibitors and dioxygen on the formation of formazan in vascular tissues of spinach hypocotyls. Cross sections of hypocotyls of 13-d-old seedlings, cut 1 mm from the top, were used, and generation of superoxide was detected as a blue precipitate of formazan that was formed as 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*-diethylthiocarbamate (DDC), 10 mM KCN, 1 mM NaN₃, 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. 3. 2. 3B.

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 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 neutrophils.

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-I₂ complex was found in the vascular tissue and its location pattern was similar to that of the formazan (Fig. 3. 2. 2A),

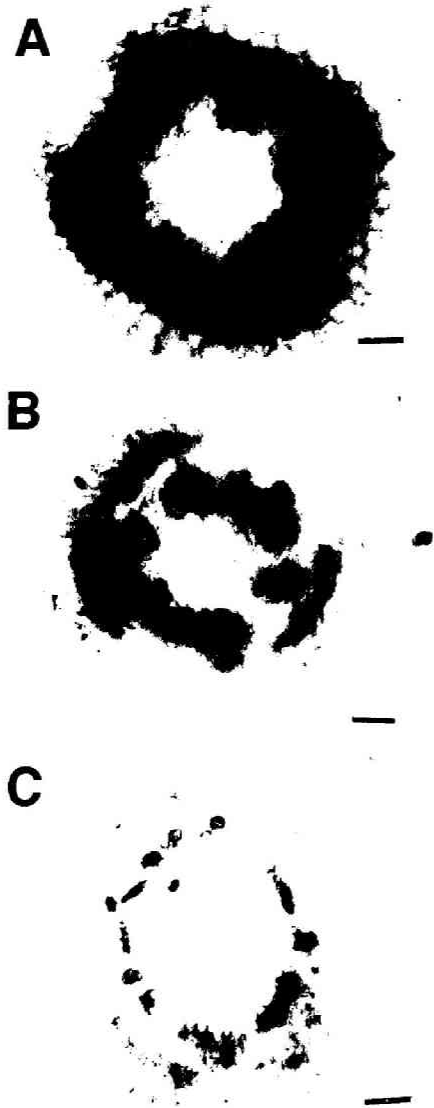
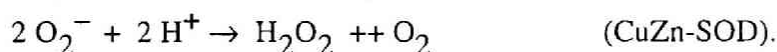


Fig. 3. 2. 2. Effects of inhibitors on the formation of starch-I₂ complex in the vascular tissue of spinach hypocotyls. The cross sections of the hypocotyl from a 13-d-old seedling were prepared as in Fig. 3. 2. 1, and generation of hydrogen peroxide was located as dark blue starch-I₂ complex, as described in Materials and Methods. A, Formation of the starch-I₂ complex in the control section; B, in the presence of DDC; C, in the presence of DPI. DDC (2 mM) and DPI (100 μ M) were applied to the sections prior to the incubation with the starch-iodide solution. Scale bars, 100 μ m.

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-I₂ complex was suppressed by the CuZn-SOD inhibitor DDC and the NAD(P)H oxidase inhibitor DPI (Fig. 3. 2. 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, I propose that in the vascular tissue of spinach hypocotyl, the hydrogen peroxide is generated via the following pathway;



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, Mn²⁺ 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 horse radish 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 NaN₃, 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. 3. 2. 1C, D). Furthermore, DPI and imidazole, which did not inhibit either peroxidase or CuZn-SOD, suppressed almost completely the generation of superoxide (Fig. 3. 2. 1F, G) and hydrogen peroxide (Fig. 3. 2. 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. 3. 2. 3A). The untreated sections showed only the pale-brown color in the vessels (Fig. 3. 2. 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. 3. 2. 3C, arrow), but not on all the thickened cell walls (Fig. 3. 2. 3C, arrowhead). After a 90-min incubation, formazan was found not only in the xylem but also in the phloem (Fig. 3. 2. 3D, arrow). Thus, the superoxide anions were generated mainly in xylem and to a much lesser extent in phloem.

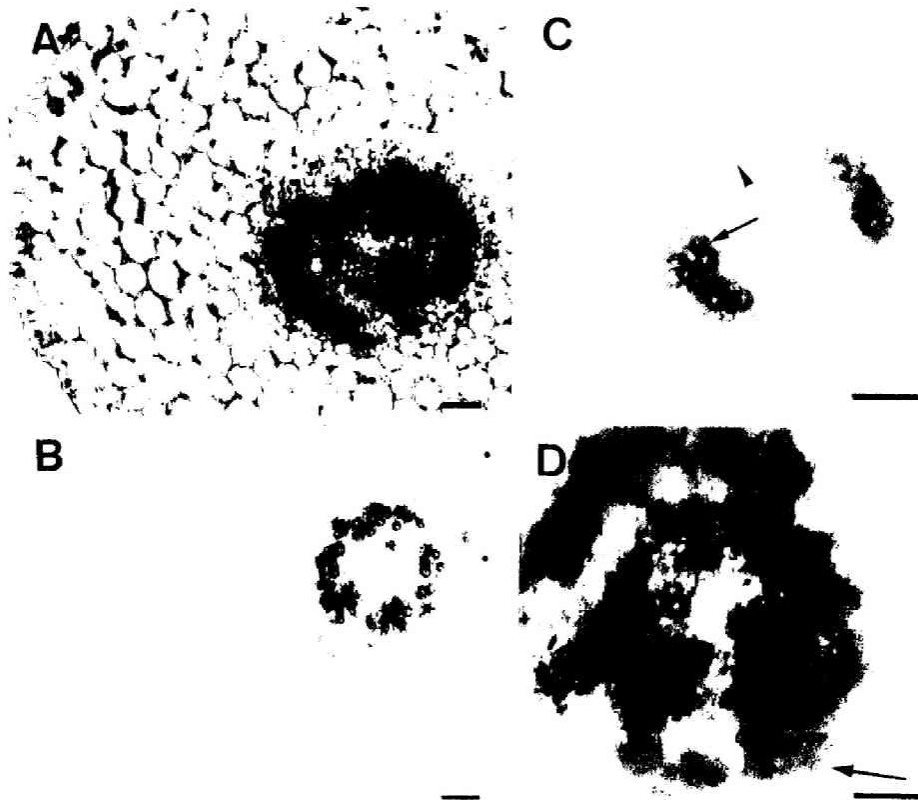


Fig. 3. 2. 3. Distribution of sites of generation of superoxide in hypocotyls of spinach, as indicated by blue precipitates of formazan. A, Precipitation of formazan in a cross section of the hypocotyl from a 13-d-old seedling that had been incubated with NBT and DDC as described in Fig. 3. 2. 1B for 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. 3. 2. 1B; D, precipitation of formazan after a 90-min incubation as in C. Scale bars, 100 μm .

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). Therefore, the cells with secondary thickenings which precipitated formazan (Fig. 3. 2. 3C, arrow) appear to be at stage (3), and those which precipitated little formazan (Fig. 3. 2. 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. 1996b). 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. 3. 2. 4A). In contrast, control cross sections without the primary antibody showed only a light brown color on the cell walls of the vessels (Fig. 3. 2. 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. 3. 2. 4A and 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. 3. 2. 4C, small arrows). Thus, CuZn-SOD in vascular tissue was distributed (Fig. 3. 2. 4A and C) at the sites of generation of superoxide (Fig. 3. 2. 1 and 3). In addition to that in the vessels, CuZn-

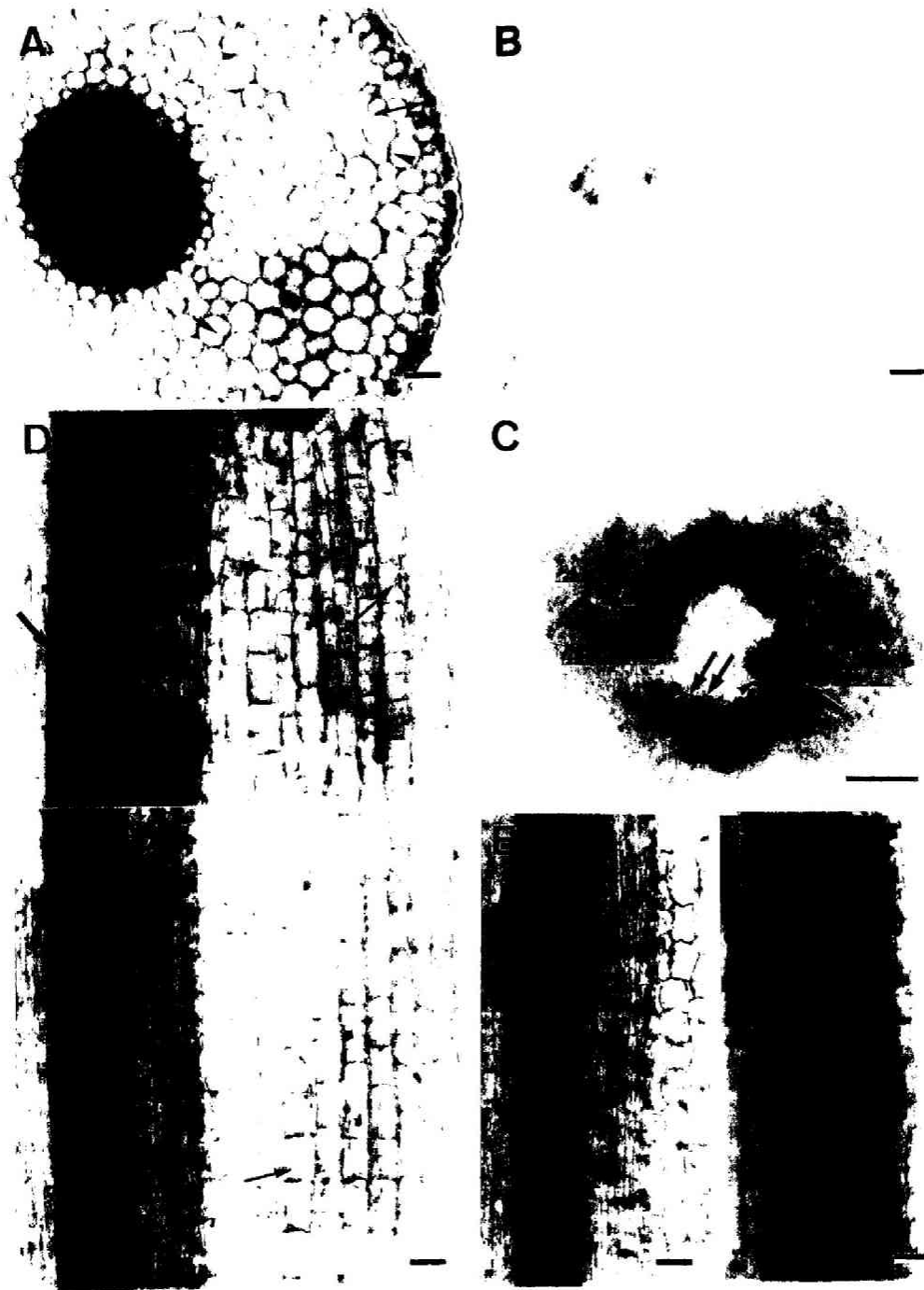


Fig. 3. 2. 4. Immunohistochemical distribution of the "cytosolic" CuZn-SOD in spinach hypocotyls. Immunohistochemical staining of "cytosolic" CuZn-SOD in hypocotyls from 13-d-old seedlings was performed as described in Materials and Methods. Immunostaining of "cytosolic" CuZn-SOD is visible as brown 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 μ m. Another control section of A (untreated) is shown in Fig. 3. 2. 3B.

SOD was distributed in the symplast of xylem parenchyma cells (Fig. 3. 2. 4C, arrowhead). CuZn-SOD was located also on the symplast in the epidermal cells (Fig. 3. 2. 4A, arrow), with a little on that in cortex (Fig. 3. 2. 4A, arrowheads), where superoxide was generated as detected by formazan (Fig. 3. 2. 3A). I have demonstrated the location of "cytosolic" CuZn-SOD in nuclei and tonoplasts of spinach mesophyll cells by immunogold electron microscopy. (Ogawa et al. 1996b). 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 1997), 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. 3. 2. 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. 3. 2. 4D, large arrows). Faint immunolabeling of CuZn-SOD was found on the cytoplasm in the cortex (Fig. 3. 2. 4D, small arrows), resembling that in the cross section in Fig. 3. 2. 4A. The insides of the parenchyma cells adjacent to the xylem were also found to contain CuZn-SOD (Fig. 3. 2. 4E, arrowheads). The parenchyma cells and their cell walls also were immunolabeled by the antibody against CuZn-SOD (Fig. 3. 2. 4D and 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 (H rouart et al. 1994). The present work confirmed the previous results obtained by the immunogold labeling and electron microscopy (Ogawa et al. 1995b, 1996b). 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. 1996b).

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. 3. 2. 5C), corresponded to the sites of distribution of CuZn-SOD (Fig. 3. 2. 5B) and to the generation site of superoxide (Fig. 3. 2. 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.

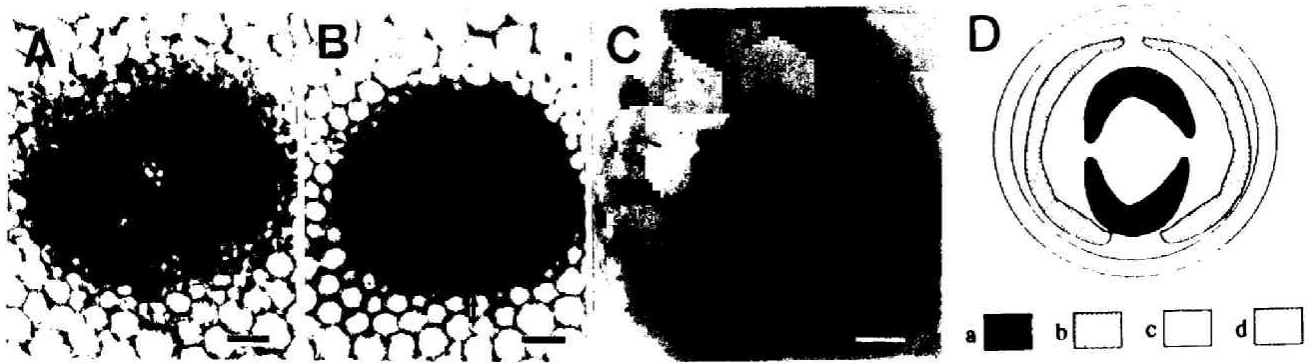


Fig. 3. 2. 5. Comparison of the distribution of lignin and the so-called "cytosolic" CuZn-SOD with that of sites of generation of superoxide in spinach hypocotyl. A, Formation of formazan at sites of generation of superoxide under the same conditions as described for Fig. 3. 2. 3A; B, immunolabeling of "cytosolic" CuZn-SOD; C, staining of lignin by phloroglucin-HCl. Scale bars, 100 μ m; 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.

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. 3. 2. 5C, arrows). The endodermal cells also contained CuZn-SOD (Fig 5B, arrows) and generated superoxide radicals (Fig. 3. 2. 5A, arrows), which may participate in the generation of hydrogen peroxide for the biosynthesis of suberin.

As schematically shown in Fig. 3. 2. 5D, in the vascular tissue of spinach hypocotyls, the sites of distribution of CuZn-SOD and of generation of superoxide (Fig. 3. 2. 1, 3 and Fig. 3. 2. 5A) and hydrogen peroxide (Fig. 3. 2. 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-I₂ 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. I 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. 3. 2. 4C and 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, I have shown that either the CuZn-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. 1997b). These results lend further support to our hypothesis that CuZn-SOD is essential for lignification (Ogawa et al. 1996b) and to the participation of NAD(P)H oxidase in the generation of superoxide for the hydrogen peroxide in the biosynthesis of lignin.

I propose the possible system for supplying hydrogen peroxide to the biosynthesis of lignin as schematically shown in Fig. 3. 2. 6. In the presence of the inhibitors of CuZn-SOD, little hydrogen peroxide was detected in spite of the generation of superoxide (Fig. 3. 2. 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 peroxidase (Metodiewa et al. 1992). Thus, CuZn-SOD is essential to lignification since it supplies hydrogen peroxide and protects the peroxidase from inactivation by superoxide.

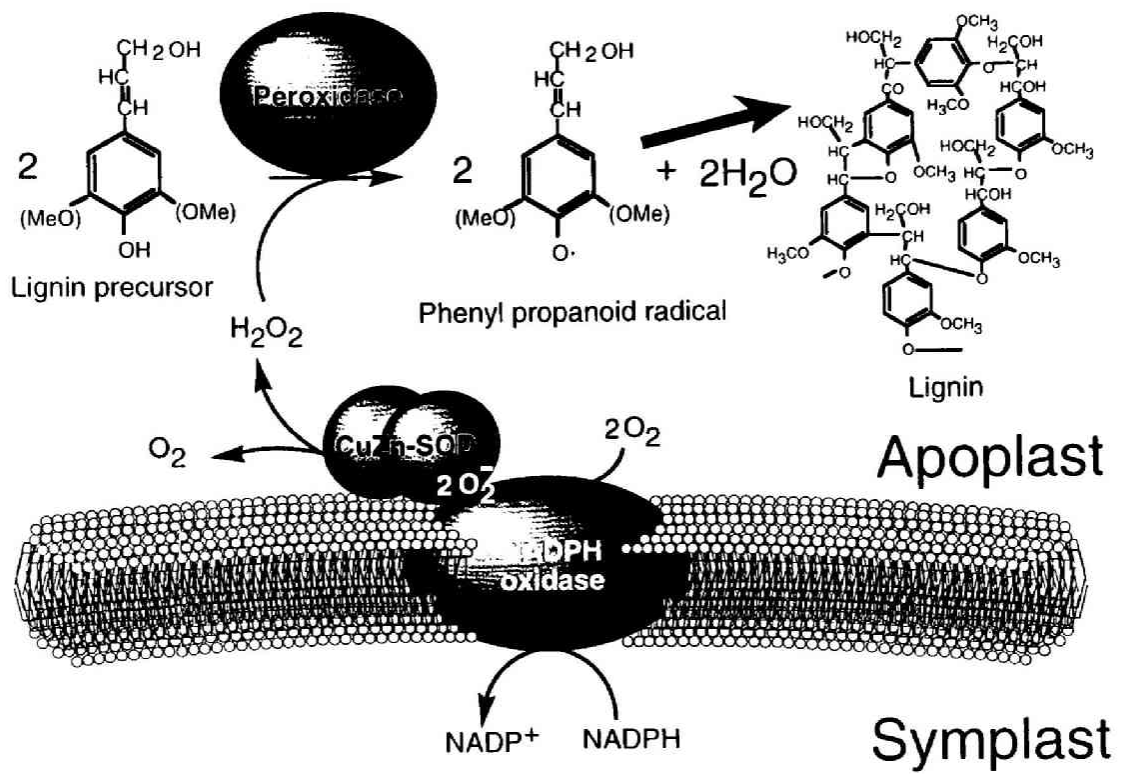


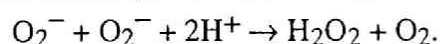
Fig. 3. 2. 6. A proposed scheme for supplying hydrogen peroxide to lignin biosynthesis.

3. 3 Superoxide Is Transiently Generated and Accompanied with Lignification of Secondary Cell Wall in the Differentiation of Tracheary Elements during Cultured Cells of *Zinnia elegans*

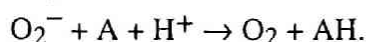
Introduction

Differentiation of tracheary element (TE) has been studied at the cytological, biochemical and molecular levels (Ye and Varner 1993, Demura and Fukuda 1994, Yoshimura et al. 1996, see a review of Fukuda 1996), and it has been shown to be a process of programmed cell death, during which a variety of events specific for the formation of TE occurs. One of the typical events is the secondary thickening of cell walls, which is characterized not only by the biosyntheses of cellulose and xylan but also by that of lignin.

Lignin is polymerized via the interaction of the phenyl propanoid radicals that are produced via the univalent oxidation of lignin precursors such as coniferyl, sinapyl and *p*-coumaryl alcohols, and peroxidases and/or laccases are involved in the univalent oxidation of the precursors (Harkin and Obst 1973, Lewis and Yamamoto 1990, O'Malley et al. 1993, Olson and Varner 1993). Phenylalanine ammonia-lyase, cinnamyl alcohol dehydrogenase, *O*-methyltransferase, cinnamate hydroxylase and 4-coumarate:CoA ligase participate in the biosynthesis of the lignin precursors phenyl propanoids, and their relation to lignification has been established (Lewis and Yamamoto 1990, Feuillet et al. 1995, Fukuda 1996, Campbell and Sederoff 1996, Kajita et al. 1996). If peroxidase is the major participating enzyme in the biosynthesis of lignin, hydrogen peroxide is indispensable for it. Elstner and Heupel (1976), Gross et al. (1977) and Halliwell (1978) have proposed that peroxidase participates also in the generation of hydrogen peroxide. Recently, I have shown that superoxide anion radicals are generated in lignifying vascular cells of spinach hypocotyls (Ogawa et al. 1996b), agreeing with the mechanism proposed by Gross et al. (1977) and Halliwell (1978) in respect of the production of hydrogen peroxide via the disproportionation of superoxide anion radicals. However, inhibitors for peroxidase do not but those for NAD(P)H oxidase inhibit the generation of superoxide in the vascular tissue, indicating the formation of superoxide anions by NAD(P)H oxidase, but not by peroxidase (Ogawa et al. 1997a). Superoxide anions are spontaneously disproportionated to hydrogen peroxide and dioxygen at a considerable rate ($10^5 \text{ M}^{-1} \text{ s}^{-1}$ at pH 7), but, when CuZn-superoxide dismutase (SOD) is inhibited, little hydrogen peroxide is accumulated in the vascular tissue of spinach hypocotyls (Ogawa et al. 1997a). Thus, the hydrogen peroxide required for the biosynthesis of lignin is produced via the SOD-catalyzed disproportionation of superoxide at a diffusion-controlled rate ($2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$);



However, if SOD could not function, the superoxide anions are oxidized by component(s) (A) in the vascular tissue to suppress the production of hydrogen peroxide,



Actually CuZn-SOD is localized in the secondary thickenings of the cell walls in vascular cells of spinach leaves, in which lignin is to be deposited (Ogawa et al. 1995b, 1996b). The cytosolic isozyme of CuZn-SOD is expressed as determined by SOD3 promoter-GUS chimeric gene in vascular tissue of tobacco (H  rouart et al. 1994) and is localized in the vascular tissues of spinach hypocotyl by immunohistochemical method (Ogawa et al. 1997a). These observations show the production of hydrogen peroxide for lignification via the SOD-catalyzed disproportionation of superoxide anions.

I here report the stage-specific generation of superoxide during the differentiation of TE in the cultured cells of *Zinnia elegans*. Superoxide anions were generated by NAD(P)H oxidase in *Zinnia elegans* cells for a short time accompanying with the accumulation of lignin. Further, I describe the effects of the inhibitors of CuZn-SOD and NAD(P)H oxidase on the accumulation of lignin in the secondary cell walls, to assess the contribution of CuZn-SOD and the superoxide generated by NAD(P)H oxidase to the biosynthesis of lignin.

Materials and Methods

Culture of mesophyll cells of Zinnia elegans — *Zinnia elegans* seeds were germinated on moistened vermiculite in a growth chamber (28  C; 14 h of 8,000 lux; humidity, 60%) for 7 d and then the seedlings were grown under a continuous light of 8,000 lux. The primary leaves from 14-d-old seedlings were used for isolation of the mesophyll cells as described by Fukuda and Komamine (1980). The isolated mesophyll cells were cultured in the TE-inducing medium containing 0.1 mg liter⁻¹   -naphthaleneacetic acid and 1 mg liter⁻¹ benzyladenine (Demura and Fukuda 1994).

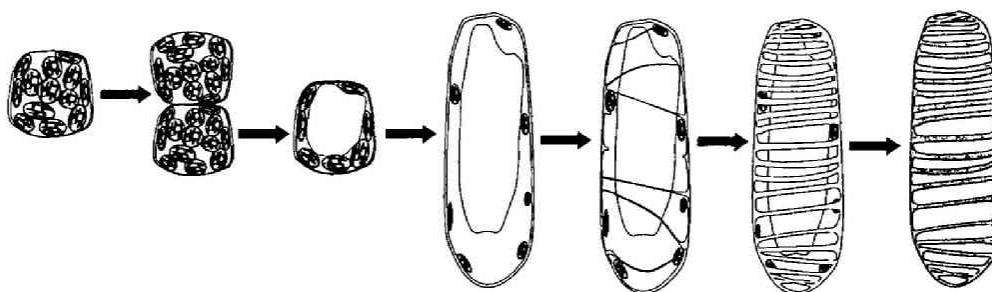


Fig. 3. 3. 1. Schematic illustration of process of the differentiation to TE in cultured cells of *Zinnia elegans*.

Under the present conditions, isolated mesophyll cells were divided and expanded, and subsequently secondary thickening of the cell walls was initiated at 60 h, followed by the appearance of the typical patterned thickenings of the cell walls (Fig. 3. 3. 1). During the differentiation, chloroplasts and Chl of isolated mesophyll cell were decreasing to null, and the deposition of lignin in secondary thickened cell walls was started at around 143 h. Finally 13% of the isolated mesophyll cells differentiated to TE.

Detection of lignin — The cultured cells mounted on a slide glass were incubated with 1% phloroglucin in ethanol and, just before ethanol was vaporized, 4 M HCl was added. Subsequently, lignin was located as a red staining under an Olympus microscope (BH and PM-20, Olympus, Tokyo, Japan). Alternatively, lignin was located as a blue autofluorescence using a fluorescence microscope (BH, Olympus, Tokyo, Japan, UV light excitation, emission $\lambda=435$ nm).

Detection of superoxide — The mesophyll cells at various stages of culture were incubated with 50 μ M nitroblue tetrazolium (NBT) and 5 mM *N,N*-diethyldithiocarbamate (DDC) in the TE-inducing medium for 20 min, and then the generation of superoxide was detected by the formation of formazan under a microscope. DDC is an inhibitor of CuZn-SOD (Asada et al. 1975), and formazan was detectable only in its presence in vascular tissue of spinach hypocotyl (Ogawa et al. 1997a). The number of superoxide-generating cells was counted using micrographs that were taken after the 20-min incubation with NBT. When the effect of the NAD(P)H-oxidase inhibitor (Auh and Murphy 1995) was tested, the cultured mesophyll cells were preincubated with 50 μ M diphenyleneiodonium (DPI) in the TE-inducing culture medium for 10 min prior to the incubation with NBT. Superoxide also was detected as 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one (MCLA)-dependent chemiluminescence using a photon counter (Hamamatsu Photonics, Japan). MCLA has been shown to be a specific probe for superoxide anion radical, and to allow its detection by chemiluminescence (Nakano et al. 1986). The cells were suspended in the culture medium (3 ml) containing 0.5 μ M MCLA, and the generation rate of superoxide was calibrated by the xanthine-xanthine oxidase system. By the reduction rate of Cyt *c* using a reaction mixture containing 10 μ M Cyt *c*, 50 μ M xanthine and various amounts of xanthine oxidase, the superoxide generation rate by the xanthine oxidase was determined. Subsequently, by replacing Cyt *c* with 0.5 μ M MCLA, the intensity of the chemiluminescence was determined by the calibrated amounts of xanthine oxidase.

Results and Discussion

Timing of superoxide generation and lignification during tracheary element differentiation in cultured cells of Zinnia elegans — To reveal an interrelation

between the timings of the generation of superoxide and the deposition of lignin, the numbers of the superoxide-generating cells and lignin-containing cells were counted at various times of culture in the TE-inducing medium (Fig. 3. 3. 2A). The typical shapes of TE-differentiating cells after the start of culture are shown in Figure 3. 3. 3, in which the lignin containing cell is red-staining with the phloroglucin-HCl reaction.

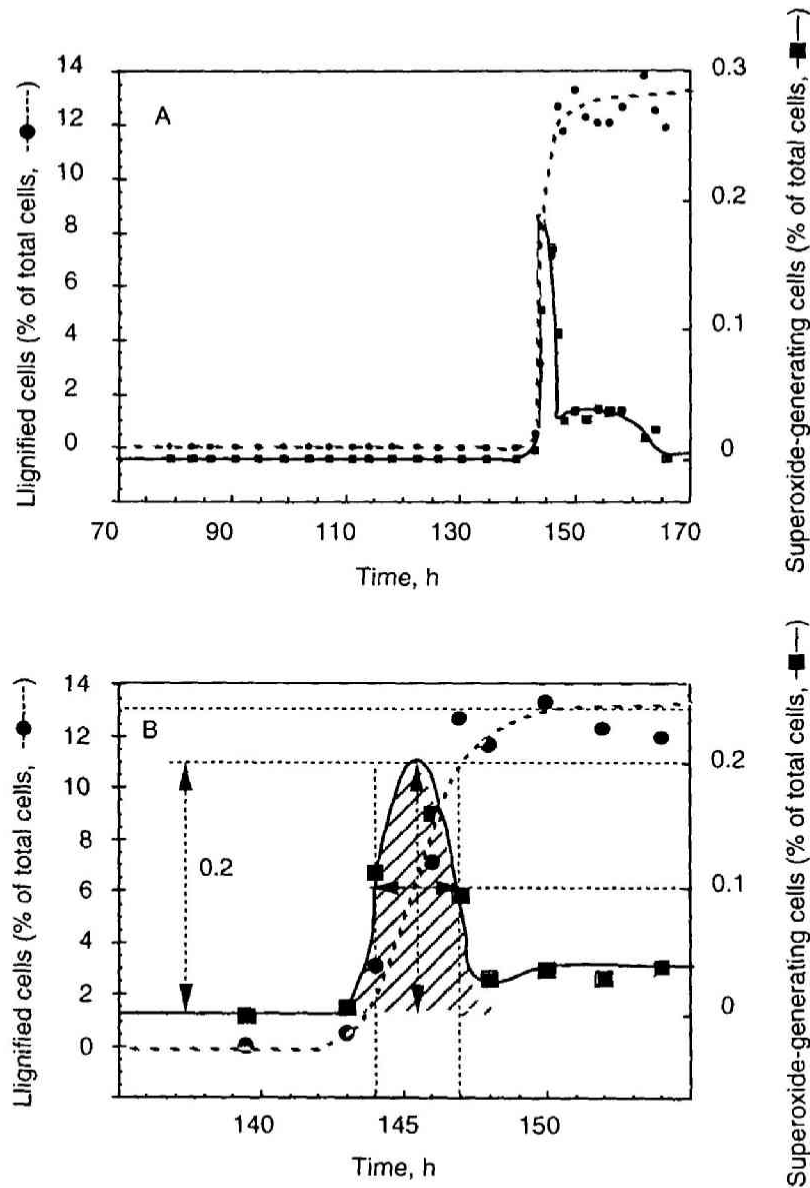


Fig. 3. 3. 2. Changes of the populations of superoxide-generating cells and lignin-containing cells during the differentiation into TE of cultured cells of *Zinnia elegans*. Populations of superoxide-generating cells and lignin-containing cells were evaluated as the percentage of the number of respective cells to the total number of the cultured cells. Over 2,000 cells at each culture time were counted using micrographs, as described in Materials and Methods. The deposition of lignin and generation of superoxide during the TE differentiation are shown in Panel A, and its expanded time scale in Panel B. Closed circles and squares represent the percentages of lignin-containing cells and superoxide-generating cells in total cultured cells, respectively.

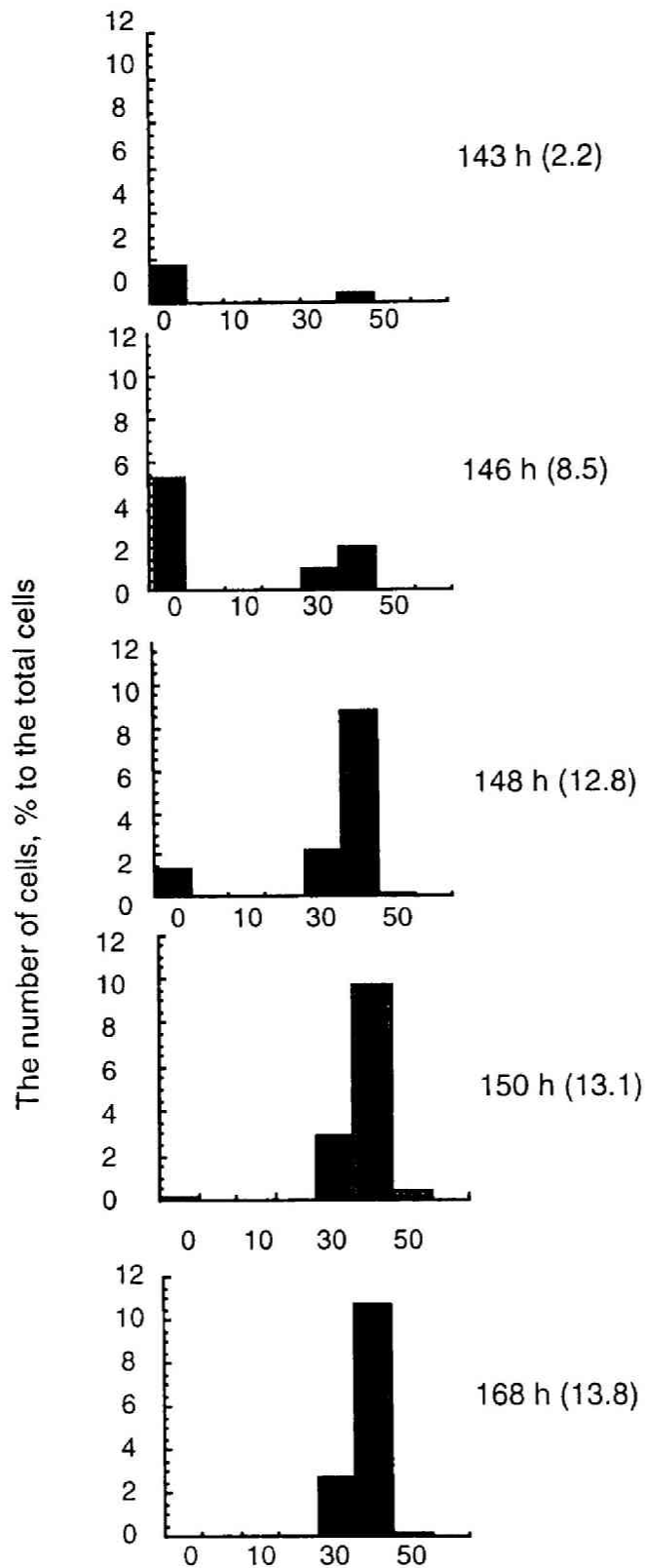
As shown in Figure 3.3.2, the lignin-containing cells was not observed until 139 h-culture (Fig. 3.3.3A to C), followed by a transient increase in number from 143 to 149 h (Fig. 3.3.3E, G). Thereafter, the number of lignin-containing cells (Fig. 3.3.3I) showed a plateau level (Fig. 3.3.2A). Under UV-fluorescence microscope, the cells showed only red fluorescent due to chlorophyll until around 139 h after the start of culture. Thereafter, whenever the phloroglucin-stained cells appeared, the cells showed blue-fluorescence with the same pattern of the secondary thickenings as the phloroglucin-staining under UV-excitation (data not shown), confirming the deposition of lignin.

With respect of superoxide in Figure 3.3.2, its generation as the formation of formazan in the differentiating cells was not observed until 139 h (Fig. 3.3.3A to C), but, subsequently, transient intensive generation of superoxide was observed between 143 to 148 h (Fig. 3.3.3D1). For assesment of the superoxide generation by formazan only, the intensively stained cells as indicated by an arrow (Fig. 3.3.3D1) were counted as the superoxide-generating cells. However, pale-stained cells as indicated by arrowheads were not counted (Fig. 3.3.3D1, D2), since this level staining was observed even when only DDC was added prior to the addition of NBT. Little generation of superoxide was found in the cells after 148-h culture (Fig. 3.3.3H). The transient generation time of superoxide was accompanied with the deposition time of lignin in the secondary thickened cell walls, strongly supporting that the biosynthesis of lignin requires the generation of superoxide. To compare between the sites of formation of formazan and deposition of lignin in a cell, the phloroglucin-HCl staining was performed just after 20-min incubation with NBT (Fig. 3.3.3F, G), in which concentration of DDC was reduced to 0.5 mM to avoid complete inhibition of lignin deposition in the secondary cell walls. The sites of formation of formazan (Fig. 3.3.3F) and deposition of lignin (Fig. 3.3.3G) were mutually identical and limited to the patterned secondary thickenings, indicating further the association of generation of superoxide with lignification.

Fig. 3.3.3. Morphological change and timings of the generation of superoxide and accumulation of lignin in TE differentiation of the cultured cells of *Zinnia elegans*. Cells cultured for indicated times (h) in the TE-inducing medium were stained for superoxide and lignin as described in Materials and Methods. A, B, C, D, F and H represent the cells which were stained with NBT in the presence of DDC for detection of superoxide, and E, G and I, the cells which were stained for detection of lignin with phloroglucin-HCl using the same sample after observation of NBT-staining. To confirm DDC effect on the superoxide generation, the cells cultured in the presence (D1) and absence (D2) of DDC in the medium were compared. An arrow (D1) represents the type of cells that was counted as the superoxide-generating cells, arrowheads (D1 and D2) the type of the cells that was not counted as the superoxide-generating cells. To see detailed deposition of formazan, the concentration of DDC was decreased to 0.5 mM in the TE-inducing medium (F and H), and the incubation time with NBT was 20 min. Scale bars: 20 μ m.



Fig. 3. 3. 3.



Relative intensities of phloroglucin staining, lignin deposition

Fig. 3. 3. 4. Deposition of lignin in the secondary cell walls of *Zinnia elegans* cells in the TE-inducing medium. The cells at the indicated times after the start of the incubation were stained by phloroglucin-HCl for 3 min, and the deposition of lignin in the secondary cell walls were evaluated as the red-staining intensities using a Macintosh soft ware (Image 1.61). The numbers in the brackets represent the percentage of the cells having the patterned secondary thickenings to the total cells..

Accumulation time of lignin and generation time of superoxide in one cell — Image analysis on the relation of the degree of deposition of lignin as determined by the intensity of phloroglucin-HCl staining and the number of the cells with patterned secondary thickenings shows that mean degree of lignin deposition was not observed at any time of culture (Fig. 3. 3. 4), indicating rapid deposition of lignin in the secondary cell walls. The maximum rate of the accumulation of the lignin-containing cells was observed between 145 and 146 h, where the superoxide generating cells also were observed at the highest percentage for 20 min (Fig. 3. 3. 2B). During microscopic observation of the mesophyll cells in NBT and DDC the formazan-containing cells successively appeared. Integration number of the superoxide-generating cells deduced for every 20 min was equal to the population of lignin-containing cells, indicating that the time of generation of superoxide anion in a cell (T) is shorter than the 20-min observation time.

Under the present culture conditions about 13% of the cultured cells (r) were differentiated to the lignin-containing cells. $T \times r$ corresponds to the area as indicated by stripe in Figure 3. 3. 2B (S) because the curve represents the rate of numeral change of the superoxide-generating cells to the total cells per time. Therefore, the following equation is given,

$$T = \frac{S}{r}$$

If S is approximated by the peak height (about 0.2%) and width at the half height (about 3 h), S value is about 0.6 h*. Thus, T is estimated to be about 3 min, indicating superoxide is transiently generated at specified time in the course of secondary thickening of the cell wall.

Generation of superoxide by NAD(P)H oxidase in the course of the TE differentiation — I have shown that generation of superoxide in the vascular tissue of spinach hypocotyls is catalyzed by the putative NAD(P)H oxidase (Ogawa et al. 1997a). To show the operation of such generation of superoxide also in the differentiation to TE in the cultured cells of *Zinnia elegans*, I tested the effect of NAD(P)H oxidase specific inhibitor DPI (Auh and Murphy 1995) on the generation of superoxide by following the superoxide-specific chemiluminescence of MCLA (Nakano et al. 1986). Table 3. 3. 1 shows comparison of the intensities of MCLA-dependent chemiluminescence between NBT-reducing cells (146-h culture, Fig. 3. 3. 3D to G) and -nonreducing cells (138-h culture, Fig. 3. 3. 3C). MCLA-dependent chemiluminescence was observed only in the NBT-reducing cells, confirming that the reduction of NBT in *Zinnia* cells was caused by superoxide. DPI inhibited the MCLA-chemiluminescence, indicating the participation of the putative AD(P)H oxidase in the generation of superoxide in *Zinnia elegans*. The MCLA-dependent chemiluminescence was completely inhibited by 50 μ M DPI (Fig. 3. 3. 5), which was

Table 3. 3. 1. Superoxide production of the cultured cells of *Zinnia elegans* as estimated from the MCLA-dependent chemiluminescence. The cultured cells for 138 h and 146 h in the TE-inducing medium were used as NBT-nonreducible cells and -reducible cells. To the cell suspension, 0.5 μM MCLA was added, and the chemiluminescence was determined by a photon counter. MCLA-dependent chemiluminescence by generated superoxide was calibrated for superoxide as described in Materials and Methods.

Cultured cell	Production rate of superoxide [pmol (10 ⁵ cells) ⁻¹ min ⁻¹]	Total superoxide production [pmol O ₂ ⁻ (cell) ⁻¹]
NBT-reducible cell	15.9	0.3 ^c
NBT-reducible cell plus 50 μM DPI ^a	0 ^b	— ^d
NBT-nonreducible cell	0 ^b	— ^d

^aSee details in Fig. 3. 3. 5.

^bBelow 0.1 pmol (10⁵ cells)⁻¹ min⁻¹.

^cDatum was calculated from the following equation:

(Production rate of superoxide) / (The ratio of superoxide-generating cells to total cells at 146 (see Fig. 3. 3. 2)) \times (Time of superoxide generation in a cell) = Total superoxide production a cell;

15.9 [pmol (10⁵ cells)⁻¹ min⁻¹] / 0.0015 \times 3 [min] = 0.3 [pmol O₂⁻ (cell)⁻¹].

^dNot calculated.

similar to the concentration of DPI for the maximum suppression of formation of formazan (data not shown). These observations indicate that NAD(P)H oxidase functions for a short time in the differentiation to TE of *Zinnia elegans* cell as a generator of superoxide as well as in spinach vascular tissue.

Different from the formazan from NBT, the detection of superoxide by MCLA-chemiluminescence was observed even in the absence of the CuZn-SOD inhibitor DDC, probably due to a higher reaction rate of O₂⁻ with MCLA than that with NBT. Since DDC suppressed the MCLA-chemiluminescence due to O₂⁻ by unknown mechanisms, I could not determine the actual generation rate of O₂⁻ under the CuZn-SOD-inhibited conditions in the *Zinnia* cells. The observed rate in Table 3. 2. 1, therefore, should be regarded as the minimum one since the disproportionated O₂⁻ by cellular CuZn-SOD was not detected. Under such limitation, 0.3 pmol O₂⁻ is estimated to be produced for 3 min for the accumulation of lignin in the secondary thickenings of *Zinnia* cells. Assuming that the size of *Zinnia* cells is 20 \times 20 \times 60 μm and the lignin occupies 1% of the cell weight deduced from cell volume, the lignin content in one cell is estimated to be 0.8 pmol on the basis of phenyl propanoid moiety, being the similar level to the total producing amount of O₂⁻. These estimations look like to indicate that the generation of superoxide is the minimum

amounts to polymerize phenyl propanoid precursor for lignin deposition in the secondary thickenings.

Effects of inhibitors on the accumulation of lignin in the secondary cell walls — The detection of superoxide by the formation of formazan depended on the presence of the CuZn-SOD inhibitor DDC (Asada et al. 1975) (Fig. 3. 3. 3D1, D2), which is consistent with the observation in the vascular tissue of spinach hypocotyl (Ogawa et al. 1996b, 1997a). Due to a low reactivity of NBT with O_2^- than that of MCLA the superoxide would have little chance to interact with NBT due to the cellular SOD. To show the actual involvement of CuZn-SOD and the putative NAD(P)H oxidase in the biosynthesis of lignin, the effects of respective inhibitors on the deposition of lignin in the patterned secondary thickenings were investigated. Either DDC or DPI was added to the 138-h culture cells, i. e., before the start of the superoxide generation (Fig. 3. 3. 3C), and then the populations of the lognin-containing cells were counted in the cells of the 146-h culture. The deposition of lignin in the cells with secondary cell walls was absolutely suppressed in the presence of either DDC or DPI, whereas the cells cultured in the absence of them could accumulate lignin (Fig. 3. 3. 6), being $56 \pm 5.1\%$ (mean \pm SD, $n=200$) of the cells with patterned secondary cell walls. These observations support further our proposal that CuZn-SOD is required for the biosynthesis of lignin and the superoxide generated by NAD(p)H oxidase is the source of hydrogen peroxide for the biosynthesis of lignin in *Zinnia* cells.

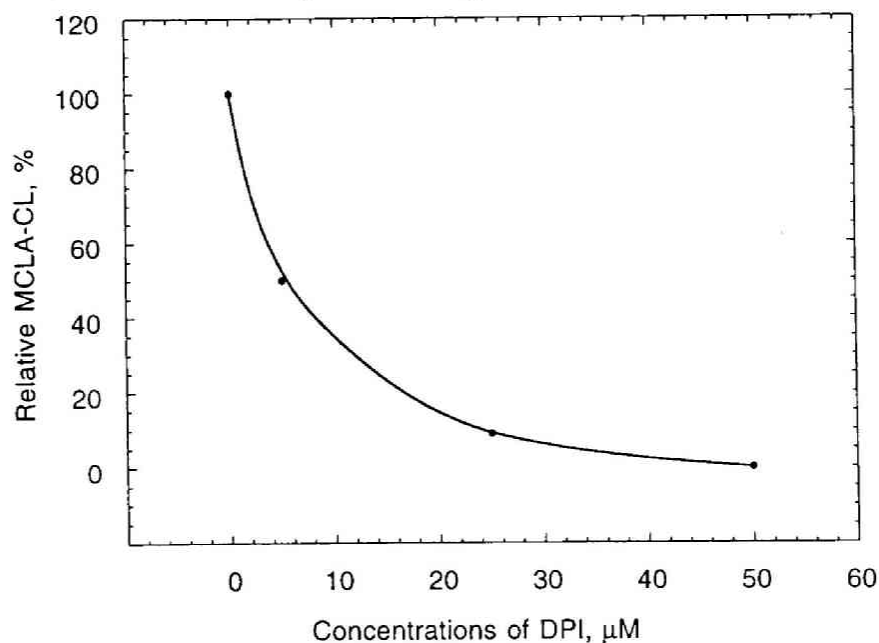


Fig. 3. 3. 5. Effect of the NAD(P)H oxidase inhibitor DPI on the generation of superoxide in the cultured cells of *Zinnia elegans* as observed by the MCLA-chemiluminescence. To the cells cultured for 146 h in the TE-inducing medium were added 0.5μ M MCLA and DPI at indicated concentrations, and the superoxide-dependent chemiluminescence was determined using a photon counter. The addition of 1μ M Mn-SOD did not affect the intensity of the chemiluminescence in the absence of DPI, indicating that the superoxide was generated at the site where Mn-SOD could not interact.

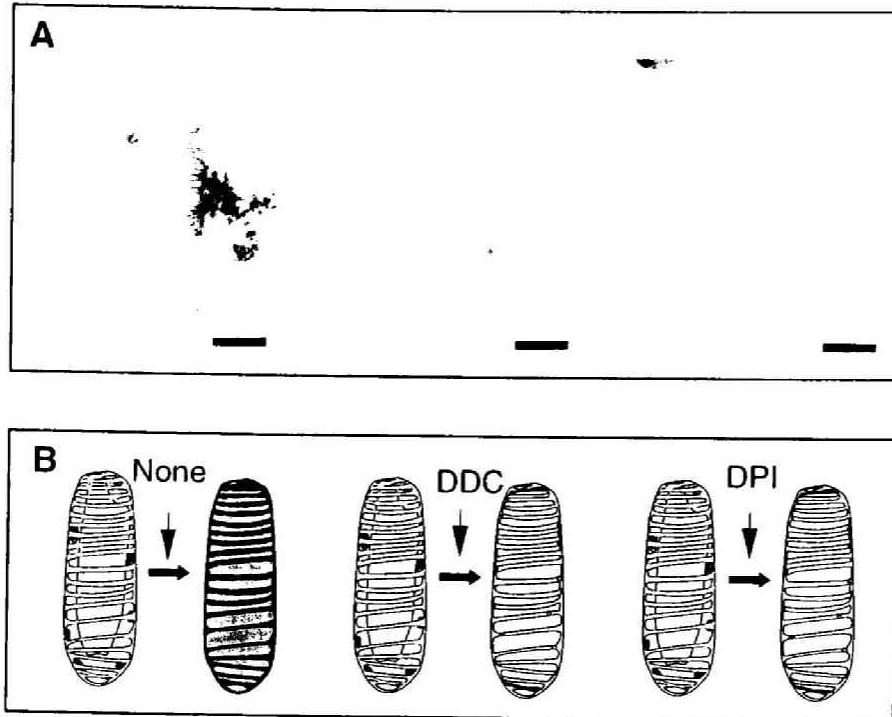


Fig. 3.3.6. Effects of inhibitors of CuZn-SOD (DDC) and NAD(P)H oxidase (DPI) on the accumulation of lignin in the patterned secondary cell walls of the cultured cells of *Zinnia elegans*. Either 5 mM DDC or 50 μ M DPI was added to the 138-h-cultured cells in the TE-inducing medium, but the control cells (none) were cultured without any addition. After 8-h-culture, the cells were stained by phloroglucin-HCl for lignin. The typical cells cultured for 146 h of each treatment are shown in (A). Effect of the inhibitors on the lignification is schematically illustrated in (B). Scale bars: 20 μ m.

Concluding remarks — I here showed the stage-specific, transient generation of superoxide during the differentiation of TE in the cultured *Zinnia elegans* cells as observed by accumulation of lignin, confirming that the generation of superoxide in the tracheary elements is regulated by the program of differentiation into TE. Such generation of superoxide is operated by the putative NAD(P)H oxidase in *Zinnia* cells. Furthermore, the effects of the inhibitors of CuZn-SOD and NAD(P)H oxidase on the deposition of lignin in the cell walls demonstrate that the superoxide generated by NAD(P)H oxidase is used as the source of hydrogen peroxide for the biosynthesis of lignin and CuZn-SOD actually contributes to the supply of hydrogen peroxide from superoxide.

In the vascular tissue of spinach hypocotyls, the CuZn-SOD inhibitor blocks the formation of hydrogen peroxide (Ogawa et al. 1997a), suggesting that generated superoxide would disappear through its interaction with cellular components if SOD were inactive. However lignin precursor coniferyl alcohol was almost unreactive with superoxide (data not shown), indicating that other oxidants of superoxide are present in apoplast. When hydrogen peroxide was supplied via the disproportionation of superoxide produced in the xanthine-xanthine oxidase system, the peroxidase-

dependent oxidation of coniferyl alcohol was accelerated by SOD, even at pH 5.5 which is the apoplastic pH (data not shown). This fact reflects that even under such low pH in apoplast, peroxidase would be inhibited by superoxide due to the formation of Compound III which is the inactive form of peroxidase, if SOD were inactive. Therefore, the SOD-catalyzing disproportionation of superoxide to hydrogen peroxide is indispensable for the efficient biosynthesis of lignin.

The present results raise several interesting questions to be solved. The first is how the transient generation of superoxide is triggered and regulated during the course of differentiation to TE. Elicitor-inducing generation of superoxide is initiated by the activation of putative NAD(P)H oxidase, but not by regulation of supply of NAD(P)H (Kauss and Jeblick 1995). However, the primary triggering factor for the generation of superoxide is remained to be determined. Secondly, if hydrogen peroxide is required for biosynthesis of lignin by peroxidase, the divalent oxidases such as glycolate oxidase and glucose oxidase apparently are suitable, since they produce hydrogen peroxide as the primary product. Why do plants use the superoxide-generating oxidase such as NAD(P)H oxidase for the biosynthesis of lignin? In this respect, it should be noted that exogenous Mn-SOD delayed the TE differentiation, but increased the ratio of the TE differentiated cells in the cultured cell of *Zinnia elegans* (Ogawa K. et al. unpublished). The above observation seems to indicate that a trace of superoxide functions as anything else other than the source of hydrogen peroxide for the lignin biosynthesis during the TE differentiation.

Chapter 4 Summaries

Summary of 2.1

In chloroplasts O_2^- is photoproducted via the univalent reduction of O_2 in PS I even under conditions that are favorable for photosynthesis. The photogenerated O_2^- is disproportionated to H_2O_2 and O_2 in a reaction that is catalyzed by superoxide dismutase (SOD). The H_2O_2 -scavenging ascorbate peroxidase is bound to the thylakoid membranes at or near the PS I reaction center [Miyake and Asada (1992) *Plant Cell Physiol.* 33: 541-553], and the primary product of oxidation in the peroxidase-catalyzed reaction, the monodehydroascorbate radical, is photoreduced to ascorbate in PS I in a reaction mediated by ferredoxin [Miyake and Asada (1994) *Plant Cell Physiol.* 35: 539-549]. Therefore, SOD should be localized at or near the PS I complex. We report here the microcompartmentalization of the chloroplastic CuZn-SOD on the stromal-faces of thylakoid membranes where the PS I-complex is located. Spinach leaves were fixed and substituted by a rapid freezing and substitution method that allows visualization of intact chloroplasts. The embedded sections were immuno labeled with an antibody against CuZn-SOD by the immuno-gold method. About 70% of the immunogold particles were found within 5 nm from the surface of the stromal-faces of thylakoid membranes. Of these particles, about 40% were found at the ends and margins of the grana thylakoids and 60% were found on the stromal side of the stromal thylakoids. From these results, the local concentration of CuZn-SOD on the stroma-facing surfaces of the thylakoid membranes was estimated to be about 1 mM. The effect of the microcompartmentalization of CuZn-SOD on the scavenging of superoxide radicals is discussed.

Summary of 2. 2

I have shown the attachment of chloroplastic CuZn-superoxide dismutase (chlCuZn-SOD) to the site of the generation of O_2^- (PS I) in spinach chloroplasts in Chapter 2. 1 (*Plant Cell Physiol.* 36: 565-573, 1995). We demonstrated the involvement of Mg^{2+} in such attachment by the binding assay of CuZn-SOD to the thylakoid membranes (*Plant Cell Physiol.* 37: s79, 1996). Then, to evaluate the effect of localization of CuZn-SOD, the wild type (SR I) and transgenic tobacco which overproduces mitochondrial Mn-SOD in chloroplasts (ov-Mn) were transformed, respectively, into transgenic tobaccos harboring the chloroplastic CuZn-SOD (an-CuZn and ov-Mn,an-CuZn, respectively). No phenotype difference between SR I and an-CuZn was observed under low-light conditions, whereas leaves of an-CuZn were bleached under high-light conditions. Furthermore, ov-Mn,an-CuZn, which has Mn-

SOD but no CuZn-SOD within the chloroplasts, also was subject to photodamage under high-light conditions. Then, PSI was more damaged than PSII was, indicating that the introduced Mn-SOD failed its targeting to the functioning site of CuZn-SOD in chloroplasts. The above observations demonstrate an importance of the localization of SOD at the site of superoxide generation within the chloroplasts in the protection from photodamage.

Summary of 3. 1

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 formazan 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.

Summary of 3. 2

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-I₂ 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.

Summary of 3. 3

The time course of generation of superoxide during the differentiation of the tracheary element in cultured cell of *Zinnia elegans* was studied as the formazan formation from nitroblue tetrazolium and the superoxide-specific chemiluminescence. Superoxide was transiently generated only at the period of the accumulation of lignin in the secondary cell walls. Formation of formazan was enhanced by the CuZn-superoxide dismutase (SOD) inhibitor, but generation of superoxide was suppressed by the NAD(P)H oxidase inhibitor. No accumulation of lignin was observed in the presence of either the CuZn-SOD inhibitor or the NAD(P)H oxidase inhibitor. These observations indicate that the generation of superoxide in xylematic cells is one of stage-specifically regulated events during the differentiation to the tracheary element formation and that the superoxide generated by NAD(P)H oxidase is the source of hydrogen peroxide for lignification by means of its catalytic disproportionation with CuZn-SOD.

Chapter 5 Conclusion

Microcompartmentation of CuZn-SOD — In the present thesis, I demonstrated the microcompartmentation of CuZn-SODs and revealed their physiological role in protection from excess photon energy in plant cells. In chloroplasts, CuZn-SOD is turned out to be localized at the site of superoxide generation on the thylakoid membranes by means of its interaction with Mg^{2+} . Transgenic experiments confirmed the significance of the microcompartmentation of CuZn-SOD on the protection of PSI from photodamages in chloroplasts. The scavenging system of photoproduced O_2^- and H_2O_2 on the thylakoid membranes is proposed as shown in Fig 5. 1, taking the results of other researchers into an consideration.

Several enzymes of the CO_2 -fixation cycle in the stroma are sensitive to H_2O_2 as described in Chapter 1. It has been shown that ribulose-1,5-bisphosphate carboxylase, ferredoxin-NADP reductase and the H_2O_2 -sensitive enzymes, except for fructose-1,6-bisphosphatase, form a multienzyme complex on the thylakoid membranes (Süss et al. 1993, 1995). The compartmentalization of the CO_2 -fixation enzymes on the thylakoid membranes would be very effective for operation of the cycle by the photoproduced NADPH and ATP, but the complex confronts the risk of inactivation by the active species of oxygen photogenerated at the same site. The microcompartmentalization of the scavenging enzymes on the surface of thylakoid membranes might be essential to reduce such a risk.

On the other hand, major "cytosolic" CuZn-SOD is revealed to be extracellularly distributed and mainly localized in the secondary thickenings of cell walls for the supply of hydrogen peroxide in lignin biosynthesis. Apoplast-localized CuZn-SOD are ionically bound to the plasma membranes or the cell walls. Oxidation of lignin precursors by peroxidase is inhibited by superoxide and facilitated by SOD. Furthermore, the inhibitor of CuZn-SOD suppresses the generation of hydrogen peroxide from superoxide that is generated by NAD(P)H oxidase, but exogenous Mn-SOD did not affect such generation in the presence of the CuZn-SOD inhibitor. These observations in the present thesis suggest that CuZn-SOD is localized at the site of superoxide generation where exogenous Mn-SOD is inaccessible. This observation also indicates a significance of the microcompartmentation of SOD.

The above observation may indicate a reason why the plants have many isoforms of SODs even though their enzymatic properties are similar. There are little information on the mechanism for such microtargeting to the fatal site of localization of each SOD, which remains to be elucidated by future studies. At least, from the present study, ionic weak interactions with the membrane plays a role in the "targeting" of CuZn-SODs to the vicinity of either the PS I complex or NADP(H) oxidase.

In this respect, it is of interest to note that in humans, a point mutation in CuZn-SOD causes amyotrophic lateral sclerosis (Deng et al. 1993). The mutation is not in the active-site domain and the mutant SOD retains activity (Gurney et al. 1994) and transgenic mice overexpressing the mutant SOD exhibits phenotypical similarity to amyotrophic lateral sclerosis in spite of no decrease of CuZn-SOD contents in the organs (Ripps et al. 1995). Down regulation of the CuZn-SOD by antisense method results in the amyotrophic lateral sclerosis (Troy et al. 1994). It seems, therefore, likely that the mutation in domains other than the reaction center disturbs proper "targeting" of soluble CuZn-SOD in nervous cells.

Operation mechanism for generation of superoxide in lignification — From the effects of inhibitors of CuZn-SOD, peroxidase and NAD(P)H oxidase on generations of superoxide and hydrogen peroxide and deposition of lignin, a mechanism schematically shown in Figure 5. 2 has been established for the supply of hydrogen peroxide at the final step of lignin biosynthesis. Generation time of superoxide in a tracheary element-differentiating cell is so short that the activation and inactivation mechanisms for the production of superoxide should be operated in the cells. Oxidative burst mechanism has been intensively studied, and several lines of evidence for its signal transduction mechanism have been proposed as described in Chapter 1. The mechanism of the oxidative burst has many similarities to the superoxide generation for lignin biosynthesis in the tracheary element-differentiating cells, and might be similar to that of mammalian phagocyte NAD(P)H oxidase. However, this mechanism remains to be elucidated by future studies.

Conclusion — Dioxygen is very essential to aerobes as an very effective electron acceptor to produce ATP, and as a substrate for the metabolite production via oxidases and oxygenases. However, these essential functions are operative only under the conditions where the oxygen toxicity to organisms is suppressed by the defense mechanism acquired during the evolution. As shown in the present thesis, plants also are not an exception. They operate mechanisms for protection from and utilization of active oxygen generated from dioxygen with a good balance. The facts to be noted in the mechanisms established in the present thesis, are the microcompartmentation of soluble enzymes at the site of production of the substrate for protection from and utilization of generated active oxygens. I hope that the present study will contribute not only to the genetic engineering of stress-tolerant plants but also to solving such a hereditary diseases as amyotrophic lateral sclerosis. In addition to it, I hope the present study will help researches concerning lignin biochemistry, bioengineering of lignin contents in woods and regulation of active oxygens during such differentiation of the plant cells as tracheary element differentiation.

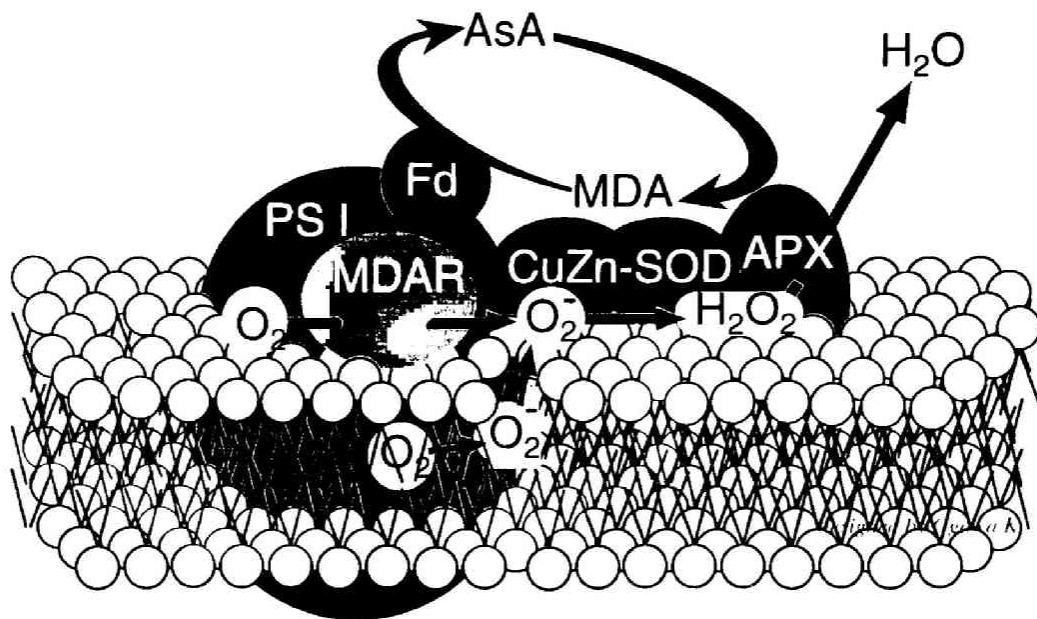


Fig. 5. 1 Thylakoidal scavenging system of superoxide and hydrogen peroxide in chloroplasts. The O_2^- photoproduced within the PS I complex is ejected to the surface of the thylakoid membranes and is disproportionated by the membrane-attached superoxide dismutase (SOD). The H_2O_2 produced via the SOD-catalyzed reaction is reduced by ascorbate (AsA) to water catalyzed with the membrane-bound ascorbate peroxidase (APX). The primary product of the APX-reaction, the monodehydroascorbate radical (MDA), is reduced to AsH by the photoreduced ferredoxin (Fd) in PSI. All of the scavenging enzymes seem to be compartmented in the vicinity of the PSI complex within 5 nm layer on the surface of the thylakoid membrane.

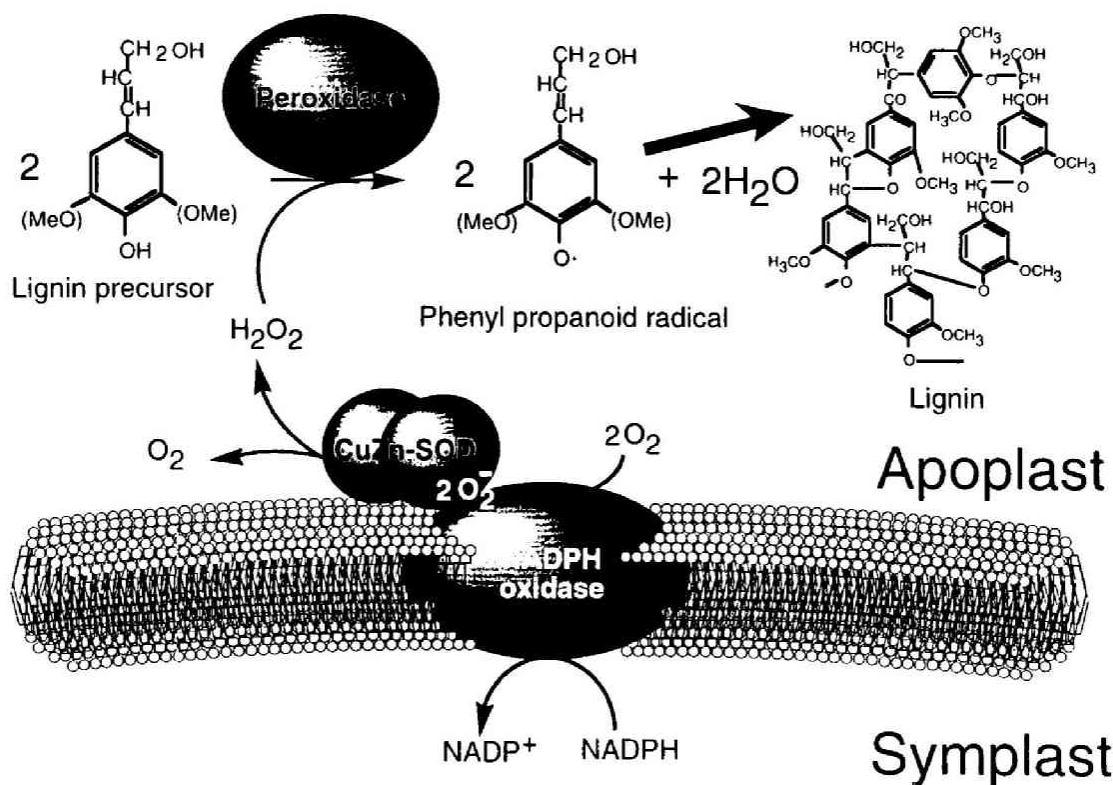


Fig. 5. 2. A proposed scheme for supplying hydrogen peroxide to lignin biosynthesis (Ogawa et al 1997).

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