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Development of Environmental Stress-Tolerant Plants by Gene Manipulation of Antioxidant Enzymes

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Oxidative stress is one of the major limiting factor in plant productivity. Reactive oxygen species (ROS) generated during metabolic processes damage cellular functions and consequently lead to disease, senescence and cell death. Plants have evolved an efficient defense system by which the ROS is scavenged by antioxidant enzymes such as superoxide dismutase (SOD) and ascorbate peroxidase (APX). Attempts to reduce oxidative damages under the stress conditions have included the manipulation of ROS scavenging enzymes by gene transfer technology. Increased SOD activities of transgenic plants lead to increased resistance against oxidative stresses derived from methyl viologen (MV), and from photooxidative damage caused by high light and low temperature. Transgenic tobacco plants overexpressing APX showed reduced damage following either MV treatment of photooxidative treatment. Overexpression of glutathione reductase (GR) leads to increase in pool of ascorbate and GSH, known as small antioxidant molecules. These results indicate that the manipulation of antioxidant system in plant through overexpression of enzymes involved in ROS-scavenging could maintain or improve the plant productivities under environment stress condition. In this study, the rational approaches to develop stress-tolerant plants by gene manipulation of antioxidant enzymes will be introduced to provide solutions for the global food and environmental problems in the 21st century.

Keywords **:** antioxidant enzyme, superoxide dismutase, ascorbate peroxidase, dehydroascorbate reductase, multiple expressions, stress-inducible promoter.

Oxygen is essential for the existence of aerobic life, but toxic reactive oxygen species (ROS), which include the superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2), are generated in all aerobic cells during metabolic processes (Foyer et al., 1994; Asada, 1999). Injury caused by these ROS is known as oxidative stress, which is one of the major damaging factors to plants exposed to environmental stress. Chloroplast is the most sensitively damaged organelle by ROS because electrons escaped from the photosynthetic electron transfer system are to react with relatively high concentration of O_2 in chloroplast. This phenomenon can lower rates of photosynthesis and diminish plant growth. Plants possess capabilities to cope with oxidative stress by scavenging ROS using antioxidants such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbic acid (AsA, reduced form), glutathione and flavonoids.

It is important to maintain and/or increase the productivity (photosynthetic capacity) under stressful environment by developing plants that have well adapted to environmental stress through manipulating antioxidant system in chloroplast. One of the well-known mechanisms, that how the antioxidants work properly at the onset of oxidative stress is the water-water cycle (Asada, 1999; Fig. 1). The most important function of this cycle is a rapid, immediate scavenging of superoxide anion radical and hydrogen peroxide at the site of generation prior to their interaction with target molecules. SOD, APX (thylakoid-bound and stroma), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) were participated in this cycle. However this antioxidative mechanism seems to be not enough to protect plants from the elevated environmental stresses. To maintain the productivity of plants under the stress condition, it is important to fortify the antioxidative mechanism of the chloroplasts by manipulating the antioxidant enzymes and small antioxidant molecules in the chloroplast.

Transgenic plants overexpressing single transgene of SOD, APX, and GR separately in chloroplast or other com-

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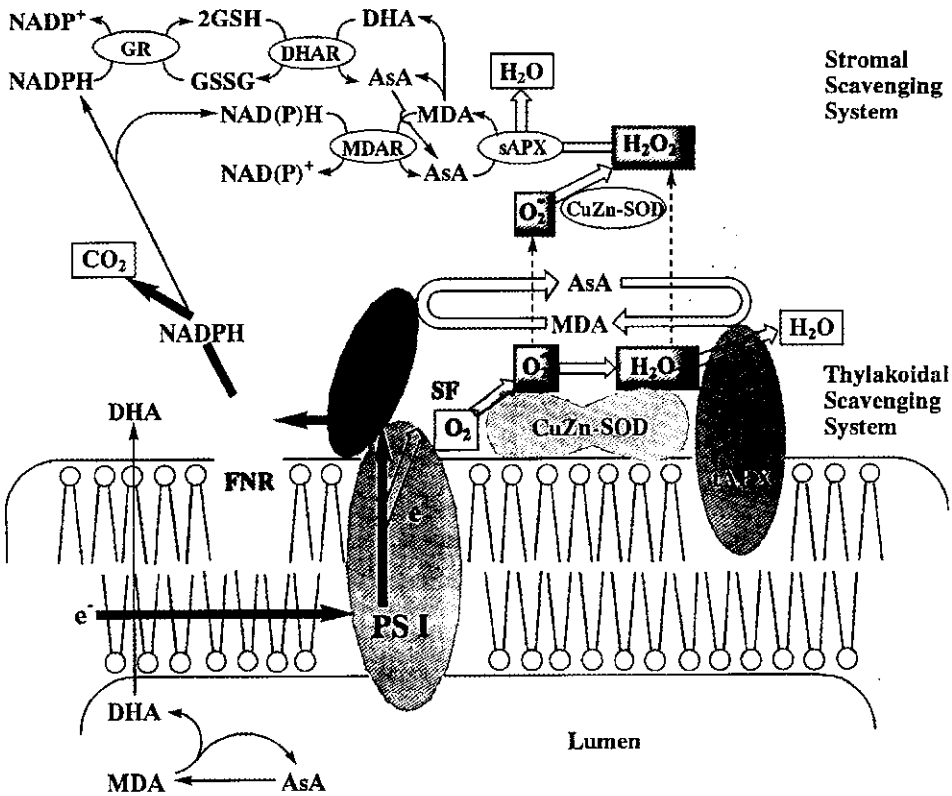

Fig. 1. The water-water cycle and microcompartmetalization of the participating enzymes (Asada, 1999).

partment of plant cell were generated and displayed increased tolerance against the oxidative stress (Table 1). It seems that multiple expressions of tolerance genes in target organelle and their efficient regulation of expressions using a stress-inducible promoter are required for the ideal transgenic plants with enhanced tolerance to environmental stresses.

Expression of SOD in transgenic plants. The SOD (EC 1.15.1.1) is a metalloprotein that catalyzes the initial step in the water-water cycle in chloroplasts, the dismutation of superoxide to H_2O_2 and molecular oxygen (Scandalios, 1993; Bowler et al., 1994; Allen, 1995; Asada, 1999). The subsequent reduction of H_2O_2 to water through the cycle in the chloroplast uses reducing equivalents from NADPH (Foyer et al., 1994). SOD enzymes are classified according to their metal cofactor and their subcellular localization. The predominant forms are a mitochondrial MnSOD, a cytosolic CuZnSOD, and a chloroplastic CuZnSOD. In a number of plant species, chloroplasts also contain FeSOD. The four forms of SOD differ in their biochemical properties and inhibition by H_2O_2 and cyanide (Scandalios, 1993; Bowler et al., 1994; Allen, 1995).

Different SODs have been expressed in transgenic plants, but the results vary (Scandalios, 1993; Foyer et al., 1994; Allen, 1995). For example, Pitcher et al. (1991), Tepperman and Dunsmuir (1990), and Payton et al. (1997) found no improvements, whereas Sen Gupta et al. (1993a, 1993b), Bowler et al. (1991), Van Camp et al. (1994, 1996), Perl et al. (1993), and McKersie et al. (1993, 1996, 1999, 2000) found significant improvements to oxidative or environmental stress tolerances. This difference has usually been attributed to the complexity of the detoxification system of ROS, because changing one enzyme may not change the capacity of the pathway as a whole.

Transgenic tobacco plants expressing a pea CuZnSOD in the chloroplasts were more tolerant to photooxidative stress and methyl viologen (MV, paraquat), an ROS-generating chemical (Sen Gupta et al., 1993a; Sen Gupta et al., 1993b). McKersie et al. (1996, 1997) reported that transgenic alfalfa (*Medicago sativa* L.) plants expressing an MnSOD had increased vigor after freezing stress and increased winter survival under field conditions. However, tolerance of freezing stress measured at the cellular level by electrolyte leakage or by vital staining with tetrazolium was not affected by elevated level of introduced MnSOD activity in these alfalfa plants. They argued that this improvement was caused by enhancement of the overall stress-defense system by the peroxide produced, as the hydrogen peroxide has been shown to elicit several stress-tolerance-conferring genes.

Another of the SOD isoenzymes, FeSOD from *Arabidopsis*, has also been expressed in tobacco (Van Camp et

Table 1. Expression of antioxidant genes in transgenic plant (Modified from Allen, 1997)

Gene constructs	Host plant	Reported phenotype	Reference
SOD			
Chloroplastic	Tobacco	No protection from MV or ozone	Tepperman and Dunsmuir (1990)
CuZnSOD	Tobacco	Reduced MV damage and photoinhibition	Sen Gupta et al. (1993a, 1993b)
	Potato	Reduced MV damage	Perl et al. (1993)
MnSOD	Tobacco	Reduced MV damage and no protection from photoinhibition	Slooten et al. (1995)
	Alfalfa	Reduced acifluorfen, freezing, and water-deficit damage	McKersie et al. (1996, 1997)
FeSOD	Alfalfa	Modified regulation of photosynthesis at low CO_2	McKersie et al. (2000)
	Maize	Enhanced tolerance to MV	Van Breusegem et al. (1999)
Mitochondrial	Tobacco	Reduced MV damage in the dark	Bowler et al. (1991)
MnSOD	Alfalfa	Reduced freezing and water-deficit damage	McKersie et al. (1996)
Cytosolic	Potato	Reduced MV damage	Perl et al. (1993)
CuZnSOD			
APX	Tobacco	Reduced MV damage and photoinhibition	Allen (1997)
Cytosolic	Tobacco	Reduced MV damage and photoinhibition	Allen (1997)
Chloroplastic	Tobacco	Reduced MV damage	Yun et al. (2000)
POD			
GR	Tobacco	Reduced MV and SO_2 damage but not O_3	Aono et al. (1993)
E. coli			
Chloroplastic	Poplar	Reduced photoinhibition	Foyer et al. (1995)
Pea GR	Tobacco	Reduced O_3 damage, variable results with MV	Broadbent et al. (1995)
GST/GPX	Tobacco	No tolerant to MV, tolerant to salt and chilling	Roax et al. (1997, 2000)
Ferritin	Tobacco	Tolerant to MV and pathogens	Deák et al. (1999)

al., 1996). When targeted to the chloroplast, this enzyme protected both the plasmalemma and photosystem II against superoxide generated during illumination of leaf dics treated with MV by scavenging radicals. However, when the FeSOD gene was introduced into alfalfa, the improvement of winter survival it was not correlated with the increased level of FeSOD (McKersie et al., 2000). They suggest that Fe SOD overexpression reduced secondary injury symptoms and thereby enhanced recovery from stresses experienced during winter.

Expression of APX in transgenic plants. The H_2O_2 produced via the disproportionation of O_2^- catalyzed with SOD is reduced to water by APX (E.C. 1.11.1.11), which use ascorbate as the electron donor in chloroplasts. APX is a heme peroxidase, and uses two molecules of ascorbate to reduce H_2O_2 to water, with the generation of two molecules of monodehydroascorbate (MDHA). It distributed in at least four distinct cell compartments, the stroma (sAPX) and thylakoid membrane (tAPX) in chloroplasts, the microbody (mAPX), and the cytosol (cAPX) (Miyake and Asada, 1992; Asada, 1999; Yoshimura et al., 2000). In addition, Zhang et al. (1997) identified an APX associated with the glyoxysomal membranes.

Allen et al. (1997) reported that transgenic tobacco plants expressing gene constructs for either cytosolic APX or a chimeric chloroplast-targeted cytosolic APX from pea have increased protection against MV-mediated membrane damage compared with untransformed control plants. These transgenic plants had the increased protection from photooxidative stress (exposure to high light intensity and chilling temperature for 4 h). These results seem to indicate that increased scavenging of H_2O_2 in either chloroplasts or the cytosol can reduce oxidative stress in chloroplasts.

Peroxisomes are one major source of ROS in plant cells. When a putative peroxisomal membrane-bound APX from *Arabidopsis* was expressed in tobacco plants, the transgenic plants had more protection against oxidative stress damage caused by aminotriazole that inhibits catalase activity that is found mainly in glyoxysomes and peroxisomes and leads to accumulation of H_2O_2 in those organelles (Wang et al., 1999).

Expression of other enzymes in transgenic plants. In the water-water cycle, it is necessary that maintaining the contents of small molecular antioxidants such as ascorbate and glutathione, that are maintained by enzymes such as GR, DHAR and MDHAR, for immediate scavenging the generated ROS by SOD and APX. The transgenic plants overexpressing GR have higher ascorbate contents and improved tolerance to oxidative stress (Foyer et al., 1991; Aono et al., 1993; Foyer et al., 1995). On the other hands, reduced GR activity resulted in increased stress sensitivity (Aono et al., 1995). Ascorbate-deficient mutant of *Arabidopsis* is sensitive to oxidative stress such as UV and pollut-

ants (Conklin et al., 1997).

Ascorbate acts as an important antioxidant in both enzymatic and non-enzymatic (reacting directly with hydroxyl radicals, superoxide, and singlet oxygen) reactions in plant cells. Some of the monodehydroascorbate (MDHA), oxidized form of ascorbate, is re-reduced by MDHAR using NAD(P)H, but the remainder undergoes spontaneous dismutation to AsA (reduced ascorbate) and dehydroascorbate (DHA, oxidized ascorbate). DHAR (EC 1.8.5.1) catalyzes the re-reduction of DHA to AsA with simultaneous oxidation of GSH to GSSG. Thus, DHAR, as well as MDHAR, is critical for protection of cellular components against oxidative injury (Asada, 1999). Also, DHAR activity is enhanced in response to various environmental stresses (Urano et al., 2000). The DHAR-overexpressing tobacco plants have been recently developed in our laboratory and have elevated level of tolerance to oxidative stress derived from various sources (Ahn et al., 1999; Kwon et al., 2000).

Roxas et al. (1997, 2000) showed enhanced seed germination and seedling growth under stressful condition by expressing plant glutathione-S transferase/glutathione peroxidase (GST/GPX). In this transgenic plant, increased glutathione-dependent peroxide scavenging and alterations in glutathione and ascorbate metabolism lead to reduced oxidative damage achieved these stress tolerances.

The most harmful ROS, the hydroxyl radical, is produced by Fenton reaction, in which hydrogen peroxide and free Fe^{2+} are involved. Because intracellular iron catalyzes oxidative reactions, the control of free iron could be a potential way to reduce oxidative damage. Deák et al. (1999) reported that the transgenic plants expressing alfalfa ferritin, an iron-binding protein, showed retained photosynthetic function under oxidative stress and tolerance to pathogens.

Multiple expressions of antioxidant genes. Several reports have shown that drought, salt, and freezing stress are also accompanied by the formation of ROS (Holmberg and Bülow, 1998). The expressions of a CuZn SOD gene from cassava and peroxidase (POD) genes from sweet potato were increased by various stresses such as MV, ozone, and chilling treatment (Huh et al., 1997; Lee et al., 1999; Kim et al., 1999). Evidence for this is that freezing- and salinity-tolerant plants also have well-developed antioxidant defenses, and by pretreating plants with one form of stress is often possible to increase the tolerance to a different stress factor.

Aono et al. (1995) showed increasing oxidative stress tolerance by expression of GR and CuZnSOD together in the cytosol of transgenic tobacco. The genes encoding these enzymes were derived from *E. coli* and rice, respectively. The plants expressing both GR and CuZnSOD exhibited less damage than GR or CuZnSOD transgenic plants. This result indicates that the expression of combinations of antioxidant enzymes in transgenic plants may have synergistic effects on stress tolerance.

We also developed transgenic tobacco plants expressing both SOD (CuZnSOD or MnSOD) and APX in chloroplasts by double transformation. The simultaneous expression of SOD and APX provided much better protection from MV-mediated oxidative stress than single expression of SOD or APX in the leaf disc level, showing the additive effect of two enzymes in ROS scavenging activity (Kwon et al., 1999). When the MV solution was sprayed on the leaves of seedling, the transgenic plants expressing both SOD and APX showed higher resistance to MV and recover quickly from the MV-induced damage. These results indicate that transgenic plants expressing both SOD and APX have higher ROS scavenging activity than transgenic plants expressing either SOD or APX.

Development of extreme tolerance of plants may be achieved by introducing genes from different stress resistance into a single plant. That is, introduction of genes for osmoprotectant-production, heat-shock protein, related membrane fluidity, and ROS scavenging enzyme in a plant by multiple transformation or by crossbreeding plants containing different stress-tolerant genes, could contribute to overcome the various abiotic stresses. But it is paramount importance to target the location, control the level and time of expression, and ensure precursor availability for each enzyme in order to avoid negative effects.

Stress-inducible promoters. A strong constitutive promoter such as CaMV 35S promoter was usually used for expression of foreign genes in plants. But more precise regulation of expression using inducible promoter, especially stress-inducible promoter, might be useful for production of proteins that have deleterious effects on plant growth (Yoshida and Shinmyo, 2000). In fact, *rd29A* promoter was used for expression of stress-inducible transcription factor, of which expression caused retardation of the plant growth if it is regulated by CaMV35S promoter (Kasuga et al., 1999).

We characterized an oxidative stress-inducible POD promoter (*SWPA2* promoter) from suspension cultures of sweet potato (Kim, 2000; Kwak et al., 2000), of which expression was induced by various oxidative stresses such as hydrogen peroxide, UV irradiation, and wounding in transgenic plant carrying GUS as reporter. In this respect, *SWPA2* promoter is very useful for the tight regulation of expression of antioxidant gene in transgenic plants and mass production of useful components including pharmaceutical protein in transgenic cultured cells and plants.

Conclusion and Prospects. The ROS, especially hydrogen peroxide, have been proved as a central component of plant adaptation to biotic and abiotic stresses (Mittler et al.,

1999; Karpinski et al., 1999). Under the stress conditions, ROS may play two very different roles: damaging the cellular components or signaling the activation of defense responses. Such a dual function was first described in pathogenesis but has recently been demonstrated during several abiotic stress responses. To allow for these different roles, cellular levels of ROS must be tightly controlled. In first, precise understanding the roles of each ROS scavenging enzyme and small molecular antioxidants in stress adaptation and accurate characterization of the complex stress tolerance phenotypes is necessary to develop stress tolerant plants.

We are trying to develop ideal transgenic plants with enhanced tolerance to environmental stress by expression of multiple antioxidant enzymes in target organelle of a plant cell under the control of stress-inducible promoter.

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