The 13th Symposium on Plant Biotechnology **13**: 79-84, 1999.
1999 Korea-Japan Join Symposium.

Manipulation of Antioxidative Mechanism in Chloroplasts

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Abstracts

Oxidative stress is one of the major environmental stresses to plants. Reactive oxygen species (ROS) generated during metabolic processes damage cellular functions and consequently lead to cell death. Fortunately plants have *in vivo* defense system by which the ROS is scavenged by enzymes such as superoxide dismutase (SOD) and ascorbate peroxidase (APX). In attempts to understand the protection mechanism of plant against oxidative stress, we developed transgenic tobacco (*Nicotiana tabacum* cv. Xanthi) plants that expressed both SOD and APX in chloroplast using *Agrobacterium*-mediated transformation and evaluated their protection capabilities against methyl viologen (MV, paraquat) -mediated oxidative damage. Three double transformants (CA1, CA2, and CA3) expressed the chimeric CuZnSOD and chimeric APX in chloroplast, and one transformant (AM) expressed the chimeric APX and chimeric MnSOD in chloroplast. In addition, we obtained three lines of transformants (C/A1, C/A2, and A/C) that expressed the APX, CuZnSOD, and MnSOD by crossing. These multiple transformants have higher enzyme activities of APX and SOD than control plants, and more resistant to oxidative stress caused by MV. Transformants (C/A and A/C) overexpressing MnSOD, CuZnSOD and APX at the same time showed the highest resistance to MV-mediated oxidative stress among the transformants.

Oxygen is essential for the existence of aerobic life, but toxic oxygen species, 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. Injury caused by these reactive oxygen species (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, glutathione and phenolic compounds.

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 (Figure 1) [3]. 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 stromal), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), 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 were generated and displayed increased tolerance against the oxidative stress (Table 1). In this report, transgenic tobacco plants expressing SOD (CuZnSOD and MnSOD) and APX simultaneously in chloroplast were developed and their protection effects against oxidative stress induced by MV were investigated. This is the first report that generation of oxidative stress resistant plants using multiple transgenes.

Transgenic tobacco plants expressing both SOD and APX

The tobacco transgenic plants simultaneously expressing SOD and APX in chloroplast were generated by double transformation using plant expression

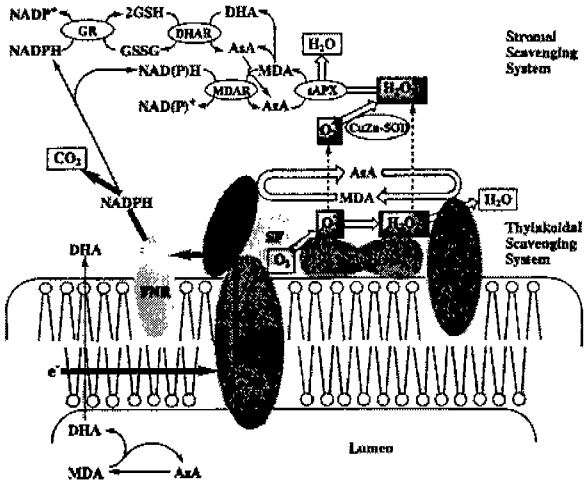

Figure 1. The water-water cycle and microcompartmentalization of the participating enzymes (Asada, 1999).

vectors with a different selection marker [kanamycin resistance (pCGN1578 or pBIN19) and basta resistance (pGPTV-Bar)] with the control of CaMV 35S promoter. Double transformants expressing both CuZnSOD and APX (designated as CA plants) were generated by transformation of the transgenic plants containing the chimeric chloroplast-targeted CuZnSOD [16] with chimeric chloroplast-targeted APX construct [1] using bialaphos as a selection agent. Transgenic plants expressing APX and MnSOD (designated as AM plants) were also developed by transformation of the transgenic plants containing the chimeric chloroplast-targeted APX with chimeric chloroplast-targeted MnSOD construct [15] using bialaphos as a selection agent. In addition, we obtained transgenic plants that expressed two kinds of SODs (CuZnSOD and MnSOD) as well as APX in the chloroplast (designated as C/A and A/C plants) by crossing CA and AM plants.

The isozyme patterns of SOD (Figure 2A, B) and APX (Figure 3A, B) in the leaves of the transgenic plants (CA, AM, C/A and A/C plants) were same as the band pattern of CuZnSOD, MnSOD, and APX transgenic plants in native gel assay. The specific activities of the SOD and APX were well corresponding to the native gel assay. The specific activities of SOD and APX in transgenic plants expressing the SOD and APX (CA, AM, C/A, A/C) were much higher than in the transgenic plants expressing SOD (CuZnSOD, MnSOD) or APX alone, respectively.

Protection of membrane damage in transgenic plants

MV treatment on leaf disks have been used for the test of tolerance to oxidative stress [6, 16]. The extent of cellular damage was quantified by solute leakage, which is a measure of membrane disruption. The membrane damage was checked by measuring the

Table 1. Expression of antioxidant genes in transgenic plant.

Gene construct	Host plant	Reported phenotype	Reference
SOD			
Chloroplastic			
CuZnSOD	Tobacco	No protection from MV or ozone	Tepperman and Dunsmuir
	Tobacco	Reduced MV damage and photoinhibition	Sen Gupta *et al.*
	Potato	Reduced MV damage	Perl *et al.*
MnSOD	Tobacco	Reduced MV damage and no protection from photoinhibition	Slooten *et al.*
	Alfalfa	Reduced acifluorfen, freezing, and water-deficit damage	McKersie *et al.*
FeSOD	Tobacco	Modified regulation of photosynthesis at low CO_2	Arisi *et al.*
	Maize	Enhanced tolerance to MV	Van Breusegem *et al.*
Mitochondrial			
MnSOD	Tobacco	Reduced MV damage in the dark	Bowler *et al.*
	Alfalfa	Reduced freezing and water-deficit damage	McKersie *et al.*
Cytosolic			
CuZnSOD	Potato	Reduced MV damage	Perl *et al.*
APX			
Cytosolic	Tobacco	Reduced MV damage and photoinhibition	Webb and Allen
Chloroplastic	Tobacco	Reduced MV damage and photoinhibition	Webb and Allen
POD	Tobacco	Reduced MV damage	Yun *et al.*
GR			
E. coli			
Chloroplastic	Tobacco	Reduced MV and SO_2 damage but not O_3	Aono *et al.*
	Poplar	Reduced photoinhibition	Foyer *et al.*
Pea GR	Tobacco	Reduced O_3 damage, variable results with MV	Broadbent *et al.*
GST	Tobacco	No tolerance to MV, tolerant to salt and chilling	Roax *et al.*
Peroxiredoxin	*Arabidopsis*	Antisense approach, important to photosynthetic machinery	Baier and Dietz

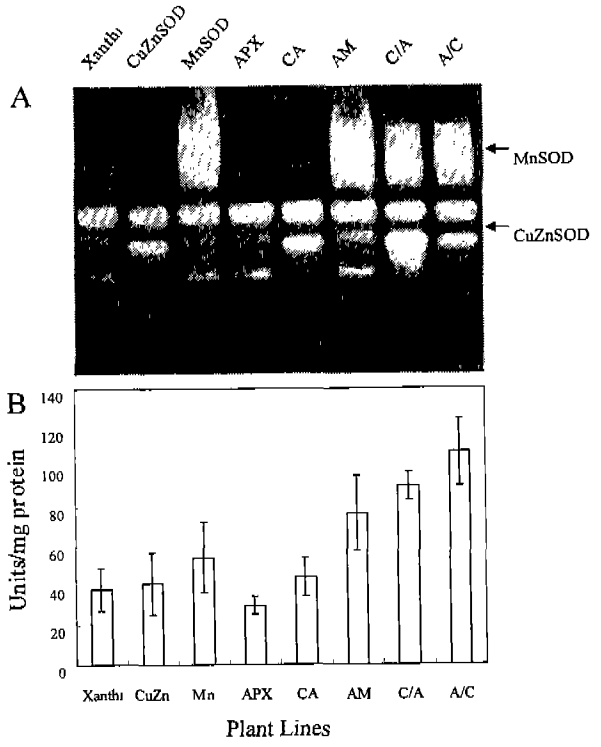

Figure 2. SOD activities in different plant lines. SOD isozyme analysis. (A) was performed by the method of Bowler et al (1991) and SOD specific activity (B) was measured using xanthine/SOD/cytochrome c according to McCord and Fridovich (1969). Data are means± S.E. of three independent plants.

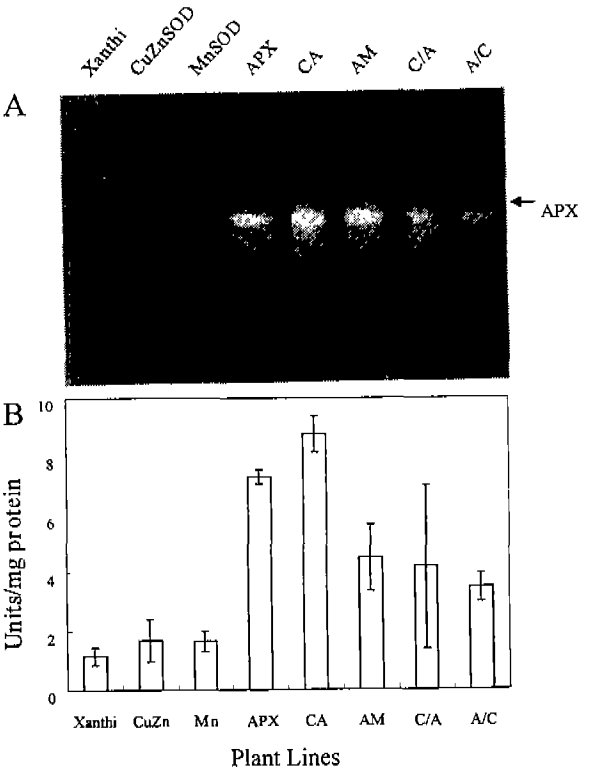

Figure 3. APX activities in different plant lines. APX activity gel asssay (A) was performed according method of Mittler and Zilinskas (1994), and APX specific activity (B) was measured according to method of Nakano and Asada (1981) by measuring rate of ascorbate oxidation. Data are means± S.E. of three independent plants.

ion leakage from the leaf disks. Leaf disks (ϕ7 mm) from T_1 plants were incubated in the 0.4 M sorbitol containing 2 μM or 5 μM MV, for 12 hours in darkness to absorb MV, exposed to the light, and measured loss of cytoplasmic solutes based on electrical conductance of the solution at 1 and 2 days after treatment (DAT).

The conductivity of the solution containing the leaf disks of transgenic plants expressing both the SOD and APX (CA, AM, C/A, A/C plants) were much lower than that of the solution containing leaf disks of control non-transformed plant (Xanthi) or the plants expressing the SOD or APX alone (Figure 4A). When tobacco leaf disks were subjected to 2 μM MV, A/C plants and C/A plants showed about 82% and 47% reduction in membrane damage compared to non-transformed plants (Xanthi) at 1 DAT, respectively. The level of necrosis was severe in the control leaf disks, but the leaf disks of CA, AM, C/A, and A/C showed partial necrosis at the boundary of leaf disks. The relative ion leakages compared to the control were 92.0% in CuZnSOD, 81.6% in MnSOD, 75.4% in APX, 58.3% in CA, and 81.7% in AM at 1 DAT. The 5 μM MV solution seems to be saturated concentration to the leaf membrane damage (Figure 4B). At 2 DAT, relative ion leakages from leaf disks of transgenic plants were as much as the control leaf disks except for C/A and A/M which had 23% and 41% reduction of ion leakage, respectively. This reduction in ion leakage from the leaf disks of plants expressing both SOD and APX indicated that the ROS was efficiently scavenged by combination of SOD and APX expressed in chloroplast simultaneously.

Although the SOD disincorporate the superoxide anion radical into hydrogen peroxide and water, CuZnSOD is inactivated by hydrogen peroxide generated from the reaction. If there were not enough hydrogen peroxide scavenging system (such as APX), CuZnSOD could not maintain the activity. However, MnSOD is not susceptible to hydrogen peroxide, and it's activity remains in presence of hydrogen peroxide. Since chloroplast does not have MnSOD naturally, the protection provided by chloroplastic MnSOD would be affected by the variety of host plant, the age of plants, the light intensity under which the plants were grown [17].

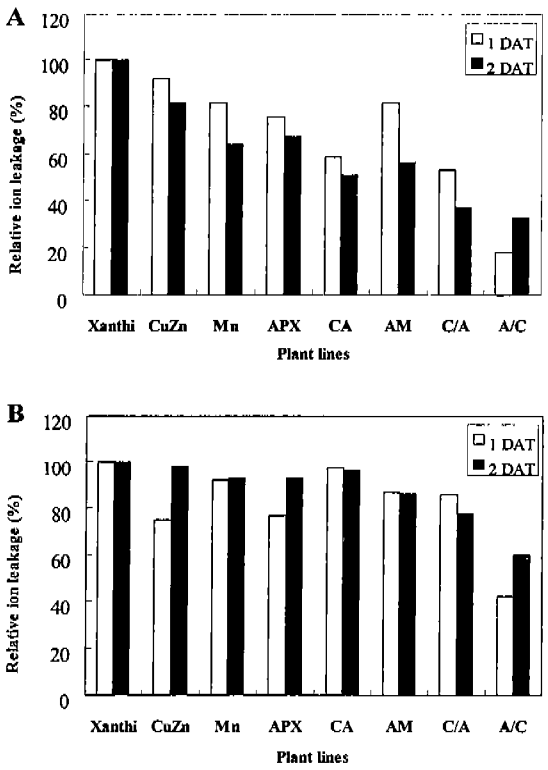

Figure 4. Effect of protection on membrane damages in transgenic plants. Relative ion leakage from the leaf-disks of control (Xanthi) and transgenic plants floated on (A) 2 μ M MV and (B) 5 μ M MV solution. The relative ion leakages of transgenic plants measured at 1 and 2 days after treatment (DAT) were compared to that of nontransgenic control plant (Xanthi).

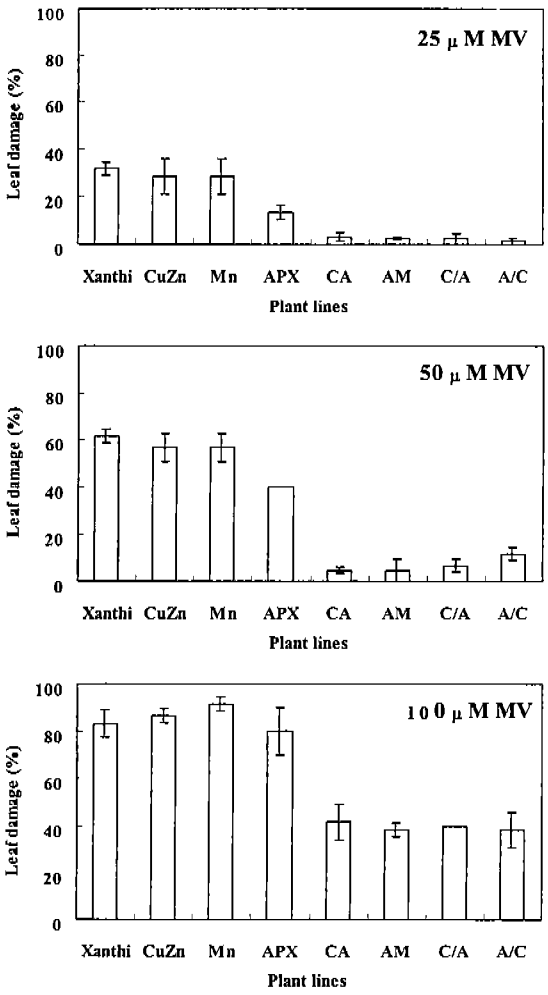

Figure 5. Visible damage appeared on leaves of control (Xanthi) and transgenic plants at 3 days after MV spraying. Data are means ± S.E. of three independent plants.

MV treatment on whole plants

In order to investigate whether transgenic plants have tolerance against MV-induced oxidative stress, we evaluated the visible damage appeared on the leaves by spraying various concentration of MV solution (0 μM, 25 μM, 50 μM, and 100 μM) at 3 DAT (Figure 5 and 6). Transgenic plants expressing both SOD and APX (CA, AM, C/A, and A/C) showed much less leaf damage (1.3～3%) by 25 μM MV, but non-transformed control plant (Xanthi) and transgenic plants expressing SOD or APX (CuZnSOD, MnSOD, and APX) alone showed damage in about 30% of leaves. When 50 μM and 100 μM MV solutions were sprayed on the leaves of transgenic plants, the visible damages were 5～12% and 40% in plants expressing SOD and APX, respectively, while those of control (non-transgenic) and transgenic plants expressing SOD or APX were 40～62% and 80%, respectively. The quantum yield of transgenic plants expressing both SOD and APX is similar to that of non-treated control plants at 3 DAT. The damaged leaves of plants expressing both SOD and APX appeared to recover quickly from the MV-induced stress. These results suggested that transgenic plants, expressing both SOD and APX, have higher ROS scavenging activity than transgenic plants expressing either SOD or APX.

Conclusion

We developed transgenic tobacco plants expressing both SOD and APX in chloroplast under the control of CaMV 35S promoter for the enhanced protection to oxidative stress. Our results indicate that the simultaneous expression of SOD and APX in chloroplast provided much better protection from MV-mediated oxidative stress than single expression of SOD or APX, showing the additive effect of two enzymes in ROS scavenging activity.

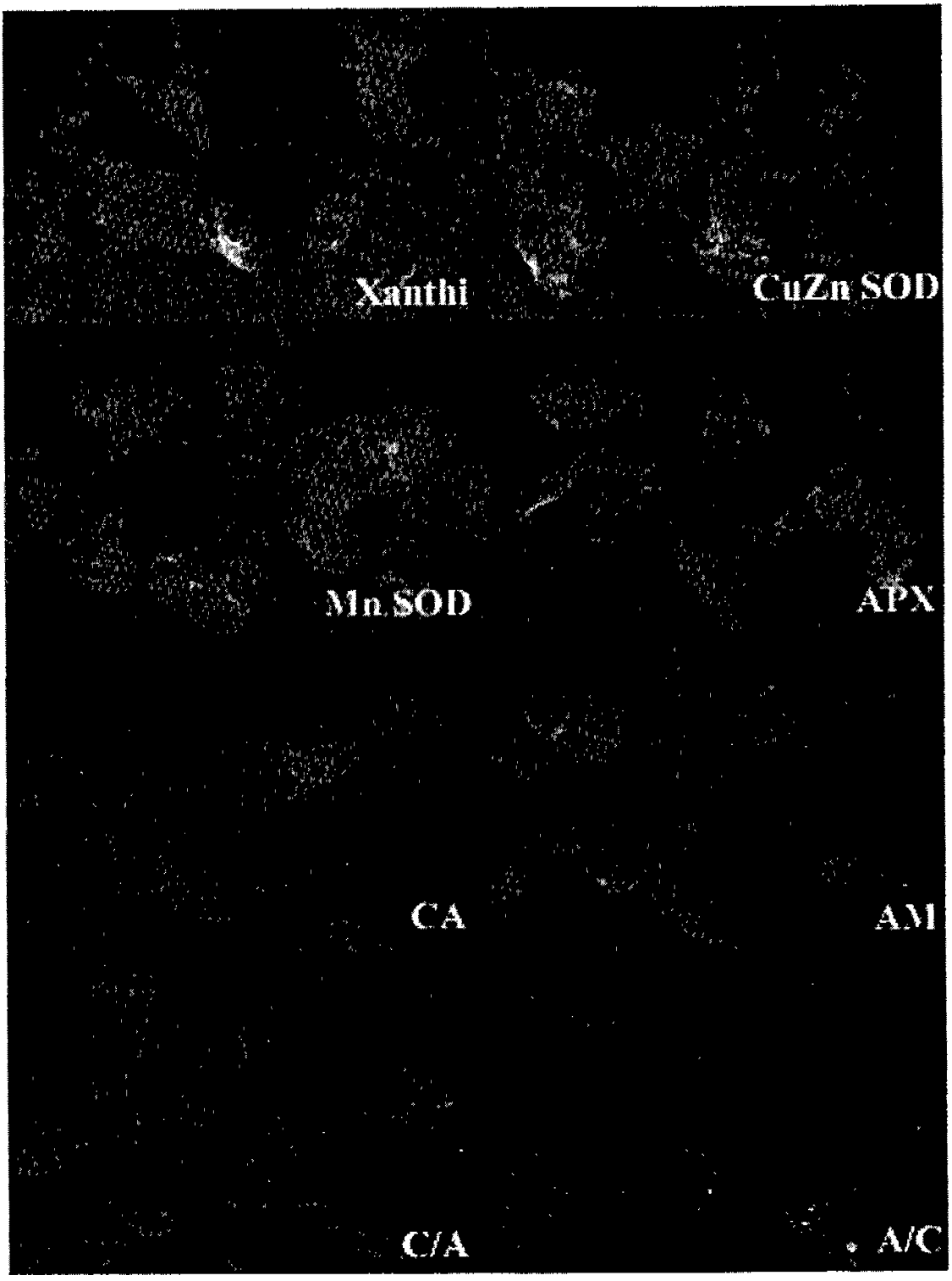

Figure 6. Photograph showing the damage on the leaves of control and transgenic plant by 50 μM MV spraying.

Taken all the results together, we propose that plants overexpressing both SOD and APX work rapidly for scavenging of superoxide anion radical and hydrogen peroxide at the site of generation prior to their interaction with target molecules. In other word, plants scavenge superoxide anion radical first by SOD (CuZnSOD and MnSOD) generating hydrogen peroxide which is sequentially scavenged by APX releasing water. In order to improve protection further from the oxidative stress in chloroplasts, manipulation of enzymes such as DHAR, MDHR and GR is required so that generation of higher concentration of small molecular antioxidants such as ascorbate and glutathione could enhance the water-water cycle enough to scavenge ROS.

A strong stress-inducible promoter is also required to develop the most efficient plants to scavenge ROS. In this respect, the stress-inducible POD promoter from suspension culture of sweet potato is under characterization [9].

In conclusion, the manipulation of antioxidant mechanisms in chloroplasts will contribute to solve the global food and environmental problems in 21st century.

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