CHAPTER III

EVALUATION OF TRANSGENIC PLANTS OVEREXPRESSING SUPEROXIDE DISMUTASE OR ASCORBATE PEROXIDASE IN CHLOROPLASTS

- Correlation between elevated activity and stress tolerance -

Introduction

In the previous chapters, the gene expressions of AOS-scavenging enzymes are up-regulated in response to various environmental stresses. These results and other previous reports indicated that the elevation of activities of AOS-scavenging enzymes under stress conditions is necessary for the mitigation of damages in plant cells. From the closely correlation between AOS-generation and the damages by environmental stresses, many transgenic plants, which have the artificially elevated activities of AOS-scavenging enzymes by gene transfer techniques, have been analyzed, so far, to expect the increased tolerance against various stresses and to indicate the importance of AOS-scavenging enzymes for the protection. The analysis of transgenic tobaccos, which overexpress Mn-SOD in chloroplasts or mitochondria, was first report of elevated tolerance against oxidative stress using methyl viologen compared with the non-transgenic plants (Bowler et al., 1991). After this report, several analyses of SOD-overexpressing plants were reported. The transgenic plants, which were overexpressed chloroplastic Cu/Zn-SOD (Perl et al., 1993; Sen Gupta et al., 1993a; b), Mn-SOD in chloroplast (Slooten et al., 1995), cytosolic Cu/Zn-SOD (Perl et al., 1993; Sakamoto and Tanaka, 1993c) or Fe-SOD (Van Camp et al., 1996), also enhanced the tolerance against oxidative stress. In contrast, the petunia chloroplastic Cu/Zn-SOD-overexpressing plant with highly increased SOD activity was not protected against methyl viologen-mediated oxidative stress (Tepperman and Dunsmuir, 1990). Transgenic alfalfas, which have elevated Mn-SOD activity in chloroplasts and mitochondria using the same constructs as used by Bowler et al. (1991), was more tolerant under freezing and drought stress (McKersie et al., 1993; 1996). Several analyses of transgenic plants, which overexpress bacterial GR in cytosol (Aono et al., 1991; Foyer et al., 1991) or chloroplasts (Aono et al., 1993) or plant chloroplastic GR (Broadbent et al., 1995), were also indicate to be more tolerant against oxidative stress. Furthermore, the availability of elevated activity of both AOS-scavenging enzymes, SOD and GR, against oxidative stress was exhibited by the cross-fertilization of the previous analyzed transgenic plants, which overexpressed SOD and GR in cytosol (Aono et al., 1995). The bacterial GR-overexpressing plant, in chloroplast, was also more tolerant to sulfur dioxide, which is known as one of air pollutant (Aono et al., 1995). In

particular, ozone, which is a major widespread air pollutant and cause the oxidative stress (Kangasjarvi et al., 1994), have been focusing as a major target for the manipulation of activity of AOS-scavenging enzyme. SOD-overexpressing plants (Van Camp et al., 1994b), which were same as those produced by Bowler et al. (1991), and transgenic plants, which expressed antisense RNA for cytosolic APX (Orvar and Ellis, 1997), indicated more tolerant against ozone damages, while overproduction of cytosolic or chloroplastic Cu/Zn-SOD and cytosolic APX confer no ozone tolerance to plants, which were done by same researchers (Pitcher et al., 1991; Pitcher and Zilinskas, 1996; Torsethaugen et al., 1997). On the other hand, overexpression of bacterial CAT in chloroplasts conferred the tolerance against oxidative and drought stress (Shikanai et al., 1998), and that of glutathione S-transferase (GST)/GPX enhanced the stress tolerance of plants under chilling and salinity stress (Roxas et al., 1997).

Like this, even if many analyses for the transgenic plants with manipulation of the AOS-scavenging ability, have already been reported, the molecular mechanisms or necessary factors associated with the stress tolerance to plant cells have, to date, been not clarified only because these studies can indicate the correlation between the elevated activity of AOS-scavenging enzymes and stress tolerance. In this chapter, the author produced the transgenic tobaccos, which is useful plant for the gene transformation, overexpressing SOD or APX in chloroplasts not only to elucidate the correlation between the elevated SOD or APX activity and stress tolerance but also to clarify the important factors for the enhancement of the stress tolerance.

Materials and Methods

Production of SOD- or APX-Overexpressing Tobacco -

For the construction of a chimeric gene for overexpression of plastidic Cu/Zn-SOD, a coding region of spinach plastidic Cu/Zn-SOD cDNA, SSOD2 (Sakamoto et al., 1993b) was used (Fig. VIII-1). was amplified by PCR using mutagenic primers, SSODFP1 (5'-AGAGGATCCAGCCAT GGCCGCACACACCATTC-3') and SSODRP1 (5'-TTCGAGCTCAAACTTATTATTACTAAAA ATGT-3'). After the digestion of *Bam*HI and *Sac*I (underlined in the sequences of primers), this fragment was ligated into the *Bam*HI and *Sac*I sites of a binary vector pBI121 (CLONTECH, Palo Alto, CA), in the place of the *uid*A (GUS) gene. For the overexpression of APX in chloroplasts, *A. thaliana* cytosolic APX cDNA, AP1 (Kubo et al., 1992), was translationally fused with the downstream of 57 amino acids transit peptide of SSOD2 (Sakamoto et al., 1993b) as follows (Fig. VIII-1). About 220 bp fragment of *Bam*HI and *Rsa*I digested SSOD2, containing transit peptide region, was ligated into the *Bam*HI and *Hin*cII sites of a plasmid vector pGEMEX-1 (Promega

Japan), and then an *Eco*RI fragment of AP1 (full-length cDNA) was ligated into *Eco*RI site of pGEMEX-1, in the downstream of transit peptide. This chimeric gene (TP-APX) was inserted into *Bam*HI and *Sac*I sites of pBI121 in the place of GUS gene. These chimeric genes were mobilized to *Agrobacterium tumefaciens* strain LBA4404 by triparental mating as follows. *E. coli* DH5α, harboring these chimeric genes, *A. tumefaciens* LBA 4404 and *E. coli* pRK2013 were mixed and co-cultured on L-Broth (LB) medium at 28 °C for 1 d. The 1:1000-diluted cultured cells with 10 mM $MgSO_4$ were re-cultured on the LB medium containing 50 μg/ml kanamycine and 300 μg/ml streptomycine at 28 °C for 2-3 d. These procedures were performed in the dark. The plasmid DNAs from the single colonies cultured in LB liquid medium were extracted by common alkaline lysis method and used to confirm as the positive clone by PCR.

Leaf disks of tobacco were transformed as described by Horsch et al. (1985) as follows. Leaf disks (1 cm x 1 cm) of wild-type tobacco (*N. tabacum* cv SR1), which was grown sterilely, were inoculated in the cultured solution of *A. tumefaciens* diluted with basal MS medium (Murashige and Skoog, 1962) for 3-5 min, and then cultured on the co-culture medium (basal SM medium containing 0.1 mg/ml NAA, 1 mg/ml 6-BAP and 0.2% (w/v) gelite) at 25 °C for 2 d under the illumination. After the co-culture, the leaf disks were washed in co-culture liquid medium, and then subjected to antibiotic selection for 3-4 weeks on co-culture medium containing 0.1 g/l kanamycine, 0.5 g/l Claforan (Hoeschst Japan, Tokyo, Japan) and 0.5 g/l carbenicillin. The regenerated shoots were transferred to MS medium containing 0.2 g/l kanamycine until the roots and shoots would be completely formed.

Approximately 10 kanamycin-resistant transgenic plants in each line were regenerated. In the SOD-overexpressing plants, the regenerated plants were assayed for alternation in SOD isozymes profile by SOD activity staining according to the methods in CHAPTER I. The independent line PSOD-7, which showed most increased SOD activity (Fig. VIII-2A), was used for further analysis. In the APX-overexpressing plants, the regeneration plants were analyzed by immunoblot analysis (Fig. VIII-2B) using monoclonal-antibody against *A. thaliana* cytosolic APX (kindly gift from Dr. Tanaka and Dr. Kubo) as described in CHAPTER II-1 (the study of rice GR). Consequently, the independent line PAPX-4 is used for the further analysis because the plant showed the high amount of exogenous APX proteins.

Plant material and growth conditions -

For the analyses of the selected transgenic plants, seeds of T_2 or T_3 generation after repeating self-pollination were used. The wild-type and selected transgenic plants of tobacco were grown on

vermiculite at 25 °C under 16 h of light at 50-170 μmol quanta m^{-2} s^{-1} and 8 h of darkness. The bolting stage (20-30 cm tall) plants were used in all experiments. The second or third fully expanded leaves at this stage were used for the assays of AOS-scavenging enzyme activities and antioxidants.

Assays of AOS-scavenging enzyme activities -

For the assays of SOD, GR, MDAR and DHAR activities, leaf tissues (1-2 g of fresh weight) were ground in a grinding buffer containing 50 mM K-phosphate (pH 7.5), 0.1 mM EDTA and 0.1% (w/v) Triton X-100 at 4 °C. In the case of APX activity, the grinding buffer was added 5 mM AsA. These homogenates were then centrifuged at 12,000 x *g* for 20 min at 4 °C, and the supernatant was used for each enzyme assay. SOD activity staining of Native-PAGE gel was carried out as described in CHAPTER I-3. The soluble protein extracts from samples (100 μg) were separated on a native 10% polyacrylamide gel at 4 °C and subjected to the activity staining. The solution assay of SOD and GR activity were measured as described in CHAPTER I and CHAPTER II, respectively. SOD or GR activity were indicated as unit mg^{-1} protein or μmol NADPH oxidized h^{-1} mg^{-1} protein. APX activity was assayed by following decrease of absorbance at 290 nm with the oxidation of ascorbate (ε=2.8 mM^{-1} cm^{-1}), according to Nakano and Asada (1981), and indicated as μmol ascorbate oxidized h^{-1} mg^{-1} protein. DHAR activity was determined by following the increase in absorbance at 265 nm due to GSH-dependent production of AsA (ε=14 mM^{-1} cm^{-1}), according to Hossain and Asada (1984a), and indicated as μmol ascorbate h^{-1} mg^{-1} protein. The protein concentration was determined colorimetically (Bradford, 1976) using BSA as a standard. Unless otherwise mentioned, all absorbance measurements in this chapter were carried out by a BioSpec-1600 spectrometer (Shimadzu).

Determination of ascorbic acid content -

The amounts of reduced and oxidized ascorbic acid (AsA and DHA) and were determined as described in Law et al. (1983). The assay of ascorbic acid is based on the reduction of Fe^{3+} to Fe^{2+} by AsA in acidic solution. The absorption from the complex formed with the Fe^{2+} and bipyridyl was measured at 525 nm. Total ascorbic acid (AsA+DHA) was determined through the reduction of DHA to AsA by dithiothreitol (DTT). Leaves (approximately 1 g of fresh weight) of samples were extracted with 200 ml of 4% (w/v) trichloroacetic acid (TCA), and then the supernatant was recovered by the centrifugation (10,000 x *g*, 30 min). After adding sixtieth volume of 5M NaOH, a part of the sample solution (100 μl) was subjected to the determination of total ascorbic acids

(AsA+DHA) or AsA content. An equal volume of 150 mM K-phosphate solution (pH 7.4) was added into the samples. For the determination of total ascorbic acids, a half volume of 10 mM DTT was also added, kept at room temperature for 15 min, and then a half volume of 0.5% *N*-ethylmaleimide was added. In the both case, the resulting solution was kept at room temperature for over 30 min, and then 200 μl of 10% TCA, 44% H_3PO_4, and 4% bipyridyl, which was dissolved in 70% ethanol, and 100 μl of 3% $FeCl_3$ were added. The resulting supernatant, which was recovered by the centrifugation (20,000 x *g*, 10 min) after the incubation at 37 °C for 60 min, was subjected to the measurement of absorbance at 525 nm. AsA or DHA (0-40 nmol) were used as a standard. The determined ascorbate contents were indicated as nmol g^{-1} fresh weight.

Northern Blot Analysis -

The preparation of total RNA from leaves of each transgenic plant and wild-type plant and Northern blot analysis were performed as described in CHAPTER I-1. Each total RNA (20 μg) was used for Northern blot analysis. For the detection of a transcript specific to the transgenes, *Bam*HI and *Rsa*I fragment of SSOD2 (Sakamoto et al., 1993b), which contains a transit peptide of spinach plastidic Cu/Zn-SOD, was used as a hybridization probe. In the case of tobacco Fe-SOD and plastidic Cu/Zn-SOD, the partial cDNAs were prepared by PCR using *N. tabacum* single-strand cDNA, which was synthesized with SUPERSCRIPT II reverse transcriptase (GIBCO BRL) according to the manufacturer's instruction, with the following primer pair; for Fe-SOD: NPFESODF1 (5'TTTGAACTCCAGCCTCCTCCTTAT-3') and NPFESODR1 (5'-CATTGTTGTC CCACACCTCAACTT-3'), which was synthesized based on the sequence of *N. plumbaginifolia* Fe-SOD (Van Camp et al., 1990), for plastidic Cu/Zn-SOD: FM-II and NotI-dT_{18} (CHAPTER I-1). To detect the transcripts specific to cytosolic Cu/Zn-SOD and actin 1, the EcoRI-fragment of rice cytosolic Cu/Zn-SOD full-length cDNA (RSODCc1) (Sakamoto et al., 1992a), and PCR-fragment of coding region of rice actin 1 cDNA (accession number X16280; McElroy et al., 1990), which was prepared by PCR with a primer pair, RAc1S (5'-CGTCTGCGATAATGGAACTGGT-3') and RAc1A (5'-GAAAATGATAACAGATAGGCCGGT-3'), were used.

Estimation of Oxidative Stress Tolerance -

For the experiment of leaf disk assay, leaf disks (0.9 cm diameter) were preincubated in various concentration (0, 0.1, 0.5, 1, 2, and 5 μM) of methyl viologen solution, containing 0.1% Tween-20. Prior to the treatment, the leaf disks were kept in the dark for 2 h and then kept under constant illumination (110 μmol quanta m^{-2} s^{-1}) at 25 °C until visible damages would be observed.

For the estimation of damages by oxidative stress treatment, the photochemical efficiency of PS II was determined as the ratio of variable to maximum chlorophyll fluorescence (F_v/F_m) with a pulse amplitude modulation fluorometer PAM-100 (Waltz, Effeltrich, Germany). The F_v/F_m ratio is known as an indicator of PS II activity and was used for the estimation of damage by stress treatments. The square leaf disks (1.5 cm x 1.5 cm) were treated with 1 μM methyl viologen in 0.1% (w/v) Tween 20 for duration of the treatment (0-10 h) under constant illumination (110 μmol quanta m^{-2} s^{-1}) at 25 °C after keeping in the dark for 2 h. Prior to the measurements, the leaf disks were kept in the darkness for 30 min.

Environmental stress treatments -

For the treatment of drought stress, the wild-type and transgenic plants were grown on vermiculite by withholding water at 35 °C under 16 h of light at approximately 1000 μmol quanta m^{-2} sec^{-1} and at 30 °C under 8 h of darkness. This stress treatment was done in Nara Institute of Science and Technology with valuable helps of Dr. Yokota and Dr. Miyake. After this treatment for 7 d, the treated plants were recovered under standard growth condition for 7 d. In the case of chilling stress, the wild-type and transgenic plants were subjected to the condition, at 4 °C under 16 h of light at 220 μmol quanta m^{-2} sec^{-1} and under 8 h of darkness for 1 d. After this treatment, these plants were grown under the standard growth condition for 1 d. These procedures were repeated once again.

Results

Production of chloroplast-localized SOD or APX-overexpressing tobaccos -

Two binary plasmids (pBI121/SPSOD and pBI121/TP-APX) for expression of SOD or APX under the control of CaMV 35S promoter were constructed for *A. tumefaciens*-mediated transformation to tobacco (Fig. VIII-1). In the case of pSPSOD, the coding region of spinach plastidic Cu/Zn-SOD cDNA (SSOD2; Sakamoto et al., 1993b) was directly fused to the downstream of CaMV 35S promoter, and the exogenous SOD in tobacco was expected to import to chloroplasts. In the case of pBI121/TP-APX, the sequence of *A. thaliana* cytosolic APX cDNA (AP1; Kubo et al., 1992) was fused for the transit peptide of SSOD2 for targeting to the same site of chloroplasts. About 10 of independent primary transformants (T_0) with pBI121/SPSOD and pBI121/TP-APX, respectively, that expressed the exogenous SOD or APX protein were obtained (data not shown). The analyses of SOD activity staining or immunoblot with anti-*A. thaliana*

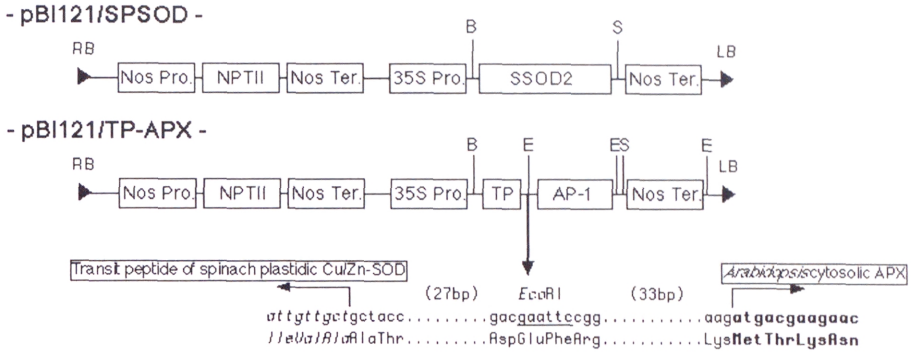

Fig. VIII-1. Constructs for the overexpression of SOD or APX in chloroplasts of transgenic plants. Plasmid pBI121/SPSOD and pBI121/TP-APX containing, respectively, spinach plastidic Cu/Zn-SOD cDNA (SSOD2; Sakamoto et al., 1993b) and the chimeric gene, *A. thaliana* cytosolic APX cDNA (Kubo et al., 1992) fused on the downstream of transit peptide region of SSOD2, under the control of CaMV 35S promoter, in a binary vector for *A. tumefaciens* transformation.

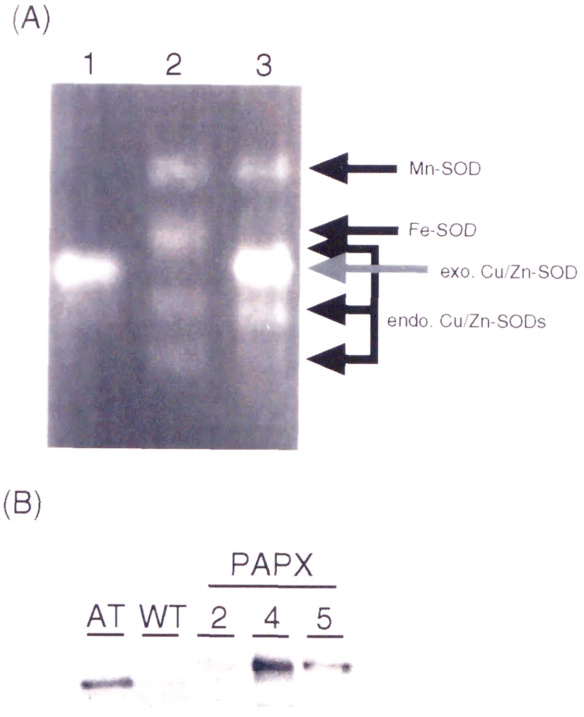

Fig. VIII-2. (A) Analysis of SOD activity staining in PSOD plants. The soluble protein extracts from leaves of spinach, wild-type and PSOD plants (100 μg) were separated on a native 10% polyacrylamide gel at 4 °C and subjected to the activity staining as described in Fig. III-5. lane1, spinach; lane 2, wild-type plant ; lane 3, PSOD plant. exo.. exogenous ; endo, endogenous. (B) Immunoblot analysis of PAPX plants. Each total protein (20 μg) extracted from the leaves of *A. thaliana*, wild-type and PAPX plants was separated by 14% SDS-PAGE, transferred to PVDF membrane and subjected to immunoblotting with the monoclonal antibody against *A. thaliana* cytosolic APX. AT, A. thaliana (ecotype Columbia); WT, wild-type plant.

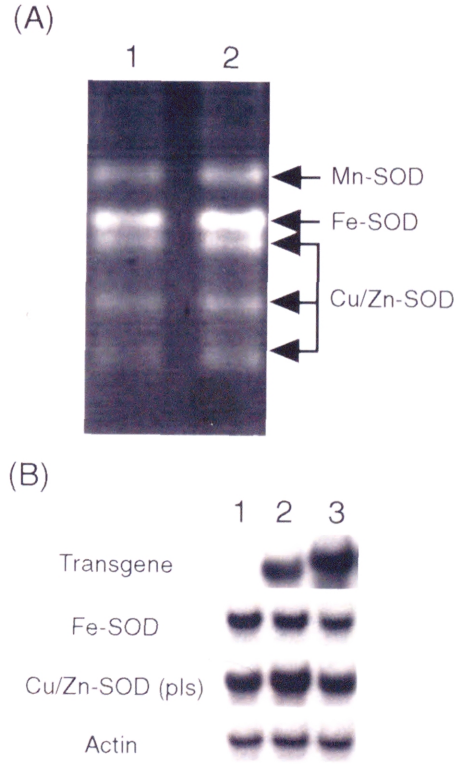

Fig. VIII-3. Effect of SOD or APX overexpression on the endogenous SOD. (A) Analysis of SOD activity staining in PAPX plants. The soluble protein extracts from leaves of wild-type and PAPX plants (100 μg) were separated on a native 10% polyacrylamide gel at 4 °C and subjected to the activity staining as described in Fig. III-5. lane1, wild-type plant ; lane 2, PAPX plant. (B) Northern blot analysis of *N .tabacum* SOD genes. Total RNA (20 μg) was isolated from wild-type, PSOD, and PAPX plants, and subjected to Northern blot as described in Materials and Methods, with ^{32}P-labeled SOD probes for the detection of *N. tabacum* SOD genes. lane1, wild-type plant ; lane 2, PSOD plant; lane 3, PAPX plant.

cytosolic APX monoclonal-antibody were carried out in the total soluble proteins extracted from the leaves of the self-fertilized lines (T_2 or T_3) for each construct. The band of exogenous SOD activity was corresponded to the spinach plastidic Cu/Zn-SOD activity (Fig. VIII-2A). The specific band reacted with the APX antibody was detected only in *A. thaliana* and transgenic plants (Fig. VIII-2B). Same results were obtained in the soluble proteins from isolated chloroplasts (data not shown). Furthermore, Northern blot analysis with transgene-specific probe, which was prepared as described in Materials and Methods, indicated that high amount of specific transcript derived from transgene was detected only in the transgenic plants (Fig. VIII-3B). These results indicated that the exogenous protein were actually expressed in the chloroplasts of transgenic tobaccos. The selected transgenic plants for pBI121/SPSOD and pBI121/TP-APX, that have most highly expressed exogenous SOD or APX, were referred to PSOD and PAPX, respectively, and used for all experiments.

Assays of AOS-scavenging enzyme activities and antioxidants -

To evaluate the changes of SOD and APX activity by the introduction of exogenous SOD or APX, both activity of total soluble fractions from the transgenic and wild-type plants were determined (Table VIII-1). SOD specific activity of PSOD plants was 2.26-fold higher than in wild-type plants, but no significant increase of APX activity was observed. Whereas APX activity of PAPX plants was 8.47-fold higher than in wild-type plants, and 1.9-fold enhancement of SOD activity was also observed. In order to clarify the effect of SOD or APX-overexpression to other AOS scavenging enzymes, activities of MDAR, DHAR, and GR were also determined (Table VIII-1). Only 10% increases of these activities in PAPX plants was observed, but there are no significant changes of the activities in PSOD plants. The contents and redox states of ascorbic acid were also determined to elucidate the effect of overexpression of SOD or APX (Table VIII-2). The pool size

Table VIII-1. Comparison of AOS-scavenging enzyme activities in wild-type and transgenic plants, PSOD and PAPX.

Specific activities of SOD (unit mg^{-1} protein), APX (μmol ascorbate oxidized h^{-1} mg^{-1} protein), GR (μmol NADPH oxidized h^{-1} mg^{-1} protein), and DHAR μmol ascorbate h^{-1} mg^{-1} protein) in leaf extracts from wild-type and transgenic plants. Valuse are means ±SD; n=3-5 for each measurement.

	SOD	APX	GR	DHAR
WT (SR1)	94.68±17.74 (1.00)	57.93±9.22 (1.00)	1.785±0.305 (1.00)	4.390±0.606 (1.00)
PSOD	213.87±53.50 (2.26)	63.23±15.63 (1.09)	1.607±0.246 (0.90)	4.442±0.683 (1.01)
PAPX	179.55±32.37 (1.90)	490.54±185.02 (8.47)	1.976±0.236 (1.11)	4.800±0.757 (1.09)

Table VIII-2. Comparison of ascorbic acid contents in wild-type and transgenic plants, PSOD and PAPX.

Total and reduced ascorbic acid contents in leaves of wild-type and transgenic plants were determined according to Law et al. (1993). The ascorbic acid contents are indicated as nmol g^{-1} fresh weight. Valuse are means ±SD; n=3.

	AsA+DHA	AsA	DHA	AsA/AsA+DHA (%)
WT (SR1)	2.602±0.497 (1.00)	0.999±0.165 (1.00)	1.603±0.340 (1.00)	38.62
PSOD	2.828±0.348 (1.09)	1.082±0.147 (1.08)	1.746±0.207 (1.09)	38.23
PAPX	2.564±0.140 (0.99)	0.864±0.076 (0.86)	1.700±0.119 (1.06)	33.72

of total ascorbic acid (AsA+DHA) was not changed, but 5% of increase in oxidized state of ascorbic acid was observed only in PAPX plants.

Alteration of endogenous SOD activities in SOD or APX-overexpressing plants -

In order to elucidate the factor for the alteration of endogenous SOD activity in PSOD and PAPX plants, the analysis of SOD activity staining of wild-type and PAPX plants was carried out (Fig. VIII-3A). Only the band of Fe-SOD activity was apparently increased by the comparison with that of wild-type plants. On the oher hand, the endogenous Cu/Zn-SOD and Fe-SOD activities in PSOD plants were lower than those in wild-type plants (Fig. VIII-2). Northern blot analysis with *N. tabacum* Fe-SOD and plastidic Cu/Zn-SOD probes indicated that there was no effect of the overexpression of SOD or APX on the expression of chloroplast-localized SOD genes (plastidic Cu/Zn-SOD and Fe-SOD) at transcriptional level. The increase of plastidic Cu/Zn-SOD mRNA in PSOD plants may due to the cross-hybridization of *N. tabacum* plastidic Cu/Zn-SOD probe to transgenes. These results indicated that the alteration of endogenous SOD activities in each transgenic plant may be occurred at post-translational level.

Reduction of the damages by oxidative stress in transgenic plants -

In order to evaluate the protective effects of overexpression of SOD or APX in chloroplasts to oxidative stress, the leaf disk assay in transgenic and wild-type plants were carried out by the treatment with various concentration of methyl viologen to observe the sensitivity of visible damages to oxidative stress (Fig. VIII-4). In this experiment, the author regarded the chlorosis in leaf disks as the damage by oxidative stress. Both transgenic plants have the more tolerance to oxidative stress than wild-type plants. In this experiments, direct comparison between PSOD and PAPX was not performed. To compare directly the damage of these transgenic plants, the

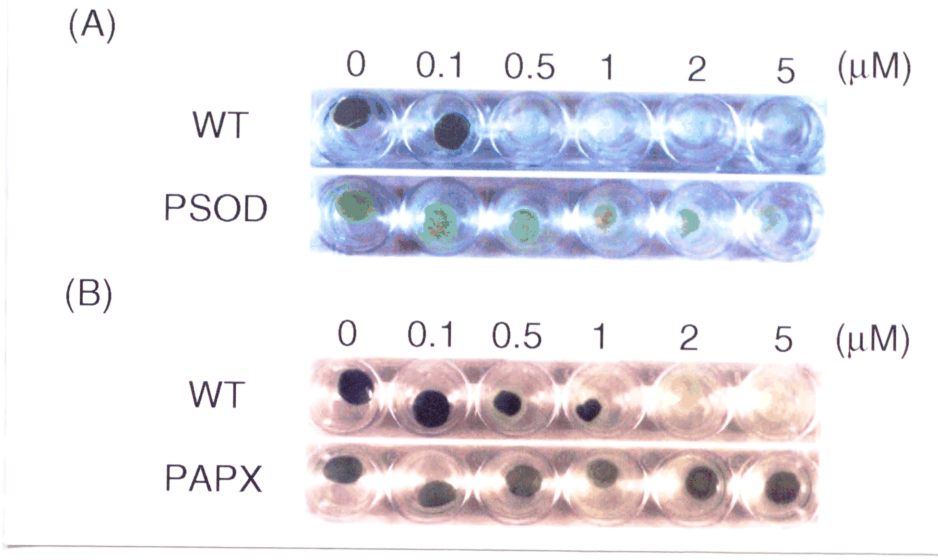

Fig. VIII-4. Leaf disk tests with various concentration of methyl viologen. Leaf disks (0.9 cm diameter) were exposed to various concentration (0, 0.1, 0.5, 1, 2, and 5 μM) of methyl viologen solution under constant illumination (110 μmol quanta m^{-2} s^{-1}) at 25 °C, after keeping in darkness for 2 h, until visible damages would be observed.

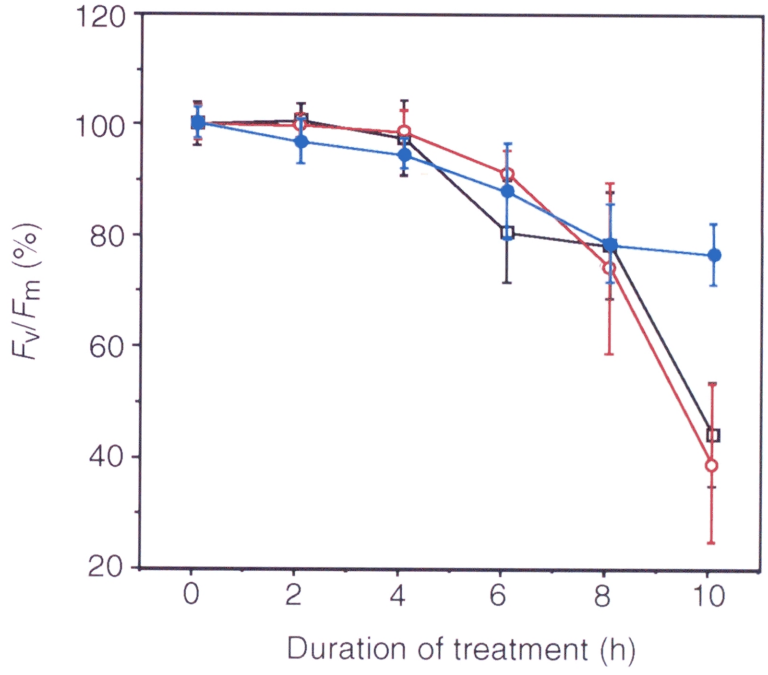

Fig. VIII-5. Changes in the activity of PS II activity in wild-type and transgenic plants under oxidative stress. The square leaf disks (1.5 cm^2) were treated with 1 μM methyl viologen at 25 °C under constant illumination (110 μmol quanta m^{-2} s^{-1}) after keeping in the dark for 2 h. The initial value for F_v/F_m in each plant were taken as relative values of 100. □-, wild-type plant; ○-, PSOD plant; ●-, PAPX plant.

chlorophyll fluorescence in the leaf disks duration of methyl viologen treatment (1 μM) for 10 h was measured and calculated the F_v/F_m ratio, which was known as an indicator of PS II activity (Fig. VIII-5). During the treatment for 8 h, the significant difference of PS II activity in wild-type and transgenic plants was not observed. However, the PS II activity of PAPX plants was retained to 76.4% of the initial activity after 10 h treatment, while those of wild-type and PSOD plants was reduced to 44% and 39%, respectively. The difference between the results of leaf disk assay and

this experiment suggested that there is no direct correlation between the reduction of PS II activity and visible damage.

Enhancement tolerance of APX-overexpressing plants against various environmental stresses more than with SOD-overexpressing plants -

To estimate the tolerance of transgenic plants to environmental stress, the visible damage in the recovery period after the drought stress with high light (1000 μmol quanta m^{-2} sec^{-1}) or chilling stress (4 °C) with relatively high light (220 μmol quanta m^{-2} sec^{-1}) was observed as described in Materials and Methods. In the recovery period (7 d) of drought stress (Fig. VIII-6), only PAPX plants (C) recovered from damages caused by drought stress and was similar to non-stressed plants, but wild-type (A) and PSOD (B) plants remained to have serious damages. On the other hand, the visible damage only on the top of wild-type leaf was observed in the recovery period of chilling stress (Fig. VIII-7).

Fig. VIII-6. Drought tolerance of PSOD and PAPX transgenic plants. The wild-type and transgenic plants were treated by withholding water at 35 °C under 16 h of light at approximately 1000 μmol quanta m^{-2} sec^{-1} and at 30 °C under 8 h of darkness for 7 d and then returned to standard growth condition for 7 d. A, wild-type plant; B, PSOD plant; C, PAPX plant.

Discussion

In this chapter, the author reported the production and evaluation of the transgenic plants with elevated activities of SOD or APX in chloroplasts. APX-overexpressing plants were more tolerant to various stresses than SOD-overexpressing plants. This is first report of enhanced tolerance in APX-overexpressing plants. In particular, the increase of H_2O_2-scavenging ability was important

Fig. VIII-7. Chilling tolerance of PSOD and PAPX transgenic plants. The wild-type and transgenic plants were subjected to the condition, at 4 °C under 16 h of light at 220 μmol quanta m^{-2} sec^{-1} and under 8 h of darkness for 1 d, and then returned to the standard growth condition for 1 d. These procedures were repeated once again. A, wild-type plant; B, PSOD plant; C, PAPX plant.

and available for the elevation of stress tolerance against various environmental stresses. Both exogenous SOD and APX was imported to chloroplasts as a targeting subcellular compartment because chloroplast is thought to be a major source of AOS under various stress conditions.

In APX-overexpressing plants, one of endogenous SOD activity, Fe-SOD activity, was also increased (Fig. VIII-3A, Table VIII-1), while endogenous Cu/Zn-SOD and Fe-SOD activities were decreased in SOD-overexpressing plants (Fig. VIII-2A). However no changes of *N .tabacum* SOD mRNAs (plastidic Cu/Zn-SOD and Fe-SOD) were observed (Fig. VIII-3B). It was suggested that the alteration of H_2O_2 concentration by SOD- (increase) or APX- (decrease) overexpressing affected to the endogenous SOD activity because of the sensitivity of both Fe-SOD and Cu/Zn-SOD to H_2O_2. That is to say that the activities of Fe-SOD and Cu/Zn-SOD, are always inhibited by H_2O_2 at intracellular level, and decrease of H_2O_2 concentration in plant cells may elevate the exogenous SOD activities at post-translational level. However, these results were inconsistent with the analysis of the transgenic tobacco overexpressing *P. sativum* plastidic Cu/Zn-SOD, which induced APX activity in chloroplasts (Sen Gupta et al., 1993b). They presumed that increase of H_2O_2 concentration by the overexpression of SOD in chloroplasts induced the endogenous APX activities. This speculation was partially supported by the evidence that cytosolic mRNA level was elevated by SOD-overexpression (Sen Gupta et al., 1993b). But this result is not enough to explain the effect of chloroplastic APX mRNA even if chloroplastic APX activity was also increased. The importance of H_2O_2 for the regulation of rice cytosolic APX gene as one of signaling factor (Morita et al., 1999) indicated that the alteration of H_2O_2 concentration affect the expression of cytosolic APX gene at transcriptional level. The elevated SOD activity in PSOD plants (2.26-fold; in Table VIII-1), which was relatively lower than that of petunia chloroplastic Cu/Zn-SOD-overexpressing plant (3.5-fold; Sen Gupta et al., 1993b), may be not enough to induce the gene expression of cytosolic APX.

The overexpression of APX in chloroplasts promoted the oxidation of ascorbic acid but not

changed the activity of ascorbate-glutathione cycle enzymes, DHAR and GR, and the pool size of ascorbic acids (Table VIII-1,2). Whereas in the transgenic plants with elevated GST/GPX activity, the further oxidization of glutathione and the increased pool size of total glutathione were observed (Roxas et al., 1997). The artificially increased pool size of ascorbic acid and glutathione induced the elevation of MDAR, DHAR, and GR activities in spinach (Hausladen and Kunert, 1990). Therefore the oxidation of ascorbic acid is not seemed to play as a signal to increase the activity of AOS-scavenging enzymes and the pool sizes of antioxidants.

The stress tolerance of transgenic plants in term of visible symptoms by methyl viologen treatment was inconsistent with that in term of the protection of photosynthesis activity (Fig. VIII-4,5). Such difference was also observed in the analyses of bacterial GR-overexpressing plants, the plants have the oxidative stress tolerance only as visible damages but not as the protection of photosynthesis activity (Foyer et al., 1991; Aono et al., 1991). Foyer et al. (1994) suggested that the greater capacity to regenerate the ascorbate pool by GR-overexpression is important for the protection against oxidative damage as visible leaf injury. In the author's results, the elevation of H_2O_2-scavenging capacity is more available to confer the stress tolerance against various stresses (Fig. VIII-5,6). Chloroplast-localized APXs are rapidly inactivated in the absence of AsA (Amako et al., 1994), which can also reduce AOS by itself, and in the presence of excess H_2O_2 (Hossain and Asada, 1984b). Therefore, it is suggested that the elevation of H_2O_2 concentration by inactivation of chloroplast-localized APXs is responsible for the stress-associated damages, such as visible symptoms and the decrease of photosynthesis activity. This hypothesis was also, actually, supported by the analyses of the transgenic plants with the elevated H_2O_2-scavenging capacity, which overexpressed *E. coli* CAT in chloroplasts (Shikanai et al., 1998) or GST/GPX (Roxas et al., 1997), that revealed more tolerance against various stresses compared with control plants. On the other hand, the transgenic tobacco with highly increased SOD activity (30 to 50-fold than control) by overexpression of petunia chloroplastic Cu/Zn-SOD was not protected against methyl viologen-mediated inhibition of $^{14}CO_2$ assimilation nor chlorophyll bleaching during photoinhibitory condition (Tepperman and Dunsmuir, 1990), nor against ozone toxicity (Pitcher et al., 1991). In contrast, the transgenic tobaccos with the relative highly increased SOD activity by the overexpression of chloroplastic Cu/Zn-SOD (Sen Gupta et al., 1993a; b), Mn-SOD in chloroplast (Slooten et al., 1995), Fe-SOD (Van Camp et al., 1996) were revealed the tolerance against methyl viologen in term of protection of photosynthesis. This difference of stress tolerance support may due to the H_2O_2 concentration at cellular level, which was affected by the SOD activity. The author concluded that the elimination of H_2O_2 in plant cells is most effective to confer the stress tolerance not only against oxidative stress but also against various environmental stresses.