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Update on Oxidative Stress

Dissection of Oxidative Stress Tolerance Using Transgenic Plants[1]

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Environmental stress is the major limiting factor in plant productivity. Much of the injury to plants caused by stress exposure is associated with oxidative damage at the cellular level. Widespread losses of forests and crops due to ozone pollution provide a highly visible example of oxidative stress (see Tingey et al., 1993, for a review), but less obvious losses caused by oxidative damage associated with periods of cold or drought also take their toll in the accumulation of incremental setbacks during a growing season. The role of ROIs in plant stress damage is indicated by the increased production of ROIs and the increased oxidative damage in tissues during stress.

In plants, the highly energetic reactions of photosynthesis and an abundant oxygen supply make the chloroplast a particularly rich source of ROIs. High light intensity can lead to excess reduction of PSI so that CO_2 fixation cannot keep pace and $NADP^+$ pools are reduced. Under these conditions, O_2 can compete for electrons from PSI, leading to the generation of ROIs through the Mehler reaction. When CO_2 fixation is limited by environmental conditions such as cold temperatures or low CO_2 availability (closed stomata), excess PSI reduction and increased ROI production can occur even at moderate light intensities. Efficient removal of ROIs from chloroplasts is critical, since H_2O_2 concentrations as low as 10 μM can inhibit photosynthesis by 50% (Kaiser, 1979). Although the toxicity of $\cdot O_2^-$ and H_2O_2 themselves is relatively low, their metal-dependent conversion to the highly toxic $\cdot OH$ via the Haber-Weiss reaction is thought to be responsible for the majority of the biological damage associated with these molecules.

Antioxidant systems of plant chloroplasts include enzymes such as SOD and APX, and nonenzymatic components such as ascorbic acid and glutathione. The proposed ROI scavenging pathway of chloroplasts is shown in Figure 1 (Asada, 1994). Superoxide radicals are produced by the reduction of molecular oxygen at PSI via the Mehler reaction. This $\cdot O_2^-$ is rapidly dismuted to H_2O_2 by SOD that is associated with the thylakoid. The H_2O_2 produced is quickly scavenged by a thylakoid-bound APX. Superoxide and H_2O_2 that diffuses away from the membrane-associated enzymes can be scavenged in the stroma. Monodehydroascorbate radicals produced by APX can be quickly reduced to ascorbic acid via Fd or by stromal monodehydroascorbate reductase. Alternatively, they can spontaneously disproportionate into ascorbic acid and dehydroascorbic acid, which is converted to ascorbic acid by dehydroascorbate reductase using reduced glutathione as an electron donor. Subsequent regeneration of reduced glutathione requires GR and NADPH.

The hypothesis that antioxidant enzymes are critical components in preventing oxidative stress in plants is based on several lines of evidence. (a) The activity of one or more of these enzymes is generally increased in plants exposed to stressful conditions, and this elevated activity correlates with increased stress tolerance. (b) Pretreatment of plants under one form of stress can increase tolerance for a different stress. This phenomenon is known as cross-tolerance. (c) Plant lines selected for resistance to ROI-inducing herbicides such as MV typically have increased levels of one or more of these enzymes and can also exhibit cross-tolerance (see Gressel and Galun, 1994, for a review). These observations indicate that exposure to environmental stress can stimulate plants to enhance their ROI scavenging systems, and this enhancement can apparently provide generalized stress protection. Although strongly suggestive, these observations still provide only circumstantial evidence for the role of these enzymes in stress protection and they say little if anything about the specific roles of individual enzymes.

Recently, manipulation of the expression of enzymes involved in scavenging ROIs by gene transfer technology has provided new insights into the role of these enzymes in chloroplasts by allowing direct investigation of their functions and interactions (Foyer et al., 1994). Although still preliminary, these experiments also indicate that modification of ROI scavenging systems of chloroplasts can lead to significant changes in oxidative stress tolerance and pro-

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Abbreviations: APX, ascorbate peroxidase; CaMV cauliflower mosaic virus; GR, glutathione reductase; MV, methyl viologen; ROIs, reactive oxygen intermediates; O_2^-, superoxide radical; SOD, superoxide dismutase.

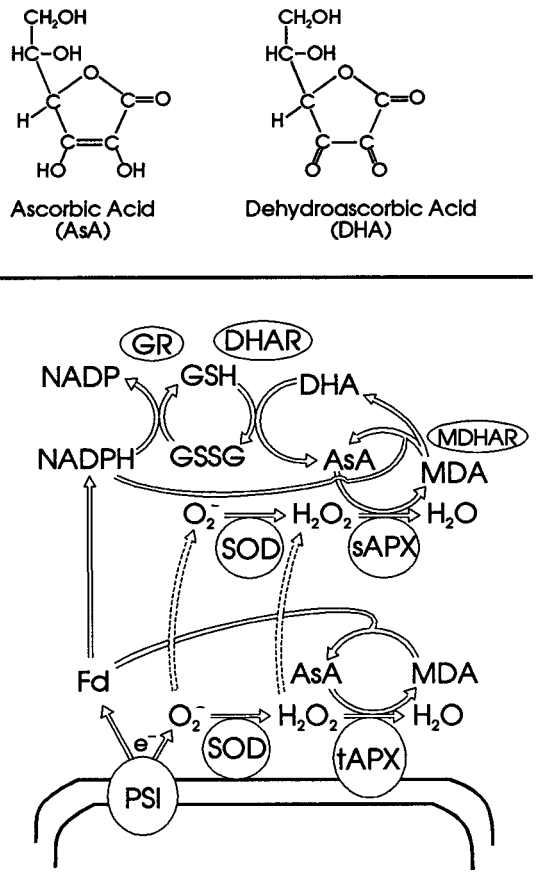

Figure 1. Structures of ascorbic acid (AsA) and dehydroascorbic acid (DHA) and proposed reactive oxygen intermediate scavenging pathway of plant chloroplasts (modified from Asada, 1994). Superoxide radicals (O_2^-) are dismuted by SOD associated with PSI and the resulting H_2O_2 is scavenged by thylakoid-bound APX (tAPX). Reactive oxygen species that escape destruction at the thylakoid are scavenged by stromal SOD and stromal APX (sAPX). Monodehydroascorbate radicals (MDA) produced by APX are converted to AsA through reactions with Fd or monodehydroascorbate reductase (MDHAR). Reduction of DHA to AsA is catalyzed by dehydroascorbate reductase (DHAR) via the ascorbate:glutathione pathway.

vide some indication that these approaches can be used to improve plant performance.

SOD

SODs are a family of metalloenzymes that catalyze the following reaction:

$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2.$$

These isoenzymes are found in various compartments of plant cells and can contain Cu and Zn, Fe, or Mn cofactors (see Bowler et al., 1994, for a recent review). Chloroplasts of virtually all plants contain Cu/Zn SOD, and Fe SOD is also found in chloroplasts of some species. Plant SODs are products of nuclear genes, and cDNAs that encode chloroplastic Cu/Zn SODs include amino-terminal chloroplast transit peptide sequences (Scioli and Zilinskas, 1988). A full-length Fe SOD cDNA from soybean also includes an apparent chloroplast targeting domain (Crowell and Amasino, 1991).

Cu/Zn SOD

Attempts to manipulate the levels of Cu/Zn SODs in plant chloroplasts by nuclear gene transfer have been successful, but inconsistencies in the phenotypes of the resulting transgenic plants have been reported. Tepperman and Dunsmuir (1990) developed transgenic tobacco plants (cv W38) that expressed a chimeric gene for the chloroplast-localized Cu/Zn SOD from petunia under control of the constitutive CaMV 35S promoter. These plants were reported to have 30- to 50-fold higher SOD activity than untransformed control plants as estimated by SOD activity gels. In spite of these very high activities, no differences could be detected in the inhibition of CO_2 fixation by the redox-cycling herbicide MV in these plants when compared with untransformed control plants, nor were these transgenic plants detectably different from control plants in symptom development after ozone fumigation (Pitcher et al., 1991). Furthermore, transgenic tomato plants that carried the same chimeric gene but with more modest increases in SOD activity were not more resistant than control plants to photoinhibition under conditions of high light intensity, low CO_2, and chilling. In this case, increased expression of SOD alone was insufficient to provide any detectable change in oxidative stress tolerance under the conditions used. These results could indicate that dismutation of O_2^- is not the rate-limiting step in the ROI scavenging pathway of tobacco chloroplasts. Alternatively, Cu/Zn SOD, which is H_2O_2 sensitive, may be deactivated under stress conditions by the accumulation of H_2O_2. In either case, overexpression of additional components of the ROI scavenging systems, in addition to SOD, were apparently necessary for increased oxidative stress protection.

Dramatically different results have been obtained by introducing similar Cu/Zn SOD transgenes into tobacco (*Nicotiana tabacum*) and potato (*Solanum tuberosum*) (Perl et al., 1993; Sen Gupta et al., 1993a). Expression of pea (*Pisum sativum*) chloroplastic Cu/Zn SOD in tobacco (cv Xanthi) resulted in a 3-fold increase in total SOD activity, which led to a significant increase in resistance to light-mediated MV-induced membrane damage (measured by electrolyte leakage). This protective effect was found only over a relatively narrow range of MV concentrations, which could indicate that Cu/Zn SOD was deactivated by H_2O_2 at higher MV concentrations. This result could partially explain the discrepancy between these results and those of Tepperman and Dunsmuir (1990).

Although MV provides a convenient means to induce oxidative stress in plant tissues under natural conditions, plants must cope with photooxidative stress associated with exposure to excess light, especially under conditions that inhibit CO_2 fixation (such as chilling, heat, or water deficit). Exposure of leaf discs from mature tobacco plants that overexpress chloroplastic Cu/Zn SOD to high light intensity and chilling temperatures for up to 6 h resulted in

very little loss in photosynthetic capacity after they were returned to ambient conditions (>90% recovery); when exposed to the same conditions, control plants lost virtually all photosynthetic activity (Fig. 2) (Sen Gupta et al., 1993b).

Mn SOD

Bowler et al. (1991) developed transgenic tobacco plants (cv SR 1) that expressed a chimeric gene derived from a Mn SOD cDNA from *Nicotiana plumbaginifolia* in which the native mitochondrial transit peptide was replaced by a chloroplastic transit peptide from the *Arabidopsis RuBP-Case* gene. Expression of this transgene was controlled by the CaMV 35S promoter. Plants that carried this novel chloroplastic Mn SOD gene expressed a unique Mn SOD isoform that was correctly targeted to the chloroplasts. The Mn SOD activity in these plants was estimated to be between 1.5- and 2-fold higher than in untransformed plants.

Light-dependent MV-mediated oxidative damage to membranes and Chl (measured by the accumulation of pheophytin) were found to be significantly decreased in plants that expressed the chloroplastic Mn SOD gene compared to untransformed control plants. Leaves of these transgenic tobacco plants also had fewer lesions than control plants when fumigated under conditions that simulate natural diurnal ozone fluctuations in industrialized regions (Van Camp et al., 1994).

Transfer of the same chloroplastic Mn SOD chimeric gene constructs used by Bowler et al. (1991) into other plant species has also been completed. Significant increases in resistance to the herbicide acifluorofen and to freezing were found to co-segregate with the expression of this transgene in alfalfa (*Medicago sativa*) (McKersie et al., 1993), and in cotton (*Gossypium hirsuitum*) preliminary analysis

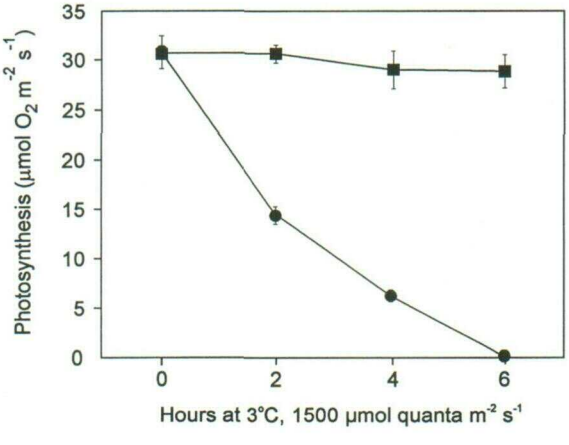

Figure 2. Photosynthesis in leaf discs of control tobacco plants (cv Xanthi) and transgenic tobacco plants that express chloroplastic Cu/Zn SOD exposed to photooxidative stress (high light intensity and low temperature). Xanthi leaf discs undergo rapid loss of photosynthetic function (photoinhibition), whereas leaf discs of transgenic Cu/Zn SOD-expressing plants show very little damage (Sen Gupta et al., 1993b).

Figure 3. Transgenic cotton plants that express chloroplast-localized Mn SOD have increased tolerance to chilling-induced oxidative stress. Plants grown to the four-leaf stage in a growth chamber at 28°C day and 15°C night were then exposed to chilling temperatures of 15°C day and 4°C night for 5 d. Immature leaves of wild-type cotton plants were visibly damaged (left), whereas those of transgenic cotton plants had no visible damage (right).

has indicated that expression of the chloroplastic Mn SOD gene may provide increased chilling tolerance (Fig. 3) (Trolinder and Allen, 1994).

Support for the hypothesis that Cu/Zn SOD is deactivated during exposure to severe oxidative stress can be seen by comparing the MV sensitivity of Cu/Zn SOD-overexpressing plants with that of transgenic tobacco plants of the same variety (cv Xanthi) that express a chloroplastic Mn SOD gene construct. This Mn SOD transgene is similar to that reported by Bowler et al. (1991), but consists of a Mn SOD cDNA from pea with a Cu/Zn SOD chloroplast targeting sequence (Schake, 1995). At low MV concentrations (0.6 and 1.2 μM), both Mn SOD and Cu/Zn SOD-expressing plants showed similar levels of damage that were significantly lower than control plants. However, at a higher MV concentration (2.4 μM), protection in the Mn SOD plants was maintained while protection in Cu/Zn SOD plants was not significantly different from that in control plants (Fig. 4). It is interesting that transgenic tobacco plants that express chloroplastic Mn SOD were found to be much more sensitive to photooxidative damage than the Cu/Zn SOD-overexpressing plants and only slightly more tolerant than control plants (Schake, 1995).

Clearly, overexpression of SOD in chloroplasts provides increased protection from oxidative stress. Elevated activity of H_2O_2-resistant Mn SOD in the chloroplast stroma apparently provides superior protection from oxidative stress caused by chemical exposure (MV, acifluorofen, ozone), but this isoenzyme is much less effective than Cu/Zn SOD at providing protection from photooxidation under photoinhibitory conditions. These differences clearly show that simply elevating SOD activity in the chloroplast stroma may not be sufficient. Rather, it appears that the type of SOD that is increased is also a critical factor. Since the Cu/Zn SOD is associated with the surface of the thy-

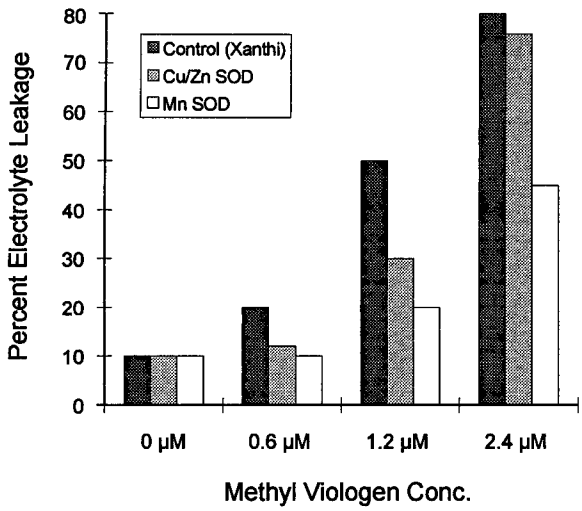

Figure 4. Comparison of membrane damage caused by exposure of leaf discs from untransformed control plants (cv Xanthi) and two transgenic tobacco lines that express either chloroplastic Cu/Zn SOD or chloroplastic Mn SOD to the free radical-generating herbicide MV. After infiltration with MV, leaf discs were illuminated for 2 h followed by dark incubation for 16 h (Sen Gupta et al., 1993a). Membrane damage was detected by electrolyte leakage. At low MV concentrations samples of both transgenic Cu/Zn SOD- and Mn SOD-expressing plants were significantly less damaged than Xanthi samples, but at the highest concentration only Mn SOD-expressing plants were protected.

lakoid membranes in close association with PSI (K. Asada, personal communication), it seems likely that the different protective activities provided by Cu/Zn SOD and Mn SOD in chloroplasts are due to differences in the biochemical characteristics and suborganellar localization of these enzymes.

OTHER ENZYMES

APX

APXs have high substrate specificity for ascorbate and are the primary H_2O_2 scavenging enzymes in the chloroplasts and cytosol of plant cells (Asada, 1992). Although catalase is capable of scavenging large quantities of H_2O_2, its location in peroxisomes and its relatively low K_m limit its ability to keep H_2O_2 concentrations low enough to prevent chloroplast damage. Cytosolic and chloroplastic forms of APX have been identified in plant cells. These enzymes differ in substrate specificity, pH optimum, and sensitivity to ascorbate depletion (Asada, 1992). Distinct thylakoid-bound and stromal forms of APX also apparently exist in chloroplasts (Miyake and Asada, 1992; Miyake et al., 1993). Cytosolic APXs are encoded by nuclear genes and a number of cDNAs for cytosolic APX have been isolated and characterized from plants, including pea (Mittler and Zilinskas, 1991) and *Arabidopsis* (Kubo et al., 1992). However, no cDNAs that encode chloroplastic APXs have yet been identified. For this reason, the APX gene family in plants has not been clearly characterized.

Transgenic tobacco plants that overexpress cytosolic APX have been developed (Pitcher et al., 1994; D. Inzé, personal communication). In addition, gene constructs that encode chimeric chloroplastic APX isoforms that consist of a 5′ chloroplast transit peptide sequence fused to cytosolic APX cDNAs have been developed and successfully expressed in tobacco plants. Increased tolerance to MV damage has been reported in tobacco plants (cv Bel W3) that overexpress cytosolic APX but not those that express a chloroplast-targeted isoform (Pitcher et al., 1994). Additional physiological analysis of these transgenic plants is currently underway to determine whether APX overexpression can provide increased protection from oxidative stress.

Sen Gupta et al. (1993b) have reported a 3-fold increase in APX activity and mRNA in transgenic tobacco plants that overexpress chloroplastic Cu/Zn SOD. Induction of APX to similar levels could also be achieved by treating untransformed tobacco leaf discs with H_2O_2 (Allen et al., 1994). These results indicate that the regulatory mechanisms that control the expression of SOD and APX in tobacco can remain intact in transgenic plants and result in changes in the activities of enzymes other than those encoded by the introduced gene.

GR

Regeneration of oxidized ascorbate is a critical component of the ROI scavenging system. Of the enzymes that are involved in this process, GR has received the most attention (see Creissen et al., 1994, for review). In pea leaves GR is present in chloroplasts, mitochondria, and cytosolic cellular compartments (Edwards et al., 1990). These enzymes are encoded by nuclear genes, and GR-encoding cDNAs from pea and tobacco have been isolated and sequenced. The derived amino acid sequences from these cDNAs have substantial homology with bacterial and mammalian GRs, but they include putative amino-terminal chloroplast transit peptide sequences. As with APXs, the genomic organization of the GR gene family has not been completely elucidated.

Initial attempts to manipulate the levels of GR in transgenic plants were carried out with the GR gene from *Escherichia coli* (*gor*) under control of the CaMV 35S promoter. Total GR specific activity in transgenic tobacco plants that expressed the chimeric *gor* gene was increased by as much as 3.5-fold, and since the *E. coli* protein lacked a transit peptide it was presumably confined to the cytosol. Plants that expressed high levels of bacterial GR showed no decrease in ozone sensitivity but were somewhat more tolerant of MV and were able to maintain the reduction state of their ascorbate pools more effectively than control plants (Aono et al., 1991; Foyer et al., 1991).

Targeting of the *E. coli* GR to chloroplasts in transgenic tobacco plants has been carried out and GR activity levels approximately 3-fold higher than control plants were achieved. These plants are reported to have increased tolerance to MV and sulfur dioxide but not to ozone (Aono et al., 1993). Transgenic tobacco plants that overexpress pea

GR have also been developed and are currently being analyzed (Creissen et al., 1994).

CONCLUSIONS

Physiological and genetic evidence clearly indicates that the ROI scavenging systems of plants are an important component of the stress protective mechanism. The ability to manipulate the levels of specific enzymes of this pathway using gene transfer technology has begun to provide insights into the specific functions of the component enzymes of this system, and has raised hopes that in the future this approach can be used to improve the stress tolerance of economically important plants. It is clear that the outcome of such experiments can be largely dependent on the particular isoform of the enzyme used, the species and variety of the host plant, and the methods used to apply stress and to detect damage. Future analyses of the capacity of ROI scavenging enzymes, either individually or in combination, to alter the stress tolerance of plants should be combined with intensive physiological, biochemical, and biophysical analyses to enable us to understand the interactions of these enzymes in cellular metabolism.

Although the protective effects provided by the overexpression of single genes are usually rather small, it should be remembered that the stress regimes used are designed to produce detectable stress damage in a short period of time. Therefore, one might speculate that in crop plants exposed to frequent periods of mild or moderate stress throughout a growing season, these small increases in tolerance could be additive and have substantial effects on yield. Field tests of transgenic plants that overexpress SODs will surely provide the answer to these and other questions about the utility of enhancing the ROI scavenging systems of crop plants.

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