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Increased Resistance to Oxidative Stress in Transgenic Plants by Targeting Mannitol Biosynthesis to Chloroplasts[1]

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To investigate the potential role of a polyol, mannitol, in oxidative stress protection, a bacterial mannitol-1-phosphate dehydrogenase gene was targeted to chloroplasts by the addition of an amino-terminal transit peptide. Transgenic tobacco (*Nicotiana tabacum*) lines accumulate mannitol at concentrations ranging from 2.5 to 7 μmol/g fresh weight. Line BS1-31 accumulated approximately 100 mM mannitol in chloroplasts and was identical to the wild type in phenotype and photosynthetic performance. The presence of mannitol in chloroplasts resulted in an increased resistance to methyl viologen (MV)-induced oxidative stress, documented by the increased retention of chlorophyll in transgenic leaf tissue following MV treatment. In the presence of MV, isolated mesophyll cells of BS1-31 exhibited higher CO_2 fixation than the wild type. When the hydroxyl radical probe dimethyl sulfoxide was introduced into cells, the initial formation rate of methane sulfinic acid was significantly lower in cells containing mannitol in the chloroplast compartment than in wild-type cells, indicating an increased hydroxyl radical-scavenging capacity in BS1-31 tobacco. We suggest that the chloroplast location of mannitol can supplement endogenous radical-scavenging mechanisms and reduce oxidative damage of cells by hydroxyl radicals.

In several studies a beneficial effect on abiotic stress tolerance against damage by drought and high salinity has been documented when transgenic plants contained or accumulated a number of metabolites, such as fructan (Pilon-Smits et al., 1995), Pro (Kishor et al., 1995), Gly betaine (Nomura et al., 1995), trehalose (Holmstrom et al., 1996), and mannitol (Tarczynski et al., 1993). As a group, these and other metabolites, which alone or in combinations accumulate in a number of stress-tolerant plants, have been termed compatible solutes (Ahdam et al., 1979; Hanson et al., 1994) because they do not interfere with normal metabolic reactions even at high concentrations. Compatible solutes include sugar alcohols, quaternary ammonia compounds, Pro, and tertiary sulfonic compounds. Some of the compounds are methylated derivatives of common metabolites (Bohnert and Jensen, 1996). The most obvious function of these compounds is in "osmotic adjustment." For example, the halophytic ice plant can accumulate more than 1 M of a methylated inositol, D-pinitol, under salt stress, which will increase the cellular osmotic potential and sustain water balance (Adams et al., 1992). However, there is doubt about whether this is the main or the only function of these metabolites.

Transgenic plants, whether they have been engineered to accumulate mannitol, Gly betaine, Pro, trehalose, or fructans, contain these compatible solutes at concentrations in the range of 5 to less than 100 mM, which seems too low to account for a significant osmotic adjustment. The demonstrated protection might be based on a different mechanism or individual compatible solutes might act through several different mechanisms. To accommodate such unknown functions a concept was proposed that viewed these compatible solutes as "osmoprotectants" (Le Rudulier et al., 1984). It is suggested that compatible solutes might, for example, stabilize the structure of proteins under stress (Galinski, 1993; Papageorgiou and Murata, 1995), that these compounds might be radical scavengers, or that they might be viewed as low-molecular-weight chaperones (Bohnert and Jensen, 1996).

Mannitol is a widely distributed sugar alcohol in organisms from bacteria to algae, fungi, and more than 100 species of higher plants, including many crops such as celery, olive, and carrot (Bieleski, 1982; Stoop et al., 1996). In vitro experiments demonstrated that mannitol, sorbitol, and Pro at low concentrations (approximately 20 mM) inhibited the hydroxylation of salicylate by hydroxyl radicals and protected enzymes from inactivation (Smirnoff and Cumbes, 1989). Although mannitol has been known as a hydroxyl radical scavenger in vitro for many years, the products of the reactions between mannitol and hydroxyl radicals are still not identified (Buxton et al., 1988; Halliwell et al., 1988; Franzini et al., 1994). Here we investigated a potential role of mannitol in radical oxygen scavenging in vivo.

Production of ROS is inevitable in chloroplasts in the light. Abundant oxygen can be reduced to generate superoxide radicals in the Mehler reaction of PSI when a reduction of PSI exceeds the demand utilized by CO_2 fixation (Asada, 1994; Allen, 1995). Efficient scavenging of active oxygen species is essential for chloroplasts to maintain photosynthesis because even low amounts of H_2O_2 will inhibit the activity of dark-reaction enzymes (Kaiser, 1979).

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Abbreviations: MSA, methane sulfinic acid; MV, methyl viologen; ROS, reactive oxygen species.

Under normal conditions, ROS are scavenged by resident enzyme systems and nonenzymatic antioxidants. The enzyme systems include superoxide dismutases, which catalyze the reaction from superoxide to H_2O_2, and ascorbate peroxidases, which are responsible for the conversion of H_2O_2 to water. Ascorbate can be regenerated by the ascorbate-glutathione cycle (Asada, 1994; Allen, 1995). Overexpression of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in transgenic plants has already been shown to lead to an increased resistance to oxidative stresses (Bowler et al., 1991; Aono et al., 1993; Gupta et al., 1993a; Van Camp et al., 1994a). Nonenzyme antioxidants include α-tocopherol, ascorbate, glutathione, and carotenoids. Botanical sources of such antioxidants not only play roles in plant stress adaptation, but also retard aging and the diseases that are related to oxidative damage in animals (Dalton, 1995).

The hydroxyl radical can be produced by either the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^*$) or by the direct interaction of superoxide and H_2O_2. In contrast to the detoxification systems for H_2O_2 and O_2^-, an enzyme system that could deal with the short-lived hydroxyl radical has not been identified and, in fact, might not have evolved (Halliwell and Gutteridge, 1990). Considering that hydroxyl radicals are even more toxic than either superoxide or H_2O_2, an abundant generation of this radical would pose a major problem and might lead to the oxidation of proteins. We have now generated tobacco (*Nicotiana tabacum*) plants that accumulate mannitol in chloroplasts to analyze the role of mannitol when ROS increase in vivo.

MATERIALS AND METHODS

Gene Constructs and Transformation

To target mannitol-1-phosphate dehydrogenase into chloroplasts, an *Escherichia coli* gene, *mtlD* (Novotny et al., 1984), was fused with the transit peptide sequence of the pea (*Pisum sativum*) *RbcS3A* gene. A *Pst*I-*Bam*HI PCR fragment containing the coding region of *mtlD* was inserted into the pJIT117 vector (Guerineau et al., 1988), resulting in plasmid pBS1. The *Sst*I-*Xho*I fragment of pBS1 was then subcloned into the *Sst*I-*Sal*I sites of the binary vector pBIN19 and was introduced into the *Agrobacterium tumefaciens* strain LBA4404 by electroporation. Transformation of tobacco (*Nicotiana tabacum* var SR-1) leaf discs was performed as described previously (Tarczynski et al., 1992). Thirty-three independent T_0 transformants were regenerated, which were selfed to produce T_1 seeds. Five T_1 plants were selfed to produced T_2 seeds, and homozygous T_2 seeds were used for these experiments.

Analysis of Mannitol in Plants

Leaf mannitol content was measured by HPLC analysis, as described previously (Tarczynski et al., 1992). Intact chloroplasts were prepared from mesophyll protoplasts. Tobacco leaves (2–4 g) were cut into sections that were incubated in 40 mL of enzyme solution containing 1.2% cellulase (Onozuka R10, Yakult Honsha Co., Ltd., Tokyo, Japan), 0.4% macerozyme R10, and 0.45 M Suc in a B5 medium. After 4 to 6 h of incubation, the cell suspension was filtered through a 100-μm nylon mesh and was centrifuged at 60*g* for 5 min. Intact protoplasts were floated on a 0.45 M Suc medium and then washed with a B5 medium containing 0.45 M Gly betaine. Chloroplasts were released from the protoplasts by passing the protoplasts through a 25-μm nylon mesh three times and were collected by centrifugation at 100*g* for 1 min. The amount of mannitol in the pellets of protoplasts and chloroplasts was measured as described previously (Tarczynski et al., 1992). Chlorophyll was determined according to Arnon (1949).

MV Treatment of Leaf Discs

MV sensitivity of leaf discs was analyzed as described previously (Bowler et al., 1991; Gupta et al., 1993a). Leaf discs (1.3 cm diameter) collected from the leaves of four to five plants in the preflowering, rapid-growth phase were transferred to 3.5-cm Petri dishes containing 3.5 mL of 2 μM MV solution. Each Petri dish contained eight leaf discs and was incubated at 25°C for 20 h in darkness. Leaf discs were then illuminated (150 μmol m^{-2} s^{-1}) for 36 h, and the chlorophyll content was determined (Arnon, 1949).

Analysis of CO_2 Fixation

Mesophyll cells were isolated as before (Jensen et al., 1971) with minor modifications. Leaf samples were collected from four to five individual plants in the preflowering, rapid-growth phase. Small leaf sections were digested in a maceration medium containing 0.5% macerase pectinase (Calbiochem), 0.3% potassium dextran sulfate, 0.6 M Gly betaine in half-strength Hoagland solution (pH 5.6), and cells were washed twice in the same solution without an enzyme. Cells equivalent to approximately 20 μg of chlorophyll were suspended in an 800-μL incubation medium (0.6 M Gly betaine, 20 mM Hepes, pH 7.4, in half-strength Hoagland solution) with or without MV, and 40 μL of 0.25 M $KH^{14}CO_3$ (specific activity, 1 μCi/μmol) was then added. The glass vials were placed in a water bath at 25°C and illuminated at 450 μmol m^{-2} s^{-1}. The amount of CO_2 fixed in cells was determined with a liquid scintillation counter after removal of excess $H^{14}CO_3^-$. Leaf CO_2 fixation was measured by using a LI-6400 photosynthesis system (Li-Cor, Lincoln, NE).

Hydroxyl Radical Production in Cells

Production of hydroxyl radicals in mesophyll cells was determined using DMSO as a probe. The mesophyll cells containing approximately 300 μg of chlorophyll were incubated with different concentrations of DMSO and 100 μM MV at 4°C in darkness for 30 min. After dark incubation, cells were placed in a water bath at 25°C and illuminated (500 μmol m^{-2} s^{-1}) for 1 h. The reaction was terminated by adding 500 μL of an extraction buffer (methanol: chloroform:water, 12:5:3, v/v). The extract was centrifuged at 10,000*g* for 5 min and the supernatant was dried in a vacuum drier at room temperature. The resulting pellet was resuspended in 1 mL of water and passed through a Sep-Pak column (Millipore) to remove hydrophobic inter-

Figure 1. Schematic outline of the pBS1 gene construct. The gene cassette was subcloned into pBIN19 and introduced into plants by *Agrobacterium*-based transformation. TP, Transit peptide sequence of the pea *RbcS-3A* gene; mtlD, coding region of the *E. coli* mannitol-1-phosphate dehydrogenase gene; CaMV Poly A, cauliflower mosaic virus poly(A).

ferences. MSA in samples was determined as described by Babbs et al. (1989; Babbs and Steiner, 1990). The recovery of standard MSA using this procedure is more than 85%.

RESULTS

Accumulation of Mannitol in Chloroplasts

The bacterial mannitol-1-phosphate dehydrogenase gene *mtlD*, under control of a double cauliflower mosaic virus 35S promoter, was fused with a pea *RbcS* transit peptide sequence to target the enzyme into the chloroplasts of tobacco (Fig. 1). This enzyme catalyzes the reaction from Fru-6-P to mannitol-1-phosphate. Mannitol-1-phosphate is then converted to mannitol by a nonspecific phosphatase in tobacco (Tarczynski et al., 1992). After *Agrobacterium*-mediated transformation of the gene construct, 16 independently transformed tobacco lines that produced mannitol were isolated. Mannitol amounts in all of the transformants were dependent on plant and leaf age, with the highest amounts found in plants before flowering. Mature leaves accumulated more mannitol than young leaves. The highest mannitol-producing line, termed BS1-31, accumulated mannitol as high as 7 μmol/g fresh weight in leaves and 3 μmol/g fresh weight in roots and was selected for further experiments.

Experiments were carried out with the homozygous T_2 generation after selfing. All individuals in the T_2 population were kanamycin-resistant and accumulated mannitol. Transgenic plants that were grown in soil showed no visual difference in phenotype compared with wild-type SR-1 and had the same leaf CO_2 fixation rate as the wild type (Table I). Determination of mannitol in isolated protoplasts and intact chloroplasts isolated from such protoplast preparations indicated that at least 60% of the total cellular mannitol was present in chloroplasts (Table I). Based on the relationships between chlorophyll content and chloroplast stromal space (Asada, 1994), the amount of mannitol detected in the chloroplast fraction is calculated to be 73 mM based on 35 μL stroma space/mg chlorophyll. In comparison, 80% of the Rubisco, which is located only in the chloroplast stroma, was recovered from comparable chloroplast preparations (data not shown). Hence, with that adjustment, the actual mannitol concentration in chloroplasts may be approximately 100 mM.

Resistance to MV-Induced Oxidative Stress in Transgenic Plants

BS1-31 plants were treated with the herbicide MV to induce oxidative stress (Bowler et al., 1991; Gupta et al., 1993a) following two different experimental strategies. In leaf disc experiments discs from the leaves of an identical developmental age were incubated with 2 μM MV and the loss of chlorophyll was determined (Fig. 2). Leaf discs from BS1-31 plants consistently retained significantly more chlorophyll at the end of the incubation period than leaf discs from wild-type plants. To eliminate the differences that might exist in the MV uptake in leaf discs between BS1-31 and SR-1, mesophyll cells were isolated and $KH^{14}CO_3$ and 100 μM MV were added to the incubation medium. In the absence of MV, cells from SR-1 and BS1-31 had indistinguishable CO_2 fixation rates but after the addition of MV cells from transgenic BS1-31 showed a higher amount of fixed CO_2 than SR-1 (Fig. 3).

Enhanced Hydroxyl Radical-Scavenging Capacity in Transgenic Plants

DMSO is an ideal molecular probe for trapping hydroxyl radicals in plant cells because of its low toxicity in concentrations up to 1 M and because DMSO penetrates cell membranes readily (Babbs and Steiner, 1990). DMSO can be attacked by hydroxyl radicals, resulting in the production of MSA, which can be quantitated in a color reaction with fast-blue BB (Sigma; Babbs et al., 1989; Babbs and Steiner, 1990). The rate constant for a reaction between DMSO and hydroxyl radicals is approximately 4-fold higher than the rate constant of a reaction between mannitol and hydroxyl radicals (Buxton et al., 1988). In a Fenton reaction mixture producing hydroxyl radicals, 100 mM mannitol competed with DMSO for hydroxyl radicals only at less than 50 mM DMSO in the reaction. With an increase of the DMSO concentration, inhibition of MSA formation by mannitol decreased (Fig. 4A). At DMSO concentrations exceeding 100 mM, the inhibition of MSA formation by mannitol was reduced to less than 2%, indicating that nearly all hydroxyl radicals are trapped by DMSO under

Table I. *Photosynthetic CO_2 fixation and mannitol accumulation in wild-type tobacco (SR-1) and the transgenic line (BS1-31)*

Chloroplasts, five repetitions with different protoplast preparations, were isolated from leaves of plants before bolting. Values are the means ± SE.

Plant	CO_2 Fixation Rate in Leaves	Mannitol Content in Leaves	Mannitol Content in		Percent CP[a]
			Protoplasts	Chloroplasts	
	μmol m^{-2} s^{-1}	μmol/g fresh wt	μmol/mg chlorophyll	μmol/mg chlorophyll	
SR-1	10.2 ± 0.2	ND[b]	ND	ND	
BS1-31	10.1 ± 0.4	2.5–7.0	4.08 ± 0.07	2.54 ± 0.08	62.3

[a] Percent CP, Percentage of total mannitol localized in chloroplasts. [b] ND, None detected.

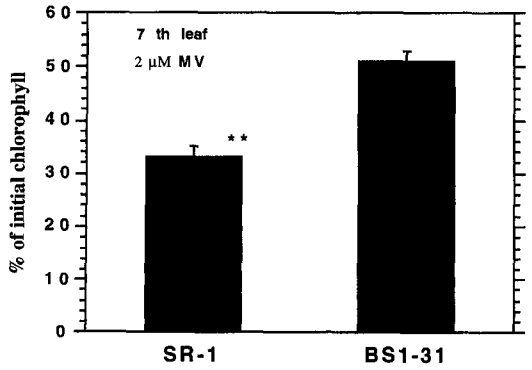

Figure 2. Loss of chlorophyll in MV-treated leaf discs from mannitol-accumulating transgenic (BS1-31) and wild-type (SR-1) tobacco. A total of 64 leaf discs were collected from five plants and randomly distributed in Petri dishes. The experiment was repeated three times with very similar results. Values are mean ± SE of one experiment. **, P = 0.01.

these conditions (data not shown). Based on such in vitro competition experiments, we used DMSO at less than 25 mM to detect whether competition for radicals between mannitol and DMSO could be shown.

To measure the total amount of hydroxyl radicals that were produced in cells, however, 300 mM DMSO was chosen. When mesophyll cells were incubated with DMSO in the dark, the production of MSA was less than 15 nmol mg^{-1} chlorophyll h^{-1}. In the absence of DMSO, MSA could not be detected in plant cells, indicating that the color reaction is specific for MSA. In the presence of 100 μM MV, BS1-31 cells showed lower initial rates of MSA formation than cells from SR-1 (Fig. 4B). This change in MSA production is correlated with a high concentration of mannitol in chloroplasts, suggesting that BS1-31 had a modified hydroxyl radical-scavenging capability and that mannitol could be responsible for the changes. At high DMSO concentrations, we expected that vast amounts of hydroxyl radicals should be captured by DMSO and not by mannitol or by endogenous radical scavengers. In this case, the amount of MSA formation would be indicative of total production of hydroxyl radicals in the experiment.

Incubation of the SR-1 and BS1-31 cells in the presence of 300 mM DMSO revealed no difference between the two cell types with respect to MSA formation, indicating that synthesis and the presence of mannitol in the BS1-31 cells did not affect hydroxyl radical production per se. When MV was added to the cells, MSA amounts and hydroxyl radical production approximately doubled in both SR-1 and BS1-31 cells (Fig. 5), again indicating that mannitol was not active in reducing the amounts of hydroxyl radicals that are formed. The doubling of radical production at the MV concentration used was obviously enough to overwhelm the endogenous scavenging systems, and mannitol may contribute to the scavenging capacity to slow down damage under such conditions.

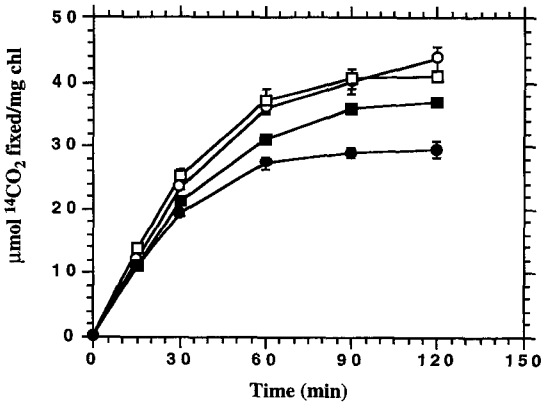

Figure 3. Time course of photosynthetic CO_2 fixation by isolated mesophyll cells from BS1-31 and wild-type tobacco. One hundred micromolar MV was added to mesophyll cells in traces ■ and ●; □ and ○, controls without MV; □, ■, BS1-31 cells; ○, ●, wild-type cells. chl, Chlorophyll.

DISCUSSION

Environmental stresses such as drought (Smirnoff, 1993), low temperature in high light (Wise, 1995), or air pollution

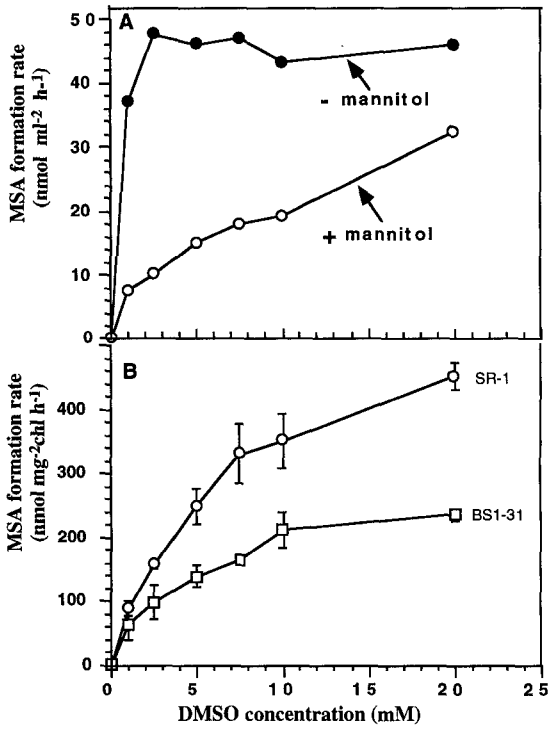

Figure 4. Kinetics of MSA formation dependent on DMSO concentration. A, Competition between DMSO and mannitol for hydroxyl radicals in vitro. Hydroxyl radicals were generated by a Fenton reaction: 5 mM phosphate buffer, pH 7.4, 0.1 mM $FeCl_3$, 0.1 mM ascorbate, 1 mM H_2O_2, and 0.2 mM EDTA. The mannitol concentration was 100 mM. The reaction mixture was incubated at room temperature for 90 min and MSA was determined as described (Babbs et al., 1989; Babbs and Steiner, 1990). B, Competition between DMSO and other hydroxyl radical scavengers in mesophyll cells from transgenic plants (BS1-31) and the wild type (SR-1). The mesophyll cells were preincubated with 100 μM MV and different DMSO concentrations at 4°C in darkness for 30 min. MSA formation was determined after cells were illuminated with 500 μmol quanta m^{-2} s^{-1} light at 25°C for 1 h. The values are the mean ± SE of three independent experiments. chl, Chlorophyll.

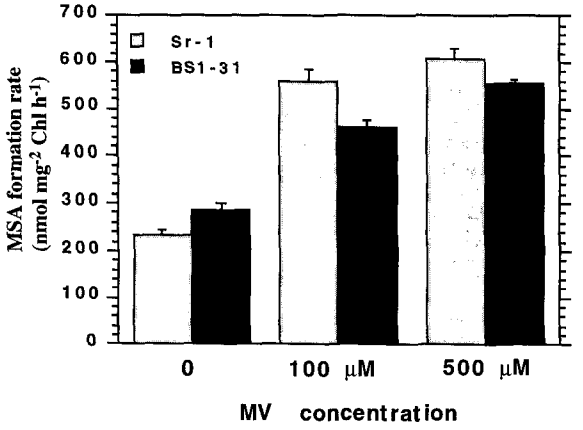

Figure 5. Effect of MV on hydroxyl radical production in mesophyll cells. The mesophyll cells were incubated with MV and 300 mM DMSO at 4°C in darkness for 30 min. MSA formation was determined after cells were illuminated with 500 μmol quanta m^{-2} s^{-1} light at 25°C for 1 h. The values are mean ± SE of four independent experiments. Chl, Chlorophyll.

(Mehlhorn and Wellburn, 1994) can directly or indirectly induce oxidative stress. Under low-temperature and drought conditions, production of ROS increases significantly (Smirnoff, 1993; Mehlhorn and Wellburn, 1994). Overexpression of radical-scavenging enzymes such as superoxide dismutase and glutathione reductase have resulted in an increased resistance to drought, ozone, and low-temperature and high-light stresses (Gupta et al., 1993b; Van Camp et al., 1994b; McKersie et al., 1996). Responding to such environmental stress, many plants accumulate compatible solutes, with betaines and polyols being the most common (Ahdam et al., 1979; Hanson et al., 1994; Bohnert and Jensen, 1996). One obvious function of compatible solutes is probably the osmotic adjustment through which water retention would be supported. However, transgenic plants with overexpression of these compatible solutes typically accumulate them only to low concentrations, yet increased resistance to stress has been demonstrated (Tarczynski et al., 1993; Kishor et al., 1995; Nomura et al., 1995; Pilon-Smits et al., 1995; Holmstrom et al., 1996). This raises the question whether compatible solutes might have functions in addition to osmotic adjustment, which was observed even at low concentrations.

One such function, suggested by in vitro experiments (Halliwell et al., 1988; Smirnoff and Cumbes, 1989; Franzini et al., 1994), is the involvement in hydroxyl radical scavenging. To test if mannitol can function as a hydroxyl radical scavenger in vivo, we introduced a mannitol-1-phosphate dehydrogenase gene into tobacco and targeted the enzyme into chloroplasts. Our rationale was that increases in hydroxyl radical production should most effectively be accomplished in the chloroplast compartment. In in vitro experiments, 20 mM mannitol scavenged approximately 60% of the hydroxyl radicals (Smirnoff and Cumbes, 1989). Our transgenic plants accumulated as high as 100 mM mannitol in chloroplasts, which would be high enough to function as a hydroxyl radical scavenger in vivo. Under normal growth conditions, antioxidant systems in plants are able to detoxify active oxygen species. However, following MV treatment, the production of hydroxyl radicals increases 2-fold (Fig. 5). This increase appears to exceed the capacity of scavenging systems, resulting in oxidative damages. Thus, it is clear that mannitol in the chloroplast does not reduce hydroxyl radical production, but that it increases the capacity to scavenge these radicals and protects cells against oxidative damages under MV treatment. When a hydroxyl radical probe, DMSO, was introduced into the cells, competition between mannitol and DMSO for available hydroxyl radicals was observed. Compared with cells from the wild-type plants, the initial rate of MSA formation was much lower in cells from mannitol-containing plants, suggesting that the ability to scavenge hydroxyl radicals was enhanced in the cells from these transgenic plants. Compared with the rate of MSA formation in the in vitro reaction without any competitor, the initial rate of MSA formation in the wild-type cells was much lower (Fig. 4), suggesting that other antioxidants such as ascorbate, α-tocopherol, and glutathione provide protection and that these endogenous systems may be sufficient under nonstress conditions.

There are several possible advantages in targeting mannitol-1-phosphate dehydrogenase to chloroplasts. First, chloroplasts are the main site of ROS production in plants (Asada, 1994). Second, mannitol-1-phosphate dehydrogenase catalyzes the reaction from Fru-6-P to mannitol-1-phosphate. Fru-6-P is a substrate for starch biosynthesis in chloroplasts. The low flux of Fru-6-P to mannitol biosynthesis will most likely be of little consequence, considering the size of the Fru-6-P pool. Third, enzymes that degrade mannitol have been localized in the cytosol in celery (Stoop et al., 1996). Synthesis of mannitol in chloroplasts will separate synthesis spatially from the degradation pathway, which may result in a reduced turnover of mannitol. These implications may explain why chloroplast targeting of mannitol biosynthesis in transgenic plants leads to higher mannitol amounts than in plants in which mannitol biosynthesis is cytosolic (Tarczynski et al., 1992). Finally, sugars such as Glc, Fru, and Suc, in which the radical-scavenging capability has been demonstrated in vitro, will inhibit the expression of photosynthesis-related genes and affect CO_2 fixation at high concentrations (Sheen, 1990). Mannitol, even at high concentrations, does not suppress photosynthesis and its presence has no harmful effects on plants (Table I). The transgenic plants showed leaf photosynthetic rates identical to the wild type and there was no visible phenotype.

Compared with superoxide and H_2O_2, hydroxyl radicals are more reactive and readily attack lipids, nucleic acids, and proteins at near-diffusion-limited rates, resulting in oxidative damage to cells (Halliwell and Gutteridge, 1990; Asada, 1994). Because of their high reactivity and short lifetimes, hydroxyl radicals produced in vivo will react at, or close to, the site of their formation. Protection from damage in vivo is then best achieved by mechanisms that prevent hydroxyl radical formation. Once produced, however, protection depends on the presence of antioxidants in the vicinity of the site of formation. Most of the hydroxyl

radicals generated in vivo originate from the decomposition of H_2O_2 in the presence of transition metals such as Fe^{2+} and Cu^{2+} (Asada, 1994; Allen, 1995). The components for this reaction are abundant in chloroplasts, because superoxide and H_2O_2 are produced in the Mehler reaction of PSI. In addition, ascorbate, which is present in concentrations of 10 mM in chloroplasts, can reduce Fe^{3+} to Fe^{2+} (Asada, 1994). Fe and Cu ions are more concentrated in chloroplasts than in the rest of the cell (Whatley et al., 1951; Terry and Low, 1982). Leaves of sugar beet, for example, contain 127 nmol Fe/mg chlorophyll and approximately 80% of the Fe is located in chloroplasts. Stromal amounts of Fe are approximately 25 nmol Fe/mg chlorophyll, which results in a concentration of approximately 700 μM, whereas the Fenton reaction requires only a trace amount of Fe (<10 μM) (Terry and Low, 1982). Considering the presence of H_2O_2, Fe^{2+}, and ascorbate in chloroplasts, hydroxyl radicals are most likely formed via a Fenton reaction. Our data demonstrated the formation of hydroxyl radicals in vivo using DMSO as a probe. In chloroplasts H_2O_2 rapidly inhibits photosynthesis via inactivation of Calvin-cycle enzymes, especially SH enzymes such as Fru-1,6-bisphosphatase and ribulose-5-phosphate kinase (Kaiser, 1979). Considering the presence of metal ions in chloroplasts, the inactivation of SH enzymes by H_2O_2 may be mediated by hydroxyl radicals.

We have recently used an isolated thylakoid system in which the hydroxyl radical scavengers mannitol and sodium formate prevented the loss of ribulose-5-phosphate kinase activity, suggesting that this enzyme is prone to inactivation by hydroxyl radicals (B. Shen, unpublished data). In contrast to the enzymes of the photosynthetic dark reactions, the thylakoid proteins and electron transport carriers are less sensitive to H_2O_2 (Asada, 1994). We suggest that the higher CO_2 fixation in transgenic plant cells under MV treatment is likely due to protection of dark reaction enzymes such as ribulose-5-phosphate kinase against hydroxyl radical damage. The precise mechanism by which mannitol decreases hydroxyl radical damages is still unknown, however. It may chemically react with the radicals within a diffusion boundary, which would be in line with the compatible solute hypothesis that considers accumulating osmolytes as osmoprotectants (Le Rudulier et al., 1984; Smirnoff and Cumbes, 1989; Bohnert and Jensen, 1996).

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