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Paraquat Sensitivity of Transgenic *Nicotiana tabacum* Plants that Overproduce a Cytosolic Ascorbate Peroxidase

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Summary

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The cDNA encoding the Arabidopsis cytosolic ascorbate peroxidase was placed under the control of the promoter for ribulose-1,5-bisphosphate carboxylase small subunit gene, and the chimeric gene was then introduced into tobacco. Leaves of the transgenic plants exhibited up to 5 to 10 fold higher ascorbate peroxidase activity than control non-transgenic plants. However, the paraquat sensitivity of these transgenic plants did not differ significantly from that of control plants as evaluated by electrolyte leakage from leaf discs. The ascorbate content of leaf discs of both transgenic and control plants rapidly decreased during paraquat treatment. The cytosolic activity of ascorbate peroxidase appears therefore, at least under the present study conditions, not to be a limiting factor in the tolerance of plants to paraquat-induced oxidative stress.

Introduction

Various environmental conditions cause oxidative stress to plants through the intracellular production of active oxygen species (AOS). These oxygen species, unless rapidly removed by the cells' scavenging system, may destroy or inactivate various organic substances and lead to cellular damage. The AOS scavenging system consists of various redox substances such as ascorbate and glutathione, and enzymes such as superoxide dismutase and those involved in the

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ascorbate-glutathione pathway. This system appears not only to be indispensable to survival under normal aerobic environments, but also to be involved in tolerance to adverse conditions. By manipulating genes that encode enzymes of the AOS-scavenging system, there has been some success in artificially controlling the resistance of plants to oxidative stress (ALLEN 1995). Transgenic plants that overproduce superoxide dismutase have been reported to exhibit enhanced tolerance to the herbicide paraquat, to the air pollutant ozone, and to low temperatures and freezing stress. Similarly, transgenic plants with enhanced glutathione reductase activity have been shown to have enhanced tolerance to paraquat and the air pollutant sulfar dioxíde, whereas plants with reduced glutathione reductase activity showed increased sensitivity to paraquat. These results not only strongly support the role of superoxide dismutase and glutathione reductase in the scavenging system, but also imply that these enzymes, under certain conditions, may be limiting factors in the plant stress response. Apart from these enzymes, however, little such work has been reported for other enzymes of the AOS scavenging system.

Ascorbate peroxidase (APX) is a component of the ascorbate-glutathione pathway and catalyzes the reduction of hydrogen peroxide to water using ascorbate as an electron donor. At least three different isoforms of the enzyme have been identified in leaf cells; thylakoid-bound, stromal and cytosolic forms (MIYAKE & al. 1993). Of these, cDNAs encoding the cytosolic form have been isolated from numerous plant species, and transgenic tobacco plants that overproduce cytosolic APX have been generated and found to be less sensitive to paraquat than the control, non-transgenic plants (PITCHER & al. 1994). However, detailed information about such transgenic plants is not yet available. We report here the generation and characterization of transgenic tobacco plants that express the cytosolic APX cDNA from *Arabidopsis thaliana*.

Material and Methods

1. Generation and growth of transgenic plants

A cDNA encoding the cytosolic APX of *A. thaliana* (KUBO & al. 1992) was placed downstream of the promoter region of a tomato gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rbcs-3B (SUGITA & al. 1987), and the chimeric gene was then inserted into plasmid pBI101 (Clontech, Palo Alto, USA) from which the *f*À-glucuronidase gene had been deleted. This construct was used to generate transgenic plants of *Nicotiana tabacum* SR1 through Agrobacterium-mediated transformation of leaf discs. Seeds, obtained by self-fertilization of the transgenic plants, were used to grow progenies that were used for various analyses. Seedlings were grown on vermiculite in pots at 25□□ under a regime of 14 h light (120 $\mu E^{-2}\ s^{-1}$) and 10 h of darkness.

2. Measurement of APX activity and immunoblotting

Crude extracts were prepared from tobacco leaves by homogenizing the tissue in 5 ml (g fresh weight)$^{-1}$,P of 0.1 M K-phosphate (pH 7.8) containing 5 mM Na ascorbate and 2% sorbitol. The homogenates were subjected to centrifugation at 6,000 x g for 20 min, and the supernatants used for the determination of APX activity and immunoblotting. APX activity was measured as

described by NAKANO & ASADA 1987. Monoclonal antibodies were prepared against Arabidopsis cytosolic APX as described by SAJI & al. 1990. Some of these antibodies were found to cross-react with tobacco APX while other antibodies did not. These two types of antibody were used to distinguish the transgene product and endogenous tobacco cytosolic APX by immunoblotting.

3. Examination of paraquat sensitivity

The sensitivity of leaf tissues to paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride; methyl viologen, Sigma Chemical Company, USA) was evaluated by electrolyte leakage from leaf discs as described by AONO & al. 1995. Discs were excised from fully expanded leaves of 1-2 month old plants and exposed to 20 μM paraquat containing 0.1% Tween 20 by vacuum infiltration. Ten leaf discs were floated in this solution in the dark for 1 h and then washed and transferred to 7 ml of deionized water in a Petri dish. They were left in the light (120 μE^{-2} s^{-1}) at 25°C, and the conductivity of the bathing water was measured with a conductivity meter B-173 (Horiba Ltd., Japan) every hour.

For the measurement of ascorbate content, these leaf discs were homogenized in 1 ml of 5% metaphosphoric acid followed by centrifugation at 6,000 x g for 5 min. The concentration of reduced ascorbate in the supernatant fraction was determined with a reflecting photometer (RQflex, Merck, Germany) according to the supplier's instructions.

Results and Discussion

1. Expression of transgene in transgenic plants

Transgenic plants with various levels of transgene expression, as determined by immunoblot analysis, were obtained. These plants showed no morphological or growth abnormalities. Of these, three plant lines with the highest APX activities were selected, designated ssAPX1, ssAPX2 and ssAPX3, and were used for further analyses. The foliar APX activity varied considerably, even among individual plants of the same line, but were generally 2-10 fold the control value in ssAPX1 and ssAPX3, and 1.5-3 fold in ssAPX2.

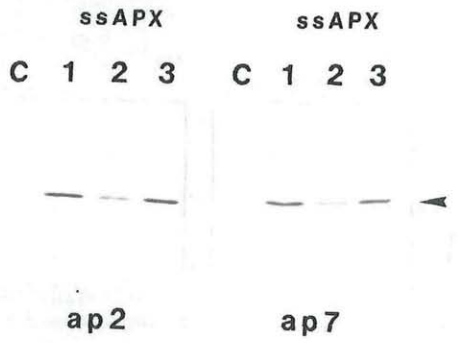

Fig. 1. Immunoblots of foliar extracts of control (C) and transgenic (ssAPX1, 2, 3) plants probed with anti-APX monoclonal antibodies. Antibody ap2, but not ap7, cross-reacts with the endogenous tobacco APX. The arrowhead indicates the position of cytosolic APX.

Results of immunochemical analyses demonstrated that the amounts of APX protein in these plants corresponded closely with these activities (Fig. 1). Furthermore, the transgene product was clearly detectable in extracts of transgenic plants using an antibody (ap7) that did not cross-react with the host enzyme.

2. Paraquat sensitivity of leaf tissues of transgenic plants

Sensitivity to the AOS-generating herbicide, paraquat, is the most extensively used index of plant tolerance to oxidative stress. The herbicide is first reduced by electrons from the photosynthetic electron transport chain in the light, so it is then able to react with oxygen to yield superoxide. We measured the leakage of electrolytes from leaf discs so as to monitor the extent of damage to the leaf tissue caused by the herbicide. Rapid electrolyte leakage was observed in the leaf discs of tobacco plants treated with paraquat in the light (Fig. 2a). In contrast, the amount of electrolytes that leaked from the discs was minimal throughout the measurement period, when they were either not treated with paraquat or left in the dark. Therefore, using the present method of detection, both paraquat and light were required to induce cellular damage. However, no significant difference in the extent of damage was observed between leaf discs of control and transgenic plants even though the APX activities of the transgenic leaf tissues were 2-7 fold those of the control (Fig. 2b). We subsequently analyzed leaf discs from plants of different ages, with different concentrations of paraquat and under different light intensities, and found that the extent of cellular damage, as expressed by the rate of electrolyte leakage, was dependent on all of these factors. However, we were unable to observe a reproducible difference in paraquat sensitivity between transgenic and control leaf tissues, under any of the conditions examined.

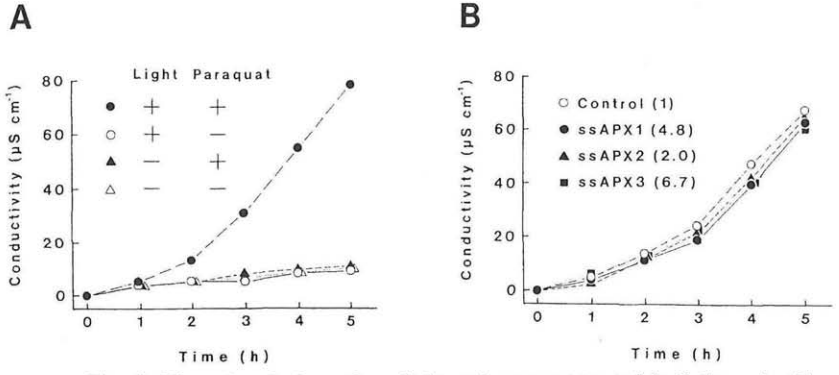

Fig. 2. Electrolyte leakage from light and paraquat-treated leaf discs. A; Discs were excised from the leaf of a control, non-transgenic plant and treated with or without 20 μ M paraquat, and then floated in deionized water either in the light or in the dark. The conductivity of the bathing water was measured at various times after the start of the floatation. B; Discs were excised from leaves of control and transgenic plants, treated with paraquat and then floated in deionized water in the light. Values in parentheses denote the relative APX activities of the leaves from which the discs were obtained.

3. Ascorbate content of leaf discs treated with paraquat

The ascorbate content of leaf tissues was about 4 mM and did not differ significantly between transgenic and control plants. Treatment of leaf discs with paraquat in the light resulted in a rapid and similar decrease in ascorbate content of both transgenic and control plants to about 30% of the initial value

1.5 h after the beginning of light irradiation (Fig. 3). Therefore, cells of these leaf discs appear to have suffered from oxidative stress before marked electrolyte leakage was observed (compare with Fig. 2). Thus, the higher APX activity of the transgenic leaf discs had no significant effect on the rate of ascorbate depletion under these conditions.

The present results do not necessarily negate the involvement of cytosolic APX in the plant defense response against oxidative stress. The enzyme may indeed be necessary for the defense system, so that even the control non-transgenic plants may contain excess amounts of this enzyme; conceivably, under the conditions used in the present work, factor(s) other than cytosolic APX activity may be limiting the tolerance to paraquat. Ascorbate, a substrate of APX, is a candidate for such a limiting factor since its amount is rapidly decreased by paraquat treatment (Fig. 3). Therefore, the supply of ascorbate may be more crucial for the defense mechanism than the enhancement of APX activity under the conditions used. This is supported by evidence that transgenic tobacco plants with enhanced activity of glutathione reductase, an enzyme that participates in the regeneration of ascorbate in the ascorbate-glutathione pathway, exhibited enhanced tolerance to paraquat under similar conditions (AONO & al. 1991).

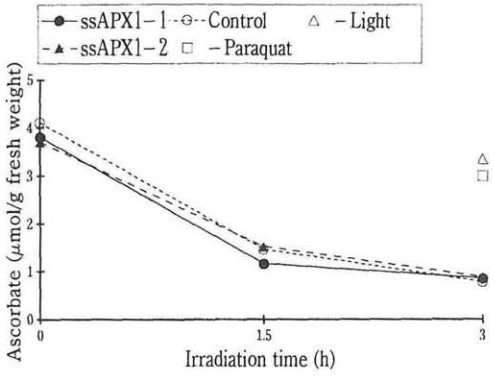

Fig. 3. Ascorbate content of leaf discs treated with paraquat in the light. Paraquat-treated leaf discs from control and transgenic plants were floated in deionized water and left in the light. „-Paraquat" and „-Light" represent results from leaf discs of control non-transgenic plants that were not treated with paraquat or were left in the dark after paraquat treatment, respectively. APX activities of leaf tissues of ssAPX1-1, ssAPX1-2 and control plants used in this experiment were 0.80, 0.44 and 0.29 μ mol ascorbate oxidised min^{-1} (mg protein) $^{-1}$, respectively.

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References

ALLEN R.D. 1995. Dissection of oxidative stress tolerance using transgenic plants. - Plant Physiol. 107: 1049-1054.

AONO M., KUBO A., SAJI H., NATORI T., TANAKA K. & KONDO N. 1991. Resistance to active oxygen toxicity of transgenic *Nicotiana tabacum* that expresses the gene for glutathione reductase from *Escherichia coli*. - Plant Cell Physiol. 32: 691-697.

— , SAJI H., FUJIYAMA K., SUGITA M., KONDO N. & TANAKA K. 1995. Decrease in activity of glutathione reductase enhances paraquat sensitivity in transgenic *Nicotiana tabacum*. - Plant Physiol. 107: 645-648.

KUBO A., SAJI H., TANAKA K., & KONDO N. 1992. Cloning and sequencing of a cDNA encoding ascorbate peroxidase from *Arabidopsis thaliana*. - Plant Mol. Biol. 18: 691-701.

MIYAKE C., CAO W.H. & ASADA K. 1993. Purification and molecular properties of the thylakoid-bound ascorbate peroxidase in spinach chloroplasts. - Plant Cell Physiol. 34: 881-889.

NAKANO Y. & ASADA K. 1987. Purification of ascorbate peroxidase in spinach chloroplasts; Its inactivation in ascorbate-depleted medium and reactivation by monodehydroascorbate radical. - Plant Cell Physiol. 28: 131-140.

PITCHER L.H., REPETTI P. & ZILINSKAS B.A. 1994. Overproduction of ascorbate peroxidase protects transgenic tobacco plants against oxidative stress. - Plant Physiol. 105: S-169 (Abstract No. 623).

SAJI H., TANAKA K. & KONDO N. 1990. Monoclonal antibodies to spinach ascorbate peroxidase and immunochemical detection of the enzyme in eight different plant species. - Plant Sci. 69: 1-9.

SUGITA M., MANZARA T., PICHERSKY E., CASHMORE A. & GRUISSEM W. 1987. Genomic organization, sequence analysis and expression of all five genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase from tomato. - Mol. Gen. Genet. 209: 247-256.