Chapter 25

Genetic Engineering for Air-Pollutant Resistance in Hybrid Aspen[1]

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Introduction

Preservation of the natural environment, a growing worldwide concern, is being approached in various ways. Forests are useful because they continuously eliminate air pollutants while remediating and preserving the natural environment. Forest trees have a high capacity to absorb air pollutants and detoxify toxic substances produced by air pollution, but they do not adequately resist harmful air pollutants and other environmental stresses. An effective way to preserve the natural environment is to improve the resistance of forest trees to air pollutants and other environmental stresses.

Damage to plant cells caused by environmental stresses, including herbicide exposure, high intensity light with low temperature, or air pollutants such as ozone and sulfur dioxide, is related to the over production of active oxygen in the cells. Active oxygen, such as singlet oxygen ($^1O_2$), superoxide (O_2^-), and hydrogen peroxide (H_2O_2), are the by-products of many biological oxidations. For example, the electron transport chain of mitochondria and chloroplasts is a well-documented source of active oxygen. In plant cells, detoxification systems for active oxygen have evolved to facilitate immediate removal of active oxygen. However, under stressful situations, the equilibrium between the oxidative and antioxidative capacity can change within the plant cells, so that over production of active oxygen is induced. As the oxygen detoxification system of higher plants has become better understood, we have attempted to improve the detoxification system by using genetic engineering to increase resistance to environmental stress.

Detoxification System of Active Oxygen

The production and destruction of active oxygen species, such as singlet oxygen ($^1O_2$), superoxide (O_2^-), and hydrogen peroxide (H_2O_2), occur during normal plant cell metabolism and are regulated processes. In plant cells, photosynthesis is the major source of active oxygen species. Chloroplasts generate highly active oxygen species by direct donation of excitation energy, or electrons, to oxygen from the photosynthetic electron transport chain. To counteract the toxicity of active oxygen species, plants possess an efficient antioxidative defense system composed of both nonenzymatic and enzymatic constituents. The typical detoxification system for active oxygen is described in figure 1. Superoxide, which is produced via the electron transport chain, is catalyzed to H_2O_2 by superoxide dismutase (SOD; Enzyme Commission number, EC 1.15.1.1). The H_2O_2 is destroyed in the ascorbate peroxidase (APX; EC 1.11.1.11) reaction, and ascorbate is regenerated either directly by the electron transport chain or by the ascorbate-glutathione cycle.

Increased activities of these antioxidative enzymes in the detoxification system may be related to additional stress-defense capacity. Thus, several research efforts are seeking to improve the natural antioxidative defense capacity by over expression of an antioxidative enzyme in transgenic plants. We are interested in improving the antioxidative defense capacity in woody plants by producing transgenic hybrid aspen with over expression of an antioxidative enzyme, glutathione reductase (GR; EC 1.6.4.2).
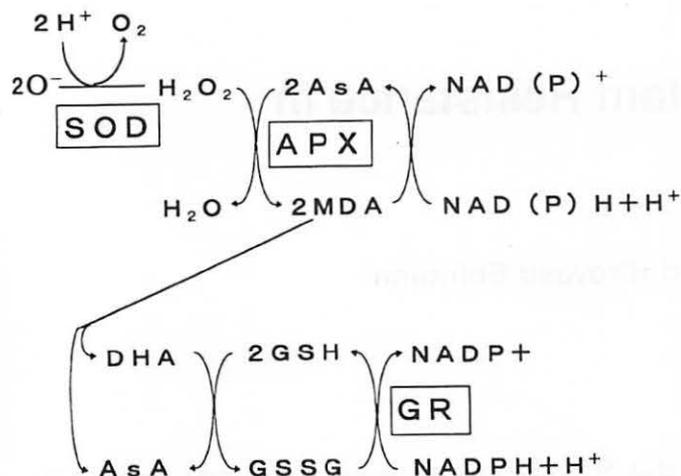
Figure 1. Detoxification system of active oxygen species in higher plants. SOD=superoxide dismutase; APX=ascorbate peroxidase; AsA=ascorbate; MDA=monodehydroascorbate; DHA=dehydroascorbate; GSH=reduced glutathione; GSSG=oxidized glutathione.
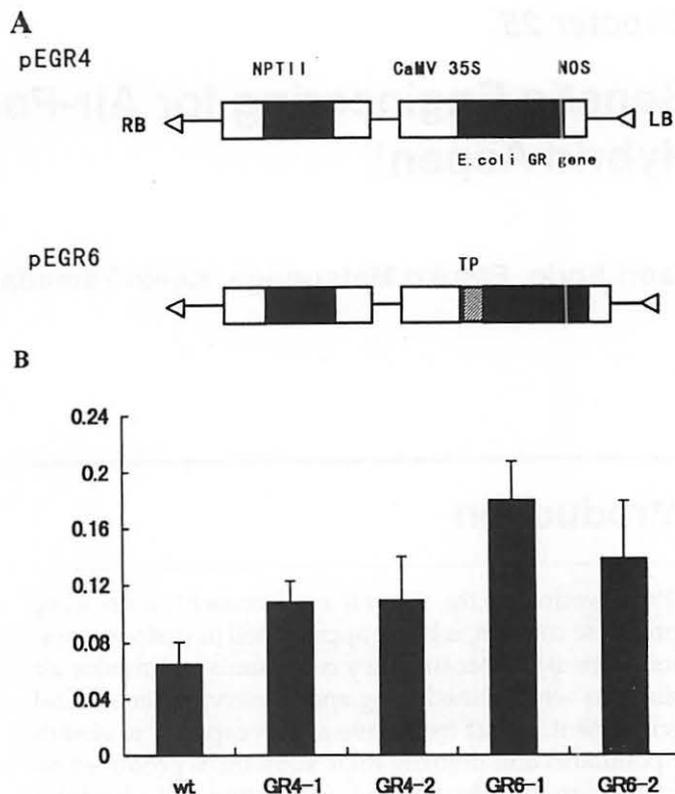

Figure 2A. Construction of the T-DNA region of pEGR4 and pEGR6. RB=right border; LB=left border; NPT II=a chimeric gene for neomycin phosphotransferase; CaMV 35S=35S promoter of cauliflower mosaic virus; NOS=nopaline synthase terminator; TP=chloroplast transit peptide.

Figure 2B. GR activity of transgenic aspen (GR4-1, GR4-2, GR6-1, and GR6-2) and nontransgenic aspen (wt). GR activity of leaf extracts was measured in a reaction mixture, which contained 0.1 M potassium phosphate (pH 7.8), 0.2 mM GSSG, and 0.2 mM NADPH in a final volume, and was monitored by the decrease in absorbance of NADPH at 340 nm.

Glutathione reductase, which exists in chloroplasts and cytoplasm of plant cells, catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) by accompanying oxidation of NADPH (Foyer and Halliwell 1976; Gillham and Dodge 1986; Rennenberg 1980). Furthermore, GR can be either a limiting factor in the recycling capacity of an antioxidant, such as glutathione, or responsible for the actual amount of antioxidant in the cell. Transgenic tobacco plants containing a transferred *GR* gene have exhibited elevated GR activity and improved resistance to environmental stress associated with active oxygen (Aono et al. 1991, 1993).

Production of Transgenic Plants With the *E. coli GR* Gene

Two derivatives of the binary vector plasmid pBI121, which contained the *E. coli GR* gene under control of the *35S* promoter (pEGR4 and pEGR6), were constructed. In the pEGR6 construct, the DNA fragment coding for a chloroplastic transit-peptide was inserted between the *35S* promoter and the *GR* gene to transport GR protein from the cytosol to the chloroplast (figure 2A). Using our transformation method (Ebinuma

et al. this volume), we introduced these genes into a hybrid aspen clone 'Y63' (*Populus sieboldii* x *P. grandidentata*). We subsequently obtained 86 transgenic plants, 28 lines transformed with GR4 and 58 lines transformed with GR6. Expression of the *E. coli GR* gene was confirmed by immunochemical analysis.

The glutathione reductase activity of transgenic plants was assayed in leaf extracts by monitoring absorbance decreases of NADPH at 340 nm. The GR activity of transgenic aspen, GR4 and GR6, ranged from 1- to 3-fold higher than that of nontransgenic plants. Seven lines of GR4 transgenic aspen and 16 lines of GR6 transgenic aspen showed significantly higher GR activity in comparison with nontransgenic aspen. The GR

188

USDA Forest Service Gen. Tech. Rep. RM-GTR-297. 1997.

activity of the transgenic plants, used in tolerance experiments, is shown in figure 2B. Elevated GR activity in the transgenic plants confirms expression of the introduced *E. coli GR* gene. These results indicate that increases in GR activity were caused by *GR* over expression.

Resistance to Environmental Stress Caused by Over Production of Active Oxygen Species

When plants are exposed to stressful conditions, such as a high intensity light, air pollutants, some herbicides, and metals, the generation of active oxygen species is increased. Increase in active oxygen production under stressful conditions results from either the inhibition of the photosynthesis pathway to decrease CO_2 assimilation, or the direct involvement in free radical production. So, plants under stressful conditions have increased susceptibility to photoinhibition with the subsequent development of chlorosis or necrosis.

Transgenic aspens with elevated GR activity (figure 2B) should exhibit higher resistance to active oxygen when compared to nontransgenic aspens. To examine the resistance of transgenic aspens to environmental stresses, we exposed transgenic aspens to paraquat, sulfur dioxide (SO_2), and high intensity light at low temperature.

Paraquat

Several herbicides may cause production of active oxygen species, either by direct involvement in radical production or by inhibition of the biosynthetic pathways. Paraquat (methyl viologen; 1,1-dimethyl-4,4-bipyridium dichloride) is a herbicide that produces active oxygen in plant cells under light. Upon accepting an electron from Photosystem I, paraquat forms a free radical. The paraquat radical is recycled to paraquat by reacting with O_2 to produce active oxygen, which then causes the disruption of cellular membranes and visible leaf damage.

To assess the resistance to paraquat, leaf discs from aspen were incubated with paraquat and illuminated. This treatment caused visible damage to leaf discs of nontransgenic plants, as exemplified by a disruption in the cellular membranes and a color change to dark brown. The extent of cellular damage was quantified by solute leakage, which is a measurement of membrane disruption. Leaf discs of transgenic and nontransgenic aspen were incubated with various concentrations of paraquat (0, 1, 10, and 100 μM) for 1 h at 25 °C in darkness. After paraquat

treatment, they were washed and floated on H_2O under light (6,000 lux). Solute leakage was assessed by conductivity of the floating solution. Increased solute leakage of cellular components is indicative of cellular membrane disruption. Leaf discs of nontransgenic aspen showed a dose-dependent increase in membrane damage at 1 μM paraquat, and were nearly completely disrupted at 10 μM. Transgenic aspen leaf discs showed no damage at 1 μM paraquat (figure 3); however, at 10 μM, the extent of damage in transgenic leaf discs was not significantly different from that of nontransgenic aspen. In addition, after the 1 μM paraquat treatment, leaf discs of nontransgenic aspen showed visible damage, whereas those of transgenic aspen did not.

These results indicate that leaf tissues of the GR transgenic aspen are resistant to paraquat when compared to nontransgenic aspen plants. Using paraquat treatment, we tested 2 lines of cytosolic transgenic aspen (GR4-1 and GR4-2) and 2 lines of chloroplastic transgenic aspen (GR6-1 and GR6-2). We obtained similar results in 4 independent experiments. However, we were unable to detect any significant difference in tolerance to paraquat between cytosolic GR transgenic aspen (GR4) and chloroplastic transgenic aspen (GR6).
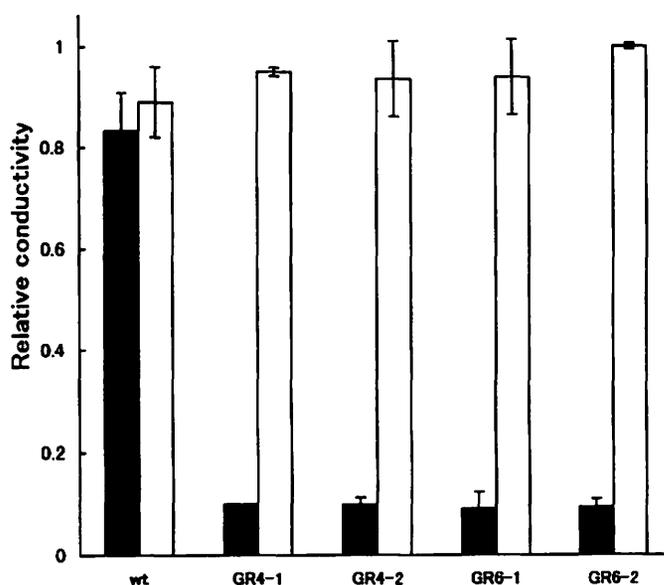

Figure 3. Electrolyte leakage from paraquat-treated leaf discs. Electrolyte leakage was assessed by conductivity of the floating solution. Conductivity was measured after illumination for 20 h, and relative values at 1 μM (filled columns) and 10 μM (unfilled columns) were determined against conductivity at 100 μM paraquat.

USDA Forest Service Gen. Tech. Rep. RM-GTR-297. 1997.

189

Sulfur Dioxide

Air pollutants, such as ozone (O_3) and sulfur dioxide (SO_2), are thought to be major factors influencing modern forest decline. Sulfur dioxide injury, which may occur in many plant species, is potentially severe under light or daytime conditions. Damage by SO_2 to biological systems is probably caused via the generation of radicals, such as O_2^-, $OH\cdot$, and SO_3^-, during SO_2 oxidation to sulfate. In the chloroplasts, SO_2 oxidation can be initiated by superoxide generated from the photosynthetic electron transport chain, this process can induce the production of active oxygen species and sulfur trioxide radicals.

We exposed transgenic and nontransgenic aspen grown in a growth chamber for 4 months to 1 ppm SO_2 at 25 °C under light (550 $\mu E \cdot m^{-2} \cdot s^{-1}$) with 70 percent relative humidity. Three samples of each nontransgenic and transgenic (GR4-1, GR4-2, GR6-1, and GR6-2) aspen line were used for the SO_2 fumigation experiment. After fumigation for 7 h, all nontransgenic plants showed symptoms of foliar damage such as necrosis and dehydration (figure 4A); however, leaves of transgenic plants with elevated GR activity (GR4 and GR6) exhibited lower damage levels (figure 4B). Similar results were obtained from independent replications. Transgenic plants with GR activity levels similar to those of nontransgenic plants also showed severe damage. The SO_2 resistance of transgenic plants is apparently dependent on the increased GR activity from over expression of the *E. coli* GR gene.

We quantified the extent of cellular damage caused by SO_2 fumigation by solute leakage, which is an indicator of membrane disruption. Leaves at the same position were removed, cut into leaf discs, and incubated in H_2O at 30 °C for 1 h. Solute leakage was then measured. After fumigation for 4 h, leaf discs of nontransgenic plants showed increased solute leakage. Nontransgenic leaves fumigated for 7 h showed complete disruption. Leaves of nontransgenic aspen had SO_2-dependent membrane damage, whereas leaves of transgenic plants had less damage. Damage exhibited by cytosolic GR transgenic plants was not significantly different from that of chloroplastic GR transgenic plants. These results indicate that over expression of the *E. coli* GR gene contributes to SO_2 resistance.

High Intensity Light With Low Temperature

When plants are exposed to high intensity light, active oxygen species are over produced. Exposure of plants to high intensity light can exceed the capacity of photosynthesis, leading to over production of active oxygen. Injury resulting from high intensity light is potentially increased with low temperature.
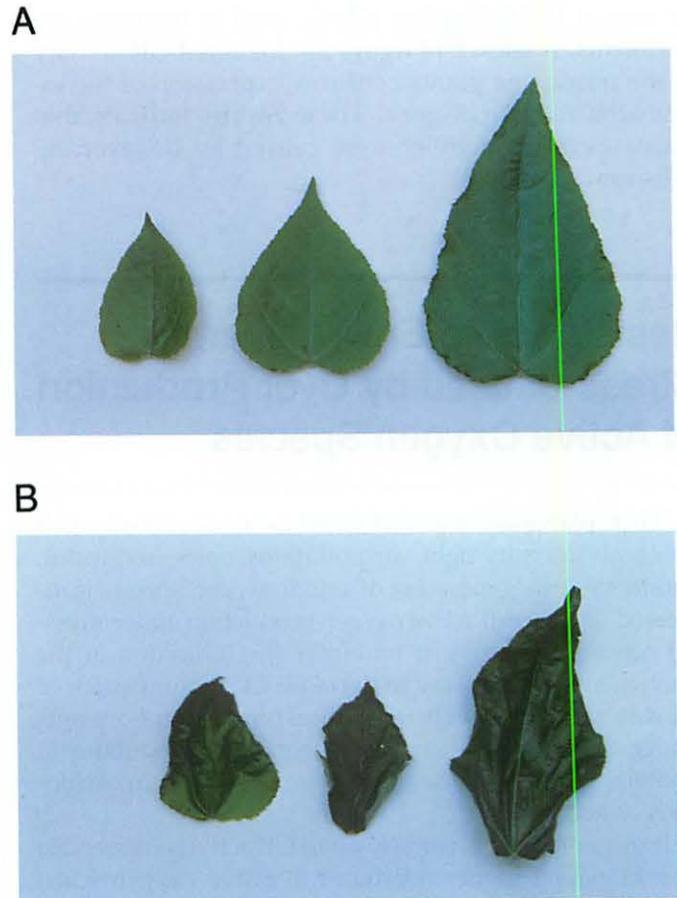
Figure 4. Effect of SO_2 fumigation. Leaves from the same position in transgenic and nontransgenic aspen after exposure to 1.0 ppm SO_2, 25 °C, light (550 $\mu E \cdot m^{-2} \cdot s^{-1}$) and 70% relative humidity for 7 h. A) Leaf of nontransgenic aspen. B) Leaf of transgenic aspen (GR6-1) with elevated GR activity.

To induce photoinhibition, we exposed leaves of transgenic and nontransgenic aspen to high intensity light (10,000 lux) with low temperature (5 °C). Leaves at the same position in transgenic (GR4 and GR6) and nontransgenic aspen (2 clones of every line) were exposed to the stress conditions that cause photoinhibition. After treatment for 6 h, leaves of the nontransgenic plants showed an increase in solute leakage. In contrast, leaves of transgenic aspen with over-expressed GR showed less (ca. 50 percent) damage. These results are similar to those from transgenic tobacco with over-expressed SOD. We believe that transgenic aspen with over-expressed GR are resistant to photoinhibition.

190

USDA Forest Service Gen. Tech. Rep. RM-GTR-297. 1997.

Conclusion

The results presented here demonstrate that *GR* transgenic aspens exhibit higher tolerance to active oxygen. *GR* transgenic aspens may also exhibit resistance to other environmental stresses, such as ozone and metal, that result in the production of active oxygen species. We believe that "environmental stress-resistant" trees produced by genetic engineering represent a very promising approach to help preserve the natural environment.

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191