Chapter 25

Genetic Engineering for Air-Pollutant Resistance in Hybrid Aspen

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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 \( (\text{O}_2^*) \), superoxide \( (\text{O}_2^-) \), and hydrogen peroxide \( (\text{H}_2\text{O}_2) \), 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 \( (\text{O}_2^*) \), superoxide \( (\text{O}_2^-) \), and hydrogen peroxide \( (\text{H}_2\text{O}_2) \), 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 \( \text{H}_2\text{O}_2 \) by superoxide dismutase (SOD; Enzyme Commission number, EC 1.15.1.1). The \( \text{H}_2\text{O}_2 \) 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).

Section IV Biotic and Abiotic Resistance

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 immunochromical 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

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**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.
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 CO₂ 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 (SO₂), and high intensity light at low temperature.

Paraoquat

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 O₂ 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℃ in darkness. After paraquat treatment, they were washed and floated on H₂O 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.
### Sulfur Dioxide

Air pollutants, such as ozone (O₃) and sulfur dioxide (SO₂), 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 SO₂ to biological systems is probably caused via the generation of radicals, such as O₂⁻, OH⁻, and SO₄⁻, during SO₂ oxidation to sulfate. In the chloroplasts, SO₂ 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 SO₂ at 25 °C under light (550 μE · m⁻² · 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 SO₂ 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 SO₂ 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 SO₂ 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 H₂O 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 SO₂-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 SO₂ 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.

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.

Figure 4. Effect of SO₂ fumigation. Leaves from the same position in transgenic and nontransgenic aspen after exposure to 1.0 ppm SO₂, 25 °C, light (550 μE · m⁻² · 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.
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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Literature Cited


