Manipulation of Superoxide Dismutase and Catalase to Enhance Sulfur Dioxide Tolerance in Transgenic Chinese Cabbage

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Abstract
To evaluate the feasibility of using engineered antioxidant enzymes as an approach to improve the tolerance of plants to ambient stress, we have engineered transgenic Chinese cabbage plants that overproduce superoxide dismutase (SOD) and catalase (CAT). These enzymes convert superoxide radicals into water, and are believed to play a crucial role in antioxidant defense. The SOD and/or CAT isolated from maize were targeted either to the cytosol or to the chloroplast. Hypocotyl and cotyledon explants of Chinese cabbage were infected with an Agrobacterium strain (LBA4404) carrying a distinct disarmed T-DNA containing sod and/or cat gene. Transgenic plants were confirmed by PCR, northern hybridization and enzyme activities. Besides, the sod and cat genes could co-transfer into the same plantlet, and transcribed into RNA. Enhanced SOD or CAT activity in the cytosol or chloroplast, when expressed on their own, had only a minor effect on sulfur dioxide (SO$_2$) tolerance. However, overproduction of both SOD and CAT in the cytosol and chloroplast resulted in a 3-4 fold reduction of visible SO$_2$ injury. These results suggest that attempts to increase stress resistance by simply raising the activity of one of the antioxidant enzymes have not always been successful presumably because of the need for a balanced interaction of protective enzymes. These results clearly indicate that alterations in the expression of enzymes involved in reactive oxygen-intermediate scavenging can have significant metabolic effects and bring about the hope that this strategy, the co-transformation system, can be used to develop plants with increasing stress tolerance.

INTRODUCTION
Environmental stress is one of the major limiting factors in plant productivity. Much of the injury to plants caused by stress exposure is associated with oxidative damage at the cellular level. Environmental conditions can cause oxidative stress damage by overproducing of toxic oxygen species, including superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), singlet oxygen (1$^1$O$_2$) and hydroxyl radical (•OH) (Halliwell and Gutteridge, 1989; Bowler et al., 1992).

To counteract the toxicity of active oxygen species, a highly efficient antioxidative defense system, composed of both non-enzymatic and enzymatic constituents, is present in all plant cells (Bowler et al., 1992). The enzymatic antioxidative components include SOD, CAT and peroxidase. SODs are metalloenzymes that are ubiquitous among aerobic organisms and are very efficient at scavenging O$_2^-$ . All the forms are nuclear encoded. CAT is a tetrameric heme-containing enzyme found in all aerobic organisms. It provides protection against reactive oxygen toxicity by converting hydrogen peroxide to water and oxygen.

Initial studies with both bacterial and plant systems using single enzyme genes produced largely negative results (Tepperman and Dunsmuir, 1990). For example, increases in SOD failed to protect the organisms unless the endogenous system can cope with the H$_2$O$_2$ produced. In order to achieve the goal, increase in several components of antioxidative system would be necessary to obtain a substantial increase in stress tolerance. Thus, co-transformation of SOD and CAT, the most important enzymatic
antioxidant system, was performed to determine whether a substantial increase in stress tolerance was possible.

MATERIALS AND METHODS

Transformation Vectors
The full-length cDNA encoding maize Cu/ZnSOD and CAT were cloned by PCR method, corresponding to the sequences of the maize Cu/ZnSOD (Cannon et al., 1987) and maize CAT2 (Redinbaugh et al., 1988), respectively. SOD and CAT fragments are 0.48kb and 1.48 kb, respectively. Two binary vectors, pKcSmn (CaMV-SOD) and pKcCmn (CaMV-CAT), were constructed to replace the GUS gene of pBI121 (Clontech, USA) with the maize sod and cat genes. Furthermore, pKrSmn (rbcS-SOD) and pKrCmn (rbcS-CAT) with sod and cat gene driven by rbcS promoter was in place of the uidA (GUS) reporter gene and CaMV 35S promoter. Moreover, a sequence of chloroplastic transit peptide was inserted into pKcSmn, pKcCmn, pKrSmn, or pKrCmn to create the pKcTSmn (CaMV-T-SOD), pKcTCmn (CaMV-T-CAT), pKrTSmn (rbcS-T-SOD), pKrTCmn (rbcS-T-CAT), respectively. The vectors were integrated into Agrobacterium LBA4404 (Clontech, USA).

Plant Materials and Agrobacterium Co-transformation
Chinese cabbage (Brassica campestris L. ssp. pekinensis cv. Tropical Pride) seeds were sterilized and sown on MS medium (Murashige and Skoog, 1962) containing 30 g/L sucrose and 15 g/L agar, and cultured for 5 days at 25°C/20°C (D/N) and 16hr photoperiod at 150 μmol m⁻² s⁻¹. The cotyledon or hypocotyl explants were infected with a mixture of two Agrobacterium (each 2×10⁶ bacteria/ml), e.g. LBA4404-pKcSmn and LBA4404-pKcCmn (CaMV-SOD+CaMV-CAT), LBA4404-pKcSmn and LBA4404-pKrCmn (rbcS-SOD+rbcS-CAT), LBA4404-pKcSmn and LBA4404-pKcCmn (CaMV-T-SOD+CaMV-T-CAT), or LBA4404-pKrSmn and LBA4404-pKrCmn (rbcS-T-SOD + rbcS-T-CAT), and then regenerated into plantlet.

Plant DNA Isolation and PCR Analysis
Total DNA was extracted from leaves using the cetyltrimethylammonium bromide (CTAB) procedure (Doyle and Doyle, 1990). PCR protocol was referred to Jayaraman et al. (1991).

RNA Isolation and Analysis
For RNA isolation, 1.0 g of fully developed leaves was homogenized in 2 volumes of cold Ultraspec™ RNA buffer (Biotecx, USA). Northern-blot hybridization was analyzed as described by Sambrook et al. (1989).

Isolation of Chloroplasts, Protein Extraction, Native PAGE, SOD and CAT Activities
Chloroplasts were isolated from leaves according to Miyake and Asada (1992). Plant materials (1.0 g) were extracted as described by Anderson et al. (1995). SOD and CAT isozymes were separated on non-denaturing polyacrylamide gels (10% T, 3% C) at 40 mA for 4 hr at 4°C according to the method of Laemmli (1970). After completion of electrophoresis, the gels were stained for the activities of SOD and CAT following the procedure of Rao et al. (1995) and Woodbury et al. (1971). The activities of SOD and CAT were determined in a method similar to that of McCord and Fridovich (1969) and Beers and Sizer (1952), respectively.

SO₂ Fumigation
Transgenic and control plants were exposed to 400 ± 10 ppb SO₂ for 7 days under cycles of 12 hr (between 9 AM and 9 PM), 80% RH, 25°C in the growth chamber. When not fumigated, plants were exposed to filter air. Pollutant was administered through mass
flow controllers into the air stream entering the growth chamber. SO₂ concentration was measured with a sulfur analyzer (Model 8850, Monitor Labs Inc.). Foliar injury was scored by assessing the visible leak damage. Except for senescent leaves, this included all leaves that displayed visible damage.

**Statistics**

The data of SOD and CAT activities expressed in terms of means ± SE of five transgenic plants with highest SOD or CAT activity together with their controls were presented, each treatment with three independent measurements. In the sulfur dioxide fumigation experiment, eight plants of each line were examined and repeated three times. The data listed below were the means of percentage of foliar injury (± SE) from three independent experiments each with eight replications. The Student’s t-test was used to determine the significance level of differences between mean values.

**RESULTS**

**Confirmation of Transgenic Chinese Cabbage by Molecular Analysis**

After 3 weeks of growth in the greenhouse, transgenic Chinese cabbage plants (more than 160 plants) grew normally, and there was no difference between transgenic and non-transgenic Chinese cabbage plants in terms of growth and morphological characters. For each different construct at least 10 independent transgenic regenerants were obtained. The presence of the transformed sod and/or cat genes in transformed plants was confirmed by PCR amplification (Fig. 1A, B). A DNA band of 0.48 kb was presented in the transformants transferred with sod gene (Fig. 1A, lane 1~4; lane 7~10), while absent in water and nontransformed controls (Fig. 1A, lane 5, 6). Similarly, 1.48 kb bands were found only in the cat gene transformed plants (Fig. 1B, lane 1~4, lane 7~10). One hundred and twenty plants out of 163 kanamycin-resistant plants examined had sod and/or cat gene. Northern blot hybridization was performed by adding both ³²P-labelled sod and cat fragments at the same time. Two hybridization bands were present in the transformants contained both sod and cat genes (Fig. 2, lane 3~4, 11~12), while only one band was present in the transformants contained either sod (Fig. 2, lane 1, 9) or cat gene (Fig. 2, lane 2, 10). Results also indicated that the sod and/or cat transcripts were about 3 times more abundant in the transformants transferred with sod and/or cat driven by the rbcS promoter rather than by the CaMV 35S promoter.

Significant amounts of maize Cu/ZnSOD were accumulated in the leaves of transgenic Chinese cabbage (Fig. 3A, Lane 3~10). The results of gel staining of maize SOD activity were two to three times higher than endogenous SODs in transgenic plants. There was no detectable maize SOD activity in nontransformed controls. As for the CAT, maize CAT isoform were detected in eight kinds of transgenic CAT plants (Fig. 3B, Lane 3~10). Expression of introduced maize sod and/or cat gene somehow did not interfere with the activity of native Chinese cabbage SOD and/or CAT isoforms. The maize SOD or CAT was also presented in the proteins extracted of the transformants transferred with the transgenes containing the sequence of chloroplast signal peptide (Fig. 3A, lane 7~10; Fig. 3B, lane 7~10).

The results of SOD or CAT activities analysis indicated that the transformants contained transferred sod gene were 3~4 times higher than those of controls, 1~2 times higher in CAT (Table 1). There were no significant differences in the SOD or CAT activities of transformants contained transgene driven either by rbcS promoter or the CaMV 35S promoter. The SOD and CAT activities in the chloroplast of transformants transferred with the transgenes containing the sequence of chloroplast signal peptide were 12~14 times or 7~9 fold higher than that of untransformed controls, respectively (Table 2).
Evaluation of Visible SO\textsubscript{2} Stress

Plants began to display visible symptoms after 3 days of exposure to 400 ppb SO\textsubscript{2} levels. The control plant showed typical symptoms of SO\textsubscript{2} damage, water soakage first appeared in the young leaves, then pale and chlorosis developed after 7 days of SO\textsubscript{2} treatment. While co-transformed plants show the resistance to SO\textsubscript{2} treatment (Fig. 4). Foliar damages were scored by assessing the visible necrosis to examine whether different forms of transgenic plants could be responsible for the observed divergence in leaf injury. The degree of protection in transgenic Chinese cabbage contained single transgene (\textit{sod} or \textit{cat}) regardless sequence of chloroplast signal peptide was not significantly different from control plants (Fig. 5). Transformants of CaMV-SOD, \textit{rbcS}-SOD, CaMV-CAT, \textit{rbcS}-CAT, CaMV-T-SOD, \textit{rbcS}-T-SOD, \textit{rbcS}-T-CAT showed 65\%, 62\%, 66\%, 64\%, 55\%, 50\%, 52\%, and 53\% of damaged leaf area, respectively. However, the extent of damage to the co-transformants of CaMV-SOD + CaMV-CAT, \textit{rbcS}-SOD + \textit{rbcS}-CAT, CaMV-T-SOD + CaMV-T-CAT, \textit{rbcS}-T-SOD + \textit{rbcS}-T-CAT was only 25\%, 18\%, 12\% or 9\%, respectively.

DISCUSSION

Two T-DNA’s co-transformed into plants by a double \textit{Agrobacterium} infection are mainly integrated at the same locus was reported by De Block and Debrouwer (1991). The results of PCR, northern hybridization, and enzyme activities assays indicated that the \textit{sod} and \textit{cat} genes could co-transfer into the same plantlet, transcribed into RNA, and translated into active enzymes. We had demonstrated that overexpression of both Cu/ZnSOD and CAT in cytosol or chloroplasts of Chinese cabbage could protect these plants from oxidative toxicity. Our results showed that co-transformation of both genes into plants by a double \textit{Agrobacterium} infection may be an efficient method for transfer two genes, simultaneously.

In this study, transgenic SOD plants expressed the maize SOD in cytosol to levels 3–4 times higher than that of controls, were not tolerant to SO\textsubscript{2} damage. This was not in agreement with that of transgenic tobacco plants overexpressing this enzyme, which were shown to be tolerant to the cellular damage caused by oxygen radicals (Bowler et al., 1991). However, transgenic SOD plants expressed the maize SOD in chloroplast were somewhat more tolerant to SO\textsubscript{2} damage than those of in cytosol or controls. This could be explained by the facts that the thylakoid membrane is the site primarily damaged by oxygen radicals (Miyake and Asada, 1992). Indeed, transgenic Chinese cabbage maintain 85\% overproducing SOD or CAT over the total activities of both enzymes within chloroplasts improving the ability to eliminate oxygen radical (Table 1, 2; Fig. 5). Furthermore, the amount of SOD presents within the different cellular compartments together with the endogenous H\textsubscript{2}O\textsubscript{2}-scavenging abilities are likely to influence the rate of turnover and accumulation of hydroxyl radical under stress conditions. It is suggested that elevating SOD without the presence of co-elevating enzymes that remove H\textsubscript{2}O\textsubscript{2} does not provide protection against oxygen toxicity (Bowler et al., 1992).

The combined actions of SOD and CAT prevented the interaction of superoxide and H\textsubscript{2}O\textsubscript{2} to form the very potent oxidant, the hydroxyl radical (\textit{OH}). Overproduction of the two antioxidant enzymes at the same time in plant, especially in chloroplasts, is an essential and proper way to guard against SO\textsubscript{2} injury. Our work showed that SOD and CAT overproduction had a positive effect on SO\textsubscript{2} tolerance in Chinese cabbage and may induce a higher level of other antioxidant enzyme (AP, GR etc.). We are hopeful that further analysis of these plants will provide regulatory relationships among antioxidant system genes and cross-tolerances to different stress conditions.

Literature Cited


Table 1. SOD or CAT activities in the leaves of *sod* and/or *cat* transformed Chinese cabbage.

<table>
<thead>
<tr>
<th>Transgenic plants</th>
<th>SOD activities (Unit)(^1)</th>
<th>CAT activities (Unit/mg)(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>0.20±0.09*</td>
<td>12.7±1.1</td>
</tr>
<tr>
<td>CaMV-SOD</td>
<td>0.94±0.08</td>
<td>16.1±1.5</td>
</tr>
<tr>
<td>CaMV-T-SOD</td>
<td>0.84±0.07</td>
<td>10.6±0.8</td>
</tr>
<tr>
<td><em>rbcS</em>-SOD</td>
<td>0.94±0.07</td>
<td>14.8±2.0</td>
</tr>
<tr>
<td><em>rbcS</em>-T-SOD</td>
<td>0.89±0.18</td>
<td>11.9±1.2</td>
</tr>
<tr>
<td>CaMV-CAT</td>
<td>0.31±0.07</td>
<td>22.7±0.7</td>
</tr>
<tr>
<td>CaMV-T-CAT</td>
<td>0.32±0.03</td>
<td>25.7±1.8</td>
</tr>
<tr>
<td><em>rbcS</em>-CAT</td>
<td>0.31±0.06</td>
<td>33.2±1.9</td>
</tr>
<tr>
<td><em>rbcS</em>-T-CAT</td>
<td>0.26±0.02</td>
<td>21.6±3.7</td>
</tr>
<tr>
<td>CaMV-SOD + CaMV-CAT</td>
<td>0.99±0.08</td>
<td>27.0±1.9</td>
</tr>
<tr>
<td>CaMV-T-SOD + CaMV-T-CAT</td>
<td>0.85±0.10</td>
<td>22.7±2.1</td>
</tr>
<tr>
<td><em>rbcS</em>-SOD + <em>rbcS</em>-CAT</td>
<td>0.99±0.12</td>
<td>31.5±4.3</td>
</tr>
<tr>
<td><em>rbcS</em>-T-SOD + <em>rbcS</em>-T-CAT</td>
<td>1.01±0.07</td>
<td>25.2±4.1</td>
</tr>
</tbody>
</table>

\(^1\) One unit is defined as the quantity of enzyme required to inhibit the reduction of cytochrome c by 50%.

\(^2\) One unit per mg is defined as ΔA₂₄₀ per min×1000 per 43.6×mg enzyme per mL reaction.

\(^*\) Values are means ± SE of five independent plants with three duplicates each.

Table 2. SOD or CAT activities in the chloroplasts of *sod* and/or *cat* transformed Chinese cabbage.

<table>
<thead>
<tr>
<th>Transgenic plants</th>
<th>SOD activities (Unit)(^1)</th>
<th>CAT activities (Unit/mg)(^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>0.06±0.02(^2)</td>
<td>2.3±0.5</td>
</tr>
<tr>
<td>CaMV-T-SOD</td>
<td>0.81±0.09</td>
<td>3.0±0.4</td>
</tr>
<tr>
<td>CaMV-T-CAT</td>
<td>0.06±0.02</td>
<td>22.8±0.8</td>
</tr>
<tr>
<td><em>rbcS</em>-T-SOD</td>
<td>0.89±0.11</td>
<td>2.9±0.3</td>
</tr>
<tr>
<td><em>rbcS</em>-T-CAT</td>
<td>0.08±0.03</td>
<td>19.6±0.7</td>
</tr>
<tr>
<td>CaMV-T-SOD + CaMV-T-CAT</td>
<td>0.77±0.08</td>
<td>20.5±0.3</td>
</tr>
<tr>
<td><em>rbcS</em>-T-SOD + <em>rbcS</em>-T-CAT</td>
<td>0.91±0.13</td>
<td>23.0±0.7</td>
</tr>
</tbody>
</table>

\(^1\) One unit is defined as the quantity of enzyme required to inhibit the reduction of cytochrome c by 50%.

\(^2\) One unit per mg is defined as ΔA₂₄₀ per min×1000 per 43.6×mg enzyme per mL reaction.

\(^*\) Values are means ± SE of five independent plants with three duplicates each.
Fig. 1. Chinese cabbage transformants were examined through PCR analysis. Part of the *sod* gene (0.48 kb) (A) or *cat* gene (1.48 kb) was amplified from a plasmid or DNA from plants and analyzed by electrophoresis.

Fig. 2. Northern-slot analysis of Chinese cabbage transformed with maize *sod* and/or *cat* gene. Hybridization was carried out using $^{32}$P-labelled *sod* and *cat* fragments at the same time. Lanes 1~3, 5, 9~11: $45\mu$ g RNA, Lanes 4, 12: $15\mu$ g RNA.
Fig. 3. Overproduction of transgenic maize Cu/ZnSOD and/or CAT in Chinese cabbage levels were quantified by activity gel analysis. SOD (A) or CAT (B) activity in protein exacts from Chinese cabbage plants. Eighty microgram of total protein was loaded into each lane.

Fig. 4. The appearances of transgenic Chinese cabbage (left) and control (right) plants were inspected after exposure to SO$_2$ at 800 ppb for 7 days.
Fig. 5. Effect of sulfur dioxide fumigation (400 ppb SO$_2$ for 7 days) on visible injury in twelve transgenic Chinese cabbage constructed to overexpress maize Cu/ZnSOD and/or CAT in cytosol or chloroplasts. The values are means ± SE of eight plants with three duplicate measurements each. Distinct letters denote significant differences (P < 0.01) according to Student’s t-test.