



Expression of functional human-cytosolic Cu/Zn superoxide dismutase in transgenic tobacco

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Abstract

Nicotiana tabacum was transformed with cDNA encoding the human cytosolic copper/zinc superoxide dismutase (human SOD) under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The cDNA sequence was present in a range of copies, from single to several copies, in the primary transformant plants and a transcript of the expected length was produced. The expression of human SOD protein in the transgenic plants was shown by *in situ* gel staining assay. Ten plants produced human SOD up to 40 ng protein per mg of fresh leaf tissue.

Introduction

Several genes encoding animal proteins, such as hormones, antibodies, and cytokines, have been expressed in plant cells (Barta *et al.* 1986, Edelbaum *et al.* 1992, Hiatt *et al.* 1989). However, although a few proteins have been successfully produced, the productivity in most cases was extremely low: the introduced animal mRNA was not efficiently translated or the synthesized proteins were not as stable as other cellular proteins. Therefore, the question still remains, whether animal genes can be accurately and stably expressed in plants. To approach this question, we set up an experiment expressing the human SOD protein in the tobacco plant.

The expression of human SOD in plant is a good model for heterologous expressional study because the molecular structure of the SOD is complicated. The human SOD enzyme, isolated from erythrocytes, is a dimeric protein (32 000 kDa) composed of identical non-covalently linked subunits with one Cu²⁺ and one Zn²⁺. It is expressed in the cytosol. SOD plays a central role in aerobic organisms in the protection against O₂ toxicity. It catalyzes the conversion of disproportionately present superoxide radicals to H₂O₂ and O₂

(Fridovich 1986). As a pharmaceutical agent or cosmetic ingredient, it is widely useful and has significant commercial value.

Despite numerous studies of human SOD, there has been no report dealing with its expression in transgenic plants. However, the human SOD has been expressed in *E. coli* (Avraham *et al.* 1988), in yeast (Eloy-Stein *et al.* 1986), and in mouse (Hallewell *et al.* 1987). In our case, a heterologous gene expression experiment was conducted with the human SOD gene in order to investigate whether the SOD protein produced in the transgenic tobacco was enzymatically active and in the correct location, namely the cytosol. In this experiment, we also investigated whether there was a post-translational process in the plant cell that could process subunits of human SOD with Cu²⁺/Zn²⁺ into the dimeric active form. As a result, the transgenic tobacco plants were indeed able to produce an enzymatically active form of human SOD. And the recombinant human SOD could have use in pharmaceuticals, and cosmetics.

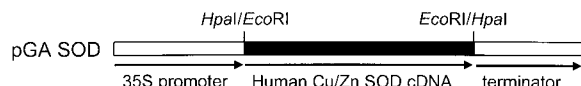


Fig. 1. pGA-SOD construct used for plant transformation. The 0.6 kb open reading frame from the human Cu/Zn SOD cDNA was inserted into the *HpaI* site of the binary transformation vector pGA643. Sites separated by a / have been destroyed by fusion during vector construction.

Materials and methods

Expression vector construction

A cDNA containing the entire human SOD coding region was prepared by polymerase chain reaction (Scharf *et al.* 1986). The primers used were based on sequences in the 5' and 3' untranslated regions of the human Cu/Zn RNA (Sherman *et al.* 1984): 5'-dGAATTCGATGGCGACGAAGGCCGTGTGCGTGCGGTG-3' and 5'-dATCAGGATAGAATTCTACCGCTAGC-3'. The amplified cDNA was cut with *EcoRI* and cloned into the *EcoRI* site of pUC 19, and its DNA sequence was verified (pSD 1). The cDNA was excised with *EcoRI*, filled-in with Klenow enzyme, and subcloned into the *HpaI* site of the binary plant vector pGA 643 (An *et al.* 1989). In this vector, the cDNA was under the transcriptional control of the CaMV 35 S promoter (Figure 1).

Plant transformation

The binary vector construct was introduced into *Agrobacterium tumefaciens* LBA4404 by the freeze-thaw method (Holsters *et al.* 1978). Transformants were selected on minimal nutrient plates containing rifamycin (100 mg l⁻¹), streptomycin (300 mg l⁻¹), kanamycin (25 mg l⁻¹), and tetracycline (15 mg l⁻¹). The T-DNA region including the human SOD cDNA was introduced into tobacco plants (*Nicotiana tabacum* L. cv. NC 82) by the leaf disc transformation method (Horsch *et al.* 1985). Regenerated plants for assays were selected by rooting on kanamycin (300 mg l⁻¹) medium. Vegetative plants used were 15 weeks old with three to four non-senescent leaves. Uniform plants were chosen within each experiment and there was no phenotypically distinguished feature between the vector transformed control and the other transformants. After 2 months, 23 kanamycin-resistant plants were potted in soil and transferred to a greenhouse for propagation.

Enzyme assay for neomycin phosphotransferase II (NPTII) activity

NPTII activity was detected by the *in situ* gel assay of Reiss *et al.* (1984). Protein was extracted from 150 mg leaf tissue and separated by electrophoresis in non-denaturing polyacrylamide gels. The gels were then exposed to kanamycin sulfate and [γ -³²P]ATP. Phosphorylated kanamycin, which was produced in the regions of the gel containing NPTII activity, was detected by autoradiography after blotting onto a phosphocellulose paper.

Nucleic acid analysis

Total DNA was isolated from the leaf tissue of untransformed as well as transformed tobacco plants according to Dellaporta *et al.* (1983). DNA (10 μ g) was restricted with *HindIII* and *ClaI* and fractionated on a 1% (w/v) agarose gel before being transferred to a nylon membrane. Based on gene reconstruction experiments, the human Cu/Zn SOD gene was present at one to five copy equivalents of the tobacco genome. The nylon filter was incubated with nick-translated, [α -³²P]dCTP labeled human SOD cDNA. The filter was then exposed to X-Omat AR film (Kodak) at -70 °C with intensifying screens (Sambrook *et al.* 1982).

Total RNA was isolated from the leaf tissue of untransformed and transformed tobacco plants as described previously (Chomczynski & Sacchi 1987). Glyoxalated total RNA (10 μ g) of each sample was resolved in a 1.4% (w/v) phosphate-buffered agarose gel, transferred to a nylon membrane, and hybridized as described above for the DNA.

SOD activity assay

The tissue was thoroughly ground with a pestle in a cold mortar in an ice bath until no fibrous residue could be seen. The grinding buffer (200 μ l/100 mg fresh weight) consisted of 0.1 M potassium phosphate buffer (pH 7.5). The homogenate was centrifuged twice at 13 000 g for 20 min at 4 °C in a refrigerated centrifuge. The supernatant, hereafter referred to as crude SOD extract, was used for electrophoresis and for the determination of SOD content in the tissue. The water-soluble protein content of all crude SOD extract was determined by the method of Bradford (1976).

SOD isozymes (480 μ g soluble protein per lane) were separated by electrophoresis on 1.5 mm thick

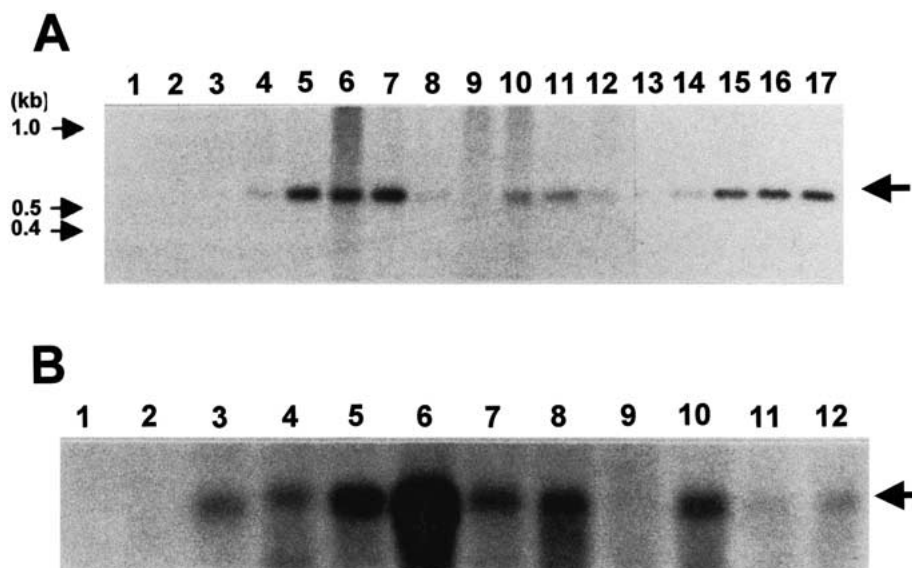


Fig. 2. DNA and RNA gel blot analysis of transgenic plants expressing human Cu/Zn SOD. (A) DNA gel blot analysis of genomic DNA from transgenic plants. Lane 1: 10 μ g of *Hind*III and *Cla*I double digested DNA from an untransformed tobacco plant, lane 2: pGA 643 transformant, lanes 3 to 12: pGA-SOD transformants, lane 3: S1 plant, lane 4: S2 plant, lane 5: S3 plant, lane 6: S4 plant, lane 7: S5 plant, lane 8: S6 plant, lane 9: S7 plant, lane 10: S8 plant, lane 11: S9 plant, and lane 12: S10 plant. Lanes 13 to 17: copy number standards. *Eco*RI digested pSD 1 DNA in amounts equivalent to one to five copies per tobacco genome. Gel migration position of the 0.6 kb fragment of the human SOD cDNA is indicated by the arrow on the right. The size in kb is shown on the left side. (B) RNA gel blot analysis of leaf RNA from transgenic plants. Lane 1: nontransformed control plant, lane 2: pGA643 transformant, lanes 3 to 12: pGA-SOD transformants the same as NPTII and DNA blot assayed samples, S1 to S10, lane 3: S1 plant, lane 4: S2 plant, lane 5: S3 plant, lane 6: S4 plant, lane 7: S5 plant, lane 8: S6 plant, lane 9: S7 plant, lane 10: S8 plant, lane 11: S9 plant, and lane 12: S10 plant. Gel migration position of about 0.7 kb expressed the human SOD is indicated by the arrow on the right.

15% (w/v) non-denaturing polyacrylamide gels without SDS. To determine SOD enzyme activity, gels were stained as described by Beauchamp & Fridovich (1971). The presence of SOD inhibited the background staining reaction in proportion to its concentration. Total enzymes were quantified by densitometer scanning with a UMAX scanner using the 1D Main program (Advanced American Biotechnology, 1166 E. Valencia Dr, #6C, Fullerton, CA 92831, USA). One unit of SOD activity was arbitrarily defined by measuring the area under the peak corresponding to one unit of human SOD activity (Sigma). One unit of SOD was equal to approx. 200 ng SOD protein.

Results

The tobacco leaf segments were transformed with the human SOD expression vector, a plasmid also containing a bacterial NPTII gene, and selected for resistance to the antibiotic kanamycin. Selected plantlets were examined for SOD activity and for the presence of integrated human SOD cDNA and NPTII sequences

in their chromosomal DNA. Transgenic tobacco plants did not exhibit a phenotype change due to the transformation.

To eliminate variations due to the position effect, we analyzed NPTII and SOD gene expression levels in at least ten independently transformed plants. The expression of the kanamycin gene in transgenic plants was confirmed by testing the activity of the NPTII gene under the control of the nopaline synthase promoter. Extracts of the kanamycin resistant plants contained the expected NPTII specific activity, which confirmed the biological function of the integrated hybrid marker gene (data not shown).

For the transgenic plants with pGA-SOD, a band of 0.6 kb corresponding to a *Hind*III and *Cla*I fragment containing the human SOD coding sequence was expected. The DNA was digested with *Hind*III and *Cla*I, fractionated, and transferred to a nylon filter. A 0.6 kb *Eco*RI fragment, which carries the SOD coding sequence, was isolated from pSD1 and labeled with [α - 32 P]dCTP by nicktranslation, and then hybridized to the blot (Figure 2A). The SOD probe did not hybridize to the DNA from the untransformed

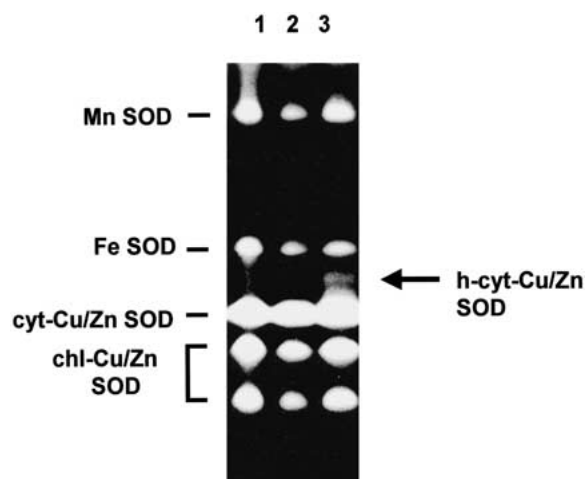


Fig. 3. Human SOD gene expression in transgenic plants tested by activity staining of non-denaturing polyacrylamide gel. Protein samples (480 μ g total protein) were separated on native protein gels and stained for SOD activity immediately. Expressed (human Cu/Zn SOD) and endogenous SODs are indicated. Lane 1: non-transformed control plant, lane 2: pGA643 transformant (expression control), lane 3: pGA-SOD transformant (S4 plant).

control plant (negative control) but the plant transformed with pGA 643 (expression vector control) did hybridize to the expected 0.6 kb DNA fragment of the 10 transgenic plants. The copy number of the gene in plants was determined by comparing with the standard genome as described above. Transgenic plants contained one to several copies of the human SOD gene in their genomes.

The relative levels of mRNA encoding human SOD in the transgenic plants were investigated by the RNA gel blot analysis. The RNA gel blot in Figure 2B represents the accumulation of SOD steady-state mRNA in total RNA isolated from transgenic tobacco leaves, which was detected by the 32 P-labeled SOD probe. Eight transgenic plants expressed high levels of human SOD mRNA (about 0.6 kb) while the remaining two (S-7, S-9) contained relatively low levels. As expected, control plants (lanes 1 and 2) did not show the human SOD mRNA. However, the SOD mRNA expression in the transgenic plants did not completely correlate with the copy number of the inserted DNA. These differences are presumed due to the influences of the surrounding plant DNA-chromatin structure, methylation, or other 'position effect' (Finnegan & McElroy 1994).

To determine whether the observed SOD activity reflects the steady state mRNA content, total protein was isolated from the same 10 representative trans-

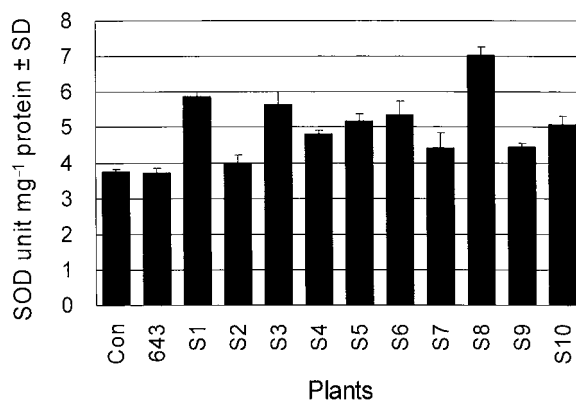


Fig. 4. SOD activities in tobacco plant transformed with pGA-SOD measured by activity gel with 1D main program. Con – nontransformed control tobacco; 643 – pGA 643 transformant; S1 to S10 – pGA-SOD transformants.

genic plants and two control plants for the enzymatic SOD activity measurement. The protein extracts were assayed by the *in situ* staining method of Beauchamp & Fridovich (1971) in order to confirm if the increase in SOD was correlated to human SOD expression in the transgenic plants. Although plants have several forms of SOD activity, these endogenous proteins did not prevent the specific detection of human SOD activity because the plants have no endogenous protein that electrophoretically migrates in the same manner with the human SOD. We have demonstrated the presence of 6 major bands of SOD activity in protein extracts derived from leaves of a non-transgenic plant and the pGA 643 transformant. Figure 3 shows that a new protein with SOD activity was present in the transgenic plants which was not observed in the proteins of control plants. Inhibitor studies with H_2O_2 and KCN, commonly used to distinguish between the different classes of SOD, identified one protein (five major bands) as Mn SOD (resistant to both H_2O_2 and KCN), four as Cu/Zn SODs (sensitive to both H_2O_2 and KCN), and other as Fe SOD (resistant to KCN but sensitive to H_2O_2). The new SOD activity could be inhibited by cyanide and hydrogen peroxide, indicated its identity as Cu/Zn SOD.

In the pGA 643 transformant, 643, the activity of SOD activity was, as expected, similar to that of the negative control plant, whereas nine transformants, except S2, had higher activities (Figure 4). The control extracts exhibited a specific activity of 3.8–3.9 units mg^{-1} protein. All other transformants exhibited a specific activity ranging from 4–6.9 units mg^{-1} . However, the expression rate did not correlate com-

pletely with the mRNA quantity. The S4 plant had the largest amount of mRNA but not the highest enzyme activity. Although S1 and S8 had less mRNA than S4, they had higher enzyme activity. Two transgenic plants (S1 and S8) showed an approx. 1.5- to 1.8-fold increase in total SOD activity. In the case of S8, about 40 μg human SOD (0.62% of soluble protein) were obtained from 1 g of fresh leaf material. The calculated specific activity of the plant-derived human SOD was about 200 ng unit^{-1} .

The plants studied here expressed the transgene as an active enzyme. They are thus capable of forming the human protein. The difference in total SOD activity between the control and the transformants is the consequence of the specific overproduction of the introduced human SOD gene.

Discussion

This paper describes a new eukaryotic system for the expression of recombinant human SOD. Several human proteins have been produced in plants. Production of animal proteins in a fused protein form in plants has been reported before (Trudel *et al.* 1995, Vandekerckhove *et al.* 1989). However, information about the production of unfused bioactive proteins in plants has been rather limited. Some researchers have tested a few genes of the animal kingdom for the production of animal bioactive peptides in an unfused form. Higo *et al.* (1993) have used specifically designed genes to transform plants for the production of human EGF (hEGF) in the cytosol. The highest hEGF peptide content per unit of total soluble protein was about 0.001%. Other attempts were made with human erythropoietin (EPO) and interferon-beta. With regard to productivity, all three human gene transformations turned out similar. The results suggested that either these mRNAs were not efficiently translated or the synthesized proteins were not as stable as other cellular proteins.

Our results, however, were similar to the report of the production of the hen egg white lysozyme in tobacco (Trudel *et al.* 1992). In that research, the introduced hen egg white lysozyme gene was expressed as an active enzyme in transgenic plants (about 30 μg per g leaf tissue). This productivity is similar to our results of about 40 μg (0.6% of soluble proteins in the leaf tissue). These two enzymes exist as many isozyme types in vertebrates as well as in plants. In the plant cell, these introduced protein and the indigenous isozymes may not be distinguished clearly. The two cases seem

to suggest that some animal genes may be efficiently translated in the plant cell as a pseudo-isozyme of the plant and stably maintained in its cytosol.

Since only a limited number of animal genes have been examined for accurate expression and processing in plants, it is difficult to draw general conclusions about the feasibility of animal genes to be expressed in plants. The differences in expression level may reflect specific properties of the individual genes or a difference in experimental set-up (e.g., unfused vs. fused forms). The accuracy of the expression of any given gene in the transgenic plant might, at this time, still be considered unpredictable.

In summary, recombinant plasmids encoding human SOD were integrated into the cellular DNA of tobacco plants and expressed at various levels as human SOD mRNA. The transcribed RNA was translated successfully to yield the enzymatically active human SOD protein. At the highest level of over-expression obtained (S8 ref.) the production of 1 mg human SOD will require just 1 transgenic plant (about 0.2–0.5 m^2 of greenhouse space). Our data indicated that the plant could be efficiently used for the production of human SOD.

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