# Efficient expression of laccase gene from white-rot fungusSchizophyllum communein a transgenic tobacco plant

Research Letter

# Efficient expression of laccase gene from white-rot fungus *Schizophyllum commune* in transgenic tobacco plant

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**Abstract**

Ligninolytic enzymes produced by white-rot fungi are effective degraders of recalcitrant aromatic environmental pollutants. However, gene sequences of these enzymes are rich in CpG dinucleotides which are particularly unfavorable to efficient expression in plants. In order to develop phytoremediation technique with ligninolytic enzymes-producing transgenic plant, laccase cDNA (*scL*) from white-rot fungus *Schizophyllum commune* was used as a model ligninolytic enzyme, and we tried the efficient expression of *scL* in transgenic tobacco plant by decrease in CpG-dinucleotide motif's content. We constructed a mutagenized *scL* sequence, *scL12*, decreasing 12% of CpG-dinucleotide motif's content, and *scL12* was introduced into tobacco plant. Much higher laccase activity was detected in transgenic *scL12* plants than in transgenic *scL* plants and wild type plants. By RT-PCR analysis, *scL12* was translated in transgenic *scL12* plants whereas mRNA of *scL* was not detected in the transgenic *scL* plants, and scL which is the product of the *scL12* gene was produced in the transgenic *scL12* plants by native-PAGE analysis. Moreover, transgenic *scL12* plants were able to remove trichlorophenol more effectively than transgenic *scL* plants and wild type plants. These results suggest that the decrease in CpG-dinucleotide motif's content in fungal target

genes is a useful method for efficient expression of these genes in transgenic plants.

**Keywords**

laccase; white-rot fungi; CpG-dinucleotide motif; trichlorophenol; transgenic plant

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## Introduction

Lignin is biologically difficult to be degraded because the free radical coupling mechanism responsible for its biosynthesis from phenolic cinnamyl alcohols results in a polymer interconnected through diverse carbon–carbon and ether bonds that are not hydrolyzable under biological conditions (Boerjan *et al.*, 2003). White-rot fungi have the apparently unique ability to degrade lignin to the level of $CO_2$ (Kirk & Farrell, 1987). Due to the heterogeneity of the substrate, white-rot degradation of lignocellulosic material involves an ensemble of extracellular enzymes. Lignin peroxidase (LiP), mangnese peroxidase (MnP) and laccase are the major extracellular ligninolytic enzymes of white-rot fungi involved in lignin biodegradation (Kirk & Farrell, 1987). There is a great interest in the lignin-degrading white-rot fungi and their ligninolytic enzymes because of their industrial potential for degrading recalcitrant environmental pollutants, such as polychlorinated dibenzodioxin, DDT, lindene (Bumpus *et al.*, 1985), chlorophenols (Joshi and Gold, 1993), and polycyclic aromatic carbons (Bezalel *et al.*, 1996; Collins *et al.*, 1996). Recently, we demonstrated that ligninolytic enzymes such as MnP and laccase were effective in degrading of methoxychlor (Hirai *et al.*, 2004) and Irgarol 1051 (Ogawa *et al.*, 2004), and in removing the estrogenic activities of bisphenol

A, nonylphenol (Tsutsumi *et al.*, 2001), 4-tert-octylphenol (Tamagawa *et al.*, 2007), genistein (Tamagawa *et al.*, 2005) and steroidal hormones (Suzuki *et al.*, 2003, Tamagawa *et al.*, 2006).

On the other hand, phytoremediation for removal of recalcitrant environmental pollutants can be an alternate/supplementary method, since plants have the advantage that they can grow independently using sunlight, water and inorganic ions and can be cultivated by germination of seeds or by vegetative propagation causing least disturbance to the contaminated sites. They are robust in growth, are a renewable resource and can be used for in situ bioremediation. Different steps in phytoremediation of recalcitrant environmental pollutants involve uptake, translocation, transformation and compartmentalization and sometimes mineralization (Schnoor *et al.*, 1995). Plant roots also secrete metabolites, which stimulate the growth of microorganisms in the rhizosphere, which in turn can degrade and mineralize the organic compounds. Although plants have several advantages over bacteria as candidates for bioremediation, they lack xenobiotic degradative capabilities not as bacteria. Hence, introduction of genes for degradation of recalcitrant environmental pollutants from microbes or other eukaryotes will further enhance their ability to degrade/mineralize recalcitrant environmental pollutants. However, microbe’s gene sequences are rich in CpG

dinucleotides and have highly skewed codon usages, both of which are particularly unfavorable to efficient expression in plants (Rugh *et al.*, 1995). MnP gene (Iimura *et al.*, 2002) or laccase gene (Sonoki *et al.*, 2005) from white-rot fungi have been already introduced into tobacco plants. However, very low activities of these enzymes were detected in transgenic plants. On the other hand, Rugh *et al.* have reported that *merA18*, in which was decreased 18% of CpG dinucleotides motif's content of bacterial mercuric reductase (*merA*) gene, was constructed, and that the *merA18*-introduced plantlets have released elemental mercury at approximately 10 times faster than untransformed plantlets (Rugh *et al.*, 1998). These results indicate that plants expressing modified *merA* constructs could provide a means for the phytoremediation of mercury pollution.

In the present study, laccase cDNA (*scL*) from white-rot fungus *Schizophyllum commune* was used as a model ligninolytic enzyme, and we tried the efficient expression of *scL* and the removal of a recalcitrant compound in transgenic tobacco plant by decreasing of CpG-dinucleotide motif's content in order to develop phytoremediation with ligninolytic enzymes-producing transgenic plant.

## Materials and methods

### Plant and microorganisms

Tobacco (*Nicotiana tabacum* L. cv. Burley 21) was used for the generation of transgenic plants. *Escherichia coli* DH5α (Nippon Gene) was used as host cells for manipulation of DNA. White-rot fungus *S. commune* (NBRC 30496) was used for the isolation of laccase (scLac). cDNA (*scL*) encoding laccase from *S. commune* (Accession No. AB015758; Hatamoto *et al.*, 1999) was used as a model ligninolytic enzyme gene.

### Reconstruction of *scL* for plant expression

A *scL* including Xba I/Sac I site was isolated, and then the fragment was inserted into Xba I/Sac I replacement region in binary plant expression vector pBI121 (Invitrogen) to produce pBI121-scL (Fig. 1). Short primers 5'S and 3'A, large mutagenic primers internal to the *scL* coding sequence 404-493A (90 bases) and 473-552S (80 bases) (Fig. 2), were used to amplify the A and B halves of the *scL* gene with PCR. The PCR amplification contained amplification buffer (Nippon Gene) with 3% dimethyl sulfoxide and 0.5 units of Taq polymerase (Nippon Gene) and was carried out for 30 cycles (94°C for 30 sec, 52°C for 1 min, and 68°C for 2 min). Once gel

purified, the A and B fragments (Fig. 2) were joined together in an overlap extension (OE)-PCR reaction (Ho *et al.*, 1989). The same PCR conditions as above were used except that the reaction was primed with the external oligonucleotides 5'S and 3'A. The mutagenized fragment was cleaved by Xba I and Kpn I, and ligated into the Xba I/Kpn I replacement region in the plasmid pBI121-scL to produce pBI121-scL12.

**Construction of transgenic plants**

T-DNA region of pBI121-scL12 was introduced into the genome of tobacco by the leaf-disk method using *Agrobacterium tumefaciens* LBA-4404 (Liang *et al.*, 1989). The control transgenic tobacco plants, into which T-DNA region of pBI121-scL was introduced, was also constructed. These transgenic tobacco plants, which have regenerated from leaf-disks and were resistant to kanamycin, were transferred to MS media (Murashige & Skoog, 1962) containing 30 g/L sucrose and 250 mg/L kanamycin and 500 mg/L carbenicillin, and then incubated in a growth chamber at 25°C.

**Measurement of laccase activity**

Each transgenic tobacco plant was incubated on MS medium containing 30 g/L sucrose and 250 mg/L kanamycin and 500 mg/L carbenicillin for 1 month, and then was

homogenized by a Polytron PT1200E with 50 ml of 20 mM phosphate buffer (pH 6.0) containing 0.05% Tween 80 and 0.004% phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 10,000 *g* for 10 min, and the supernatant was used as cell-free extract. Laccase activity in the cell-free extract was determined by monitoring the oxidation of 2,6-dimethoxyphenol (DMP) at 470 nm at 30°C. The reaction mixture contained 1 mM DMP and 20 mM phosphate buffer (pH 6.0). One katal (kat) of enzyme activity is the amount of the enzyme producing 1 mol of the quinine dimer (49.3 $mM^{-1}$ $cm^{-1}$) from DMP per second.

**RT-PCR**

Total RNA was extracted from 100 mg (wet weight) of leaf tissue using Plant RNeasy extraction kit (Qiagen), and then 200 ng of total RNA was applied to RT-PCR. PrimeScript RT-PCR Kit (Takara Bio) was used together with gene-specific primers; *scL12* (forward, 5’ ATTGGCACCAACAATTATAGGA 3’; reverse 5’ AAGGACACTATTCTGAGTCT 3’), *scL* (forward, 5’ GTTGGCACCGACGATTATAGGC 3’; reverse, 5’ AAGGACACTATTCTGAGTCT 3’), and tobacco actin (forward, 5’ TGGACTCTGGTGATGGTGTC 3’; reverse, 5’ CCTCCAATCCAAACACTGTA 3’).

**Native PAGE**

The cell-free extract was concentrated by a Ultrafilter unit (10 kDa cut-off, Advantec) to 1.0 nkat/ml. Ten μl of the concentrate was separated by native PAGE (gel conc. 12.5%), and was examined by activity staining using DMP. scLac prepared by the method described by Vries *et al.* (Vries *et al.*, 1986) was also applied to native PAGE.

**Removal of trichlorophenol by hydroponically cultured transgenic plants**

Each transgenic tobacco plant was incubated on MS medium containing 30 g/L sucrose and 250 mg/L kanamycin and 500 mg/L carbenicillin for 1 month, and then was transferred to 100 ml fresh MS liquid medium containing 30 g/L sucrose and 250 mg/L kanamycin and 500 mg/L carbenicillin. After 4-days incubation, 20 μmol of trichlorophenol (TCP, 0.5 ml of 40 mM TCP/DMF soln.) was added to the liquid medium, and then the plant was incubated for 5 days. One ml aliquot of the liquid culture was added to 2 ml of acetonitrile, and the solution was filtrated. The filtrate was subjected to analysis by HPLC. Chromatographic separations were performed with a Wakosil-II 5C18HG (length, 250 mm; i.d., 4.6 mm; Wako). The mobile phase consisted of a 1% acetic acid : acetonitrile (1 : 1, vol/vol) with a flow rate of 1.0 ml/min.

## Results

### Construction of transgenic plants

The original 1557-nt *scL* coding sequence is G+C rich (57%), contains 147 CpG dinucleotides (Hatamoto *et al.*, 1999), and is skewed toward GpC-rich codons, which are uncommon in plant (Murray *et al.*, 1989). Therefore, we constructed a modified *scL* gene, *scL12*, using an OE-PCR strategy, which is shown in Fig. 2. Codons 404-552 (i.e. 12% of the coding region) were replaced with nucleotide combinations and codons more common to highly expressed plants (Fig. 3).

The *scL12* or *scL* was subcloned into a plant expression vector, which placed *scL12* or *scL* under control of the constitutive plant cauliflower mosaic virus 35S promoter in a T-DNA binary vector. Each construct was transformed into tobacco leaf-disk from an *A. tumefaciens* bacterial host. Six independent lines of *scL12*-introducing transgenic tobacco plants (T line) and 3 independent lines of *scL*-introducing transgenic tobacco plants (C line) were obtained.

### Detection of laccase activities in cell-free extract from transgenic tobacco plants

In order to confirm whether laccase activity in T line was higher than in C line or

wild type tobacco (WT), each cell-free extract was prepared from each plant, and laccase activity in each cell-free extract was assayed. As shown in Fig. 4, much higher laccase activities were detected in T line than in C line or WT. The average of laccase activities in T line (6 lines, n=5) was 13.1 nkat/g dry weight plant, whereas those in C line (3 lines, n=5) and WT (n=5) were 6.7 and 5.9 nkat/g dry weight plant, respectively (Table 1).

**Translation analysis by RT-PCR and laccase production analysis by native PAGE**

In order to establish that the translation of *scL12* in T line accounted for the increase in laccase activity, the expression of *scL12* in T line was confirmed by RT-PCR analysis (Fig. 5). A clear band was detected in T line although no band was observed in C line. It was evident that the *scL12* was expressed in T line whereas the expression of *scL* was not occurred. To confirm the production of laccase, the product of *scL12*, in T line, the concentrate of cell-free extract from T line was analyzed by native PAGE and active staining with DMP (Fig. 6). Positive bands in lane 2 (T line) and lane 3 (scLac) could be detected, whereas WT produced no positive band (lane 1). In native PAGE analysis of WT, a broad- and weak band, which was proceed from endogenous laccase in WT, was observed at different position.

**Removal of TCP by transgenic tobacco plants**

Since much higher laccase activities were detected in T line than in C line or WT, the removal ability of TCP by each hydroponically cultured transgenic tobacco plant was examined for the application of the transgenic plant to phytoremediation of recalcitrant environmental pollutants. T line was able to remove TCP more effectively than C line and WT (Fig. 7). The average of removal of TCP by T line (6 lines, n=2) was 36.5 μmol/g dry weight plant, whereas those by C line (3 lines, n=2) and WT (n=4) were 20.5 and 20.7 μmol /g dry weight plant, respectively (Table 2).

## Discussion

Phytoremediation is the term used to describe those methodologies that employ living higher organisms, which include green vegetation, plants, aquatic plants, trees and grasses, to remove toxic compounds. This technology has the advantage of in situ treatment of contaminated soils, sediments, groundwater, surface water and external atmosphere (Shimp *et al.* 1993; Cunningham *et al.* 1995; Macek *et al.* 2000). To some extent, molecular biology approaches have already been in use to evaluate phytoremediation and reveal elimination of toxicity from contaminated sites (Rugh *et al.* 1998; Bizily *et al.* 1999; Krämer & Chardonnens 2001). However, these sequences of microbe's gene, which might be introduced into plants, are rich in CpG dinucleotides and have highly skewed codon usages, both of which are particularly unfavorable to efficient expression in plants (Rugh *et al.*, 1995). Practically, these MnP gene- or laccase gene-intoroducing transgenic plants have produced very low amounts of these enzymes (Iimura *et al.*, 2002; Sonoki *et al.*, 2005). In the present study, we tried the efficient expression of *scL* in transgenic tobacco plant by decrease in CpG-dinucleotide motif's content. As shown in Fig. 4 and Table 1, much higher laccase activity was detected in the transgenic T line than in C line or WT. These results

suggested that the decrease in CpG-dinucleotide motif's content in *scL* gene accelerated the production of laccase in the transgenic plant. Moreover, the detected laccase was not produced by the activation of endogenous laccase gene in the tobacco plant, but by effective translation and transcription of *scL12* in transgenic plant T line, as shown in Figs. 5 and 6. Most of microbe's enzyme activities could not be detected in microbe's gene-introducing transgenic plants, and most of studies in these transgenic plants have used western blot analysis to detect these target proteins (ex. Karavangeli *et al.*, 2005; Nagata *et al.*, 2006; Mohammadi *et al.*, 2007). Until now, very little is known about the assayable detection of microbe's enzyme in transgenic plans, and the high production of ligninolytic enzyme in transgenic plants. To our best knowledge, this is the first report that the ligninolytic enzyme laccase can be produced effectively in a transgenic plant. Moreover, the removal ability of TCP, which is one of recalcitrant environmental pollutants, in T line was increased whereas that in C line was almost same as that in WT, as shown in Fig. 7 and Table 2. This result suggests that the produced scL in T line degraded TCP. By using this genetical engineering, transgenic plants in which ligninolytic enzymes are expressed will be useful tools for phytoremediation of recalcitrant environmental pollutants. Our next target is root-specific overexpression of ligninnolytic enzymes in transgenic plants for development of ecological

phytoremediation technology.

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Table 1 Laccase activity in each cell-free extract from transgenic tobacco plants.

| | Laccase activity (nkat/g dry weight) |
|---|---|
| T line | 13.1 ± 10.8 * |
| C line | 6.7 ± 4.9 |
| WT | 5.9 ± 2.4 |

* $P < 0.05$

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Table 2 Removal of TCP by each transgenic tobacco plants.

| | Removal of TCP (μmol/g dry weight) |
|---|---|
| T line | 36.5 ± 17.1 * |
| C line | 20.5 ± 5.0 |
| WT | 20.7 ± 4.4 |

* $P < 0.05$

Figure titles

Fig. 1. T-DNA region of the Ti plasmid pBI121-scL. RB, Right boarder of T-DNA; LB, left boarder of T-DNA; $Km^r$, neomycin phosphotransferase gene; 35S-P, CaMV 35S promoter sequence; N-ter, nopaline synthase terminator.

Fig. 2. Construction of *scL12* gene for efficient expression in plant. Strategy to mutagenize the *scL* gene. A map of the *scL* gene with base numbers is shown. The A and B halves of *scL12* were amplified in two separate PCR reactions, using pairs of sense (S) and antisense (A) mutagenic oligonucleotides, 5'S/404-493A and 473-552S/3'A, respectively.

Fig. 3. The DNA and protein sequences in the internal region altered by the mutagenic oligonucleotides 404-493A and 473-552S. The first line shows scL12 protein sequence. The second line gives shows the G + C-rich nucleotides found in wild type *scL*. The third line gives the synthetic sequence incorporated into *scL12*.

Fig. 4. Laccase activity in each cell-free extract from transgenic tobacco plants. The

preparation of cell-free extract and the assay of laccase activity were described at Materials and methods.

Fig. 5. RT-PCR analysis of *scL* and *scL12*. Equal amount of total RNA from T line, C line, and WT was applied to RT-PCR. Actin gene was used as internal control.

Fig. 6. Native PAGE (active-staining with DMP) of each cell-free extract from plants. Equal activity of laccase from T line, WT, and *S. commune* was applied to native PAGE. Lane 1, WT; Lane 2, T line; Lane 3, scLac.

Fig. 7. Removal of TCP by each transgenic tobacco plants. This experimental procedure was described at Materials and methods.

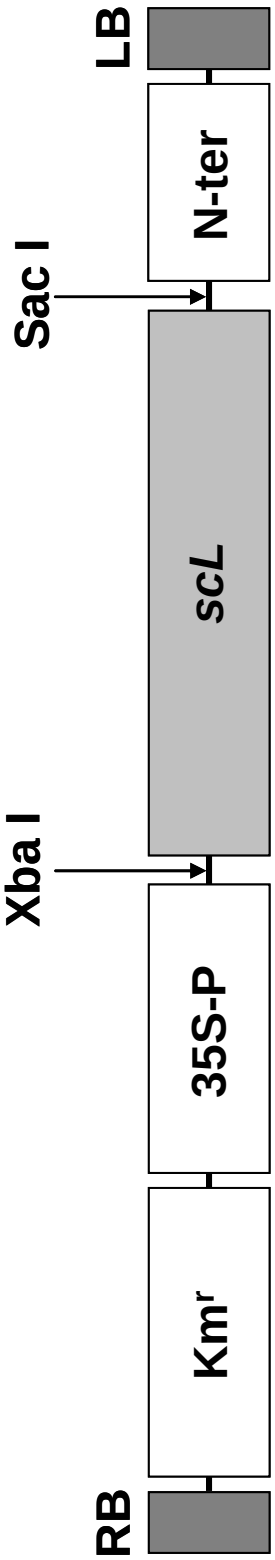


Fig. 1

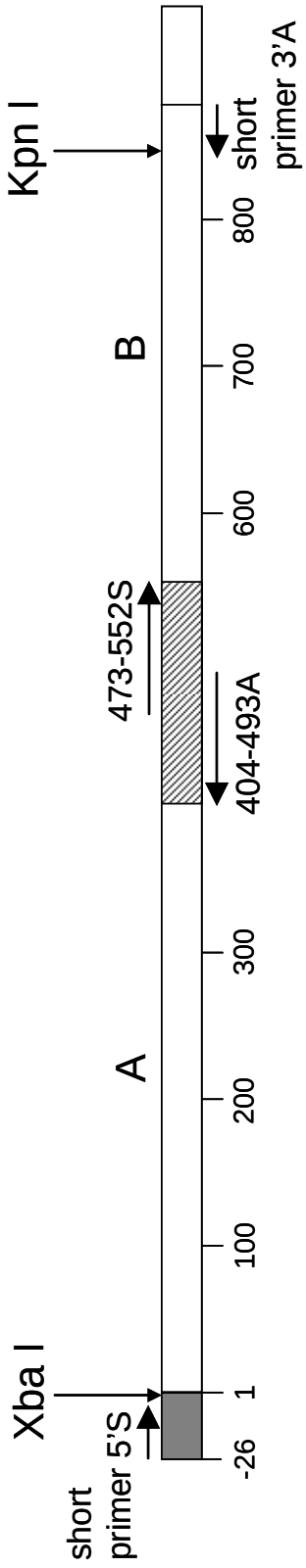
Kpn I
short
primer 3'A
800
B
700
600
473-552S
404-493A
300
A
200
100
Xba I
1
-26
short
primer 5'S

Fig. 2

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 135 | Gly | Leu | Arg | Gly | Ala | Met | Val | Val | Tyr | Asp | Pro | Ala | Asp | Pro | 148 |
| 403 | GGA | CTA | CGT | GGT | GCG | ATG | GTG | GTA | TAC | GAT | CCC | GCA | GAC | CCT | 444 |
| | | | A A | | A | | | | T | | A | | | | |
| 149 | His | Leu | Ser | Leu | Tyr | Asp | Val | Asp | Asp | Asp | Asn | Thr | Val | Ile | 162 |
| 445 | CAC | CTT | TCG | CTG | TAC | GAT | GTC | GAC | GAC | GAC | AAT | ACT | GTG | ATC | 486 |
| | | | A | | T | | A | T | T | | | | | | |
| 163 | Thr | Leu | Ala | Asp | Trp | Tyr | His | Ala | Leu | Ala | Pro | Thr | Ile | Ile | 176 |
| 487 | ACT | CTT | GCG | GAT | TGG | TAT | CAT | GCG | TTG | GCA | CCG | ACG | ATT | ATA | 528 |
| | | | A | | | | | A | | | A | A | | | |
| 177 | Gly | Val | Gly | Thr | Pro | Asp | Ser | | | | | | | | 183 |
| 529 | GGC | GTC | GGC | ACG | CCC | GAC | TCC | | | | | | | | 549 |
| | A | A | | A | A | | | | | | | | | | |

Fig. 3

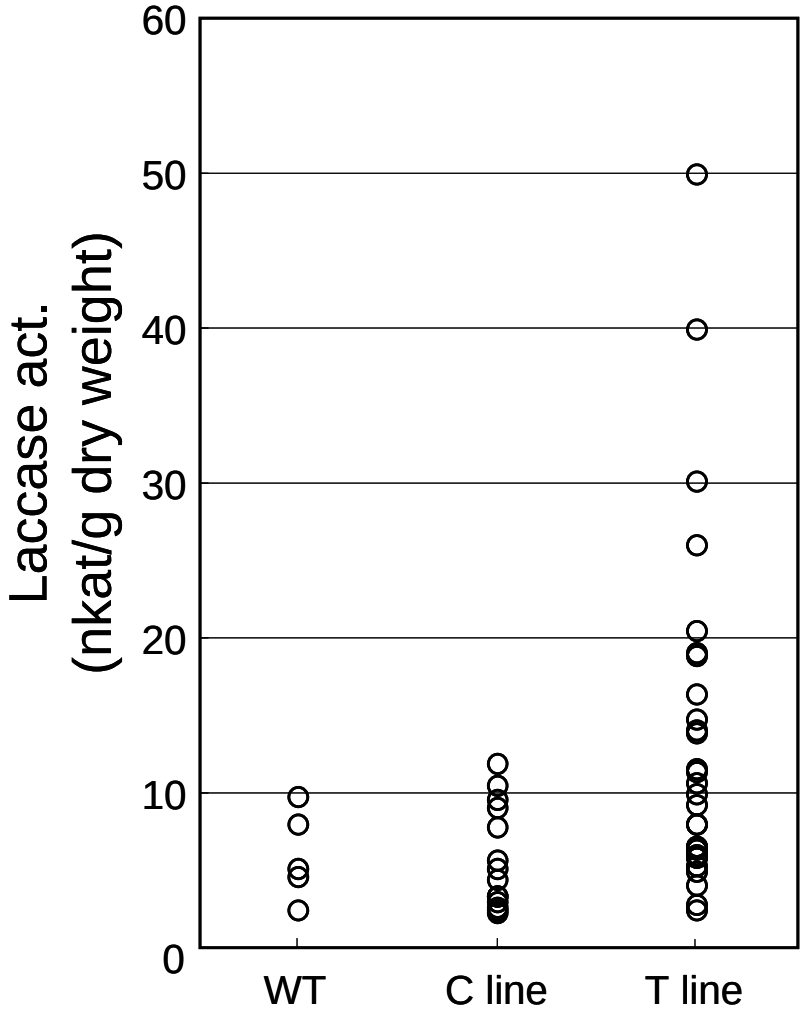


Fig. 4

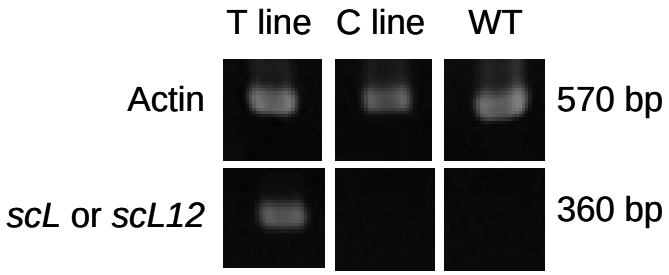


Fig. 5


Fig. 6

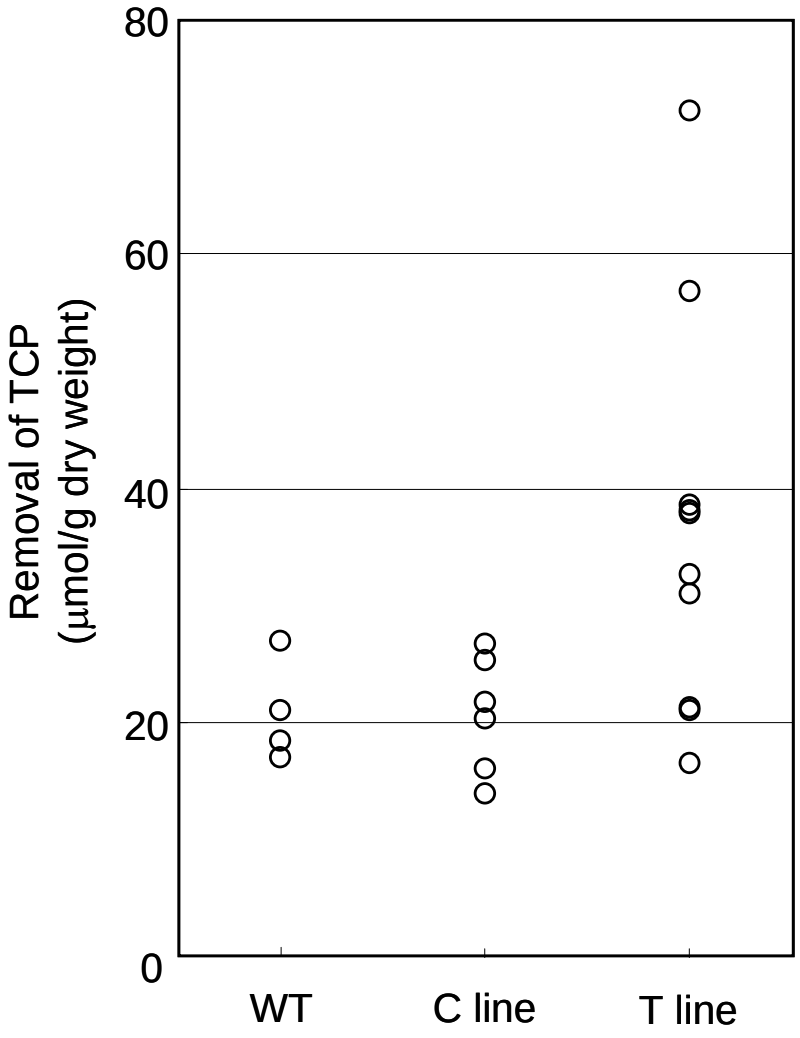


Fig. 7