引き抜き性状に対する土とジオシンセティクスとの相乗効果について

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要　旨

いろいろな補強土構造物の設計に際して生じる問題点の一つとしてここでは土及びジオシンセティクスの引き抜き挙動に関する研究を行った。砂質土及び粘性土に対してフォートラックとスタビランカの2種類のジオシンセティクスを用い，それぞれ6つの垂直応力の下で一連の引き抜き実験を行った。実験結果の解析によると粘性土ではフォートラックの方がスタビランカより大きな摩擦抵抗を持つのに対して，砂質土のそれは低い値を，また，両試料に対しスタビランカの方がフォートラックより明らかに高い粘着力を示すことが分かった。ジオシンセティクスと土との組み合わせに関係なく垂直応力及び引き抜き変位の増加とともに引き抜き応力が増加するという一般的傾向が見られる。この傾は他の文献に見られる研究結果と一致する。

キーワード：ジオシンセティクス，補強土，引き抜き性状，フォートラック，スタビランカ，室内実験，相乗効果

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Expression of *Thlaspi caerulescens* phytochelatine synthase gene and its role in Cd tolerance in yeast

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Abstract

Thlaspi caerulescens is recognized as a typical model for studying hyperaccumulation of cadmium (Cd) and zinc (Zn), and has been interested to apply its ability for extraction of Cd from contaminated soil. However, Cd tolerance ability of *T. caerulescens* has not been applied for developing of the technique of phytoremediation, because of a lack of understanding of the mechanism involved in Cd^{2+} tolerance and hyperaccumulation of this plant. In order to know about participate role of metal detoxication mechanism for hyperaccumulation, a cDNA of phytochelatin synthase, which synthesis the metal chelating peptide phytochelatin, was cloned from *Thlaspi caerulescens*, and investigated its work for Cd^{2+} tolerance. The deduced amino acid sequence of cloned PCS gene, named TcPCS, was 475 amino acid length and showed high homology with PCSs from Ni hyperaccumulator plant *Thlaspi japonicum* and *Arabidopsis thariana*. Expression of *TcPCS* gene carried the strong Cd^{2+} tolerance to yeast, as shown as normal growth on the YNB-Ura- agar plate containing 100 μM $CdCl_2$, and growth in the culture medium up to 300 μM Cd^{2+}. These data suggest that PCS have some role for Cd^{2+} tolerance in *T. caerulescens*.

Key Words: cadmium, phytochelatin synthase, phytoremediation, *Thlaspi caerulescens*, yeast
Running title: Phytochelatin synthase in *Thlaspi caerulescens*

Introduction

Cleaning-up soils contaminated with heavy metals is a difficult and complicated task, but Cd pollution is a serious problem worldwide, especially in Japan[1]. Widespread Cd pollution causes contamination of food crops such as rice and maize, posing a grave threat to the health of Japanese people[2, 3].

One solution to this problem that has been developed and studied vigorously[4, 5] is phytoremediation, a new soil clean-up technique using plants to bioaccumulate the heavy metal contaminants. The plant tissue with the accumulated heavy metal are then harvested and treated for disposal. Some methods of phytoremediation studied include using Indian mustard (*Brassica juncea*), which accumulates many kinds of toxic metals in its tissues[6]. However, certain problems such as the low biomass of metal accumulating plants and secondary pollution by chelater chemicals prevent this technique from being widely adopted. To improve the utility of phytoremediation, investigations of metal absorption, accumulation, and detoxification systems in plants are demanded in conjunction with low-toxicity chelators.

Some plants that accumulate unusual level of metals (metal hyperaccumulators) have been investigated and analyzed as appropriate models for phytoremediation[7]. Among them, Thlaspi is a genus with many

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species of hyperaccumulators of Cd, Zn, Ni and other metals, and many scientists have been studying these species for their metal resistance and accumulation. In particular, Cd detoxification in the Cd hyperaccumulator species *Thlaspi caerulesecens* has been investigated vigorously by many researchers[8)-10)], revealing its role for surviving in Cd contaminated soil[11)]. For metal hyperaccumulation, there are several mechanisms that are thought to be important: 1) strong resistance to metal toxicity, 2) constant expression and production of metal transporter proteins in the roots, and 3) separation of metals into storage tissues such as vacuoles and cell walls. Resistance and/or detoxification are very important for accumulating toxic metals such as Cd.

For metal detoxification in plants, some peptide and proteins such as glutathione, phytochelatin and metallothionein have been reported to act as biological chelator peptides, and their application for phytoremediation has been tested[12-16)]. Phytochelatin, which is enzymatically synthesized by phytochelatin synthase (PCS), has a specific detoxification ability for Cd, a metal which is extremely thiol reactive[17, 18)]. In one study, an *Arabidopsis thaliana* mutant with PCS deficiency was reported to be hypersensitive to Cd[19)], and in another, expression of *A. thaliana* PCS in bacterial cells resulted in Cd accumulation[20)]. These results suggest that PCS has a role in Cd resistance and detoxification in plants. Similarly, it is speculated that PCS provides resistance to and hyperaccumulation of Cd in *T. caerulescens*.

In order to gain information on the role of phytochelatin synthase in phytoremediation, we cloned the PCS gene from *T. caerulescens* and investigated its role in Cd tolerance by expression in yeast.

Materials and Methods

Plant material

The seeds of *T. caerulescens* ecotype Ganges used in this paper were obtained from Dr. R. R. Brooks (Massey Univ., New Zealand). The seeds were germinated in potting compost in a growth chamber at 23℃ with a scheduled (16-h day/8-h night) artificial light period. After one month, plants were grown in a glass house under ambient light within a range of 20-30 ℃.

Cloning of the Tcpcs gene

Cloning and DNA sequence determination of *Tcpcs* cDNA was performed as described in our previous paper, which reported the cloning of a PCS gene from Japanese Ni-hyperaccumulator plant *Thlaspi japonicum*[21)]. In short, total RNA was isolated from Cd stimulated *T. caelurescens* leaves and used for cDNA template synthesis by reverse transcriptional reaction with M-MLV reverse transcriptase (Wako chemicals, Osaka, Japan).

Two primers, FWD2 {5'-ATGYTNGAYTGYTGYGARCC-3' (Y; C or T, N; A, C, G or T, R; A or G)} and REV1 {5'-ACCCARTGNGGNGGRTAYTT-3'}, designed by the consensus regions of reported phytochelatin synthase sequences, were used for partial amplification of *Tcpcs* cDNA. The fragments of *Tcpcs* were determined by 5'- and 3'-RACE methods, with sequence determination performed by an automated DNA sequencer (Model 3100, Applied Biosystems Japan, Tokyo). The DDBJ/EMBL/GenBank database was searched for homologous sequences using BLAST[22)], and alignments of DNA/protein sequences were performed using the CLUSTAL W multiple sequence alignment program[23)].

Tcpcs expression in yeast

The *Tcpcs* expression plasmid was constructed by insertion downstream of the Gal1 promoter of the

inducible expression vector pKT10-Gal-HA-BS with fusion into two hemagglutinin (HA) genes, which encoded a HA tag amino acid sequence GYPYDVPDYA[24]. Transformation of the resulting plasmid (pTcPCS) and an empty plasmid to the *S. cerevisiae* strain BJ1824 (*MaTa, leu2, ura3, trp1, pep4, cir*$^+$) was performed by the lithium acetate method[25], and transformants were cultured in a yeast nitrogen base medium supplied with tryptophan and leucine (YNB-ura$^-$ medium) and appropriate carbon sources.

Western blotting for detection of recombinant TjPCS

Cell-free extracts (5 μg) of recombinant yeasts were subjected to 8% SDS-PAGE and blotted onto PVDF membrane. Recombinant TcPCS fused with two HA-tags was detected by immunoblotting using 1:1000 diluted anti-HA tag monoclonal antibody (Santa Cruz, CA, USA) and Amplified Alkaline Phosphatase Immno-Blot Assay Kit (Bio-Rad, Alfred Novel Drive Hercules, CA, USA) containing anti-mouse IgG-biotinated goat IgG, streptavidin, biotinated alkaline phosphatase, and a coloring reagent.

Cd tolerance of yeast displaying *Tcpcs* expression

Plate culture experiment: Yeast cells transformed with empty or pTcPCS plasmids were pre-cultured with YNB-Ura$^-$ at 30 ℃ and 140 rpm until OD_{600}=0.6. Cells were diluted to OD_{600}= 0.01 in distilled water and spotted (5 μl) on agar plates of YNB-ura$^-$ medium containing 0, 50 and 100 μM of $CdCl_2$ and supplied with 1% galactose and 1% sucrose (gene expression promoting condition) or 2% glucose (gene expression suppressing condition). Cell growth was measured after incubation at 30 ℃ for 3 days.

Liquid culture experiment: Pre-cultured cells were diluted to OD_{600}=0.001 with YNB-Ura$^-$ medium containing 0-600 μM of $CdCl_2$ and supplied with 1% galactose and 1% sucrose or 2% glucose and cultured at 30 ℃ with shaking at 140 rpm. Cell growth was determined as the values of OD_{600}.

Results

A cDNA fragment of PCS that had a length of about 300 bp was successfully amplified from *T. caerulescens* cDNA template, and a 1585 bp cDNA sequence containing 1455 bp of phytochelatin synthase gene (*Tcpcs*) was determined with the continuous 5'- and 3'-RACE. The deduced amino acid sequence of *Tcpcs* (TcPCS) had 485 amino acids (Fig. 1A), the same as the phytochelatin synthases from *A. thaliana, A. halleli* and *T. japonicum*. TcPCS showed a high degree (98.4%) of similarity to TjPCS of *T. japonicum*, and about 90% similarity to PCSs of the Brassicaceae plants *Brassica juncea, Arabidopsis thaliana*, and *A. halleri*. The PCSs of the ferns *Athyrium yokoscence* (Cd hyperaccumulator) and *Pteris vittata* (As hyperaccumulators) showed similarity only at N-terminal region. The phylogenetic tree of these PCS proteins is shown in Fig. 1B.

Production of TcPCS fused with two HA-tags was detected by Western blotting using an anti HA-Tag monoclonal antibody (Fig.2). Recombinant TcPCS was detected only in the yeast cultured with 1% galactose and 1% sucrose, which induced *Tcpcs* expression. To clarify the meaning of *Tcpcs* expression for Cd tolerance, recombinant yeast containing the expression plasmid of TcPCS was spotted on the yeast nitrogen base (YNB-Ura$^-$ medium) supplied with 0-100 μM $CdCl_2$. The growth of yeast containing a control plasmid, or pTcPCS-HA when *Tcpcs* expression was suppressed by glucose, was strongly suppressed when Cd concentration exceeded 100 μM. In contrast, recombinant yeast expressing *Tcpcs* showed obvious tolerance to Cd and grew the same at a concentration of 100 μM Cd as at 0 and 50 μM (Fig. 3).

The Cd tolerance of the yeast expressing *Tcpcs* was also recognized in the liquid culture. The recombinant yeast containing the *Tcpcs* gene showed apparent growth improvement in the medium

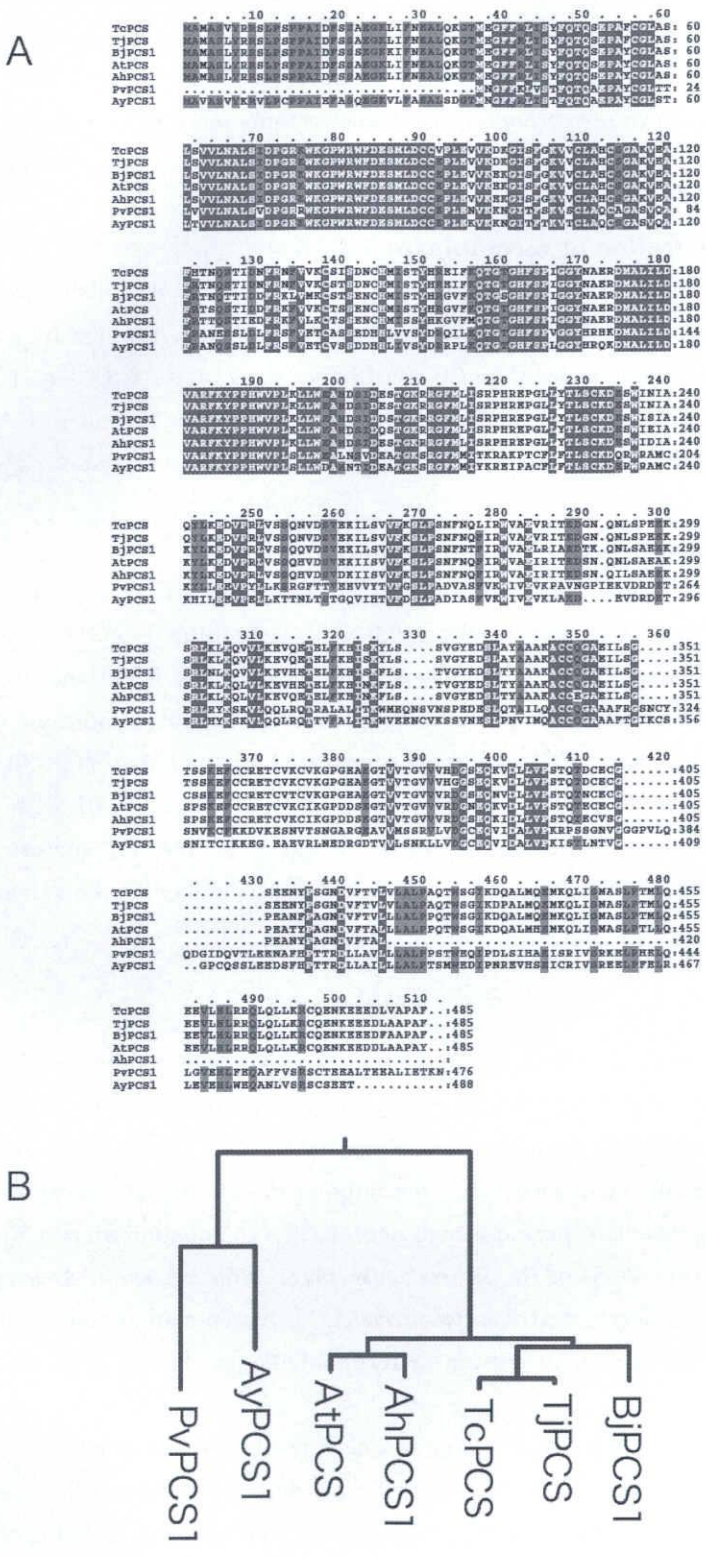

Fig. 1 Sequence conservation in plant phytochelatin synthase
(A) Multiple alignments of deduced amino acid sequences of phytochelatin synthase of *Arabidopsis thaliana*, metal hyperaccumulator and phytoremediation plants. Tc: *Thlaspi caerulescens*, Tj: *Thlaspi japonicum*, Bj: *Brassica juncea* At: *Arabidopsis thaliana*, Ah: *Arabidopsis halleri*, Pv: *Pteris vittata*, Ay: *Athyrium yokoscence*. GenBank accession numbers: AtPCS; BAB09067; BjPCS1, BAB85602; AyPCS1, BAB64932; TjPCS, BAB93119; TcPCS, BAB93120; PvPCS1, AAT11885; AhPCS1, AAS45236. (B) Phylogenetic tree of the PCSs.

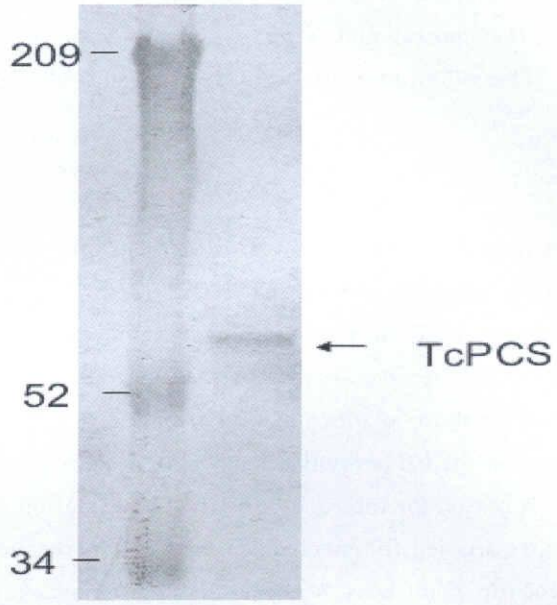

Fig. 2 Western analysis of TjPCS in recombinant yeasts
The recombinant yeast cells carrying pTcPCS were cultured with YNB-Ura$^-$ medium containing 1% galactose and 1% sucrose. Crude cell-free extract containing 5 μg protein was subjected to SDS-PAGE on 8% gel, electrotransferred, and probed with anti-HA monoclonal antibody.

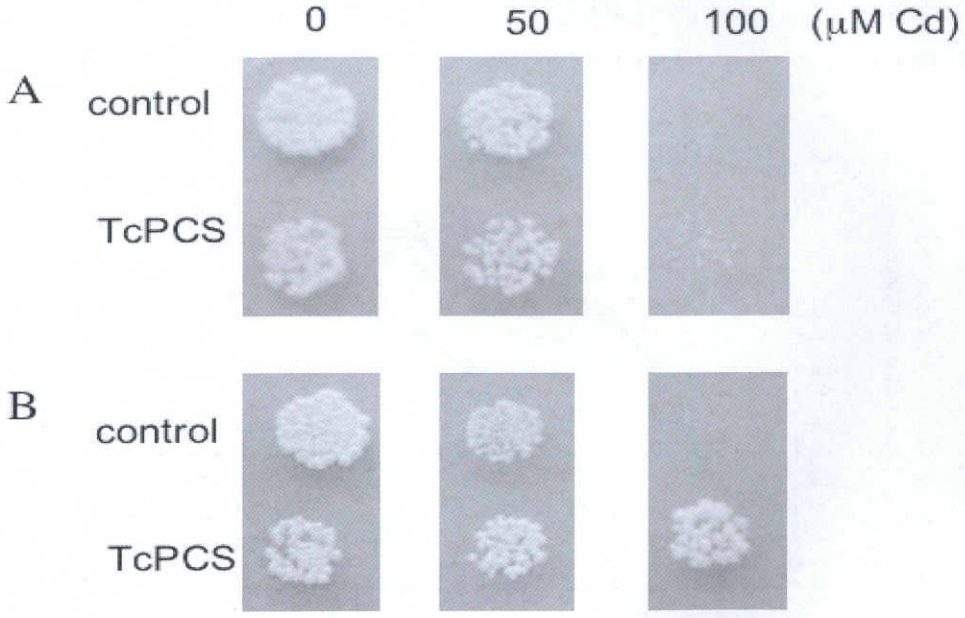

Fig. 3. Growth of TcPCS-induced yeast on Cd^{2+} containing plates
The recombinant yeast carrying the empty vector pKT10-Gal-HA-BS (Control) or pTcPCS (TcPCS) were cultured on the YNB-Ura$^-$ medium containing various concentrations of Cd^{2+}. A: *Tcpcs* induction plates (1% sucrose and 1% galactose). B: *Tcpcs* repression plates (2% glucose). Five μl of diluted yeast broth ($OD_{600}=0.01$) was spotted and incubated at 30℃for 3 days.

containing 100 μM Cd (Fig. 4) and survived in the gene expression medium with 300 μM Cd (Fig. 5). Growth differences between the control and *Tcpcs* expressing yeast were not recognized when gene expression was not induced. These data indicated that TcPCS lends Cd detoxification ability.

Discussion

We cloned the phytochelatin synthase gene from *T. caerulescens* and measured its ability to confer Cd tolerance. Expression of TcPCS provided obvious Cd resistance to yeast, indicating that phytochelatin production results in Cd tolerance in *T. caerulescens* and has some role in Cd hyperaccumulation in this plant. However, the degree of Cd tolerance was no better than that of *TjPCS* and *TaPCS1*, which were isolated from non-Cd hyperaccumulator plants and tested for their Cd tolerance under the same conditions[21, 26]. This result suggests that the Cd detoxification ability of PCS does not differ between plants, and that expression is not the main reason for the extraordinary accumulation of Cd in *T. caerulescens*.

To date, many scientists have studied the mechanism for Cd hyperaccumulation in *T. caerulescens*, and some unique characteristics of this plant have been highlighted. It was reported that a gene of the ZIP family transporter homolog (TcZNT) which is important for Cd/Zn transport, is strongly and constitutively expressed in the *T. caerulescens* root[27]. This species is also reported to store Cd principally in the less metabolically active parts of the leaf cells[28, 29]. These mechanisms are speculated to be the main reason for the extraordinary Cd accumulation in *T. caerulescens* shoots. Detoxification and resistance to Cd by PCS may work as a valuable adjunct to Cd transport and isolation in this plant.

To date, several transgenic plants overexpressing metal tolerance-related genes have been produced, and their abilities for depuration of polluted soil has been tested[30-32]. Furthermore, PCS expression in plants has been found to be related to enhanced arsenic and lead tolerance[33, 34]. These investigations strongly support

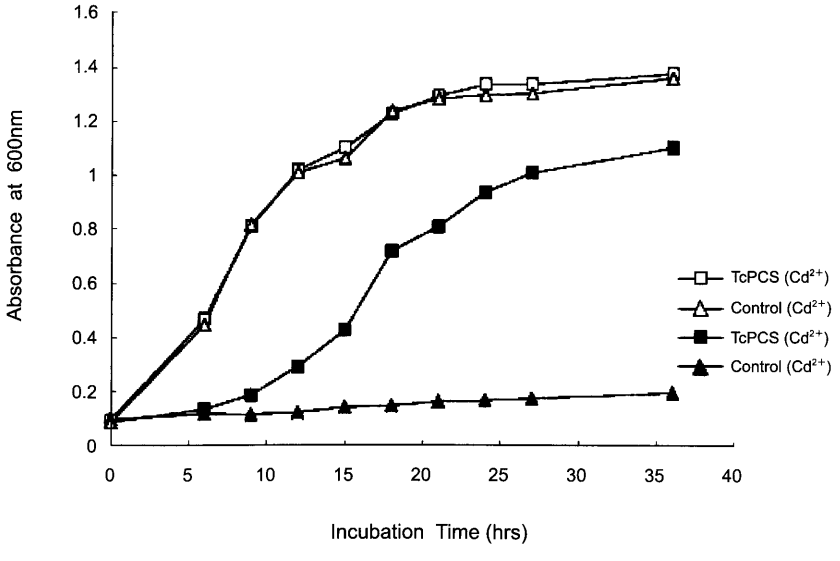

Fig. 4 Growth recovery of *Tcpcs* expression yeast in high Cdd^{2+} condition.
Yeast cells carrying the control vector (pKT10-Gal-HA-BS, triangles), or TjPCS expression vector (pTjPCS, squares) were grown in YNB-Ura^- (1% galactose and 1% sucrose) containing no Cd^{2+} (open symbols) or 100 μM C d^{2+} (filled symbols). The average values of OD_{600} of each sampling time (n=3) are shown.

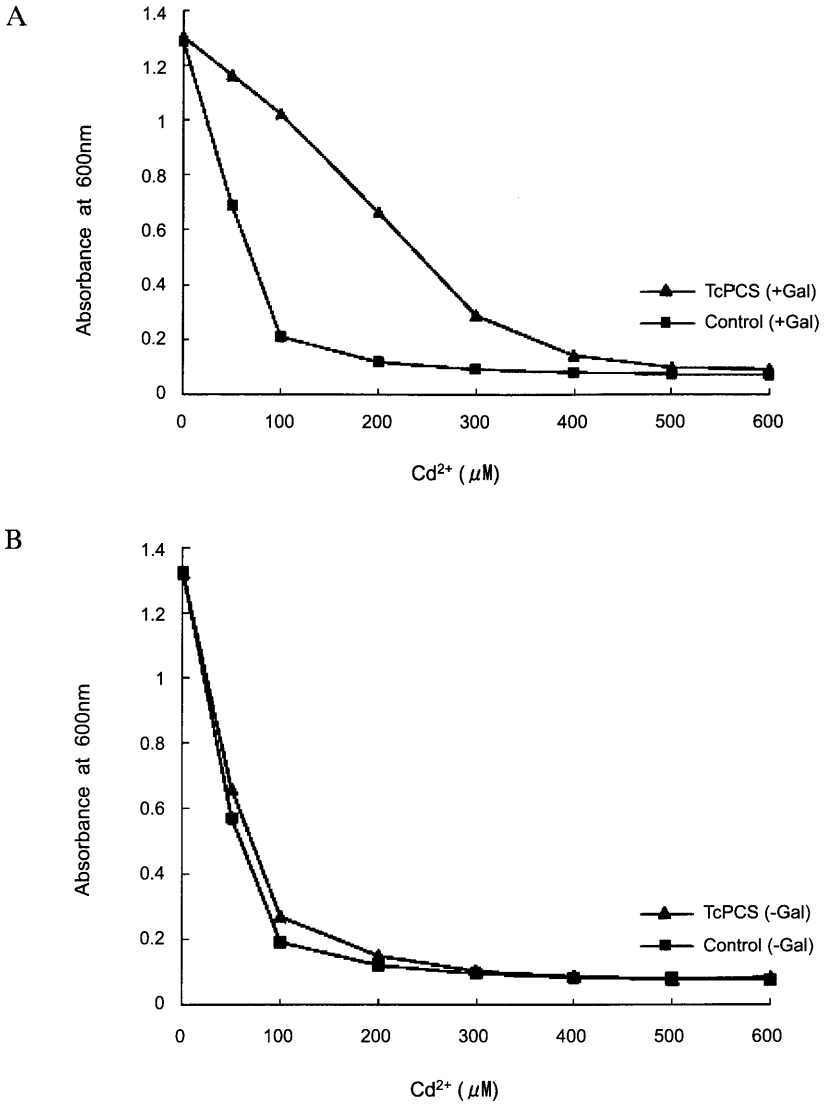

Fig. 5 Growth limit of *Tcpcs* expression yeast in high Cd^{2+} condition.
The yeast cells carrying the control vector (filled triangles) and pTjPCS-HA (filled squares) were grown at various Cd^{2+} concentrations. A: Cells were cultured in *Tcpcs* expressing condition with YNB-Ura^- supplied 1% galactose and 1% sucrose. The average OD_{600} after a 24-hr incubation (n=3) is shown. B: Cells were cultured without *Tcpcs* expression in YNB-Ura^- supplied 2% raffinose. The average values of OD_{600} after a 40-hr incubation (n=3) are shown.

the utilization of transgenic plants for actual phytoremediation applications, provided they are accompanied by appropriate risk assessment studies.

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