Expression of the Novel Wheat Gene \textit{TM20} Confers Enhanced Cadmium Tolerance to Bakers’ Yeast

Yu-Young Kim, Do-Young Kim, Donghwan Shim, Won-Yong Song, Joohyun Lee, Julian I. Schroeder, Sanguk Kim, Nava Moran, and Youngsook Lee

From the \textsuperscript{4}POSTECH-UZH Cooperative Laboratory, Division of Molecular Life Sciences, Pohang University of Science and Technology, Pohang, 790-784, Korea, \textsuperscript{5}Cell and Developmental Biology Section, Division of Biological Sciences, and Center of Molecular Genetics, University of California, San Diego, La Jolla, California 92093-0116, \textsuperscript{6}Structural Bioinformatics Laboratory, Division of Molecular Life Sciences, Pohang University of Science and Technology, Pohang, 790-784, Korea, \textsuperscript{7}Faculty of Agricultural, Food and Environmental Quality Sciences, Hebrew University of Jerusalem, Rehovot 76100, Israel, and \textsuperscript{8}Environmental Biotechnology National Core Research Center, Gyeongsang National University, Jinju 660-701, Korea

Cadmium causes the generation of reactive oxygen species, which in turn causes cell damage. We isolated a novel gene from a wheat root cDNA library, which conferred Cd(II)-specific tolerance when expressed in yeast (\textit{Saccharomyces cerevisiae}). The gene, which we called \textit{TaTM20}, for \textit{Triticum aestivum} transmembrane 20, encodes a putative hydrophobic polypeptide of 889 amino acids, containing 20 transmembrane domains arranged as a 5-fold internal repeating unit of 4 transmembrane domains each. Expression of \textit{TaTM20} in yeast cells stimulated Cd(II) efflux resulting in a decrease in the content of yeast intracellular cadmium. \textit{TaTM20}-induced Cd(II) tolerance was maintained in yeast even under conditions of reduced GSH. These results demonstrate that \textit{TaTM20} enhances Cd(II) tolerance in yeast through the stimulation of Cd(II) efflux from the cell, partially independent of GSH. Treatment of wheat seedlings with Cd(II) induced their expression of \textit{TaTM20}, decreasing subsequent root Cd(II) accumulation and suggesting a possible role for \textit{TaTM20} in Cd(II) tolerance in wheat.

Cadmium is one of the most dangerous heavy metals in the environment and poses a significant risk to human health (1). Cadmium induces oxidative stress, which in turn mediates cellular damage in many plants and animals (2, 3). One of the effects of oxidative stress is disruption of the permeability control of the plasma membrane, leading to membrane breakage and subsequent cell death (4). Humans and livestock can be exposed to cadmium via many different routes, but the predominant one is food intake. Agricultural products can become contaminated by cadmium when grown in cadmium-contaminated soils. In addition to farm products, cadmium exists in materials used in our daily lives, including paint and batteries.

Previous studies have shown that certain plant species are much more tolerant to cadmium than other plants (5, 6). Cadmium-tolerant plants have the ability to prevent the absorption of cadmium or detoxify cadmium after it has been absorbed. Plants secrete organic acids, such as oxalate, that precipitate cadmium in rhizospheres, preventing its absorption (7). Translocation of cadmium from roots to shoots is inhibited by the Casparian strip of the plant endodermal layer (8). Upon entry into the cell, plants employ diverse mechanisms of cadmium detoxification. Plants synthesize metal-chelating peptides, such as GSH and phytochelatins, or Cys-rich proteins, such as metallothioneins, that bind to Cd(II) and reduce its toxicity (9, 10).

Another mechanism of detoxification involves transport of cadmium or Cd(II)-conjugated molecules across the plasma membrane and tonoplast. Cd(II)-GSH complexes are compartmentalized into vacuoles by transporters localized in the tonoplast (11). AtMRP3, an ABC transporter, has been implicated in the sequestration of Cd(II)-GSH complexes in vacuoles (12, 13), and AtCAX2, an H\textsuperscript{+}/Cd\textsuperscript{2+} exchanger (14), mediates Cd(II) accumulation in vacuoles. The ABC transporter AtPDR8 is involved in Cd(II) efflux at the plasma membrane (15) and so is the plasma membrane multidrug efflux carrier AtDTX1 (16).

Antioxidants and antioxidant-synthesizing enzymes have also been implicated in enhancing plant tolerance to heavy metals, particularly Cd(II) and nickel. Heavy metals induce ROS\textsuperscript{5} generation and increase the expression of genes that encode the antioxidant-synthesizing enzymes (17–19). Expression of genes that encode enzymes involved in repairing the damage

\textsuperscript{5}The abbreviations used are: ROS, reactive oxygen species; AAS, atomic absorption spectroscopy; EV, empty vector; RT, reverse transcription; BSO, buthionine sulfoximine; WT, wild type; AChR, acetylcholine receptor; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

\textsuperscript{*}This work was supported, in whole or in part, by National Institutes of Health Grant P42ES010337 (NEHS) (to J. I. S.). This work was also supported by grants from the Global Research Program of the Ministry of Science and Technology, Korea (to Y. L. and N. M.), by MOST/KOSEF, Environmental Biotechnology National Core Research Center Grant R15-2003-012-02003-0, Korea (to Y. L.), and by Korea Research Foundation Grant KRF-2005-070-C00095 (to S. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{†}The on-line version of this article (available at http://www.jbc.org) contains supplemental Experimental Procedures, additional references, Table 1, and Figs. 1–4. The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\textsuperscript{TM}/EBI Data Bank with accession number(s) DQ323065.

\textsuperscript{‡}Present address: Carnegie Institution of Washington, 260 Panama St., Stanford, CA 94305.

\textsuperscript{¶}Present address: Institute of Plant Biology, University of Zurich, Zollikerstrasse 107, 8008 Zurich, Switzerland.

\textsuperscript{§}Present address: Dartmouth College, Biological Science Department, 6044 Gilman, Hanover, NH 03755.

\textsuperscript{‡**}To whom correspondence should be addressed. Fax: 82-54-279-2199; E-mail: ylee@postech.ac.kr.
caused by ROS improves Cd(II) tolerance. For example, over-expression of Arabidopsis aldehyde dehydrogenase, a scavenger of lipid peroxidation products, enhances tolerance to ROS-inducing stimuli, such as Cd(II), salt, and drought (20). In support of this mechanism of tolerance to heavy metals, many plants that are hyperaccumulators of nickel contain high antioxidant levels and high expression levels of the antioxidant-synthesizing enzymes (21).

In this study, we isolated a novel gene involved in Cd(II) detoxification from a wheat root cDNA library. The gene encodes a protein with a structure indicative of a transporter molecule, with 20 putative transmembrane domains, hence we named this gene TaTM20. TaTM20 enhanced export of Cd(II) from yeast cells, and it conferred Cd(II) tolerance to yeast. In wheat, Cd(II) pretreatment up-regulated TaTM20 expression, and this wheat accumulated subsequently less Cd(II) in the root than control, which was not pretreated with Cd(II). These results suggest a potential role for this protein in Cd(II) tolerance in planta.

EXPERIMENTAL PROCEDURES

Plant Growth Conditions—Seeds of wheat (Triticum aestivum Lcv. Atlas 66) were surface-sterilized, placed in the dark at 4 °C for 2 days, and then sown on ½ MS agar plates (22). The plants were grown under long day conditions (16 h light/8 h darkness) at 22/18 °C for 1 week.

Isolation of TaTM20 from a Wheat Root cDNA Library—To isolate Cd(II) tolerance genes, we used a wheat root cDNA library constructed using mRNA isolated from 4-day-old wheat roots grown in K-D-lacking medium (23), and we selected genes that functionally complement the YCFI-null, a Cd(II)-sensitive yeast strain DTY167 (MAT uRA3-52 leu2-3,-112 his-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ycf1::hisG; see Ref. 24). The genes in the cDNA library were introduced into the cells by the lithium acetate method (25), and transformants were plated on one-half synthetic galactose (1⁄2 SG) agar plates containing 60 μM CdCl2, and spot-forming transformants were selected and tested for Cd(II) tolerance. Plasmids DNA from Cd(II)-resistant transformants were isolated and sequenced, and then the full mRNA sequences of the genes were isolated using 5’-rapid amplification of cDNA ends PCR (Ambion Inc., Austin, TX). Full CDS-containing pYES2 (Invitrogen) plasmids were re-introduced into Δycf1 cells to confirm complementation by Cd(II) tolerance test.

Generation of N- or C-terminal Truncated TaTM20 Constructs and Complementation Tests in Yeast—All N- or C-terminal truncations of TaTM20 were generated by PCR amplification and restriction enzyme digestion. The fidelity of the DNA sequences of the constructs was verified by automated DNA sequencing (ABI 3100, PerkinElmer Life Sciences). The yeast Δycf1 and Δcup1 (MATα trp1-1, leu2-3, leu2-112, gal1, His-, ura3-50, cup1::ura3; see Ref. 26) strains and their isogenic wild type strains, DTY165 and DTY3, respectively, were transformed with the indicated constructs using the lithium acetate method. Transformants were selected on minimal medium plates lacking uracil (27). For the metal ion tolerance test, yeast containing the indicated constructs were grown on ½ SG agar plates in the absence or presence of 40 μM CdCl2, 1 mM Pb(NO3)2, 100 μM CuCl2, or 0.8 mM Na2HAsO4 at 30 °C for 3 days.

Cadmium Content Measurement and Cd(II) Flux Assay in Yeast—For measurement of cadmium content in yeast, cells were incubated in SG broth containing 20 μM CdCl2 for 5 h at 30 °C with shaking and then harvested. Cells were briefly rinsed with ice-cold 0.1 M CaCl2 and ice-cold water, collected by centrifugation, and completely digested with 11 M HNO3 at 200 °C. Digested samples were diluted with deionized water and briefly vortexed. The diluted samples were then analyzed for ion content using AAS (SpectrAA-800, Varian, Palo Alto, CA) or inductively coupled plasma emission spectroscopy (Optima 4300DU, PerkinElmer Life Sciences).

For the 109Cd(II) uptake assay, cells were incubated in SG medium supplemented with 10 μM CdCl2 containing 4.1 kBq liter1 of 109Cd(II) for 0, 15, 30, 60, 120, 180, or 240 min at 30 °C with shaking. At each time point, cells were harvested and rinsed twice with ice-cold 1 mM CaCl2. Radioactivity was measured using an automatic gamma counter (1470 Wizard, PerkinElmer Life Sciences). 109Cd levels were counted at the energy windows of 22–88 keV. For the efflux assay, cells were incubated in SG medium supplemented with 6 μM CdCl2 containing 4.1 kBq of 109Cd(II) for 1 h at 30 °C with shaking and then harvested and rinsed with ice-cold 1 mM CaCl2 and SG medium. Cells were resuspended in SG medium and incubated for 0, 5, 15, 30, or 60 min at 30 °C with shaking. At each time point, cells were harvested and rinsed, and then radioactivity was measured as described for the uptake assay.

Cd(II) Flux Assay in Wheat Seedlings Pretreated with Cd(II)—The pretreatment consisted of immersing the root parts of 7-day-old wheat seedlings into ½ MS liquid media supplemented with or without 100 μM CdCl2 for 6 h. They were then immersed into ½ MS liquid medium containing 2.4 kBq liter1 of 109Cd(II). After 3 h of 109Cd(II) exposure to roots, shoot and root parts of wheat seedlings were harvested separately and rinsed twice with ice-cold distilled water. Radioactivity was measured as described above.

Reverse Transcription (RT)-PCR and Real Time RT-PCR—Total RNA was extracted from 7-day-old wheat seedlings with TRizol reagents. Five micrograms of total RNA was subjected to cDNA synthesis using a PowerscriptTM RT-kit (Clontech) and oligo (dT) primers, according to the manufacturer’s instructions. TaTM20 cDNA was amplified by PCR using the following primer pair: TaTM20 RTF (5’-AAGGTTTGCTCCTTCTTGATCTGTGTTG3’) and TaTM20 RTR (5’-GTATAGGGCCAGCACCAGTTAGGGTTG3’). As a control, glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was amplified in wheat seedlings (primers: TaG3PDHF, 5’-CAACGCTAGCCTGCAGGCTAACT-3’; TaG3PDHR, 5’-ACTCCTCTTTGATAGCAGCCTT-3’), together with TaTM20. Real time PCR was performed in triplicate using SYBER Green Master Mix (Applied Biosystems, Foster City, CA). The sequences of the primers designed to amplify TaTM20 were as follows: 5’-CCGATCTTCCTTGACAAACT-3’ and 5’-ATGGGACAGCATGAAGCTC-3’; for G3PDH were as follows: 5’-CTACCCACCGAGTACATGACC-3’ and 5’-TCGTCCTTGAGCTTGTGAT-3’. Real time PCR analysis was...
performed on an Applied Biosystems Prism 7900 sequence detection system (Applied Biosystems).

RESULTS

Isolation of a Cd(II) Tolerance Gene, TaTM20, from a Wheat Root cDNA Library—To screen for Cd(II) tolerance genes, a wheat root cDNA library (23) was introduced into the YCF1-null mutant yeast strain DTY167 (Δycf1). We identified two novel genes that conferred strong Cd(II) tolerance to Δycf1 mutant yeast, and we selected one of these genes for further characterization. We called it TaTM20, because it encoded a putative wheat protein with 20 transmembrane domains.

To confirm the role of TaTM20 in Cd(II) tolerance, Δycf1 cells were transformed with pYES2-TaTM20 and plated on 1/2 SG agar medium supplemented with 40 μM CdCl₂ for qualitative assessment, or grown in suspension cultures for quantitative assays. Δycf1 cells that expressed TaTM20 grew much better than those transformed with the control empty vector (EV) (Fig. 1A). They also grew better than wild type (WT) yeast transformed with EV (WT-EV, Fig. 1A). To characterize the Cd(II) tolerance phenotype of TaTM20-expressing yeast, cultures were grown in liquid SG media containing various concentrations of CdCl₂ until they reached stationary phase (56 h), and cell suspension absorbance at 600 nm (A₆₀₀) was measured using a spectrophotometer. In medium lacking Cd(II), the growth of TaTM20-transformed Δycf1 cells was similar to WT-EV cells or EV-transformed Δycf1 cells (Fig. 1B). In the presence of 40, 50, and 60 μM Cd(II), cell density decreased in a concentration-dependent manner. TaTM20-expressing Δycf1 Na₂HAsO₄ or 1 mM Pb(NO₃)₂. EV-transformed cells did not grow as well as wild type yeast on 1/2 SG media containing As(V) or Pb(II) (supplemental Fig. 1A), which was in agreement with previously published results (24, 28, 29). TaTM20 expression did not alter the sensitivity of Δycf1 cells to As(V) and Pb(II) (supplemental Fig. 1A). To determine whether TaTM20 conferred copper tolerance, the Δcup1 (copper metallothionein)-null mutant yeast strain DTY4 was transformed with pYES2-TaTM20, and cells were spotted onto 1/2 SG agar medium. EV-transformed Δcup1 cells grew less than WT-EV yeast on 100 μM CuCl₂-containing media (supplemental Fig. 1B), as expected based on previously published results (26). Expression of TaTM20 did not alter the growth of Δcup1 cells on Cu(II)-containing medium (supplemental Fig. 1B), indicating that TaTM20 does not play a role in tolerance to excess Cu(II). These results demonstrated that TaTM20 confers tolerance to Cd(II) but does not affect the tolerance of yeast cells to As(V), Pb(II), or Cu(II).

TaTM20 Sequence Analysis and Prediction of TaTM20 Structure—The cDNA sequence and predicted amino acid sequence of TaTM20 are shown in Fig. 2A. TaTM20 encodes a novel gene (GenBank™ accession number DQ523065), based on examination of expressed sequence tags (ESTs) and cDNA sequences deposited in the GenBank™ data base. We screened the TIGR data base for homologues of TaTM20 in rice, and found that at the amino acid level TaTM20 has 24% identity and 33% similarity with ZmTM20 (dek34 embryogenesis protein) from Zea mays (30), and 26–50% identity and 36–60% similarity with 20 rice homologues. Phylogenetic tree shows that
TaTM20 is a member of a gene family with many members exclusively from monocotyledonous plants (supplemental Fig. 2). No member of this gene family has yet been shown to be involved in heavy metal tolerance.

The open reading frame of TaTM20 was 2,670 bp in length and encoded a hydrophobic polypeptide of 889 amino acids, which contained 20 putative transmembrane domains (colored in Fig. 2A). The topology of TaTM20 was predicted using a web-based program, the Kyte-Doolittle hydrophobicity scale of TopPred. Based on the predicted structure, the 20 transmembrane domains of TaTM20 had several interesting structural features, including a 5-fold tandem repeat units of four transmembrane domains each, and consensus motifs for protein kinase C phosphorylation (Fig. 2B). The five repeat units are highly homologous in nucleotide and amino acid sequence (Fig. 2C), suggesting that they arose by gene duplication events, followed by variation (31). The repeat units and the 5-fold structural symmetry of TaTM20 are similar to the nicotinic acetylcholine receptor (AChR), which has five symmetrical subunits that form a ring structure in the membrane around a central ion-conducting pore (32). Unlike TaTM20, however, which encodes a single polypeptide with five symmetric units and 20 transmembrane domains, the subunits of the nicotinic AChR are encoded by different genes. Recent data from membrane protein crystal structures indicated that repeat units and structural symmetry play a major role in protein function (33, 34).

Therefore, we investigated whether all of the five tandem repeat units of TaTM20 were essential for its ability to confer Cd(II) tolerance.

![FIGURE 2. Analysis of the sequence of TaTM20. A, nucleotide and deduced amino acid sequences of TaTM20. The putative transmembrane domains are in colored boxes; the stop codon is marked with an asterisk, and the ending points of the constructs described for Fig. 3A are indicated by arrows. Transmembrane domains in the same color have a similar amino acid sequence. B, schematic representation of the hypothetical structure of TaTM20. Note the 5-fold repeat of similar sequences, forming five transmembrane units. Circled P represents the consensus sequence for phosphorylation by protein kinase C. C, similarity assessment of the five repeated transmembrane units in the TaTM20 open reading frame using RADAR. Colored bars represent predicted transmembrane domains. Asterisk represents identical amino acids. Colon and dot represent strongly and weakly conserved amino acids, respectively, with respect to the higher order structure of proteins.]

N- or C-terminal Deletion Mutants of TaTM20 Fail to Confer Cd(II) Tolerance to Yeast—We examined whether truncated TaTM20 were sufficient to confer Cd(II) tolerance, similar to full-length TaTM20. Several TaTM20 constructs were generated, in which one or more of the repeat units were deleted, abolishing the Cd(II) tolerance property of full-length TaTM20. The open reading frame of TaTM20 was 2,670 bp in length and encoded a hydrophobic polypeptide of 889 amino acids, which contained 20 putative transmembrane domains (colored in Fig. 2A). The topology of TaTM20 was predicted using a web-based program, the Kyte-Doolittle hydrophobicity scale of TopPred. Based on the predicted structure, the 20 transmembrane domains of TaTM20 had several interesting structural features, including a 5-fold tandem repeat units of four transmembrane domains each, and consensus motifs for protein kinase C phosphorylation (Fig. 2B). The five repeat units are highly homologous in nucleotide and amino acid sequence (Fig. 2C), suggesting that they arose by gene duplication events, followed by variation (31). The repeat units and the 5-fold structural symmetry of TaTM20 are similar to the nicotinic acetylcholine receptor (AChR), which has five symmetrical subunits that form a ring structure in the membrane around a central ion-conducting pore (32). Unlike TaTM20, however, which encodes a single polypeptide with five symmetric units and 20 transmembrane domains, the subunits of the nicotinic AChR are encoded by different genes. Recent data from membrane protein crystal structures indicated that repeat units and structural symmetry play a major role in protein function (33, 34).

Therefore, we investigated whether all of the five tandem repeat units of TaTM20 were essential for its ability to confer Cd(II) tolerance.
the potential 5-fold symmetry of the protein (Fig. 2A, arrows, and Fig. 3A). To examine whether a single repeat unit was functional, we generated Δ721–2667, which expressed only the first repeating unit (the first four transmembrane domains of TaTM20). When we introduced this construct into Δycf1 cells and spotted colonies on medium supplemented with 40 μM CdCl2, we found that their growth was similar to EV-transformed yeast expressing each construct on a 1/5 SG agar plate containing 40 μM CdCl2. From top to bottom, the constructs are as follows: positive control (WT yeast, EV-transformed), negative control (Δycf1 yeast, EV-transformed), indicated truncated constructs, and full-length TM20. The plate was incubated at 30 °C for 3 days. Percentage (%) represents the relative size of TaTM20, compared with the full-length open reading frame. B, RT-PCR analysis of expression of truncated TaTM20 constructs in yeast.

Therefore, the TaTM20-mediated decrease in cadmium content in yeast did not appear to be due to alterations in the homeostasis of other metal ions.

**Cadmium Content Is Specifically Reduced in TaTM20-transgenic Yeast**—To determine the mechanism of Cd(II) tolerance by TaTM20 in yeast, we measured intracellular cadmium content in EV- and TaTM20-transformed Δycf1 cells cultured in liquid SG medium containing 20 μM CdCl2, using AAS. TaTM20-expressing yeast accumulated less cadmium than EV-transformed yeast (Fig. 4A). This result suggests that expression of TaTM20 enhances Cd(II) tolerance in yeast by decreasing intracellular cadmium content.

To examine whether the reduction of cadmium content upon expression of TaTM20 was coupled to the regulation of essential metal ions, yeast strains were cultured in liquid SG medium without CdCl2 or supplemented with 20 μM CdCl2, and then calcium, copper, iron, potassium, magnesium, manganese, sodium, and zinc content, as well as cadmium content, were measured using inductively coupled plasma emission spectroscopy. With the exception of cadmium and regardless of the presence or absence of supplemented Cd(II), there was no significant difference in the ion content of any of the ions examined between TaTM20- and EV-expressing cells (supplemental Fig. 3).

**TaTM20-expressing Cells Remove Cd(II) More Rapidly Than EV-transformed Cells**—To understand the mechanism of TaTM20-induced reduction of cadmium content, we monitored time-dependent changes in Cd(II) content in Δycf1 cells using the radioactive isotope 109Cd(II). Yeast strains were cultured in liquid SG medium containing 109Cd(II), and 109Cd(II) activity in the cells was measured using a gamma counter. Two hours after the onset of 109Cd(II) uptake, it was apparent that TaTM20-expressing Δycf1 cells had accumulated less Cd(II) than the same strain of yeast transformed with EV. After 4 h of exposure to Cd(II), Cd(II) content in TaTM20-expressing Δycf1 cells was 80% that in EV-transformed cells (Fig. 4B). This result was consistent with the observed decrease in cadmium content in TaTM20-expressing cells measured using AAS (Fig. 4A).

To determine whether TaTM20 enhanced Cd(II) efflux, we monitored the kinetics of Cd(II) efflux from the two strains of yeast. Cells were cultured in 109Cd(II)-containing liquid SG-
medium for 1 h, washed, then transferred to SG medium without Cd(II), and 109Cd activity was monitored during the following 1 h. 109Cd activity decreased more rapidly in TaTM20-expressing Δycf1 cells compared with EV-transformed cells (Fig. 4C). Thirty minutes after the onset of the efflux phase, this difference was significant (p < 0.001). At this time point, TaTM20-expressing cells exported 37% of the total amount of Cd(II) loaded, whereas EV-transformed cells removed only 16%. Thus TaTM20 expression enhanced the export of Cd(II) out of cells, causing the decrease in Cd(II) content.

TaTM20 Improves Cd(II) Tolerance of Δycf1 Cells under Reduced GSH Conditions—To investigate whether TaTM20-mediated Cd(II) efflux required GSH, we examined the growth of yeast on 1/2 SG plates containing Cd(II) and buthionine sulfoximine (BSO). BSO effectively blocks the synthesis of GSH by inhibiting γ-glutamylcysteine synthetase (γ-ECS) in yeast cells and renders them sensitive to oxidative- or heavy metal-induced stress (35). We spotted TaTM20 or EV transformants of Δycf1 together with WT-EV yeast as a control on plates containing 35 μM CdCl2 and/or 1.5 mM BSO. BSO alone did not alter the growth of the cells (Fig. 5A, 1st and 2nd lanes). In medium containing 35 μM Cd(II), TaTM20-expressing yeast clearly showed improved growth compared with Δycf1 cells
(Fig. 5A, 3rd panel). In medium containing BSO in addition to Cd(II), the growth of all three yeast strains was reduced further compared with growth on medium without BSO, possibly because of an increased sensitivity to Cd(II)-induced oxidative stress. However, even with GSH levels reduced by BSO, TaTM20-transgenic Δycf1 mutants still maintained a better tolerance to Cd(II) than EV-transformed Δycf1 or WT-EV cells (Fig. 5A, 4th panel). Under these conditions, GSH levels were reduced by BSO, as evidenced by the similar growth pattern of the two strains of yeast, Δycf1-EV and WT-EV, that differ in expression of YCF1, a GSH-dependent Cd(II) tolerance gene (Fig. 5A, 1st and 2nd lanes of the 4th panel).

To quantify the GSH dependence of Cd(II) tolerance by TaTM20, we examined the growth of WT-EV, EV-transformed, and TaTM20-expressing Δycf1 mutant cells in liquid SG media in the absence and presence of BSO and Cd(II). 2 mM of BSO did not change the growth of the cells in SG medium, and the three strains grew similarly (Fig. 5B, top panel). When 35 μM Cd(II) was added to the medium without BSO, the three strains grew at different rates; WT-EV faster than Δycf1-EV, and TaTM20-expressing Δycf1 the fastest of all (Fig. 5B, middle panel). When Cd(II) was added to the medium in addition to BSO at a concentration of 35 μM (Fig. 5B, bottom panel) or 40 μM (data not shown), TaTM20-expressing Δycf1 mutants also grew better than the other two yeast strains, but the growth difference between WT-EV and Δycf1–EV cells (Fig. 5B, middle panel) was abolished (Fig. 5B, bottom panel). Thus, the mechanism of tolerance conferred by TaTM20 seems to differ from that of YCF1; YCF1-mediated Cd(II) tolerance depends directly on GSH but TaTM20-mediated Cd(II) tolerance depends on GSH to a lesser extent, possibly including a decrease in oxidative stress.

Expression of TaTM20 Increases in Response to Cd(II) in Wheat Seedlings—To investigate whether TaTM20 functions in Cd(II) tolerance in wheat, we examined Cd(II)-induced changes in TaTM20 expression. Wheat seeds were germinated on 1/2 MS agar medium and grown for 7 days. They were transferred to 1/2 MS liquid medium for 12 h, and then exposed to 1/2 MS medium containing 100 μM CdCl2 for 6 h. The expression of TaTM20 was analyzed in shoots and roots by RT-PCR and quantified by real time RT-PCR. TaTM20 was expressed both in shoots and roots under control conditions, with higher expression levels in shoots than in roots (Fig. 6A). Treatment of seedlings with 100 μM CdCl2 resulted in an increase in TaTM20 expression, both in the shoots and roots (Fig. 6A). Transcript levels increased by Cd(II) more than 2- and 6-fold in the shoots and roots, respectively (Fig. 6B), suggesting a potential function of TaTM20 in the Cd(II) response in wheat.

Cd(II)-pretreated Wheat Seedlings Accumulate Less Cd(II) in Roots—To obtain a clue to the in planta function of TaTM20 in relation to Cd(II) tolerance, we elevated the expression level of TaTM20 in wheat seedlings using Cd(II) pretreatment, and we then compared their uptake of radioactive Cd(II) with those of control non-pretreated ones. Root part of wheat seedlings was pretreated with or without 100 μM CdCl2 for 6 h and then transferred to a medium containing 109Cd(II). After 3 h of exposure of the root to 109Cd(II), 109Cd activity in the shoot and root was measured. Shoot 109Cd activity in the shoot and root did not differ between the pretreated and control groups of plants and were less than 8% of those in the root (data not shown). This result is consistent with previous findings that reported that only a small portion of heavy metals in the root is translocated to the shoot (36, 37). In the root, 109Cd(II) content of Cd(II)-pretreated wheat was 75% that in control without pretreatment (Fig. 7). This diminished Cd(II) content in Cd(II)-pretreated seedling roots correlates.
well with the relatively high TaTM20 induction in the root by Cd(II) pretreatment (Fig. 6), suggesting that TaTM20 may decrease Cd(II) accumulation in wheat roots.

**DISCUSSION**

In this study, we identified and characterized a novel Cd(II) tolerance protein, TaTM20, that enhances Cd(II) export at the cell membrane. We isolated TaTM20 by screening a wheat cDNA library (23, 38) for functional complementation of the yeast Dyepl mutant (Fig. 1). TaTM20 encodes a transmembrane protein of 889 amino acids (98 kDa), which contains 20 putative membrane-spanning domains (Fig. 2). TaTM20-expressing yeast accumulated less cadmium and exported Cd(II) from the cell more rapidly than EV-transformed control cells (Fig. 4). In wheat, TaTM20 was expressed mainly in shoots and was strongly induced in roots by Cd(II) (Fig. 6). Moreover, TaTM20-induced wheat accumulated less Cd(II) in roots than control uninduced wheat seedlings (Fig. 7). These results suggest that TaTM20 may enhance export of Cd(II) from the wheat root cell, thereby decreasing cadmium content and mediating Cd(II) tolerance in planta.

The mechanisms of heavy metal tolerance have been studied primarily in Arabidopsis and in heavy metal hyperaccumulators, such as Thlaspi caerulescens and Arabidopsis halleri. To date, there have been only several reports of toxic heavy metal tolerance in monocots. They include reports on organic acid secretion and consequent immobilization of Pb(II) by tolerant rice varieties (39), isolation of the phytochelatin synthase gene from wheat (40), induction of OsPDR9 expression by Cd(II) and Zn(II) in the rice root (41), and hypersensitivity to Cu(II), Zn(II), and Pb(II) of rice mutant plants that are deficient in expression of OsHMA9 (42). TaTM20 is a new transporter protein of monocot that can contribute to toxic heavy metal tolerance. Although we were not able to obtain TM20 mutant wheat, a recent physiological study on wheat describes the effects of pretreatment with Cd(II), which could be interpreted as supporting our findings. Protoplasts isolated from wheat seedling grown on Cd(II)-containing media accumulated less Cd(II) than protoplasts from plants grown in the absence of Cd(II) (43). We observed a similar result in whole seedlings pretreated with Cd(II) (Fig. 7); wheat seedlings pretreated with cold Cd(II) took up less radioactive cadmium than nontreated control. At least one interpretation could be an up-regulation of a cadmium export system in the plasma membrane of these wheat protoplasts, and wheat seedlings. TaTM20 could be a component of this Cd(II) export system induced in wheat exposed to Cd(II), because TaTM20 transcript level was elevated 6-fold in roots by Cd(II) treatment (Fig. 6B). Reduced radioactive Cd(II) activity in Cd(II)-pretreated wheat compared with untreated control (Fig. 7) may reflect, at least in part, the function of TaTM20 in reducing cadmium uptake. TaTM20 may provide an efficient cadmium tolerance mechanism to wheat because it is highly up-regulated in a relatively short time in response to Cd(II) exposure (Fig. 6) and reduces cadmium uptake into the root (Fig. 7). Protection of roots from the heavy metal toxicity is important, because it could reduce heavy metal accumulation in the shoot during long term exposure, and root growth is important for growth of the whole plant.

**Homologues** of TaTM20 exist in Oryza sativa and Z. mays (30, 44), but not in Arabidopsis, which indicates that this family of proteins is unique to monocotyledonous plants. Ectopic expression of TaTM20 in Arabidopsis had little effect on the tolerance to Cd(II) or accumulation of Cd(II) (data not shown). Interestingly, Cd(II) pretreatment of wild type Arabidopsis actually induced higher Cd(II) net influx in a 109Cd(II) uptake assay (45). Therefore, the mechanisms of Cd(II) tolerance mediated by TM20 may be unique to monocotyledonous plants, unlike those mediated by metallothionein or phytochelatin synthase, which are common to both monocots and dicots (44, 46–48). Only one protein of the TM20 family, ZmTM20 in Z. mays, has been characterized to date. ZmTM20 is expressed during embryogenesis (30) and was shown to transport auxin when expressed in Xenopus oocytes (44). However, transport of auxin by ZmTM20 has not been demonstrated in planta. For further investigation on the roles of TaTM20 in wheat, generation of mutant plants using techniques such as RNA interference would be helpful.

Although it is not highly homologous in amino acid sequence to TaTM20, the AChR channel protein bears a striking degree of similarity in structure to TaTM20. The AChR channel has a pentameric ring structure consisting of five subunits with a pore in its center, through which small cations, such as K⁺, Na⁺, and Ca²⁺, pass from one side of the plasma membrane to the other. Based on structural analogy, TaTM20 may be involved in transporting cadmium ions through a central pore region. Full-length TaTM20 is required for Cd(II) tolerance in yeast, as N- or C-terminal deletions of the repeat units abolished the protective effect of the protein (Fig. 3). The first repeat unit of TM20 also failed to enhance Cd(II) tolerance in Cd(II)-sensitive yeast (Fig. 3). This is in contrast to the M2 transmembrane segment of the δ subunit of the AChR, which forms pentamers and transports cations when expressed alone (49). These results indicate that the structural integrity of TaTM20 is essential for its function in Cd(II) tolerance, and suggest that the intact pentameric ring structure is required for its function.

Based on the predicted structure of TaTM20 and the reduced cadmium content in TaTM20-expressing yeast (Fig. 4), we hypothesized that TaTM20 may function as a transporter. Previous literature suggests that expression of an essential ion transporter can alter transport of Cd(II) indirectly. Cd(II) uptake into rice roots is inhibited by exogenously supplied essential divalent ions such as Ca²⁺ and Mg²⁺ (37). The deficiency of another essential heavy metal, iron, causes cadmium accumulation in maize (50). Therefore, we examined whether the content of any essential ion was altered by expression of TaTM20 in yeast cells. None of the ions assayed (calcium, potassium, sodium, copper, magnesium, zinc, iron, and manganese) were affected in TaTM20-expressing yeast compared with EV-transformed yeast (supplemental Fig. 3). Therefore, it does not seem likely that TaTM20 transports one or more of the essential divalent metal ions that would inhibit cadmium entry into the cell by competition. The specific alteration of cadmium content is in line with the specific effect of TaTM20 on tolerance to Cd(II) but not to As(V), Pb(II), or Cu(II) (supplemental Fig. 1). These results suggest that TaTM20 confers a tolerance...
to cadmium via a mechanism that reduces cadmium accumulation and that TaTM20 expression does not affect the transport and homeostasis of As(V), Pb(II), or Cu(II). We also examined whether TaTM20 decreased cadmium content by improving Cd(II) efflux from the cell. If the decreased cadmium content was because of inhibition of Cd(II) uptake from the media by TaTM20, the rate of Cd(II) release from TaTM20-expressing and EV-transformed yeast would be similar. However, the rate of Cd(II) release was more rapid in TaTM20-expressing cells compared with EV-transformed cells (Fig. 4C). Moreover, the difference between the two strains of yeast became apparent much earlier during efflux than during uptake, suggesting that the difference observed in the uptake of Cd(II) was an indirect effect, originated from the difference in the rate of Cd(II) release. These results suggest that TaTM20 enhances Cd(II) efflux at the plasma membrane. To enhance the export of Cd(II) from the cytosol to the extracellular environment, the protein should be located at the plasma membrane. We found that TaTM20:GFP, expressed in the Arabidopsis protoplast, is co-localized with RFP:AtAHA2, a plasma membrane marker protein, which suggests that TaTM20 localizes to the plasma membrane (supplemental Fig. 4). In addition, the efflux of Cd(II) was practically abolished when extracellular pH was elevated to 8.0 (supplemental Table 1), suggesting the potential importance of the pH gradient across the plasma membrane for the efflux. Based on these results, we suggest that TaTM20 functions at the plasma membrane enhancing Cd(II) export from the cell.

Although it may resemble AChR in pore arrangement, TaTM20 cannot be a Cd²⁺ channel because it transports Cd(II) against Cd(II) electrochemical gradient. A potential energy source for its transport could be protonmotiv force, i.e. TaTM20 could be a H⁺-coupled transporter similar in function to AtCAX2 and AtCAX4, H⁺/Cd²⁺ exchangers, which mediate Cd(II) exclusion from the cytoplasm (albeit, into the vacuole) using the protonmotive force (14, 51). H⁺-dependent mechanisms of Cd(II) extrusion exist also in the microbial cells. Bacterial H⁺/X antiport mediating Cd(II) efflux is also a part of toxic metal resistance mechanism. This includes three polyepitope RND chemi-osmotic complexes consisting of an inner membrane pump, a periplasmic-bridging protein, and an outer membrane channel. The best studied among these is the Czc system (Cd²⁺, Zn²⁺, and Co²⁺) (52) and ZitB, a member of the cation diffusion facilitator family that mediates obligatory H⁺ antiport efflux of cadmium and zinc across the plasma membrane of Escherichia coli (53). Another explanation for the pH effect, valid in case where there is very little leak of other ions across the plasma membrane to accompany the extruded Cd²⁺, is that a back-leak of protons into the cell cancels the extra-negative charges remaining behind. In the absence of this proton leak, negative charge accumulation eventually impedes the extrusion of Cd²⁺. Future experiments will be aimed in distinguishing between these two possibilities.

The Cd(II)-GSH complex, a well characterized substrate of Cd(II)-detoxifying transporters (54), is most likely not the substrate of TaTM20, because TaTM20 conferred Cd(II) tolerance to yeast in the presence of BSO (Fig. 5), an effective GSH synthesis inhibitor in yeast (35). Under these conditions, YCF1-mediated Cd(II) tolerance was abolished, indicating that intracellular GSH levels were indeed reduced by BSO (Fig. 5). It is not surprising that BSO inhibited the overall growth of the cells in the presence of Cd(II). GSH is an important antioxidant against ROS, and under conditions of reduced GSH, cells are not fully protected against cadmium-induced ROS. It remains to be determined whether Cd(II) is exported in free ionic form in some other conjugated forms.

In conclusion, we have shown that TaTM20 is a novel gene that contributes to Cd(II) tolerance when overexpressed in Δycf1 mutant yeast. To our knowledge, this is the first report of a transporter from any organism, with tandem 5-fold repeat units structure that extrudes Cd(II) and confers Cd(II) tolerance on yeast. TaTM20 may have other important functions in wheat. However, because in wheat roots the transcription of TaTM20 was strongly induced upon Cd(II) treatment (Fig. 6) and Cd(II) accumulation decreased after pre-exposure to Cd(II) (Fig. 7), we suggest that TaTM20 functions in Cd(II) detoxification in planta.

Acknowledgments—We thank Dr. Dennis Thiele for the kind donation of the mutant yeast lines and Dr. Sun-Hee Leem for modification of DTY4 strain of yeast. We thank Dr. Sichul Lee for help with in silico search. We also thank Aekyung Han for management of the seedlings. Inductively coupled plasma measurement of ions was conducted at the Korea Basic Science Institute in Daejeon, Korea.

REFERENCES

1. Jarup, L., Berglund, M., Elinder, C. G., Nordberg, G., and Vahter, M. (1998) Scand. J. Work Environ. Health 24, 1–51
2. Hart, B. A., Lee, C. H., Shukla, G. S., Shukla, A., Osier, M., Eneman, J. D., and Chiu, J. F. (1999) Toxicology 133, 43–58
3. Sandalio, L. M., Dalurzo, H. C., Gomez, M., Romero-Puertas, M. C., and del Rio, L. A. (2001) J. Exp. Bot. 52, 2115–2126
4. Kolzumi, T., Shirakura, H., Kumagai, H., Tatsumoto, H., and Suzuki, K. T. (1996) Toxicology 114, 125–134
5. Boominathan, R., and Doran, P. M. (2003) Biotechnol. Bioeng. 83, 158–167
6. Kupper, H., Lombi, E., Zhao, F. I., and McGrath, S. P. (2000) Plant Cell 12, 75–84
7. Nigam, R., Srivastava, S., Prakash, S., and Srivastava, M. M. (2001) Plant Soil 230, 107–113
8. Lux, A., Sottnikova, A., Opatrna, J., and Greger, M. (2004) Physiol. Plant. 120, 537–545
9. Palmiter, R. D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 428–430
10. Raineri, A., Castagna, A., Scabba, F., Careri, M., Zagnoni, I., Predieri, G., Pagliari, M., and di Toppo, L. S. (2005) Plant Physiol. Biochem. 43, 45–54
11. Petrovic, S., Pascolo, L., Gallo, R., Cupelli, F., Ostrow, J. D., Goffeau, A., Tiribelli, C., and Bruschi, C. V. (2000) Yeast 16, 561–571
12. Rea, P. A., Li, Z. S., Lu, Y. P., Drozdowicz, Y. M., and Martinoia, E. (1998) Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 727–760
13. Tommasini, R., Vogt, E., Fromenteau, M., Hortenstein, S., Matile, P., Arnheim, N., and Martinoia, E. (1998) Plant J. 13, 773–780
14. Hirschi, K. D., Korenenk, V. D., Wilganowski, N. L., and Wagner, G. J. (2000) Plant Physiol. 124, 125–133
15. Kim, D.-Y., Bovet, L., Maeslima, M., Martinsena, E., and Lee, Y. (2007) Plant J. 50, 207–218
16. Li, L., He, Z., Pandey, G. K., Tsuchiya, T., and Luan, S. (2002) J. Biol. Chem. 277, 5360–5368
17. Dixit, V., Pandey, V., and Shyam, R. (2001) J. Exp. Bot. 52, 1101–1109
18. Hsu, Y. T., and Kao, C. H. (2004) Plant Growth Regul. 42, 227–238
19. Vitoria, A. P., Lea, P. J., and Azavedo, R. A. (2001) Phytochemistry 57, 701–710
20. Sunkar, R., Bartels, D., and Kirch, H. H. (2003) Plant J. 35, 452–464
21. Freeman, J. L., Persans, M. W., Nieman, K., Albrecht, C., Peer, W., Pickering, I. J., and Salt, D. E. (2004) *Plant Cell* **16**, 2176–2191
22. Murashige, T., and Skoog, F. (1962) *Physiol. Plant.* **15**, 473–497
23. Schachtman, D. P., and Schroeder, J. I. (1994) *Nature* **265**, 655–658
24. Szczypka, M. S., Wemmie, J. A., Moye-Rowley, W. S., and Thiel, D. J. (1994) *J. Biol. Chem.* **269**, 22853–22857
25. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) *J. Bacteriol.* **153**, 163–168
26. Hamer, D. H., Thiele, D. J., and Lemontt, J. E. (1985) *Science* **228**, 685–690
27. Covello, P. S., and Reed, D. W. (1996) *Plant Physiol.* **111**, 223–226
28. Preveral, S., Ansoborlo, E., Mari, S., Vavasseur, A., and Forestier, C. (2006) *Biochimie (Paris)* **19**, 1651–1663
29. Song, W. Y., Sohn, E. J., Martinova, E., Lee, Y. J., Yang, Y. Y., Jasinski, M., Forestier, C., Hwang, I., and Lee, Y. (2003) *Nat. Biotechnol.* **21**, 914–919
30. Shimizu, T., Mitsuke, H., Noto, K., and Arai, M. (2004) *J. Mol. Biol.* **339**, 1–15
31. Miyazawa, A., Fujiyoshi, Y., and Unwin, N. (2003) *Nature* **423**, 949–955
32. Hunte, C., Screpanti, E., Venturi, M., Rimon, A., Padan, E., and Michel, H. (2005) *Nature* **435**, 1197–1202
33. Yamashita, A., Singh, S. K., Kawate, T., Jin, Y., and Gouaux, E. (2005) *Nature* **437**, 215–223
34. Mehdi, K., and Penninckx, M. J. (1997) *Microbiology* **143**, 1885–1889
35. Eun, S. O., Youn, H. S., and Lee, Y. (2000) *Physiol. Plant.* **110**, 357–365
36. Kim, Y. Y., Yang, Y. Y., and Lee, Y. (2002) *Physiol. Plant.* **116**, 368–372
37. Clemens, S., Antosiewicz, D. M., Ward, J. M., Schachtman, D. P., and Schroeder, J. I. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 12043–12048
38. Yang, Y. Y., Jung, J. Y., Song, W. Y., Suh, H. S., and Lee, Y. (2000) *Plant Physiol.* **124**, 1019–1026
39. Clemens, S., Kim, E. J., Neumann, D., and Schroeder, J. I. (1999) *EMBO J.* **18**, 3325–3333
40. Moons, A. (2003) *FEBS Lett.* **23**, 370–376
41. Lee, S., Kim, Y. Y., Lee, Y., and An, G. (2007) *Plant Physiol.* **145**, 831–842
42. Lindberg, S., Landberg, T., and Greger, M. (2007) *Plant Physiol. Biochem.* **45**, 47–53
43. Jahrmann, T., Bastida, M., Pineda, M., Gasol, E., Ludevid, M. D., Palacin, M., and Puigdomenech, P. (2005) *Planta* **222**, 80–90
44. Larsson, E. H., Asp, H., and Bornman, J. F. (2002) *J. Exp. Bot.* **53**, 447–453
45. Ha, S. B., Smith, A. P., Howden, R., Dietrich, W. M., Bugg, S., O’Connell, M. J., Goldbrough, P. B., and Cobbett, C. S. (1999) *Plant Cell* **11**, 1153–1164
46. Howden, R., Andersen, C. R., Goldsbrough, P. B., and Cobbett, C. S. (1995) *Plant Physiol.* **107**, 1067–1073
47. VatamanuIuk, O. K., Mari, S., Lu, Y. P., and Rea, P. A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **8**, 7110–7115
48. Oiki, S., Danho, W., Madison, V., and Montal, M. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 8703–8707
49. Meda, A. R., Scheuermann, E. B., Prechsl, U., Erenoglu, B., Schaaf, G., Hayen, H., Weber, G., and von Wiren, N. (2007) *Plant Physiol.* **143**, 1761–1773
50. Koren’kov, V., Park, S., Chen, N. H., Sreevidya, C., Lachmansingh, J., Morris, J., Hirschi, K., and Wagner, G. J. (2007) *Planta* **225**, 405–411
51. Silver, S., and Phung, L. (2005) *J. Ind. Microbiol. Biotechnol.* **32**, 587–605
52. Chao, Y., and Fu, D. (2004) *J. Biol. Chem.* **279**, 12043–12050
53. Li, Z. S., Szczypka, M., Lu, Y. P., Thiele, D. J., and Rea, P. A. (1996) *J. Biol. Chem.* **271**, 6509–6517