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Identification of the Cadmium-Inducible *Hansenula polymorpha SEO1* Gene Promoter by Transcriptome Analysis and Its Application to Whole-Cell Heavy-Metal Detection Systems▽†

Jeong-Nam Park,[1]‡ Min Jeong Sohn,[1,2]‡ Doo-Byoung Oh,[1] Ohsuk Kwon,[1] Sang Ki Rhee,[1] Cheol-Goo Hur,[1] Sang Yup Lee,[2] Gerd Gellissen,[1]§ and Hyun Ah Kang[1]*

Omics and Integration Research Center, Korea Research Institute of Bioscience and Biotechnology, 52 Eoeun-dong, Yuseong-gu, Daejeon 305-806, Korea,[1] and Department of Chemical and Biomolecular Engineering, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Korea[2]

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The genomewide gene expression profiling of the methylotrophic yeast *Hansenula polymorpha* exposed to cadmium (Cd) allowed us to identify novel genes responsive to Cd treatment. To select genes whose promoters can be useful for construction of a cellular Cd biosensor, we further analyzed a set of *H. polymorpha* genes that exhibited >6-fold induction upon treatment with 300 μM Cd for 2 h. The putative promoters, about 1,000-bp upstream fragments, of these genes were fused with the yeast-enhanced green fluorescence protein (GFP) gene. The resultant reporter cassettes were introduced into *H. polymorpha* to evaluate promoter strength and specificity. The promoter derived from the *H. polymorpha SEO1* gene (Hp*SEO1*) was shown to drive most strongly the expression of GFP upon Cd treatment among the tested promoters. The Cd-inducible activity was retained in the 500-bp deletion fragment of the Hp*SEO1* promoter but was abolished in the further truncated 250-bp fragment. The 500-bp Hp*SEO1* promoter directed specific expression of GFP upon exposure to Cd in a dose-dependent manner, with Cd detection ranging from 1 to 900 μM. Comparative analysis of the *Saccharomyces cerevisiae SEO1* (Sc*SEO1*) promoter revealed that the Sc*SEO1* promoter has a broader specificity for heavy metals and is responsive to arsenic and mercury in addition to Cd. Our data demonstrate the potential use of the Hp*SEO1* promoter as a bioelement in whole-cell biosensors to monitor heavy metal contamination, particularly Cd.

Industrial activities lead to large-scale environmental contamination with toxic heavy metals, such as cadmium (Cd) and mercury (Hg). These metals are toxic even at low levels and tend to accumulate in the body over an extended period of time, which can eventually cause serious health problems in humans. Therefore, the development of monitoring systems for these metal ions in the environment has become increasingly important in order to prevent chronic exposure to these pollutants (2). As a response to this need, numerous biological systems and nonbiological sensors based on the emerging nanotechnology have been developed to monitor heavy metal contamination (38, 43). In particular, biosensors that use unicellular microorganisms as analytical tools to monitor heavy metals have drawn attention because of several practical advantages (1): the large population size, rapid growth rate, low cost, and easy maintenance. Moreover, the feasibility of genetic manipulation makes microbial cells an attractive choice as environmental bioreporters.

Many current microbial whole-cell sensors are based on genetically modified microorganisms (43, 28). In general, microbial biosensors comprise the molecular fusion of two linked genetic elements: a sensing bioelement and a reporter gene. In most cases, the sensing element is a promoter that specifically responds to the presence or absence of the target molecule, and the reporter gene, which is fused to the sensing element, encodes a quantifiable molecule such as a bioluminescent or fluorescent protein (13). In creating the promoter-reporter biosensor system, identification of the proper promoter is considered the most crucial step. With the recent advent of functional genomics technology, such as DNA and protein microarray technology, the selection and evaluation of multiple candidate promoters for biosensor construction is quicker and more efficient. Functional genomics techniques have proven especially useful in identifying genes that are directly regulated by changes in metal ion status (8, 13).

The methylotrophic yeast *Hansenula polymorpha*, which can utilize methanol as its sole carbon and energy source, has attracted increasing interest as a useful system to convert methanol to higher-value products (10). Particularly, it has gained popularity as a promising host for heterologous protein production using the strong methanol-inducible promoters (21). One of the peculiar characteristics of *H. polymorpha* is that expression vectors, even in a circular form containing the autonomous replication sequence, are multiply integrated into the host chromosomes with a high frequency, which makes this yeast an ideal host to stably maintain the high-level expression

* Corresponding author. Mailing address: Omics and Integration Research Center, Korea Research Institute of Bioscience and Biotechnology, 52 Eoeun-dong, Yuseong-gu, Daejeon 305-806, Korea. Phone: 82-42-860-4378. Fax: 82-42-860-4594. E-mail: hyunkang@kribb.re.kr.
‡ J.-N.P. and M.J.S. contributed equally to this study.
§ Present address: PharmedArtis GmbH, Forckenbeckstr. 6, 52074 Aachen, Germany.
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of foreign genes (20). In addition, *H. polymorpha* can grow at high temperatures, up to 48°C, and can tolerate various environmental stresses induced by heavy metals, xenobiotics, and environmental pollutants (3). Thus, this yeast has been used as a model organism to study resistance mechanisms to heavy metal ions or as a bioelement to detect toxic compounds. For example, oxidative stress responses and cellular reorganization associated with vanadate tolerance in *H. polymorpha* have been investigated (31, 32). Metabolically engineered *H. polymorpha* strains were also developed as the sensing bioelements in a whole-cell biosensor to detect alcohols and formaldehyde (12, 26).

The availability of sequence information on the genome of *H. polymorpha* has allowed for postgenomic approaches to obtain comprehensive information on gene function and regulatory mechanisms utilized by *H. polymorpha* (20, 39). We previously fabricated a partial cDNA microarray of *H. polymorpha* based on random sequence tag information and demonstrated the sensitivity and reliability of our microarray in analyzing global gene expression changes of *H. polymorpha* under defined environmental conditions (37). After completion of *H. polymorpha* whole genome sequencing (39), we developed *H. polymorpha* whole-genome cDNA microarrays by the same strategy as was used in the construction of the partial cDNA microarray. In the present study, we identified Cd-inducible novel *H. polymorpha* genes by using the *H. polymorpha* whole-genome cDNA microarray and analyzed some of these genes for their promoter activities. We show that the promoter of the *H. polymorpha SEO1* gene displayed a highly specific, dose-dependent response to Cd. Furthermore, we show that the *Saccharomyces cerevisiae SEO1* promoter can also direct Cd-responsive expression but has broader specificity for other heavy metals, including arsenic (As) and mercury (Hg), than the *H. polymorpha* promoter.

MATERIALS AND METHODS

Strains and culture conditions. *H. polymorpha* NCYC495 (ATCC 14754) was used for Cd-specific transcriptome profiling, promoter cloning, and reporter cassette expression. *S. cerevisiae* L3262 was used as a source of the 1,000-bp Sc*SEO1* gene promoter, and *S. cerevisiae* Y2805 was used for reporter cassette expression. *H. polymorpha* and *S. cerevisiae* were incubated at 37 and 30°C, respectively. Yeast transformation was carried out as described by Hill et al. (16). *H. polymorpha* and *S. cerevisiae* transformants were selected in minimal medium (2% [wt/vol] glucose, 0.67% [wt/vol] yeast nitrogen base without amino acid, 2% [wt/vol] agar) supplemented with leucine drop-out supplement or uracil drop-out supplement (BD Biosciences, Palo Alto, CA), respectively. To investigate the effects of metal ion treatment, yeast culture grown in 3 ml of YPD broth (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, 2% [wt/vol] glucose) for 16 h was inoculated into 100 ml of YPD broth medium at an optical density at 600 nm (OD_{600}) of 0.1. When the yeast cells reached an OD_{600} of 0.3, the cells were incubated with heavy metals for 2 h (or 3 h if necessary). Stock solutions of each metal—300 μM sodium arsenate ($Na_2HAsO_2 \cdot 7H_2O$), 300 μM cadmium sulfate ($CdSO_4 \cdot 8/3H_2O$), 300 μM chromium(III) chloride ($Cl_3Cr \cdot 6H_2O$), 300 μM cupric sulfate ($CuSO_4$), 300 μM iron(II) sulfate ($FeSO_4 \cdot 7H_2O$), 300 μM lead(II) nitrate [$Pb(NO_3)_2$], 300 μM mercury(II) chloride ($HgCl_2$), 300 μM nickel(II) sulfate ($NiSO_4$), and 300 μM zinc sulfate ($ZnSO_4 \cdot 6H_2O$)—were prepared in distillated water.

DNA microarray experiment and data processing. The whole-genome *H. polymorpha* cDNA microarray was constructed based on the manually annotated *H. polymorpha* complete genome sequence (20, 39), which predicted 5,848 open reading frames (ORFs). For PCR amplification of *H. polymorpha* ORFs, gene-specific primer pairs were designed by using the eprimer3 program (ftp://emboss.open-bio.org/pub/EMBOSS/). Detailed description on the design of *H. polymorpha* whole-genome cDNA microarrays and information on *H. polymorpha* ORFs spotted on the arrays were submitted to the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) at the National Center for Biotechnology Information with GEO platform accession numbers GPL4818 and GPL4802. Time course microarray experiments were carried out in duplicate with dye swapping using total RNAs obtained from *H. polymorpha* NCYC495 cultivated in the presence of 300 μM Cd for 0, 15, 30, 60, and 120 min (Fig. 1A). Yeast cells were cultured in YPD broth at 37°C to early exponential phase (OD_{600} of 0.3), and then cadmium sulfate (C2919; Sigma-Aldrich, St. Louis, MO) was added. After the addition of Cd, cells were harvested at the indicated times by centrifugation, and pellets were immediately frozen in liquid nitrogen. Total RNA was isolated by hot phenol method (5) and further purified with RNeasy mini purification kits (QIAGEN, Valencia, CA). The quality and amount of total RNA were checked by electrophoresis on 1.2% formaldehyde agarose gels and by UV/Vis spectrophotometry (serial no. 2598; NanoDrop Technology, Wilmington, DE). Target cDNAs were labeled with a 3DNA Submicro EX expression array detection kit (Genisphere, Hatfield, PA) as previously described (37). Briefly, a mixture of total RNA (50 μg) and oligo(dT) tagged with either Cy3- or Cy5-specific 3DNA capture sequence was heated at 80°C for 10 min and quickly chilled on ice for 2 to 3 min. A 5× reverse transcriptase buffer containing 0.1 mM dithiothreitol, deoxynucleoside triphosphate mixture, RNase inhibitor, and 200 U of reverse transcriptase (Invitrogen, Carlsbad, CA) was added to the mixture and incubated at 42°C for 2 h. The resultant cDNAs were purified, incubated at 80°C for 10 min and at 50°C for 20 min, and then applied to the prehybridized slide. The array was incubated in a dark humidified chamber at 43 to 53°C for 6 to 12 h. Hybridized slides were scanned with a ScanArray 5000 scanner (Packard, Billerica, CA) using the appropriate photomultiplier tube value to obtain the highest signal intensity without saturation. Using GenePix Pro 4.0 software (Axon, Foster City, CA), the spot intensity was quantified for each channel and transferred to Microsoft Excel spreadsheets. All arrays were analyzed by using Quintet, an R-based unified cDNA microarray data analysis system with GUI (6). The clustering of genes with the similar expression patterns across the time points was carried out by using the K-Means clustering program (42).

Accession number. The microarray data have been deposited in the GEO database with an accession number of GSE6989.

Construction of green fluorescent protein (GFP) reporter cassettes. The putative 1,000-bp promoters of the *H. polymorpha HSP12*, *FEN2*, and *SEO1* genes were PCR amplified from the template *H. polymorpha* NCYC495 genomic DNA using the each primer pairs described in Table 1. Boldface characters in Table 1 represent the restriction enzyme recognition sites, BamHI (GGATCC) and HindIII (AAGCTT). The PCR products were digested with BamHI and HindIII and ligated with BamHI-HindIII-digested pMOX-GFP2 (Table 1) to replace the methanol oxidase promoter (P_{MOX}). This procedure yielded pHHSP-GFP, pHFEN-GFP, and pHSEO-GFP, respectively. To construct YEpSSEO-GFP for expression of the GFP reporter in *S. cerevisiae*, a 1,000-bp DNA fragment containing the putative Sc*SEO1* promoter was PCR amplified from *S. cerevisiae* genomic DNA with the primer pair, ScSEO1p-F and ScSEO1p-R (Table 1). The PCR product was digested with BamHI and HindIII and subcloned into the corresponding sites of YEp352 (15), resulting in YEp352-SSEO. Then, the *GFP* gene, isolated by HindIII digestion of pMOX-GFP1 (Table 1), was cloned into the HindIII site of YEp352-SSEO, resulting in YEpSSEO-GFP. Subsequently, pSSEO-GFP was constructed by replacing the Hp*SEO1* promoter fragment of pHSEO-GFP with the Sc*SEO1* promoter fragment. YEpHSEO-GFP was constructed by stepwise cloning of the Hp*SEO1* promoter fragment and the *GFP* gene into YEp352 as described for the construction of YEpSSEO-GFP. A set of deleted Hp*SEO1* promoter fragments was amplified from the template pHSEO-GFP using the primers HpSEO1p-F1, HpSEO1p-F2, HpSEO1p-F3, and HpSEO1p-R (Table 1), digested with BamHI and HindIII, and exchanged with the 1,000-bp Hp*SEO1* promoter fragment of the pHSEO-GFP, generating pHSEO(1)-GFP, pHSEO(2)-GFP, and pHSEO(3)-GFP, respectively.

Bioassay procedures. After exposure to metals for 2 h, yeast cells were harvested, and the cell pellets were suspended in distillated water at OD_{600} of 1 to be normalized for fluorescence intensity per cell. The inducible activities of the yeast promoters upon metal exposure were quantified by measuring the fluorescence intensity of the cells with a spectrofluorometer (RF 5310PC; Shimadzu, Kyoto, Japan) at an excitation wavelength of 488 nm and an emission wavelength of 510 nm. The fluorescence intensity of recombinant yeast exposed to metal ions was expressed in arbitrary units, which were obtained by subtracting the fluorescence value for untreated recombinant cells. For microscopic observation, yeast cells treated with Cd for 2 h were harvested, washed, and resuspended with water. About 8 μl of cell suspensions were mounted on microscope slides and visualized with a confocal microscope (LSM 510 META; Carl Zeiss GmbH, Jena, Germany) using an argon laser (458, 477, 488, and 514 nm) and META

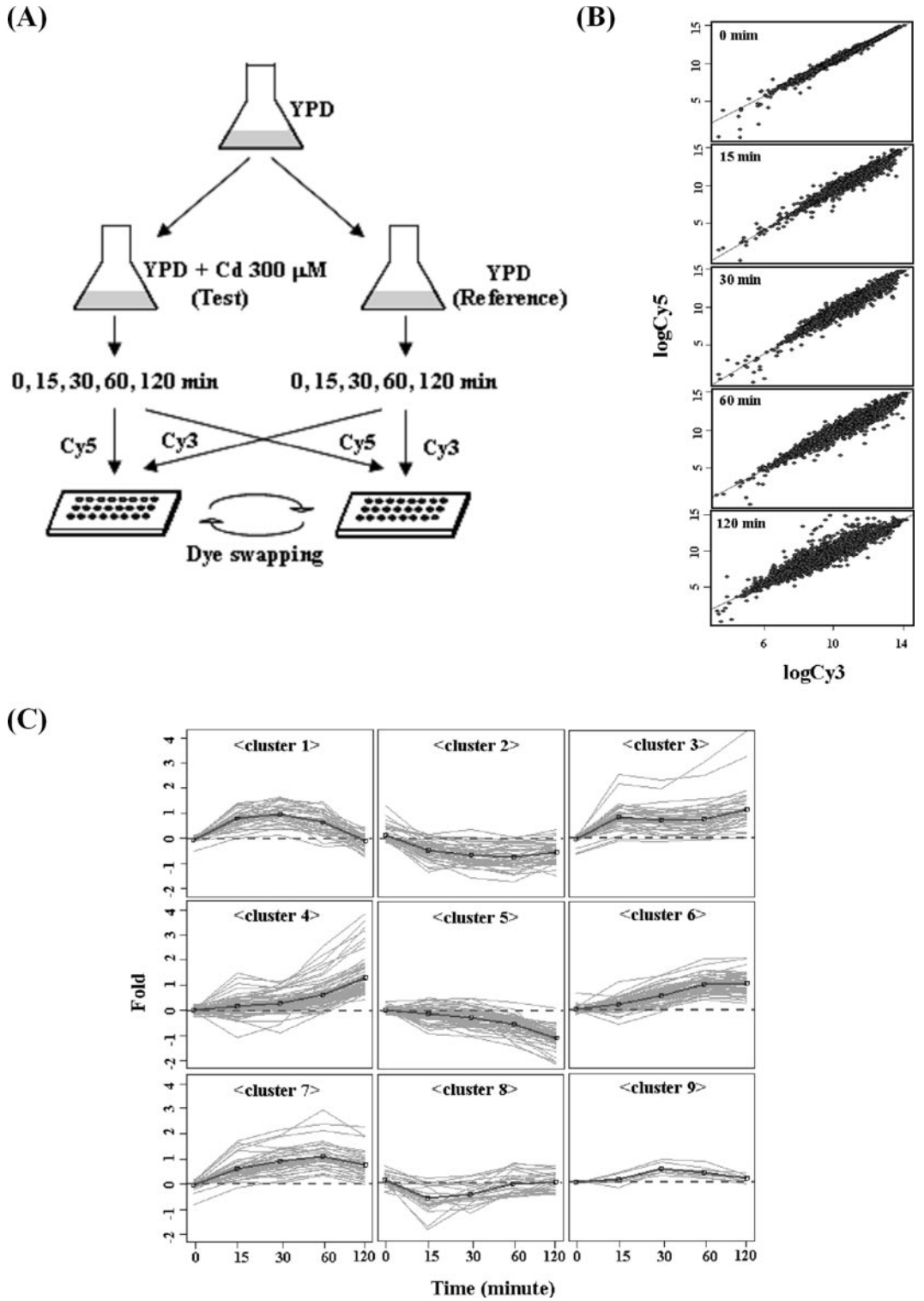

FIG. 1. Transcriptome profiling of *H. polymorpha* in response to Cd exposure. (A) Schematic diagram on microarray analysis. The test RNA samples from the Cd-treated cells and the reference RNA samples from the untreated cells were obtained at the indicated incubation times and labeled alternatively with Cy3 or Cy5 for the dye-swapping experiments. (B) Scatter plots of the time course transcriptome analysis of *H. polymorpha* cells treated with 300 μM Cd for 0, 15, 30, 60, and 120 min. (C) Cluster analysis displaying distinct temporal patterns for induction or repression of *H. polymorpha* genes during 300 μM Cd exposure for 2 h. The graph represents relative expression level (*y* axis) versus incubation time points (*x* axis).

TABLE 1. Oligonucleotides, plasmids, and strains used in this study

Oligonucleotide, plasmid, or strain	Sequence or description[a]	Source or reference
Oligonucleotides		
HpHSP12p-F	ATGC**GGATCC**TCTGCCTCTCTCGTTGGC	This study
HpHSP12p-R	ATGT**AAGCTT**GTTTGATTCTGTGGTAAAAG	This study
HpFEN2p-F	ATGC**GGATCC**TGCGAGCGCGTTCTGCTA	This study
HpFEN2p-R	ATGT**AAGCTT**GGTTGGAGGAGGAATGATT	This study
HpSEO1p-F	GGTA**GGATCC**AAGTGTATGTTACCATTCCG	This study
HpSEO1p-F1	AATT**GGATCC**CGGCAACTCTCCCTTGCTCT	This study
HpSEO1p-F2	AATT**GGATCC**CAGGTGTACCTGCTTGCTCTGC	This study
HpSEO1p-F3	AATT**GGATCC**CAAGACCGGTTGCAGCCTC	This study
HpSEO1p-R	ATGG**AAGCTT**TTGGGATTGAACAAACCCG	This study
ScSEO1p-F	ATGC**GGATCC**TATATCCGATTCATTCTTCTTTG	This study
ScSEO1p-R	ATGC**AAGCTT**GTATGTTATTTATATATGCAGTAG	This study
Plasmids		
pMOX-GOD	*MOX* promoter (P_{MOX}), Amp^r; glucose oxidase (*GOD*), *HLEU2, HRAS36*	25
pMOX-GFP1	Exchange of *GOD* with *GFP* in pMOX-GOD	This study
pMOX-GFP2	Removal of HindIII site at the 3′ end of *GFP* in pMOX-GFP1	This study
pHHSP-GFP	Exchange of P_{MOX} with $P_{HpHSP12}$ in pMOX-GFP2	This study
pHFEN-GFP	Exchange of P_{MOX} with P_{HpFEN2} in pMOX-GFP2	This study
pHSEO-GFP	Exchange of P_{MOX} with $P_{HpSEO1(F0)}$ in pMOX-GFP2	This study
pHSEO(1)-GFP	Exchange of $P_{HpSEO1(F0)}$ with $P_{HpSEO1(F1)}$ in pHSEO-GFP	This study
pHSEO(2)-GFP	Exchange of $P_{HpSEO1(F0)}$ with $P_{HpSEO1(F2)}$ in pHSEO-GFP	This study
pHSEO(3)-GFP	Exchange of $P_{HpSEO1(F0)}$ with $P_{HpSEO1(F3)}$ in pHSEO-GFP	This study
pSSEO-GFP	Exchange of $P_{HpSEO1(F0)}$ with P_{ScSEO1} in pHSEO-GFP	This study
YEp352	pBR322 origin, Amp^r; 2μ origin, *URA3*	15
YEp352-SSEO	YEp352 containing P_{ScSEO1}	This study
YEp352-HSEO	YEp352 containing $P_{HpSEO1(F0)}$	This study
YEpSSEO-GFP	YEp352 containing P_{ScSEO1}-GFP	This study
YEpHSEO-GFP	YEp352 containing $P_{HpSEO1(F0)}$-GFP	This study
Strains		
H. polymorpha NCYC495	*leu1-1*	3
HHSP	NCYC495 transformed with pHHSP-GFP	This study
HFEN	NCYC495 transformed with pHFEN-GFP	This study
HSEO	NCYC495 transformed with pHSEO-GFP	This study
HSEO1	NCYC495 transformed with pHSEO(1)-GFP	This study
HSEO2	NCYC495 transformed with pHSEO(2)-GFP	This study
HSEO3	NCYC495 transformed with pHSEO(3)-GFP	This study
HSSEO	NCYC495 transformed with pSSEO-GFP	This study
S. cerevisiae L3262	*MAT***a** *ura3-52 leu2-3,112 his4-34*	19
Y2805	*MAT***a** *pep4*::*His3 prb-1.6R can1 his3-20 ura3-52*	19
SSEO	Y2805 transformed with YEpSSEO-GFP	This study
SHSEO	Y2805 transformed with YEpHSEO-GFP	This study

[a] Amp^r, ampicillin resistance.

photomultiplier tube detector (polychromatic 32-channel detector). Cell images were obtained with LSM 510 META software.

RESULTS

Transcriptome profiling of *H. polymorpha* in response to Cd exposure. To counteract Cd toxicity, living organisms have evolved several defense mechanisms that are mainly mediated by massive changes in gene expression (34, 35). To gain insight into the basic mechanism of Cd detoxification in the methylotrophic yeast *H. polymorpha*, we analyzed temporal changes in transcriptional profiles in response to Cd exposure (Fig. 1A). We used *H. polymorpha* whole-genome cDNA microarrays, which contain 5,837 spots corresponding to 99% of all *H. polymorpha* ORFs in duplicate or triplicate (GEO platform accession numbers GPL4818 and GPL4802). Total RNA samples were collected from *H. polymorpha* cells after 15, 30, 60, and 120 min incubation with 300 μM Cd. Using the RNA samples obtained from the unstressed cells cultivated at the same incubation time as a reference, the differential fluorescence intensities of each RNA sample prepared at the indicated times were measured after labeling with Cy3 or Cy5 fluorochromes. For all analyses, we performed dye-swapping experiments to avoid dye bias (30), thus four or six intensity values were generated for each ORF and averaged for analysis. Scatter plots of the time course transcriptome analysis of *H. polymorpha* cells treated with Cd showed that Cy5 and Cy3 signals were more widely distributed with longer exposure time, indicating that the extent of gene expression change increased with the increase of exposure period (Fig. 1B).

A total of 159 genes were induced (105 genes) or repressed (54 genes) by greater than a factor of 2 after 2-hour exposure to 300 μM Cd. As listed in Table S1 in the supplemental material, several subsets of genes relevant to the oxygen stress response, protein degradation, sulfur amino acid metabolism,

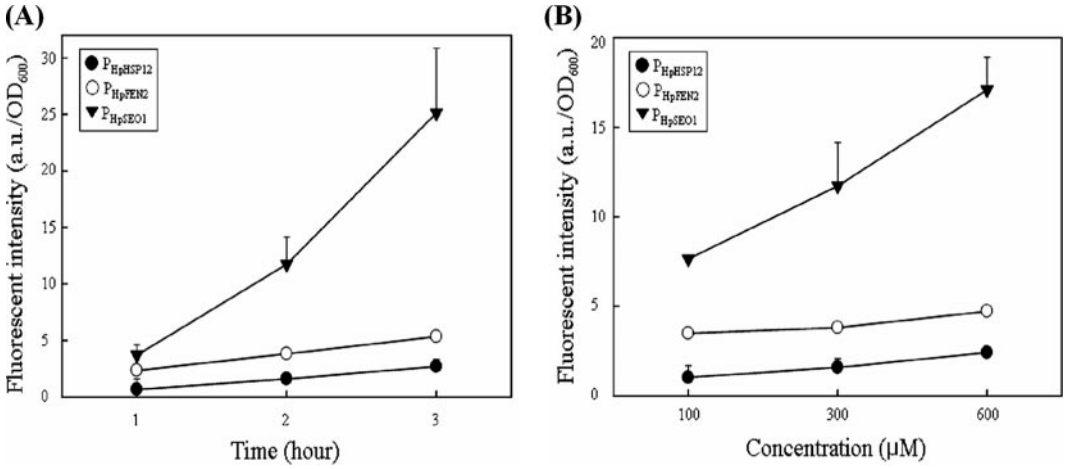

FIG. 2. Analysis of Cd-inducible activities of Hp*HSP12*, Hp*FEN2*, and Hp*SEO1* promoters. (A) Time course analysis of the Hp*HSP12*, Hp*FEN2*, and Hp*SEO1* promoter activities. Recombinant yeast cells, HHSP, HFEN, and HSEO, were inoculated into 100 ml of YPD at an OD_{600} of 0.1 and grown to an OD_{600} of 0.3, and then 300 μM Cd was added. The cells were incubated and harvested at 1-h intervals for 3 h. $P_{HpHSP12}$, Hp*HSP12* promoter; P_{HpFEN2}, Hp*FEN2* promoter; P_{HpSEO1}, Hp*SEO1* promoter. (B) Analysis of Cd concentration-dependent activities of three promoters. Recombinant *H. polymorpha* cells were inoculated into 100 ml of YPD at an OD_{600} of 0.1 and grown to an OD_{600} of 0.3. After the addition of 100, 300, or 600 μM Cd, the cells were incubated for 2 h and harvested. The OD_{600} of the cells was adjusted to 1, and fluorescence was measured with a fluorometer. a.u., arbitrary unit. The fluorescence value was normalized with the fluorescence value at zero time, and the error bars indicate the standard deviation of the mean fluorescence for two independent trials.

and sulfur salvage pathway were highly upregulated in *H. polymorpha*. As reported in a previous transcriptome analysis study with *S. cerevisiae* under the same Cd treatment conditions (35), several of the highly induced genes in *H. polymorpha* during Cd exposure were involved in sulfate and methionine transport (*SUL1*) and the sulfur assimilation pathway for glutathione biosynthesis (*MET3* and *MET5*). Several common stress genes (*HSP12*, *HPS26*, and *PRX1*) were also highly induced. Notably, ca. 30% of the genes upregulated upon Cd treatment have no currently recognized function. Relatively small numbers of *H. polymorpha* genes were repressed by 2-h exposure to 300 μM Cd. Most of the repressed genes were involved in two representative functions: protein biosynthesis and the electron transport chain (see Table S2 in the supplemental material). The predominant repression of genes encoding ubiquinol cytochrome *c* complex and succinate dehydrogenase subunits, which couple the oxidation of succinate to the transfer of electrons to ubiquinone, and the induction of antioxidant genes in *H. polymorpha* agree with the recent report that Cd inhibits the electron transfer chain and induces reactive oxygen species in animal cells (45).

Identification of Cd-inducible genes from transcriptome data. Cluster analysis of genes, whose expression levels were changed by ≥2-fold in at least one time point after Cd treatment, classified a total of nine clusters with similar expression patterns (Fig. 1C). Since our aim was to find novel genes activated in the presence of Cd to construct a new Cd detection biosensor, we were initially interested in genes with an increased expression pattern after exposure to Cd; these genes were grouped into clusters 3, 4, 6, and 7. Because the time-dependent response to reagents is another important factor in developing cellular biosensors, we focused especially on the genes in cluster 4. Cluster 4 genes exhibited progressively increased expression during the course of Cd exposure, while the genes in clusters 3, 6, and 7 displayed saturated expression patterns as the exposure time progressed. Among the genes in cluster 4, four *H. polymorpha* genes (Hp*HSP12*, Hp*FEN2*, Hp*ADH3*, and Hp*SEO1*) were induced >6-fold during cadmium treatment (see Table S1 in the supplemental material) and were chosen for further confirmation of Cd-inducible expression by semiquantitative reverse transcription-PCR (data not shown). As suggested from cDNA microarray analysis data, the increased expression of these genes was clearly shown upon Cd exposure, despite some quantitative differences between the two methods.

To examine the strength of promoters derived from these Cd-responsive genes, approximately 1,000-bp sequences upstream of Hp*HSP12* (homolog of the *S. cerevisiae* heat shock protein 12 encoding gene), Hp*ADH3* (homolog of the *Pseudomonas aeruginosa* alcohol dehydrogenase PA5427 encoding gene), Hp*FEN2* (homolog of the *S. cerevisiae* fenpropimorph resistance protein encoding gene), and Hp*SEO1* (homolog of the *S. cerevisiae* suppressor of sulfoxide ethionine resistance encoding gene) were fused with the *GFP* gene. The resultant reporter cassettes, pHHSP-GFP, pHFEN-GFP, pHADH-GFP, and pHSEO-GFP, were introduced into *H. polymorpha* NCYC495 and analyzed for their responses to Cd. The fluorescence intensities of the recombinant yeasts increased with time (Fig. 2A) and Cd concentration (Fig. 2B). Compared to recombinant yeasts HHSP and HFEN carrying pHHSP-GFP or pHFEN-GFP, the recombinant strain HSEO carrying pHSEO-GFP had the highest increase in fluorescent intensity upon Cd exposure. The expression level of GFP from recombinant yeast harboring pHADH-GFP was too low for further analysis even after Cd treatment (data not shown).

Evaluation of the specificity of Hp*SEO1* promoter to Cd induction. Smaller-sized promoters are advantageous both in the construction of expression cassettes with various reporter genes and in the multiple integration of expression cassettes into host chromosomes. Thus, we roughly identified the mini-

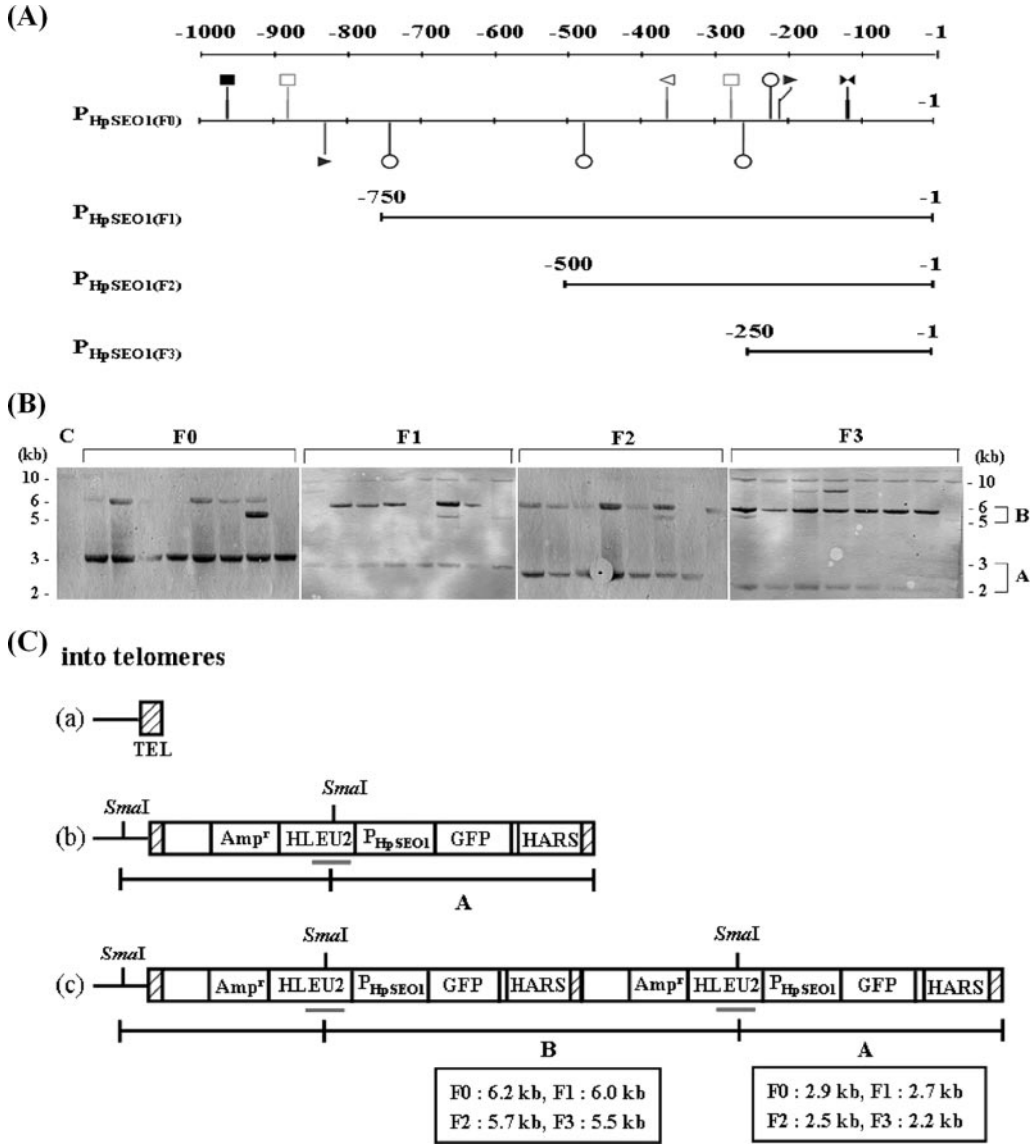

FIG. 3. Deletion and regulatory sequence analysis of the *H. polymorpha SEO1* promoter. (A) Stepwise deletion of *H. polymorpha SEO1* promoter (1,000 bp; F0). F1, Deleted 750-bp promoter fragment; F2, deleted 500-bp promoter fragment; F3, deleted 250-bp promoter fragment. The sequence analysis of 1,000-bp Hp*SEO1* promoter was carried out by using RSAT (Regulatory Sequence Analysis Tools; http://rsat.ulb.ac.be/rsat/). Some binding sites for representative transcriptional regulator (22) are presented; ■, SWI5; □, ACE2; ▶, GCR1; ○, ADR1; ◁, GCRE/ARE, ⋈, CBF1. The 1,000-bp Hp*SEO1* promoter sequence has been deposited to GenBank with an accession number of AY792972. (B) Southern blot analysis. Genomic DNAs of individual transformants HSEO, HSEO1, HSEO2, and HSEO3 were digested with SmaI and probed with the 1.2-kb digoxigenin-labeled Hp*LEU2* gene fragment, as previously described (21). Lane C, DNA from nontransformed host cell as a control. (C) Schematic diagrams explaining the integration of recombinant plasmids into the telomeres. Preintegration (a), single-copy integration (b), and two-copy tandem integration (c) patterns are shown, and the expected sizes of the SmaI-treated plasmid fragments (boxes A and B) are listed.

mum promoter size required for the Cd-inducible response by simple stepwise deletion analysis of the Hp*SEO1* promoter. The 750-bp (F1), 500-bp (F2), and 250-bp (F3) fragments of the Hp*SEO1* promoter were fused with the *GFP* gene to construct the expression vectors pHSEO(1)-GFP, pHSEO(2)-GFP, and pHSEO(3)-GFP, respectively (Fig. 3A). Recombinant *H. polymorpha* strains, designated HSEO1, HSEO2, and HSEO3, were analyzed for their responses to 300 μM Cd and for the integration copy number of the GFP cassette to consider the effect of copy number on the expression levels of GFP. The average values of fluorescence intensity obtained from randomly chosen eight transformants, harboring each pHSEO-GFP expression vector, were divided by the average copy numbers of the GFP cassette analyzed by Southern blotting (Fig. 3B). Integration patterns of the pHSEO-GFP vectors, which contain the telomeric HARS sequence as a replication origin, suggested that the GFP cassettes were mostly integrated in tandem into the telomere locus (Fig. 3C), as previously observed on other expression vectors using the telomeric HARS (21). Integration copy numbers were roughly

TABLE 2. Average copy numbers and fluorescence intensities of *H. polymorpha* transformants harboring each pHSEO-GFP expression vector

Promoter fragment	Size (bp)	Avg[a]		Fluorescence/ copy no.[b]	Relative activity (%)[c]
		Copy no.	Fluorescence		
F0	1,000	2.4	7.48	3.12	100
F1	750	3.5	11.06	3.16	101
F2	500	3.9	14.16	3.63	116
F3	250	6.3	4.01	0.64	20

[a] The GFP expression vectors under the control of a set of the Hp*SEO1* promoters—pHSEO-GFP, pHSEO(1)-GFP, pHSEO(2)-GFP, and pHSEO(3)-GFP—were introduced into the *H. polymorpha* NCYC4795 strain. Eight individual transformants containing each vector were randomly chosen and analyzed for the copy number of pHSEO-GFP vectors and for GFP expression. The transformants were grown for 2 h in YPD medium supplemented with 300 μM Cd.

[b] The expression levels from each deleted promoter were calculated by dividing the average fluorescence by the average copy number to obtain the specific activity of each promoter.

[c] The relative activity of each Hp*SEO1* promoter fragment (F1, F2, and F3) was compared to that of the 1,000-bp Hp*SEO1* promoter (F0).

estimated based on the relative intensities of B fragments, which correspond to the whole fragments of each expression vector generated by tandem integration, to those of A fragments corresponding to the partial fragments of each expression vector integrated just at the end of telomere (Fig. 3B). It was revealed that the F2 construct, containing the 500-bp promoter upstream of Hp*SEO1* promoter, retained all Cd-inducible activity, but the more truncated F3 construct, the 250-bp promoter, greatly reduced its response to Cd (Table 2).

We further analyzed the response of the 500-bp Hp*SEO1* promoter to several other metals. The recombinant strain HSEO2 was exposed to diverse metal ions, including arsenic (As), mercury (Hg), chromium (Cr), copper (Cu), iron (Fe), nickel (Ni), lead (Pb), and zinc (Zn), at 300 μM for 2 h. The choice of 300 μM as an assay condition for every metal was based on the observation that the extent of growth inhibition was relatively mild and comparable among the tested metals at 300 μM during 2-h exposure (see Fig. S1 in the supplemental material). The GFP expression was negligible in response to other metals except to As (Fig. 4A). Interestingly, the response of the Hp*SEO1* promoter to As and to Cd was found to be similar at low concentrations. However, the detection range for As was quite limited compared to that for Cd. In the case of Cd, the Hp*SEO1* promoter activity increased steadily up to 900 μM. In contrast, the Hp*SEO1* promoter activity increased only up to 100 μM As and decreased gradually thereafter (Fig. 4B). The difference in detection ranges for As and Cd appears to reflect the different tolerance ranges of *H. polymorpha* to these metals. *H. polymorpha* growth was not much inhibited by 300 μM Cd but was severely prevented by 100 μM As for 2 h incubation (see Fig. S1 in the supplemental material). The Hp*SEO1* promoter did not respond to the other metal ions even at low concentrations or at high concentrations up to 900 μM (data not shown).

Comparison of the *SEO1* promoters from *H. polymorpha* and *S. cerevisiae*. The *SEO1* gene was first isolated as a gene encoding a suppressor of ethionine sulfoxide resistance in a methionine uptake mutant in *S. cerevisiae* (18). The *S. cerevisiae SEO1* gene (Sc*SEO1*) product shows significant similarities to

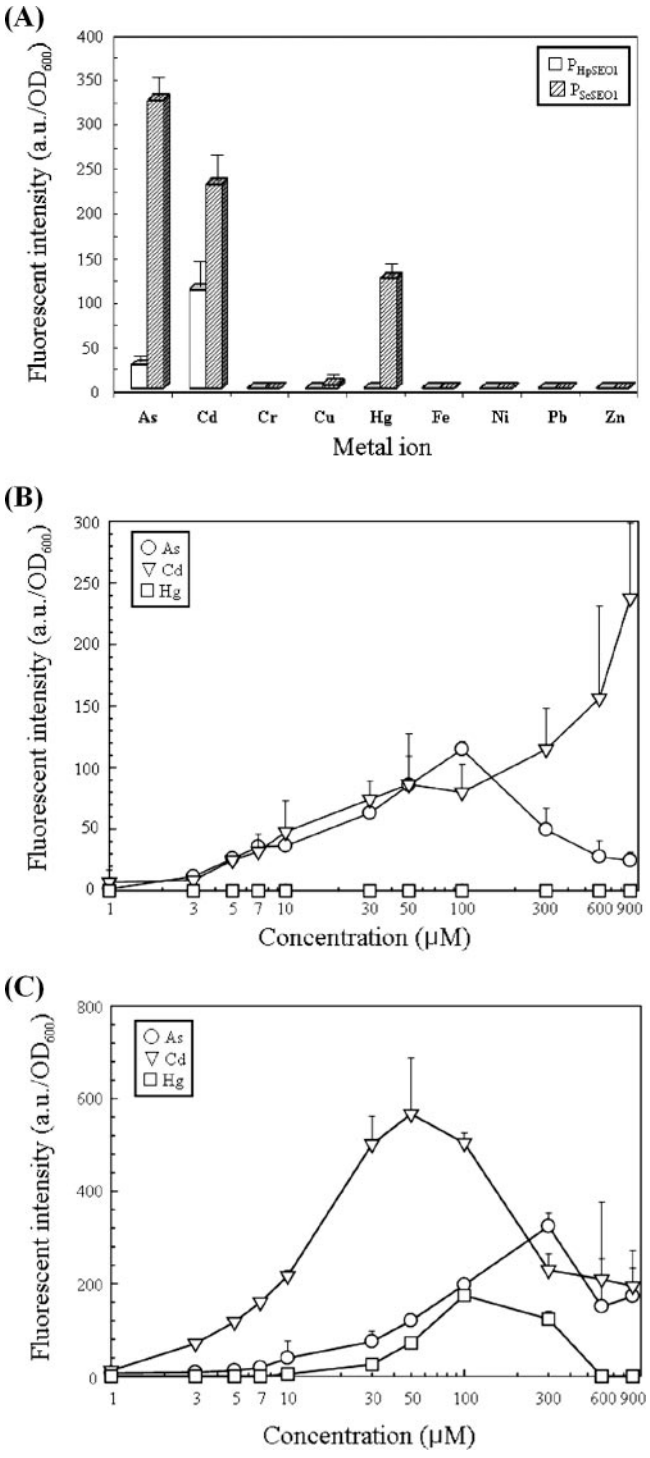

FIG. 4. Assay of the yeast *SEO1* promoter-inducible responses to toxic heavy metals. (A) Response of the yeast *SEO1* promoters to several metal ions at 300 μM. P_{HpSEO1}, the Hp*SEO1*(F2) promoter; P_{ScSEO1}, the Sc*SEO1* promoter. (B) Detection ranges of the Hp*SEO1*(F2) promoter to As and Cd. (C) Detection ranges of the Sc*SEO1* promoter to As, Cd, and Hg. Yeast cells were inoculated into 50 ml of YPD at an OD_{600} of 0.1 and grown to an OD_{600} of 0.3. After the addition of each metal at the indicated concentration (1 to 900 μM), the cells were incubated for 2 h and then harvested. The OD_{600} of the cells was adjusted to 1, and fluorescence was measured by using a fluorometer. a.u., arbitrary unit. The fluorescence value was normalized with the fluorescence value at zero time, and the error bars indicate the standard deviation of the mean fluorescence for two independent trials.

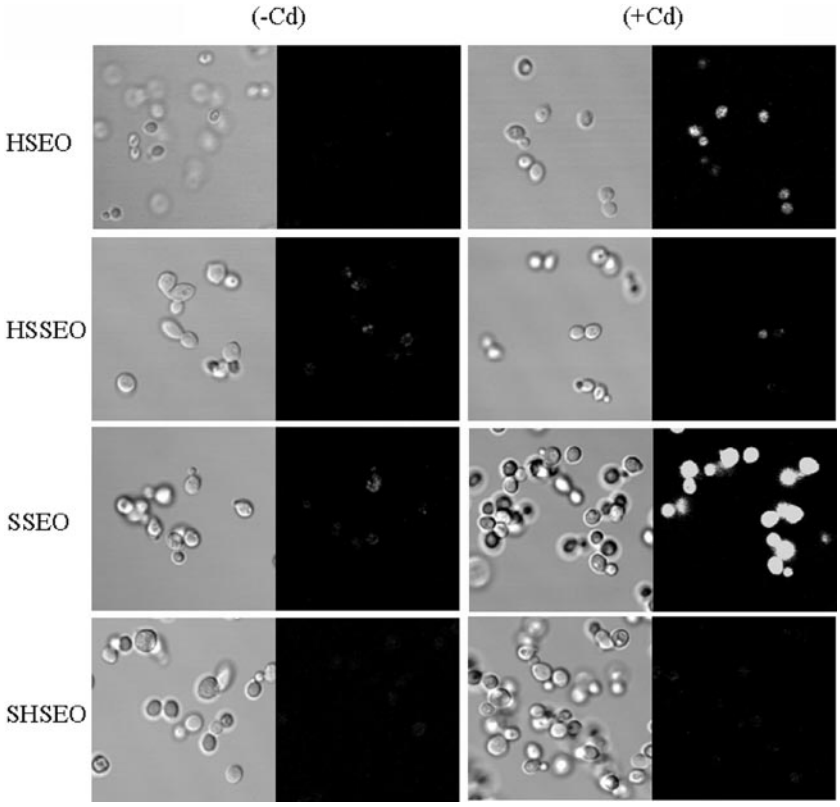

FIG. 5. Interchangeability test of two yeast *SEO1* promoters for Cd-inducible response. Confocal microscope images of yeast transformants expressing GFP under each yeast promoter are shown. HSEO, *H. polymorpha* transformant containing pHSEO-GFP; HSSEO, *H. polymorpha* transformant containing pSSEO-GFP; SSEO, *S. cerevisiae* transformant YEpSSEO-GFP; SHSEO, *S. cerevisiae* transformant containing YEpHSEO-GFP. The recombinant yeasts were inoculated into YPD at an OD_{600} of 0.1 and grown to an OD_{600} of 0.3. After the addition of 300 μM Cd, the cells were incubated for 2 h and harvested for image analysis. (−Cd), No Cd addition; (+Cd), Cd addition.

allantoate permease homologs, belonging to Dal5 (degradation of allantoin) family among the major facilitator superfamily (36), but its accurate cellular function is not defined yet. Although the Sc*SEO1* gene was included among upregulated genes in previous transcriptome analyses of *S. cerevisiae* treated with Cd (35), no detailed analysis of the Cd-inducible activity of the Sc*SEO1* promoter has been reported. Thus, we investigated the responses of Sc*SEO1* promoter to various metal ions by analyzing the inducible activity of a 1,000-bp fragment upstream of the Sc*SEO1* gene and compared the results to those for the Hp*SEO1* promoter. *S. cerevisiae* Y2805 strain was transformed with YEpSSEO-GFP, which contains the GFP reporter cassette fused with the Sc*SEO1* promoter in the backbone of YEp352, and the resultant transformant SSEO was analyzed for inducible activity in response to heavy metals. The *S. cerevisiae* promoter directed strong inducible expression of GFP, not only in response to Cd but also in response to As and Hg, along with a mild response to Cu (Fig. 4A). Interestingly, the dose-dependent response range of the *S. cerevisiae* promoter to Cd was only up to 50 μM (Fig. 4C), which is quite limited compared to the broad response range of the Hp*SEO1* promoter of up to 900 μM (Fig. 4B). In contrast, the detection range of the Sc*SEO1* promoter for As appeared much broader than that of the Hp*SEO1* promoter (Fig. 4C). The difference might be largely attributable to the different degree of sensitivity of the two yeasts to these toxic metals: *S. cerevisiae* is more sensitive to Cd, but more resistant to As, compared to *H. polymorpha* (data not shown).

We then tested whether the two yeast *SEO1* promoters could retain the Cd-responsive activation when introduced into the heterologous host. The expression vectors pSSEO-GFP and YEpHSEO-GFP were constructed and transformed into *H. polymorpha* and *S. cerevisiae*, respectively, resulting in the recombinant strains HSSEO or SHSEO. As shown in Fig. 5, neither of the *S. cerevisiae* and *H. polymorpha SEO1* promoters exhibited Cd-inducible responses in the reciprocal host. The results indicate that different regulation mechanisms operate for each *SEO1* promoter. The higher fluorescence intensities in the recombinant *S. cerevisiae* SSEO strains might be partly attributed to the higher copy number of reporter cassettes in the episomal 2μ plasmid of *S. cerevisiae* (more than 25 copies) than in the integrative plasmid of the *H. polymorpha* HSEO strains (2 to 3 copies).

DISCUSSION

Living organisms have developed various defense mechanisms to cope with environmental stresses, mainly by regulating the expression of several subsets of genes involved in the defense response (40). Exploring how transcriptional mechanisms are modulated in response to stress conditions caused by toxic materials has become more effective with the advent of

DNA microarray technology (29). Genome-wide transcriptional profile analysis with DNA microarrays has led to high-throughput screening of target-specific stress-responsive genes and facilitates the selection of appropriate promoters applicable to the development of cell-based biosensors (4, 24).

To identify novel *H. polymorpha* genes responsive to cadmium exposure, we analyzed the time course of transcriptome profiles of Cd-treated *H. polymorpha* cultures using our homemade *H. polymorpha* whole-genome cDNA microarray. A total of 159 *H. polymorpha* genes were differentially expressed with >2-fold changes in expression after 2 h of exposure to 300 μM Cd. We chose several genes exhibiting >6-fold induction upon Cd treatment and performed promoter strength analyses by measuring GFP reporter expression (Fig. 2). The Hp*SEO1* gene promoter was selected as the most suitable promoter for the development of a Cd-specific whole-cell detection system based on the concentration- and time-dependent response to Cd.

The Hp*SEO1* gene encodes a protein with 59% identity to the Sc*SEO1* protein, which has an unknown cellular function. The Sc*SEO1* protein has eight putative membrane-spanning regions and might be involved in the transport of sulfur compounds other than methionine sulfoxide (18). Thus, defining the physiological function of the *SEO1* gene product in yeast is an intriguing subject, especially as it relates to Cd detoxification. Based on previous studies carried out with *S. cerevisiae* (9, 44), upon Cd exposure, most sulfur amino acids are thought to be directed to the massive production of glutathione, the major sulfur compound involved in Cd detoxification, thus leading to depletion of sulfur amino acids in the cell. This, in turn, induces the expression of genes for sulfur compound-importing transporters, probably such as *SEO1*, and genes involved in sulfur amino acid metabolism. As shown in Fig. 3A, the regulatory sequence analysis of the 1,000-bp Hp*SEO1* promoter revealed two putative *cis*-regulatory elements, which might be involved in Cd-inducible expression: the GCN4 and activator protein 1 (AP-1) recognition element core sequence (GCRE and ARE core sequence [TGACT]) located at positions −367 to −363 and the CBF1 core binding sequence (CACRTG motif [ATCACATG]) located at positions −123 to −116 in the upstream region. Gcn4p (for general control nonderepressible) is a member of the basic leucine-zipper protein family and is required for the transcriptional activation of many genes involved in amino acid biosynthesis under conditions of amino acid starvation (17). Yap1p (for yeast AP-1), a homolog of the mammalian transcription factor AP-1, reportedly induces a variety of genes, including *GSH1* (for γ-glutamylcysteine synthetase 1), *YCF1* (for yeast cadmium factor 1), and *SOD1* (for superoxide dismutase 1), under cadmium and oxidative stresses in *S. cerevisiae* (44, 46). Cbf1p (for centromere-binding factor 1) is a member of the basic helix-loop-helix protein family and is involved in both the regulation of sulfur amino acid metabolism and chromosome remodeling (23, 27). Cd-inducible expression of *S. cerevisiae GSH1* was mediated by the interaction of Cbf1 and the Met4 transcription factor (7). Although further detailed analyses are required, the two putative cadmium-responsive *cis* elements—the GCRE/ARE core sequence and the CBF1 binding motif—might be related to Cd-inducible activation of the Hp*SEO1* promoter. Our stepwise deletion analysis of the 1,000-bp Hp*SEO1* promoter (F0) suggested that at least the GCRE/ARE core sequence was required for the Cd-inducible response of the Hp*SEO1* promoter (Fig. 3A and Table 2).

It is interesting that the Hp*SEO1* promoter, which was selected solely based on DNA microarray data from *H. polymorpha* cells treated with Cd, also responded to another well-known heavy metal, As (Fig. 4A), although the detection range for As was limited compared to that for Cd. Several studies reported that pathways for As and Hg detoxification in *S. cerevisiae* are similar to those for Cd detoxification (11, 14, 40, 47), which is consistent with our observation that the Sc*SEO1* promoter is highly induced not only by Cd but also by As and Hg. The negligible response of the Hp*SEO1* promoter to Hg suggests that *H. polymorpha* has similar detoxification responses for As and Cd but uses a different defense mechanism against Hg toxicity.

One possible explanation of the different behavior of two yeast *SEO1* promoters is provided by the structural organization of each promoter. The sequence analysis of the Sc*SEO1* promoter indicates that it contains the stress response element (STRE, CCCCT [−450 to −446]), which is recognized by the transcription factor MSN2/MSN4 (for multicopy suppressor of SNF1 mutation) (33). However, the Sc*SEO1* promoter lacks the cadmium-responsive *cis* elements, such as the putative GCRE/ARE core sequence and the CBF1 core binding sequence, that were observed in the Hp*SEO1* promoter. This structural difference in the yeast promoters generate distinctive regulatory systems in *H. polymorpha* and *S. cerevisiae*, as was partly demonstrated by the incompatibility of these *SEO1* promoters in the heterologous host (Fig. 5). The different metal ion-inducible activities between *H. polymorpha* and *S. cerevisiae* also result from distinctive physiological characteristics of the two yeasts. For example, *H. polymorpha* cells are very insensitive to Cd compared to *S. cerevisiae* (10). In fact, compared to the number of *S. cerevisiae* genes (632 [total] = 310 [up] + 322 [down]) differentially regulated by 300 μM Cd (35), we observed that fewer *H. polymorpha* genes (351 [total] = 245 [up] + 106 [down]) responded to even higher concentrations of Cd, up to 600 μM Cd (data not shown). The broad detection range of the Hp*SEO1* promoter for Cd could make it more advantageous than the Sc*SEO1* promoter as the bioelement of a whole-cell biosensor to monitor Cd under heavily contaminated conditions.

To date, several biosensors based on genetically engineered bacteria have been developed for heavy metal detection using metal-specific gene promoters (43). The most sensitive recombinant bacterial strain reported for measuring heavy metals is the recombinant *Staphylococcus aureus* that carries firefly luciferase under the control of the *cadA* promoter and detects both Cd and Pb. The maximum response was obtained within 2 to 4 h of incubation and the lowest detectable limits were 10 and 33 nM for Cd and Pb, respectively (41). However, the maximum concentration at which the recombinant *S. aureus* could detect Cd was 1 μM. At higher Cd concentrations, the luminescence decreased and reached the background value by 10 μM. This is a stark contrast to the recombinant yeast strains carrying GFP under the control of *SEO1* promoters. In the case of the recombinant *S. cerevisiae* developed in the present study, the detection range for Cd was 1 to 50 μM, whereas the recombinant *H. polymorpha* had a Cd detection range of 1 to

900 μM for 2 h of incubation (Fig. 4B). The difference in the sensitivity ranges between bacterial and yeast systems may be partly attributed to the different reporting elements: bioluminescence versus fluorescence. Since bioluminescence is a measure of enzymatic activity, detection of the target analyte is much faster and more sensitive than with fluorescence (2). The medium composition for cell cultivation also affects sensitivity to metal stresses; the minimal medium HMM was used for recombinant *S. aureus* to achieve the highest sensitivity (41), while the rich medium YPD was used for recombinant yeast in the present study to mimic contaminated industrial water, which is full of other nutrient sources. Another consideration is the difference in the organisms' resistance to Cd toxicity. Yeasts appear to be more resistant to Cd toxicity than are bacteria. Whereas the severe growth inhibition of *S. aureus* strain RN 4220 began to occur at 100 nM Cd (43), the growth inhibition of *S. cerevisiae* Y2805 and *H. polymorpha* NCYC495 was not significant even at 100 μM Cd under the culture conditions used for Cd detection (data not shown).

In the present study, we identified a novel Cd-inducible *H. polymorpha SEO1* promoter by transcriptome analysis and showed its high potential for yeast whole-cell biosensor systems to detect heavy metal contamination. In particular, the *H. polymorpha* system was highly specific for Cd at high concentrations. The detection threshold of the yeast system could be improved by rational genetic manipulation, which will become more effective as we understand more about the regulatory circuits and defense mechanisms involved in *H. polymorpha* responses to Cd stress. Engineering the information processing systems of yeast cells, such as manipulating transcription factors involved in putative regulatory sites in the Hp*SEO1* promoter, could be attempted to develop recombinant yeast strains with more sensitivity and selectivity to Cd.

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