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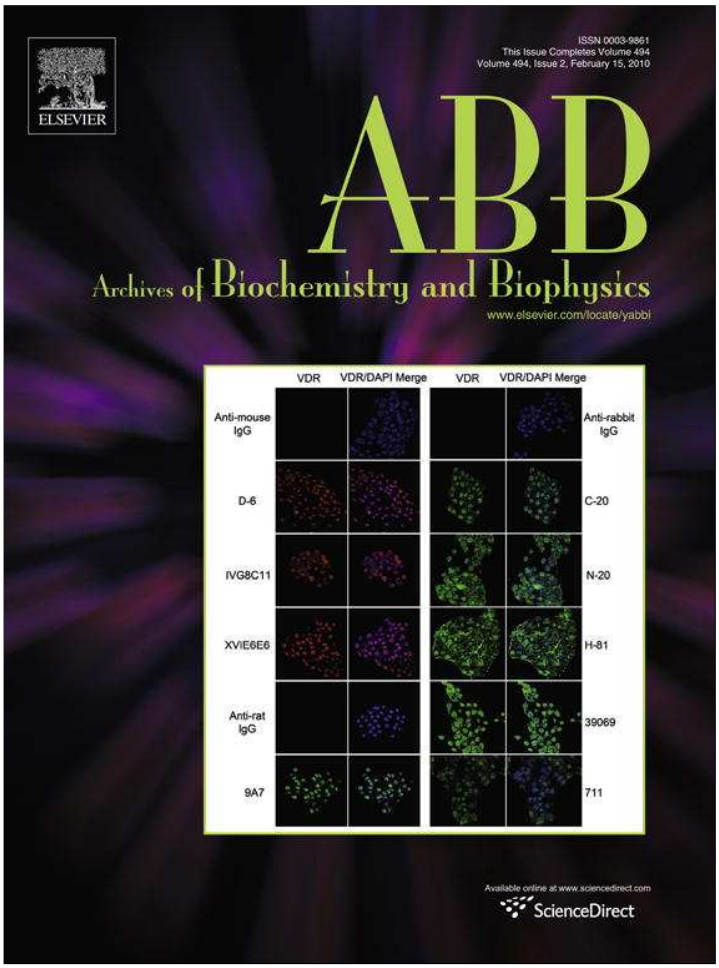

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Decolorization of malachite green by cytochrome *c* in the mitochondria of the fungus *Cunninghamella elegans*

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ABSTRACT

We studied the decolorization of malachite green (MG) by the fungus *Cunninghamella elegans*. The mitochondrial activity for MG reduction was increased with a simultaneous increase of a 9-kDa protein, called CeCyt. The presence of cytochrome *c* in CeCyt protein was determined by optical absorbance spectroscopy with an extinction coefficient ($E_{550-535}$) of $19.7 \pm 6.3\ mM^{-1}\ cm^{-1}$ and reduction potential of + 261 mV. When purified CeCyt was added into the mitochondria, the specific activity of CeCyt reached $440 \pm 122\ \mu mol\ min^{-1}\ mg^{-1}$ protein. The inhibition of MG reduction by stigmatellin, but not by antimycin A, indicated a possible linkage of CeCyt activity to the Qo site of the *bc1* complex. The RT-PCR results showed tight regulation of the *cecyt* gene expression by reactive oxygen species. We suggest that CeCyt acts as a protein reductant for MG under oxidative stress in a stationary or secondary growth stage of this fungus.

Introduction

Triphenylmethane (TPM)[1] dyes are synthetic organic dyes, which are widely used in various purposes for treatment of infectious diseases in food-producing animals and fish as well as for staining of textiles, plastics and biomaterials. The use of malachite green (MG) in industry and aquaculture is strictly controlled in many countries, not only because it is acutely toxic to aquatic organisms [1,2], but also because its biotransformed product, colorless leucomalachite green (LMG), is suspected carcinogen and mutagen [3,4].

There are several biological systems which are involved in the decolorization of TPM dye molecules. *Citrobacter* sp. strain KCTC 18061P contains a NADH-dependent reductase for the decolorization of MG, crystal violet and basic fuchsin [5,6]. Lignin-degrading fungi produce peroxidases and laccases, which degrade MG and various structurally related TPM dyes apparently with broad specificities [7,8]. Under anaerobic conditions in animal and human guts, a number of intestinal microorganisms participate in the reduction of MG and gentian violet [9–14]. Otherwise, cytochrome *b* is involved in the reduction of TPM salts like tetrazolium redox-indicators, thereby resulting in the formation of intensively colored, insoluble formazan dyes [15,16].

Cunninghamella elegans is a non-ligninolytic zygomycete fungus, which is able to degrade MG to various *N*-demethylated MG and LMG derivatives [17]. It was previously proposed that a cytochrome P450 activity was involved in the *N*-demethylation reactions of MG [17] and gentian violet [18]. There was, however, no substantial evidence of a reductive pathway for the decolorization of TPM dyes in the eukaryotic cells.

Here, we reported for the first time that purification of a *c*-type heme-containing protein, named as CeCyt from the fungus *C. elegans*. This protein was able to decolorize MG in the presence of NADH and mitochondria as the electron donor, and catalyzed the decolorization of Coomassie Brilliant Blue R-250 (CBB) with dithiothreitol (DTT). Mitochondrial inhibitors such as KCN, antimycin A, stigmatellin and Triton X-100 were used to find an electron transport chain system of the mitochondria. We also tested if mitochondria could serve as electron donor of purified CeCyt for the reduction of MG. During cultivation of the fungus, the expression levels of CeCyt were examined under various oxidative conditions such as dimethylsulfoxide (DMSO) and paraquat. Quantitative RT-PCR was performed to determine the gene expression levels of CeCyt and a cytochrome c_1 (CeCyc1) which is supposed to participate in the *bc1* complex. From this study, it was elucidated that the gene expression of CeCyt was tightly regulated by cellular content of reactive oxygen species (ROS). It will be an effective way to reduce

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[1] *Abbreviations used*: TPM, triphenylmethane; MG, malachite green; LMG, leucomalachite green; CeCyt, a cytochrome *c* of the fungus *Cunninghamella elegans*; apoCeCyt, apoprotein of CeCyt.

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the acute toxicity of MG particularly under oxidative stress at a stationary growth or secondary production stage of the fungus *C. elegans*.

Materials and methods

Chemicals and materials

Antimycin A, CHAPS, CBB, DMSO, MG (oxalate), β-NADH, β-NADPH, 2,6-dichloroindophenol sodium salt hydrate (DCIP), paraquat (methyl viologen), 6-phosphogluconate, potassium cyanide (KCN), cytochrome *c* reductase [NADPH] assay kit, cytochrome *c* reductase (type I from a porcine heart), stigmatellin and Triton X-100 were obtained from Sigma–Aldrich, St Louis, MO. A Protean II mini gel kit and protein reagents were purchased from Bio-Rad, Hercules, CA, and a FPLC system and columns used were supplied by GE Healthcare, Uppsala, Sweden. Potato dextrose (PD) media (BD-Diagnostic Systems, Franklin Lakes, NJ) and buffers used were prepared with Milli-Q water (>18.2 MΩ cm), and were sterilized by autoclave at 121 °C for 15 min or by 0.2-μm membrane filtration.

Fungal strain and cultivation

C. elegans strain ATCC36112 was kindly supplied by Dr. Carl E. Cerniglia, US FDA-NCTR, Jefferson, AR. About 10^4 outgrowing spores of this fungus were inoculated into 25 ml of PD media in Ø 150 mm petri dishes. They were incubated in zipper bags to keep the moisture during the cultivation for a week at 30 °C. In order to examine effects of ROS mediated oxidative stress on the mitochondrial activity for MG decolorization, 2% (v/v) DMSO or 100 μM paraquat was included under the same conditions. Three or more petri dishes were sacrificed timely to harvest sufficient amounts of mycelia over 47-mm Corning 25981-PF glass fiber filters by vacuum-filtration. All experiments were performed at least three times and results were reported as mean and standard deviation.

Purification of mitochondria

Harvested mycelia (4–5 g by wet weight) were suspended in three volumes of ice-cold 0.27 M sucrose–PBS buffer, and cells were disrupted using 2-mm glass beads by repeated strokes at 1000 rpm for 30 s and cooling on ice for 30 s. Purification of mitochondria and proteins was carried out at 4 °C unless otherwise stated. Glass beads and debris were removed by centrifugation at 500*g* for 30 min, and the supernatant was then centrifuged at 3000*g* for 30 min to obtain a mitochondrial pellet. The mitochondrial pellet was suspended in 2 ml of 0.27 M sucrose–PBS buffer, and subjected to discontinuous Percoll® gradient centrifugation as described elsewhere [19]. A mitochondrial fraction was gently removed and transferred to an Amicon® filtration set (Millipore Co., Bedford. MA) in order to clean up soluble protein contaminants using a 20-nm Anodisc 25 filter (Whatman, Maidstone, England). The mitochondrial fraction was washed twice with 5 volumes of the same buffer under the pressure of nitrogen gas at 3 bar. During the purification of mitochondria, no reducing reagent like DTT was used to avoid the spontaneous reduction of MG. A purification fold of mitochondria was determined by the enzyme activities of 6-phosphogluconate dehydrogenase [20] and complex II succinate dehydrogenase [21]. Protein concentration was determined using a Coomassie Plus™ Protein Assay Reagent (Pierce, Rockford, IL) and bovine serum albumin as a standard.

Inhibition of mitochondrial MG decolorization

To find an electron transport chain system for the reduction of MG, purified mitochondria (2 μg amount of protein) were treated with 1 mM KCN, 20–100 μM antimycin A, 40–200 μM stigmatellin, or 1% (v/v) Triton X-100 for 5 min under ambient conditions. The activity for MG decolorization was measured at 616 nm ($\varepsilon_{616\ nm}$ = 148,900 M^{-1} cm^{-1}) for 5 min after the addition of 1 mM NADH and 0.0001% (w/v) MG. Because it was considered that a non-enzymatic reduction of MG could occur under reduced conditions in the mitochondria, the mitochondria were treated at 80 °C for 5 min, and used as parallel controls of the respective experiments.

Purification and identification of proteins

Mitochondrial proteins were solubilized overnight in 20 mM sodium phosphate buffer (pH 6.8) containing 4% (w/v) CHAPS. After centrifugation at 120,000*g* for 1 h, the solubilized proteins were applied on a hydroxyapatite column (1.6 × 5 cm) at 1 ml min^{-1}. The chromatography with a 30-min linear gradient to 0.5 M sodium phosphate (pH 6.8) was monitored at 280 and 405 nm on a Waters 2478 dual absorbance detector (Waters Co., Milford, MA), which reported protein absorbance at 280 nm and heme signal at the ratio of A_{405}/A_{280} simultaneously. Each fraction (100 μl portion) was briefly assayed for the decolorization of CBB (final concentration, 0.0002% w/v) with 10 mM DTT in a 96-well microplate at room temperature. The specific activity for MG decolorization was determined in a single day, when 2 μg protein amount of highly purified mitochondria was given as electron donor in the presence of 1 mM KCN, 0.5 mM NADH and 0.0001% MG in the same buffer. Fractions showing dye decolorization were combined, and concentrated using a Centriprep-3 (Millipore, Bedford, MA) before the application to Superdex™ 200 gel filtration (1.6 × 60 cm) equilibrated with 20 mM sodium phosphate buffer (pH 7.0) at 0.5 ml min^{-1}.

From the differential centrifugation at 3000 *g*, the supernatant was reserved to obtain the cytosolic fraction after centrifugation at 120,000*g* for 1 h. From the cytosol, a probable apoprotein of CeCyt (apoCeCyt) without showing heme absorbance at 405 nm was purified by sequential column chromatography on DEAE–sepharose, Superdex™ 200 and Phenyl-Superose. The *N*-terminal amino acid residues of PVDF membrane-blotted proteins were determined by automated Edman degradation, and the obtained sequences were identified by BLAST searches in the cDNA library of the fungus *C. elegans* at the Functional Genomics Center, Concordia University, Montreal, Canada.

Analysis of protein properties

To examine a possible electron donor, purified CeCyt was tested for the MG decolorization with the following: 1 mM β-NADH or β-NADPH for NAD(P)H-dependent oxidoreductase; or 1 mM β-NADPH and 0.1 U cytochrome *c* reductase [NADPH] for cytochrome P450; or 1 mM β-NADH and 0.1 U porcine heart cytochrome *c* reductase for cytochrome *c* oxidoreductase; or 10 mM H_2O_2 for peroxidase activity. An isoelectric point (*p*I) of native CeCyt was determined by a vertical mini-gel method using a Bio-Lyte ampholyte (pH 3–10) and Bio-Rad IEF standards [22]. The heme absorbance spectrum was analyzed by the pyridine hemochrome assay [23]. A cuvette containing reduced CeCyt was flushed with carbon monoxide (CO) for 1 min at room temperature, and the spectrum was analyzed to examine effect of CO binding on the Soret band. To determine a reduction potential of CeCyt, a cuvette containing CeCyt (final concentration, 20 μM) and 20 μM $K_3Fe(CN)_6$ in 20 mM HEPES buffer (pH 7.0) was analyzed by a modified method from Urban and Klingenberg [24]. As an internal redox reference, 20 μM DCIP ($E^{\circ\prime}$ = +217 mV at pH 7, Ref. [25]) was included. The optical absorbance changes of CeCyt ($A_{550-535}$; extinction coefficient $E_{550-535\ nm}$ = 19.7 mM^{-1} cm^{-1} in this study) and DCIP (A_{600}; extinction coefficient $\varepsilon_{600\ nm}$ = 21.0 mM^{-1} cm^{-1}, Ref. [26]) were recorded after 1 min equilibration with the continuous additions of

3 μM sodium ascorbate at room temperature, and the fully reduced state was recorded after the addition of 0.2 mM DTT.

Reverse transcriptase-polymerase chain reactions

RT-PCR analyses were performed four times during cultivation of the fungus in the presence or absence of 2.5% DMSO or 100 μM paraquat. Total RNA was extracted by the hot phenol extraction method, and quantified by UV absorbance at 260 nm. The cDNA synthesis was carried out using a TakaraPrimeScript™ 1st strand cDNA Synthesis kit, 70 ng RNA extract and 1 pmol each of the sense primers; 5′-AAGCTTTATTTAAAACAATT for *cecyt* and 5′-AGAAAGATCTGGG GGTAAAGCACC for *cecyc1*. Using template cDNA, PCR was performed with Dr. Taq Master Mix (BioRnD, Korea) by the addition of 20 pmol each primer; forward 5′-AAGTGTCCTGCTGAGCAG and reverse 5′-AAGCTTTATTTAAAACAATT for a 406-bp *cecyt* DNA fragment, and forward 5′-TGTTCTGCTTGTCACTCTTTA and reverse 5′-AGAAAGATCTGGGGGTAAAGCACC for a 234-bp *cecyc1* DNA fragment. The PCR conditions are 2-min hot start at 94 °C and repeated 28 and 38 cycles of 94 °C/30 s, 50 °C/30 s and 72 °C/45 s for *cecyt* and *cecyc1*, respectively. To ensure no DNA contamination and normalize RT-PCR results, PCR and RT-PCR against each RNA sample were performed parallel with the universal primers LR0R and LR5 targeted for 28S ribosomal RNA of fungi. Gel images of 406-bp amplicons in a 38-cycle PCR for *cecyt*, 234-bp amplicons in a 28-cycle PCR for *cecyc1* and about 900-bp amplicons in a 30-cycle PCR for 28S rRNA were analyzed by a Gel Doc EQ system installed with a Quantity One program (Bio-Rad, Hercules, CA). The reference cDNA sequences were obtained from a cDNA library of the fungus *C. elegans* at the Functional Genomics Center, Concordia University, Montreal, Canada.

Results

Growth curves and malachite green decolorization of the fungus C. elegans

The biomass of strain ATCC36112 increased exponentially for 3 days after the cultivation with PD media (Fig. 1A). The fungus increased its mitochondrial activity for MG decolorization at late exponential or stationary growth phase with a simultaneous increase of a 9-kDa protein (Fig. 1B). In contrast, no specific activity for MG decolorization was detected from the cytosolic fractions during this cultivation period (data not shown).

A nanofiltration technique was employed to purify the mitochondria more extensively because the mitochondrial fraction obtained from differential centrifugation and Percoll gradient ultracentrifugation contained a soluble protein activity of 6PGD. The applied nanofiltration was useful for the removal of contaminated 6PGD. By this method, specific activity of complex II SDH was increased by 1.26 folds compared to the former Percoll gradient centrifugation step (Table 1). The specific activity of highly purified mitochondria for the reduction of MG was 3.6 ± 0.4 μmol min^{-1} mg^{-1} protein. The increase in specific activity for MG reduction was equivalent to the increase of SDH activity, which indicated that the two enzyme activities were located in the same sub-cellular fraction.

The mitochondrial activity for MG reduction was about 68% inhibited by treatment with 200 μM stigmatellin, whereas the MG reduction was enhanced by more than 200% in the presence of 100 μM antimycin A or 1 mM KCN. This activity was completely abolished by treatment with 0.1% Triton X-100 which solubilized the membrane. KCN, antimycin A and stigmatellin are supposed to inhibit one-electron transfer reactions of cytochrome *c* oxidase, Qi site and Qo site of the *bc1* complex, respectively. Therefore, it was thought that a specific activity for the MG reduction linked through the proton-coupled electron transfer at the Qo site of the

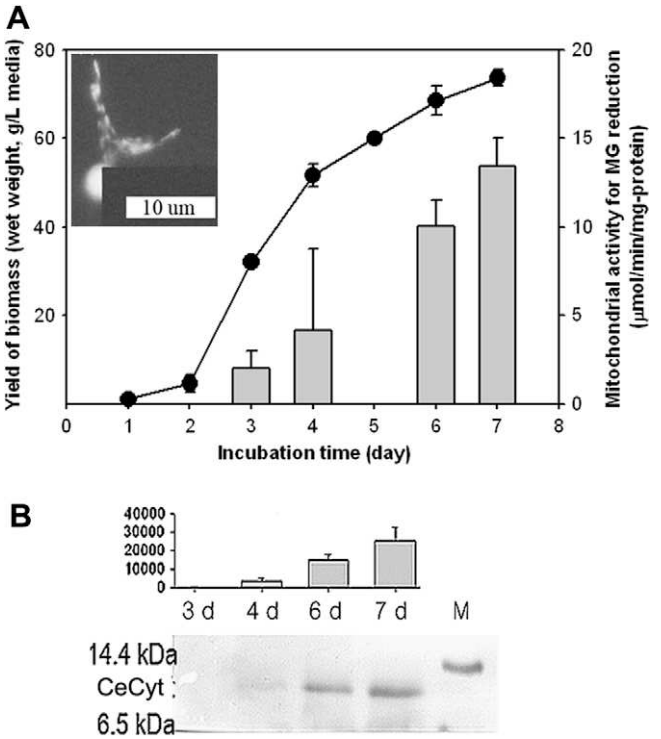

Fig. 1. (A) Growth curve (wet weight biomass) of the fungus *C. elegans* strain ATCC36112 and specific activity (bar graphs) of the mitochondria for the decolorization of malachite green. (*Inset*) An epifluorescence microscopy image of a 2-h outgrowing spore stained with rhodamine 123 in PD medium used as inocula. (B) A gel image of a 9-kDa protein (CeCyt) in the mitochondrial fractions (2 μg amount of protein loading per lane). Band intensity of CeCyt is shown with bar graphs (arbitrary units) on the lithium dodecylsulfate–PAGE gel image. M lane, molecular size markers.

bc_1 complex in the intact mitochondria. In the presence of 100 μM antimycin A, the specific activity of highly purified mitochondria for MG decolorization was 6.7 ± 0.5 μmol min^{-1} mg^{-1} protein.

Purification and characterization of CeCyt

When the fungus *C. elegans* was grown with PD media for 6 days, the intensity of CeCyt at ~9 kDa increased remarkably during a stationary or secondary growth period of 5–6 days (Fig. 2A). Beneath this protein band, a putative apoCeCyt was consistently presented by the expected size of ~700 Da lower than CeCyt in the whole cell extracts. The 6-day culture was used for the purification of CeCyt and apoCeCyt from the fractionated mitochondria and cytosol, respectively. By the expected size of ~700 Da lower than CeCyt, a protein band of putative apoCeCyt was purified to an apparent homogeneity by sequential column chromatography on DEAE–sepharose, Superdex™ 200 and Phenyl-Superose as shown in a denaturing SDS–PAGE gel (Fig. 2B). From sequential column chromatography on hydroxyapatite and Superdex™ 200, the purification of CeCyt was indicated by continual increase in the A_{405}/A_{280} ratio up to 2.0. The gel filtration of native protein showed a single peak of CeCyt at ~9 kDa, which was matched to the monomeric size of CeCyt in a denaturing SDS–PAGE gel (Fig. 2C). Using the fractions, it was confirmed that CeCyt catalyzed the decolorization of chemically stable CBB in the presence of 10 mM DTT at room temperature. The *N*-terminal amino acid residues of CeCyt and apoCeCyt were determined as XCPAEQNSQSYCN and KCPAEQNSQSY, respectively. The *N*-terminal residue X of CeCyt was determined as an *N*-acetylated lysine (acK) by com-

Table 1
Purification folds of mitochondria from fungus *Cunninghamella elegans*.

Purification steps	Total protein (mg)	Specific activity (nmol/min/mg-protein)[a]			% Recovery (SDH)[b]	Purification fold (SDH)[b]
		6PGD	SDH	MG		
Differential centrifugation (3000*g*)	7.91	76.8 ± 15.5	103 ± 8.54	2.29 ± 1.18	100	1
Percoll gradient centrifugation (22,000*g*)	0.18	268 ± 379	2470 ± 156	131 ± 36.0	54.6	24
Ø20 nm-filtration (under N_2 gas at 3 bar)	0.11	ND	3110 ± 6.95	174 ± 17.6	42.0	30

[a] Specific enzyme activities: 6PGD, 6-phosphogluconate dehydrogenase measured by the addition of 1 mM 6-phosphogluconate and 0.5 mM NADP (ε_{340nm} = 6220 M^{-1} cm^{-1}); SDH, succinate dehydrogenase (complex II) measured by the addition of 1 mM KCN, 1 mM sodium succinate, 0.5 mM NAD, 10 μM PMS and 20 μM MTT (ε_{610nm} = 11,300 M^{-1} cm^{-1}); MG, malachite green decolorization activity measured by the addition of 1 mM KCN, 0.5 mM NADH and 0.0001% (w/v) malachite green oxalate (ε_{616nm} = 148,900 M^{-1} cm^{-1}). Results from triplicate measurements are reported as mean ± standard deviation of the mean. ND, not detected.
[b] Purification fold and recovery (%) are based on the activity of SDH.

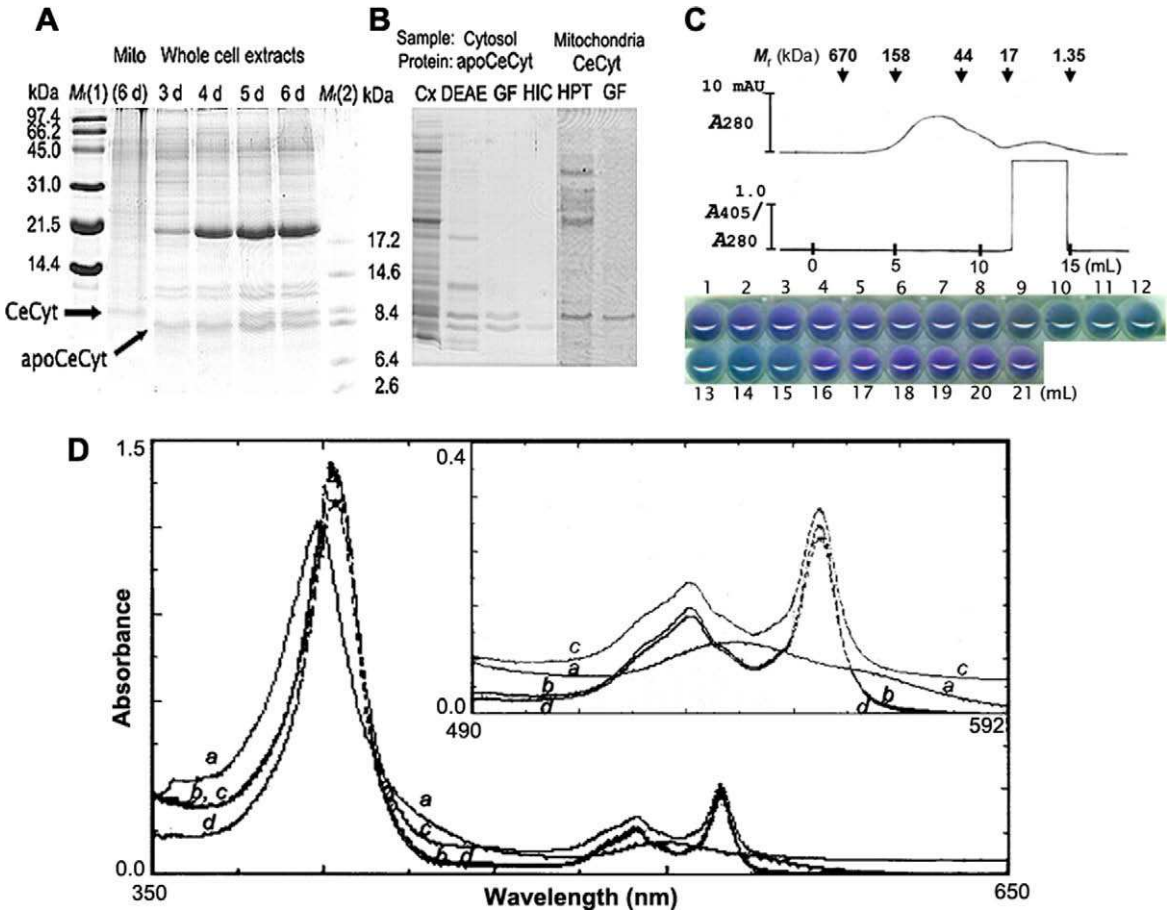

Fig. 2. (A) Expression patterns of CeCyt and apoCeCyt in whole cell extracts (Cx) and the mitochondrial fraction (Mito) prepared at day 6. Each lane was loaded with 2 μg amount of protein. (B) Purification steps of apoCeCyt from the cytosolic fraction and CeCyt from the mitochondrial fraction. The column chromatography was performed on DEAE–sepharose (DEAE, 1.6 × 20 cm); Superdex™ 200 (GF, 1.6 × 60 cm); Phenyl-Superose HR5/5 (HIC, 1 mL); and hydroxyapatite (HPT, 1.6 × 5 cm). (C) Gel filtration of native protein and a microplate test of each fraction for the decolorization of Coomassie Brilliant Blue R-250 (final concentration, 0.0002%w/v) in the presence of 10 mM DTT at room temperature. Elution and purity of a heme-containing protein was recorded with the absorbance ratio of A_{405}/A_{280}. Absorbance unit at 280 nm (A_{280}) and A_{405}/A_{280} ratio are indicated at the left of the chromatograms. (D) Absorbance spectra of CeCyt (0.1 mg ml^{-1}) between 350 and 650 nm. (*Inset*) An enlargement between 490 and 592 nm. *a*, CeCyt + 0.1 mM $K_3Fe(CN)_6$; *b*, CeCyt + 10 mM sodium ascorbate; *c*, CeCyt + 10 mM DTT; *d* = *c* + carbon monoxide (gas).

parison with phenylthiohydantoin (PTH) derivative of an authentic *N*-acetyl-L-lysine. Therefore, both proteins contained the identical *N*-terminal amino acid sequences. However, apoCeCyt displayed neither heme absorbance nor catalytic activity for the decolorization of CBB and MG. That is, the heme attachment to apoCeCyt and the location to the mitochondria may be essential steps for maturation of the catalytic activity of CeCyt.

As shown in Fig. 2D, the spectrum of oxidized CeCyt (*a*) showed two absorbance maxima at 408 nm (Soret-band) and 538 nm (α-band). When CeCyt was treated with 10 mM sodium ascorbate (*b*) or DTT (*c*), the Soret-band shifted to 413 nm, and the two peaks of β-band and γ-band were shown at 520 nm and at 549.5 nm, respectively. A carbon monoxide treatment (*d*) did not affect the spectrum of CeCyt in the ferrous state. These optical characteristics were similar to Class I cytochrome *c*, which contains a sixth ligand from an amino acid residue at the iron center. The extinction coefficient ($E_{550-535}$) of CeCyt was 19.7 ± 6.3 mM^{-1} cm^{-1}. The heme of CeCyt was covalently bound because it was not released by the denaturation process at 100 °C under reduced SDS–PAGE conditions.

From a log–log plot of $[DCIP_{ox}]/[DCIP_{red}]$ vs $[CeCyt_{ox}]/[CeCyt_{red}]$, the reduction potential of CeCyt was determined to be + 261 mV in 20 mM HEPES buffer, pH 7.0 (Fig. 3A). The calculated slope

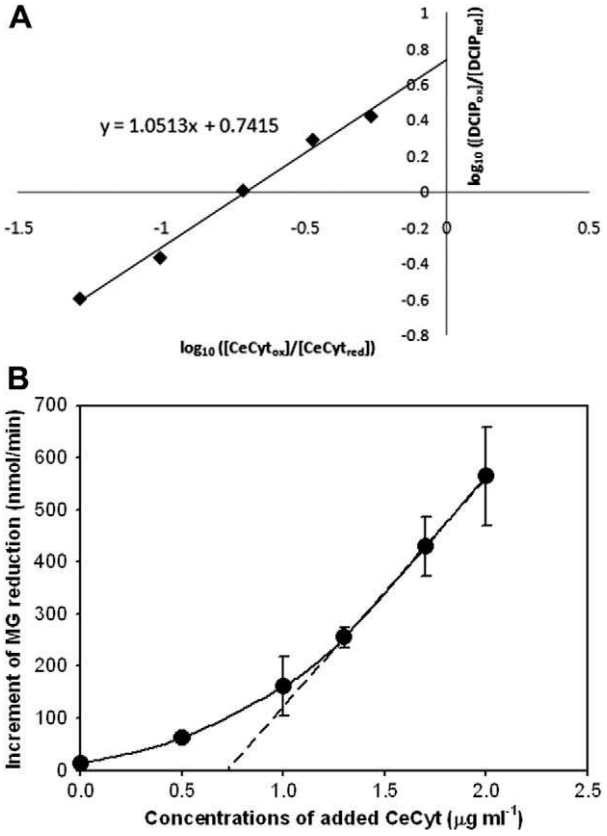

Fig. 3. (A) Log–log plot of calculated mole fractions of $[DCIP_{ox}]/[DCIP_{red}]$ and $[CeCyt_{ox}]/[CeCyt_{red}]$ between 20% and 80% of the fully reduced states. The best-fit to the Nernst equation (R^2 = 0.99) results in the slope (n_{DCIP}/n_{CeCyt}) of 1.05 and the *y*-intercept ($n_{DCIP} \times (E^{o\prime}{}_{CeCyt} - E^{o\prime}{}_{DCIP})/59.1$ mV) of 0.74. (B) Specific activity of added CeCyt for malachite green reduction in the mixture with 2 μg amount of protein of highly purified mitochondria. The mixture was incubated for 5 min at room temperature before the measurement was started by the addition of 0.0001% malachite green, 0.5 mM NADH and 1 mM KCN. The average values of triplicate determinations at above 1 μg ml^{-1} of added CeCyt result in the best-fit to a straight line (R^2 = 0.99) with a slope of 440 nmol min^{-1} μg^{-1} protein.

(n_{CeCyt}/n_{DCIP}) of 1.05 indicated one-electron transfer between CeCyt and DCIP. When various concentrations of purified CeCyt were added into the mitochondria (protein amount, 2 μg ml^{-1}), the specific activity of CeCyt for MG reduction was determined to be 440 ± 122 μmol min^{-1} mg^{-1} in a linear range above 1 μg ml^{-1} of added CeCyt (Fig. 3B). A lower concentration of added CeCyt at below 1 μg ml^{-1} resulted in a concave slope of the MG reduction, probably because the given CeCyt was not properly localized to the mitochondria. Otherwise, the other tested oxidoreductases (a NADH/NADPH-dependent oxidoreductase, a cytochrome *c* reductase for cytochrome P450 monooxygenase, a terminal cytochrome *c* reductase and a peroxidase) and reducing/oxidizing substances (β-NADH, β-NADPH and H_2O_2) did not enable CeCyt to reduce MG.

Identification and expression patterns of the cecyt gene and a cytochrome c_1 (cecyc1) gene

The *N*-terminal amino acid sequence of apoCeCyt showed perfect match to the deduced amino acid sequences from three cDNA sequences in the cDNA library of the fungus *C. elegans* (Fig. 4). The *cecyt* gene without intron was confirmed by chromosome walking on the genomic DNA sequence of *C. elegans* strain ATCC36112 (GenBank accession No., DQ389156). The translated protein sequences of the retrieved *cecyt* genes consist of an 18-residue signal peptide at the *N*-terminus and a mature portion of 81 amino acid residues that starts from a lysyl group (K) at the 19th position being consistent with the Edman degradation results. A predicted sequence of mature apoCeCyt has the theoretical molecular weight of 8970.11 and *p*I value of 8.66 as similar to the observed M_r and *p*I values of 9 kDa and 8.4. However, the apoCeCyt sequence contains no canonical CXXCH motif site or any conserved sequence of cytochrome *c* in publicly available genomic databases. Some cytochromes *c* and c_1 isolated from the mitochondria of *Euglena*, *Trypanosoma* and *Leishmania* contain only single CH motif sites for the cytochrome *c* maturation [27]. But, it was not yet verified if the fungus *C. elegans* possesses a specific heme lyase system to use a single CH motif at the 32nd or 45th position of CeCyt.

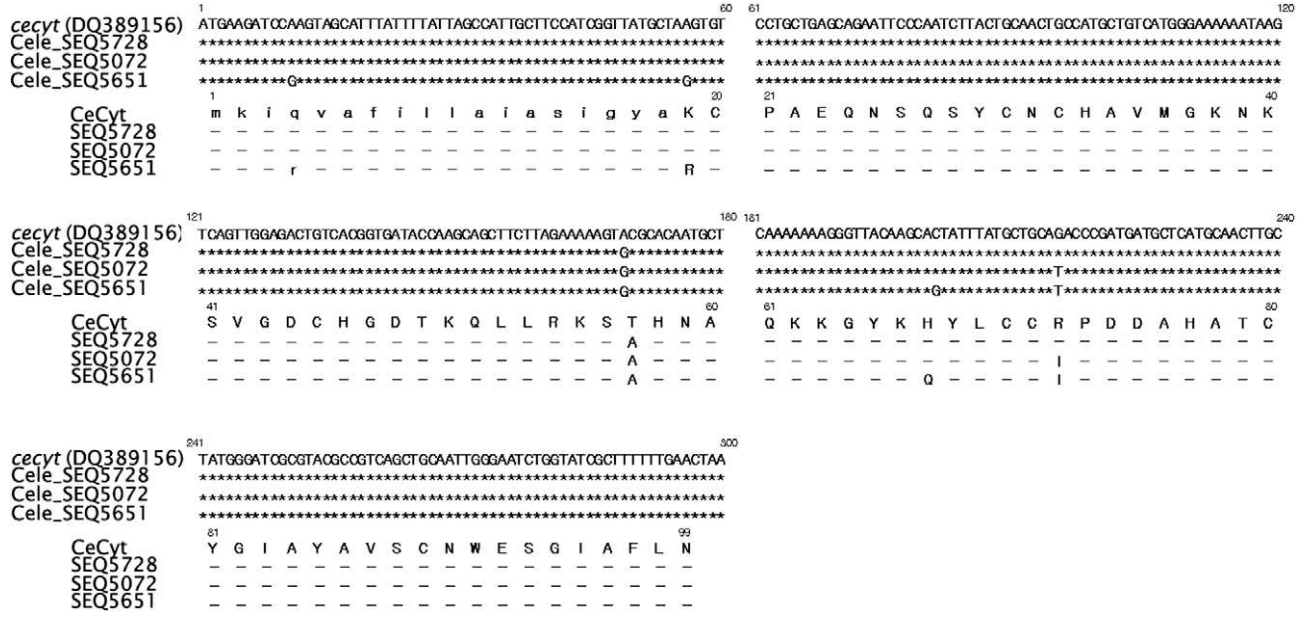

Fig. 4. Variations in copy number and DNA sequence of the *cecyt* genes in the fungus *C. elegans*. The gene sequence for CeCyt (DQ389156) was obtained from genomic DNA of *C. elegans* ATCC36112. The reference cDNA sequences, Cele_SEQ5728, Cele_SEQ5072 and Cele_SEQ5651, were obtained from a cDNA library of the fungus *C. elegans*. Identical sequences of nucleotides and amino acids are indicated with asterisks and bars, respectively. The *N*-terminal signal peptide consisting of 18 amino acid residues is shown with the lowercase letters.

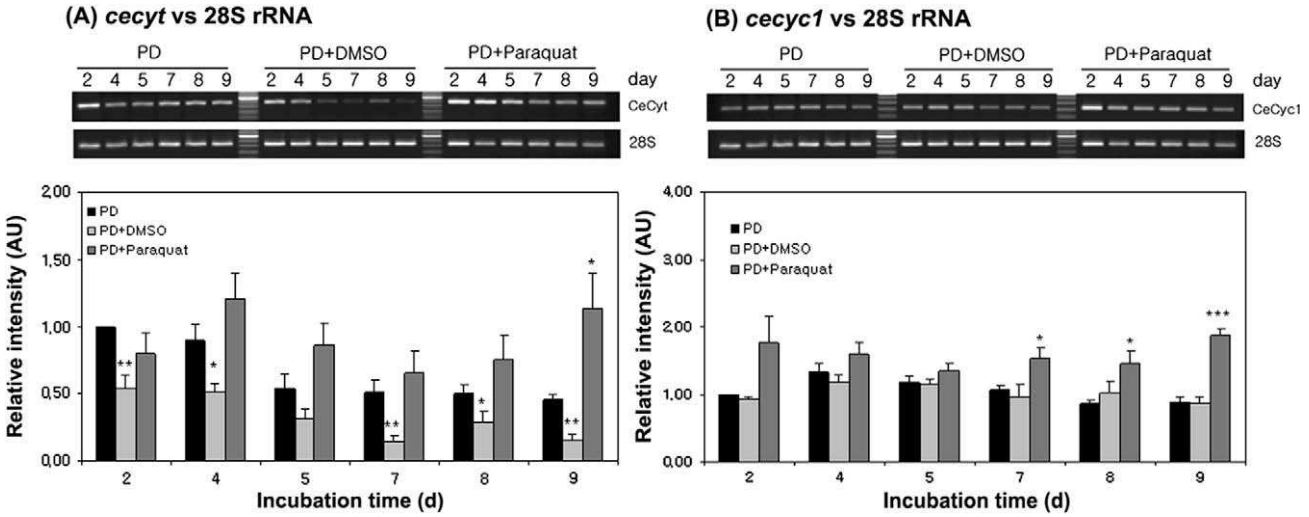

Fig. 5. Representative gel images of quantitative RT-PCR analyses for the determination of (A) *cecyt* and (B) *cecyc1* gene expression levels. Cells were grown with PD media in the presence or absence of 2% DMSO or 100 μM paraquat. Bar graphs show the normalized average and standard deviation (error bars) of four determinations of mRNA levels relative to 28S ribosomal RNA, and normal mRNA levels obtained from PD media at day 2 are expressed as one unit. Statistical differences between treated and non-treated cells were analyzed by *t*-tests with the significance levels, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

To analyze expression profile of the *cecyt* gene under various oxidative conditions, the fungus was cultivated in the presence of 100 μM paraquat or 2.5% DMSO. The level of the *cecyt* mRNA was increased in the presence of 100 μM paraquat, but it was significantly decreased in the presence of 2.5% DMSO (Fig. 5A). Also the gene expression of a cytochrome c_1 (CeCyc1, GenBank accession No. GQ415053) was analyzed because it is supposed to play a pivotal role in the electron transfer from the *bc1* complex. By using a consensus sequence of fungal cytochrome c_1, the RT-PCR primers were designed to obtain a partial *cecyc1* cDNA (234 bp). The deduced amino acid sequence of CeCyc1 was 83% identical to a putative cytochrome c_1 of *Cryptococcus neoformans* var. *neoformans* JEC21 (AAW44407.1). The RT-PCR results showed that the *cecyt* mRNA levels were increased along with the increase of *cecyc1* mRNA levels in the presence of 100 μM paraquat, whereas *cecyc1* mRNA levels were almost constant when cells were exposed to 2.5% DMSO (Fig. 5B). The expression levels of the two genes were simultaneously increased under oxidative stress, although they were regulated independently.

Discussion

This study demonstrates that a novel *c*-type cytochrome, CeCyt, was involved in the decolorization of TPM dyes including MG and CBB in the mitochondria of the fungus *C. elegans*. The inhibition by stigmatellin, which binds to the heme b_1 domain of cytochrome *b* and the iron–sulfur protein at the Qo site, indicates that reducing power of CeCyt is replenished through the proton-coupled electron transfer at Qo site of *bc1* complex. Incidentally, the bc_1 complex transports electrons from an inner membrane NADH: ubiquinone oxidoreductase through the bifurcated Qo site to a variety of oxidized and ionized molecules at the surface of the outer membrane [28–30]. Although the location of CeCyt was not yet confirmed, the inhibition of the MG reduction by stigmatellin indicates that it probably serves as a reductant of MG in the intermembrane space or at the surface of the outer membrane of the mitochondria.

The addition of purified CeCyt to the mitochondria increased the rate of MG decolorization. Considering that a low concentration of the given CeCyt resulted in a concave slope of the MG reduction, a high concentration of CeCyt is seemingly needed for the proper efficiency of successive one-electron transfers ($2 \times e$) to MG at the vicinity of the catalytic sites. A respiratory chain complex of *Shewanella oneidensis* MR-1 was similarly involved in the decolorization of a diazo dye Remazol Black B (Reactive black 5) and an oxazine dye Levafix Royal Blue E-FR (Reactive blue 224) directly by the outer membrane cytochromes or indirectly via electron-mediating melanin [31]. In *S. oneidensis*, multiheme *c*-type cytochromes, CymA and MtrA, function to transfer electrons from the inner membrane quinone/quinol pool through the periplasm to the outer membrane, and the metal (hydr)oxides are reduced most likely by translocating decaheme *c*-type cytochromes, MtrC and OmcA, across the outer membrane to the surface of bacterial cells [32].

During cultivation of the fungus *C. elegans*, the *cecyt* gene expression appeared to be tightly regulated by cellular content of reactive oxygen species such as superoxide and hydroxyl radical. Superoxide-generating paraquat increased the *cecyt* mRNA levels, which made equivalent to the expression levels of a cytochrome c_1 gene (*cecyc1*). The calculated reduction potential of CeCyt ($E^{o\prime}$ = +261 mV at pH 7) is close to the literature value of cytochrome c_1 in a range of +220 to +290 mV [33]. It seems that the coupled reaction of CeCyt and cytochrome c_1 is effective to reduce MG, since the redox potential of MG is accounted for +489 mV at pH 7 [34]. A cytochrome c_1 is supposed to participate in the electron leakage from the bc_1 complex to oxygen thereby resulting in the formation of superoxide. If CeCyc1 is upregulated by oxidative stress, the concomitant upregulation of CeCyt may be an important means for the prevention of complex III-mediated reactive oxygen species generation by the scavenging of such electrons. It was previously reported that the presence of a ROS scavenger in *C. neoformans* [35] or the deficiency of a cytochrome c_1 in *Podospora anserine* [36] were effective in sustaining fungal growth for a prolonged period by the reduction of cellular ROS content. However, more studies are still needed to elicit a biological function of CeCyt particularly in relation to the protection of cells against ROS generation and oxidative damage in a stationary growth or secondary production stage of this fungus.

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