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ORIGINAL RESEARCH PAPER

Expression of a mitochondrial gene *orfH79* from the CMS-HongLian rice inhibits *Saccharomyces cerevisiae* growth and causes excessive ROS accumulation and decrease in ATP

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Abstract Cytoplasmic male sterility (CMS) has often been associated with abnormal mitochondrial open reading frames (ORF), *orfH79* is a mitochondria chimeric gene being responsible for the CMS trait in Honglian (HL) rice. Weakly expressed ORFH79 strongly inhibits the growth of yeast cells. In addition, the content of reactive oxygen species (ROS) in the transformants that expressed ORFH79 was increased by 31%, and ATP was decreased by 41% compared with the control. These results showed ORFH79 peptide is toxic to yeast cells.

Keywords ATP level · CMS · Reactive oxygen species (ROS) · Rice · *orfH79* · Yeast growth

Introduction

Cytoplasimc male sterility (CMS) is a widespread phenomenon in plant kingdom, which is maternally inherited and characterized by a failure to produce functional pollen (Young and Hanson 1987). In most cases, the failure of pollen development in CMS background is associated with chimeric mitochondrial open reading frames (ORFs) arising from unusual recombination events (Hanson and Bentolila 2004). In previous studies, many CMS-associated genes such as T-maize *urf13*, sunflower *orf522,* Brassica Ogura radish *orf138* and BT-rice *orf79* have been shown to encode peptides which are lethal to *E. coli* (Dewey et al. 1988; Duroc et al. 2005; Nakai et al. 1995; Wang et al. 2006). However, the toxicity of this kind of peptide in eukaryotic cell is seldom studied.

Honglian cytoplasmic male sterility (CMS-HL) rice (*Oryza sativa*) is one of the three typical CMS systems of rice. Hybrid rice varieties based on CMS-HL have been widely grown in China and South Asia since the beginning of this century. Molecular dissection reveals the mitochondrial chimeric gene *orfH79*, which is located downstream of *atp6*, has been proposed be responsible for the CMS trait in CMS-HL rice (Yi et al. 2002). Li et al. previous reported that excessive ROS levels was found during microgenesis in CMS-HL, furthermore, the depletion of ATP and NADH were also testified in the CMS-HL rice (Li et al. 2004; Wan et al. 2007). However, whether the abnormal physiological characters is caused by ORFH79 remains unclear.

In this paper, we introduced *orfH79* gene into *Saccharomyces cerevisiae,* an efficient eukaryotic host for heterologous gene expression, and we found that weakly expressed ORFH79 strongly inhibits the growth of yeast. In addition, excess content of reactive oxygen species (ROS) and decreased ATP level were

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detected in the transformant that expressed ORFH79. To our knowledge, this is the first report that the product of CMS associated gene is toxic to yeast cells.

Materials and methods

Yeast strains and media

The strain used in this work was the wild-type SEY6210 (Robinson et al. 1988) from *Saccharomyces cerevisiae,* phenotype: *MAT α suc2-9 ura3-52 leu2-3,113 his3-200, trp1-901 lys2-801*. The strain was routinely grown on YPD medium (10 g yeast extract/l, 10 g peptone/l, and 20 g glucose/l) at 30°C. Yeast cells were grown on supplemented synthetic complete medium (SC-ura medium [0.67 g yeast nitrogen base/l, 20 g glucose/l and appropriate amino acids]) lacking uracil to screen positive transformants, or YPGal medium (0.67 g yeast nitrogen base/l, 20 g galacose/l, and appropriate amino acids) when inducing expression.

Plasmid construction for expression of *orfH79* in yeast

DNA fragment encoding *orfH79* was amplified from the CMS-HL rice DNA by PCR using a primer set (forward, 5′-GCCGGATCCATGACAAATCTGCTC CGATGGC TC-3′; reverse, 5′-GCCCTCGAGTTA CTTAGGAAAGACTACACG-3′). The *orfH79* gene was ligated to the pYes2 vector (Invitrogen) digested with *Bam*HI and *Xho*I to construct the plasmid pYesH79. The plasmids pYesH79 and pYes2 empty vector were transformed into *Saccharomyces cerevisiae* strain SEY6210.

Preparation of total protein extract from yeast

One ml yeast cell culture was centrifuged at 10,000 × g for 10 min, the pellet was taken up in 150 µl extraction buffer (1.85 M NaOH, 2% v/v 2-mercaptoethanol) and vortexed repeatedly for 3 min. The mixture was added to 64 µl 100% trichloroacetic acid, set on ice for 10 min and then centrifuged at 18,000 × g for 5 min at 4°C. The supernatant was discarded and the protein pellet was washed twice with 1 ml ice-cold acetone. After the remaining acetone was removed by air drying at room temperature, the protein pellets were suspended in 40 µl PBS buffer and placed on ice.

Antibody preparation

A peptide corresponding to the amino acid residues from 37 to 55 of ORFH79 was synthesized as an antigen. Antisera were prepared in rabbits by Alpha Diagnostic International (San Antonio, Tex., USA).

Western blot

Equal amounts of proteins from each fraction were separated by 18% SDS-PAGE, and then transferred onto an Immobilon-PSQ transfer membrane (PVDF type; Millipore). The ORFH79 antibody with 1:500 dilution and a goat anti-rabbit IgG conjugated with alkaline phosphatase (AP) with a dilution of 1:2000 were used for western blot. Finally, signal was detected with a method described in Yue et al. (2004) with brief modifications: the membrane was washed with AP 7.5 buffer (0.1 M Tris/HCl pH 7.5, 0.1 M NaCl, 2 mM $MgCl_2$) twice, and once with AP 9.5 buffer (0.1 M Tris/HCl pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) for 10 min each. Then the membrane was incubated with 2.5 mg Nitroblue Tetrazolium (Promega) and 1.25 mg 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Promega) in 7.5 ml AP 9.5 buffer at room temperature until the signal appeared. Finally, TE buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA pH 8.0) was added to stop the reaction.

Yeast viability and growth curve assay

To assay the viability of these transgenic yeast, the overnight cultures were adjusted to OD_{600} of 1, and ten-fold serial dilutions were spotted onto SC-ura and YPGal agar plates respectively. These plates were incubated for 48 h at 30°C. To test the growth of yeast cells, the cultures were grown overnight at 30°C with shaking (220 rpm), and the OD_{600} was monitored.

Measurement of intracellular oxidation levels

The oxidant-sensitive probe, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) was utilized in the measurement of intracellular oxidation levels in yeast. After cultured for 10 h in SC-ura and YPGal medium respectively, the recombinant cells were

collected and resuspended in Tris/HCl (pH 8.0, 1 mM) buffer. The cells were incubated with 10 μg H_2DCFDA/ml for 2 h with shaking at 30°C (Madeo et al. 1999). After being briefly rinsed three times with Tris/HCl buffer to remove the dye, yeast images were collected with a confocal microscope. The percentages of H_2DCFDA-stained cells were account at least three visual field. To detect the ROS content, the H_2DCFDA-stained cell suspensions were gently passed through a 40 μm nylon mesh filter to remove large aggregates and then analyzed with a FACVantage flow cytometer (Beckman Counter-Epics XL, Beckman Coulter Inl. SA, Nyon, Switzerland) as described in Hensley and Floyd (2003). ROS content in the yeast cells was measured by the fluorescence intensity of H_2DCFDA excited at 488 nm.

Detection of ATP

The amount of ATP was measured by the luciferin-luciferase method (John 1970) following the protocol of ATP detection kit (Beyotime, China). After being cultured in either SC-ura and YPGal medium for 10 h, the recombinant cells were adjusted to OD_{600} of 1.0. Then, 2 ml of each cell culture were collected and centrifuged at 12,000 × g for 5 min. The pellets were ground with 200 μl lysis buffer from the ATP detection kit. After centrifuged at 12,000 × g for 5 min at 4°C, the supernatant was transferred to a new tube for ATP test. The luminescence from a 100 μl sample was assayed in a luminometer (Perkin Elmer) together with 100 μl ATP detection buffer from the ATP detection kit. The standard curve of ATP concentration was prepared from a know amount (1 nM–1 μM).

Results and discussion

Transformants construction and expression of *orfH79*

Expression cassette of pYesH79 was transformed into *Saccharomyces cerevisiae* (Fig. 1a). We extracted the total protein of yeast transformants after induction and then carried out SDS-PAGE and western blot analysis. ORFH79 protein was not observed on Coomassie Blue gels (data not shown) while the expression of ORFH79 was confirmed by western

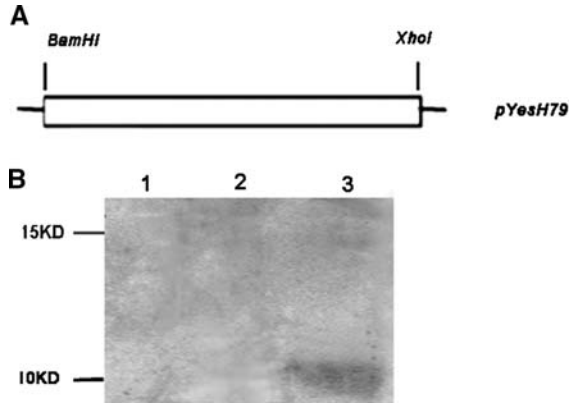

Fig. 1 The *orfH79* transform cassette, and its expression in yeast. **a** Plasmid construction for expression in yeast. **b** Expression of *orfH79* in yeast was confirmed by western blot. Lane 1: Total protein of the empty *Saccharomyces cerevisiae* strain SEY6210; lane 2: Total protein from the tranformant pYeas2, YPGal medium; lane 3: Total protein of the transformant pYesH79 YPGal medium

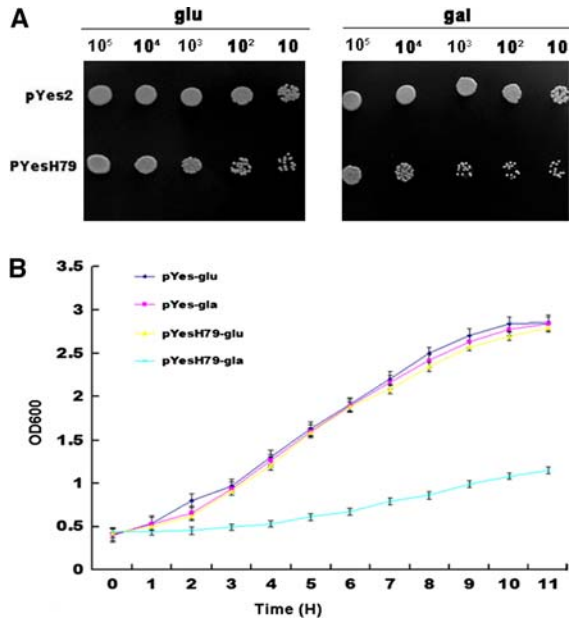

Fig. 2 The effect of *orfH79* expression on yeast growth. **a** Analysis of transformed yeast cell growth. Yeast cells carrying pYeas2 and pYesH79 were incubated in liquid SC-ura medium overnight at 30°C. The overnight cultures were adjusted to OD_{600} at 1.0, and then diluted 10-, 100- 1,000-, 10,000- fold with SC-ura medium, and then each dilution series were spotted on SC-ura and YPGal agar plates respectively. The cells were grown at 30°C for 3 days. **b** Growth curve of tansformants in liquid culture. Cell density was determined at various time points at OD_{600}. Cells were cultured at 30°C glu, glucose; gal, galactose. Data were presented as mean ± SE of triplicate

blot assay (Fig. 1b). A band about 9 kDa was detected in the total protein of pYesH79 transformed yeast. No signal was either detected in the empty plasmid (pYes2) transformed yeast or the empty *Saccharomyces cerevisiae* strain.

Expression of *orfH79* inhibit the growth of yeast

In the processes of inducing *orfH79* expression, we found the growth of pYesH79 transformant was inhibited compared with the control, and then we tested the yeast viability and cell growth curve (Fig. 2). As shown in Fig. 2a, transformants of pYes2 and pYesH79 exhibited the similar viability on the SC-ura plate, while on the YPGal plate, the pYesH79 transformant revealed reduced cell viability compared with the pYes2 transformant. Meanwhile, we also preformed experiments using liquid medium to monitor growth curve of these transformants (Fig. 2b) and got the same result that the expression of *orfH79* inhibit the growth of yeast cells.

Excessive reactive oxygen species (ROS) produced causing by expression of *orfH79*

The ROS level is abnormal in CMS-HL rice during microsporogenesis (Li et al. 2004). To address the question as to whether the expression of *orfH79* is associated with the abnormal ROS content, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), an oxidant-sensitive probe was used for the ROS detection in these transformants. As shown in Fig. 3, about 19% of the pYesH79 transformed cells were fluorescent after cultured in YPGal medium for 10 h. In contrast, pYes2 transformants only showed 5% of H_2DCFDA-stained cells. To quantify the ROS dose of these transformants, flow cytometer was used (Fig. 4). The data showed that H_2DCFDA fluorescence intensity in induced pYesH79 transformant was increased by 31% compared with basal level, while the H_2DCFDA fluorescence intensity of the pYes2 showed almost no change in SC-ura medium and YPGal medium. These results here showed that expression of ORFH79 can cause excessive ROS content in yeast.

The ATP level is reduced in the *orfH79* expressed transformant

Excessive ROS increases in general under conditions of mitochondria dysfunction (Bras et al. 2005), ATP content is one of the important indexes of mitochondrial activity. To find out whether the mitochondria activity is normal, the ATP content of these transformants were monitored. The data showed that the ATP content in ORFH79 expressed transfrmant was decreased by 41% than that in the control

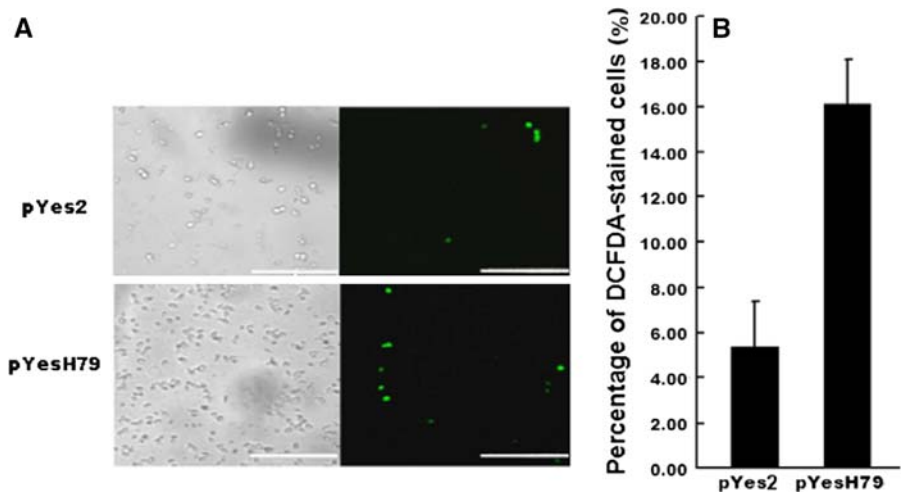

Fig. 3 ROS determine in the transformants. **a** Confocal micrographs illustrating ROS production in yeast cells grown in YPgal medium. Transformants (pYes2, pYesH79) were cultured in YPGal medium for 10 h at 30°C. Then, the cell were stained with H_2DCFDA and examined by confocal micrographs. Bar, 100 μm. **b** Percentage of ROS-accumulating cells of the total yeast cells. H_2DCFDA positive cells were counted in fluorescence images and total cells in corresponding bright images. Each value represents the mean ± SE of three different visual field with a confocal microscope

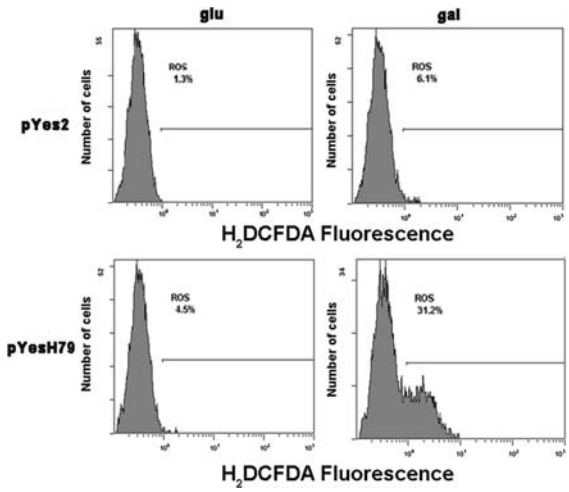

Fig. 4 Comparison of ROS generation of the transformants. The transforments were cultured in SC-ura and YPGal medium respectively for 10 h, and then, the cell suspension were stained with H_2DCFDA. The ROS dose of these transformants were detected using flow cytometer. x-axis, H_2DCFDA fluorescence; y-axis, number of yeast cells

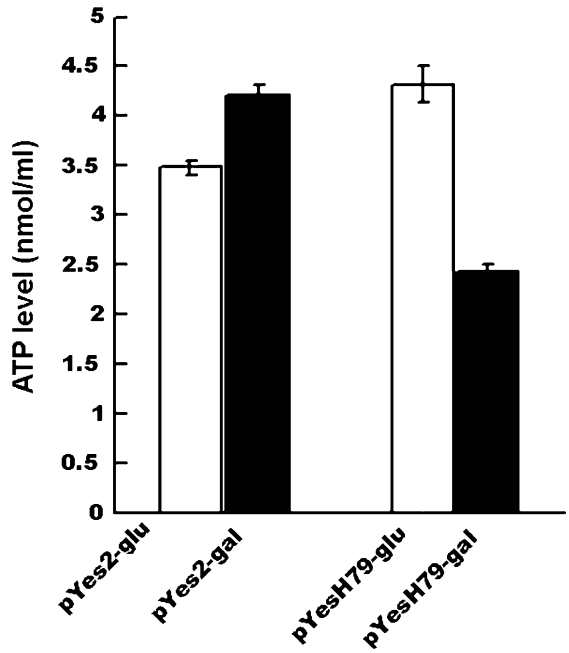

Fig. 5 ATP content assay in transformants. Tranformanrs carrying carrying pYeas2 and pYesH79 were incubated in liquid SC-ura and YPGal medium respectively for 10 h at 30°C. Then the cultures were adjusted to OD_{600} at 1.0 and the ATP content of these transformants were detected. Data were shown as the mean ± SE of triplicate

transformant (Fig. 5). These results indicate that the mitochondria function is impaired in the transformants expressed ORFH79.

Previously, a few of CMS-associated genes (maize *urf13*, sunflower *orf522*, radish *orf138* and rice *orf79*) have been shown to encode peptides which are lethal to *E. coli*, and the heterologous expression of *orfH79* in *E. coli* also inhibits bacterial growth (data not shown). However, report about the kind of protein being toxic to the yeast cell growth has not been seen. Sometimes, over-expression of heterologous protein is toxic to yeast, however, this is very unlikely for ORFH79 in the present experiment, although ORFH79 can be readily detected by western blot, too little protein is produced for direct test by SDS-PAGE gel. Therefore, ORFH79 is toxic to yeast at low concentrations.

Induced pYesH79 transformants were found produced excessive content ROS and reduced ATP level compared with the control, these results were in accordant with Wan et al. (2007) previously report in microgenesis of CMS-HL rice, showing that the physiological features in yeast expressing ORFH79 mimic that in microspore of CMS-HL rice during microgenesis. The pollen-specific phenotype in CMS systems and not technically feasible to handle in plant increase the difficulties in studies of the mechanism of CMS of plant, therefore, our find in the experiment may provide a clue to get some information about the mechanism on CMS of Honglian rice using the *Saccharomyces cerevisiae* model system.

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