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Research paper

Peculiar genes for thermostable bifunctional catalase-peroxidases in *Chaetomium thermophilum* and their molecular evolution

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ABSTRACT

Catalase-peroxidases represent one important subfamily of ancestral antioxidant enzymes originally evolved in bacteria for the protection against various forms of oxidative stress. *KatG* genes coding for these bifunctional catalase-peroxidases were during their peculiar evolution transferred from Bacteroidetes to the fungal phylum Ascomycota via a horizontal gene transfer event. Here we analyse a newly discovered fungal *katG* gene without introns coding for a thermostable catalase-peroxidase from *Chaetomium thermophilum* var. *dissitum* and compare it with closely related thermophilic and mesophilic *katGs* and their translation products. We show that *CthediskatG* gene resembling its bacterial counterparts has a typical eukaryotic transcription start site and also contains a conserved eukaryotic polyadenylation signal behind its 3′ terminus. Moreover, we have detected polyA tails in corresponding transcripts of *katG* from two different mRNA libraries of *C. thermophilum* var. *distum*. Although otherwise highly conserved, only in *katG* genes of two *C. thermophilum* variants a unique 60 bp long deletion leading in the translated product with high probability to a modified loop and thus access to the prosthetic heme group was observed. We also present an updated molecular phylogeny revealing the evolutionary position of fungal thermostable catalase-peroxidases within a robust phylogenetic tree of the whole KatG subfamily.

1. Introduction

Hydrogen peroxide is one of the most frequently occurring reactive oxygen species (ROS) in the biosphere. H_2O_2 is regularly generated in most known organisms mainly as a by-product of the aerobic metabolism. Generally, ROS are harmful or even toxic for all living organisms and their production and degradation must be tightly coordinated and regulated in all cells (e.g. Camejo et al., 2016; Gammella et al., 2016). However, the molecules of hydrogen peroxide can also possess several important physiologic roles mainly in signal transduction pathways (Apel and Hirt, 2004). Catalase-peroxidases are unique bifunctional enzymes (E.C. 1.11.1.21) containing heme *b* as the prosthetic group and capable of efficient removal of peroxide bonds thus contributing to ROS equilibrium in various cells. From the phylogenetic point of view, they represent a basal evolutionary line leading to a divergent evolution of a large and ubiquitous peroxidase-catalase superfamily (Zamocky et al., 2015). Their peculiar fused genes mostly abbreviated *katGs* were proposed earlier as unique relicts of a very ancestral gene duplication event from a primordial peroxidase gene (Welinder, 1991; Zamocky, 2004). Although they were for a longer time known and analysed only among bacteria and archaea (Faguy and Doolittle, 2000) later it was shown that corresponding highly similar genes are also present and distributed in the fungal kingdom (Klotz and Loewen, 2003) where they were apparently transferred by horizontal gene transfer from bacteria (Zamocky et al., 2012a). Additionally, also several other eukaryotic catalase-peroxidase genes were detected in the meantime so a novel reconstruction of KatG phylogeny (i.e. the phylogeny of the monophyletic catalase-peroxidase gene family) can provide interesting insights in the peculiar evolution of such enzymes mainly involved in active defence against oxidative stress with the evolutionary advantage of their real bifunctionality (e.g. Vega-García et al., 2018).

Chaetomium thermophilum is an ascomycetous, thermophilic fungus (Amlacher et al., 2011) belonging to the order of Sordariales. Thermophilic fungi are unique among eukaryotic organisms as they can

Abbreviations: CDS, coding sequence; GB, GenBank; *katG*, gene coding for a bifunctional catalase-peroxidase; HGT, horizontal gene transfer; ML, maximum likelihood phylogenetic method; PDB, protein data bank; PolA, polyadenylation site; ROS, reactive oxygen species; RT-qPCR, reverse-transcription quantitative real-time polymerase chain reaction; TBE, Tris–Borate–EDTA; TSS, transcription start site; UTR, untranslated region

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withstand elevated temperatures that are harmful for majority of rather sensitive eukaryotic cells. Currently, thermophilic fungi are attracting interest for their possible biotechnological potential due to fact that they can be sources of a wide variety of divergent thermostable enzymes (van den Brink et al., 2015). Only a few species of fungi have the ability to thrive at temperatures between 45° and 55 °C, and most of them are placed into the Ascomycota orders Sordariales, Eurotiales and some of them in the Mucoromycota order Mucorales (Morgenstern et al., 2012; Maheshwari et al., 2000). However, in comparison to their mesophilic counterparts like *Chaetomium cochliodes* (Zamocky et al., 2016) only very little is known about their response to oxidative stress that together with elevated temperatures can possess a challenge for growth of fungal mycelia. Therefore, we have focused our here updated phylogeny for the subfamily of bifunctional catalase-peroxidases mainly on eukaryotic thermophilic representatives and we perform here also the analysis of a native thermostable fungal *katG* by the means of RT-qPCR for a newly sequenced member with potential future application of the corresponding recombinant enzyme in biotechnologies.

2. Materials and methods

2.1. Fungal strain and cultivation

The strain *Chaetomium thermophilum* var. *dissitum* (Cooney & R. Emerson) was obtained from the internal collection of the Institute of Molecular Biology, Slovak Academy of Sciences in Bratislava, Slovakia. For a long-term storage of this strain two preservation methods were chosen, either an overlay of the fungal culture with paraffin (Nakasone et al., 2004) or drying of its stock on a sterile filter paper at 4 °C. During all here described experiments this fungus was cultivated on Malt extract–Peptone–Glucose (MPG) liquid medium (20 g Malt extract, 1 g Peptone, 20 g Glucose/L; pH 5.4) or on MPG-agar plates (additionally supplemented with 20 g agar/L) mostly at 45 °C. Obtained mycelia were harvested from the submerged cultures by vacuum filtration and immediately frozen in liquid nitrogen and stored at −70 °C.

2.2. Isolation of genomic DNA; total RNA and preparation of cDNA library

Genomic DNA (gDNA) was isolated from 70 to 100 mg of fresh or frozen mycelium using peqGOLD Fungal DNA Mini Kit (PeqLab, Erlangen, Germany) according to the manufacturer's instructions with a slight modification in the homogenization step. Investigated mycelium was homogenized using glass beads with 1.0 mm diameter and the BeadBug microtube homogenizer (Benchmark Scientific, USA). Samples were milled for 3 min at maximum speed (4000 rpm).

Total RNA was isolated from 30 mg of fresh or frozen mycelium using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Samples were homogenized by using liquid nitrogen and BeadBug homogenizer with the same procedure as for the genomic DNA isolation. Complementary DNA (cDNA) was synthesized from 3 to 6 μL (0.5 μg) of total RNA using AMV First Strand cDNA Synthesis Kit (New England BioLabs; USA).

2.3. PCR amplification and cloning

Obtained DNA samples (gDNA and/or cDNA) were used as a template in PCR to amplify various fragments. Either two typical molecular markers for fungal phylogenetics and exact taxonomic classification (*β-tubulin* and large ribosomal subunit — 28S rDNA) were amplified or parts of a gene coding for the bifunctional catalase-peroxidase (*CthediskatG1*). PCR amplifications were preformed using the AccuPrime *Pfx* DNA Polymerase (Invitrogen, Carlsbad, USA). Each PCR reaction contained 1.5 U *Pfx* Polymerase, 1× *Pfx* Reaction mix and 10 pmol of each primer. In this work several universal primers of fungal barcode markers (*β-tubulin*, 28S rDNA genes) and specific primers designed by us (all details in Table 1) were used. Novel primers were designed by using Primer3 software (Untergasser et al., 2012). Reactions were amplified in LabCycler thermocycler (Göttingen, Germany) with the following program: initial denaturation for 2 min at 95 °C; 35 cycles with 20 s at 95 °C, 30 s. at annealing temperature (dependent on Tm of primer pairs; Table 1), extension 1 min per kb at 68 °C and the last extension for 10 min at 68 °C.

Table 1
DNA primers used for the amplification of *CthediskatG1*; β-tubulin, 28S RNA, ITS1 gene fragments of *Chaetomium thermophilum* var. *dissitum*.

Primer ID	Sequence in 5′ → 3′ direction	Tm [°C]
bt2a	GGTAACCAAATCGGTGCTGCTTTC	62.7
bt2b	ACCCTCAGTGTAGTGACCCTTGGC	66.1
LR0R	ACCCGCTGAACTTAAGC	52.8
LR5	ATCCTGAGGGAAACTTC	50.4
SordarITS1Fwd	CCGCCGGAGGTTCAAAACTC	61.4
SordarITS1Rev	CACTGAATTCTGCAATTCACATTAC	58.1
Oligo28dT	TTTTTTTTTTTTTTTTTTTTTTTTTTTT	46.1
CthekatG1.1Fwd	CACCTATCGTGTCTTTGATGGA	58.4
CthekatG1.1Rev	AGAGCCAAACTTATTGCTCCAG	58.4
CthekatG1.2Fwd	TTTAGGCTGGAGCAATAAGTTC	54.7
CthekatG1.2Rev	TCCCAGTCAAACTTGAAGAGGT	58.4
CthekatG1.3Fwd	ATGGAGTACCAATTTCCTGGAGTA	59.3
CthekatG1.3Rev	GGTAGCAAGGATCGCCTGCTTGAG	59.3
CthekatG1.4Fwd	CTCAAGCAGGCGATCCTTGCTACC	59.3
CthekatG1.4Rev	TCCAGCAAGTTGACGAAGAAATCG	57.6
CthekatG1.5bFWD	ATGGGTGAATGCCCCGTTCGTCAT	64.4
CthekatG1.1RevRT	TCTTCTCACCATACTTCTGCTTCA	59.3
CthekatG1.6bREV	TCAGCGCTTGCCGAGATCAAAGCGAT	66.4
CthekatG1.7aFWD	GAACCTGTGGTAGGTTGACTTTCTA	61.3
CthekatG1.7REV	ATATCCTGCTTTAGAGCCTCGTAGT	61.3
CthekatG1.8FWD	GAAATCTTTGAAGGCTATGA	51.1

PCR products were analysed with electrophoresis in 1% agarose gel in TBE buffer and stained with GelRed Nucleic Acid Gel Stain (Biotium, Fremont, USA) as recommended by the manufacturer. Relevant and correct amplicons were cloned into the pCR-Blunt II-TOPO vector and transformed into One Shot TOP10 Chemically Competent *E. coli* cells using Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Clones were selected on LB plates with kanamycin (50 μg/mL) and in a further step they were verified by Sanger sequencing with M13 or gene-specific primers. All sequencing was carried out by GATC Biotech, Constance, Germany.

2.4. Sequence analyses

The bioinformatics analysis of sequenced clones was first performed with FGENESH 2.6 program in Softberry suite (www.softberry.com; Solovyev et al., 2006) with parameters optimized for *Chaetomia* genomes. Overall similarities and identities of related DNA sequences were analysed with pairwise sequence alignments at EMBOSS-Needle site with DNAfull matrix (https://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html; Rice et al., 2000). Translated protein sequences were compared with BlastX algorithm either in GenBank or in a specialized database, PeroxiBase (http://peroxibase.toulouse.inra.fr; Fawal et al., 2013).

2.5. Collection of related sequence data, multiple sequence alignments and structural alignment & modelling

DNA sequences coding for β-tubulin and 28S-rDNA genes in corresponding regions were collected from 34 distinct GenBank files of Ascomycetes (overview in Table 2). Joined DNA sequences from these two highly conserved regions were aligned with the Muscle program (Edgar, 2004) implemented in MEGA 6 package with its default parameters for DNA sequences and 100 iterations. Translated full-length protein sequences coding for 250 catalase-peroxidases proportionally selected from all known phyla where they occur as well as for one Hybrid A heme peroxidase were collected from PeroxiBase (http://

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