Identification and functional analysis of a novel mitochondria-localized 2-Cys peroxiredoxin BbTPx-2 from *Babesia bovis*

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Abstract

Cysteine-based peroxidases, known as peroxiredoxins (Prx) or thioredoxin peroxidases (TPx), are important antioxidant enzymes that prevent oxidative damages caused by reactive oxygen species (ROS). In this study, we identified a novel 2-Cys Prx, BbTPx-2 from a bovine *Babesia* parasite, *B. bovis*. BbTPx-2 cDNA encodes a polypeptide of 254 amino acid residues. This protein has mitochondrial-targeting peptide at N-terminal and two conserved cysteine residues of the typical 2-Cys Prx. By using a thiol mixed-function oxidation assay, the antioxidant activity of *Escherichia coli* expressed recombinant BbTPx-2 was revealed and this antioxidant activity was comparable to that of known cytosolic 2-Cys Prx from *B. bovis*, BbTPx-1. Notably, we confirmed that BbTPx-2 was expressed in the mitochondria of intra-erythrocytic *B. bovis* merozoites. Altogether, these results suggest that the mitochondrial BbTPx-2 is an antioxidative enzyme in scavenging ROS for *B. bovis*.

Keywords

Antioxidant activity / *Babasia bovis* / Mitochondria / Peroxiredoxin

Introduction

Babesia parasites are tick-borne intra-erythrocytic protozoa in the phylum Apicomplexa. They infect a wide range of mammalians, such as cattle, sheep, and horses, and the majority of them are responsible for serious economic losses in the livestock industry (Brown and Palmer 1999; Dewaal 2000; Schnittger et al. 2012). Similar to other parasites of this phylum, such as *Plasmodium*, *Babesia* undergoes a complex life cycle involving both tick and mammalian host. *Babesia* parasites initiate infection in mammalian hosts by sporozoites, which are transmitted through the bites of infected ticks; subsequently, the merozoites invade and replicate within the infected erythrocytes, eventually leading to babesiosis (Hunfeld et al. 2008; Schnittger et al. 2012).

Babesia bovis is one of the most important species because of its impact on the cattle industry. *B. bovis* causes bovine babesiosis with clinical features such as anemia, fever, renal failure, and in severe case, cerebral babesiosis which is characterized by sequestration of infected erythrocytes in the microvasculature of the brain (Homer et al. 2000). Cattle that have recovered from acute infection become asymptomatic carriers, where the parasites persist in blood for years and recrudescence of parasitemia can occur at unfixed intervals (Bock et al. 2004). Although bovine babesiosis can be controlled by treatment with antiparasitic drugs, many drugs have been withdrawn from the market due to safety issues or emergence of resistance (Bork et al. 2005a; Vial and

77 Gorenflot 2006). Therefore, to develop new therapeutic strategies for bovine babesiosis,
78 more detailed analysis of mechanisms essential for survival of *Babesia* parasites in the
79 host is important.

80 Since *Babesia* parasites proliferate in their mammalian hosts and the tick vectors
81 which are oxygen-rich environments, the parasite is likely to be under the toxic effects
82 of reactive oxygen species (ROS) that could cause damages to membrane lipids, nucleic
83 acid, and proteins (Robinson et al. 2010). For parasites which live in the host
84 erythrocytes, redox balance control is considered as an important biological property
85 (Müller et al. 2003; Becker et al. 2004; Bosch et al. 2015). A major source of ROS in
86 the parasite cell is heme, which is produced as a byproduct of hemoglobin digestion.
87 Moreover, these parasites have a mitochondrion with a functional electron transport
88 chain which may product ROS. To protect themselves from the damages caused by ROS,
89 malaria parasites are equipped with antioxidant enzymes, such as peroxiredoxins (Prxs)
90 (Becker et al. 2004; Kawazu et al. 2008). Prxs are known collectively as thioredoxin
91 peroxidase (TPx) and are widely distributed among both eukaryotes and prokaryotes
92 (Rhee et al. 2005). The family is classified into three groups based on the number and
93 the position of highly conserved active cysteine residues; namely, 1-Cys, typical 2-Cys
94 and atypical 2-Cys type (Vaca-Paniagua et al. 2009; Wood et al. 2003). In recent years,
95 several TPxs of malaria parasites were characterized and the structural and functional

properties of the enzymes have been determined as key factors for development of new drugs (Kawazu et al. 2000, 2008; Richard et al. 2011; Hakimi et al. 2012, 2014, 2015; Jortzik and Becker 2012). In *Plasmodium falciparum*, two typical 2-Cys Prxs, PfTPx-1 and PfTPx-2 are well characterized (Kawazu et al. 2008; Yano et al. 2005; Boucher et al. 2006). While PfTPx-1 is expressed in cytoplasm of parasite, PfTPx-2 is located in mitochondria. It is suggested that the presence of PfTPx-2 in mitochondria is imperative for the malaria parasites to maintain the integrity of the organelle (Boucher et al. 2006).

In *Babesia*, the presence of some antioxidant proteins, including superoxide dismutase (SOD), catalase and glutathione peroxidase (Gpx), thioredoxin (Trx) and thioredoxin reductase (TrxR) has been reported (Becuwe et al. 1992; Clarebout et al. 1998; Regner et al. 2014). Moreover, recently, BbTPx-1 and BgTPx-1, cytoplasmic 2-Cys Prxs of *B. bovis* and *B. gibsoni*, respectively, were identified and their antioxidant activities were demonstrated (Tanaka et al. 2009; Masatani et al. 2014). In this study, we identified a novel 2-Cys peroxiredoxin from *B. bovis*, BbTPx-2 localized to mitochondria of the parasite. To our knowledge, this is the first report on the mitochondrial peroxiredoxin from *Babesia* parasites.

Materials and methods

Parasites

115 The Texas strain of *B. bovis* (Hines et al. 1992) was maintained in purified bovine
116 red blood cells with GIT medium (Wako, Osaka, Japan) by a microaerophilic
117 stationary-phase culture system (Asada et al. 2012; Bork et al. 2005b).
118
119 Multiple sequence alignment analysis
120 Multiple sequence alignment of BbTPx-2 (GenBank accession no.
121 XM_001609049) with BbTPx-1, 2-Cys Prxs from *Theileria parva* (TpTPx:
122 XM_760611), *B. gibsoni* TPx-1 (BgTPx-1: AB829722), *Plasmodium falciparum* TPx-1
123 and TPx-2 (PfTPx-1: BAA97121 and PfTPx-2: XM_001350518, respectively) was
124 performed using GENETYX ver. 10 (Genetyx Co., Tokyo, Japan).
125
126 Cloning of the genes coding for TPxs of *B. bovis*
127 The RNA of *B. bovis* was prepared from cattle erythrocytes infected with *B. bovis*
128 by using TRI reagent (Sigma, St. Louis, MO, USA). Parasite cDNA was synthesized
129 from the extracted RNA by using Transcriptor First Strand cDNA Synthesis Kit (Roche
130 Diagnostics, Basel, Switzerland). The sequences of the DNA encoding *B. bovis*
131 BbTPx-1 and BbTPx-2 were amplified using the PrimeSTAR MAX enzyme (Takara
132 Bio Inc., Otsu, Japan) with the following sets of primers; forward primer (5'- CGT GCT
133 CGA GAA TTG CTG TTG GTC AAC CTG CAC-3') and reverse primer (5'- GCT

134 CGA ATT CTT ATG AGT GCT TGC TAG TAA GG -3') for BbTPx-1, and forward
135 primer (5'- CGT GCT CGA GAA ACG GTG TGT TGC GTC TAC C -3') and reverse
136 primer (5'- GCT CGA ATT CTT AAG AAA AGG TCT TGA AAA GG -3') for
137 BbTPx-2. *Xho*I and *Eco*RI sites are underlined. The PCR products were digested with
138 *Xho*I and *Eco*RI, and then ligated to pRSET-B vector (Invitrogen, Carlsbad, CA, USA).
139 The plasmids were designated as pRSET-BbTPx-1 and pRSET-BbTPx-2, respectively
140 and the nucleotide sequences were analyzed with ABI Prism 3100 Genetic Analyzer
141 (Applied Biosystems, Carlsbad, CA, USA).
142
143 Expression and purification of recombinant proteins
144 The pRSET-BbTPx-1 and pRSET-BbTPx-2 were transformed into *Escherichia*
145 *coli* strain BL21 (DE3). Recombinant BbTPx-1 and BbTPx-2 (rBbTPx-1 and rBbTPx-2,
146 respectively) were expressed as a His-tagged fusion protein in *E. coli* and purified using
147 HisTrap (GE Healthcare, Piscataway, NJ, USA). To express recombinant
148 glutathione-*S*-transferase (rGST), pGEX-6P1 (GE Healthcare) was transformed into *E.*
149 *coli* strain BL21 (DE3). Expressed rGST was purified using Glutathione-Sepharose 4B
150 beads (GE Healthcare). After dialysis in phosphate buffered saline (PBS), protein
151 concentrations were measured using a BCA protein assay kit (Pierce Biotechnology,
152 Rockford, IL, USA). The expressions of the recombinant proteins were confirmed by

153 performing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
154 using 12% gel and standard coomassie brilliant blue staining.
155
156 Antioxidant activity assay
157 The antioxidant activity of rBbTPx-2 was evaluated by a mixed-function
158 oxidation (MFO) assay (Hakimi et al. 2012; Sauri et al. 1995; Masatani et al., 2014).
159 The reaction mixture containing 40 μM $FeCl_3$, 10mM dithiothreitol (DTT), 20 mM
160 EDTA, 25mM HEPES was pre-incubated with or without the rBbTPx-1, rBbTPx-2
161 protein (200, 100, 50 and 25 μg/ml) or rGST (200 μg/ml) at 37 °C for 1 hour. We used
162 rGST as negative control because molecular weight (26 kDa) is similar to rBbTPx-2.
163 After the pre-incubation period, 0.5 μg of pBluescript SK (+) plasmid DNA was added
164 and the reaction mixture was incubated for another 3 hours. Nicking of the supercoiled
165 plasmids was evaluated by 1 % agarose gel electrophoresis and ethidium bromide
166 staining.
167
168 Production of mouse anti-BbTPx-2 serum
169 For production of antisera against BbTPx-2, 10-week-old female ICR mice (Clea
170 Japan, Tokyo, Japan) were used in this study. One hundred micrograms of the
171 rBbTPx-2 was mixed with TiterMax Gold (TiterMax USA Inc., Norcross, GA, USA)

172 and subcutaneously injected into mice. On day 14 after first injection, boost
173 immunization was performed. The mouse sera were collected 20 days after the second
174 immunization. The mice were housed, fed and given clean drinking water in accordance
175 with the stipulated rules for the care and use of research animals promulgated by Obihiro
176 University of Agriculture and Veterinary Medicine, Japan (approval number: 24-118).
177
178 Western blotting
179 *B. bovis*-infected RBCs were pelleted by centrifugation, supernatants discarded,
180 and hemolyzed with 0.05% saponin. After centrifugation, the pellets were washed three
181 times by PBS and resuspended in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl,
182 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 1% NP-40). The
183 solubilized parasite pellet was mixed with equal amount of SDS-PAGE loading buffer
184 (0.1 M Tris-HCl pH 6.8, 5% SDS, 15% glycerol, 4.5% dithiothreitol, and 10%
185 2-mercaptoethanol) and heated at 100 °C for 5 min. Parasite proteins were separated by
186 SDS-PAGE (15% gel) and subsequently transferred onto a polyvinylidene difluoride
187 membrane (Amersham Hybond PVDF Blotting Membrane; GE Healthcare).
188 Membranes were blocked with a blocking solution (Blocking One; Nacalai tesque) and
189 reacted with anti-BbTPx-2 mouse serum at 1:200. Afterward the membrane was washed
190 with PBS containing 0.05% Tween 20 (PBS-T), and the

horseradish-peroxidase-conjugated goat anti-mouse IgG (W402B; Promega, Madison, WI, USA) was reacted at 1:25,000. The signals were developed with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) and detected by LAS-4000 mini luminescent imaging analyzer (Fujifilm, Tokyo, Japan).

Indirect immunofluorescent antibody test (IFAT)

B. bovis-infected RBCs (IRBCs) were incubated with 250 nM of Mitotracker Red CM-H_2XRos (Invitrogen) for 20 min at 37°C and washed three times with PBS. Thin smear was prepared from the IRBCs and fixed with 4% Paraformaldehyde-containing 0.0075% glutalaldehyde. The smear was blocked with 10% normal goat serum (Life technologies, Rochester, NY, USA) in PBS, and incubated with anti-BbTPx-2 mouse serum at 1:200. After washing with PBS-T, Alexa-Fluor 488 conjugated goat anti-mouse IgG (Invitrogen) was used at 1:1000. Finally, nuclei were stained by Hoechst 33342 at 0.3 μg/ml and fluorescence was detected using a laser scanning confocal microscope (Nikon A1R, Nikon, Tokyo, Japan) equipped with a × 60 objective lens (Nikon).

Results and discussion

The BbTPx-2 gene (762 bp) coded for a protein comprised to 253 amino acid residues with the predicted molecular weight and theoretical isoelectric point of 28.02

kDa and 9.06, respectively. The multiple sequence alignment of BbTPx-2 with 2-Cys Prxs from other Apicomplexan parasites revealed that two VCP motifs of the 2-Cys Prx active sites (Cys 108 and Cys 227) were conserved (Fig. 1). Amino acid sequence analysis by using SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) showed that the protein had no signal peptide. Notably, both PSORT II server (http://psort.hgc.jp/cgi-bin/runpsort.pl) and TargetP 1.1 server (http://www.cbs.dtu.dk/services/TargetP/) showed that the expected values for mitochondrial localization of this protein are 60.9 % and 0.816 (the maximum values are 100% in PSORT II and 1 in TargetP 1.1, respectively). Moreover, TargetP 1.1 predicted that this protein has mitochondrial targeting peptide at N terminal (aa 1 to 54) (Fig. 1). Thus, it is expected that the mature BbTPx-2 protein comprised to 199 amino acid residues with the predicted molecular weight and theoretical isoelectric point of 21.8 kDa and 6.29, respectively.

In order to demonstrate the enzymatic activity of this BbTPx-2, the coding sequence of BbTPx-2 gene was amplified by RT-PCR (Fig. 2a) and cloned into the prokaryotic expression vector pRSET-B. Then, recombinant rBbTPx-2 was expressed in *E. coli* as a soluble protein. The purified rBbTPx-2 had an apparent molecular weight of approximately 28-kDa, as determined by SDS-PAGE (Fig. 2b). Then, the antioxidant activity of rBbTPx-2 was evaluated by MFO assay (Fig. 3). In this assay, the hydroxyl

229 radicals that are generated by $FeCl_3$ and DTT damage DNA (Sauri et al. 1995). In the
230 reaction mixture containing both $FeCl_3$ and DTT but not rBbTPx-1 or rBbTPx-2,
231 hydroxyl radicals nick the supercoiled plasmid DNA, thereby apparent size of the
232 plasmid was increased (Fig. 3, lane 4). However, the presence of 200 and 100 μg/ml of
233 rBbTPx-2 in the reaction mixtures prevented nicking of the supercoiled plasmid DNA
234 (Fig. 3, lanes 11 and 12) and this antioxidant activity was comparable to that of
235 rBbTPx-1 (Fig. 3, lanes 7 and 8). These results strongly suggested that BbTPx-2 has
236 antioxidant activity similar toBbTPx-1 and acts as an antioxidant enzyme catalyzing
237 hydrogen peroxide in the antioxidant system of *B. bovis*.

238 Next, we produced an antiserum against rBbTPx-2 and analyzed the expression of
239 BbTPx-2 in *B. bovis* by Western blotting and IFAT (Fig. 4). Western blot analysis
240 showed that an antiserum against rBbTPx-2 bound to a protein of the expected
241 monomeric size of about 24-kDa in extracts of *B. bovis*-infected RBCs (Fig. 4a). This
242 band, which is smaller than 28 kDa from rBbTPx-2, suggests that most BbTPx-2
243 proteins in the parasite are expressed as a mature protein with cleaved mitochondrial
244 targeting signal. The lack of any additional bands in the Western blotting experiment
245 shows the specificity of the mouse polyclonal antiserum in detecting only the target
246 protein. Collectively, this data indicates that BbTPx-2 is expressed in *B. bovis*
247 merozoites. IFAT with antiserum against rBbTPx-2 (Fig. 4b) revealed that the dots-like

248 strong fluorescence with some diffused signal in the cytoplasm of the parasites. Notably,
249 co-localization of anti-BbTPx-2 signal (green) with Mitotracker (red) indicates that
250 most of BbTPx-2 was present in mitochondria of parasites as predicted by bioinfomatics
251 analysis (Fig. 4b, Merged). Taken together, our results indicate that BbTPx-2 is
252 expressed in mitochondria during the asexualstage of *B. bovis*.

253 In conclusion, we have characterized a novel functional antioxidant typical 2-Cys
254 Prx, BbTPx-2 in *B. bovis* mitochondria. Because BbTPx-2 has the antioxidant activity,
255 we believe that the BbTPx-2 plays a vital role in the reduction of ROS produced in
256 mitochondria. On the other hand, *Babesia* parasites have other antioxidant proteins,
257 including SOD, catalase, Gpx and another 2-Cys Prx, BbTPx-1 (Becuwe et al. 1992;
258 Clarebout et al. 1998; Tanaka et al. 2009). Thus, it would be interesting to elucidate
259 relation between BbTPx-2 and these antioxidant proteins. Further experiments
260 dissecting the precise roles of the BbTPx-2 using knock-out system (Asada et al. 2012,
261 2015) will help to gain a better understanding of the antioxidant system of the parasite.
262 Recently it was reported that the expression of a mitochondrial peroxiredoxin in
263 *Leishmania donovani* protects the parasites against hydrogen peroxide-induced cell
264 death (Harder et al. 2006). Moreover, Teixeira et al. (2015) revealed that mitochondrial
265 Prx of *L. infantum* functions as a chaperon reservoir which allows parasites to deal with
266 protein unfolding conditions during the transition from insect-stage to mammalian-stage.

Thus, it is interesting to reveal other functions of mitochondrial BbTPx-2 in future studies. Our data may provide important information on BbTPx-2 that can be used as a base for future studies to investigate its precise role in the parasite and its potential as a drug target against bovine babesiosis.

Acknowledgements

This work was partially supported by a grant from the Global COE program (Grant No. J02), Sasakawa Scientific Research Grant from The Japan Science Society (Grant No. 24-404) and Grant-in-Aid for Scientific Research (KAKENHI) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan (Grant Nos. 25850199 and 15K18783).

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409

410

411 **Figure captions**

412 **Fig. 1**

413 Multiple sequence alignment of the deduced amino acid sequences of BbTPx-2 with the
414 sequences of other 2-Cys Prxs from *T. parva* (TpTPx), *B. bovis* (BbTPx-1), *B. gibsoni*
415 (BgTPx-1), and *P. falciparum* (PfTPx-1 and PfTPx-2). Black boxes with white letters
416 show identical residues among all the sequences, and gray boxes with black letters show
417 common residues in many of the sequences. The dashes indicate gaps introduced
418 between the sequences. Red box shows predicted mitochondrial targeting peptide of

419 BbTPx-2.

420

421 **Fig. 2**

422 Amplification of BbTPx-2 gene and expression of BbTPx-2 in *E. coli*. (a) Agarose gel
423 electrophoresis images of the RT-PCR-amplified BbTPx-2 gene (765 bp). M, 100 bp
424 DNA ladder marker. (b) SDS-PAGE images of recombinant BbTPx-2 (rBbTPx-2). M,
425 protein marker.

426

427 **Fig. 3**

428 MFO assay to evaluate rBbTPx-2 antioxidant activity. Nicking of the supercoiled
429 plasmids by the MFO was evaluated on 1.0% agarose gels stained with ethidium bromide.
430 The nicked form (NF) and supercoiled form (SF) of the plasmid are indicated on the right.
431 M, 100 bp DNA ladder marker. Lane 1, pBluescript plasmid DNA only; lane 2,
432 pBluescript plasmid DNA and $FeCl_3$; lane 3, pBluescript plasmid DNA and DTT; lane 4,
433 pBluescript plasmid DNA, $FeCl_3$ and DTT; lanes 5–8, pBluescript plasmid DNA, $FeCl_3$,
434 DTT, and 25, 50, 100 and 200 μg/ml of rBbTPx-1 protein, respectively; lanes 9–12,
435 pBluescript plasmid DNA, $FeCl_3$, DTT, and 25, 50, 100 and 200 μg/ml of rBbTPx-2
436 protein, respectively; lane 13, pBluescript plasmid DNA, $FeCl_3$, DTT, and 200 μg/ml of
437 recombinant glutathione-*S*-transferase (rGST) from *Schistosoma japonicum* as a negative

438 control. Triangles show the increasing concentration of the recombinant protein.

439

440 **Fig. 4**

441 Molecular characterization of native BbTPx-2 in parasites. (a) Western blot analysis of

442 native BbTPx-2. The positions of molecular mass standards are indicated on the left. (b)

443 Indirect immunofluorescence microscopy to determine the cellular localization of

444 BbTPx-2 in the parasite cells. Brightfield and fluorescent images (green, BbTPx-2; red,

445 mitochondria; blue; nuclear) are merged on the one panel (Merged).

446

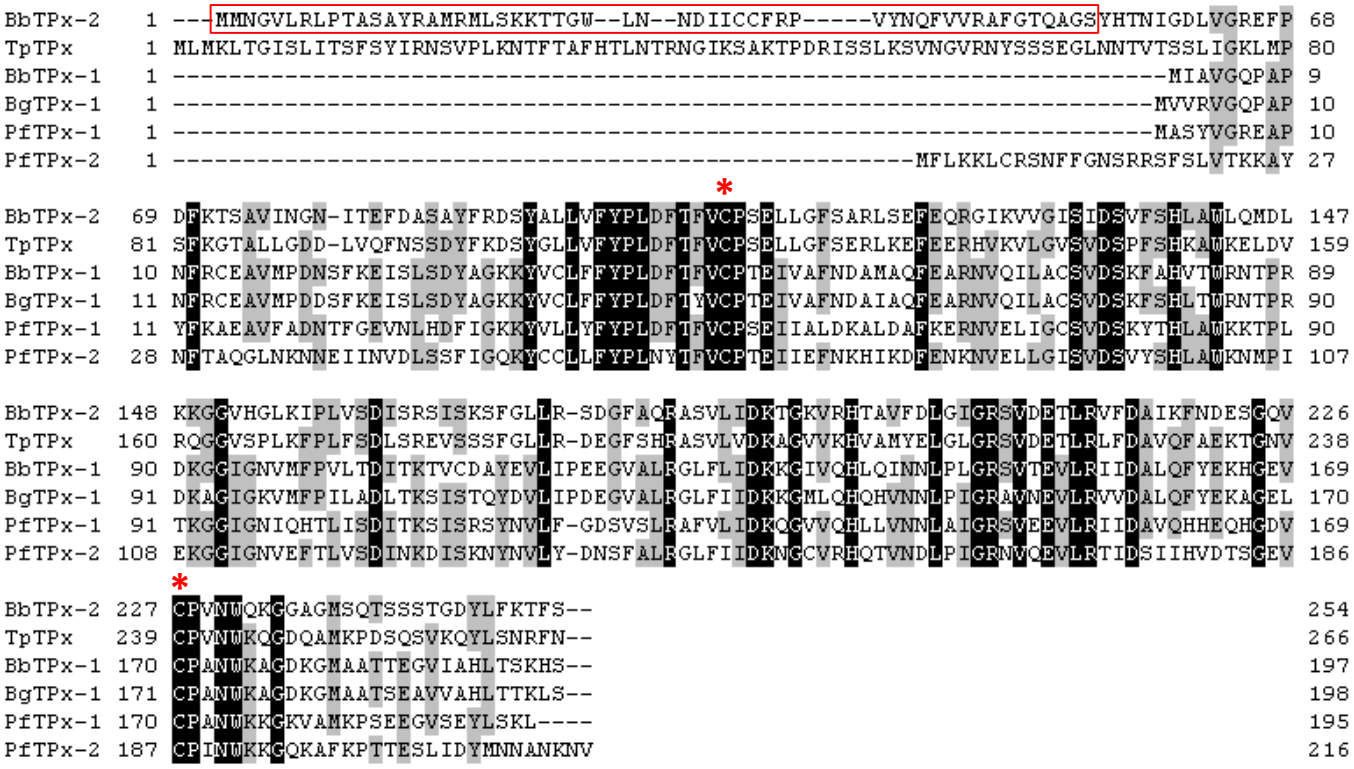

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Fig. 1

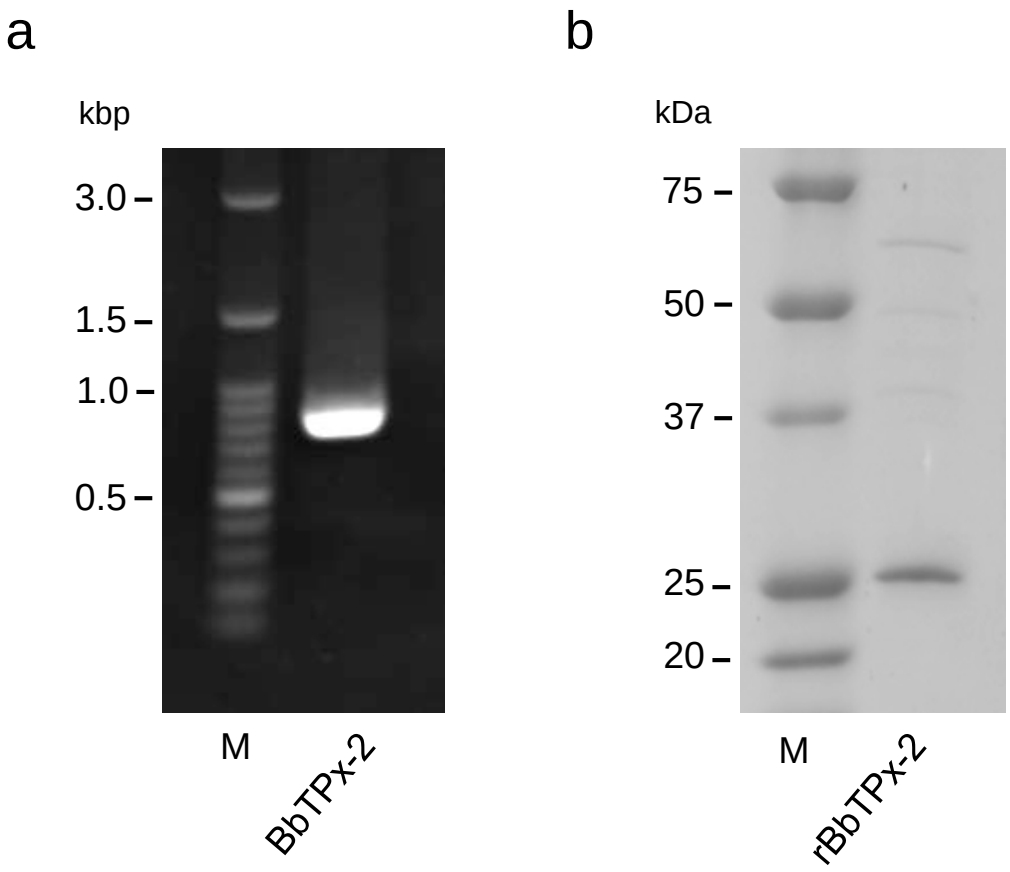

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Fig. 2

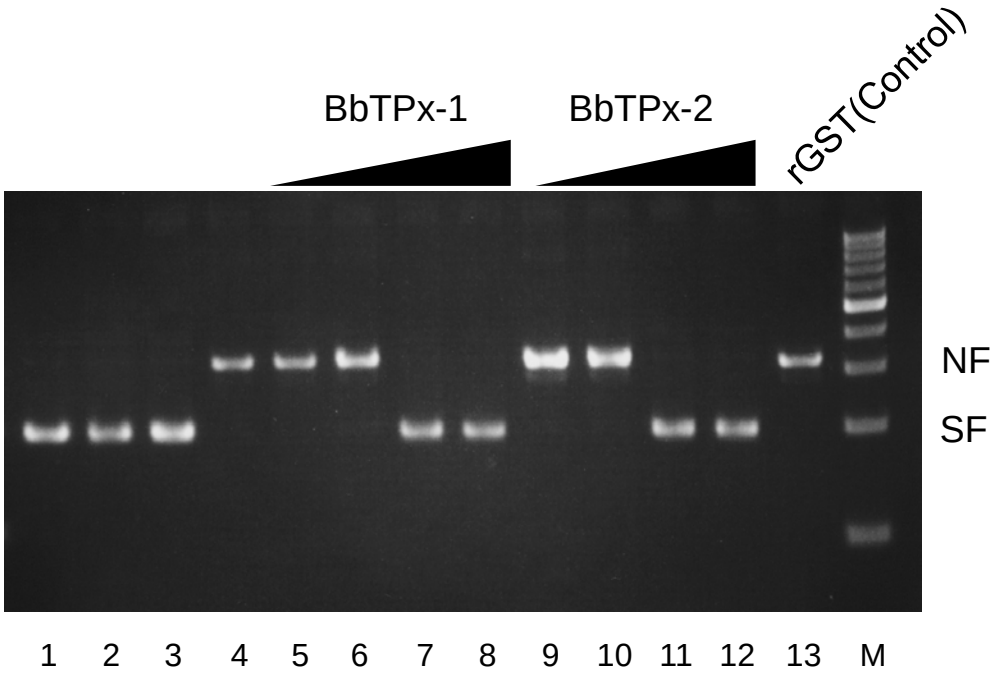

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Fig. 3

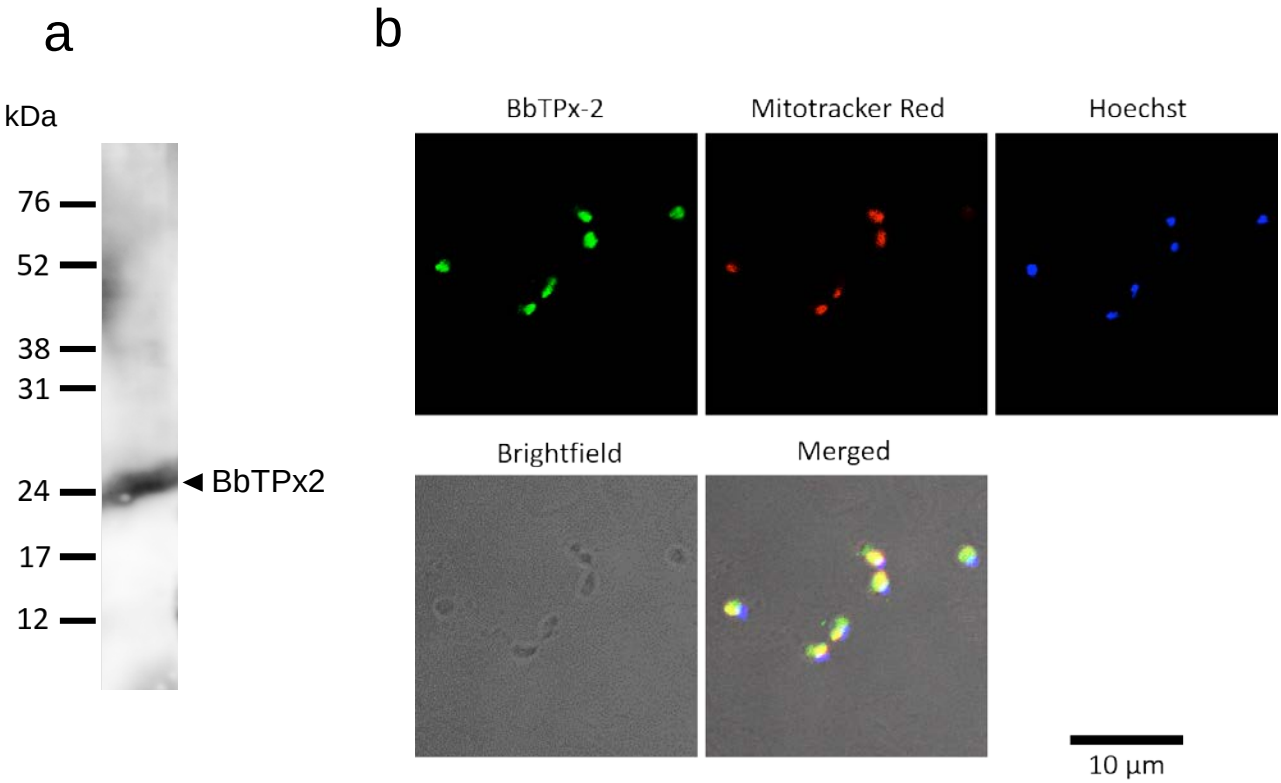

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Fig. 4