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Faculty of Science

Department of Molecular Biology and Biochemistry

Bachelor thesis

Functional analysis of the YCF 45 gene in procyclic *Trypanosoma brucei*

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Anotation:
In this study was shown by RNA interference that YCF 45 gene of putative chloroplast origin is not essential for surviving of procyclic *Trypanosoma brucei.* Three different construct bearing HA-tagged YCF 45 protein that differs on their N-terminus were generated to reveal the localization of the protein in the cell. Spliced leader acceptor site of YCF 45 mRNA was identified and in comparison to annotated data new start codon was proposed. Fylogeny tree was made to shed some light on the origin of this gene.

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I hereby declare that I did all work, summarized in this thesis, on my own or on the basis of consultations with my supervisor, and only using the cited literature.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své bakalářské práce, a to v nezkrácené podobě, Přírodovědeckou fakultou, elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách.

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Jiří Týč

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CONTENTS

1. INTRODUCTION:

Trypanosoma brucei brucei is a subspecies of the *Trypanosoma brucei* complex and a member of the protozoan order Kinetoplastida (Simpson et al., 2006; Moreira et al., 2004). Kinetoplastid flagellates are causative agents of several serious diseases that are spread worldwide – *T. brucei* is responsible for African sleeping sickness and livestock disease Nagana (Barrett et al, 2003), related *Trypanosoma cruzi* causes Chagas disease and *Leishmania* spp. is the causative agent of leishmaniasis.
T. brucei is famous for several distinctive oddities such as surface antigenic variation, kinetoplast DNA, trans-splicing or mitochondrial RNA editing (Lukes et al., 2005). It is also a model species for which a number of molecular methods are available, including functional analysis using RNA interference (RNAi)(Wang et al., 2000).

Together with Euglenoids, kinetoplastids constitute the phylum Euglenozoa. All euglenoids bear secondary chloroplasts, while this organelle is absent from kinetoplastids (Hannaert et al., 2003). It is a long debated question whether the predecessors of kinetoplastids contained a chloroplast and lost it after their separation from euglenoids, or euglenoids engulfed it only after the separation of both lineages (Cavalier-Smith, 1999; Martin and Borst, 2003). Some authors gathered molecular evidence to support the fact that trypanosomes at some point had a plastid (Hannaert et al., 2003), some raised opposing arguments based on ultrastructure (Leander, 2004).
The question whether kinetoplastids had or had not a plastid is highly relevant. It would shed light on the long evolutionary history of the whole phylum Euglenozoa. Moreover, if these parasitic flagellates ever harbored a plastid, some remnant metabolic pathways totally different from those of their hosts including human may still be present in their cells. Such pathways would represent an excellent drug target (Waller et al., 2004). In fact, a reduced plastid termed apicoplast currently represents the most promising target in the malaria parasites (McFadden et al., 1999). Whole genome sequencing heralded the post genomic era of *T. brucei* (Berriman et al., 2005). Unfortunately the TriTryp genome-sequencing project (*Leishmania major, Trypanosoma cruzi, T. brucei*) has not revealed any substantial evidence for the existence of remnant plant or plastid genes in the trypanosomatid genomes (El-Sayed et al., 2005).

YCF stands for h**Y**pothetical **C**hloroplast open reading **F**rame (Hallick and Bairoch, 1994). YCFs is an arteficial group of ORFs connected with each other only because of their at present unknown function and their connection with chloroplasts. They are encoded either in chloroplast genomes (Steane, 2005) or they have been transferred into the nucleus (Hackett et al., 2004). Their functions gradually become known (Mäenpää et al., 2000, Ohtsuka et al., 2004), but unforutnately so far no one becomes interested in YCF 45. The gene YCF 45 is the only member of its kind very surprisingly found in all TriTryps genomes and is very likely of cyanobacterial or chloroplast origin (Opperdoes and Michels, 2007). It is highly conserved, has an unknown function and was so far found only in cyanobacteria and chloroplast-bearing eukaryotes (algae, plants and diatoms). YCF 45 is, as others YCFs, coded either by the chloroplast genome itself, or has been transferred into the nucleus and obtained the chloroplast targeting sequence (Fig. 1). This fact strongly indicates that its function is associated with the chloroplast.

YCF 45 gene of kinetoplastids may be either a remnant of a chloroplast or was acquired by horizontal transfer. Alternatively, lateral transfer can be considered following the "You are what you eat" theory (Doolittle, 1998), since there are still many extant free-living kinetoplastids which feed on bacteria or small unicellular eukaryotes using fagocytosis (Vickerman, 1976).
In this work I have tried to establish the intracellular localization of the protein product of YCF 45, as well as whether it is essential for the procyclic (insect form) *T. brucei.*

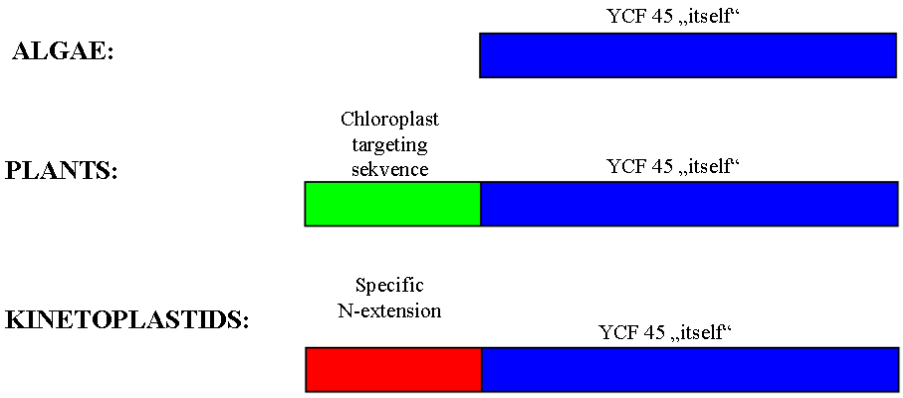


Fig. 1 Difference between N ending of YCF 45 among different groups of organisms bearing YCF 45 gene.

2. OBJECTIVE:

Create and characterize YCF 45 knockdown cell line in procyclic stage of *T. brucei.*

Create HA-tagged YCF 45 protein producing cell line.

Compare distribution of HA-tagged YCF 45 with different N terminal peptides using digitonin fractionation and imunolocalization.

3. MATERIALS AND METHODS:

3.1. RNA interference

3.1.1. Preparation of p2T7-177 construct for RNAi

1) Using the annotated YCF 45 sequence from www.genedb.org primers for RNAi were designed. Forward primer was artificially elongated with the XhoI restriction site and accordingly was reverse primer enriched with restriction site for SpeI (Tab. 1).
2) An amplicon was subsequently obtained by PCR in Mastercycler machine (Eppendorf) with the genomic DNA of *T. brucei* used as a template. Genomic DNA was isolated with DNeasy® Tissue Kit (QIAGEN).
   PCR conditions: denaturing at 94°C, 60 s, annealing at 52°C, 60 s, extending at 72°C, 60 s (30 cycles)
3) PCR product of expected size (540 bp) was extracted from a 0.75% agarose gel with the QIAquick® Gel Extraction kit (Quiagen) following manufacturer's instructions, and than cloned into the pCR® 2.1-Topo vector (Invitrogen) (Fig. 2).
4) Ligation mixture was subsequently transformed into XL1 Blue competent cells (*E. coli*) and transferred on LB agarose plates treated with 100 µg/ml ampicilin and 700 µg X-Gal.
5) Positive white colonies were picked up and grown at 200 rpm overnight at 37°C in 3 ml liquid LB medium supplemented with 100 µg/ml ampicilin.
6) Plasmids containing the insert were isolated by QIAprep® Spin Miniprep Kit (QIAGEN) or by alkaline lysis (TENS).
7) To verify if the target fragment is really present, was the product sequenced using M13 reverse primer and Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).
8) After confirmation that we really have YCF 45 fragment was this fragment cut out with XhoI and SpeI enzymes and cloned into the p2T7-177 vector using T7 ligase (Fig. 3).
9) XL1 Blue *E. coli* cells were transformed with the p2T7-177 vector bearing the insert, which was once more verified with restriction analysis.
10) Clone bearing right sized fragment was grown in 100 ml liquid LB medium with ampicilin.
11) DNA was isolated using MIDI prep method.
12) Approximate 10 µg of DNA was digested with NotI and this linearized plasmid was prepared for electroporation by precipitating in ethanol.

name	Sequence 5' → 3'	Restriction site
YCF- KD-FP	G**CTCGAG**AGAAGTATCGGACAGAAGAA	Xho I
YCF-KD-RP	G**ACTAGT**TGGAGAGTATCAAAACACTA	SpeI

Tab. 1 primers used for amplification of the fragment of YCF 45 used for RNAi, in bold the restriction site is marked.

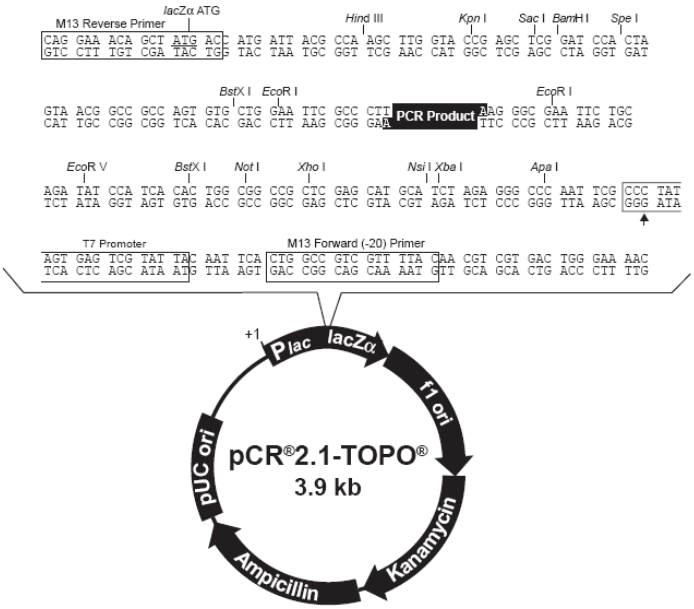


Fig. 2 pCR® 2.1-Topo vector (Invitrogen) used for amplification and verification of our fragment

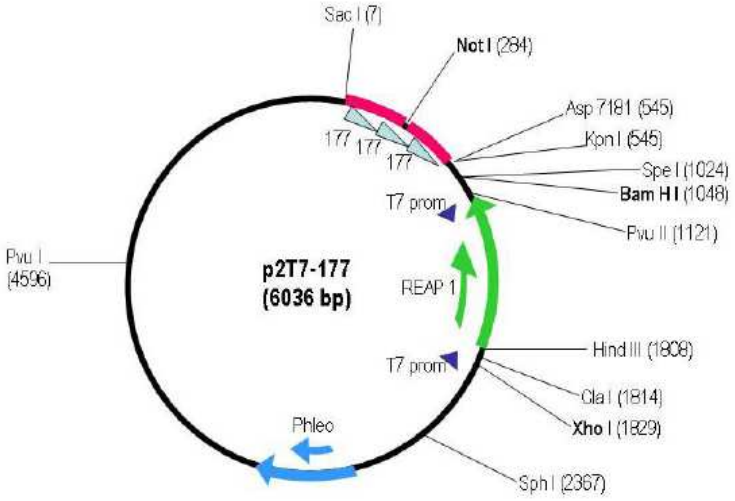


Fig. 3 p2T7-177 vector used for expression of dsRNA in the *T. brucei* (Wickstead et al., 2002)

3.1.2. Cultivation of procyclic *Trypanosoma brucei*

For our experiments we were using procyclic stage of *T. brucei* strain 29-13 (Wirtz et al., 1999). For cultivation of this strain SDM-79 medium with addition of 15 µg/ml G418 and 50 µg/ml hygromycin is used (Brun and Schonberger, 1979). *T. brucei* is cultivated under 27°C and 5% carbon dioxide (CO_2 is not essential) in densities between 10^6-10^7 cells/ml. For selecting transformants mostly phleomycin resistance is used (2.5 µg/ml phleomycin). Inducible synthesis is usually triggered by 1 µg/ml tetracycline.

3.1.3. Electroporation of procyclic stage of *T. brucei*

1) 10 ml of culture with 10^7 cells/ml density was harvested at 1300G for 10 min at 4°C.
2) Cells were washed in 10ml of cytomix solution and spun down again at the same conditions as mentioned above.
3) Pelleted cells were resuspended in 1ml of cytomix.
4) This suspension was transferred into 2mm electroporation cuvette previously loaded with 10 µg linearized p2T7-177 vector.
5) Cells were electroporated in ECM 630 BTX Electroporator by two pulses, the first one of 1500 V, 25 Ω and 25 µF followed in 10 s with second of 1700 V, 25 Ω and 25 µF.
6) Electroporated suspension was then transferred into 5ml SDM-79 medium containing hygromycin and G418.
7) After 24 hours another 5ml of medium was added together with 2.5 µg/ml phleomycin to select the successfully transformed cells.
8) This culture was transferred into 24 wells plate following this scenario:
   A: The first row was loaded with 1.5 ml of our culture.
   B: The second and third row were loaded with 1 ml fresh SDM-79.
   C: The fourth row was loaded with 0.5 ml fresh SDM-79.
   D: Starting at first column, 0.5 ml of the first well was transferred into the second, pipetted up and down, 0.5 ml was than transferred into the third well, and so on.
9) This experiment was done in parallel with another group of cells which were not added any DNA into the cuvette. Those cells served as negative control.
10) Pseudoclones for future analysis were obtained.

3.1.4. Searching for fenotype

3.1.4.1. Northern blot

1) Pseudoclones were divided into two flasks, one of which was induced with 1 µg/ml tetracycline (tet +) to start transcription of the YCF 45 dsRNA.
2) After 4 days induction 10 ml of each culture with 10^7 cells/ml density was harvested at 1300 G for 10 min at 4°C (cultures – WT, tet-, tet +).
3) RNA was extracted using Tri Reagent (Sigma) according to manufacturer's instructions.
4) Approximately 10 µg of RNA was mixed 1:2 with a loading buffer containing 62% formamide, 1% formaldehyde, 1X MOPS and 10 µg/ml ethidium bromide and loaded on a 1% formaldehyde-MOPS gel (Sambrook et al., 1989) with standard RNA markers (Promega).
5) The gel was run at 60 V for 3 hrs.
6) RNA was transferred from the gel to a Nylon membrane (Biodyne B Membrane, 0.45 µm, Pall Corporation) by Northern blot overnight by capillary elution using 5x SSC.
7) RNA was immobilized on the membrane by UV crosslinking (UV Stratalinker, Stratagene).
8) The membrane was prehybridized in NaPi (0.5 M NaH_2PO_4, 0.5 M Na_2HPO_4, 7% SDS, 1mM EDTA, pH 7.2) at 55°C for one hour.
9) While membrane was prehybridizating, radioactive probe was prepared (below).
   - Preparation of probe: template DNA was obtained by PCR or digested out of the vector, this template was used for radioactive labeling with α-P^{32} dATP (ICN) by random priming using the HexaLabel™ DNA Labeling Kit (MBI Fermentas). Probes were purified in spin columns filled with G-25 Sephadex beads (Amersham).
10) The probe was added to the solution and hybridization underwent at 60°C overnight.
11) The membrane was washed first at 2X SSC + 0.1% SDS for 20 min at room temperature and subsequently with 0.2X SSC + 0.1% SDS for 20 min at 55°C.
12) The exposure was done in a phosphor-screen cassette (Molecular Dynamics) overnight and image was visualized in a Typhoon phosphoimager (Amersham).
13) Alternatively was membrane exposed to BioMax film (Kodak) using intensification screens at -80°C for 48 hours.

3.1.4.2. Growth curves

Cultures of *T. brucei* were grown in 5ml of SDM-79 medium containing appropriate antibiotics. After tetracyclin induction of cells we measured growth rate and growth fenotype and compare cultures with and without tetracycline. Cell densities were measured every 24 hours using the Beckman Coulter Z2 Particle Counter for 9 days.

3.1.5. Spliced leader mediated 5' rapid amplification of cDNA ends PCR

1) RNA from *T. brucei* 29-13 was isolated.
2) This RNA was reverse transcribed into cDNA using Superscript RNAse H$^-$ Reverse Transcriptase (Invitrogen) and Oligo $(dT)_{12-18}$ (Invitrogen) accordingly to the manufacturer.
3) cDNA served as template for the SL-RACE PCR. Primers are shown in Tab. 2. PCR conditions: denaturing at 94°C, 30 s, annealing at 52°C, 30 s, extending at 72°C, 60 s (30 cycles).
4) Bands of expected size were cloned into pCR 2.1-Topo vector (Invitrogen) (Fig. 4).
5) Ligation mixture was subsequently transformed into XL1 Blue competent cells (*E. coli*) and transferred on LB agarose plates treated with 100 µg/ml ampicilin and 700 µg X-Gal.
6) Positive white colonies were picked up and grown at 200 rpm overnight at 37°C in 3ml liquid LB medium with 100 µg/ml ampicilin.
7) Plasmids containing target insert were isolated by QIAprep Spin Miniprep Kit (QIAGEN).
8) To verify if the insert is really present we digested plasmids with EcoRI and than sequenced those bearing our product by the company Macrogen (www.macrogen.com).
9) Obtained sequences were compared in MEGA3.1 (Kumar et al., 2004).
10) SL acceptor site was defined and obtained N-terminal sequence was put into the program for prediction the target compartment of proteins. A) Mitoprot http://ihg.gsf.de/ihg/mitoprot.html (Claros and Vincens, 1996), B) TargetP http://www.cbs.dtu.dk/services/TargetP/ (Emanuelsson et al., 2000).

name	Sequence 5' → 3'
YCF45_SLPCR_Rv1	GAAAGACCACAATCCTGAGA
YCF45_SLPCR_Rv2	ACAAGACATCGAAGTGAGAG
Tb SL1	AACTAACGCTATTATTAGAACAGTTTC
Tb SL2	TATTATTAGAACAGTTTCTGTACTATATTG

Tab. 2 Primers used for SL-RACE PCR

3.2. Localization of the YCF 45 protein

3.2.1. Preparation of pJH 54 constructs containing HA-tag

The same steps as described in: Preparation of p2T7-177 construct for RNAi, were performed. Here I point out just major differences.

1) Different set of primers was designed to amplify the whole gene. In total one reverse primer and three different forward primers were made (Tab. 3). Reverse primer contains XbaI restriction site while forward primers were elongated with HindIII specific site.
2) pGEM®-T Easy vector (Promega) was used instead of pCR® 2.1-Topo (also cloning steps into this vect were slightly different – procedure was performed following users guide.) (Fig. 4).
3) For sequencing samples were sent to the company Macrogen (T7 and SP6 sequencing primers were used to obtain whole sequences of all three constructs).
4) Different set of endonucleases were used – see step no. 1.
5) pJH 54 plasmid (Fig. 5) was used instead of p2T7-177.

name	Sequence 5' → 3'	Restriction site
YCF45-RP-end	GGCCGG**TCTAGA**ATTAAAGTTATTCAGTTCGT	Xba I
YCF45-FP-I-end	GGTCT**AAGCTT**ATGTGCACGTCATATGCGGAAGT	HindIII
YCF- FP-II-end	GGTCT**AAGCTT**ATGTGCACGTCATATGCGGAAGT	HindIII
Ycf45-L-FP	T**AAGCTT**ATGCTCTTGGGATGCCGT	HindIII

Tab. 3 primers used for amplification of the YCF 45 that differ in N terminal sequence. YCF45-FP-I-end is for amplification of annotated gene (N+), YCF- FP-II-end is for construction N terminal absent form (N-) and with Ycf45-L-FP we had amplified YCF 45 containing newly revealed part (NW). In bold the restriction site is marked and blue start codon is artificially added to the N- sequence.

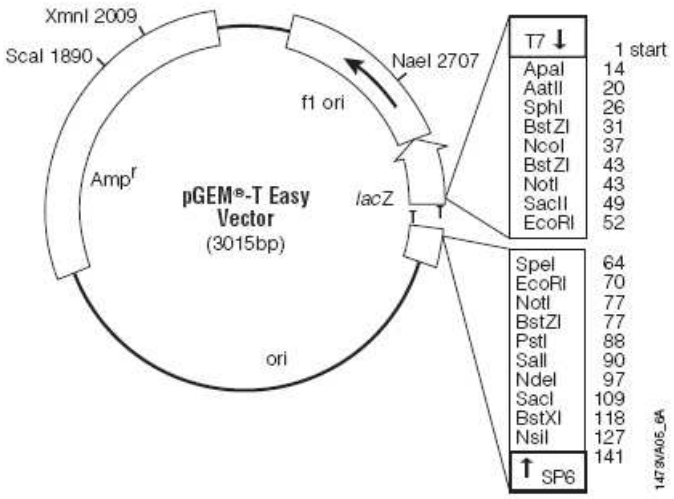


Fig. 4 pGEM®-T Easy vector (Invitrogen) used for amplification and verification of our fragment

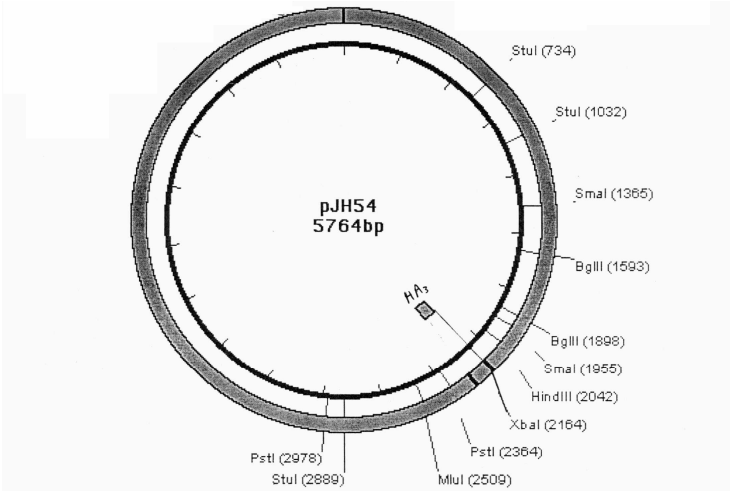


Fig. 5 pJH 54 vector used for expression of HA-tagged forms of YCF 45. Sequence for special high immunogenous epitope is added before the stop codon - the hemagglutinin (HA) peptide (YPYDVPDYA).

3.2.2. Digitonin fractionation

1) Pseudoclones were divided into two flasks one of them was induced with 1 µg/ml tetracycline (tet +) to start transcription of YCF 45 fragment.
2) After 1 days induction $5x10^8$ cells was harvested at 1300 G for 10 min at 4°C (cultures – WT, tet-, tet +).
3) Pelleted cells were washed with cold SHE (250 mM sucrose, 25 mM HEPES pH 7.4, 1 mM EDTA) and spun down as described previously.
4) Pellet was diluted to $5x10^9$ cells/ml with SHE and cells were kept on ice.
5) Protein concentration was determined with Bradford method (Bradford, 1976).
   - To construct calibrating curve BSA (Bovine serum albumin) was used.
   - To 2 µl of cells suspension 18 µl HBSS (136.9 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 4.2 mM $NaHCO_3$, 5.5 mM glucose; adjust pH to 7,3) were added.
   - 5 µl of mixture mentioned above were diluted with 245 µl Mili-Q water and 1 ml of Bradford reagent and subsequently vortexed.
   - After 10 min the absorbance at 595 nm wavelength was measured on spectrophotometer Biphotometer (Eppendorf).
6) To 1 mg of proteins 200 µl HBSS and 8 µl digitonin (10 mg/ml) were added, mixed and incubated at 25°C for 5 min.
7) Suspension was spun down at 13000 rpm for 2 min. Supernatant is a cytosolic fraction. (all fractions were stored on ice).
8) Pellet was washed with HBSS and centrifuged again.
9) To pellet 200 µl HBSS and 2 µl of 10% Triton X-100 were added, mixed and kept on ice for 5 min.
10) Suspension was spun down at 13000 rpm for 2 min. Supernatant is a mitochondrial fraction.
11) To obtain the whole cell lysate, 200 µl HBSS and 2 µl of 10% Triton X-100 were added directly to 1 mg of protein suspension.
12) The mixture was incubated on ice for 5 min and centrifuged for 2 min at 13000 rpm.

3.2.3. Western blot

1) For standard western blot total lysate of 5 x 10^6 cells per line is used. Another option is to use samples prepared by digitonin fractionation.
2) Protein samples were mixed with 2x Sample Buffer (125 mM Tris-HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue) and denatured for 10 min at 100°C.
3) 12% SDS-PAGE gel was used to separate samples. As a marker SeeBlue® Plus2 Pre-stained Standard (Invitrogen) was used.
4) Proteins were transferred to the Bio Trace NT Pure Nitrocellulose Membrane (Pall Corporation Life Sciences) by electroblotting on the Trans-Blot Semi-Dry Transfer Cell (Bio-Rad) with semidry buffer (39 mM glycine, 48 mM Tris-HCl, 0.038% SDS, 20% methanol) for 1 hour at 20 V.
5) Successful blotting and equal loading was checked with Ponceau S staining.
6) The membrane was blocked in 5% nonfat milk in 1x PBS-Tween overnight.
7) The membrane was incubated for 1-2 hours with primary antibody at room temperature.
8) The membrane was washed 5x5 min with 1x PBS-Tween.
9) The membrane was incubated for 1 hour with secondary antibody conjugated to horseradish peroxidase at room temperature.
10) The membrane was washed 5x5 min with 1x PBS-Tween.
11) The signal was visualized using the PIERCE ECL Western Blotting Substrate (PERBIO).
12) Images were visualized in FUJIFILM Luminescent Image Analyzer LAS-3000 (FUJIFILM Life Science).

Remark: As primary antibodies were mostly used antibodies against the control proteins and commercial anti-HA-tag monoclonal antibody (Sigma). We also obtained a specific polyclonal rabbit commercial antibody from GenScript Corporation (www.genscript.com) generated against the specific peptide derived from YCF 45 sequence: NKIKKTILERPHSSC**.**

3.2.4. Immunolocalization

1. Fixation
   - Poly-L-Lysine solution (Sigma) was applied on the microscope slides and let to dry.
   - Slides were covered with approximately 800 µl of cell culture.
   - Slides were let dry in 27°C for 35 min and the rest of the medium was poured off.
   - Slides were incubated at methanol (-20°C, 5 min).
   - Slides were transferred into acetone and incubated under the same conditions (-20°C, 5 min).
   - Slides were let dry.
2. Slides were covered with BSA and gelatin solution (1x PBS, 0,25% BSA, 0,25% gelatin from porcine skin) and incubated for 1 hour in the wet chamber at room temperature.
3. The solution was poured off and the same solution containing primary antibody (1:200 dilution) was placed atop. Slides were incubated for 1 hour in the wet chamber at room temperature.
   - Remark: for colocalization 2 different antibodies can be used at the same time if they are derived from different animals and secondary antibody against those is available.
4. Unbound primary antibody was washed away in PBS (3x 5 min).
5. BSA and gelatin solution containing secondary antibody (1:1000 dilution) was applied on the slides and incubated in the dark in the wet chamber for 1 hour at room temperature.
6. Unbound secondary antibody was washed away in PBS (3x 5 min). This process was performed also in the dark because secondary antibody labeled with fluorescent Alexa Fluor® (Invitrogen) was used.
7. The solution was poured off but slides were not let completely dry.
8. Samples were stained with DAPI and covered by cover slides degreased in ethanol.
9. Varnish coat was used to seal the samples.
10. Slides were stored overnight at 4°C.
11. Photos were taken by DP71 digital camera (Olympus) using BX60 Olympus microscope equipped with U-RFL-T fluorescent lamp (Olympus).
    - Remark: for negative controls some slides were not treated with secondary or primary antibody or both to reveal autofluorescence and unspecific signals.

4. RESULTS:

All YCF 45 genes contain the AAA-type ATPase and cytochrome P450 domain,while the homologues from plants also bear the chloroplast targeting sequence on their N-terminus. Interestingly the YCF 45 open reading frames of kinetoplastids also have N-terminal extensions, but it is not clear whether this serves in any routing towards an intracellular compartement (see below) (Fig. 6). In *T. brucei* the gene is localized on chromosome 6 and its length is 1413 bp (470 AA) with predicted mass of 52.7 kDa and in genome databases is referred as Tb927.6.3350 (www.genedb.org, http://www.ncbi.nlm.nih.gov/).

ATGCTCTTGGGATGCCGTCGGTTTGTCACACACGCCGTCGGACAATCTTTCCCCCACGTA
ACTTCTCTATTACGTGGTAAGCGGTTGCTGCGCTTGCGCCATGGCTGCCCTCGCGCTAAA
CCACTGCTCTCCGCCGAAGGGGATTTGCACACAACGCCGTTGGACCGTCCCCCGCCAACA
GATATGCCACTCGCGTACTTGCAGGGGGAGATTTTGGATATGGAGAAGTATCGGACAGAA
GAAGCTGTTTGGTCGAGAAACACAGTGCGGGAAGCATGGAATGCAATTAACCACACACTG
CACGATACGACCCCCGGCGTTGATGCAACGAAGAAGAAGGAGATAATTGAAAAAGACCTT
GTAGGCTTGGCTCTCTGCACGTCATATGCGGAAGTGAAGCACCTTCTTTCTCTGTTGACG
CCCTCGCTTCGTTCACATATTGTTTCTCAGCCCTCCTATAAACAAACCGAGGTGGAGGAG
TTTTTTCTCCATCTTGGACAAGACATCGAAGTGAGAGGTGCGGATTGGGTGATTCAGGTT
CCGCCCCCGTCGGTGACCGATTTGCGTTATACCCTTGAGCGGGTGGGCCGATTTGGCGAT
GACGGCCGTGGGTGTATTGGACACACGCCGCATCGAGTAGCCGTGTGGCGCGGGCGGTTG
GGGGAACCGTTAGGACTCACTATTCGTGTGGGACGTTATGTGCCGAATGTTGCCAGGGCA
TTGGTTCCTCTTGCGCGACGAGGTAGTGTTTTGATACTCTCCAAGGCGGGTATGGGAAAG
ACCACAATCCTGAGAGACCTCGCTGCTTCCCTCTCTCGTGATCCGGCGAAACCCCGCGTT
ATGGTCGTTGATACCTCGAACGAAATTGGAGGTGATAGCCCGATGCCGCTGCCTTTTCTA
GGGCGTTGTCGCCGTATCCAGGTCCCGCGTCGCGAGGAGCAACAAGAAGTAATGAAACAA
ATTCTTCAAAACCACTCACCGGAGTACGTAATTGTTGATGAGGTTTCCACTACCGCCGAA
GCAGAAGTAACGTGGTCCATTTCCCAACGGGGAGTTCATCTCGTTGCCACATGCCATGGC
GAAAGTTTAGCGGGGTTATTGCAAAACCAGTCACTGAACTTGTTGGTGGGAGGCACCGCG
CATGCATTTCTTAGTAACGAGGAGCGGCGGTTACGGAACAAGATCAAGAAAACTATCCTC
GAGAGGCCGCATTCATCACCGTTTAAGTTTGTTGTGGAACTCCATTCACGGAAATTCGCT
CATCTTTATGTGGATGTAAACCAGGCTGTTGATTGTCTTTTGGACCAGGGGGATGCGAGA
CGAAGTGCACGGATTGGGAAAACAATTGCCATTGATGAGCCACTGCCAGATGAGGTGATG
GGTTTAGTTATGTCTCAAGAAAAGGAAGGTTCATATGCAAGAGAGCCGTCACACGAATCG
TGGAGTGAGTGCAACGATGATGACAGTTGCGCCCTCGCCGGGCGGGAGGGGTGGCGGCCA
AACAACCGGCCCGAGAGGCAGCGAGGGACTCGGTCGCATGGATGTCGAAAAAGATCAAAT
GACGACTTGTTGGACGAACTGAATAACTTTAATTAA

Fig. 6 The red sequence is the mature form of the YCF 45 protein, black sequence is the N-extension of YCF 45, according to the comparison of among the algae *Cyanidium caldarum, Porphyra purpurea*, the diatom *Odontella sinensis*, the plant *Arabidopsis thaliana* and the tritryps *T.brucei, T.cruzi* and *L.major*. Green sequence was added after sequencing of mRNA.

4.1. RNA interference experiment

4.1.1. Northern analysis

Because normally used exposure method at typhoon scanner did not show any mRNA transcript we decided to use classical film and special exposition conditions (48hrs at -80°C). Fig. 7 shows that double stranded RNA was detected in all three chosen clones. That means that RNAi was successful. However clones 1 and 2 exhibit a leakage. That is why results obtained from clone 3 exhibiting no signal of leakage are the most trustful. No dsRNA was detected in both controls WT (procyclic, 29-13 strain) and in bloodstream (strain 920). Bloodstream control was added after first mRNA transcript negative results. In spite of the wide accepted conviction that all genes at *T. brucei* are transcribed all the time (Clayton, 2002), there is still the possibility that some genes are transcribed just at some specific stages of the *T. brucei* life cycle. Long exposure using conventional film nevertheless showed that YCF 45 mRNA is indeed present also in procyclic stage. Because the signal was weak, second approach to prove transcription of YCF 45 was performed - SL-RACE PCR.

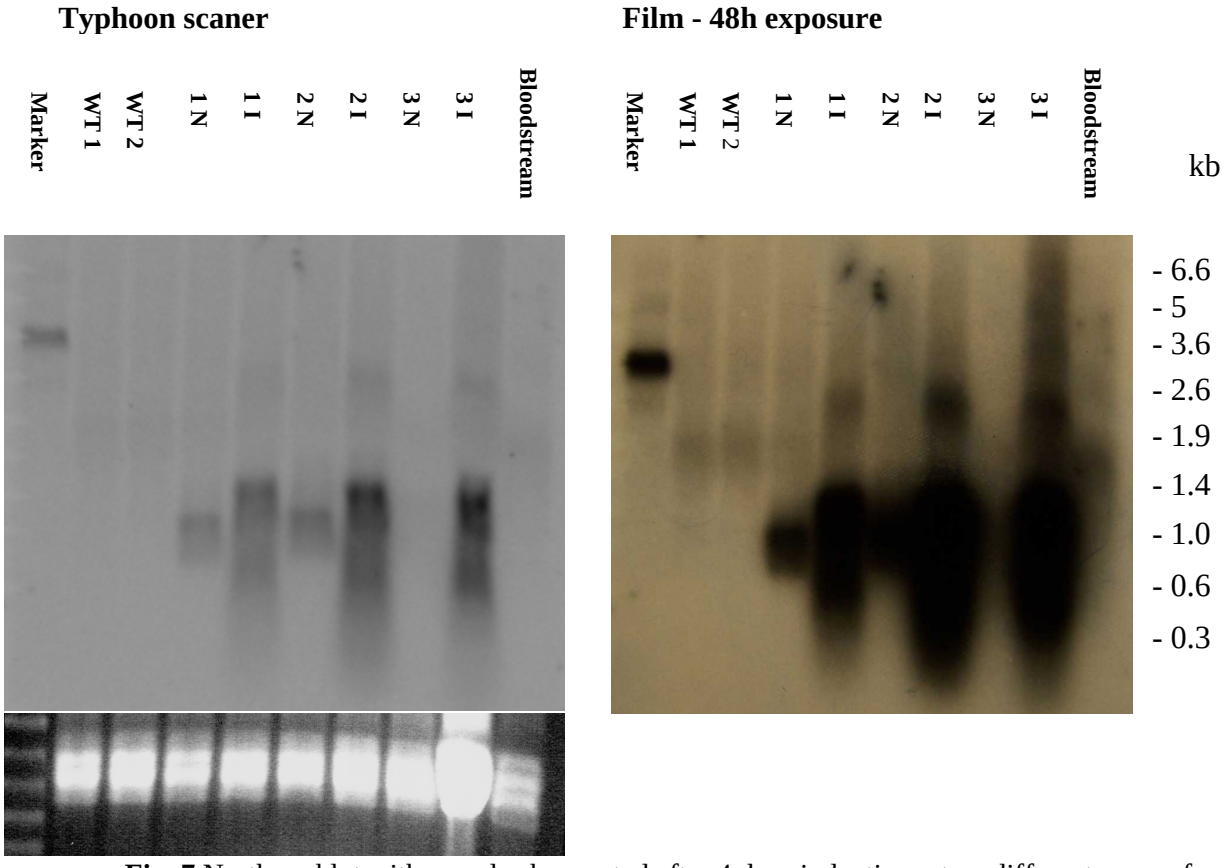
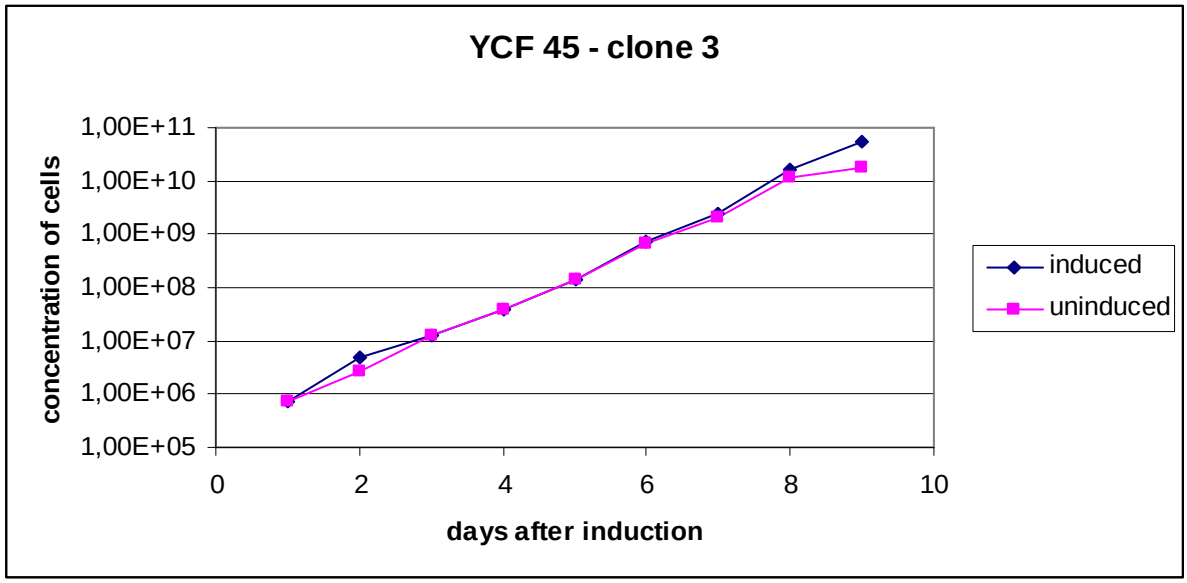
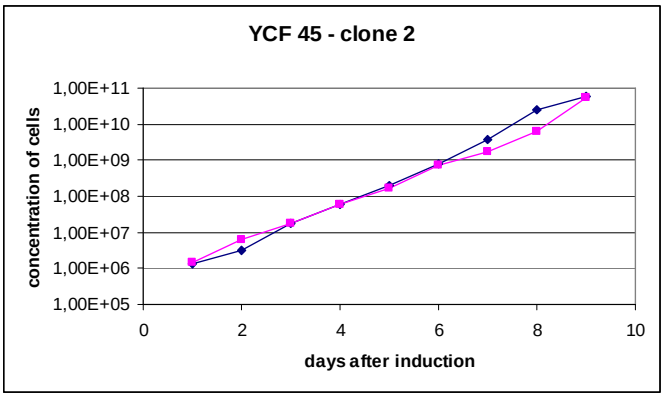


Fig. 7 Northern blot with samples harwested after 4 days induction – two different ways of explosure are shown. WT – procyclic 29-13 strain, 1,2,3 – different clones, I – induced, N – noninduced, Bloodstream – 920 strain. mRNA transcript migrates at about 1.8 kb.

4.1.2. Growth curves

Northern blot serves to prove RNAi works. Growth curves are than used to reveal the importance of silenced gene for cell survival. Unfortunately, no growth fenotype was detected after YCF 45 was knocked down (Fig. 8).





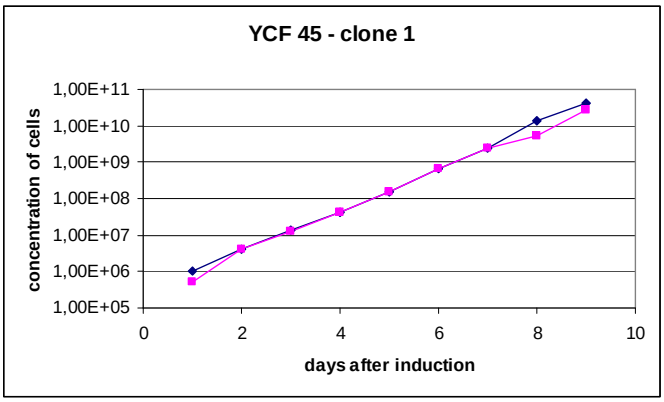


Fig. 8 Curves showing growth phenotype in YCF 45 knockdowns.

4.1.3. SL-RACE PCR

SL-RACE PCR was performed to proof that the YCF 45 gene is indeed transcribed, and also to identify the splice leader (SL) acceptor site of its premature mRNA. Primers against splice leader sequence were generously provided by Zdeňka Číčová.
Every nuclear-encoded mRNA of *T. brucei* undergoes trans-splicing and the same splice RNA sequence is added to its 5' end (Siegel, 2005). Because all mRNAs have the SL leader, the PCR is practically semi-specific. Nested PCR was performed with two primers against SL and two against the YCF 45 gene (Fig. 9).
Nested PCR resulted in the amplification of several bands of size ranging from 1500 bp to100 bp (Fig. 10). The expected size of the amplicon obtained with external primers ranged from 651 bp to 1151 bp, while the next nested PCR with inner primers was supposed to produce bands between 361 bp and 861 bp. The first number shows the shortest possible version - in this case mRNA would consist only of SL-RNA and transcript itself would start with the predicted start codon, while the second number is the longest possibility that was deducted from the database. In other words, it is the distance to the next gene located upstream of the YCF 45 on the chromosome 6. Bands that fulfill expected size were excised from the gel and sequenced. The obtained sequence was compared with the genomic sequence (Fig. 11).
The obtained result showed that YCF 45 is indeed transcribed and processed as other mRNAs in the procyclic stage of *T. brucei*. Moreover, we surprisingly found that our N-terminal sequence is even longer than the predicted one (Fig. 12). Alignment with the *T. cruzi* homolog strongly points to another methionine as a start codon, leading to the prediction of a new N-terminus. Such an N-terminus has a very strong prediction to be targeted into the mitochondrion (Mitoprot program output was 0.9946 probability that the new N terminus will be targeted into the mitochondrion, TargetP results are shown in the Tab.4). This finding was highly relevant to the following part of my work – localization using the HA-tagging strategy.

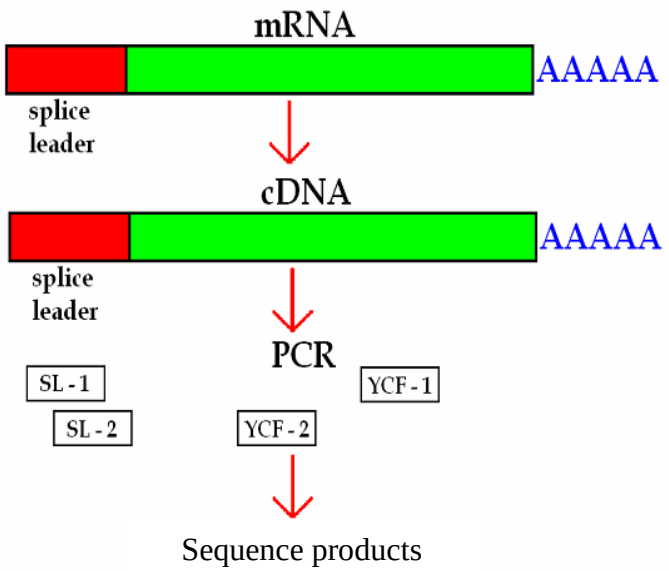


Fig. 9 Simplified scheme of SL-RACE PCR

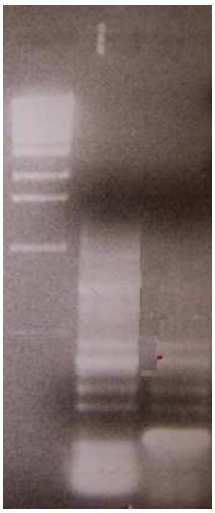
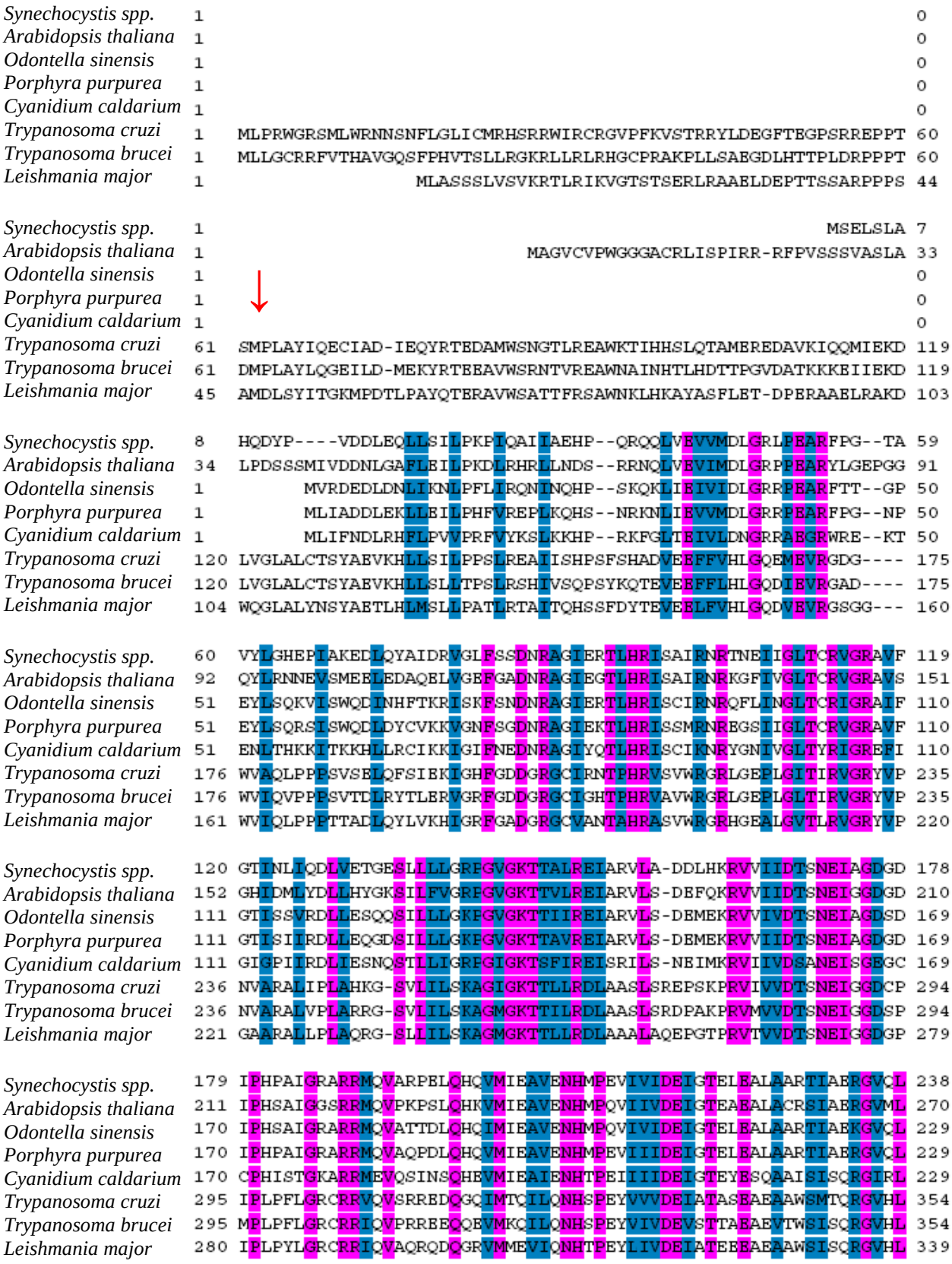


Fig. 10 Output of nested PCR performed on cDNA. 1.5% agarose gel was used. In the first row is marker, second shows products of PCR with outer primers and in the third row inner primers were used for the reaction.

```
                  [          Tb SL 2 →          ]
    [      Tb SL 1 →          ]
1   aactaacgctattattagaacagtttctgtactatattgTACAGAAGAGAGGGTTGATCTATAAGTTGTTGCTGCGAAAGA  81

82  GTTGTAGCACAA**ATG**CTCTTGGGATGCCGTCGGTTTGTCACACACGCCGTCGGACAATCTTTCCC  146

147 CCACGTAACTTCTCTATTACGTGGTAAGCGGTTGCTGCGCTTGCGCCATGGCTGCCCTCGCGCTA  211

212 AACCACTGCTCTCCGCCGAAGGGGATTTGCACACAACGCCGTTGGACCGNCCCCCGCCAACAGA  275

276 T**ATG**CCACTCGCGTACTTGCAGGGGGAGATTTTGGATATGGAGAAGTATCGGACAGAAGAAGCT  339

340 GTTTGGTCGAGAAACACAGTGCGGGAAGCATGGAATGCAATTAACCACACACTGCACGATACGA  403

404 CCCCCGGCGTTGATGCAACGAAGAAGAAGGAGATAATTGAAAAAGACCTTGTAGGCTTGGCTCT  467

468 CTGCACGTCATATGCGGAAGTGAAGCACCTTCTTTCTCTGTTGACGCCCTCGCTTCGTTCACATAT  533
                                                                    [
534 TGTTTCTCAGCCCTCCTATAAACAAACCGAGGTGGAGGAGTTTTTTCTCCATCTTGG*ACAAGACA*  589
    ← YCF 45_Rv 2    ]
590 *TCGAAGTGAGAG*GTGCGGATTGGGTGATTCAGGTTCCGCCCCCGTCGGTGACCGATTTGCGTTAT  663

664 ACCCTTGAGCGGGTGGGCCGATTTGGCGATGACGGCCGTGGGTGTATTGGACACACGCCGCATC  727

728 GAGTAGCCGTGTGGCGCGGGCGGTTGGGGGAACCGTTAGGACTCACTATTCGTGTGGGACGTTA  791

792 TGTGCCGAATGTTGCCAGGGCATTGGTTCCTCTTGCGCGACGAGGTAGTGTTTTGATACTCTCCA  856
                          [          ← YCF 45_Rv 1          ]
857 AGGCGGGTATGG*GAAAGACCACAATCCTGAGA*CCTCGCTGCTTCCCTCTCTCGTGATCCGGCGAAACCCC  926
```

Fig. 11 – The partial sequence of cDNA for YCF 45 mRNA from *T. brucei*. Positions and orientations of amplification primers are given above the sequence. SL RNA is given in small letters and both potential start codons are in bold and unederlined.


Synechocystis spp.
Arabidopsis thaliana
Odontella sinensis
Porphyra purpurea
Cyanidium caldarium
Trypanosoma cruzi
Trypanosoma brucei
Leishmania major

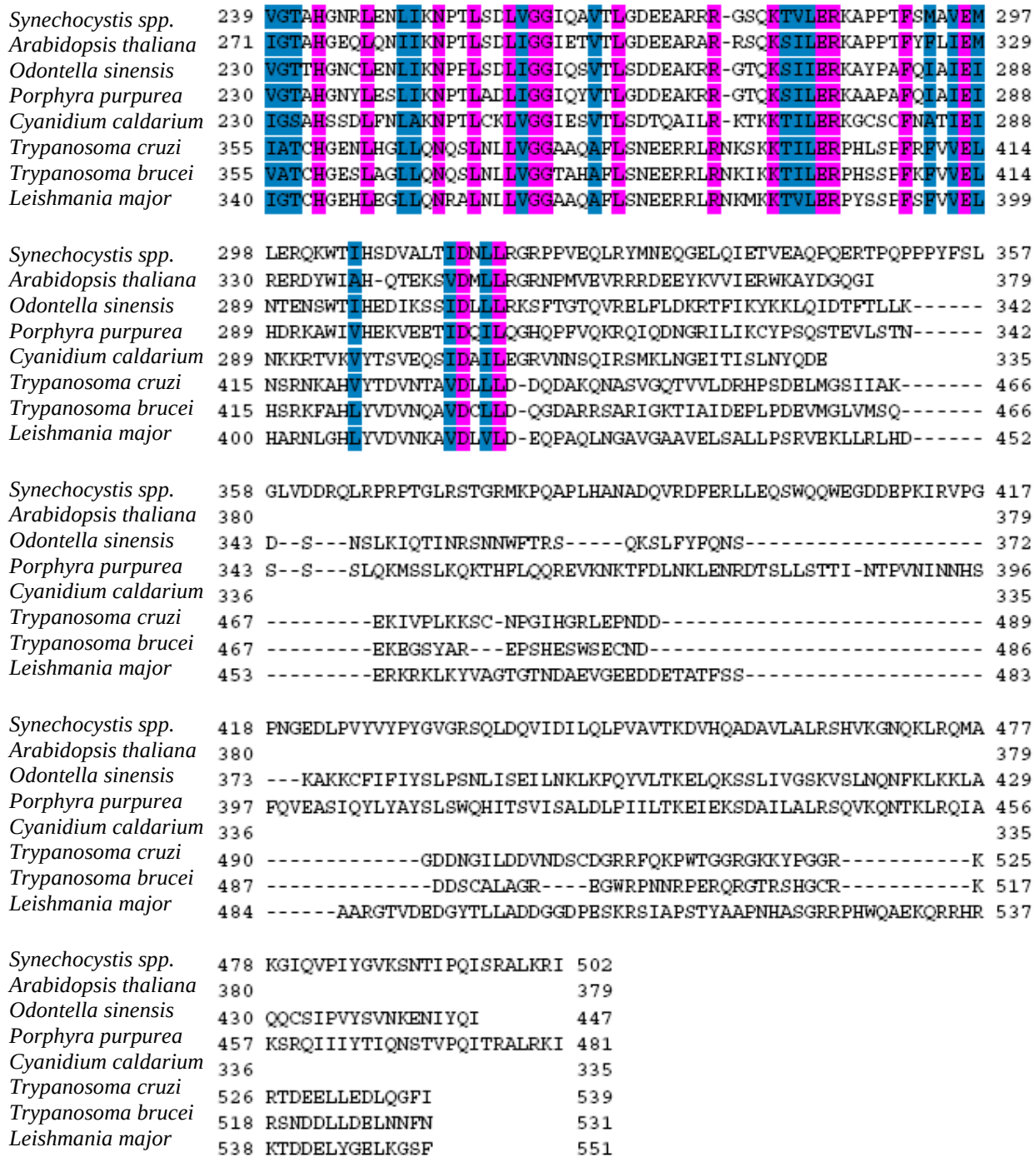


Fig. 12 Alignment of YCF 45 homologs from *Synechocystis spp.*, *Arabidopsis thaliana*, *Odontella sinensis*, *Porphyra purpurea*, *Cyanidium caldarium*, *Trypanosoma cruzi*, *Trypanosoma brucei* and *Leishmania major*. Red arrow points at second potential start codon (the original one). In blue the same aminoacids are shown, in violet are the conserved ones.

	cTP	mTP	SP	other
probability	0,145	0.974	0,014	0,022

Tab. 4 Results from TargetP prediction program. For prediction of targeting of the new N-extension to the cell compartements, to include chloroplast compartement prediction for plant like organisms was used. cTP – chloroplast targeting peptide, mTP – mitochondrial targeting peptide, SP – secretory pathway signal peptide

4.1.4. YCF 45 specific antibody

The purpose of RNAi is to silence functional product of the gene – the protein. To show that the target protein is really downregulated or completely eliminated, a specific antibody is needed. Unfortunately the commercial antibody from GeneScript Corporation seems not to work as shown in Fig. 13.

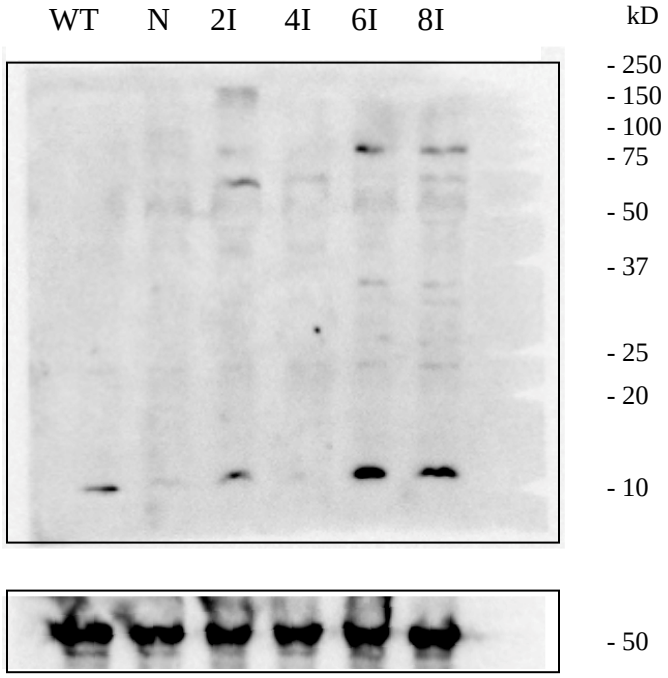


Fig. 13 Western blot prepared for testing the YCF antibody. Lower panel shows control marked by anti-enolase antibody (dilution 1:150 000). Upper panel was incubated with anti-YCF45 antibody (dilution 1:25). Each lane was loaded with approximately $1x10^7$ cells. WT – strain 29-13, in other lanes are cells bearing p2T7-177 vector with fragment for knockdown YCF 45, N – noninduced cells, I – induced (Number says how many days after induction cells were harvested.) YCF 45 specific signal should appear at about 50 kD.

4.2. Localization of the YCF 45 protein

For localization of the YCF 45 protein fusion with HA-tag was chosen. This approach was selected because no specific antibody against YCF 45 itself is available. This method also could reveal whether the N-extension serves in *T. brucei* as a specific targeting sequence of some kind, in case they would have different distribution in the cell - that was the scenario at the beginning of the experiment. Two different constructs were made - one with whole annotated gene (N+) and second without the N-extension (N-). After new potential beginning of YCF 45 was identified new prolonged construct was added (NW).
Both first constructs were prepared by ShaoJun Long and I started working on them at the stage N- before electroporation to the *T. brucei* and construct N+ I already get as clones of cells. Third construct was prepared completely by myself.
Localization was done by two different approaches – digitonin fractionation and immunolocalization.

4.2.1. Digitonin fractionation

Digitonin is a plant glycoside used as detergent, which interact with cholesterol in membranes. In lower concentrations it corrupt primarily cholesterol-rich membranes such as cytoplasmatic membrane and gives rise to big pores, in high concentrations digitonin destroys all cell membranes like a typical non-ionic detergent (Schultz, 1990).
At the procyclic stage *T. brucei* has one big mitochondrion, which makes it relatively easy to separate it from the cytosol. On westerns are shown the results. So far I got result only for the first two constructs that both seem to be targeted into the cytosol (Fig. 14).

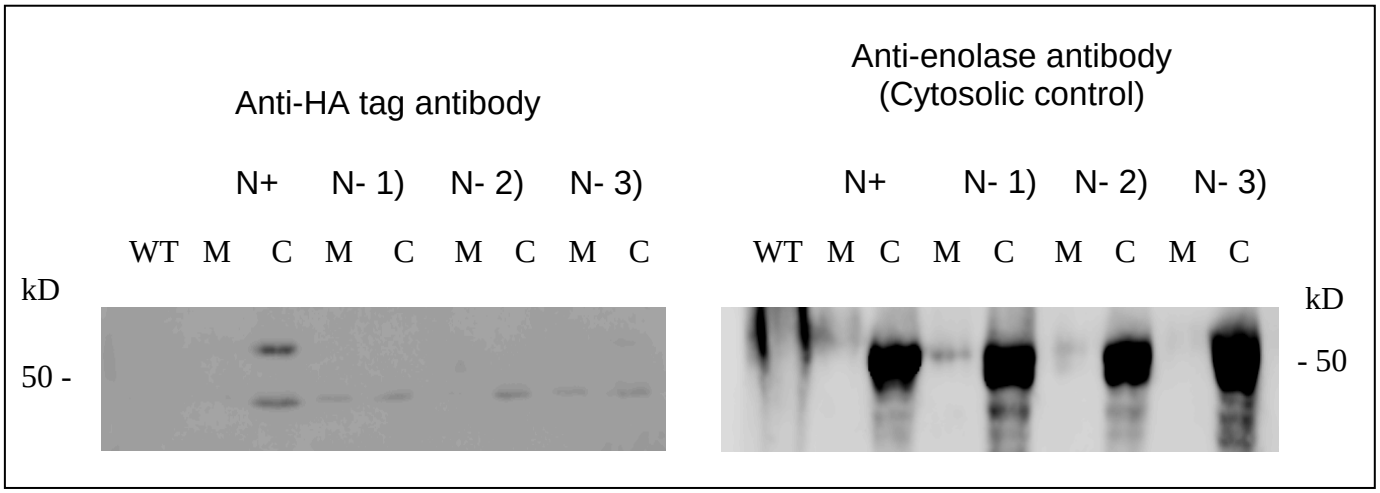


Fig. 14 western showing results of digitonin fractionation. WT – procyclic 29-13, M – mitochondrial fraction, C – cytosolic fraction, N+ - construct containing annotated N-extension, N- - different clones bearing construct without the N-extension.

4.2.2. Immunolocalization

Immunolocalization took place directly in the whole intact cells. So far only results for clone N+ are available (Fig. 14). Immunolocalization confirmed result from digitonin fractionation that N+ version of YCF 45 is targeted into the cytosol. With further experiments I was waiting for the third clone to be ready to use. Immunolocalization of all three constructs is under way.

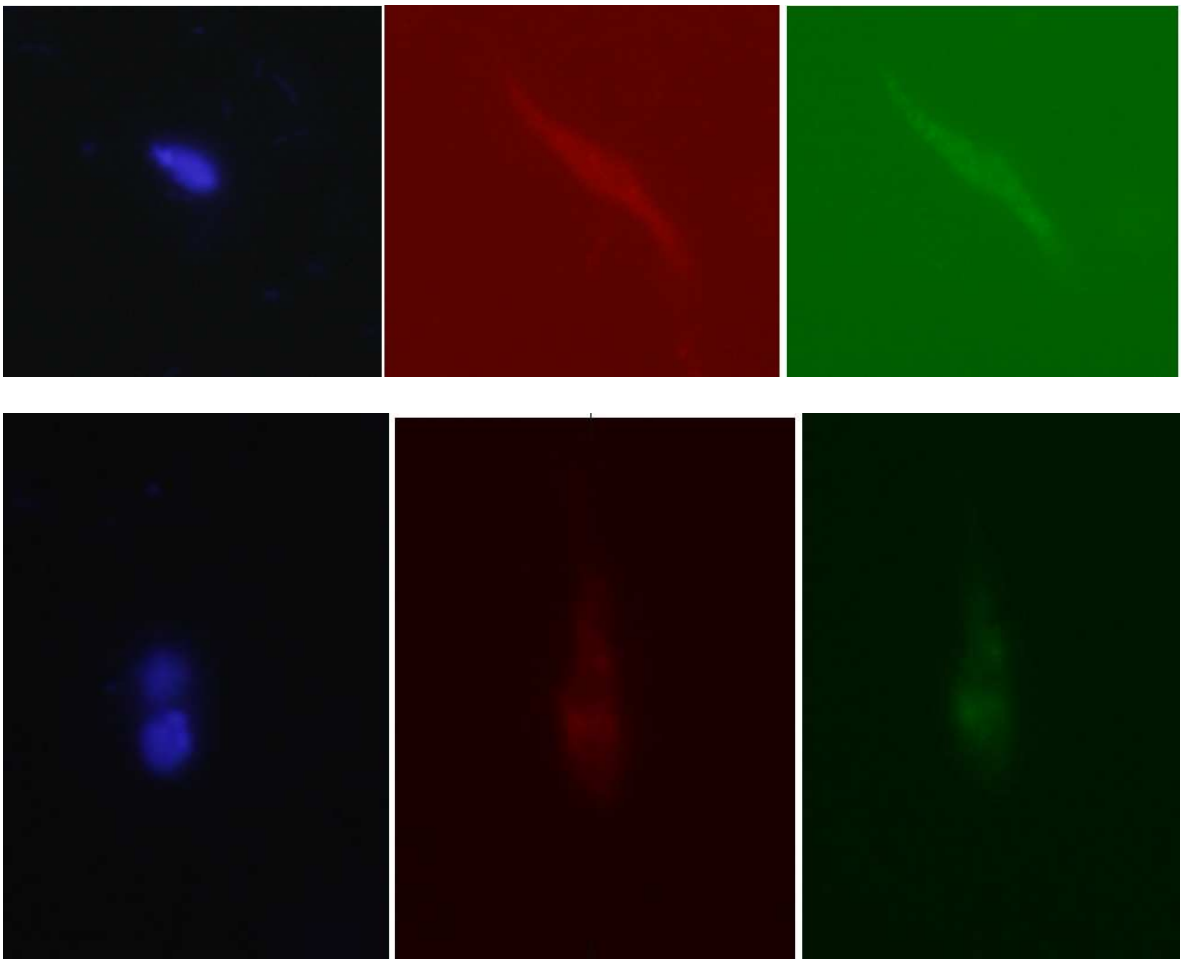


Fig. 14 Immunolocalization of HA-tagged annotated YCF 45. Each row shows one cell. In blue is Dapi stained DNA of nucleus and kinetoplast. Red secondary antibody reveals distribution of cytosilic enolase and in green N+ HA-tagged YCF 45 itself is shown. 100x magnification was used.

4.3. Phylogeny of the YCF 45 gene

The purpose was to find out whether the kinetoplastid YCF 45 genes are associated with some other particular group of organisms. That might tell us where the *T. brucei* YCF 45 comes from – which group was the donor of this weird chloroplast gene (Fig. 16). Phylogenetic tree was inferred from YCF 45 amino acid sequences. Homologues of YCF 45 were aligned using Kalign program at http://www.ebi.ac.uk/kalign/. Ambiguously aligned regions as well as gaps were excluded from further analysis. Tree was computed using Neighbor-joining (weighted) as implemented in AsaturA program (Van de Peer et al., 2002), with cutoff value = 1.163. Appropriate ML tree (loglk = -9477.97519) was computed by WAG model with discrete gamma distribution in 4+1 categories as implemented in PhyML. Particular substitutional model was chosen by PROTTEST according to AIC. ML bootstraps were computed from 200 replicates, NJ bootstraps from 1000 replicates. Numbers above branches indicate NJ bootstrap support/ maximum likelihood bootstraps.

Since so far no euglenid genome was sequenced, we tried another technique to find out whether euglenoids do have YCF 45 homologue and how close it is to those in trypanosomatids. We have run PCR on *Euglena gracilis* genomic DNA with primers used against *T. brucei.* However, no positive results were obtained. That is probably because of low similarity between the genes from Euglena and used primers (data not shown).

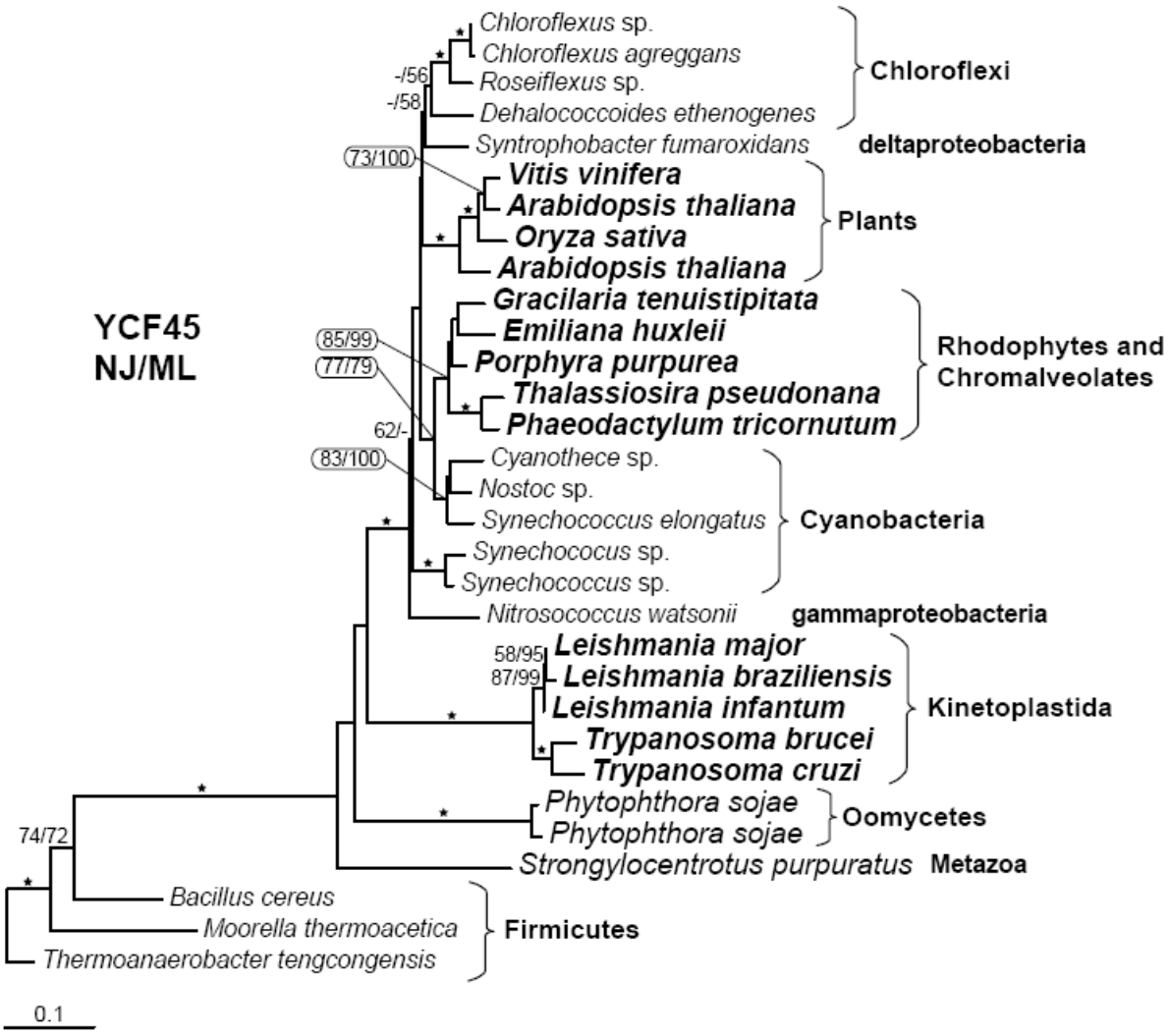


Fig. 16 Phylogeny tree of YCF 45 homologues.

5. DISCUSSION

In this work I performed functional analysis of the weird *Trypanosoma brucei* gene YCF 45 that seems to be of chloroplast origin (Opperdoes and Michels, 2007).

Two main lines of experiments have been performed in the procyclic stage of *T. brucei*. First one was knockdown of this gene, whereas in the second, intracellular localization of the protein was established.

For knockdown YCF 45, RNA interference was used. Northern analysis showed successful inducible expression of dsRNA. Some leakage was detected in the non-induced cells as well, but at least one clone exhibited no detectable dsRNA at non-induced conditions. Therefore results obtained from this clone can be considered as valid. Unfortunately, no growth phenotype was detected in any of the clones after the gene was silenced.

Because of the lack of YCF 45 specific antibody, we cannot directly show that the protein was downregulated. Therefore, polyclonal antibodies were generated against stretch of aminoacid of the protein. Preliminary Westerns blots with commercial antibodies, delivered only recently, failed to detect a bend of expected size, so different conditions have to be used. Moreover, Western analysis with bloodstream stages is yet to be performed.

Since from Northern analysis indicates that YCF 45 is a rare transcript, SL-RACE PCR was performed. This approach not only proved that YCF 45 is really transcribed in the procyclic stage of *T. brucei,* but also the splice leader acceptor site was identified. However, the most intriguing fact with a major impact on the whole project was the identification by SL-RACE PCR of a new potential start codon found downstream of the one predicted in databases (Fig. 11). For several reasons we consider this new methionine as the true start of the protein. The alignment showed that in *T. cruzi* homologue, the annotation program prioritizes this start codon as well. Originally a targeting function of some kind of the N-extensions was considered. Importantly homologues from plants and alga do not have any extensions or they are equipped with chloroplast signal peptide, accordingly to YCFs connection with chloroplast (Hallick and Bairoch, 1994). No convincing results were detected by prediction programs for any targeting signal, whereas the long N-extension has really strong prediction to be targeted into the mitochondrion. Ultimate prove is, however, localization of the YCF 45 protein in the cell.

Since the antibody is so far not available, we have decided to add an HA-tag to the protein in question, against which a monoclonal antibody is commercially available. (Long et al., 2008)
After sequencing the third construct, we have found two aminoacids differences from the genomic sequence. Because they were close to the C terminus, while our attention is focused on the targeting peptide, we found them as acceptable risk to our project. At their position they should not interfere with the targeting mechanism.

The HA- tagging strategy has also the advantage that it should be possible to distinguish between distribution of the N-extension-containing and the N-extension-lacking proteins. This specific approach shall reveal whether the weird N-extension serves as a targeting sequence to a cellular compartment. For that purpose, two survey techniques were used - digitonin fractionation and imunolocalization. Since the results for the third constructs are still in the process, only preliminary data are available.

Digitonin fractionation showed that the two tested constructs (N+, N-) are localized in the cytosolic fraction. (This observation strengthens the argument supporting first methionine as the genuine start codon of the protein, because if the N-terminal sequence is not a targeting peptide, what would be its function, as it is absent from all the other YCF 45 homologues?) Experiments performed with the third construct shall be the key for solving this issue.

Phylogenetic analysis was performed in order to get some clue as to the origin of YCF 45 and identification of its closest relatives. Indeed kinetoplastid YCF 45 homologues are related to each other but do not cluster with any other group of organisms. Therefore, it is not possible to establish where did the kinetoplastids obtain their chloroplast gene. Moreover its proximity to oomycetes and metazoa raises the possibility that YCF 45 may be originally eukaryotic and its chloroplast function is secondary and therefore derived. This fact is in variance with the currently published data (Opperdoes and Michels, 2007). These authors were working only with genes from cyanobacteria, diatoms, algae, plants and trypanosomatids without any proper outgroup. That may lead to early misjudgement in marking the YCF 45 as the chloroplast gene.

6. CONCLUSIONS

YCF 45 was successfully knocked down. The protein does not seem to be essential in the procyclic stage of *Trypanosoma brucei* according to the growth phenotype.

Three clones bearing different HA-tagged versions of YCF 45 were successfully obtained. So far original N-extension containing clone and N-extension lacking clone are targeted into the cytosol. To finish this experiment – third clone has to be added and immunolocalization of all three clones is a necessary step to settle the issue of origin and intracellular localization of YCF 45.

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