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Research Article

Increased expression of recombinant human tissue plasminogen activator in *Leishmania tarentolae*

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Recombinant tissue plasminogen activator (rt-PA) is one of the most important thrombolytic agents for treating cardiovascular obstructions such as stroke. Glycoprotein rt-PA is a serine protease, consisting of 527 amino acids of which 35 are cysteine residues. A variety of recombinant protein expression systems have been developed for heterologous gene expression in prokaryotic and eukaryotic hosts. In recent years, *Leishmania tarentolae* has been considered because of its safety aspects and special attributes in expression of complex proteins. In this study, two expression cassettes, each one including two copies of t-PA cDNA, were used for integration into the *L. tarentolae* genome by electroporation. Transformed clones were selected in the presence of appropriate antibiotics. Expression of active rt-PA was confirmed by Western blot and Zymography tests. Real-time PCR analysis was applied to investigate the presence of multiple t-PA gene copies in the parasite genome. Correlation of t-PA gene dosage and production rate was confirmed with real-time PCR. It was shown that the expression level of rt-PA in *L. tarentolae* is at least 480 IU/mL of culture media. This concentration of rt-PA is seven times higher than what was reported in previous studies in *L. tarentolae* and some other eukaryotic systems.

Keywords: Biochemical Engineering · Expression · Gene copy number · *Leishmania tarentolae* · Tissue plasminogen activator

1 Introduction

Plasminogen activators are enzymes that are used in the treatment of cardiovascular and cerebrovascular obstructions resulting from heart attacks and strokes. These proteolytic enzymes convert the catalytically inactive plasminogen to its active form, plasmin, which in turn dissolves fibrin, thereby clearing the circulatory system. Of the three major plasminogen activators in clinical use, streptokinase is produced by various strains of streptococci whereas urokinase and tissue plasminogen activator (t-PA) are found in humans. Unlike t-PA, which activates plasminogen in the presence of fibrin, thrombolysis with urokinase and streptokinase is due to systemic activation of plasminogen, which can cause unwanted digestion of coagulation proteins and considerable increase in the risk of hemorrhage during treatment [1, 2].

Human t-PA is a serine protease with 17 disulfide bonds, 3 main N-glycosylation and one O-glycosylation sites and a molecular weight of approximately 68 kDa. T-PA is encoded by a 1681-bp gene and consists of five distinct structural domains: a finger-binding domain, an epidermal growth factor (EGF) domain, two kringle structures and a catalytic domain [3, 4].

A broad range of different expression systems such as bacteria, fungi, insect and mammalian cells, transgenic animals and plants have been used for production of recombinant (r)t-PA [2]. Bacteria are generally unsuitable for the expression of recom-

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Abbreviations: APRT, adenine phosphoribosyltransferase; **CHO**, Chinese hamster ovary; **r**, recombinant; **t-PA**, tissue plasminogen activator

binant multi-disulfide polypeptides such as rt-PA as they do not fold properly in this host and accumulate in misfolded structures [5–7]. Attempts to produce rt-PA in insect cells and *Saccharomyces cerevisiae* have been unsatisfactory due to poor secretion of rt-PA into the culture media, hyperglycosylation and improper folding [8, 9]. In addition, the expression of rt-PA in transgenic plants has not provided satisfactory yields [10].

At present, human rt-PA is produced commercially in Chinese hamster ovary (CHO) cells. Despite significant advances, there is still a considerable degree of variability and very little understanding of the sources of variation in mammalian cell culture processes. This makes cell line development laborious and extensive screening of clones often takes several months. Moreover, high costs of cell culture media and the possibility of contamination with viruses and prions, depending on the source of media components, are additional problems associated with the use of mammalian cell cultures [11, 12].

In recent years, parasitic organisms of the Trypanosomatidae family have come under consideration for biotechnological purposes due to their genetic characterization. Subsequently, methods for genetic manipulation have been well-established [13]. One specific Trypanosomatidae family member, *Leishmania tarentolae*, a parasite of gecko *Tarentolae anularis*, has been found to be useful as a new expression system. Suitable properties in post-translational modifications, inexpensive growth condition, fast growth rate, safety for humans and successful expression of unimmunogenic glycoproteins make this parasite potentially attractive for expression of heterologous recombinant proteins [14–18]. Hence, *L. tarentolae* has been evaluated as an alternative to mammalian cells for expression of rt-PA.

In most eukaryotic cells, transcription is a critical point in the expression of recombinant proteins. Among several strategies for improving transcription rates such as utilizing a strong promoter and the incorporation of matrix attachment regions, gene copy number has also been shown as an effective factor in transcription modulation and protein expression [19–21]. Hence, in an effort to enhance the expression level of active rt-PA, cassettes containing two copies of t-PA gene were constructed and transferred to *L. tarentolae* for integration into the chromosomal 18S ribosomal RNA locus (ssu). Ssu is a repetitive locus of the *L. tarentolae* genome with high rates of transcription by the host RNA polymerase I [22], which could affect the level of expression as well. In addition, this strategy would help to design a new expression vector for high-level production of complex human recombinant proteins in non-pathogenic *Leishmania*.

2 Materials and methods

2.1 T-PA gene amplification

Genomic DNA of the CHO 1-15 cell line (CRL-9606. A.T.C.C), which was cultivated in Ham's F12 medium (Sigma, USA) and contained t-PA cDNA, was extracted using the DNA tissue kit (BILLATEC, Germany). The first t-PA copy was amplified with the primers FH-tPA 5′CAACTAGTGGATCC ATGGATGCAATGAAGAGAGG3′ containing *Spe*I-*BamH*I restriction sites (underlined) and RH-tPA 5′TGTAAGCTTTCA**CACCACCACCACCACCAC** CGGTCGCATGTTGTCACG3′ with *Hind*III restriction site (underlined). For amplification of the second t-PA copy, primers FM-tPA 5′CCATCTAGA ATGGATGCAATGAAGAGAGG3′ containing *Xba*I restriction site (underlined) and RM-tPA 5′TGTG TCGACTCA**CACCACCACCACCACCAC**CGGTCG CATGTTGTCACG3′ with *Sal*I restriction site (underlined) were used. Primers were designed according to the sequence of the human t-PA gene (GenBank accession number I01047). Both reverse primers contained 18 nucleotides encoding six His-tags (bold) for protein purification using the Ni-NTA agarose column. To ensure the accuracy of the sequence and produce blunt end products PWO DNA polymerase was used (Roche, Germany).

2.2 Plasmid construction

The PCR products were individually cloned in pJET1.2/blunt using the ClonJET PCR Cloning Kit (Fermentas, Lithuania) following the manufacturer's instructions. The resulting plasmids were analyzed by restriction-enzyme analysis and sequencing. The first cloned t-PA gene was digested with *Spe*I/*Hind*III restriction enzymes (Roche, Germany) and transferred to upstream of α-tubulin non-translated region that had been cloned in *Hind*III/*Xba*I site in pGEM-9Zf(-) cloning vector (Promega, USA). The second cloned t-PA gene was digested with *Xba*I/*Sal*I restriction enzymes and subcloned downstream of the α-tubulin non-translated region and recombinant pGEMt1-α-t2 plasmid was constructed. The presence and correct order of the genes were confirmed by *BamH*I/*Sal*I restriction-enzyme analysis. To construct the two expression cassettes for *L. tarentolae*, pGEMt1-α-t2 was digested with the *BamH*I/*Sal*I restriction enzymes and the resulting fragment t1-α-t2 was cloned in biocompatible sites *Bgl*II/*Xho*I of

pFX1.4sat and pFX1.4hyg (Jena Bioscience, Germany). Presence of the t1-α-t2 gene fragment in pFX1.4sat-2tPA and pFX1.4hyg-2tPA was confirmed by PCR and restriction-enzyme analysis. Throughout this work, plasmids and DNA fragments were purified using a miniprep kit (Core Bio, Korea) and a gel extraction kit (Qiagen, USA).

2.3 Transfection of *L. tarentolae* cells

Leishmania tarentolae promastigotes were cultivated as a static suspension in Brain Heart Infusion (BHI) media (Merck, Germany) supplemented with 15 μg/mL hemin (Sigma, USA), 50 IU/mL penicillin and 50 μg/mL streptomycin (Sigma, USA) at 26°C. For integration of the expression cassette into the 18S ribosomal RNA (ssu) locus, the pFX1.4hyg-2tPA plasmid was digested with the *Swa*I restriction enzyme and the hyg-2tPA fragment obtained was eluted from agarose gel. Of this purified fragment, 10 μg was transformed into *Leishmania* cells by electroporation [23] and transgenic clones were selected on supplemented BHI agar medium containing 15% fetal calf serum and 40 μg/mL hygromycin (Fluka, USA). Transformed colonies were cultured in supplemented BHI containing 30 μg/mL hygromycin and used for the second transfection. This was performed by the same procedure on two randomly chosen hyg-resistant clones with the sat-2tPA cassette resulting from digestion of the pFX1.4sat-2tPA plasmid with *Swa*I restriction enzyme and transformants were selected on BHI agar medium containing 15% serum, 80 μg/mL nourseothricin and 40 μg/mL hygromycin. Acquired colonies cultured in BHI medium containing 30 μg/mL hygromycin and 60 μg/mL nourseothricin. Two doubly resistant progeny clones, each from one singly transformed parent, were selected for further assays. Integration of each expression construct into the ssu locus was confirmed by PCR on the genomic DNA with specific primers.

2.4 Western blotting

Cells were harvested from a 6-day cell culture (10^8 cell/mL) and suspended in lysis buffer containing 50 mM tris (MP Biomedical, France), 10% glycerol (Merck, Germany), 0.1% Triton X-100, 1 mM PMSF (Sigma, USA) and 2x protease inhibitor (*FAST* Protease Inhibitor, Sigma, USA) prior to incubation at room temperature for 1 h. Afterwards, the cells were sonicated twice with 70 Hz for 10 s and centrifuged at 9000 rpm for 10 min. Total protein from the supernatant of the lysed cells (35 μg) was loaded on 12% SDS-PAGE and Western blotting was performed according to standard techniques [24]. Rabbit polyclonal antibody to t-PA (Abcam, USA) was used as the primary and goat polyclonal anti-rabbit IgG HRP (Dako, UK) as the second antibody. The ECL detection system (GE Healthcare, Norway) was used for chemiluminescence development.

2.5 Zymography test

Zymography was performed by using 20 μL of a 6-day-old culture media of both singly and doubly transformed clones. A 12% resolving polyacrylamide gel (Sigma, USA) was co-polymerized with plasminogen (Chromogenix, Italy) and gelatin (Sigma, USA). The 4% stacking gel was prepared without plasminogen and gelatin. Electrophoresis was performed at 4°C at a constant current of 10 mA. The residual SDS in the gel was removed in 2.5% (w/v) triton X-100 solution by shaking at room temperature for 1 h and subsequently incubated in 0.1 M glycine/NaOH (pH 8.3) for 3 h at 37°C and stained with CBB R-250 [25]. After the staining and destaining steps, the gel's clear area represented the active rt-PA while the background remained blue.

2.6 Amidolytic activity test

Production of active rt-PA by both singly and doubly transformed clones was measured quantitatively using the Biopool Chromolyze t-PA kit (Biopool, Ireland). Diluted supernatants taken from 1- to 7-day-old cultured cells agitated at 130 rpm in 50 mL total volume of single transformants and their doubly transformed progenies were used for analyzing the level of active t-PA secreted into the culture media at different time intervals. The growth curve of the transgenic and wild-type clones was also plotted. Active t-PA in culture media was captured by sp-322 mAb at pH 5.9. After three washing steps and addition of the plasminogen and chromogenic substrate as instructed by the manufacturer (Biopool, Ireland), the light absorbance at 405 nm wavelength was measured. The amidolytic activity of the secreted rt-PA was calculated based on the standard curve from the kit.

2.7 Quantitative real-time PCR

A 126-bp fragment located on the t-PA gene and a 118-bp fragment on the adenine phosphoribosyltransferase (APRT) gene as a single copy reference gene [26] were amplified for analyzing the copy number variations in the different transformed clones. Primers were designed using Primer Ex-

press software V.3.0 (Applied Biosystems, USA) and selected ones underwent an extensive search using BLAST tool (www.ncbi.nlm.nih.gov/blast) to avoid any significant homology with other sequences (Table 1). The genomic DNA of the singly and doubly transformed cells was extracted according to the method described by Medina *et al*. [27]. The concentration of the isolated DNA was measured using the NanoDrop_ND-1000 Spectrophotometer (NanoDrop Technologies, USA) by recording the absorbance at 260- and 280-nm wavelengths. DNA samples with the A260/A280 ratios higher than 1.7 were selected for quantitative analysis.

SYBR Green I real-time PCR assay was carried out in final reaction volumes of 25 µL with 12.5 µL of SYBR Green I Master mix (Applied Biosystems, USA), 1 µM each of forward and reverse primers and 25 ng of genomic DNA. Thermal cycling was performed on the ABI 7300 Sequence Detection System (Applied Biosystems, USA) using the following cycling conditions: 10 min at 95°C as a first denaturation step, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Each complete amplification stage was followed by a dissociation stage at 95°C for 15 s, after which the temperature dropped to 60°C for 30 s before ramping up the temperature from 60 to 95°C (at the rate of 0.03°C/s). The fluorescence intensity was continuously measured over the ramping stage for 20 min [28]. Melting curve analysis was performed according to the dissociation stage data and reactions with a single peak at expected melting temperature (*T*m) were considered for further analysis.

A validation experiment was performed on the genomic DNA of a doubly transformed clone to determine the PCR efficiencies of the test and reference gene. The standard curve was drawn by plotting the logarithmic input of template DNA *vs*. the related threshold cycle (C_T) values. The C_T parameter was defined as the cycle number at which the amplification plot passed a fixed threshold. The corresponding real-time PCR efficiencies were calculated according to the slope of the standard curve and the formula: Efficiency = $[10^{(-1/\text{Slope})}] - 1$. Data evaluation was carried out using the ABI PRISM 7300 Sequence Detection System and its software v.1.2.3 (Applied Biosystems, UK) [29]. Statistical analysis including mean, SD, correlation coefficients (R^2) and graph preparation were carried out using Excel® software.

The gene copy number was calculated for four different singly and doubly transformed clones by using the comparative threshold cycle. The mean threshold cycle (mC_T) was obtained from triplicate amplifications during the exponential phase and the mC_T value of the reference gene (APRT) was subtracted from mC_T value of the target gene (t-PA gene) to obtain normalized ΔC_T. The calculated ΔC_T was converted to a ratio using the ratio formula (Ratio = $2^{\Delta CT}$) [30].

3 Results

3.1 Construction of expression vectors

Two expression cassettes containing nourseothricin and hygromycin antibiotic resistance genes and two copies of t-PA gene were constructed to express rt-PA in *L. tarentolae* (Fig. 1). Since the primary transcripts of most coding genes in Trypanosomatidae are polycistronic, processing of pre-mRNA including *trans*-splicing and polyadenylation are necessary to produce monocistronic mRNA [31, 32]. The α-tubulin non-translated region (NTR4) between two copies of t-PA gene and other NTR (NTR1, 2, 3) in the vectors provide the proper signals for these post-transcriptional modifications. The 5′ssu and 3′ ssu are the 5′ and 3′ portions of the small subunit of *L. tarentolae* rRNA gene for homologous recombination. The sequence of the t-PA genes was confirmed by sequencing.

3.2 Transformation of *Leishmania* cells

After sequence verification, the hyg-2tPA fragment obtained from digestion of pFX1.4hyg-2tPA with the *Swa*I restriction enzyme was transformed into *L. tarentolae* by electroporation. Hyg-resistant clones were selected on BHI agar containing 40 µg/mL hygromycin. Two of these clones were subsequently transformed with a sequence-verified sat-2tPA construct obtained by digestion of

Table 1. The oligonucleotide primers designed for real-time PCR assay.

Gene	Primer	Sequence	*Tm* (°C)	Amplicon size (bp)
t-PA	tPA-F	5′GGCAAGGTTTACACAGCACAGAAC3′	60.9	126
t-PA	tPA-R	5′CGTCAGCCTGCGGTTCTTC3′	60.5	
APRT	APRT-F	5′TGGTGCTAATCGATGACGTTCTG3′	61	118
APRT	APRT-R	5′AGGAAAGGAATGGTCAAGATCGAG3′	60.7	

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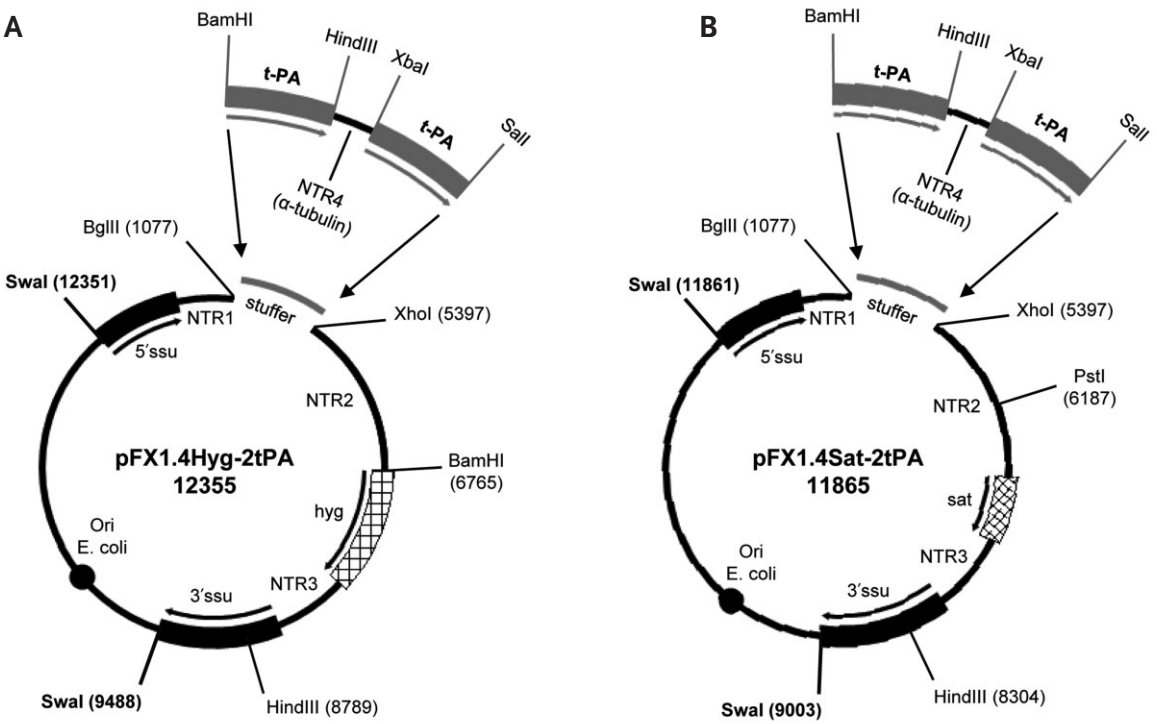

Figure 1. Schematic diagram of two vectors used for expression of t-PA in *L. tarentolae* cells. (A) pFX1.4Hyg-2tPA expression vector; (B) pFX1.4Sat-2tPA expression vector. Abbreviations: 5′ssu, 5′-portion of the small subunit of the *L. tarentolae* rRNA gene; NTR1 (0.4 k-IR cam BA), NTR2 (1.4 k-IR cam CB) and NTR3 (1.7 k-IR) are optimized gene flanking non-translated regions providing the splicing signals for post-transcriptional mRNA processing in *L. tarentolae*; NTR4 (α-tubulin), a non-translated intergenic region of *L. donovani*; tPA2, cDNA of the second copy of t-PA; sat, norseothricin resistance gene; hyg, hygromycin resistance gene; 3′ ssu, 3′-portion of the small subunit of *L. tarentolae* rRNA gene.

pFX1.4sat-2tPA with *Swa*I restriction enzyme. BHI containing hygromycin-nourseothricin was used for the selection of doubly transformed clones. Integration of the t-PA-containing cassette was confirmed by PCR using genomic DNA of the parasite as a template and primers for genes encoding hygromycin (1050 bp), nourseothricin (500 bp) and t-PA (1700 bp) (Fig. 2). For further confirmation, an amplification of the fragments with hyg or sat forward and ssu-specific reverse primers was performed, which generated 2800- and 2300-bp PCR products, demonstrating the occurrence of homologous recombination integration in the 18S rRNA site (Fig. 2, lane 4 and 8, respectively). After the initial screening by PCR, two singly transformed clones and two in total of hyg-sat positive progenies were chosen for the following assays.

3.3 Western blot analysis

The commercial rt-PA reacted with rabbit anti-t-PA antibody and produced a 66-kDa band representing the intact structure of the protein (Fig. 3, lane 5). Lysate from non-transformed cells did not react with t-PA antibody (Fig. 3, lane 4) whereas blot analysis results of the expressed t-PA reacted with anti-t-PA showed the presence of a single band with an apparent MW of 64 kDa (Fig. 3, lane 1, 2).

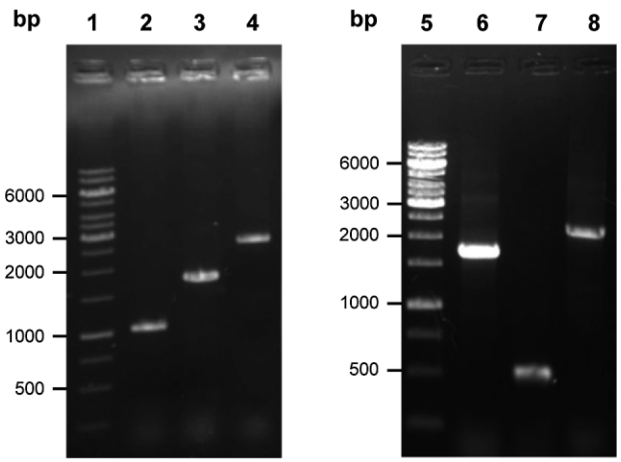

Figure 2. Agarose gel electrophoresis of PCR amplification in a doubly transformed *L. tarentolae* clone. PCR amplification was performed on t-PA, *hyg* and *sat* genes to confirm the integration of the expression cassettes into the parasite genome. Lane 1, 1-kb size marker; lane 2, *hyg* gene (1050 bp); lane 3, t-PA gene (1.7 kb); lane 4, 2.8-kb band related to the integration of *hyg*-tPA2 cassette into the ssu locus; lane 5, 1-kb size marker; lane 6, t-PA gene (1.7 kb); lane 7, *sat* gene (500 bp); lane 8, 2.3-kb band related to the integration of *sat*-tPA2 cassette into the ssu locus.

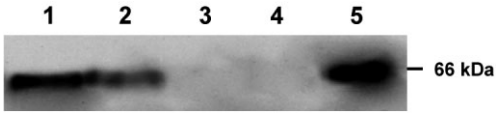

Figure 3. Western blot analysis of cytosolic fraction of *L. tarentolae* cells. Lane 1, 35 μg of total protein from cell lyses of a doubly transformed clone; lane 2, 35 μg of total protein from cell lyses of a singly transformed clone; lane 3, 10 μL of blank (loading buffer); lane 4, 35 μg of total protein from non-transformed clone cell lyses; lane 5, 250 ng of pure commercial rt-PA (Actylase).

3.4 Zymography

The Zymography test was performed to assess the secretion of rt-PA into the culture media and its serine-protease activity. After 6 days of culturing, the media of untransformed, singly and doubly transformed clones were analyzed under non-reducing conditions to retain the correct conformation of secreted rt-PA. Similar to the commercial rt-PA (Fig. 4, lane 5), the cell culture supernatant of transformed cells showed the clear zones related to the activity of expressed rt-PA (Fig. 4, lanes 2 and 3). The similarly obtained sample from non-transformed cells did not show any activity (Fig. 4, lane 1).

3.5 Amidolytic activity measurement

As active t-PA converts plasminogen into plasmin, the culture media of singly and doubly transformed cells were used for the assessment of the protein's amidolytic activity. The plasmin produced reacts with the substrate producing a chromogenic substance with a maximum absorbance at 405-nm wavelength. Figure 5 shows the growth curve of transformed *L. tarentolae* in an agitated culture and the enzymatic activity of the recombinant t-PA produced by a singly transformed cell and its progeny. The growth curves of transformed and non-transformed cells were almost identical and, therefore, only the average growth curve has been plotted. The maximum activity observed in culture media of the singly transformed colony was 182 IU/mL compared to 230 IU/mL for the doubly transformed cells. The highest activity in the other family was 375 IU/mL and 480 IU/mL for the singly and doubly transformants, respectively. No amidolytic activity was observed by non-transformed cells at the same conditions.

3.6 Real-time PCR and t-PA gene dosage analysis

In order to evaluate the feasibility of *L. tarentolae* cells to endure multiple copies of foreign genes in the ssu locus and to further study the coherency

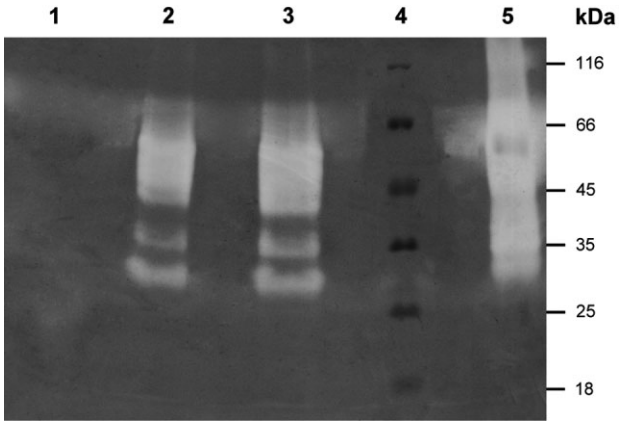

Figure 4. Zymography test results from culture supernatant of *L. tarentolae* clones. Lane 1, 20 μL supernatant of non-transformed cell culture; lane 2, 20 μL supernatant of singly transformed cell culture; lane 3, 20 μL supernatant of doubly transformed cell culture; lane 4, size markers; lane 5, 2 μg of standard rt-PA (Actylase).

between the expression rate and the gene dosage of recombinant t-PA, SYBR Green I real-time PCR was optimized to determine the copy number of the t-PA gene integrated into the *L. tarentolae* genome in comparison to APRT as a single copy reference gene using the threshold cycle method. The mean C_T values corresponding to each concentration of serially diluted template DNA were plotted against the log input of DNA. The slopes were within an acceptable range (–3.6< slope < –3.1), indicating the validity of the comparative threshold cycle method for relative quantitation (Fig. 6).

The amplification of the t-PA and APRT genes was performed in triplicate (the SD of C_T values was in the range of 0.01–0.03). The number of copies of the t-PA gene in the genomic DNA of singly and doubly transformed cells was deter-

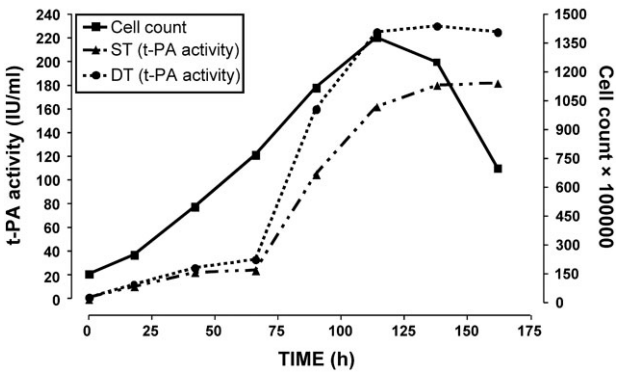

Figure 5. Amidolytic activity of secreted t-PA in *L. tarentolae* culture media is plotted against the average cell count during 7 days agitated culturing of a singly transformed parent clone (ST) and its doubly transformed progeny (DT).

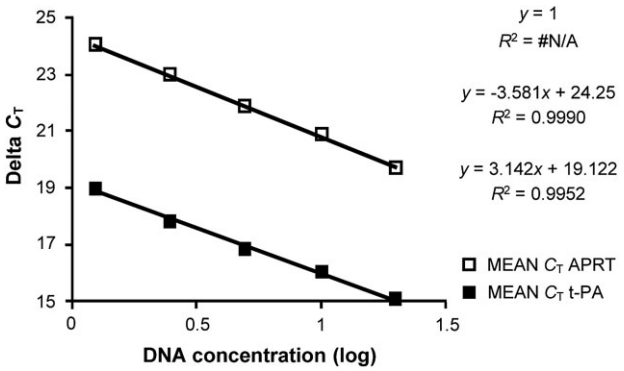

Figure 6. Validation of real-time PCR assays for analysis of t-PA gene copy number integrated in *Leishmania* cells. C_T quantifications measured over a wide range of the genomic DNA concentrations. Evaluation of mean C_T values for t-PA and APRT genes measured over series of genomic DNA dilutions (from 20 to 1.25 ng/μL genomic DNA). The resulted equations were: $y = -3.142x + 19.122$, $y = -3.581x + 26.256$, for t-PA and APRT genes, respectively. All trendlines slopes showed linear correlation (R^2 of 0.9952 and 0.9997, respectively).

mined using a single-copy gene (APRT) as a reference (Fig. 7). According to the formula, the copy number ratio between the t-PA and the APRT gene in two investigated families was 4 and 8 for the parent clones whereas the ratio was 7 and 32 for their progenies, respectively.

4 Discussion

Here, we aimed to enhance the expression of recombinant t-PA in non-pathogenic Trypanosomatidae, *Leishmania tarentolae*, as a beneficial expression system with several unique features (as mentioned above). Because of the therapeutic potential of t-PA as a fibrinolytic agent, clinical use requires a consistent and efficient method that could provide a high-yield production. Compared to the other recombinant expression systems, the rt-PA produced in *E. coli* is a non-glycosylated single-chain polypeptide accumulated as insoluble inclusion bodies. Although *E. coli* does not seem feasible as an rt-PA producer, the specific activity of the bacterial rt-PA was comparable with rt-PA from CHO. In addition, glycosylation is non-essential for the biological function of the rt-PA and the non-glycosylated form has a longer half life [33, 34]. Hence, rt-PA production in bacteria remains desirable if the refolding procedure becomes more efficient. Reteplase is a truncated form of rt-PA with fewer disulfide bonds produced successfully in a bacterial system. However, this rt-PA activates plasminogen directly and does not require plasminogen complexing, which causes fibrinogen depletion and could result in higher frequency of bleeding complications [4]. Recombinant mammalian cells transformed with different copies of human t-PA have generally been better producers than the other hosts, such as natural mammalian cell lines, bacteria, yeast and insect cells. Cloning and expression have been successfully performed in rat myeloma, CHO cells and C127 fibroblasts.

Several studies have already shown a direct correlation between the gene copy number and the expression level of heterologous proteins in *Leishmania* and some other eukaryotic hosts [21, 22, 35]. Therefore, in order to obtain high expression levels, additional copies of the t-PA gene were integrated into the *L. tarentolae* genome. Since the intergenic NTR play an important role in ensuring proper post-transcriptional modifications in Try-

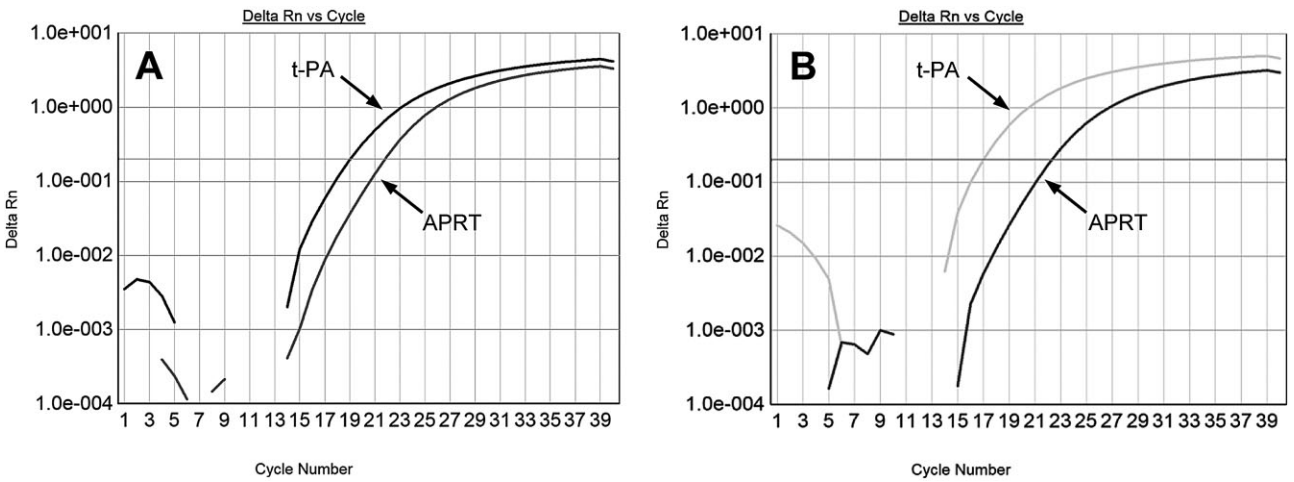

Figure 7. Amplification plots of the t-PA and reference genes in singly and doubly transformed cells containing the highest copy number. Relative fluorescence (Δ*R*n) is plotted against the number of PCR cycles. (A) Amplification curve analysis for a singly transformed cell, t-PA gene in comparison with APRT. (B) Amplification curve analysis for doubly transformed cell (progeny of the above singly transformed cell), t-PA gene in comparison with APRT.

panosomidae [36], the α-tubulin NTR from *Leishmania donovani* was inserted between two copies of t-PA gene. Two constructs, each one with two copies of t-PA gene, were respectively transformed into *Leishmania* cells to create singly and doubly transformed clones. Each transformed fragment contained twice the number of copies of t-PA gene as compared to the construct used for transformation of *L. tarentolae* in a previous study [25].

Zymography is a sensitive test with the ability to detect as low as pictogram amounts of a protein. In a gelatin zymogram matrix related to the singly and doubly transformed clones, three distinct bands were observed; a band with an apparent MW of approximately 64 kDa corresponding to the full-length t-PA and two lighter bands of 35 and 30 kDa, which may represent the two-chain form or may have resulted from proteolysis of the full-length protein. Similar observations have been reported previously by Soleymani *et al*. [25]. The results observed were the same as the commercial t-PA and verified the expression of the properly folded t-PA in this system.

Southern blotting and dot blotting are two main methods used for copy number determination in eukaryotic cells. However, they are laborious, time-consuming and semi-quantitative methods [37]. Therefore, a quantitative real-time PCR was established to quantify the t-PA gene copy number in the transformed cells. Results confirmed that it is possible to introduce up to 32 copies of the t-PA gene in *L. tarentolae* cells without any fundamental changes in the growth properties of the transgenic compared to the wild-type cells, as shown by similar growth curves (Fig. 5).

The data obtained from real-time PCR indicated that in two examined families, singly and doubly transformants of the lower productive family with expression level of 182 and 230 IU/mL contain 4 and 7 copies of the t-PA gene whereas parent and progeny of the higher productive family with productivity of 375 and 480 IU/mL contain 8 and 32 copies, respectively. As control of the gene expression in eukaryotic hosts partly happens post-transcriptionally, there are several bottlenecks, including protein translation, post-translational modifications, assembly and secretion that correlate with expression of foreign genes in high copy strains. On the other hand, it was shown that an increase in gene copy number is accompanied by a corresponding rise in the mRNA level [21, 33, 38–40]. Therefore, it seems that accumulation and degradation of unfolded protein and limitation at the secretory pathway might be the factors mostly responsible for reduced foreign protein production in high copy number eukaryotic transformants. It could be an explanation for a non-linear correlation between t-PA gene dosage and expression rate in *L. tarentolae*. For example, in the higher productive family the gene copy number increased fourfold between parent and progeny clones (from 8 to 32) while the expression level was only boosted by 30% (from 375 to 480 IU/mL). However, these data confirm that the expression level is related to the gene copy number in this host albeit in a non-linear way.

In this study, transgenic clones of *L. tarentolae* were obtained with a wide range of integrated t-PA gene copy numbers. The highest production from the doubly transformed clone (480 IU/mL) was seven times higher than previously reported for this expression system (70 IU/mL) [25]. This level of enzymatic activity is comparable with rt-PA produced in non-modified recombinant CHO (50 IU/mL) [2] and *E. coli* (3–7 IU/mL) [41]. The results are very promising and with some further optimization of our approach, we expect an even higher yield of rt-PA expression in this host.

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