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Functional evaluation of *ToxA* promoter in *Trichoderma reesei* Rut-C30

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ABSTRACT

In order to expand the collection of promoters studied in *Trichoderma reesei* Rut-C30, a functional study of the *ToxA* promoter was carried out by analysis of GFP expression in *T. reesei* Rut-C30. For this purpose, the binary pCBCT expression vector was employed to transform *T. reesei* Rut-C30. The transformants obtained were evaluated by means of fluorescence microscopy, fluorometry, dot blot and Western blot analysis. The low levels of cytoplasmic GFP protein in fungal hyphae suggest that *ToxA* promoter works as a weak constitutive promoter which can drive successfully the expression of heterologous proteins in *T. reese*i Rut-C30

KEYWORDS

*Trichodermareesei*Rut-C30;
Recombinant protein expression;
ToxA promoter;
Weak promoter.

INTRODUCTION

The genus Trichoderma is composed of several species of filamentous fungi whose natural habitat is the soil. Of all species included in this genus,*T. reesei* is the species with major industrial importance[1]. It has been successfully employed for decades in the cellulolytic enzyme production and it is currently used as a host to recombinant proteins production[2]. *T. reesei* is an interesting expression system because it has the capacityto grown in cheap fermentation systems and culture media with the particular ability to generate and secrete large amounts of recombinant protein with conventional eukaryotic post-translational modifications[3, 4]. Particularly, the mutant *T. reesei* Rut-C30 has been used successfully in the production of several recombinant proteins of different origins, for example glucoamylase[5], endochitinase[6], β-glucosidase[7], laccase[8], xylanases[9] fromfungalorigin; bacterialxylanases; vegetable endopeptidases; bovinechymosin and human erythropoietin[10]. However, vectors used for the production of these proteins are based on the use of strong promoters such as cbh1, cbh2, or their modifications. This small set of promoters available, restricted the plasticity of the expression system in *T. reesei*[11, 13]. In order to overcome this drawback, numerous efforts have been

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focused upon the search fornew functional promoters in *T. reesei*[10].

The *ToxA* promoter controls the expression of toxin A in the fungus *Pyrenophoratritici-repentis*, this promoter has been successfully used for constitutive expression of recombinant proteins in various fungi such as *Colletotrichum magna*, *P. tritici-repentis*, *Sclerotiniasclerotiorum*, *Colletotrichumtrifolii*, *Verticilliumdahliae*, *Alternariaalternata*, *Botrytis cinerea*, *Cochliobolussativus*, *Fusarium sambucinum*[14].

Taking into account the need to find new functional promoters in *T. reesei* Rut-C30 and considering that *ToxA* promoter has shown functionality in a broad host range; it was decided to study it functionality in*T. reesei* Rut-C30. Due to this, the aim of this work is to study the functionality of *ToxA* in *T. reesie* Rut-C30 and expand the repertoire of promoters studied in this strain.

MATERIALS AND METHODS

Expression vector

The binary plasmid pCBCT[15] was used for*T. reesei* Rut-C30 transformation, mediated by *Agrobacteiumtumafaciens*. This plasmid contains the following elements: hygromycin B resistance gene (hph) under control of the *Aspergillusnidulans*trpC promoter, the gene of green fluorescent protein (GFP) under control of *ToxA* promoter from *Pyrenophoratritici-repentis*, the left (LB) and right (RB) borders of T-DNA from *A. tumefaciens*, the origin of replication (RK2 oriV), the neomycin resistance gene (nptIII) and the backbone of mini binary vector pCB301. The pCBCT vector was amplified in *Escherichia coli* DH5α and isolated from it using the plasmid DNA preparation described by Sambrook[16].

Microorganisms and culture conditions

Forconidiation, *T. reesei* Rut-C30 was grown at 28°C in agarose medium potato glucose. The conidia were collected using a solution of 80% Tween 0.05% and counted in a Neubauer chamber. For expression assays, Mandels[17] liquid medium supplemented with 2 g/L glucose was inoculated with *T. reesei* Rut-C30 and *T. reesei* Rut-C30-GFP to a final concentration of $1x10^6$ spores/mL and incubated in an orbital shaker at 28°C, 250 rpm for 48 hours. The *E. coli* DH5αwere grown in Luria-Bertani medium containing 0.1 mg/mLampicillin for 24 h at 37°C and 250 rpm. *Agrobacterium tumefaciens*LBA1100 were grown in minimal media according to protocol described by Pardoand coworkers[18].

Trichoderma transformation and selection

Conidia were resuspended in 0.08% Tween 80, counted and immediately employed for the transformation following the protocol described by Michielse[19].

Protein extracts preparation and quantification

The mycelia were recovered from the liquid cultures by filtration. Then, 2 g of that wet biomass was milled using liquid nitrogen and mortar. Finally, the resulting powder was resuspended in 1 mL of distilled water and stored until use at -20°C. Total protein was quantified using the Bradford method.

SDS-PAGE and Western blot:

Protein extracts were diluted in loading buffer and 20 μg of total protein per lane was loaded on a gel 10% SDS-PAGE, the electrophoresis was performed at 160 V for 1 h. Subsequently, the proteins were transferred to a nitrocellulose membrane and the transfer verified by Ponceau red staining. The membrane was washed with water, blocked with 1% skim milk in TBS-0.1% Tween 20 during 60 min at room temperature and incubated with primary anti-GFP (rabbit) and secondary (IRDye® 800CW anti-rabbit, LI-COR Biosciences) antibody diluted 1:1000 and 1:10000 in blocking buffer with 0.05% Tween 20 respectively. For immunodetection the membrane was analyzed using infrared scanner (LI-COR Biosciences)

Fluorescence microscopy

A portion of mycelium was hydrated with distilled water and analyzed by microscopy using a fluorescence microscope Nikon Eclipse E600 equipped with SPOT-RT camera. The merged images were obtained by superposition of corresponding channels using the ImageJ v1.44 software.

Measurement of green fluorescent protein by fluorometry

For measurement of green protein,*T. reesei* Rut-C30 strains were culture and processed according the protocol described by Dandan Lv[13]. The mycelia suspension was analyzed by fluorometry for GFP expression using a microplate reader (Filtermax F5; Molecular Device, LLC.).

Dot Blot analysis

Protein extracts and purified GFP protein were diluted in phosphate saline buffer and 2 µL of each sample were dropped in nitrocellulosic membrane (Bio-Rad). The membrane was dried at room temperature and blocked with 1% skim milk in TBS-0.1% Tween 20 during 60 min at room temperature. Finally, the membrane was incubated with primary anti-GFP (rabbit) and secondary (IRDye® 800CW anti-rabbit, LI-COR Biosciences) antibody diluted 1:1000 and 1:10000 in blocking buffer with 0.05% Tween 20 respectively. For immunodetection the membrane was analyzed using infrared scanner (LI-COR Biosciences)

RESULTS AND DISCUSSION

Previous reports have shown the correct expression of GFP gene under control of different promoters in the filamentous fungus *T. reesei*Rut-C30[11]. To assess the ability of *ToxA* promoter to drive expression of the green fluorescent protein, the binary pCBCT vector was transformed into *T. reesei*Rut-C30. After transformation, the positive transformants of *T. reesei*Rut-C30 were selected for their ability to grow in the presence of hygromycin in the growth medium. One of these positive transformants was selected toGFP expression confirmation byblue light test. As shown in Figure 1A, the morphology shown by thetransformant is similar to wild-type strainsuggesting that the integration of the T-DNA cassette in the fungus genome does not disrupt genes essential for fungus viability. Fluorescence microscopy studies were carried out to evaluate the cellular localization and distributionof GFPprotein in selected*T. reesieRut-C30*transformants. The fungal transformants showed a GFPcytoplasmatic localization with a regular fluorescent signal along all hyphaestructure,but some of this hyphae display a higher expression of GFP in growing hypha tips Figure 1B. These results indicate that transformants were able to express the GFP gene under *ToxA* promoter.

To confirm the presence of GFP in *T. reeseiRut-C30* transformants Western blot analysis was performed. For this purposeintracellular fungal extracts were electrophoresed and electroblotted as described above (see materials and methods) andthePonceau staining was performed to verify the

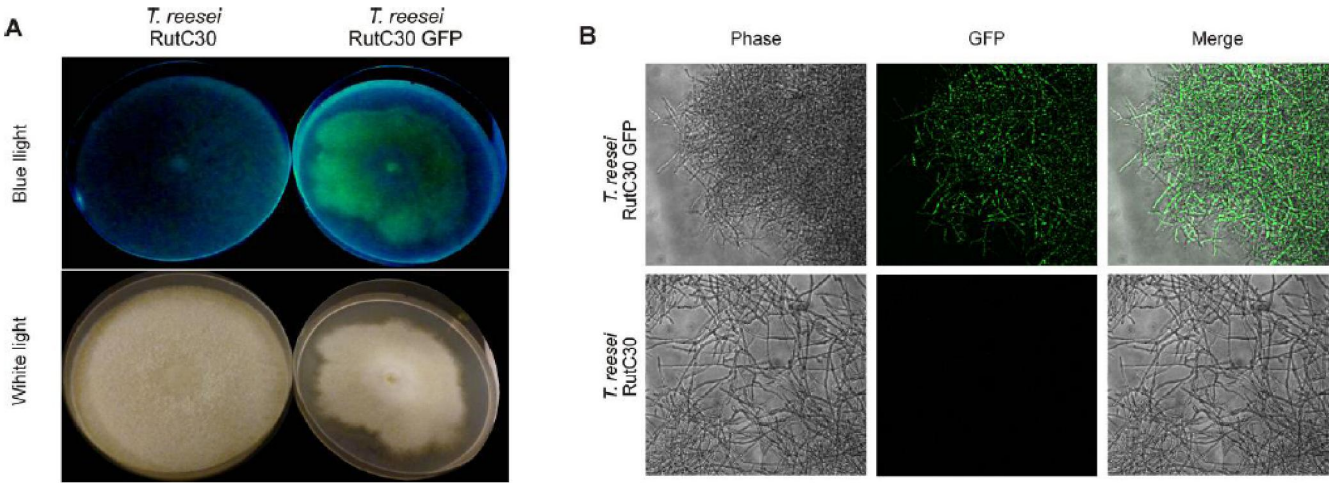

Figure 1 : Fluorescence analysis for GFP expression under the *ToxA* promoter regulation. A: Blue light test, right panels *T. reesei*Rut-C30 transformed withpCBCT in left panels *T. reesei*RutC-30 untransformed. B: Fluorescence microscopy, upper panels *T. reesei*Rut-C30 transformed withpCBCT in lower panels *T. reesei*Rut-C30 untransformed

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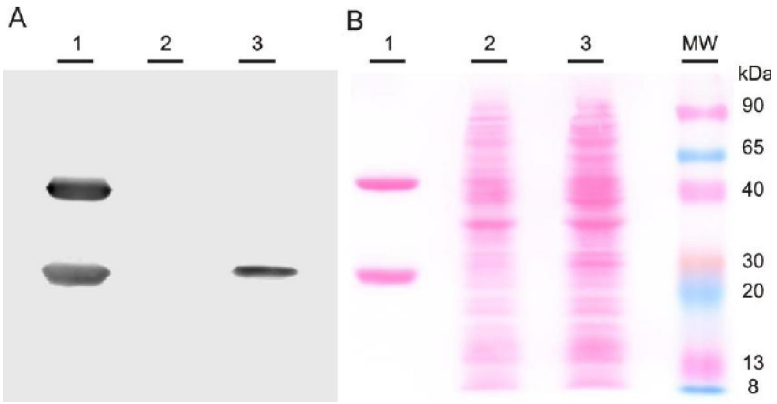
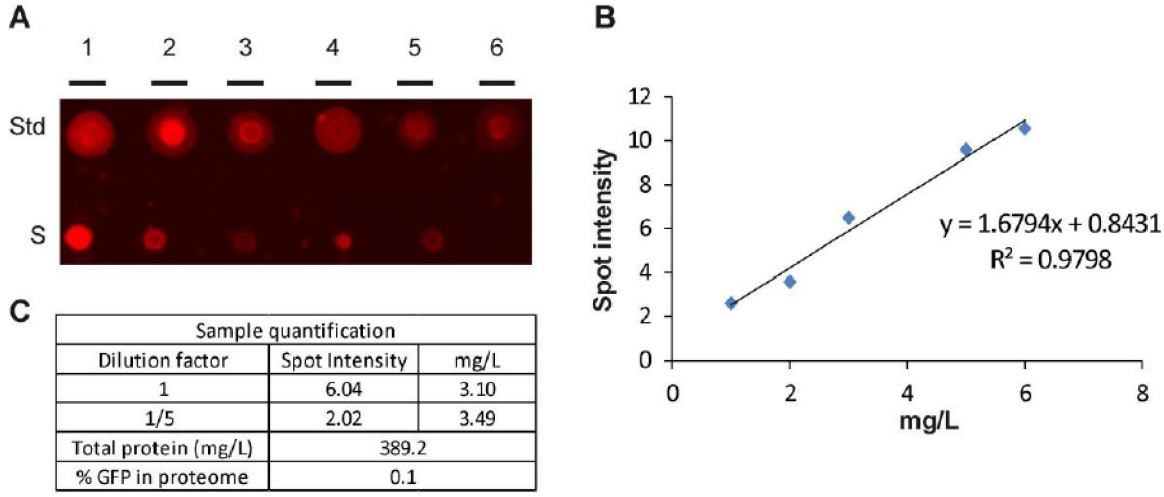


Figure 2 : Expression analysis of GFP by Western blot. A: Western blot using anti-GFP. B:Ponceau red staining. In both cases, lane 1- Recombinant GFP expressed in E. coli, lane 2- protein extract from *T. reesei*Rut-C30 untransformed, lane 3- protein extract from *T. reesei*Rut-C30 transformed with pCBCT



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Sample quantification		
Dilution factor	Spot Intensity	mg/L
1	6.04	3.10
1/5	2.02	3.49
Total protein (mg/L)	389.2	
% GFP in proteome	0.1	

Figure 3 : Quantification of intracellular GFP content. A: Dot blots using specific antibody (anti-GFP). Lane 1: 6 mg/L of standard purified GFP (Std) and undiluted sample (S). Lane 2: Std (5 mg/L) and sample diluted 1/5. Lane 3: Std (4 mg/L) and sample dilution 1/10. Lane 4: Std (3 mg/L) and sample dilution 1/5. Lane 5: Std (2 mg/L) and sample dilution 1/10. Lane 6: Std (1 mg/L). C: Calibration curve plotted from the intensities values obtained by quantification of signal for each standard spot. D: The chart shows the values obtained for the sample and their percentage in the proteome

transfer of total intracellular proteins into the nitrocellulose membrane prior to antibodies hybridization Figure 2B. After Western blotting the membrane was infrared scanned and the resulting signal showed the presence of an unique band of 26.9 kDa consistent with the GFP molecular weight in the fungal transformant extract Figure 2A. It is important to note thepresence of dimer aggregates (53.8 kDa)in recombinant GFP purified from *E. coli* extract Figure 2. These results indicate that GFP protein is produced in the*T. reeseiRut-C30* as a single in tracellularly protein without presence of aggregates.

To assess promoter strength, a fluorometric quantitative assay was performed. For this purpose, the wild type strain and the recombinant strain were grown and processed under conditions previously reported byDandan Lv[13]. The GFP fluorescence was measuredby means of fluorometric assay and the intensities obtained were compared with data previously reported for recombinant *T. reesei*Rut-C30 expressing DsRed2 under control of CBHI strong promoter[13]. As shown in TABLE 1 the strength of CHBI promoter results 6.5 fold higher than *ToxA* promoter in *T. reesie* Rut-C30. In discordance with previouslyreported for *ToxA* promoter strength in other fungus genus, this promoter work as a weak promoter in *T.reesei*Rut-C30[20]. To confirm the strength of *ToxA* promoter, a quantitative dot blot

TABLE 1 : The comparative study of total intracellular protein production by the parent strain *T. reesei*Rut-C30 and the recombinant strain

T. reesei strain	Intracellularfluorescent (fluorescentunits)	Fluorescent ratio (Transformant / wild type)	Reference
RutC30	55	97	In thiswork
RutC30-GFP	5342		
RutC30	66	637	[13]
F1*	42061		

***F1 is a *T. reesei* RutC30 transformed with pWEF31-red vector**

analysis of GFP level expression was conducted. As shown in Figure 3, GFP content is 0.1% of total intra cellular protein. In *T. reesei*Rut-C30 the extra cellular protin is 8% of total proteomeand CBHI protein represent 60% of extracellular protein[21, 22]. From these values, it is possible to estimate that CBHI protein amount is around 4.8% of proteome, this value is significantly higher than the values obtained for GFP expression under control of *ToxA* promoter.

CONCLUSION

From the results presented, is possible to conclude that*ToxA* promoter is functional in *T. reesei* Rut-C30. In addition, the study of the expression level suggesting that *ToxA* promoter is working in this strain as a weak promoter and can be employed as good promoter for co-expression of accessory proteins as chaperon proteins. The results presented here extend the repertoire of functional promoters described for*T. reesei* Rut-C30.

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