Truncated, strong inducible promoter *Pmcl1* from *Metarhizium anisopliae*

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Abstract

In this study, *Metarhizium* collagen-like protein (MCL1) promoter from *Metarhizium anisopliae* was analysed and truncated into different sizes through series of targeted and random deletions based on the presence of various transcription factor-binding sites. Synthetic Green Fluorescent Protein (sGFP) was being utilized as a reporter gene to study the relative expression driving capability of unmodified and truncated promoters. Conserved promoter sequence analysis revealed similarity between the paralogous promoters from *M. brunneum* and *M. acridum*. sGFP expression in the haemolymph was directed with the help of *mcl1* signal peptide sequence. Deleting the promoter region from – 2764 to – 1583 bp increases the promoter *mcl1* (*Pmcl1*) activity by twofolds, while deletions of the regions upstream of – 1150 bp and – 840 bp caused a decrease of sGFP expression level (80% and 70%, respectively). Transcriptional binding sites predicted for the deleted region revealed the loss of upstream repressing sequences such as Matalpha2 along with ROX1 and Rap1 repressor-binding sites located – 2234 bp, – 1754 bp and – 1724 bp from the TSS. Compared with *Pmcl1*-wild type (2.7 kbp), *Pmcl1*-1583 bp had a shorter sequence and showed statistically significant expression in *M. anisopliae*. This study introduces a highly efficient strong inducible promoter for over-expression of target genes in *M. anisopliae*.

Keywords *Mcl1* promoter · *Metarhizium* collagen-like protein · Promoter truncation · Gene expression · Entomopathogenic fungi

Introduction

Inherently, most entomopathogenic fungi infect insects by direct penetration of insect cuticle, and ingestion is not necessary unlike other entomopathogenic organism, thus making them valuable tool as biological control agent (Prior and Greathead 1989; Lomer et al. 2001; Zhao et al. 2016). *Metarhizium* spp. and *Beauveria* spp. are the best-qualified environmental friendly entomopathogenic fungi (Xiao et al. 2012), the principal alternatives of chemical insecticides, which are effectively being used for controlling agricultural pests and vectors of disease. However, these biological

agents could not compete with the chemical insecticides because of inconsistencies in their performance and low virulence (Fang et al. 2012; Wang and Leger 2007).

With the help of genetic engineering tools, scientist was able to increase the virulence and performance of entomopathogenic fungi by expressing endogenous as well as heterologous toxic gene under the control of various promoter (Wang and Leger 2007). Availability of different types of promoters ease target gene expression in particular organisms. Eukaryotic promoters are few hundreds to thousands of base pair in length compared to smaller size prokaryotic promoters (Yanofsky et al. 1981; Dudley et al. 1999; Redden and Alper 2015). Heterologous eukaryotic promoters such as *Aspergillus nidulans* glyceraldehyde 3-phosphate dehydrogenase promoter (*Pgpd*) and TrpC promoter have previously been used to express drug-resistant marker genes (Punt et al. 1990); however, the genetic transformation was not sufficient to express heterologous genes (Ruiz-Diez 2002). Jin et al. (2010) exploited the use of JEN1 promoter (2.8-kilo base pair) from *B. bassiana* to study the fungal pathogenesis related to JEN1 gene. Wang

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and St. Leger (2007) employed *Metarhizium* collagen-like protein (Mcl1) promoter (2.8 kbp) to express the heterologous scorpion neurotoxin Aa IT1 in *M. anisopliae* to increase fungal toxicity against tobacco hornworm (*Manduca sexta*) caterpillars and adult yellow fever mosquitoes (*Aedes aegypti*). To express target gene sufficiently and effectively, the host cell must recognize the heterologous promoter sequence by its transcriptional machinery (Kuo et al. 2004). Additional disadvantage of using larger heterologous promoter is that the difficulty in cloning larger gene fragment along with larger promoter sequence. It would increase the vector size dramatically, thus reducing the transformation efficiency particularly in fungal transformation.

Metarhizium anisopliae harbors collagen-like protein (Mcl1) promoter used to drive the expression of *mcl1* gene and *mcl1* signal peptide sequence helps direct secretion of Mcl1 protein to insect hemolymph. The sequence region (NCBI accession number: DQ238489) corresponding to *mcl1* gene is 4840 bp and one open-reading frame of 2076 nucleotide is coding for 605 amino acid protein. The regulatory sequence upstream of transcription start site is 2764 bp (Wang and Leger 2006). The *Pmcl1* is a strictly regulated and hemolymph-inducible promoter with target gene expression observed within 20 min post induction (Wang and Leger 2006). However, the promoter size represents a major drawback in genetic engineering, which makes it very difficult to use in any other entomopathogenic fungi due to less integration efficiency (Zhao et al. 2016).

Liao et al. (2008) studied bigger size *A. nidulans* *gpd* promoter (*Pgpd*) by truncating it to various length to improve the promoter activity. Based on the β -glucuronidase (*gus*) quantitative assay, deletions spanning between –2118 and –1153 bp demonstrated three times higher transcriptional activity when compared with *A. nidulans* *Pgpd*. This could be due to the presence of transcriptional regulatory element such as *gpd* box. Studies conducted by Cao et al. (2012) on *M. acridum* *PMagpd* promoter deletions, some increased the transcriptional level of *gus* gene by 1.38 times higher than that of *Pgpd* of *A. nidulans* and also observed that the absence of *gpd* box within the deleted regions did not reduce the activity significantly.

In the present study, to obtain a homologous promoter with a reduced size and higher expression level in entomopathogenic fungi, *M. anisopliae* *mcl1* gene promoter-based expression vector was constructed. This promoter was truncated into different lengths based on the presence of TATA box, CAAT box, and UAS and then transformed into *M. anisopliae*. This promoter was able to drive the expression of a reporter gene synthetic Green Fluorescent Protein (sGFP) (Berepiki et al. 2010) and its relative expressional capability was analysed.

Materials and methods

Bacterial and fungal strains

Metarhizium anisopliae MTCC 892 was maintained in Potato Dextrose Agar (PDA-Himedia) at 28 °C incubation. *Escherichia coli* DH5 α and *Agrobacterium tumefaciens* EHA105 were employed for cloning and transformation. All the bacterial cultures were maintained in Luria Bertani Medium (Himedia) at 37 °C incubation.

Promoter conserved sequence analysis and transcription factor-binding site prediction

Pmcl1 (DQ238489.1) and other orthologous as well as paralogous *Pmcl1* DNA sequences from other fungi species (NW_014574687.1, NW_011942149.1, NW_014574713.1, NW_006916701.1, NW_006916689.1, NW_017264037.1, C_035796.1, and NW_011942182.1) retrieved directly from NCBI–nucleotide section. For conserved sequence analysis, –3 to –5 kbp region upstream of Transcription Start Site (TSS) was taken. Initially, multiple sequence alignment and evolutionary analysis was performed with the retrieved *Pmcl1* DNA sequences using Clustal Omega online tool (Sievers et al. 2011; Sievers and Higgins 2014). For identification of conserved transcription factor-binding sites in the promoter region, the TRANSFAC professional software was employed (Matys et al. 2003).

Vector construction and *Agrobacterium*-mediated Transformation

The genomic DNA of *M. anisopliae* was used as a template to amplify the promoter region of *mcl1* gene using the primers: FP-5'AATCATGCAGCGCTATGAGAGC-3' and RP-5'CTCCTCGCCCTTGCTCACT-3'. Different regions of the promoter sequence upstream of ATG were amplified with the help of primers through Polymerase Chain Reaction (PCR). The *mcl1* signal sequence was amplified and fused with sGFP. The *Pmcl1* was placed upstream to the signal sequence and sGFP to drive the expression in the haemolymph.

All three fragments were fused together and cloning was performed in pCAMBIA 3300 binary vector by Gibson assembly method. Final construct was transformed into *M. anisopliae* by *Agrobacterium*-mediated method with limited modifications (Reis et al. 2004). *A. tumefaciens* culture carrying truncated as well as full-length pCAM-*Pmcl1* sGFP was washed with sterile water and re-suspended to the OD₆₆₀ of 0.15 in Induction Medium (IM) containing acetosyringone (200 μ M) and incubated at 28 °C until OD₆₆₀ reaches 0.5.

Both the *Agrobacterium* and *Metarhizium* (2×10^7 spores/ml) cultures were mixed in 1:1 ratio and plated onto filter paper placed on co-cultivation medium. Following the incubation for 48 h, culture was transferred to M-100 minimal media with top layer soft agar containing cefotaxime 300 µg/ml and glufosinate ammonium 300 µg/ml for the selection. The positive clones were selected after 7–14 days of culture at 28 °C. Screening for positive clones was performed with PCR from genomic DNA as templates.

Haemolymph induction

The spores of *M. anisopliae* were cultured in Potato Dextrose Broth (PDB) for 48 h at 28 °C in the incubator shaker at 150 rpm. For sGFP induction, *Antheraea assamensis* haemolymph was inoculated with 1 g of water washed mycelia. The sGFP expression was observed in real time under fluorescence microscope from 0 to 6 h postinduction.

Real time quantitative PCR analysis

The Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR) analysis was performed to quantify the transcriptional level of sGFP under the control of *mcl1* wild type and modified truncated promoters. The spores of *M. anisopliae* were cultured in Sabouraud Dextrose Broth (SDB) for 48 h and then induced by *A. assamensis* haemolymph for 12 h. Total RNAs were extracted from the mycelia of *Pmcl1* sGFP transformants using Trizol reagent (RNAiso Plus kit from TaKaRa). The RNA was treated with DNase I (NEB) to remove traces of genomic DNA contamination and the first-strand cDNA was synthesized from 1 µg total RNAs with oligo dT primers using prime script First-Strand cDNA Synthesis Kit (Clontech, TAKARA). The reaction mixtures (20 µl) for RT-qPCR contained 1 µl template cDNA, 10 µl SYBR Green mix (2×, Applied biosystem) and 0.6 µl of each primer (GFP-qPCR FP /GFP-qPCR RP, 10 µM). The reaction was performed with the AB7500 system (Applied biosystem) using *Gapdh* as an endogenous control (primers

gpd/FP and gpd/RP). Three replicates were performed for each reaction and the experiment was repeated three times.

Results and discussion

Conserved sequence analysis

To identify conserved regulatory sites in the *Pmcl1*, – 3 to – 5 kb upstream region of *mcl1* gene sequences from different species was aligned with Clustal Omega (MUSCLE) multiple sequence alignment tool. No significant sequence similarity was found between the analysed *Metarhizium* (DQ238489.1) *Pmcl1* and other orthologous *Purpureocillium lilacinum* (NW_017264037.1) *Pmcl1*, *Pochonia chlamydosporia* (C_035796.1) *Pmcl1*, and surprisingly, no sequence similarity was observed between the *M. anisopliae* *Pmcl1* against with *M. robertsii* *Pmcl1* (NW_011942182.1) itself. We have also noted that there was no sequence similarity even within the *M. brunneum* *Pmcl1* DNA sequences (NW_014574687.1 and NW_014574713.1). However, based on results of the evolutionary phylogenetic tree analysis, *M. anisopliae* *Pmcl1* (DQ238489.1) was found to be more closely related to *M. acridum* *Pmcl1* (NW_006916689.1) and *M. brunneum* *Pmcl1* (NW_014574687.1) (Supplementary file 1: S1). Multiple sequence alignment with the evolutionarily closely related *Pmcl1* revealed well-conserved region from the proximal transcription start site. Many transcriptional factor sites such as TATA box-binding protein (TBP), zinc-finger domain-based transcriptional factor (Stp4p), and RNA polymerase-specific transcription factor (Bas1p) were accumulated near the proximal starting site (Supplementary file 1: S2). TATA box-binding site was located approximately – 150 bp upstream of TSS. When *Pmcl1* sequence was predicted for transcription factor-binding sites, notable 165 reported binding sites were distributed along the promoter sequence (supplementary file 2: S1) and supplementary Table 1 summarizes the predicted transcriptional factors with their specific role.

Fig. 1 Schematic representation of full length and truncated *mcl1* promoter sequence along with *mcl1* signal peptide sequence and synthetic green fluorescent protein

Vector construction

To construct different truncated *PmclI*, initially, potential regulatory sequence was identified using the Promoter 2.0 software (Knudsen 1999) which predicts the presence of transcription start sites of Pol II, TATA, CAAT boxes, and transcription factor-binding sites and CT-rich regions in DNA sequence (Supplementary file 1: S3). Liao et al. (2008) deleted the *B. bassiana* glyceraldehyde 3-phosphate dehydrogenase promoter (PBbgpd-3942 bp) sequence based on 5' upstream region containing gpd box, C + T rich regions, *cis*-acting transcript elements and some inverted repeats. In this study, different regions of the *PmclI* sequence upstream of transcription start site were successfully amplified using designed primers

(Supplementary file 1: S4) and yielded three modified promoters with the following lengths: T1 = − 1583 bp, T2 = − 1150 bp, and T3 = − 840 bp. The full length and modified promoters were used to drive the expression of sGFP which was fused with *mclI* signal sequence (Fig. 1). Successfully fused and amplified fragments of full length and truncated promoters with sGFP were cloned in pCAMBIA 3300 binary vector for *Agrobacterium*-mediated transformation (Supplementary file 1: S5).

Genomic integration confirmation

Metarhizium anisopliae transformation was successfully performed via *Agrobacterium* EHA 105 strain bearing pCAMBIA3300-*PmclI*-sGFP with *bar* gene as a selectable

Fig. 2 Fluorescence microscopy analysis of sGFP expression in full length and truncated *M. anisopliae* clones induced with hemolymph 30 min postinduction. Data represent two independent analysis. Bar represents 20 μ M

marker, which is driven by Cauliflower Mosaic Virus (CaMV) promoter. The positive *M. anisopliae* clones which were able to grow on glufosinate ammonium (300 µg/mL) were subcultured for five consecutive generations on PDA medium without any selection agent to check the genomic integration or mitotic stability. Colonies from the selected clones showed different type of mutations, which might have been introduced as a result of *Agrobacterium* T-DNA random integration into the fungal genomic DNA. Random integration caused changes in the mycelial growth, sporulation rate, and spore colour (data not shown). The genomic DNA (gDNA) extraction was successfully done and used as template for sGFP amplification (supplementary file 1: S6-A) and Agarose gel electrophoresis analysis showed the presence of sGFP gene fragments from the selected clones which further confirmed successful integration (supplementary file 1: S6-B).

Fluorescence microscopic analysis of sGFP expression

The sGFP expression in wild type and modified promoter of fungal clones started after about 20–30 min of induction in *A. assamensis* haemolymph, as shown in Fig. 2a–h. As a result, full length showed marginal intensity sGFP expression, whereas in T1, sGFP intensity was higher when compared with other two truncated promoter driven expression. Marginal expression of wild-type promoter could be due to presence of ROX1 (Balasubramaniam et al. 1993) and Matalpha2 (Laney et al. 2006) transcriptional repressor-binding site upstream to the TSS (supplementary file 2). Moreover, the presence of haemocyte aggregates and increased fluorescence intensity around the fungal mycelium proved that *Pmcl1* was induced by haemocytes rather than haemolymph.

Wild type and truncated *Pmcl1* activity analysis

In the present study, to analyse the activity, the transcription level of sGFP was determined with RT-qPCR in wild type as well as truncated clones. The results (Fig. 3) show that deletions spanning the region from – 2764 to – 1583 bp (T1) led to an increase in the GFP expression by twofolds compared to the wild-type promoter. This could be due to the loss of transcription factor-binding sites that play role as repressing elements. Transcriptional binding sites predicted for the deleted region revealed the loss of upstream repressing sequences (URS) such as Matalpha2 along with ROX1 and Rap1 (Buck and Shore 1995) repressor-binding sites located – 2234 bp, – 1754 bp and – 1724 bp from the TSS (Supplementary file 2: S1). The loss of these repression factor-binding sites in a way increases the promoter activity. Another explanation could

Fig. 3 Relative transcription level of the sGFP gene. The transcription level of sGFP gene with full-length (F) promoter was calibrated as 1 and compared with other truncated promoters. T1, T2, and T3 indicate truncated promoters 1583, 1150, and 840, respectively. T1 promoter activity is significantly (P value < 0.0001) higher than the wild-type promoter. T2 and T3 promoter activity is significantly lower (for T1 P < 0.0001 and T2 P = 0.0001) than the wild-type promoter

be that due to sequence deletion, the bendability, and curvature of promoter sequence may change, and subsequently, its binding to transcription factors will be affected (Kanhare and Bansal 2005). Deletions of the regions upstream of – 1150 bp (T2) and – 840 bp (T3) caused 80% and 70% decrease in GFP activity, respectively, suggesting that strong positive *cis*-acting elements were located in the region upstream of these promoters. The results show that *Pmcl1* – 1583 bp fragment was necessary to direct heterologous gene expression in *M. anisopliae*. This suggests that the sequence upstream of – 1150 is a binding site for a strong positive *cis*-acting element, transcription factors, and/or regulatory proteins.

Conclusion

In conclusion, the present study provides an efficient, strong, and shorter promoter (1.5 kbp) to be used in expression of heterologous genes in entomopathogenic fungi and engineering fungal strains with higher toxicity against insect pests.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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