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Research Article

RNAi Mediated Silencing of Host Specific Protease in Fission Yeast *Schizosaccharomyces Pombe*

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ABSTRACT

The fission yeast *Schizosaccharomyces pombe*, is an attractive host model for high-level protein production and functional analysis of eukaryotic proteins as it shares many molecular, genetic and biochemical features with higher eukaryotes such as plants and animals. Furthermore, *S. pombe* has a developed Golgi apparatus and galactosyltransferase that is not found in other yeast cells. Moreover many types of human proteins have been successfully expressed in *S. pombe*, and it has also been used effectively for production of many types of heterologous proteins. However, one of the major hurdles in efficient production and purification of heterologous proteins from *S. pombe* is proteolytic degradation of the recombinant gene products by host-specific proteases. The problem becomes significant when the recombinant protein under production, is secretory and proteolytically sensitive in nature. Present study aims at controlling the protease activity by gene silencing approach. A Protease silencing cassette was designed to impede the protease enzyme post trascriptionally. Since all proteases do not attack all proteins, only protein specific protease is sought to be silenced as a test case in this study.

Keywords: *Schizosaccharomyces pombe;* Gene silencing and proteolytic degradation; Host proteases.

1. INTRODUCTION

Amongst yeasts, the fission yeast *Schizosaccharomyces pombe*, is an attractive host model for high-level protein production and functional analysis of eukaryotic proteins as it shares many molecular, genetic and biochemical features such as mRNA splicing, post-translational modification, cell-division and cell cycle control with higher eukaryotes such as plants and animals, and is distinguishable from other yeasts through its ability to proliferate by fission rather than budding [1-5]. The fission yeast is therefore an ideal host for high-level production of eukaryotic proteins. Moreover many types of human proteins have been successfully expressed in *S. pombe,* such as human antithrombin III [6], human papillomavirus E7 protein[7], and human D2S dopamine receptor[8]. It has also been used effectively for production of many types of heterologous proteins [9-13].

However, one of the major hurdles in efficient production and purification of heterologous proteins from *S. pombe* is proteolytic degradation of the recombinant gene products by host-specific proteases. The problem becomes significant when the recombinant protein under production, is secretory and proteolytically sensitive in nature such as recombinant mouse α-amylase[14]. In order to overcome this limitation, a number of attempts have been made, for e.g. control of cultivation conditions e.g. culture pH, temperature and time; and changing medium contents[15,16]. But, these methods cannot ultimately prevent the proteolytic degradation of recombinant proteins or are limited by the specificities of the host species or heterologous protein molecules.

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Alternatively, Genetic manipulation of the recombinant protein molecule itself offers protection from proteolysis, but often gives rise to serious problems for practical application. Furthermore, such genetic modification will also cause structural or functional changes of the protein molecules.

Consequently, Genetic manipulation of the host strain is being developed as a potent method for controlling undesirable proteolytic activity of host strains. It has been done by the disruption of *S. pombe* protease genes using specialized vectors for the purpose. This method has been used to develop many protease-deficient host strains, such as *Escherichia coli* [17,18] and the yeast species *Yarrowia lipolytica*[19] and *Saccharomyces cerevisiae*[20,21,22,23] and *Schizosaccharomyces pombe* [24].

Present study aims at controlling the protease activity by gene silencing approach. A Protease silencing cassette was designed to impede the protease enzyme post trascriptionally. Since all proteases do not attack all proteins, only protein specific protease is sought to be silenced as a test case in this study.

2. MATERIALS AND METHODS

2.1 Microbial Strains and culture condition: *Escherichia coli* DH5α MTCC1652 was used as a host strain for the Amplification of plasmid pRep2. The vector was isolated from *E. coli* DH5α for DNA manipulation reaction purposes. The culture was revived and maintained at 37 ^{o}C in LB broth in 250 ml under shaking conditions at 150 rpm for 24h. *Escherichia coli* DH5α was also a suitable host for GeneArt vector in which synthesized genes was recieved. Both the vectors were isolated from *E. coli* DH5α for DNA manipulation reaction purposes and further use.

Schizosachharomyces pombe FY12854 *(h-ura4-D18leu1-32)* and its vector pREP2 *(URA4 marker; strong nmt1 promoter)* used in the study were received from Yeast Genetic Resource Center of Japan (NBRP/YGRC). The culture so obtained was revived and maintained at 30 ^{o}C for 36 hours in Yeast Extract with Supplements (YES) Broth. Culturing was carried out in 250 ml under shaking conditions at 150 rpm for 30h and preserved at -70 °C as 15 % glycerol stock for further studies. MB was used for culturing of *S. pombe* FY12854 cells before transformation. Edinburgh minimal media (EMM) with lucine was used for culturing *S. pombe* FY12854 cells transformed with Rep2 plasmid.

2.2 Designing of Protease silencing Cassette and its Synthesis

The main problem for less expression of hPTH is its proteolytic cleavage by host proteases. The protease, "aspartic protease, yapsin (Yps1), responsible for cutting human Parathyroid Hormone was predicted using CutDB tool. To control the protease degradation of Human Parathyroid hormone by Yps1 a silencing trigger was designed against it.

To trigger silencing of yps1, a plasmid borne Yps1 hairpin was engineered. The 653-bp Yps1 ORF was cloned as an inverted repeat, with the sense and antisense arms of the repeat separated by a 53-bp spacer containing the first intron of the rad9 gene. The intron was included, because intron-containing hairpin RNAs induce PTGS in plants with nearly 100% efficiency[25]. The construct used here, when spliced, is presumed to leave a loop of 14 unpaired nucleotides (nt). Yps1 silencing cassette carrying dsRNA hairpin corresponding to yps1 gene was designed in the following steps for cloning in pREP2 vector.

The Sequences of Yps1 was taken from Uniprot : as UniprotKB O59774.

A 300 bases long sequence was picked from coding region (901-1200) of yapsin, inverted repeat of the same was designed using Complementary Sequence Conversion Tool and applied at 3' terminus separated by 53-bp spacer.

Then on 5' terminus Restriction Enzyme SalI is added and on 3' RE BamHI is added to ensure orientation of the insert for expression.

Transcription of the Yps1 hairpin was under the control of the thiamine-repressible nmt1 promoter[1]. The plasmid also contained the ura4 gene, to permit selection for retention of the plasmid in the absence of uracil. (The reporter strain contains a ura4 mutation and thus cannot synthesize uracil.)

The designed construct was synthesized from GeneArt Germany. Construct was received in a 3.2 kb H-pth_pMA vector. Vector was multiplied in *E. coli* DH5α from where it was isolated and for further experiments.

2.3. Cloning of yps1-silencing Cassette into Rep2 plasmid

Yps1-silencing cassette was cut out of yps1_Si_pMA vector using SalI and BamHI restriction enzymes and was ligated into

Sal1/BamH1 digested 8.9 kb Rep2 vector of *S. pombe* using T4 DNA ligase (Fig. 1). Standard methods of Sambrook[26] were followed for above experiments.

2.4 Transformation of *Schizosachharomyces pombe* FY12854 with Rep2 recombinant vector Rep2-Yps1SI

Rep2-yps1Si (Fig. 2) was introduced into *S. pombe* FY12854 cells, by Lithium Acetate method[27]. The transformed cells were selected on EMM (with Lue) medium plates, as and Uracil synthesis gene is supplied by pRep2. Transformation efficiency was calculated by using following formula:

Transformation Efficiency = No. of cfu / [(dilution factor) x (µg of plasmid DNA)]

2.5 Quantitaion of % silencing of yps1 protease

Extent of Yps1 silencing in *S. pombe* bearing yps1-silencing cassette was quantified by analyzing the Relative Gene Expression using Real Time Quantitative PCR and $2^{-\Delta\Delta C}$T method[28]. To analyze the relative gene expression total RNA was isolated from both the strains, one with the yps1-silencing cassette (Spv2) and other without it (Spv1), and analyzed by Quantitative RT—PCR in a Realplex2 (Eppendorf) using the SYBR Green PCR Kit (Sigma). Analysis was performed using Realplex2 Monitor (Eppendorf), Excel (Microsoft) software. Relative steady-state mRNA levels were determined from the threshold cycle for amplification using the $2^{-\Delta\Delta C}$T method. Yps1 from spv1 was selected as calibrator (untreated control) and hPTH was selected as internal control gene. Data Analysis Using the $2^{-\Delta\Delta C}$T Method: The CT values provided from Real-Time PCR instrumentation were imported into a spreadsheet program of Microsoft Excel. The fold change in expression of the Yps1 gene normalized to internal control was analyzed using following Equation.

CtGene of Interest –CtInternal Control = ΔCt

ΔCtSample -ΔCtCalibrator= ΔΔCt

Relative quantity = 2-ΔΔCt

Real-Time PCR of samples was performed in triplicates, the mean and S.D. of ΔCt values was then determined from them. The mean fold change in Expression was then determined.

2.6 Monitoring the effect of gene silencing cassette on cell growth

To monitor the effect of yps1 gene silencing cassette on *S. pombe* cell growth, the growth curves of both Spv1 and Spv2 were plotted. Spv1 and Spv2 cultures were plated on EMM+U Agar and EMM Agar media respectively. A single colony of Spv1 and Spv2 was picked and transferred to 50 ml each of EMM+U and EMM media respectively. They were further subcultured to 200 ml media. Flasks were incubated at 30 ^{o}C temperature under shaking conditions. Samples were taken after every 2 hrs of inoculation and Optical Density was measured at 600 nm, for 48 hours. Growth curve was plotted as Time verses Optical Density at 600 nm. Growth curves of both the cultures were compared for any change in profile.

3. RESULTS AND DISCUSSION

3.1 Quantitaion of % silencing of yps1 protease : Total RNA isolated from Spv1 and Spv2 were quantified from the OD obtained at 260 nm, a good conc. of 1040 µg/ml and 2560 µg/ml was obtained respectively from Spv1 and Spv2 (Table 1). The quality of isolated RNA samples was checked on 10 % denaturing PAGE, Two clear bands of 28S and 18S were obtained (Fig. 3).

The mRNA level of Yps1 in Spv1 and Spv2 was determined by analyzing the Relative Gene Expression using Real Time Quantitative PCR and 2-ΔΔCT method. A 152 bp DNA fragment of Yps1 mRNA was amplified by RT—PCR in Realplex2 using the SYBR Green PCR Kit.

The fold change in expression of the Yps1 gene normalized to internal control was analyzed using CT values provided from Real-Time PCR on spreadsheet program of Microsoft Excel. As shown in Table 2 there was approximately three fold decrease in Yps1 expression level in Spv2 strain as compared to Spv1 (Fig. 4).

RNA silencing is a homology-dependent repression of gene expression mediated by RNA mainly by means of post-transcriptional destruction of sequences homologous to the RNA, inhibition of their translation, or reduction in transcriptional rates. Small interfering RNAs and microRNAs are two types of 22–nucleotide (nt) noncoding RNAs that play

important roles as regulators of gene expression in eukaryotes[29]. A third class of small RNAs, Small heterochromatic RNAs, are thought to be similar in structure to siRNAs[30] and have been proposed to be derived from dsRNA transcripts representing heterochromatic chromosomal regions, such as the centromeric repeats in *Schizosaccharomyces pombe*[31]. The *Schizosaccharomyces pombe* genome encodes only one of each of the three major classes of proteins implicated in RNA silencing: Dicer (Dcr1), RNA-dependent RNA polymerase (RdRP; Rdp1), and Argonaute (Ago1) implying that siRNAs, miRNAs, or another class of small RNAs might play an important role in fission yeast. These three proteins are required for silencing at centromeres and for the initiation of transcriptionally silent heterochromatin at the mating-type locus. Schramke and Allshire[32] demonstrated that a hairpin transcript, corresponding to the ura4 locus, could trigger transcriptional silencing while Sigova *et al.,*[33] demonstrated that a dsRNA derived from a hairpin transcript can trigger posttranscriptional silencing of a corresponding mRNA in *S. pombe*. They proved that RNAi machinery of *S. pombe* requires dcr1, rdp1, and ago1, but does not require chp1, tas3, or swi6, genes required for transcriptional silencing. They suggested that the RNAi machinery in *S. pombe* can direct both transcriptional and post-transcriptional silencing using a single Dicer, RdRP, and Argonaute protein.

In the present study, the strategy used to generate the silencing trigger is same as the one followed by Sigova *et al.*[33], here the most possible reason for reduction in Yps1 mRNA concentration is that introduction of dsRNA hairpin corresponding to Yps1 protease gene triggers classical RNAi pathway in *S. pombe*, which generates small double stranded siRNAs that guide degradation of target Yps1 mRNAs via base pairing with complementary sequences.

3.2 Monitoring the effect of gene silencing cassette on cell growth: The effect of yps1 gene silencing cassette on *S. pombe* cell growth was monitored by comparing the growth curves of both Spv1 and Spv2. As evident in Fig. 5, the growth pattern of both Spv1 and Spv2 was same which proves the gene silencing cassette has no effect on growth of *S. pombe* cells. The siRNAs generated by Gene silencing cassette has no off target effect hence it is not hampering any vital activity of the strain so showing no effect on its growth.

Table 1: Absorbance ratio of total RNA samples from Spv1 and Spv2

Sample	λ260	λ280	λ260/λ280	Conc. (µg/ml)
Spv1	0.026	0.014	1.857	1040
Spv2	0.064	0.036	1.722	2560

Table 2: Spreadsheet of data analysis using the $2^{-\Delta\Delta C}$T method

S No.	Gene	Primer	Ct	Mean Ct	ΔCt	mean ΔCt	ΔΔCt	2^-ΔΔCt	1.8^-ΔΔCt
1	Yps1Control	P1	29.09	29.07	0.21				
2	Yps1Control	P1	28.97	29.07	1.16	0.92 ± 0.09	0	1	1
3	Yps1Control	P1	29.15	29.07	1.39				
4	Yps1 Test	P1	31.12	31.12	2.94				
5	Yps1 Test	P1	30.83	31.12	1.95	2.52 ± 0.29	1.6	0.32988	0.390449
6	Yps1 Test	P1	31.41	31.12	2.67				
7	Int Control	P2	28.88	28.15					
8	Int Control	P2	27.81	28.15					
9	Int Control	P2	27.76	28.15					
10	Int Control	P2	28.18	28.6					
11	Int Control	P2	28.88	28.6					
12	Int Control	P2	28.74	28.6					

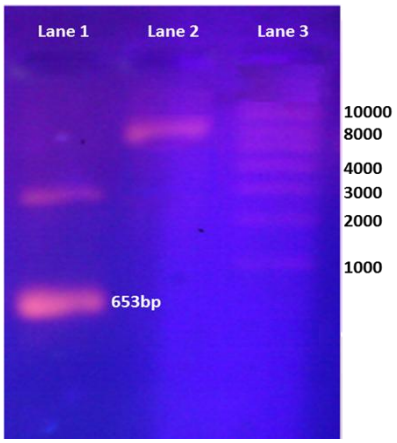

Fig. 1: Lane1: Digested Yps1-Si_pMA vector; Lane2: Digested Rep2 vector; Lane3: 1kb marker.

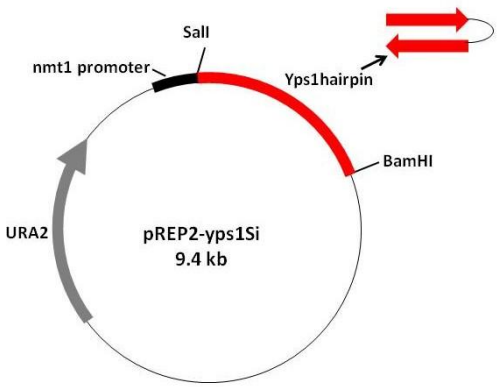

Fig. 2: The pRep2-yps1Si vector resulting after ligation of Yps1 silencing cassette with pRep2 vector.

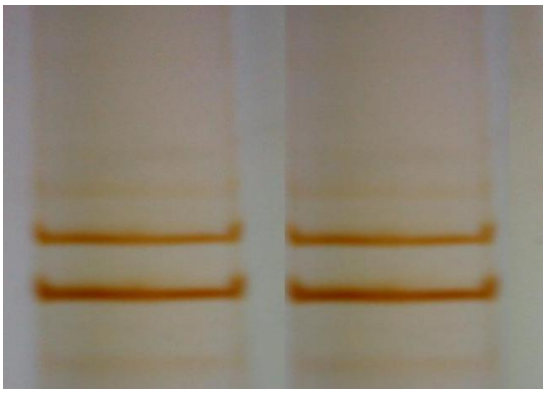

Fig. 3: 10% Denaturing PAGE profile of RNA

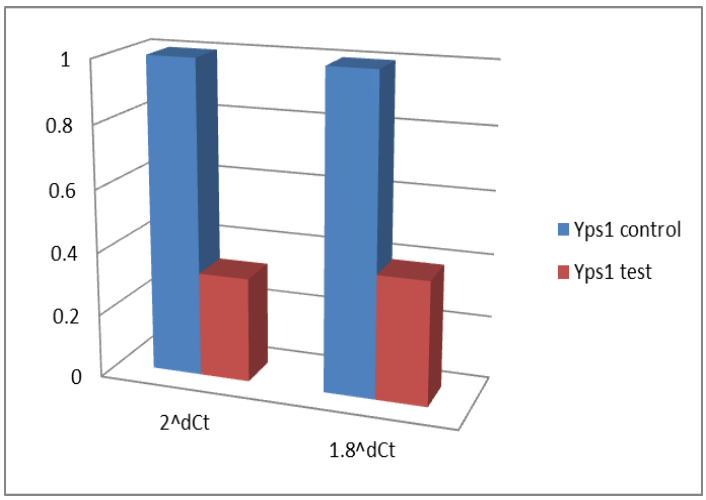

Fig. 4: The repressed expression of yps1 in the Test (Spv2) relative to Control (Spv1).

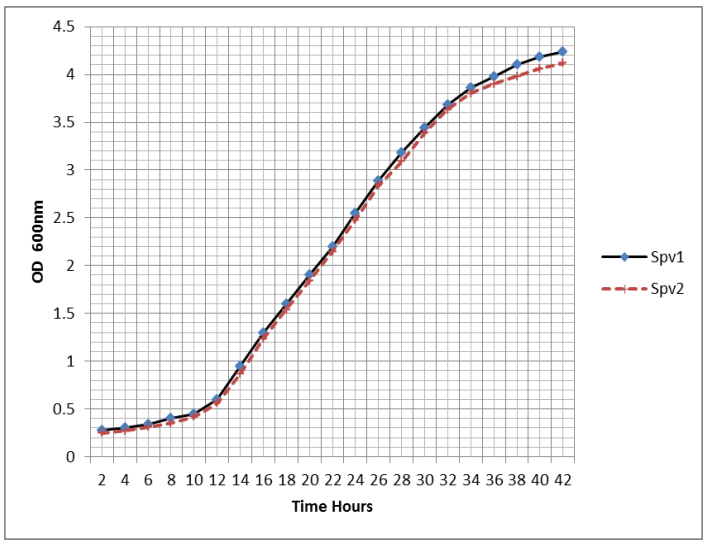

Fig. 5: Cell growth Pattern of Spv1 and Spv2.

4. CONCLUSION

There was approximately three fold decrease in Yps1 expression level in Spv2 strain as compared to Spv1, hence here the particular protease (Yps1) is knocked down instead of being completely knocked out, so that the actual function of the protease in the cell is not disturbed, as there is no effect of Yps1 silencing on growth rate of Spv2 strain.

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