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Full Length Article

Genetic Analysis and Post-Translation Modifications of 60S Ribosomal Protein *L30* in *Sordaria fimicola*

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Abstract

Ribosomes are extremely conserved organelles whose essential composition has usually been viewed as invariant. Though, recent studies have characterized remarkable variations in the expression of some ribosomal proteins (RPs) through species and very much particular effects on translation of individual mRNAs. So far, no genetic diversity and prediction of post translation modifications (PTMs) in large ribosomal subunit *L30* (*RpL30*) protein were investigated in *S. fimicola* in any region of the world. To bridge this gap during the current work an attempt was made to predict post translational modifications on *RpL30* protein. During the current study we used *S. macrospora* and *Neurospora crassa* as reference organisms. Of the 329 nucleotide, polymorphism was observed on five nucleotide positions. The *O*-glycosylation was predicted on serine and threonine while glycosylation on S6 and S8 residues was found to be conserved among *S. fimicola*, *S. macrospora* and *N. crassa*. Altogether, total 14 sites (8Ser/4Thr/2Y) of phosphorylation modifications were observed in *N. crassa* and *S. fimicola* while *S. macrospora* exhibited two additional modifications *i.e.*, Ser66 and Ser91.

Keywords: Diversity; Glycosylation; Methylation; Nucleotides; Phosphorylation

Introduction

The family *Sordariaceae* includes large number of fungi that can be used for biological, ecological and genetic studies. To produce the phylogenies within *Sordariaceae* diverse gene datasets were analysed separately and in addition combined (Eriksson *et al*., 2004). There are three demonstrative genera of *Sordariaceae i.e*., *Gelasinospora*, *Neurospora* and *Sordaria*. These fungi, though firmly correlated, retain specific common natural habitats. The family *Sordariaceae* holds 7–10 genera (Kirk *et al*., 2008). The genus *Sordaria* has three important species that are genetically most studied i.e. *S. fimicola*, *S. macrospore* and *S. brevicollis* (Saleem *et al.*, 2001). The genus *Sordaria* plays an important role as a saprophyte. Members of this genus recycle natural organic material and release the nutrients in the soil which increases the soil fertility. So far, the genome of *S. fimicola* is not sequenced and little information is available about the genome of this fungus. Therefore, here an attempt was made to highlight the effectiveness of 60S ribosomal large subunit protein (*RpL30*) and different post-translational modifications predictor tools to gauge the genetic diversity of *S. fimicola* with reference to *N. crassa* and *S. macrospora*.

Ribosomes are the organelles which are responsible for synthesis of protein (Ban *et al*., 2000; Yusupov *et al*., 2001) and account for the 90% of the transcriptional activity in cell (Rudra and Warner, 2004). In the course of evolution, the ribosomal proteins are found to be extremely conserved and are directly involved in the process of translation (Wool *et al*., 1995). The RNA-binding capabilities of these proteins are involved in controlling ribosome turnover in the cell (Stark *et al*., 2002; Schmeing *et al*., 2003).

In the nucleus, genes for ribosomal protein are transcribed and exported for translation to the cytoplasm (Milgrom *et al*., 2007). The Large subunit of ribosomes in eukaryotes comprised of 5S and 28S rRNAs and 34 diverse proteins (L1–L34) subsequently, L30 is the protein of 60S subunit of ribosome, encoded by *RpL30* gene in eukaryotes (Nesterchuk *et al.*, 2011). In fungi the modifications of pre-rRNA in nucleolus are occupied by *L30* protein, the splicing of the transcript is also auto regulated. The *L30* protein of fungi is homologous to the *L30* protein of mammals (Yoganathan *et al*., 1993;Vilardell *et al*., 2000). The *RpL30* gene plays various molecular and biological roles in the cells of fungi *e.g*., as binding of RNA, ribosomal structural constituent along with rRNA processing and cytoplasmic translation (Ben-Shem *et al*., 2012) and negative regulation of mRNA splicing *via* spliceosome. The spliceosome removes introns from transcribed pre mRNA (Macias *et al*., 2008).

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A typical structure of protein has many domains; these domains are responsible for various modifications which are controlled by a range of enzymes such as kinases and these modifications lead to the formation of diverse classes of proteins (Lothrop *et al*., 2013). The small ribosomal subunit (SSU) attaches to the messenger RNAs and translates the coded message by picking similar amino acyl-transfer RNA. The large subunit (LSU) covers the ribosomal catalytic position that is called the peptidyl transferase center (PTC), which catalyses the development of peptide bonds, ultimately polymerizing the peptide chains. The emerging polypeptides leave the ribosome by a channel in the large sub unit and act together with protein factors that help in enzymatic processing of the ribosomal tunnel (Khoury *et al*., 2011).

Various biological functions have been described for the eukaryotic ribosomal protein *L30*. It makes many inter-subunit and intra-subunit connections with protein or RNA constituents of the 80S ribosome (Dobrenel *et al*., 2016). The *RpL30* protein (Ban *et al*., 2000) is regulated by certain ecological conditions and it was found that heat and a low energy balance cause de-phosphorylation of this protein (Williams *et al*., 2003; Nukarinen *et al*., 2016) while certain growth hormones like auxin, and physical factors like low temperature and various carbohydrates like sucrose increase its phosphorylation (Turck *et al*., 2004). Post-translational modifications are of various types like phosphorylation, acetylation, methylation, and O-glycosylation (Vodermaier, 2004; Zeidan and Hart, 2010; Beltrao *et al*., 2012).

In eukaryotic proteins the regulation and stabilization of structure is controlled by post translational modifications (Halushka *et al*., 1999; Din *et al*., 2010; Minguez *et al*., 2012). Ribosomal protein phosphorylation takes place in all organisms and it has been studied in detail in yeast to plants and humans, but in spite of thorough study, the biochemical significance of ribosomal proteins phosphorylation for translation is basically unidentified (Yerlikaya *et al*., 2016). Phosphorylation of the vast majority of ribosomal proteins in cells on Ser, Thr, and Y residues may play important role in intracellular signal transduction and may be an effective and operational way to regulate most physiological activities, including metabolism, transcription, DNA replication and repair, cell proliferation, and apoptosis (Arnold *et al*., 1999; Pawson and Scott, 2005; Raman *et al*., 2007).

Variations in ribosomal proteins might be responsible for the enormous number of modifications in ribosome (Cascorbi *et al*., 2001; Warner and McIntosh, 2009). Based upon the above mentioned significance, we directed to take advantage of accessible bioinformatics tools to calculate post translational modifications for *S. fimicola* and to investigate population structure in the Evolution Canyon (EC). The hypothesis of the current study was that more genetic variations and modifications are directed to occur in strains that were isolated from the harsh environment.

Materials and Methods

Experimental Organism

All the stock cultures of *S. fimicola* strains used in the present study were provided by Molecular Genetics Research Laboratory, Department of Botany, University of the Punjab, Lahore. All the cultures were sub-cultured on Potato Dextrose Agar (PDA) growth medium at 18°C under aseptic conditions.

Genomic DNA Extraction

Modified Pietro *et al*. (1995) method was adopted for the extraction of genomic DNA of all the strains. The 15–20 mg mycelium of *S. fimicola* scratched out and crushed into powder by using liquid nitrogen. Sample crushed by adding 600 μL lysis buffer, incubated at 65ºC in hot water bath and centrifuged at 13000 g for 15 min. Supernatant was taken out, 600 μL phenol: chloroform: Isoamyl-alcohol (25: 24: 1 v/v) was added and centrifuged. Then added 300 μL of chloroform: Isoamyl-alcohol (25:1 v/v) in the supernatant and centrifuged. After this, supernatant was taken out and 1 mL ice chilled isopropanol was added followed by overnight incubation at -20ºC and centrifugation. The pellet of DNA obtained at bottom, double washed the pellet with 150 μL 70% ethanol, dried the pellet by inverting it on blotting paper and dissolved the pellet in 150 μL Tris TE buffer. The DNA was stored at -20ºC for later use. The extracted DNA was quantified on 1% agarose gel by gel electrophoreses (GE-100 Mini-run Electrophoresis hangzhoubioer technology., Ltd.) stained with ethidium bromide and photographed under gel documentation system.

Oligonucleotides

The forward and reverse primers used were RpL30-F (TGGCCCCCAAGAAGTCCAAG) and RpL30-R (AATGACGACGACCAGCCGCT): targeting exonic regions flanking introns. The primers were designed using Primer3plus software. 10 m*M* working solution of each primer was prepared from 100 m*M* stock primers, purchased from Macrogen, Korea.

Polymerase Chain Reaction (PCR) Conditions

PCR amplifications were performed for the large ribosomal subunit gene *L30* on a GeneAmp PCR 9600 (Perkin Elmer) in 50 μL of PCR master mix containing 5 μL 10 X PCR Buffer, 5 μL dNTPs, 5 μL $MgCl_2$, 2 μL rpl30 primer (forward and reverse), 1 μL *Taq* polymerase, 3 μL template DNA and 29 μL dd.H_2O under the following touchdown (TD) cycling conditions. Initial denaturation was set on 94°C for 0.15 sec, second step was annealing at 65°C for 0.30 sec, and third step was extension/elongation at 94°C for 0.15 sec and final elongation at 55°C for 0.30 sec.

Sequencing of *RpL30* Gene

The PCR products of targeted regions were purified by gel electrophoresis in 1.5% (w/v) agarose gel followed by a GeneClean (BIO 101, Vista, C.A., U.S.A.) procedure conferring to the manufacturer's guidelines and sent for sequencing to Macrogen, Korea (Geumcheon-gu, Seoul).

***RpL30* Gene Sequence Analysis**

RpL30 gene sequences were aligned with reference organism *Sordaria macrospora* published sequences from NCBI, GenBank by using basic local alignment search tool (BLAST) (Altschul *et al*., 1990). Multiple sequence alignment software CLUSTAL *O* was used to evaluate polymorphic sites between different strains of *S. fimicola*.

Results

Calculation of Base Substitutions

During this study, we calculated base substitutions in the exonic region of the *RpL30* gene in six parental strains (S_1, S_2, S_3, N_5, N_6 and N_7) of *S. fimicola*. These strains were isolated from the South slope (S-slope) and the North slope (N-slope) of EC. On the South slope there were three assigned stations i.e. 1, 2 and 3. S_1, S_2, S_3 strains were collected from these three stations of the South slope. There were also three assigned stations on the North slope *i.e*., 5, 6 and 7. N_5, N_6 and N_7 strains were sampled from 5, 6 and 7 station of the N-slope. Base substitutions on three nucleotides i.*e*., A(101)T; A(197)G and C(198)T with 50% of percentage prevalence was observed in S_1, S_2, S_3 strains of *S. fimicola* which were isolated from the S-slope of EC. Genetic polymorphism on position T(180)A with 60% of percentage prevalence was calculated in S_1, S_2, S_3 and N_5 strains but not found among N_6 and N_7 strains whereas genetic variation on position C(244)G with 100% of percentage prevalence was found to be conserved in all the strains of *S. fimicola* (Table 1).

Prediction of Glycosylation

Glycosylation predicted sites by YinOYang 1.2 (www.cbs.dtu.dk/services/YinOYang) showed glycosylation on serine and threonine residues. No threonine glycosylation modification sites were predicted in any of the filamentous fungi *i.e., S. fimicola*, *N. crassa* and *S. macrospora*. In these three above mentioned fungi glycosylation on S6 and S8 postions were found to be conserved while in case of *N. crassa* one additional sites on serine at position S7 was also predicted (Table 2).

Prediction of Phosphorylation

NetPhos 3.1 server (www.cbs.dtu.dk/services/NetPhos/) was used to predict the phosphorylation on serine/threonine/tyrosine residues *in N. crassa*, *S. fimicola* and *S. macrospora*. In *N. crassa* serine phosphorylation modifications were predicted on eight sites (S6, S8, S12, S23, S56, S62, S65, S91) threonine modifications on four sites (T33, T50, T75, T92). In *S. fimicola* phosphorylation was also predicted on eight serine residues (S6, S7, S8, S12, S23, S39, S57, and S63) but slightly on different postions; threonine modification on T51, T68, T76 and T92 while in *S. macrospora* serine modification on ten positions were predicted (S6, S7, S9, S12, S23, S39, S57, S63, S66, S91) and T33, T51, T76, T92 were predicted on threonine residues. Tyrosine modifications on residues Y61 and Y62 are common in all the three fungi except on Y60 position that was predicted only in *N. crassa*. S7, S39, S57, S63 modifications were common in *S. fimicola* and *S. macrospora* (Table 2). Among these sites S6, S12, S23, T92 and Y61 phosphorylation were found to be conserved (Fig. 1) in the above mentioned three filamentous fungi.

Prediction of Sumoylation

We also predicted the sumoylation sites for *RpL30* proteins during the present investigation by online server available at http://sumosp.biocuckoo.org (Table 3). This Table depicts the modified position, modified peptide, predicted score, prediction cut-off value, P-value and regulation type. Sumoylation on position 7 on peptide (*MAPKSSKSDAQSIG) were predicted in *S. fimicola* and *S. macrospora* while it is absent in *N. crassa*. Sumoylation on position 47 is predicted only in *S. macrospora* but not predicted in *S. fimicola* and *N. crassa*. Similarly, sumoylation on position 48 is predicted only in *N. crassa* and *S. fimicola* but not in *S. macrospora*.

Prediction of Nuclear Export Signals

To predict the nuclear export signals (NES), NetNES 1.1 Server (http:// www.cbs.dtu.dk/services/NetNES/) was used. NES were predicted on L, K, A, R and G residues on positions 34–45 in all the three above mentioned fungi (Table 4). NetNES predictor server predicted the NES score from the HMM and Artificial Neural Network (ANN) scores.

Discussion

In this study, we found genetic polymorphisms on five nucleotides and three of five polymorphic sites were present *i.e*., A(101)T; A(197)G and C(198)T in those strains of *S. fimicola* which were isolated from the stressed environmental conditions (stressed conditions are the harsh environmental conditions with high temperature, more UV lights, high pH from where the strains of the south slope were collected used throughout the studies for more detail about the stressed conditions and strains please see Saleem *et al.* (2001),

Table 1: Polymorphisms detection in the exonic region of *RpL30* genes amplified in the different strains of the *S. fimicola* in comparison with the *RpL30* gene of *S. macrospora*

Sr. No.	Base Substitutions in exon of the *RpL30* gene	*RpL30* S_1	*RpL30* S_2	*RpL30* S_3	*RpL30* N_5	*RpL30* N_6	*RpL30* N_7	Percentage Prevalence (%)
1	A(101)T	+	+	+	-	-	-	50
2	T(180)A	+	+	+	+	-	-	60
3	A(197)G	+	+	+	-	-	-	50
4	C(198)T	+	+	+	-	-	-	50
5	C(244)G	+	+	+	+	+	+	100

Table 2: Prediction of Phosphorylation and Glycosylation in filamentous fungi

Organism	Phosphorylation			Glycosylation	
	Serine	Threonine	Tyrosine	Serine	Threonine
Neurospora Crassa	S6,S8,S12,S23,S56,S62, S65,S91	T33,T50 T75,T92	Y60 Y61	S6,S7,S8	nil
Sordaria fimicola	S6,S7,S8,S12,S23,S39,S57,S63,	T51,T68,T76 T92	Y61 Y62	S6,S8	nil
Sordaria macrospora	S6,S7,S9,S12,S23,S39,S57,S63, S66,S91	T33, T51 T76,T92	Y61 Y62	S6,S8	nil

Key: Highlighted area = conserved modification regions

Table 3: Prediction of Sumoylation in *S. fimicola*, *N. crassa* and *S. macrospora*

Organism	Position	Peptide	Score	Cutoff	P-Value	Type
S. fimicola	7	*MAPKSSKSDAQSIG	3.426	3.32	0.153	Sumoylation Nonconcensus
S. fimicola	44 - 48	LRSGKAK LILIS ANTPPLR	47.423	29.92	0.024	SUMO Interaction
N. crassa	44 - 48	LRSGKAK LILIS ANTPPLR	47.423	29.92	0.024	SUMO Interaction
S. macrospora	7	*MAPKKSKSDAQSIG	3.866	3.32	0.132	Sumoylation Nonconcensus
S. macrospora	44 - 47	LRGKAK LILIS ANTPPLR	47.423	29.92	0.024	SUMO Interaction

Arif *et al.* (2017). We have found 1.5% polymorphisms in *RpL30* gene and according to Carlson *et al.* (2004) there is a chance to have 20–30% genomic differences in ribosomal proteins that act as candidate genes. Urban *et al.* (2007) also found 19 % of polymorphism in nucleotide sequences of ribosomal proteins.

Many advanced methods for the prediction of post-translational modifications (PTMs) exist and many are made publicly available. These modern PTM-specific databases aid to predict phosphorylation and glycosylation sites among different proteins (Butt *et al.*, 2012). Similar server is used in the present study to determine phosphorylation modifications in *RpL30* protein. These predictor tools give novel ideas for PTM visualization and improvements (Blom *et al.*, 2004) for prediction specificity (by using ESS – evolutionary stable sites)/. NetPhosK database is a new method for kinase specific prediction of phosphorylation sites. The new server, NetPhosK, is made publicly available at the URL http://www.cbs.dtu.dk/services/NetPhosK/.

No acetylation and methylation was predicted on any amino acid residues of *L30* protein during current investigation in any organism. Methylation was present in six ribosomal proteins (S11, L3, L16, L11, L30, and L12), the acetylation was present in three ribosomal proteins (L7, S18, and S5) and one protein (S12) was methylthiolated (Nesterchuk *et al.*, 2011).

In this investigation we found T92 and Y61 phosphorylation were found to be conserved in *S. fimicola*, *N. crassa* and *S. macrospora*. In contrast to the current findings (Lai and Pelech, 2016), have recognized three conserved phosphorylation modification sites (T198, T207 and Y210 from ERK1) by mass spectrometry. The Y210 site might be vital for appropriate conformational organization of the active site. The auto-phosphorylation of T92 and Y61 as predicted during this study may enhance the catalytic activity and stability of *RpL30* protein in filamentous fungi. As we have found S6, S12, S23, T92 and Y61 conserved phosphorylation sites of *RpL30* protein within the three members' *N. crassa*, *S. fimicola* and *S. macrospora* of *Sordariaceae* family, we can propose that hyper-phosphorylation in kinase action T-loop may act as common mechanisms for down regulation of protein kinase when the upstream kinases get activated. Likewise, Lai and Pelech (2016), proposed the same function of T207 and Y210 phosphorylation of ERK1protein in humans.

Sumoylation (SUMO) is one of the most significant and ubiquitous PTMs that takes place by the covalent linkage of proteins by SUMOs (Gill, 2005). Up to that time, experimental studies proposed that most of these sites are present on A, I, L, M, P, F, V amino acid residues. By using online database we manually predicted three Sumoylation sites in case of *S. fimicola* and *S. macrospora* on positions *i.e.*, 7, 44 and 48 while two sumoylation sites *i.e.*, 44–48 were predicted for ribosomal protein *L30* in *N. crassa*. We have found two type of Sumoylation *i.e.*, Non consensus SUMOs and SUMO interaction.

Here, we also present highly conserved nuclear export signal for *RpL30* protein in all three studied fungi on residue

Table 4: Prediction of Lucien rich nuclear export signals *in S. fimicola, N. crassa* and *S. macrospora*

Seq-Pos-Residue	ANN	HMM	NES
34-L	0.140	0.835	0.546
35-K	0.080	0.851	0.564
36-A	0.095	0.851	0.566
37-L	0.121	0.862	0.586
38-R	0.087	0.862	0.588
39-G	0.099	0.862	0.586
40-K	0.076	0.862	0.573
41-A	0.082	0.862	0.574
42-K	0.114	0.862	0.577
43-L	0.153	0.862	0.584
44-I	0.181	0.862	0.603
45-L	0.254	0.863	0.635

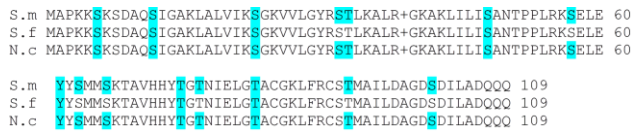

Fig. 1: Multiple sequence alignment of RpL30 protein of filamentous fungi to align conserved potential phosphorylation sites on Ser/Thr/Y residues
S. m= *Sordaria macrospora; Sordaria fimicola; Neurospora crassa*
Highlighted region= conserved potential phosphorylation sites

34–45 amino acid (Table 4). Nuclear export signals (NESs) are particularly essential to regulate the location of protein inside the cell that has ultimate impact on transcription and other nuclear processes, which are important to the sustainability of the cell (Cour *et al*., 2004). In Table 4, the NES score from the HMM and ANN scores is given for residue 34–45. The Artificial Neural Network (ANN) is specified to identify the hydrophobic position mostly on the C-terminal of the NES motif.

Conclusion

Based upon the above mentioned results it was concluded that T92 and Y61 are two most conserved phosphorylation sites on threonine/tyrosine residues that may play an important role to enhance the catalytic activity and stability of *RpL30* protein.

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