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CLONING, MUTATION AND EXPRESSION OF *PIORF4* CDNA GENE IN *ESCHERICHIA COLI* CELLS AND PURIFICATION OF *PI*ORF4(E9D)-6HIS RECOMBINANT PROTEIN

In plant cells exposed to stress, 18S rRNA has been observed to cleave as part of the small ribosomal subunit and accumulate a 135 nucleotide 5′-terminal fragment, termed 5,3S rRNA. However, the physiological significance of this phenomenon remains unknown. The aim of this study was to investigate the role of discrete 18S rRNA fragmentation in plants using the yeast toxin *Pi*ORF4, which can induce a similar cleavage in yeast. In this work, a mutated cDNA gene encoding a form of the *Pi*ORF4(E9D) toxin with increased specificity for 18S rRNA was generated. The mutated gene was cloned into the pET23c vector, followed by expression in *Escherichia coli* BL21(DE3) cells. The resulting recombinant PiORF4(E9D)-6His protein was successfully purified by metal ion affinity chromatography and its identification confirmed by immunoblotting. In addition, preliminary *in vitro* studies demonstrated the ability of *Pi*ORF4(E9D) to specifically target 18S rRNA under controlled conditions, supporting its potential as a model system for plant stress research. The results lay the groundwork for studying the mechanisms of regulation of protein biosynthesis in plants under stress conditions. The practical significance of the work lies in the possibility of using the data obtained to develop approaches for the selection of plants resistant to stress.

Keywords: Genetic construct, *Pi*ORF4, *in vitro* mutagenesis, 18S rRNA, recombinant protein.

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Esherichia coli жасушаларындағы *PIORF4* генінің кДНҚ-ын клондау, мутациялау мен экспрессиялау, және *Pi*ORF4(E9D)-6His рекомбинантты протеиндың тазарту

Стресстік жағдайдағы өсімдік жасушаларында 18S рРНҚ-ның кіші рибосомалық суббөлшек құрамында ажырап, 135 нуклеотидтен тұратын 5′-ұштық фрагментті жинақтайтыны, оны 5,3S рРНҚ деп атайтыны анықталған. Алайда, бұл құбылыстың физиологиялық маңызы әлі белгісіз. Бұл зерттеудің мақсаты өсімдіктердегі 18S рРНҚ-ның дискретті фрагментациясының рөлін ашу болды. Ол үшін ашытқыда осындай ажырату процесін тудыруы мүмкін *Pi*ORF4 ашытқы токсині пайдаланылды. Осы жұмыс аясында 18S рРНҚ-ға ерекше жоғары спецификалық қасиеті бар *Pi*ORF4(E9D) токсинін кодтайтын мутацияланған кДНҚ гені құрылды. Мутацияланған ген pET23c векторына клондалып, содан кейін *Escherichia coli* BL21(DE3) жасушаларында экспрессияланды. Алынған рекомбинантты *Pi*ORF4(E9D)-6His протеинды металл-ионды аффинді хроматография арқылы табысты тазартылып, иммуноблотинг әдісімен оның сәйкестігі расталды. Сонымен қатар, алдын ала жүргізілген *in vitro* зерттеулер PiORF4(E9D)-нің 18S рРНҚ-ға ерекше әсер ететін қабілетін бақыланатын жағдайларда көрсетіп, оны өсімдіктердің стресс жағдайындағы зерттеулерінде үлгілік жүйе ретінде пайдалану мүмкіндігін қолдады. Алынған нәтижелер өсімдіктердегі стресс жағдайында протеин биосинтезін реттеу механизмдерін зерттеуге негіз қалайды. Жұмыстың практикалық маңызы алынған деректерді стресс жағдайларына төзімді өсімдіктерді іріктеудің жаңа тәсілдерін дамыту үшін пайдалану мүмкіндігінде жатыр.

Түйін сөздер: Генетикалық конструкция, *Pi*ORF4, *in vitro* мутагенезі, 18S рРНҚ, рекомбинантты протеин.

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Клонирование, мутирование и экспрессия в клетках *Escherichia coli* кДНК гена *PIORF4* и очистка рекомбинантного белка *Pi*ORF4(E9D)-6His

Было замечено, что в клетках растений, находящихся в состоянии стресса, 18S рРНК расщепляется как часть малой рибосомной субчастицы и накапливает 135-нуклеотидный 5′-терминальный фрагмент, называемый 5,3S рРНК. Однако физиологическое значение этого явления остается неизвестным. Целью данного исследования было изучить роль дискретной фрагментации 18S рРНК в растениях с помощью дрожжевого токсина PiORF4, который может вызывать аналогичное расщепление в дрожжах. В данной работе был создан мутированный кДНК-ген, кодирующий форму токсина *Pi*ORF4(E9D) с повышенной специфичностью для 18S рРНК. Мутированный ген был клонирован в вектор pET23c, после чего он был экспрессирован в клетках *Escherichia coli* штамма BL21(DE3). Полученный рекомбинантный белок PiORF4(E9D)-6His был успешно очищен с помощью металл-ионной аффинной хроматографии, а его идентификация была подтверждена иммуноблотингом. Кроме того, предварительные исследования *in vitro* продемонстрировали способность *Pi*ORF4(E9D) специфически воздействовать на 18S рРНК в контролируемых условиях, что подтверждает его потенциал в качестве модельной системы для изучения стресса растений. Полученные результаты закладывают основу для изучения механизмов регуляции биосинтеза белка в растениях в условиях стресса. Практическая значимость работы заключается в возможности использования полученных данных для разработки подходов к селекции растений, устойчивых к стрессу.

Ключевые слова: Генетическая конструкция, *Pi*ORF4, мутагенез *in vitro*, 18S рРНК, рекомбинантный белок.

Abbreviations and notations

DNA – deoxyribonucleic acid, cDNA – complementary DNA, PAA-gel – polyacrylamide gel, PCR – polymerase chain reaction, RNA – ribonucleic acid, RT – reverse transcription, Tris – tris(hydroxymethyl)aminomethane, ATP – adenosine triphosphoric acid, BSA – bovine serum albumin, EDTA – ethylenediaminetetraacetate, 6His-tag – amino acid sequence of six histidines, *Pi*ORF4 – *Pichia inositovora* toxin encoded by the open frame ORF4.

1. Introduction

Plants are living organisms devoid of the capacity for movement. Like any other living organism, they are subject to periodic stress due to unfavourable influences such as drought, decreased or increased ambient temperature, increased salt concentrations, and hypoxia (overwatering). These factors have the capacity to alter gene expression at the level of transcription and also affect the process of protein biosynthesis [1]. Protein biosynthesis is one of the most energy-consuming processes in the cell; therefore, the ability of living organisms to suppress this process under stresses to conserve resources and prevent the accumulation of misfolded proteins is critical [2]. One way in which this negative regulation of protein biosynthesis in eukaryotic cells is mediated is by so-called ribosome inactivating proteins, RIPs (from Ribosome Inactivating Proteins). Notable examples of RIPs include ricin and abrin, which are regarded as some of the most potent biologically derived poisons [3]. The mechanism of action of RIPs is based on the cleavage of the 28S rRNA molecule along the so-called ricin-sarcin loop due to apurinisation of one of its residues. This results in the inability of 60S ribosomal subunits to bind the elongation factor eEF2, and thus to blocking the elongation stage of the polypeptide chain [4, 5]. A number of prokaryotic toxins, such as shigatoxin (shigatoxine), act in a similar manner [6].

The cleavage of rRNA is not invariably associated with ribosome inactivation; during ribosome biogenesis, several steps of cutting of the initial pre-rRNA molecule (45S) occur to form 18S, 28S and 5.8S rRNA [7]. Subsequent to the initial assembly of pre-40S particles and their transportation into the cytoplasm, the 3′-end of 18S rRNA is cleaved by the Nob1 endonuclease, thereby completing the maturation of the 40S subunit [8].

Previous studies have demonstrated that in plant cells experiencing specific forms of stress, 18S rRNA undergoes cleavage as part of the small ribosomal subunit, resulting in the accumulation of the 135-nucleotide 5'-end fragment (5,3S rRNA) within the cytoplasm [9]. The molecular mechanism and physiological significance of such discrete fragmentation of 18S rRNA remains to be elucidated [10]. One hypothesis suggests that this cleavage may lead to the suppression of translation for all mRNAs or specific mRNAs [11].

In yeast *Pichia inositovora* cells, the *Pi*ORF4 toxin has been found to cleave a 130-nucleotide fragment from yeast 18S rRNA, a process analogous to the cleavage of 5.3S rRNA molecules found in plant cells [12]. Bioinformatic analysis revealed that the 18S rRNA region where the break occurs is conserved in yeast and plants [13]. Consequently, the decision was taken to utilise the recombinant yeast toxin *Pi*ORF4 to investigate the role of discrete 18S rRNA fragmentation in plants.

The present study presents data on the cloning of a mutated cDNA gene encoding the yeast toxin *Pi*ORF4(E9D) into the expression vector pET23c, optimisation of it is expression in *Escherichia coli* cells, isolation of the recombinant *Pi*ORF4(E9D)-6His protein and confirmation of its identity by immunoblotting. In the future, the purified recombinant protein is planned to be used for site-directed cleavage of 18S rRNA as part of ribosomal subunits in a model cell-free system of protein synthesis *in vitro* from wheat embryos in order to study the role of discrete fragmentation of 18S rRNA in plants.

2. Materials and methods

The study focused on *Escherichia coli* bacteria cultivated in liquid LB medium at a temperature of 30°C [14].

Computer analysis was conducted utilising bioinformatic programmes such as MEGA-X, Blast, and RNA-structure 6.0.1. The nucleotide sequence of the *P. inositovora* plasmid pPin1-3, which contains the ORF4 gene (GenBank: AJ564102), was retrieved from the NCBI database.

Creation of the pET23c-PiORF4(E9D)-6His genetic construct. The nucleotide sequence of the *P. inositovora* ORF4 cDNA gene was optimised for optimal expression in *E. coli* cells, and a mutation was introduced into this open reading frame resulting in the replacement of a glutamic acid residue with an asparagine acid (D) residue at position 9. The mutated cDNA gene was synthesised de novo and subsequently cloned by *NdeI/XhoI* restriction sites into the vector plasmid pET23c, utilising T4 DNA ligase (Thermo Fisher Sci.) in accordance with the manufacturer's protocol.

Polymerase chain reaction (PCR) was performed for clone screening. The primers utilised were 'PiORF4-Rev-Xho' (5'-ttactcgagagagttggagttggggacatttttttgg) and 'PiORF4-FW-Nde' (5'-atcggatccatatgaatcaacaacaagctgctcca). Reactions were performed using Hot Start Taq-DNA polymerase (NEB) kits, following the temperature conditions outlined below: Stage 1 – 10 minutes at 95°C; Stage 2 – 20 seconds at 94°C, 20 seconds at 55°C, 1 minute and 30 seconds at 72°C – 35 cycles; Stage 3 – 5 minutes at 72°C – 1 cycle [15]. The amplification products were analysed by electrophoresis in a 1.0% agarose gel containing ethidium bromide, followed by band detection in transmitted UV light in a Quantum gel-documentation system (Vilber).

The DNA sequencing of constructs was performed using gene-specific primers and the Big Dye Terminator v.3.1 kit (Applied Biosystems) in accordance with the manufacturer's instructions. Subsequently, the samples were subjected to capillary electrophoresis on a 310 genetic analyser (Applied Biosystems) [16]. The obtained data were then analysed using Sequencing Analysis 5.2 software [17].

Expression and isolation of recombinant proteins. Plasmid *pET23c-PiORF4(E9D)-6His* was used to transform cells of the expression strain *E. coli* BL-21(DE3) by heat shock (42° C 90 sec). The subsequent growth of the plasmid-containing *E. coli* cells was undertaken in 200 ml of liquid LB medium at 30° C to an optical density of OD^{600}= 0.5, after which they were precipitated at 4000 g for 3 min [18]. The cells were then resuspended in 200 ml of fresh LB medium containing IPTG to a final concentration of 1 mM and cultured at 30° C for 4 hours. The recombinant *Pi*ORF4(E9D)-6His protein was isolated under native conditions by immobilised metal ion affinity chromatography (IMAC) using a PerfectPro Ni-NTA Agarose kit (5-Prime) in accordance with the manufacturer's procedure.

The one-dimensional electrophoresis of proteins in polyacrylamide gel according to Lammli [19] was performed in 12.5% PAA gel in the presence of 0.1% SDS (C = 0.5%) on a Mighti-small device (Hoefer). The gels were then stained with 0.125% Coomassie brilliant blue G-250 solution (Serva).

Immunoblotting. Protein transfer from gel to nitrocellulose membrane was performed using a

semi-dry blotting apparatus (C.B.S. Scientific). This process was carried out in transfer buffer containing 102 mM glycine, 25 mM Tris and 20% ethanol using a current of 0.8 mA/cm2 for 1 hour. Following this, the membrane was washed twice for 10 minutes each in TBS buffer (20 mM Tris-HCl at pH 7.6; 140 mM NaCl), followed by two further washes of 10 minutes each in TBST buffer (TBS supplemented with 0.05% Tween-20). The membrane was then incubated in blocking buffer (5% Sigma skimmed milk in TBST) at 4°C overnight. Following three 20-minute washes in TBST, the membrane was subjected to an incubation at room temperature for 1 hour with a secondary antibody, namely anti-mouse HRP-conjugate (5-Prime), prepared at a 1:2000 dilution in blocking buffer. Thereafter, the membrane underwent two additional 20-minute washes in TBST, followed by two 10-minute washes in TBS. Finally, the membrane was treated with a peroxidase chemiluminescent substrate, utilizing the SuperSignal West Pico Chemiluminescent Substrate kit (Promega) in accordance with the protocol provided by the manufacturer.

3. Results and discussion

To clone *Pi*ORF4 cDNA gene into expression vector pET23c, bioinformatic analysis was performed in MEGA-X programme and primers 'PiORF4-Rev-Xho' and 'PiORF4-FW-Nde' were designed and tested in RNA-structure 6.0.1 programme to check self-complementarity and cross-complementarity to avoid synthesis of non-specific PCR products and primer duplexes during PCR. DNA amplification was performed using Pwo high-precision polymerase to reduce the chance of incorporation of unforeseen mutations into *Pi*ORF4 cDNA [20]. The amplicon was treated with NdeI/XhoI restriction endonucleases and cloned into the pET23c vector treated with the same enzymes. The map of the obtained DNA construct *T7-PiORF4-6His_Pet23c* is presented in Figure 1.

The DNA clones were subjected to a polymerase chain reaction (PCR) analysis, utilising the same primers employed for the amplification of *Pi*ORF4 cDNA. The results of the analysis are presented in Figure 2.

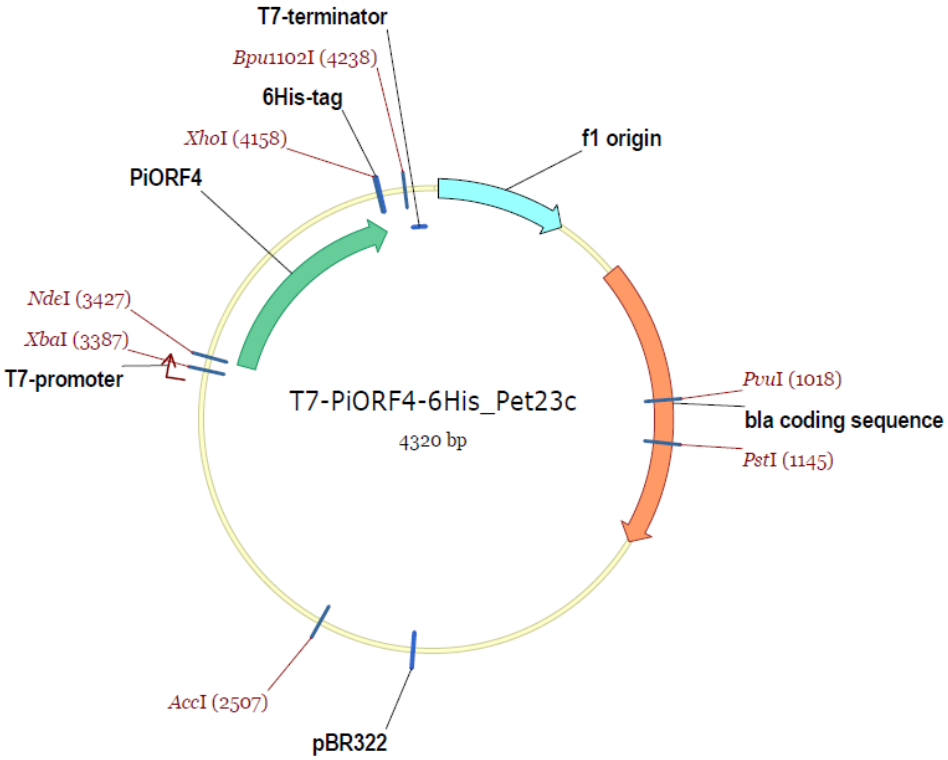

Figure 1 – T7-PiORF4-6His_Pet23c plasmid map

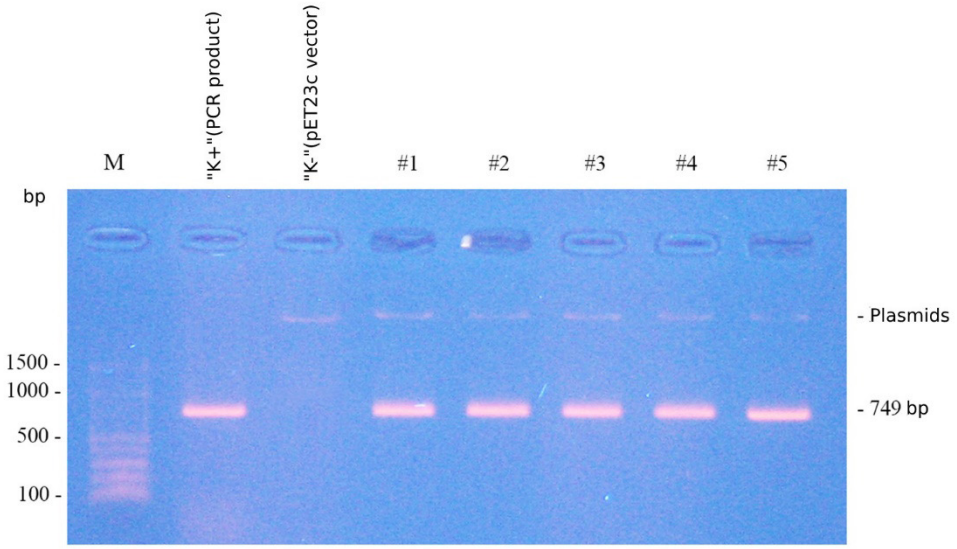

Figure 2 – PCR screening with primers of DNA clones "PiORF4-FW-Nde" / "PiORF4-Rev-Xho"

Electrophoresis was conducted in a 1.0% agarose gel containing ethidium bromide, followed by the detection of bands in transmitted UV light in a Quantum gel-documentation system (Vilber). M – marker 100bp DNA Ladder, (obtained from Thermo Fisher Sci.).

In addition, the PCR-positive clones were subjected to restriction analysis using restriction endonucleases *Nde*I and *Xho*I in order to verify them. The results of this analysis are presented in Figure 3.

According to Kast et al., replacement of a glutamic acid residue with an asparagine acid residue at position 9 of the yeast toxin *Pi*ORF4 resulted in a significant increase in the specificity of cutting the 18S rRNA molecule within the small ribosomal subunit [9]. Therefore, it was decided to mutate the cloned *Pi*ORF4 cDNA accordingly. The primers 'PiORF4-D-Rev' (5'-aatacatcaaAtcgtgtatggggagcagcttgttgttg) and "PiORF4-D-FW" (5'-tccatacgaTttgatgatgtatttgttgttgttg) were synthesised for *in vitro* mutagenesis (Figure 4).

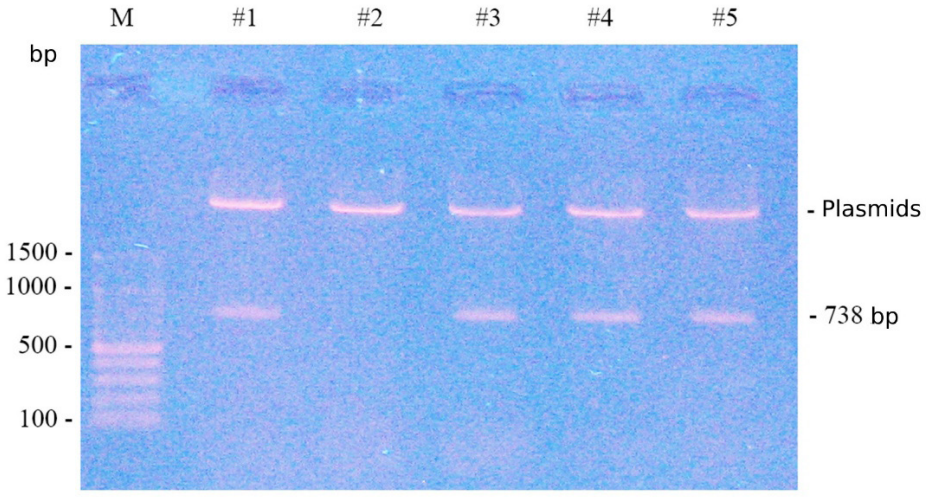

Figure 3 – Restriction analysis of NdeI / XhoI DNA clones

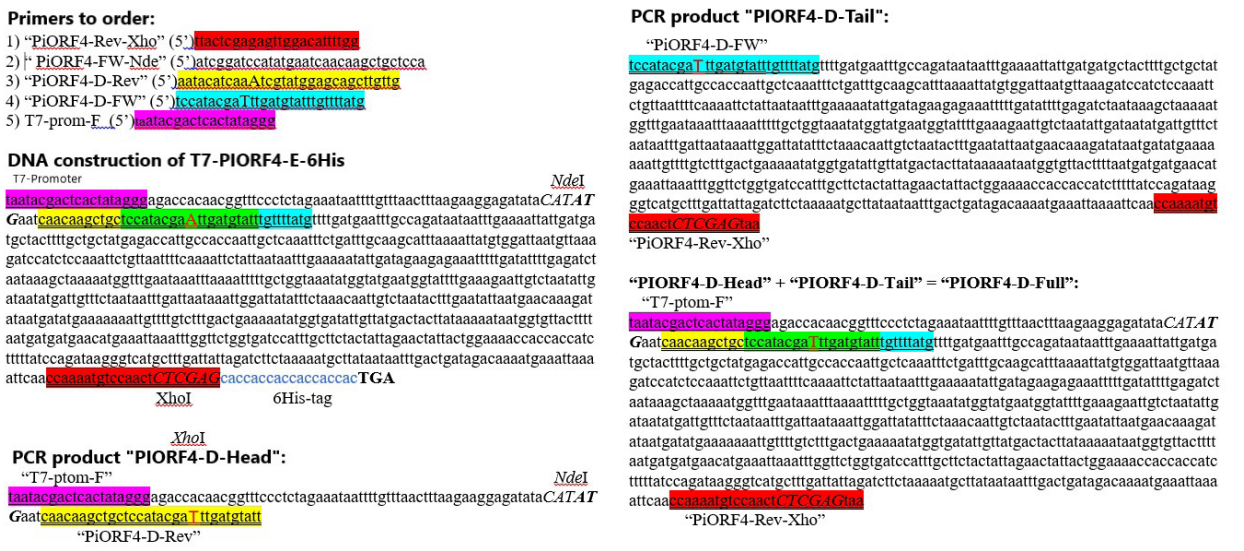

Figure 4 – Nucleotide sequences of PCR products at different stages of in vitro mutagenesis

The mutagenesis process was executed through the utilisation of a polymerase chain reaction (PCR) method, employing overlapping primers and a high-precision Pwo polymerase [21]. The mutation produced two fragments, designated PiORF4-D-Head (117 bp) and PiORF4-D-Tail (721 bp) (Figure 5), each containing a targeted nucleotide substitution. These were then combined to form a common product, PiORF4-D-Full (818 bp). The final composite PCR product was then eluted from the gel, treated with restriction endonucleases NdeI and XhoI, and cloned into the empty vector pET23c. The selection of DNA clones was carried out in the same way as for the cloning of the original PiORF4 cDNA gene.

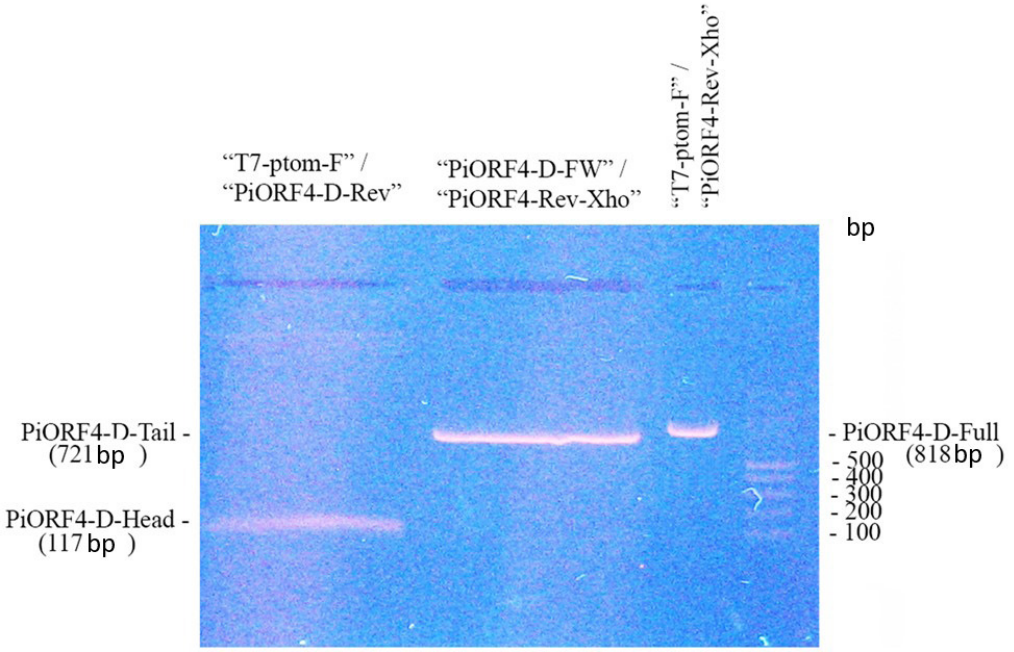

Figure 5 – The results of the mutagenesis of the overlap extension PCR

In order to verify that the nucleotide substitution had been correctly inserted at the designated site of the cloned cDNA and that no unforeseen mutation had occurred in parallel, Sanger sequencing [22] was performed using primer T7 (5'-taatacgacgactcactataggg). The analysis of the sequencing data of the obtained DNA construct *T7-PiORF4(E9D)-6His_Pet23c* confirmed that the adenine nucleotide was replaced by a thymine nucleotide at position 9 of the *Pi*ORF4 gene. The mutated construct *T7-PiORF4(E9D)-6His_Pet23c* was then utilised to develop a recombinant protein, with the objective of enhancing the specificity of the recombinant *Pi*-ORF4 protein.

A bacterial expression system was selected for the production of yeast toxins. The anticipated molecular weight of the recombinant protein was approximately 28 kD, and it was found to be active without the need for glycosylation. Furthermore, it was determined that the yeast gene expression system was not conducive to *Pi*ORF4 gene expression, owing to the toxicity of the translation product for yeast cells.

The *T7-PiORF4(E9D)-6His_Pet23c* plasmid was employed as a means of transforming cells of the expression strain *E. coli* BL-21(DE3), which contains the T7 bacteriophage RNA polymerase gene within its genome, under the control of the lacUV5 promoter. The *E. coli* cells that had been transformed with this plasmid were then grown, after which the synthesis of phage T7 polymerase was activated by adding IPTG to the medium. Following several hours of expression, the synthesis products were analysed by immunoblotting using antibodies to the 6-His amino acid sequence. Following confirmation of *PiORF4(E9D)-6His* cDNA expression in bacterial cells, its purification by IMAC affinity metal ion chromatography using Ni-NTA agarose was performed [23, 24]. The protein fractions obtained during the isolation of the recombinant protein were analysed by immunoblotting [25]. The results of this analysis are presented in Figure 6.

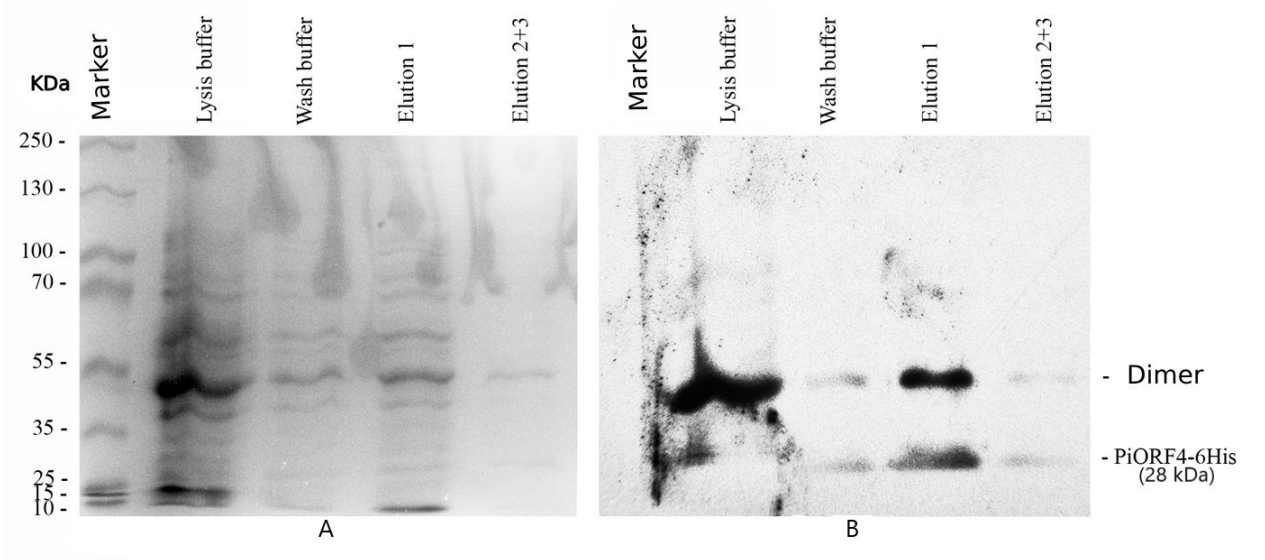

Figure 6 – Isolation of PIORF4-6His from E. coli
A – Electrophoresis in 12.5% polyacrylamide gel. B – Immunoblotting (Penta-His-Ab).
M – Protein marker, PageRuler™ Plus Prestained Protein Ladder (Thermo Fisher Sci.).

As demonstrated in Figure 6, in addition to the protein of the anticipated size (28 kDa), an additional product of approximately 50kDa was released, which, according to the calculated parameters, corresponds to the dimer of PiORF4(E9D)-6His protein. The formation of dimers in *E. coli* has been observed during mass expression [26], thus validating the outcome. The protein was purified by dialysis and concentrated in concentrating columns by centrifugation. The protein concentration was determined to be 571,9 ± 13.3 μg/ml (total volume, 450 μl). The yield of the recombinant protein was 2,574 mg from 1 litre of nutrient medium.

Conclusion

A genetic construct, designated *pET23c-6His-PiORF4*, was obtained. This construct contains an open reading frame of the yeast toxin *Pi*ORF4, which is capable of producing targeted, specific breaks in rRNA molecules. Through *in vitro* mutagenesis, a nucleotide substitution was introduced into the cloned cDNA, resulting in an E9D substitution in the *Pi*ORF4 protein, thereby increasing its specificity for 18S rRNA and preventing the yeast 28S rRNA molecule from being cut. The recombinant *Pi*ORF4(E9D)-6His protein was then produced in bacterial cells and successfully purified by means of the Immobilized Metal Affinity Chromatography (IMAC) method. The purified *Pi*ORF4(E9D)-6His protein will be further used to evaluate the role of discrete 18S rRNA fragmentation in the regulation of protein biosynthesis in plants.

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