

**ISOLATION AND IDENTIFICATION
OF A NEW PROTEIN FOR
CAPPARIS SPINOSA RPS4 GENE**

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Abstract

Capparis spinosa is an arid plant species growing in the desert of Egypt. It's one of the most important species in family capparidaceae. *Capparis spinosa* is one of the medicinal plants established to have high diversity range of economic and medicinal value. Recently, the pharmacology and chemistry of this plant have been extensively studied. Chemical studies of the different parts of *Capparis*, both fermented and non-fermented, have shown the presence of many beneficial compounds. In this study the author selected a 606bp ribosomal protein 4s (rps4) chloroplast gene of *Capparis spinosa*, where the complete sequence of rps4 gene was amplified, sequenced, cloned, and expressed. rps4 gene is a gene in the land plant chloroplast genome and it is one of very popular marker in this genome. The forward primer started with the start codon ATG of rps4 and the reverse primer was designed to align a region of 18nt upstream of the stop codon. The isolated gene is approximately 606 bp in length. After data alignment it was found that maximum identity was 98% with *Capparis flexuosa* ribosomal

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protein S4 (rps4) gene and 96% with Nasturtium officinale ribosomal protein S4 (rps4) gene. The information of this gene was found to be a new gene in comparison to the reported genes in the gene bank. According to the gene bank it's a new gene. Therefore it was given an accession number. The isolated gene was a complete gene and this gives a complete protein in expression where the accurate translation of the genetic message is an essential step in gene expretion. The clone was found to encode an 202 amino-acid protein fragment. It was expressed in Escherichia coli as a fusion protein of calculated molecular mass of about 48 kDa with C-terminus of glutathione-S-transferase (about 26 kDa). The 202 amino-acid open reading frame encodes a protein with a calculated molecular mass of about 22 kDa.

Key words: *Capparis spinosa*, *rps4* gene, sequencing, cloning, expression.

INTRODUCTION

Capparis spinosa L. (Caper) is the type species of the genus. It is a spiny, prostrate, and perennial little shrub. It has a wide distribution in the old world from South Europe, North and East Africa, Madagascar, Southwest and Central Asia to Australia and Oceania ^[1,2]. *Capparis spinosais* well known with its common name 'Capers' in different countries ^[3]. It is known by various names, e.g. Caper (English), Kabbar (Arab), Alcaparro (Spain), and Gollaro (Pakistan) ^[4]. In cultures worldwide, natural products such as medicinal plants, minerals, and materials from animal sources were used as traditional remedies by man due to their healing properties. A medicinal plant is that used by people for medicinal purposes - to build or maintain health, stave off disease, or promote recovery from illness or misfortune. *Capparis spinosais* one such plant established to have highly diverse economic and medicinal value in different systems of medicine like in Iranian, Unani, Chinese, Ayurvedic and Greco Arabi System of medicines ^[5].

The aqueous extract of *Capparis spinosais* have: Anti-inflammatory ^[6], antiallergic ^[7], anti-diabetic ^[8], antifungal activity ^[9],

hypolipidemic activity ^[10], the genotoxic and antimutagenic effects ^[11], and antihepatotoxic ^[12], etc. .

Plant cells are characterized by the presence of three genomes, i.e. nuclear, chloroplast and mitochondrial, each of them is present at a different rate of evolution ^[13]. It was demonstrated in the 1960s and early 1970s that chloroplasts contain their own DNA molecule ^[14]. Chloroplast genomes typically comprise a single circular molecule (although there are usually multiple copies per chloroplast) of 120–200 kb in photosynthetic organisms ^[15].

The rps4 gene which encoding for small subunit of chloroplast ribosomal protein 4 & the rps4 gene coding sequence has a length of 606 bp in higher plants ^[16]. rps4 gene is one of five markers having quantitative importance in chloroplast genome, i.e. more than 85% of all DNA sequence analyses were based on the ‘prime markers’ which are trnL-F, rbcL, rps4, 18S, and ITS in which the rps4 gene is one of five markers ^[17].

Protein synthesis is coordinated by the ribosome in all cells ^[18]. This central protein synthesis factory is often viewed as a finely tuned machine that functions as a static, reliable component of higher-order cellular processes ^[19].

In fact, the ribosome has a responsibility to correctly make newly synthesized protein efficiently in the cell. Recently, several ribosomal proteins have been reported to possess other functions aside from the traditional ribosomal function including induction of apoptosis ^[20], suppression of tumors^[21], regulation of development ^[22], and DNA repair ^[23]. The genes coding for these ribosomal proteins are coordinately regulated in order to accommodate the different cell requirements for protein synthesis ^[24].

Several S4 genes have been cloned from dicotyledonous and monocotyledonous plants ^[25]. However little is known about the structure, subcellular transportation and function of plant S4 proteins ^[26]. Thus, there is shortage in the data available about the rps4 gene and therefore, this work was conducted to deal with such a shortage, and to give more details about rps4 gene and its coding protein. This work considered a first trial for dealing genetically with *Capparis spinosa*. The aim of this study was focused on isolating, investigating

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and cloning of a gene for the first time that may be biologically useful and give us an idea about the medicinal role of this plant.

Materials and methods:

Plant material:

Capparis spinosa leaves were collected from different fields, then these fresh samples were frozen quickly in liquid N₂ and stored at -80 °C.

DNA Isolation from plant material:-

Total DNA was extracted from 100 mg of the dry material using the Dneasy plant mini kit of Qiagen (Hilden, Germany) according to the manufacturers protocol [27]. The DNA was eluted in 100mM Tris-buffer (PH 8.5) and stored in the freezer until amplification. Then the quality and the concentration of the extracted DNA was checked by 1% agarose gel electrophoresis and nanodrop spectrophotometer [28].

Amplification of rps4 gene:-

The chloroplast rps4 gene was amplified in a 50 µl final volume using the standard amplification technique with HotStarTaq Plus Master Mix Qiagen (Hilden, Germany) (PCR; [29]). An initial activation step 5 min at 95 °C, followed by 30 cycles (30 s 94 °C, 30 s 58 °C, 60 s 72 °C) and a final step 10 min of extension time at 72 °C. Amplified fragments were visualized on 1.5 % agarose TAE gels by EthBr staining. PCR product was purified with QIAquick PCR product extraction kits of Qiagen (Hilden, Germany) following the manufacturers protocol. The PCR product was eluted in 100mM Tris-buffer (PH 8.5) and stored in the freezer until amplification. Then the quality and the concentration of the PCR product was checked by 1.5% agarose gel electrophoresis.

Sequencing of the PCR product and Alignment and Analysis of sequence data [30]:

After purification, the PCR product was directly sequenced in both directions (MacroGen Company, South Korea) using ABI 3730XL DNA sequencer (Applied Biosystem, USA). Sequencing primers were those used for PCR. Sequencing of the purified PCR product Database 'homology' searches were conducted on the

sequence data. The basic information is to take a query sequence and compare it consequently with all known sequence in a database. The goal is to find sequences that are sufficiently similar to the query sequence. The searches were conducted using the BLAST algorithm. BLAST an acronym for "Basic local Alignment searching tool" represents a family of programs for database similarity search tool [31], for example, BLASTN searches the homology between the nucleotide query sequence and nucleotide sequence database. BLAST searches (<http://www.ncbi.nlm.nih.gov/Blast/>) were conduct to confirm the identity of generated sequences.

Ligation of PCR product to pTZ57R/T vector, Preparation of plasmid DNA, Amplification of rps4 gene insert in vector, Restriction enzyme digestion for cuttig rps4 gene and purification of linear DNA : The rps4 gene was ligated into PTZ57R/T vector, then the E.coli (XL1-blue) were transformed with this recombinant plasmid by using InsTA™ PCR cloning kit (Fermentas, #K1214), [32] and selected on LB- ampicillin X-Gal/ IPTG agar plates. After that the plasmid was purified from white colonies Genejet plasmid miniprep kit (Fermentas, #K0502), [33], and amplified by using M13/PUC sequencing as a forward primer and the reverse primer of rps4 gene as reverse primerof rps4 gene. Finally, the positive recombinant plasmid was digested with EcoRI and SaII (promege) restriction enzymes and the EcoRI- rps4- SaII fragment was purified.

Ligation into pGEX-4T-3 expression vector, selection of expression positive recombinant pGEX-4T-3 and detection of position of rsp4 gene: The rps4 gene was expressed in E.coli by using PGEX-4T-3 expression vector in which the linear EcoRI- rps4- SaII fragment was ligated into PGEX-4T-3 vector at EcoRI and SaII, where the vector cut by the same resriction enzymes to produce PGEX-4T-3-rps4 plasmid, then the E.coli (JM109) were transformed with this recombinant plasmid and selected on LB media containg ampicillin (100µg/ml). After that the plasmid was purified from white colonies Genejet plasmid miniprep kit (Fermentas, #K0502), [33], and amplified by using PGEX 5' sequencing primer of PGEX-4T-3 plasmid as forward primer and reverse primer of rps4 gene as a reverse primer in order to verify the presence of insert

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Production of the recombinant fusion protein ^[34]: A bacterial colony, with positive inframe recombinant plasmids was cultured overnight with shaking in LB- ampicillin medium. The overnight culture was diluted 1 : 10 in fresh medium and grown for 1-2 hrs. at 37°C before adding IPTG to a final concentration of 1mM in order to induce the transcription of the rps4 and consequently, the the translation of Glutathion-S-transferase (GST) fusion proteins. In order to examine the induction, 1 ml aliquots of the induced culture were taken at 1, 2, 3, 4, and 5 hrs. post induction. The cells were spun down, resuspended in 20 ml of 1X SDS-gel loading buffer and electrophoresed onto 10% SDS-PAGE.

RESULTS

DNA Isolation from plant material: For detection of the rps4 gene, we first isolated chloroplast DNA from leaves of *Capparis spinosa*, Gel electrophoresis revealed the presence of intact band at the top of gel near the well and above the marker which means that it was chloroplast DNA and it was good enough to use in the subsequent procedures where there was not any smearing in it, as illustrated in Fig.(1) .

After the isolation of DNA, Nanodrop (uv-vis spectrophotometer Q5000, Quawell, USA) was used to measure DNA concentration and purity. The Nanodrop reading revealed that the isolated DNA is pure (the ratio of 260/280 and 230/260 ranged between 1.8 and 2.0) and with a considerable concentration (ranged from 450 to 1100 ng/μl), the isolated DNA showed a clear absorbance peak at 260 nm, as illustrated in Fig.(2). Therefore, it can be used successfully as a template for PCR to isolate rps4 gene.

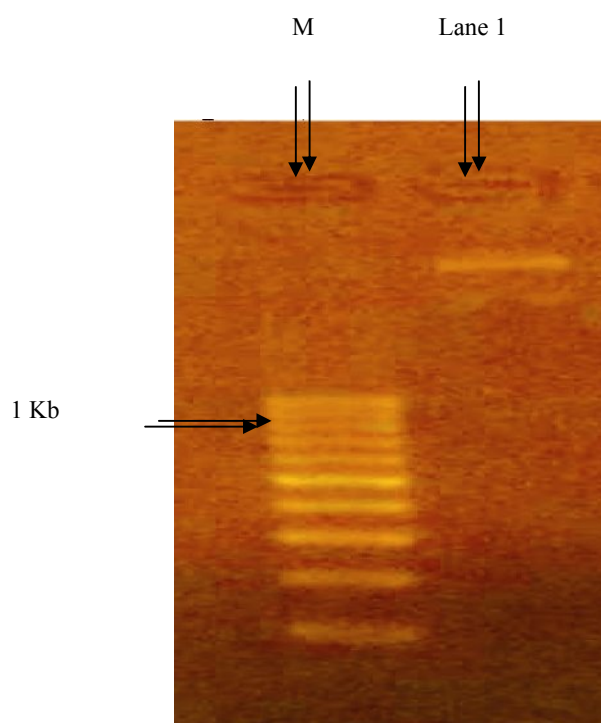


Fig (1) : Electrophoresis of DNA Isolation from plant material : 1% agarose gel showed a band above marker band. *M* : marker (10 kb ladder); *Lane 1*: 10µl the isolated DNA.

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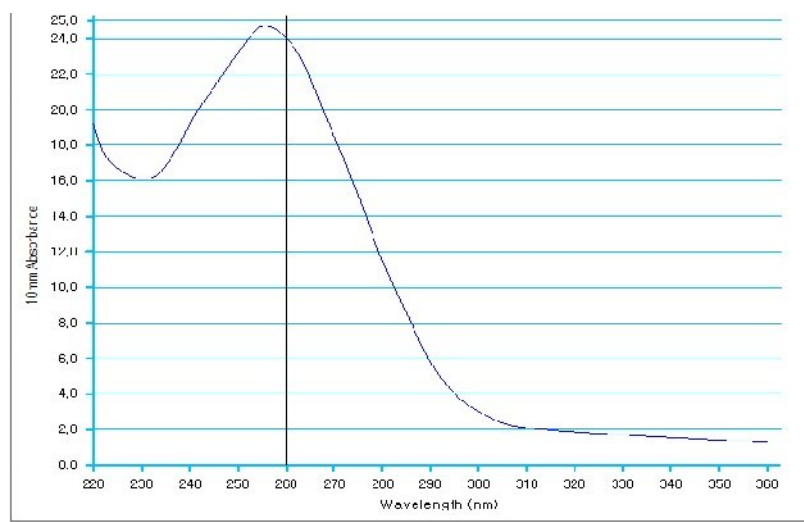


Fig (2): Quality and the concentration of DNA extracted: Nanodrop curve showing concentration of extracted DNA from a representative sample which is 970 ng/ul. The curve indicates the presence of pure DNA as the upper top of curve present at 260 and its bottom at 230.

Amplification of rps4 gene by using PCR, Purification of rps4 gene and the determination of its concentration: The extracted DNA was used as a template for PCR to amplify rps4 gene. As expected, agarose gel electrophoresis revealed the presence of 600 bp PCR products, as illustrated in Fig.(3). Before sending for sequencing, PCR product with expected size was purified by using PCR purification kit to remove primer dimers, primers, nucleotides, proteins, salt, agarose, ethidium bromide and other impurities, as illustrated in Fig.(4). After the purification of PCR product, it's concentration was measured. The Nanodrop reading revealed that the purified gene is pure (the ratio of 260/280 and 230/260 ranged between 1.8 and 2.0) and with a considerable concentration (ranged from 150 to 350 ng/μl), as illustrated in Fig.(5). This rang is sutable for using in cloning. Therefore, it can be used successfully as a template in TA cloning vector.

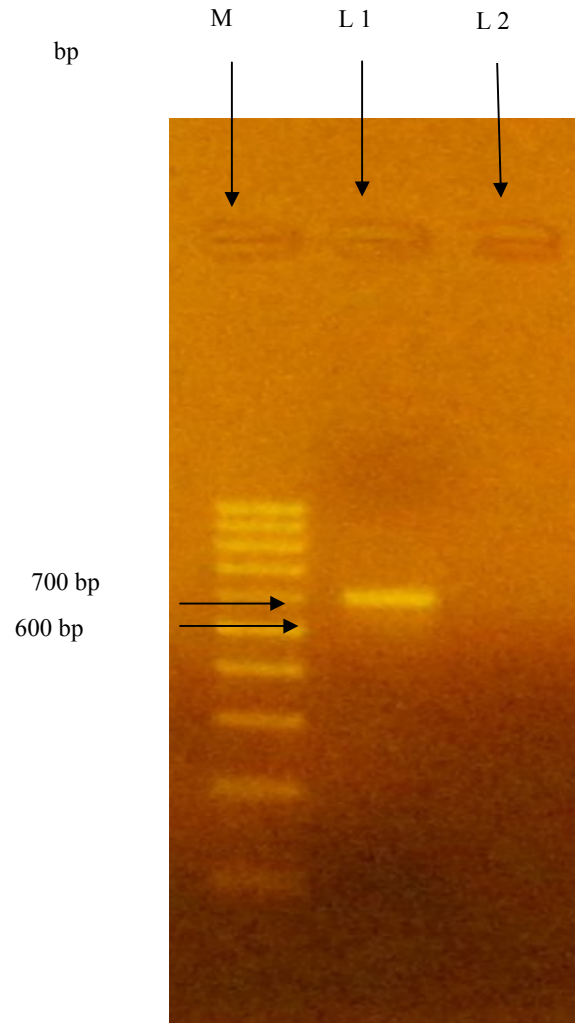


Fig (3): Electrophoresis of PCR product of rps4 gene: Ethidium bromide stained agarose gel of PCR products representing amplification of the rps4 gene in *Capparis spinosa* : 1 % agarose gel showing a band of 606 bp, *M* : marker (1 kb ladder); *Lane 1* : the PCR product; *Lane 2*: the –ve control (without DNA) to show any contamination.

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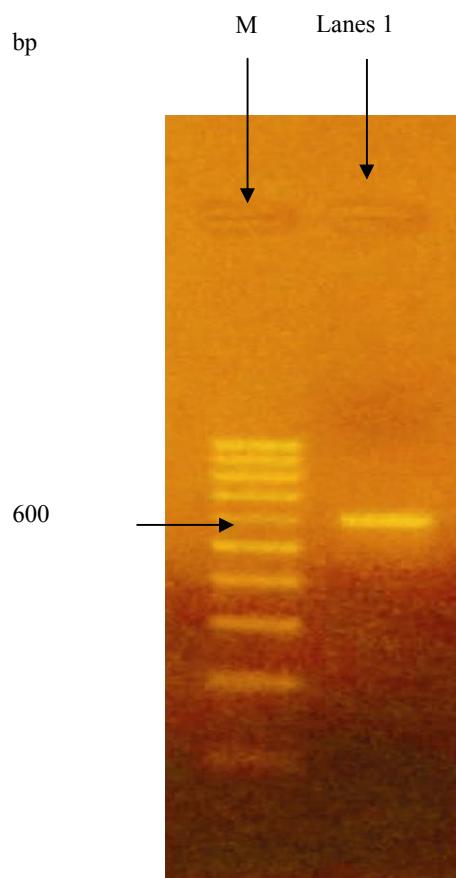


Fig (4): Electrophoresis of purified PCR product of rps4 gene: Ethidium bromide stained agarose gel of purified rps4 gene: 1% agarose gel showing a band of 606 bp, *M* : marker (1 kb ladder); *Lane 1*: purified PCR products.

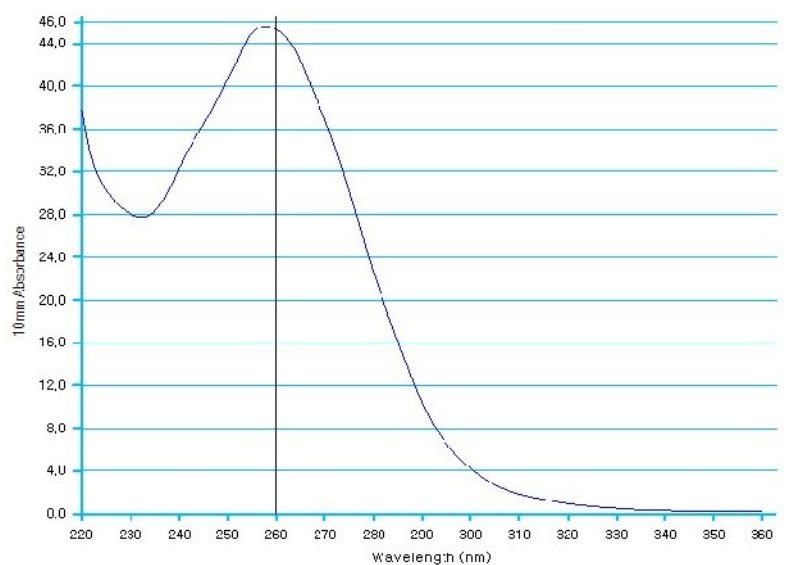


Fig (5): Quality and the concentration of PCR product: Nanodrop curve showing concentration of purified PCR product which is 300 ng/ul. The curve indicates the presence of pure DNA as the upper top of curve present at 260 and its bottom at 230.

Sequencing of the PCR product, and alignment and analysis of sequence data : Sequencing of the purified PCR product in both directions (MacroGen Company, South Korea) using ABI 3730XL DNA sequencer (Applied Biosystem, USA) confirmed the PCR results, The sequence from both ends overlapped in 606bp, so sequencing reaction yielded 606bp. We have submitted rps4 ORF into GenBank databases with accession number JQ665718 (<http://www.ncbi.nlm.nih.gov/Blast/>), as illustrated in Fig.(8). The transcription and the translation of rps4 gene in order to give a complete protein in which it was demonstrated that rps4 gene was 606 bp and the ORF of it was 202 amino acid, as illustrated in Fig.(9). The nucleotide sequence of rps4 gene of *Capparis spinosa* was used in non- redundant database for homology to sequences encoding known protein, using basic local alignment search tool (BLAST) programed at National center for Biotechnology (NCBI).

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Sequences analysis of rps4 gene observed a highest homology with 2 rps4 genes sequences with GeneBank. Fig.(10) shows alignment of our rps4 gene with GeneBank sequence of *Capparis flexuosa* rps4 gene, in which our sequence shows 98% identity with the *Capparis flexuosa* sequence where there was 569bp was identical from 582bp. The interruption in the alignment between the two sequences show the difference between individuals and the highest homology as both are from the same family. An identity of 96% is also revealed on alignment with GeneBank rps4 gene of *Nasturtium officinale*. But the homology between the the *Capparis spinosa* rps4 gene and *Nasturtium officinale* rps4 gene is less than homology between the the *Capparis spinosa* rps4 gene and *Capparis flexuosa* rps4 gene as they are from different families.

ATGTCAGGTTACCGAGGGCCTCGTTTTAAAAAATACGGC
GTCTGGGGGCTTTACCGGGACTAACTAGTAAAAAGCCTAG
AGCAGGAAGCGATCTTAGAAACCAATCACGCTCCGGAAAA
AAATCTCAATATCGTATTCGTTTAGAAGAAAAACAAAAT
TGCGCTTTCATTATGGTCTTACAGAACGCCAATTACTTAAA
TATGTTTCGTATCGCCGAAAAGCCAAAGGGTCAACGGGTC
AAGTTTTACTACAATTACTTGAAATGCGTTTAGATAACATC
CTTTTTCGATTGGGTATGGCTTTGACTATTCCTCAAGCGCG
CCAATTAGTTAACCATAGACATATTTTAGTTAATGATCGTA
TAGTAGATATACCAAGTTATCGCTGCAAACCCCAAGATATT
ATTACAGTCAAGGATGAACAAAAATCTAGAACTTTGGTTC
AAAATCTTCTTGATTCATCCGCACACCAGGAATTGCCAAAA
CATCTGACTCTTCACACATTCCAATATGAAGGATTAGTCAA
TCAAATAATAGATAGGAAATGCGCCGGTTTGAAAATAAAT
GAATTGCTTGTCGTAGAATATTACTCTCGACAGACTTAA

Fig (8): Nucleotide Sequence of the rps4 gene: The submitted complete chloroplast rps4 gene into GeneBank databases with accession number JQ665718 (<http://www.ncbi.nlm.nih.gov/Blast/>), the length of the gene was 606 bp where it contain 208 A, 117 C, 111 G, and 170 T in which G + C = 228 and A + T = 378 .

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      20              40              60
MSGYRGPRFKKIRRLGALPGLTSKKPRAGSDLRNQSRSGKKSQYRIRLEEKQKLRFHYGLTERQLLKYYRIAG
      80              100             120             140
KAKGSTGQVLLQLLEMRLDNILFRLGMALTIPQARQLVNRHILVNDRIVDIPSYRCKPQDIITVKDEQKSRT
      160             180             200
LVQNLLDSSAHQELPKHLTLHTFQYEGLVNQIIDRKCAGLKINELLVVEYYSRQT*
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Fig.(9): The translated protein and the ORF

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Capparis flexuosa ribosomal protein S4 (rps4) gene, partial cds; and rps4-tmS intergenic spacer, partial sequence; chloroplast
Sequence ID: [gb|EU002302.1|](#) Length: 585 Number of Matches: 1

Range 1: 1 to 582 [GenBank](#) [Graphics](#) [Next Match](#) [Previous Match](#)

Score	Expect	Identities	Gaps	Strand
1003 bits(543)	0.0	565/582(98%)	0/582(0%)	Plus/Plus
Query 25	TTTaaaaaaTACGGCGTCTGGGGCTTTACCGGGACTACTAGTAAAAGCTAGASCA	84		
Subject 1	TTTAAAAAATACGGCGTCTGGGGCTTTACCGGGACTACTAGTAAAAGCTAGASCA	60		
Query 85	GGAGCGAATCTTAGAACCAATCACGCTCCGaaaaaaTCTCAATATGTATTGTITA	144		
Subject 61	GGAGCGAATCTTAGAACCAATCACGCTCCGAAAAAATCTCAATATGTATTGTITA	120		
Query 145	GAAGAAAACAAAATTCGGCTTTCATTATGGTCTTACAGAGCGCAATATCTTAAMAT	204		
Subject 121	GAAGAAAACAAAATTCGGCTTTCATTATGGTCTTACAGAGCGCAATATCTTAAMAT	180		
Query 205	GTGTATTCGCGGAAAAGCCAAAGGGTCACGAGGTCAAGTTTACTCAATACTTGA	264		
Subject 181	GTGTATTCGCGGAAAAGCCAAAGGGTCACGAGGTCAAGTTTACTCAATACTTGA	240		
Query 265	ATGGTTTAGATAACATCTTTTTCGATGGGTATGGCTTTGACTATTCTCAAGGGGAC	324		
Subject 241	ATGGTTTAGATAACATCTTTTTCGATGGGTATGGCTTTGACTATTCTCAAGGGGAC	300		
Query 325	CAATTAGTTAACCATAGACATATTITAGTTAATGATGTATAGTATACCAAGTAT	384		
Subject 301	CAATTAGTTAACCATAGACATATTITAGTTAATGATGTATAGTATACCAAGTAT	360		
Query 385	CGTCGAACCCCAAGATACTATTACAGTCAAGGATGACAAAATCTAGACTTTGGTT	444		
Subject 361	CGTCGAACCCCAAGATACTATTACAGTCAAGGATGACAAAATCTAGACTTTGGTT	420		
Query 445	CAAAATCTTCTTGATTCATCCGACACAGGAAITGCAAAACATCTGACTTTCACACA	504		
Subject 421	CAAAATCTTCTTGATTCATCCGACACAGGAAITGCAAAACATCTGACTTTCACACA	480		
Query 505	TTCCATATGAGGATTAGTCRAATCAATATAGATAGGAATGCGCGGTTTGAAATA	564		
Subject 481	TTCCATATGAGGATTAGTCRAATCAATATAGATAGGAATGCGCGGTTTGAAATA	540		
Query 565	AATGAATGCTTGTCTAGAAATATATCTCTGACAGACTTAA	626		
Subject 541	AATGAATGCTTGTCTAGAAATATATCTCTGACAGACTTAA	582		

Fig. (10) : BLAST N alignment of the rps4 gene sequence of *capparis spinosa* with the gene bank sequence of rps4 gene of *Capparis flexuosa*: Query: rps4 sequence of *capparis spinosa*; Subject : rps4 gene of *capparis flexuosa*

TA cloning of PCR product to pTZ57R/T vector, selection of white recombinant plasmid and Preparation of plasmid DNA: In this step we first ligate the rps4 ORF inside the pTZ57R/T plasmids, then pTZ57R/T plasmids containing rps4 ORF were transformed into competent E.coli (XL1, blue bacteria). After culturing, the white colonies which represent the transformation were picked up and the plasmids containing rps4 were extracted from genomic DNA of bacteria using plasmid miniprep kit. The result of this cloning was detected by gel electrophoresis as shown in Fig.(11).

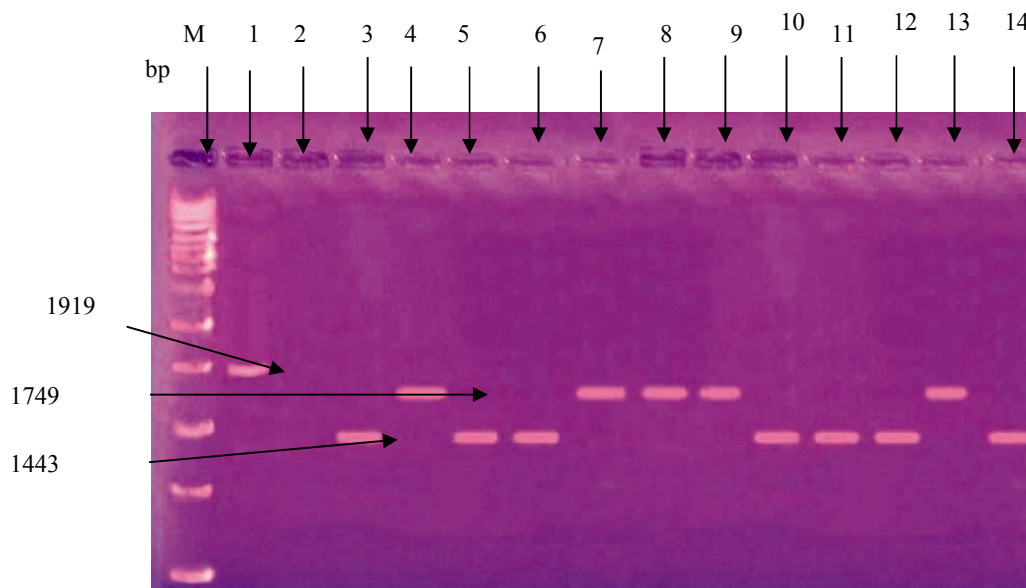


Fig.(11): Ethidium bromide 1% stained agarose gel for plasmid extracted from white colonies picked up from plate A: M: Molecular weight marker (1 Kb); Lane1: +ve control vector which was 3839 bp (1919bp); Lane 3: -ve control vector which was 2886 bp(1443bp); Lane 4,7,8,9,&13: +ve recombinants which was 3492bp (1749bp) vector with rps4; Lane 5, 6, 10, 11, 12, & 13: empty vector without rps4 gene; Lane 2: Non recombinant cell.

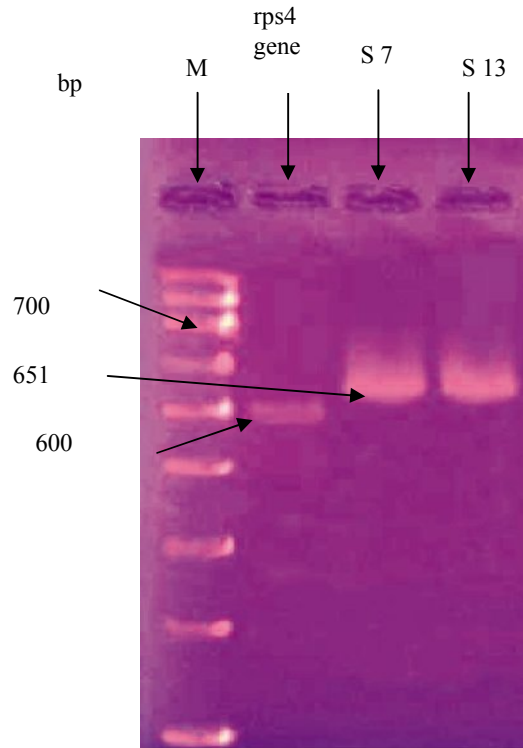


Fig.(13): Ethidium bromide 1% stained agarose gel for double digestion of pTZ57R/T plasmid: M: Molecular weight marker (100 bp); Lane 1: rps4 gene; samples 7 & 13: Linearized EcoRI- rps4- SalI

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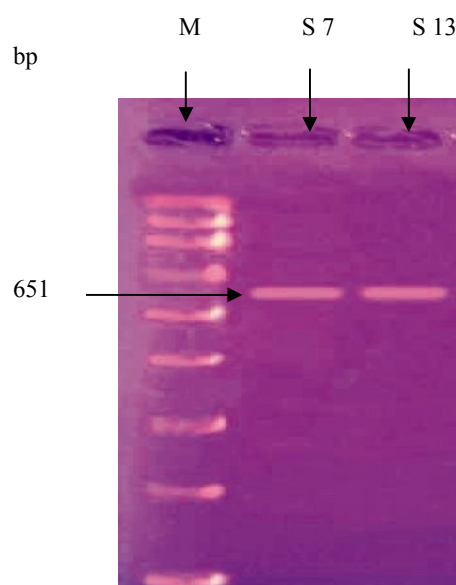


Fig.(14): Ethidium bromide 1% stained agarose gel for purification of Linearized EcoRI-rps4- SalI: *M*: Molecular weight marker(100bp); *sample7* & *13*: purified Linear EcoRI- rps4- SalI

Cloning of the insert into pGEX-4T-3 expression vector, selection of expression positive recombinant pGEX-4T-3 and detection of position of rps4 gene: The purified EcoRI-rps4- SalI clones were ligated into pGEX-4T-3 expression vector digested by the same 2 restriction enzymes, to generate pGEX-4T-3-rps4. Following transformation of E. Coli JM109 competent cells, ampicillin resistant colonies were examined by plasmid miniprep purification method to identify colonies with recombinant plasmid. The result of this cloning and miniprep purification was detected by gel electrophoresis as shown in Fig.(15). The PGEX-4T-3 plasmids containing rps4 which was prepared from a positive colonies, Firstly; were amplified with PGEX 5' sequencing primer of PGEX-4T-3 plasmid as forward primer and reverse primer of rps4 gene as a reverse primer in order to verify the presence of insert of the correct size and correct

orientation. and only the insert with correct orientation was amplified, as illustrated in fig.(16).

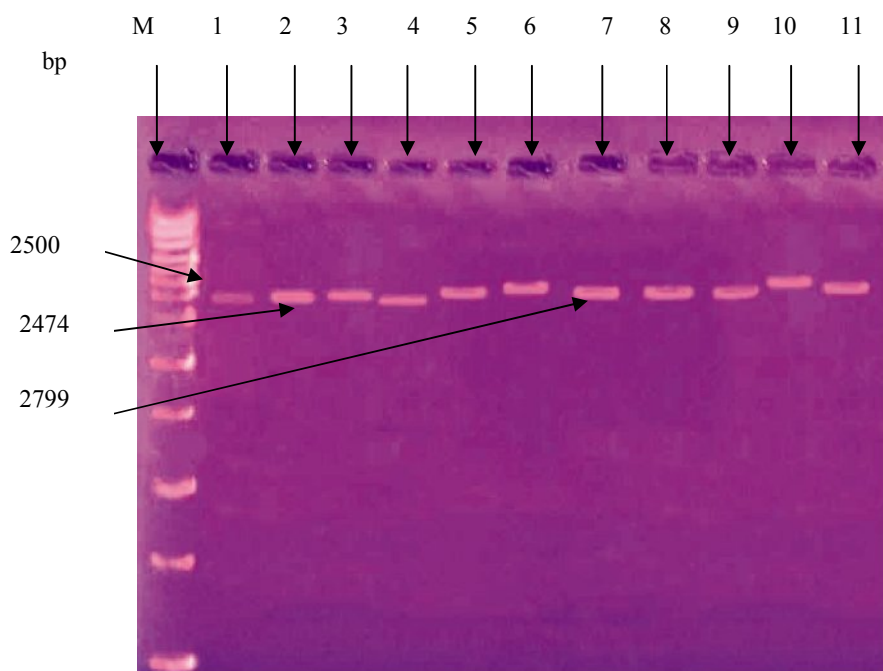


Fig.(15): Ethidium bromide 1% stained agarose gel for plasmid extracted from ampicillin resistant colonies: *M*: Molecular weight marker (1 Kb); *Lane 1*: PGEX-4T-3 which was 4948 bp (2474bp); *Lane 5, 6, & 10*: + ve recombinants which was 5599 bp (2799bp) vector with *rsp4*; *Lane 2, 3, 4, 7, 8, 9, & 11*: empty vector without *rps4* gene (2474).

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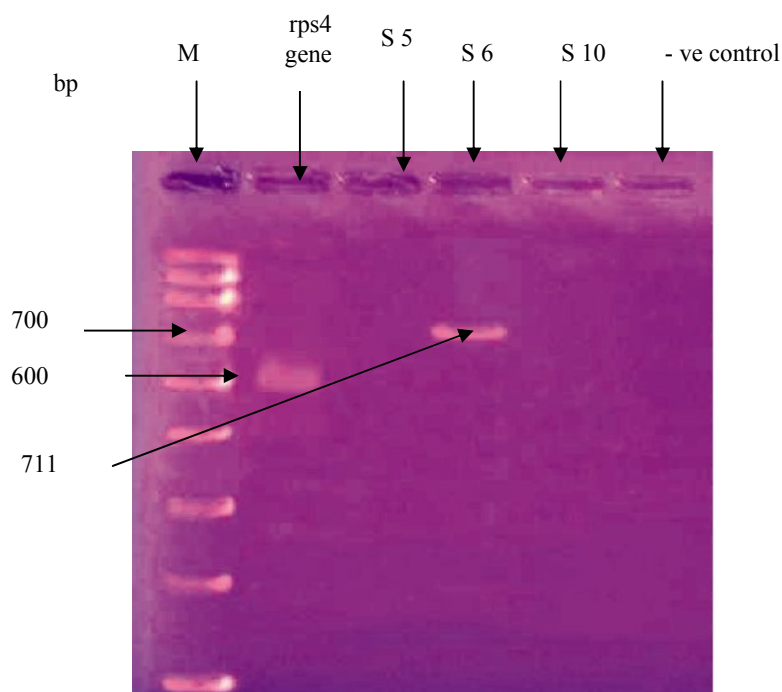


Fig.(16): Ethidium bromide 1% stained agarose gel for amplification of to EcoRI-rps4- SalI inserted in PGEX-4T-3 plasmid: *M*: Molecular weight marker (100bp); Samples 5 & 10: empty, not in correct orientation; Sample 6: PCR product of correct size and correct orientation at 711 bp; *Lane 5*:-ve control

Production of the recombinant fusion protein: A single bacterial colony contain the plasmid bearing insert (PGEX-4T-3-rps4) was grown in LB- ampicillin medium and induced with 1 mM IPTG, 1ml aliquots of the induced culture were taken at 1, 2, 3, 4, and 5 hrs. post induction. The cells were spun down, resuspended in 20 ml of 1X SDS-gel loading buffer and electrophoresed onto 10% SDS-PAGE. the induced parent PGEX-4T-3 plasmid produce a protein 26 KDa,

therefor the molecular weight of the peptide encoded by rps4 gene appear to be more than 19 KDa, as illustrated in fig.(17).

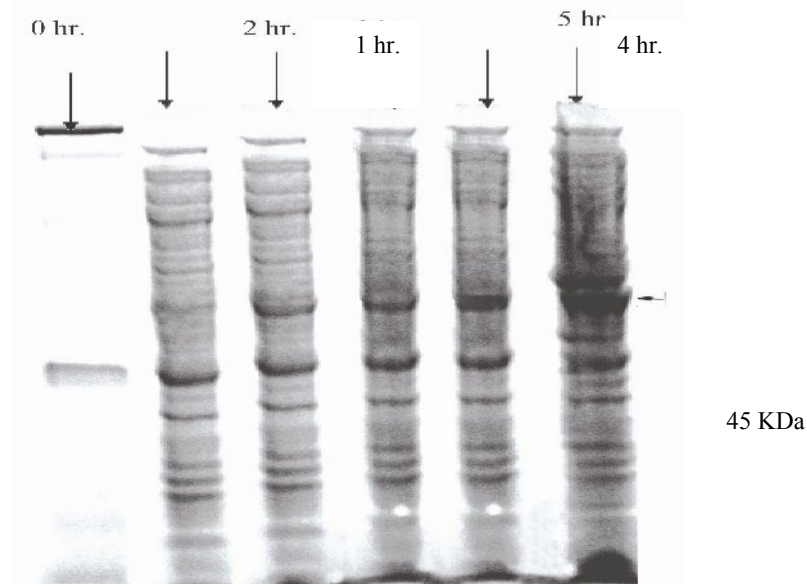


Fig.(17): SDS-PAGE for the induction of fusion protein: The figure shows the induction of protein in bacteria transformed by PGEX-4T-3-rps4 vector. The protein with molecular weight more than 45KDa referring to the expected size for a protein of 22KDa fused with GST (26 KDa). The figure shows the induction of protein in transformed bacteria at 0 hr, 1hr, 2hr 3hr, 4hr and 5 hr. The culture at 0 time of the induction was used as a negative control, where as the arrow shows the appearance of GST- rps4 fusion protein at more than 45KDa where there was a band appeared after 1hr at more than 45KDa and increased with time referring to expression of GST-rps4 fusion protein

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DISCUSSION

The present work is the first to describe the sequence of rps4 gene of *Capparis spinosa* which is the first step to determine its function. Herbal and aromatic plants are attracting more attention among plant researches because some human diseases can be treated by them. These plants contain exceptionally high amount of polysaccharids, polyphenols and other secondary metabolites which have medicinal properties, and by applying the molecular technology would increase and facilitate production of these substances [35]. However these substances that make the herbal and aromatic plants have medicinal importance, and hinder molecular approaches used with these plants because these polysaccharids and polyphenols bind firmly to the nucleic acids during DNA isolation [36]. Various molecular techniques like PCR and restriction digestion require isolation of genomic DNA of suitable purity [37].

The key point is to isolate a pure DNA from such aromatic plants. To do this successfully, we have used a famous DNA isolation kit from Qiagen. This kit was designed specifically to obtain highly pure DNA from plants as it efficiently removes polyphenols, polysaccharids, proteins and RNA contaminations as it contains CTAB which is a strong detergent used to disrupt the cell membranes (cell membrane and the nuclear membrane) to release DNA in which the detergent and the membranes molecules consists of 2 parts: hydrophilic heads and hydrophobic tails, so when the detergent comes in close to membrane, the similar structure makes them to combine and capture the lipids and protein inter in membrane structure so it disrupts the structure of membrane [37]. Moreover, the CTAB forms an insoluble complex with nucleic acid, the nucleic acid-CTAB complex is only soluble in high salt concentration and precipitation of nucleic acid, then the residual CTAB is removed by washing nucleic acid pellet with washing buffer containing ethanol where the CTAB is more soluble [38]. The CTAB method contains EDTA which chelate magnesium ions, a necessary co-factor for nucleases enzymes so prevent degradation of DNA, PVP is used to

bind and remove polyphenols, and RNase enzyme [37]. For all this the CTAB method is the most specific method for the isolation of highly pure DNA.

After some trials, we have finally succeeded to isolate total DNA "whole chloroplast genome" from *Capparis spinosa*. We have confirmed the integrity of the obtained DNA through running on gel. It gives an intact (not smeared) band at the top of the gel higher than the band of the used marker and close to the well. This result comes in agreement with [15] who reported that the general chloroplast genomes was of 120 Kb to 200 kb in higher plants and so appeared close to the wells of the gel. In consistence, some other previous studies have shown that the complete chloroplast genome of Brown seaweed is 130,584 bp [39], of *Smilax china* is 157,878 bp [40], of *Elodea canadensis* is 156,700 bp [41], of oil palm is 156,973 bp [42], and of rubber tree is 161,191 bp [43]. In addition, we did not detect any smear in the isolated DNA band in gel. This indicated that the isolated DNA was intact without any RNA contamination. In consistence [37] also reported that the clear band confirmed the quality of isolated DNA in which the smearing below the band indicated mechanical or chemical degradation and smeared band towards bottom was an indication of the presence of RNA in the extract.

The second most important step is to check the purity and the concentration of the isolated DNA. To achieve this, we measured the isolated DNA by nanodrop which revealed high purity and concentration as shown by the absorbance peak at 260 nm and 230nm. Our results are in line with [37] and [44] who reported that DNA should show a clear absorbance peak at 260 nm and measures of DNA purity could be determined by the OD260: OD280 and OD260: OD230 ratios. These ratios provided indication of protein, and polyphenol and carbohydrate contamination respectively. A pure DNA solution had an OD260: OD280 ratio of 1.8- 2 that indicated absence of protein contamination and an OD260: OD230 ratio of 1.8 that indicated absence of polysaccharids and polyphenols contaminations.

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Taken together, we successfully isolated DNA with high purity, very low contamination of polysaccharids and polyphenols, showing no smear, and did not contain RNA contamination.

The purified DNA was then used as a template to isolate the ORF of rps4 gene. We first used the web based tool, Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) to design forward and primers based on the highly similar sequences identity with published sequences of rps4 genes in other related plants. To ensure primer sequence is unique for the template sequence, we checked similarity to other known sequences with BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi). The PCR product with the expected size of 606bp was identified by gel electrophoresis indicating presence of amplified rps4 gene. This result agrees with [16] who reported that rps4 gene coding sequence has a length of 606 bp in higher plants. For further confirmation for the PCR product, we sent the purified PCR products to be sequenced in both directions (MacroGen Company, South Korea) using ABI 3730XL DNA sequencer (Applied Biosystem, USA). Before being sent for sequencing, the PCR was purified to get rid of the primer dimmers, primers, nucleotides, proteins, salt, agarose, ethidium bromide and other impurities. After purification, the electrophoresis showed a clear 606 bp band without primer dimmers, or other impurities.

The result of sequencing was compared for homology to DNA deposite in non redundant database using basic local alignment search tool (BLAST) programed at the National center for Biotechnology (NCBI). Sequence analysis showed a high homolgy between rps4 gene of *Capparis spinosa* and *Capparis flexuosa* and also with *Nasturtium officinale*. Alignment data showed that it was given maximum identity which is 98% with *Capparis flexuosa* rps4 gene and then 96% with *Nasturtium officinale*.

DNA Fragments produced through amplification or through restriction enzyme digestion were separated by using electrophoresis. Agrose gel acted as a selective filter so that DNA molecules having different molecular sizes were separated into specific bands as they migrated to positive anode where DNA was negatively charged. Because DNA molecules have a uniform charge and mass ratio, they

exhibit similar electrophoretic migration properties in resistance to free medium. The migrated DNA was visualized under UV light with the help of an intercalating dye, ethidium bromide, which fluoresced when irradiated with UV. The size of the fragment estimated by comparing unknown DNA molecule with DNA molecules for which the sizes are known and was migrated with it. The migration of DNA depended upon the molecular size of the DNA, the agarose concentration, the conformation of the DNA, and the applied current [37].

In the present study, PTZ57R/T & PGEX-4T-3 vectors were used for cloning and expression of the *rps4* protein in *E. coli*. The Gram-negative bacterium *E. coli* remains one of the most attractive expression system because of its ability to grow rapidly and high density on inexpensive substrates, its well characterized genetics and availability of an increasingly large number of cloning vectors and mutant strains [45]. Although bacterial expression system lack most posttranslational processing mechanisms found in eukaryotic cells, their expression level is usually in the order of magnitude higher than eukaryotic expression systems, especially mammalian cell systems. This constitutes a big advantage for purification and cost reduction [46]. *E. coli* have been widely used in the cloning and expression of fusion proteins from different ligated genes [47].

As when primer was prepared restriction sites at both ends were not added, so there should be restriction sites at both ends of *rps4* gene in order to ligate it into expression vector.

So that to overcome this problem PTZ57R/T vector was used in which this vector had single 3'-ddT overhangs and the PCR product had single 3'-A overhang due to Taq DNA polymerase had terminal transferase activity and such enzyme added a single 3'-A overhang to both ends of the PCR product. Therefore, such overhang facilitate cloning [32].

This vector also had an important characteristic to facilitate the selection of recombinant plasmid transformed into *E. coli* in which recombinant clones are selected based on blue/white screening. The incorporation of the *rps4* insert into the plasmid vector in one of the unique sites of the plasmid's multiple cloning located in the gene

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encoding the lacZ α - peptide of the vector results in an interruption of the gene and consequently upon induction, produces a non-functional gene product. This result in an easy screening system which utilizes the IPTG as lacZ inducer and X-gal as β - galactosidase substrate producing a blue color on digestion. Therefore the colonies that contain non-recombinant (-ve) plasmids would have non-interrupted lacZ α -peptide which, by complementing the product of the host cell lacZ Δ M15 gene on an F' episome, leads to production of functional β - galactosidase and have a blue color. In the bacterial colonies that contain the recombinant (+ve) plasmid and cosequently a disrupted lacZ α -peptide, no complementation occurs and an inactive β -galactosidase is produced (white color) [48].

The E.coli which had white color meant +ve recombinant plasmid, so that the plasmid purification occurred from these bacterial cells. The +ve recombinant plasmid with the expected size of 3492bp was identified by gel electrophoresis to indicate their presence. As the size of empty vector was 2886bp and the insert rps4 gene was 606bp so the size of +ve recombinant plasmid was 3492bp and due to the vector was colied the band of the +ve recombinant plasmid appeared between 1500bp and 2000bp, which meant the presence of +ve recombinant plasmid. But if the transformation not occurred the band appeared near 1500bp as the empty vector was 3492bp.

The purified +ve recombinant plasmid was checked for accurate insertion by using PCR. The amplication occured by using one primer from vector which was M13/PUC sequencing primer as forward primer and reverse primer of rps4 gene as a reverse primer. If the gene was inserted in the correct orientation, the electrophoresis would show a band between 600bp and 700bp as the expected size of the amplified fragment was 660bp due the the amplification included 17-mer of M13/PUC sequence which was also 37bp upstream to rps4 and the 606bp of rps4 gene so the size would be 660bp and the presence of the band between 600bp and 700bp indicated the correct orientation and the correct size of inserted rps4 gene. But If the gene was not in correct orientation, it would not be amplified. Knowing the orientation of rps4 gene is an important step in expression of the

rps4 gene as any change in the orientation will make change in ORF of the protein. Therefore, it will alter the structure of produced protein or prevent the expression of protein.

After the determination of accurate orientation of rps4 gene, the gene inserted into the vector was cut by restriction enzymes in order to produce a gene with restriction sites at both ends, so it could be easily ligated into the expression vector. The digestion of the +ve recombinant plasmid occurred by using EcoRI and SalI restriction enzymes in which the EcoRI restriction site presented 30bp upstream to the insert and SalI restriction site presented 15bp downstream to the insert, to excise the full length insert which was 606bp with about 45 extra nucleotides, so that the expected size of the linear EcoRI- rps4- SalI fragment was 651bp. The linear EcoRI- rps4- SalI fragment with the expected size of 651bp was identified by gel electrophoresis indicating presence of linear EcoRI- rps4- SalI fragment at a band between 600bp and 700bp and this came in agreement with the expected size. Before being ligated linear EcoRI- rps4- SalI fragment into the expression vector, the linear EcoRI- rps4- SalI fragment was purified to get rid of nucleotides, salt, enzymes, vector and any other impurities. After purification, the electrophoresis showed a clear band between the 600bp and 700bp which confirmed the expected size of 651bp.

All the previous steps were done in order to produce the gene in correct orientation and with restriction sites at both ends in order to be ligated into the expression vector. To produce rps4 protein encoding by rps4 gene, the rps4 gene was expressed in E. Coli by using the expression vector PGEX-4T-3 producing the recombinant protein fused to glutathione-S- transferase (GST) [49].

The ligation of linear EcoRI- rps4- SalI fragment into PGEX-4T-3 expression vector occurred at EcoRI and SalI restriction sites of PGEX-4T-3 which was cut by the same restriction enzymes. The E.coli (JM109) were transformed with this recombinant plasmid were selected on LB media containing ampicillin (100µg/ml).

The E.coli which had white color meant +ve recombinant plasmid, so that the plasmid purification occurred from these bacterial cells. The +ve recombinant plasmid with the expected size of 5635bp was

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identified by gel electrophoresis to indicate their presence. As the size of empty vector was 4984bp and the inserted linear EcoRI- rps4-SaII fragment was 651bp, so the size of +ve recombinant plasmid was 5635bp and due to the vector was colied the band of the +ve recombinant plasmid appeared near 3000bp which meant the presence of +ve recombinant plasmid. But if the transformation not occurred the band appeared between 2000bp and 3000bp as the empty vector was 4984bp.

The purified +ve recombinant plasmid was checked for accurate insertion by using PCR. The amplification occurred by using one primer from vector which was PGEX 5' sequencing primer of PGEX-4T-3 plasmid as forward primer and reverse primer of rps4 gene as a reverse primer. If the gene was inserted in the correct orientation, the electrophoresis showed a band above 700bp as the expected size of the amplified fragment was 712bp due to the amplification included 22-mer of PGEX 5' sequencing primer which was also 47bp upstream to inserted EcoRI- rps4 fragment and inserted EcoRI- rps4 fragment was 643bp, so the size would be 712bp and the presence of band above 700bp indicated the correct orientation and correct size of inserted rps4 gene. But If the gene was not in the correct orientation, it would not be amplified. Knowing the orientation of rps4 gene is an important step in expression of the rps4 gene as any change in the orientation will make change in ORF of the protein. Therefore, it will alter the structure of produced protein or prevent the expression of protein.

All these steps were done to prepare + ve recombinant plasmid containing rps4 gene at the correct orientation. Therefore, it could be used for the expression of protein. The E.coli were transformed with this recombinant plasmid were selected on LB- broth media containing ampicillin to produce GST-rps4 fusion protein. The SDS-PAGE showed the production of this fusion protein from zero time of adding IPTG till the maximum production at 5 hrs. E.coli transformed with this recombinant plasmid PGEX-4T-3-rps4 and induced with IPTG expressed a protein with 48KDa, as the molecular weight of GST protein 26KDa and the molecular weight of rps4 protein was 22 KDa. So this result comes in accordance with

expected molecular weight for the fusion protein containing GST and rps4 protein.

Conclusion

It was concluded that the present work is the first to isolate and identify the complete coding sequence (also known as open reading frame or ORF) of *Capparis spinosa* *rps4* gene. We applied an advanced molecular approach to isolate and identify the complete coding sequence of *rps4* gene using PCR technique. Subsequently, this ORF was cloned to expression vector carrying a suitable promoter and then transformed into bacteria to detect the rps4 protein using PAGE. Isolation and identification of rps4 gene and protein is the first step to determine its function. Therefore, this study provides researchers with basic and raw information about rps4 gene and protein which together with further experiments (especially, western blotting and gain of function) can give more details about the actual role played by this molecule.

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