Characterization of chloroplast gene encoding ribosomal protein S4 and trnV/rm16s spacer gene in *Capparis spinosa* L.

Mai A. Abdallah[1], Faten Z. Mohamed[1], Hala. EL- Atroush[3] and Magdy M. Mohamed*[2]

[1]Biochemistry Department, Faculty of Science, Zagazig University, Egypt
[2] Biochemistry Department, Faculty of Science, Ain-Shams University, Egypt
[3] Botany Department, Faculty of Science, Ain Shams University, Egypt
Hala_elatroush@hotmail.com

Abstract: *Capparis spinosa* is a medical threatened plant species growing in Egyptian desert. It's one of the most important species in family Capparidaceae, which established pharmaceutical and medicinal properties. A complete coding sequence of 606bp chloroplast ribosomal protein 4s (rps4) gene of *C. spinosa* was amplified, sequenced, cloned, and expressed. The isolated gene showed a maximum identity with *C. flexuosa* ribosomal protein S4 (rps4; 98%) gene and 96% with *Nasturtium officinale* ribosomal protein S4 (rps4) gene. The information of ORF of this gene was found to be a new gene encodes an 202 amino acids protein fragment with a calculated molecular mass of about 19 kDa. *C. spinosa* trnV/rm16s spacer is noncoding cpDNA region and highly predictive that might offer a phylogenetic information for comparison between different noncoding cpDNA regions due to its highly conservative region among uncoding genes. Sequence analysis of trnV/ rrn16s spacer observed a 100% identity with 12 plant sequences in GenBank of different phylogenic loci. This data might be useful for determine the localization of *C. spinosa* among Capers species.
[Mai A. Abdallah, Faten Z. Mohamed, Hala. EL- Atroush and Magdy M. Mohamed. **Characterization of chloroplast gene encoding ribosomal protein S4 and trnV/rm16s spacer gene in *Capparis spinosa* L.** *Life Sci J* 2015;12(7):163-173]. (ISSN:1097-8135). http://www.lifesciencesite.com. 17

Key words: *C. spinosa*, rps4 gene, *C. flexuosa, Nasturtium officinale*

1. Introduction:-

Capparis spinosa L. (Caper) (Family Capparidaceae) is one of the most common aromatic plants growing in wild in the dry regions around the west or central Asia and the Mediterranean basin **(Sultan and Celik, 2009)**.

C. spinosa has a wide distribution in the old world from South Europe, North and East Africa, Madagascar, Southwest and Central Asia to Australia and Oceania **(Jacob, 1965 and Fici, 2004)**.

It was established as a plant that have highly diverse economic and medicinal value in different systems of medicine like in Iranian, Unani, Chinese, Ayurvedic and Greco-Arabi System of medicines **(Sher and Alyemeni, 2010 and Tlili *et al.*, 2011)**. The aqueous extract of total areal parts of *C. spinosa*is have: Anti-inflammatory **(Panico *et al.*, 2005)**, antiallergic **(Trombetta *et al.*, 2005)**, anti-diabetic **(Eddouks *et al.*, 2004)**, antifungal activity **(Shtayeh and Abu Ghdeib 1999)**, antihypolipidemic activity **(Eddouks *et al.*, 2005)**, the genotoxic and antimutagenic effects of flower buds extract on *Allium cepa* root tips **(Sultan and Celik 2009)**, and antihepatotoxic **(Gadgoli and Mishra 1999)**.

Plant cells are characterized by the presence of main three genomes, nuclear, chloroplast and mitochondrial, each of them is characterized by a different rate of evolution **(Wolf *et al.*, 1987)**, however, chloroplasts have their own DNA molecule **(Lopez-juez and Pyke, 2005)**. Chloroplast genomes typically comprise a single circular molecule (although there are usually multiple copies per chloroplast) of 120–200 kb in photosynthetic organisms **(Koumandou *et al.*, 2004)**. Several ribosomal proteins in chloroplast have been reported to possess other functions aside from the traditional ribosomal function including induction of apoptosis **(Naora *et al.*, 1998)**, suppression of tumors **(Ziemiecki *et al.*, 1990)**, regulation of development **(Lijsebettens *et al.*, 1994)**, and DNA repair **(Green *et al.*, 2005)**. The genes coding for these ribosomal proteins are coordinately regulated in order to accommodate the different cell requirements for protein synthesis **(Wool *et al.*, 1990)**. Ribosomal protein S4 plays a central role in the biogenesis of the small ribosomal subunit. It regulates its own translation and that of other ribosomal proteins **(Naora *et al.*, 1998)**.

The rps4 gene which encoding for small subunit of chloroplast ribosomal protein S4 with a gene coding sequence of 606 bp in higher plants **(Nadot *et al.*, 1994)**. 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 nuclear trnL-F, rbcL, and ITS as well as chloroplast rps4, and 18S, in which the rps4 gene is more suitable one among five markers **(Stech and Quandt, 2010)**.

Few S4 genes have been cloned from dicotyledonous and monocotyledonous plants **(Turley *et al.*, 1995)**. However little is known about the structure, sub-cellular transportation and function of plant S4 proteins **(Gao *et al.*, 1994)**. 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.

The present study concerns about the biological activities of rps4 chloroplast protein in caper plant as a useful tool for phylogeny and medicinal properties as well as nuclear ITS gene for phylogeny characterization. We reported here cloning of rps-4 complete gene and expressed its corresponding protein for the first time of this plant. Characterization of chloroplast-ribosome s4 gene and deduced amino acids were carried out together with the previously reported results.

2. Material and methods:

Plant material:

C. spinosa leaves were collected from different localities from Abou Galoum Protected area from southern Sinai Egypt. Fresh samples were frozen quickly in liquid nitrogen 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 **(Doyal and Doyal, 1987)**. The DNA was eluted in 100mM Tris-buffer (PH 8.5). The quality and concentration of the extracted DNA was checked by 1% agarose gel electrophoresis and nanodrop spectrophotometer **(Sambrook *et al.*, 1989)**.

Amplification of rps4 gene:-

The purified DNA was used as a template to amplify the ORF of *rps4* gene using Primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) to design forward and reverse primers based on the highly similar sequences identity with published sequences of rps4 genes in other related plants. The exclusivity of primer sequence was checked with other similar known sequences with BLAST (www.ncbi.nlm.nih.gov/blast /Blast. cgi). The chloroplast rps4 gene and nuclear ITS gene were amplified in a 50 µl final volume using the standard PCR **(Saiki *et al.*, 1988)** amplification technique with Hot Star Taq Plus Master Mix (Qiagen; Hilden, Germany). An initial denaturation step for 5 min at 95°C, followed by 30 cycles (30 s 94°C, 30 s 58°C, 60 s 72°C) for both genes and a final step 10 min of extension time at 72°C. Sequence of forward primer for rps4 is 5"ATGTCAGGTTACCGAGGGCC3" where reverse primer is 5"TATTACTCTCGACAGACTTAA3" and 5"AGTTCGAGCCTGATTATCCC3" where reverse primer is 5"GCATGCCGCCAGCGTTCATC3" for ITS gene. Amplified of PCR products were purified from the agarose gel with QIA quick PCR extraction kits (Qiagen; Hilden, Germany) following the manufacturers protocol. The PCR product was finally reconstituted in 100mM Tris-buffer (PH 8.5). The size and quality and concentration of the PCR products were checked using 1% agarose gel electrophoresis.

Cloning of PCR product to pTZ57R/T vector, and Restriction enzyme digestion:

The rps4 gene was ligated into PTZ57R/T vector, then transfect *E.coli* (XL1-blue) with recombinant plasmid by using InsTA™ PCR cloning kit (Fermentas, #K1214; Rand 1996). Positive colonies were selected from LB- ampicillin X-Gal/ IPTG agar plates according to **(Sambrook *et al.*, 1989)**. Recombinant plasmids were purified by Genejet plasmid miniprep kit (Fermentas, #K0502; **Birnboim and Doly, 1979)**. Confirmation of insert orientation and size in recombinant plasmid were done by amplification of rps4 gene by using universal M13/PUC primer as a forward primer and rps4 gene as reverse primer.

Sequencing of the PCR product and Alignment and Analysis of sequence data:

Purified PCR product of both genes were directly sequenced in both directions (MacroGen Company, South Korea) using ABI 3730XL DNA sequencer (Applied Biosystem, USA). Sequencing primers were those used for InsTA™ PCR amplification. Homology of purified PCR product were conducted on sequences of NCBI database **(Altschul *et al.*, 1990)**. The basic information is to take a query sequence and compare it consequently with all known sequence in a database (**Ausubel *et al.*, 1995**). The alignment was conducted in non-redundant database using basic local alignment search tool (BLAST) programed at National centre for Biotechnology (NCBI; http://www.ncbi.nlm. nih.gov/Blast/).

Cloning of rps4 fragment into pGEX-4T-3 expression vector and:

The correct oriented positive recombinant plasmid of rps4 gene was double digested with *EcoRI* and *SaII* (promege) to isolate the *EcoRI*- rps4-*SaII* fragment in purified form. This fragment was cloned into PGEX-4T-3 expression vector, transfect *E.coli* (JM109) and plated on LB media containing ampicillin (100µg/ml). White colonies were selected and the recombinant plasmid was purified from by using Genejet plasmid miniprep kit (Fermentas, #K0502; **Birnboim and Doly, 1979**), and amplified by using PGEX 5' universal sequencing primer of PGEX-4T-3 plasmid as forward primer and rps4 gene reverse primer in order to verify the presence of insert in correct orientation.

Production of the recombinant fusion protein according to (Li *et al.*, 2009):

A bacterial colony with positive in-frame 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 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 20ml of 1X SDS-gel loading buffer and electrophoresed onto 12% SDS-PAGE.

3. Results:

Isolation and amplification of rps4 gene:

The extracted chloroplast DNA from leaves of *Capparis spinosa* was used as a template for PCR to amplify rps4 gene using specific primers. Agarose gel electrophoresis revealed the presence of about 600 bp PCR products, as illustrated in Fig. (1). PCR product was purified to be used as a template for cloning into TA cloning vector.

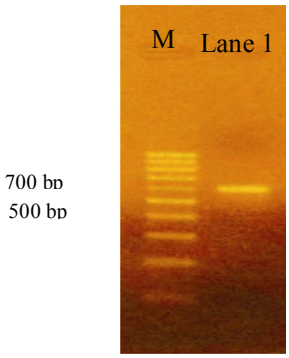

Fig (1): Electrophoresis of purified PCR product of rps4 gene: Ethiduim bromide stained agarose gel of purified rps4 gene: 1% agraose gel showing a band of 600 bp, *M: marker* (1kb ladder); *Lane 1*: purified PCR products.

Cloning of PCR product into pTZ57R/T vector:

The *rps4* gene was cloned into pTZ57R/T plasmid vector, then transfected into competent *E.coli* (XL1, blue bacteria). White colonies which contain the recombinant vector were picked up and the extracted plasmids were amplification using M13/PUC universal primer of pTZ57R/T plasmid as forward primer and rps4 gene as a reverse primer to verify the correct insert size of about 660bp including the flanking regions as illustrated in fig.(2).

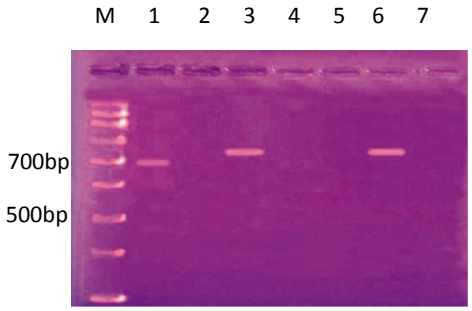

Fig.(2): Ethidium bromide 1% stained agarose gel for amplification of rps4 gene in pTZ57R/T plasmid: *M*: Molecular weight marker (100 bp); *Lane 1*: rps4 gene; *lanes* 2, 4, and 5 are incorrect orientation so they were not amplified; lanes 3 and 6:are PCR product of correct size and correct orientation; *Lane 7*:-ve control

Fig. (3): BLAST N alignment of the rps4 gene sequence of *C. spinosa* (*Query)* with and *C. flexuosa (Subject).*

Sequencing of the PCR product, alignment and analysis of data:

Sequencing of the purified PCR fragment in both directions revealed an ORF with 606bp which submitted to GenBank databases (accession number JQ665718; http://www.ncbi.nlm.nih.gov/Blast/). The nucleotide sequence of *Capparis spinosa* rps4 gene was aligned in non- redundant database. Alignment analysis showed a 98% homology with the same family *Capparis flexuosa* rps4 genes (Fig. 3), where there was 569bp identical from 582bp. However, alignment identity shows 96% with rps4 gene of *N. officinale* which located in a different family. The deduced amino acid sequence of rps4 gene proved that it is a complete protein with 202 amino acid and molecular weight of about 20KDa.

Restriction digestion and purification of rps4 gene:

The pTZ57R/T recombinant plasmid containing *rps4* was double digested with *EcoRI* and *SaII* restriction endonucleases to ligate with expression vector, as seen in fig.(4).

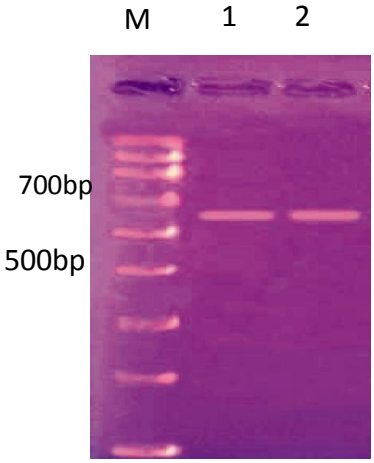

Fig.(4): Ethiduim bromide 1% stained agarose gel for purification of Linearized *EcoRI*-rps4- *SaII*: lane 1 is M: Molecular weight marker (100bp);lane2 purified PCR product and lane 3 purified Linear *EcoRI*- rps4- *SaII*

Cloning of insert into pGEX-4T-3 expression vector:

The purified *EcoRI*–rps4- *SaII* fragment was ligated with pGEX-4T-3 double digested expression vector to generate pGEX-4T-3–rps4. Positive colonies were picked up and checked for *EcoRI*–rps4- *SaII* fragment by preparation of plasmid miniprep and PCR using PGEX 5' sequencing primer of PGEX-4T-3 plasmid as forward primer and rps4 gene as a reverse primer in order to verify the presence of insert of the correct size and orientation. The only insert with correct orientation was amplified as illustrated in fig.(5).

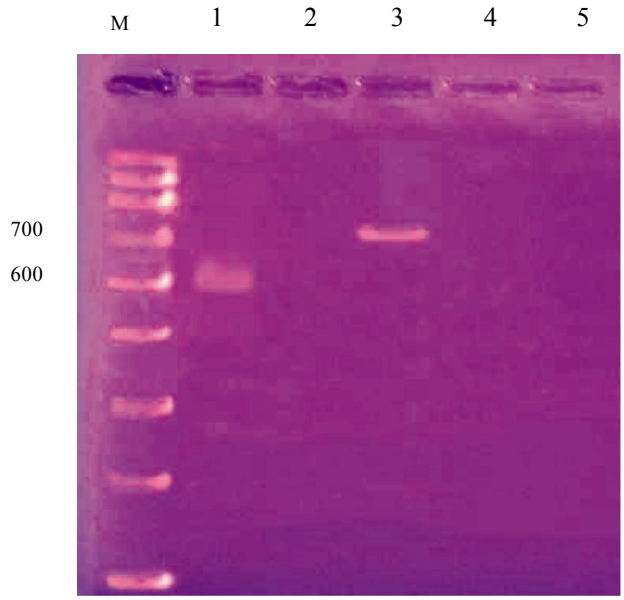

Fig.(5): 1% stained agarose gel electrophoresis for amplified *EcoRI*–rps4- *SaII* fragment in PGEX-4T-3 plasmid: *M*: Molecular weight marker (100bp); lane 1 is rps4 gene as positive control; lanes 2 and 4: plasmids in an incorrect orientation; lane 3 PCR product of correct size and orientation at 711 bp; *Lane 5*: negative 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 1mM IPTG. One 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. 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.(6).

Amplification, Purification, sequencing and alignment of trnV/ rrn16s spacer:

The extracted DNA was used as a template for PCR to amplify trnV/ rrn16s spacer. Agarose gel electrophoresis showed the presence of PCR products band at about 300 bp as illustrated in Fig.(7) which was purified for sequencing using the same PCR primers. The nucleotide sequence of the purified trnV/ rrn16s spacer of *C. spinosa* in both directions shows a fragment of 301 bp. Sequence analysis of trnV/ rrn16s spacer observed a 100% identity with 12 chloroplast plant sequences in GeneBank of different phylogenic loci. Alignment of trnV/ rrn16s spacer *C. spinosa* gene

with GeneBank sequence of *Pachycladon enysi* (Sequence ID: gb|JX205495.1|Length: 154896 from position 100492 to 100792).

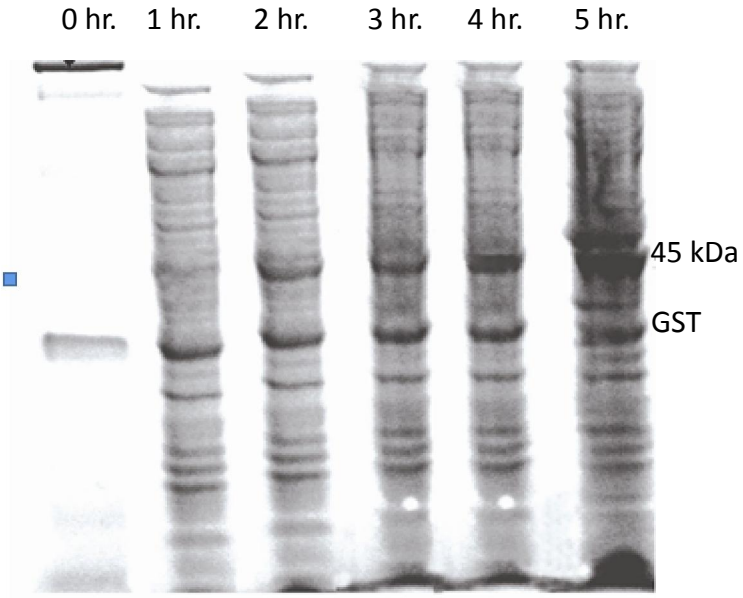

Fig.(6): SDS-PAGE for induction of fusion protein: The induction of fusion protein in bacteria transformed by PGEX-4T-3-rps4 vector. at 0 hr, 1hr, 2hr 3hr, 4hr and 5 hr. The recombinant protein (GST- rps4) with molecular weight about 48KDa referring to the expected size for a protein of 22KDa fused with GST 26 KDa.

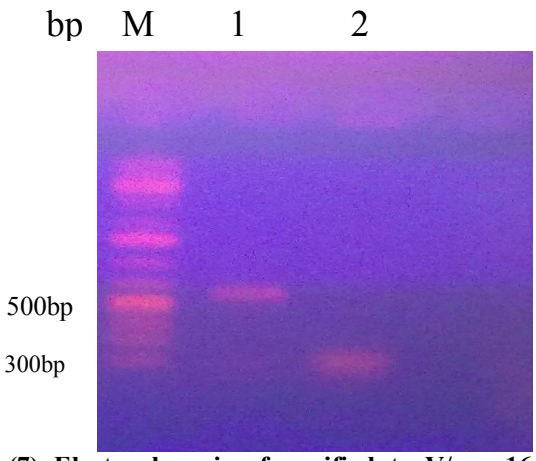

Fig (7) Electrophoresis of purified trnV/ rrn16s spacer. Ethiduim bromide stained 1% agarose gel of purified trnV/ rrn16s spacer showing a band of 300 bp, (lane 1); Lane2, purified rps4 PCR product as a control and M: marker (1 kb ladder),

4. Discussion:

Green plant cells contain three distinct protein-synthesizing systems (ribosome); one located in the cytoplasm that is very similar to the cytosolic ribosomes of other eukaryotes, one located in mitochondria whose properties are not well defined, and one located in the chloroplast that is remarkably similar to ribosomes found in eubacteria **(Boynton and Doly, 1979)**. The similarity of the chloroplast and eubacterial ribosomes extends to the nucleotide sequences of the rRNAs **(Edwards and Kossel, 1981)** and to the amino acid sequences of many of the ribosomal proteins **(Phua *et al.*, 1989)**. The chloroplasts carry most of their own translational system of ribosomes, tRNAs, and accessory factors in order to synthesize the limited number of proteins which are encoded in the chloroplast DNA. Chloroplast ribosomes are typically more abundant than mitochondrial ribosome; also it has been known that the products of the chloroplast translational system amount to over 50% of the soluble proteins in the leaf cell **(Edwards and Kossel, 1981)**. However, about one third of chloroplast ribosomal proteins are encoded in the chloroplast genome **(Shinozaki *et al.*, 1986)**. The remaining two-thirds are apparently encoded in the nuclear genome. The number of proteins encoded in the chloroplast DNA, -90 different polypeptides encoded by the organelle's own small genome **(Schmidt *et al.*, 1993)**, which is quite small compared to the total loading capacity of the plant cell. Thus, the protein synthetic activity of the chloroplast ribosome is of considerable importance to the biosphere.

Recent molecular genetic studies have revealed much about the genomic ribosomal proteins. In contrast, there is little information about the expression of genes coding for chloroplast

housekeeping proteins like ribosomal proteins (RPs). Functions of the PSRPs as active participants in translational regulation and the key feature of chloroplast protein synthesis. Some of these genes have now been cloned and characterized, including several novel protein components whose homologues are not present in the eubacterial, or *E. coli* ribosome indicating a complex evolution of some land plants chloroplast translational apparatus **(Schmidt *et al.*, 1993)**. One of those proteins who have a different function in many plants is RPS4 protein which regulates rRNA expression levels by acting as a general transcription antiterminator **(Billur *et al.*, 2009)**. The present work is the first study to describe the sequence of rps4 gene of *C. spinosa* in order to characterize its function and role in the plant cells.

Isolation of whole chloroplast genome from *C. spinosa* was characterized with high molecular weight which comes to about 120 Kb to 200 Kb in most higher plants **(Liu *et al.*, 2012 and Wang *et al.*, 2013)**. In addition to the conservative range of chloroplast genome size, there is also a relatively high degree of conservation in structure, gene content, and linear order of the genes **(Downie and Palmer, 1992)**.

The amplified fragment of *C. spinosa* rps4 gene size was about 600 bp which is within the expected size of other identified chloroplast rps4 gene and is in an agreement with previous published data **(Nadot *et al.*, 1994)**. They studied the phylogenetic analyses by sequencing the chloroplast rps4 gene of 72 species as representative of two different taxonomic levels of monocot species in addition to other 6 species located in databank. They confirmed that, rps4 gene were in different fragment sizes ranging from 470bp to 948bp. **Randolph *et al.* (1995)** cloned and sequenced the ribosomal rps4 gene from the chloroplast genome of *Chlamydomonas reinhardtii.* They found that the its size is 771bp that is considerably longer (by 153-177 bp) than rps4 gene of other photosynthetic species and *E. coli*, due to the presence of two internal insertions and a C-terminal extension. Gassmann *et al.,* 1999 also cloned the Arabidopsis species rps4 gene in 474bp. Warner *et al.* (2004) sequenced chloroplast rps4 gene of 72 taxa available and after exclusion of primers and spacers, the aligned sequences had a length of 588bp corresponding to the coding region which is less by 18 bp than our rps4 chloroplast gene.

Purified 606 bp PCR product was sequenced in both directions which showed a highest homology between *rps4* gene of *C. spinosa* compare with both *C. flexuosa* (98%) and (96%) with *Nasturtium officinale*.

Alignment sequence analysis of rps4 gene among *C. spinosa* and *C. flexuosa* revealed that, our rps4 gene is first identified in this species with a complete coding sequence that exceeds 24 bases at the 5` end of *C. flexuosa* gene which missed its starting codon, while *Nasturtium officinale* sequence represents the first starting coding region. This means that, our gene is complete gene specially when ending with stop codon TAA. There are 13 different mutations between *C. Spinosa* and *C. flexuosa* rps4 gene, most of them are transition as shown in the table (1), where G<→A type represents 7/13; 54% followed by C <→T type which represents 3/13; 23% of total base substitution.

Table (1) mutational analysis between *C. spinosa* and *C. flxuosa* rps4 chloroplast gene

No	position	base change	No	position	base change
1	39	G to C	8	369	A to G
2	74	A to G	9	414	C to G
3	165	C to T	10	501 - 454	C to T
4	237	G to A	11	468	A to C
5	252	A to G	12	551	C to T
6	273	A to G	13	660	G to A
7	359	A to G			

Sequence alignment of *C. spinosa* and *N. officinale* gene shows that our 606 bp is matched with rps4 gene on the chloroplast genome which starts with ATG as starting codon at position 45507 and end at stop codon TAA at position 44902. There are 22 different mutations were identified, some of them are base substitutions identified previously in *C. flexuosa* as represented in the table (2).

It is clear that, 3` region is stable in the three sequences where 5` end specially in the region 465 to 473 containing 5 mutants out of eight bases in comparison to rps4 gene of the *N. officinale*.

The size of *EcoRI* rps4 *SaII* fragment digested with *EcoRI* and *SaII* restriction enzymes is 651bp, in which the *EcoRI* restriction site located 30bp upstream to the insert and *SaII* restriction site located 15bp downstream to the 606bp insert size which explain fragment band between 600bp and 700bp. To express rps4 protein, the rps4 gene was ligated into the expression vector PGEX-4T-3 producing the recombinant protein fused protein with glutathione-S-

transferase (GST) as described previously (Smith and Johnson 1998). The purified +ve recombinant plasmid was checked by PCR using PGEX 5' of PGEX-4T-3 plasmid as forward primer and rps4 as a reverse primer. If the gene was inserted in the correct orientation, it showed a band above 700bp where the amplification included 22-mer of PGEX 5' sequencing primer which was 47bp upstream to inserted *EcoRI*-rps4 fragment. The SDS-PAGE showed the production of fusion protein from zero time of adding IPTG up to 5 hrs. of 1 hr. intervals. The expressed protein was 48KDa, as the molecular weight of GST protein is 26KDa, therefore the molecular weight of rps4 protein was about 22 KDa which is in agreement with calculated molecular weight of rps4 protein.

Table (2) mutational analysis between *C. spinosa* and *N. officinale* rps4 chloroplast gene

No	position	Base change	No	position	Base change
1	7	G to C	12	414	C to G
2	39	G to C	13	429	A to C
3	74	A to G	14	441	G to A
4	80	G to A	15	465	C to T
5	165	C to T	16	468	+A
6	237	G to A	17	470	A to C
7	303	T to G	18	444 - 472	C to T
8	340	A to G	19	473	-G
9	359	A to G	20	486	A to C
10	369	A to T	21	519	A to T
11	397	A to G	22	551	C to T

A search of the NBRF, GenBank, and EMBL protein data bases reveal a high homology between *C. spinosa* rps4 protein with large number of protein that carry one or more conserved domains of rps4 protein in GenBank. Therefore, the 606bp with an ORF encoding a putative 202 amino acid residues expressed as matured plastid ribosomal protein type s4 (PRPS4) with 22kDa being a basic positively charged protein due to presence of many basic amino acids as leusine, and argentine, and hydrophobic proline-rich motifs as well conserved c-terminal regions with other chloroplast ribosomal protein which is consistence with **Yamaguch *et al.*, (2003)**. Comprehensive studies interested in completing a proteome analysis and protein identification were classified the six different chloroplast-ribosomal 30S subunit proteins of higher plants (designated PRPS1 to PSRP-6) into two groups according to the net charge on protein; an acidic proteins group (PRPS1, PRPS2 and PRPS3) and a small/basic proteins group (PRPS4, PRPS5 and PRP6). They also divided these chloroplast ribosomal proteins into two groups in terms of their post-translational modifications; where, PRPS1, PRPS2, PRPS4 and PRPS6 were activated without any post-translational modification (other than transit peptide cleavage/removal), and PRPS3 and PRPS5 were activated after post-translation modification. PRPS3 were located in two forms and PRPS5 were expressed in three forms (Yamaguch *et al.,* 2003). A homology search using the BLASTP program revealed a significant sequence similarity of PRPS4 to a large number of protein that carry one or more conserved domains. The highest alignment score of PRPS4 was actually to a 1RR4_AETCO Rec 30S ribosomal protein S4 chloroplastic present in the chloroplast stroma. Other high-alignment hits were: Chloroplast 30S ribosomal protein S4 [Brassica oleraceal], ribosomal protein S4 [Draba nemorosa], ribosomal protein S4, partial (chloroplast) [Reseda lutea] with identities of 93%, 93% and 92%, respectively. It also shows a strong homology with Thx of *Thermus thermophilus* 30S subunit which is a small basic ribosomal protein and identifiable in the complete genome sequence of *A. thaliana* and in the higher plant expressed sequence tag database.

C. spinosa rps4 protein shows 99% similarity with *A. thaliana* rps4 chloroplast protein starts with residue 11 and ends with residue 211 with a high similarity was notice in N terminal of first 126 amino acid of which encoded a Toll/interleukin-1 receptor (TIR)-nucleotide binding site (NBS)-Leu-rich repeat (LRR). TIR is a class of gene family accounts for the largest number of known disease resistance (R) genes **(Zhang and Gassmann, 2003)** like other family members in different plant species. The Arabidopsis RPS4 is a disease-resistance locus on chromosome 5 of *A. thaliana* specifying resistance to strains of *Pseudomonas syringae* pv *tomato* strain avrRps4 expressing DC3000 (Gassmann *et al.,* 1999). Our high similarity data together with high leucine amino acid residues (30/202; 15%), among them leucine repeats represents 5 times within 30 located residues reveal that rps4 of *C. spinosa* containing a domain belongs to the that bacterial and pathogen resistance gene and

nucleotide binding site (fig.8). **Gassmann *et al.*, (1999)** clarified that Toll family not only plays a key role in innate antibacterial and antifungal immunity in plants, insects as well as in mammals. It contains three highly-conserved regions, and mediates protein-protein interactions between the Toll-like receptors (TLRs) and signal-transduction components.

Query = *c. spinosa* subject = *c. fleuosa* Core	**Expect**	**Method**	**Identities**	**Positives**	**Gaps**
399 bits(1026)	3e-147	Compositional matrix adjust.	198/201(99%)	199/201(99%)	0/201(0%)

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Query 11  FKKIRRLGALPGLTSKKPRAGSDLRNQSRSGKKSQYRIRLEEKQKLRFHYGLTERQLLKY  70
          FKKIRRLGALPGLTSK+PRAGSDLRNQSRSGKKSQYRIRLEEKQKLRFHYGLTERQLLKY
Sbjct 1   FKKIRRLGALPGLTSKRPRAGSDLRNQSRSGKKSQYRIRLEEKQKLRFHYGLTERQLLKY  60
                                -

Query 71  VRIAGKAKGSTGQVLLQLLEMETRLDNILFRLGMETALTIPQARQLVNHRHILVNDRIVD  130
          VRIAGKAKGSTGQVLLQLLEMETRLDNILFRLGMETALTIPQARQLVNHRHILVN*RIVD
Sbjct 61  VRIAGKAKGSTGQVLLQLLEMETRLDNILFRLGMETALTIPQARQLVNHRHILVNGRIVD  120
                                                                      -

Query 131 IPSYRCKPQDIITVKDEQKSRTLVQNLLDSSAHQELPKHLTLHTFQYEGLVNQIIDRKCA  190
          IPSYRCKPQDIITVKDEQKSRTLVQNLLDSSAHQELPKHLTLHTFQYEGLVNQIIDRKC*
Sbjct 121 IPSYRCKPQDIITVKDEQKSRTLVQNLLDSSAHQELPKHLTLHTFQYEGLVNQIIDRKCV  180
                                                                       -

Query 191 GLKINELLVVEYYSRQTSTOP  211
          GLKINELLVVEYYSRQTSTOP
Sbjct 181 GLKINELLVVEYYSRQTSTOP  201
```

Fig (8) Amino acid similarity between C. spinosa rps4 protein with A. thaliana.

A comparison between amino acid sequence of rps4 protein of *C. spinosa* and *N. officinale* revealed that a homology is about 93% with 15 different amino acid as represented in the fig (8) and summarized in table (3), where *C. spinosa* starts with residue 2 and ends with residue 201 with 2% conservative replacements (3/4 are basic amino acids substitutions at position 25, 27, 133, and ¼ acidic amino acid substitution at position 158). It is interesting to notice the deletion of glutamic and therionine amino acid twice within 15 bases (4 -residue gaps) at positions 90--91 and 103-104 (2%), and 7 mismatch bases are observed as in fig (9).

Alignment statistics for match #1					
Query = *N. officinale* subject = *c. spinosa* Score	**Expect**	**Method**	**Identities**	**Positives**	**Gaps**
372 bits(956)	2e-136	Compositional matrix adjust.	189/204(93%)	193/204(95%)	4/204(2%)

```
Query 4   SGYRGPRFKKIRRLGALPGLTSKKPRAGSDLRNQSRSGKKSQYRIRLEEKQKLRFHYGLT  63
          S YRGPRFKKIRRLGALPGLTSK+P+AGSDLRNQSRSGKKSQYRIRLEEKQKLRFHYGLT
Sbjct 2   SRYRGPRFKKIRRLGALPGLTSKRPKAGSDLRNQSRSGKKSQYRIRLEEKQKLRFHYGLT  61
Query 64  ERQLLKYVRIAGKAKGSTGQVLLQLLEMETRLDNILFRLGMETALTIPQARQLVNHRHIL  123
          ERQLLKYVRIAGKAKGSTGQVLLQLLEM RLDNILFRLGM ALTIPQARQLVNH HIL
Sbjct 62  ERQLLKYVRIAGKAKGSTGQVLLQLLEM--RLDNILFRLGM--ALTIPQARQLVNHGHIL  117
Query 124 VNDRIVDIPSYRCKPQDIITVKDEQKSRTLVQNLLDSSAHQELPKHLTLHTFQYEGLVNQ  183
          VN RIVDIPSYRCKP+DIITVKDEQ SRTLVQNLLDSSA +ELP HLTLHTFQYEGLVNQ
Sbjct 118 VNGRIVDIPSYRCKPRDIITVKDEQNSRTLVQNLLDSSAPEELPNHLTLHTFQYEGLVNQ  177
Query 184 IIDRKCAGLKINELLVVEYYSRQT  207
          IIDRKC GLKINELLVVEYYSRQT
Sbjct 178 IIDRKCVGLKINELLVVEYYSRQT  201
```

Fig (9). Alignment between amino acid sequence of rps4 protein of *C. spinosa* and *N. officinale*

The chloroplast genome can be divided into three functional categories including (1) protein-coding genes, (2) introns, and (3) intergenic spacers; the latter two do not encode proteins and are referred to as

noncoding regions. According to the physical chloroplast map **(Tsudzuki, 2000)**, approximately 43% of the long sequence chain (LSC) and short sequence chain (SSC) is noncoding. Fifteen introns make up approximately 10.6% of the single-copy chloroplast DNA, while 92 intergenic spacers comprise 32.3%. However, most of studies have used some portion of the trnL-trnL-trnF or trnK-matK-trnK regions and very few investigators have sampled other noncoding regions of the cpDNA molecule for phylogenic and barcoding studies **(Agrawal *et al.*, 2014)**. Because of this, little is known about the relative rates of evolution among the different noncoding cpDNA regions, and most investigators continue to rely on these two very popular regions.

Table (3) mutated bases among rps4 protein for *C. spinosa* and *N. officinale*

No	position	Type	No	Position	type
1	3	R to G	9	120	D to G
2	25	K to R	10	133	Q to R
3	27	R to K	11	143	K to N
4	90	-E	12	157	H to P
5	91	-T	13	158	Q to E
6	103	-E	14	162	K to N
7	104	-T	15	184	A to V
8	114	R to G			

The use of noncoding chloroplast DNA sequences to generate plant phylogenetics, population genetics, and phylogeography began early **(Gielly and Taberlet, 1994)** with over 50 completely sequenced land plant chloroplast genomes and several more on the horizon. However, relatively few noncoding chloroplast DNA regions have been directly compared in sequence-based investigations. The phylogenicity of noncoding DNA regions was studied to provide an idea about the genetic order of *C. spinosa*. Thus trnL-trnL-trnF or trnK-matK-trnK region spacers would be of greater value to systematic studies that often used. Therefore, we used a portion trnV/ rrn16s spacer of *C. spinosa*-species to surveys representing the most phylogenetic lineages of phanerogams.

Originally, the ribosomal protein 16 small subunit gene (rps16) intron was suggested to be a valuable tool for investigation at the family level and below due to its highly conservative region among the uncoding genes, but the accumulated literature suggests that it will often not provide enough characters to resolve relationships below generic levels. The *rpS16* contains a group II intron that was first used in a phylogenetic context by Yan et al. (2014). Therefore, an alignment of *C. spinosa* trnV/rm16s spacer with different noncoding cpDNA regions is highly predictive that might offer an effective means to the phylogenetic investigation and they may eventually lead to a better understanding of the mechanisms for cpDNA evolution based on comparative frequency of various types of mutational events. The non-coding trnV/rm16s spacer marker is useful as the rps4 coding gene in phylogenetic reconstruction at the high level of orders and families in higher plants and in relation to the number of aligned positions the non-coding marker was even more effective. Phylogenic analysis were be needed for determining the exact classification of *C. spinose* depending on this information for first time. These data might contribute to a broader understanding of plastid evolution across flowering plants.

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