J. Agric. Sci. Mansoura Univ., 29 (3): 1215 - 1226, 2004

AFFINITY PURIFICATION OF *ORF256-COXI* RNA BINDING PROTEINS FROM WHEAT (*Triticum aestivum*) MITOCHONDRIA

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ABSTRACT

Cytoplasmic male sterility (CMS) is associated with the presence and expression of the *orf256* gene in the hybrid plants between *Triticum aestivum (Ta)* and *T. timopheevi (Tt)*. The gene is upstream of *coxI* gene in *T. timopheevi* mitochondrial DNA (mtDNA), whereas it is not present in *T. aestivum* mtDNA. It is expressed with *coxI* in the same mRNA in *T. timopheevi*, fertility restored lines (FR), and CMS lines. Expression as a 7 kD protein occurs only in CMS lines. Full length-(3.1 kb) transcripts as well as processed transcripts with 5' termini within the *orf256* coding region have been detected in both CMS and FR lines. In *T. timopheevi* mitochondria, there is only a trace of full-length 3.1 kb transcript, and major transcripts have 5' termini within the *orf256* coding region. This prevents any significant expression of the *orf256* protein product. *Orf256-coxI* RNA binds many proteins in mitochondrial extracts from CMS, Ta, FR, and Tt, but binds to 42 and 39 kD proteins from Ta extract only (El-Shehawi *et. al.*, 2003).

We used *orf256-coxI* mRNA to test the effects of phosphorylation as well as phosphatase inhibitors (sodium vanidate) on the RNA binding to mitochondrial proteins. In addition, the RNA was used in affinity purification experiment to isolate RNA binding proteins from Ta mitochondrial extract. In this report, we conclude that binding of *orf256-coxI* RNA to mitochondrial proteins is not dependent on the 5'- or 3'-untranslated regions of the RNA. The phosphorylation state of mitochondrial proteins appears to affect their binding to the RNA. The 42 and 39 kD proteins fractionate in the 0-20% ammonium sulphate fraction. Six proteins were affinity purified from the 0-20% fraction with biotin-labeled RNA. N-terminus sequencing revealed that the 42 kD protein is similar to the coatmer complex epsilon chain protein of *Arabidopsis thaliana*.

Keywords: wheat, cytoplasmic male sterility, mitochondria, phosphorylation, RNA binding protein, affinity purification.

INTRODUCTION

A chimeric open reading frame, *orf256*, upstream of *coxI* was found in fertile *Triticum temopheevi* (Tt), cytoplasmic male sterile (CMS), and fertility restored (FR), but not in *Triticum aestivum* (Ta) mitochondria (Rathburn and Hedgcoth, 1991; Rathburn *et. al.*, 1993). The 5' flanking sequence from -228 to -1 and the first 33 nucleotides of the coding sequence of the *orf256* are identical to those of *coxI* of Ta, but the rest of the *orf256* sequence is not related to that of coxI (Rathburn and Hedgcoth, 1991). Antibodies raised against a predicted *orf256* peptide detected a 7 kD protein in mitochondrial proteins of CMS lines using Western blots. The protein was not detected in mitochondrial proteins from Ta, Tt, or FR lines (Song and Hedgcoth, 1994).

The *orf256* sequence was found in various species of wheat relatives and progenitors, but was expressed as RNA only in Tt and *Aegilops speltoides*. None of the tested plants have the expression as 7 kD protein except the CMS plants (Hedgcoth *et. al.* 2002). o*rf256-coxI* RNA binds to various mitochondrial proteins from CMS, Ta, FR, and Tt, but the 42 and 39 kD proteins were detected only in Ta mitochondrial extract that could have been involved in elimination of the *orf256* sequence from Ta mtDNA (El-Shehawi *et. al.* 2003).

Phosphorylation/dephosphorylation was shown to affect the affinity of some nucleic acid binding proteins to their ligand (Harter *et. al.*, 1994; Li and Zassenhaus, 2000; Shteiman-Kotler and Schuster 2000). G-box binding factors from parsley cytosol lost their binding activity to the G-box DNA element after dephosphorylation with immobilized alkaline phosphatase and activity was restored after rephosphorylation (Harter *et. al.*, 1994). Dephosphorylation of dodecamer binding protein in yeast was shown to abolish RNA binding activity of the protein to the dodecamer sequence (Li and Zassenhous, 2000).

Although most of the characterized RNA binding proteins bind to the 3' or 5' untranslated regions, there are some proteins that show binding to the coding region of the studied RNA molecules (Lin *et. al.*, 2000; Kash and Menon, 1999). Lutropin receptor binding protein-1, LRBP-1, was shown to bind to a polypyrimidine sequence in its mRNA indicating a role in posttranscriptional regulation of the receptor expression (Kash and Menon, 1999). Also, there are some proteins that show affinity to different types of nucleic acid molecules (Gallia *et al.*, 2000; Satoh *et. al.*, 1999; Ruzanov *et. al.*, 1999). Purα is a highly conserved protein in eukaryotic cells that binds to dsDNA, ssDNA, RNA, and cellular proteins. Y-box binding proteins are a group of proteins that have a wide variety of biological functions. They have very conserved nucleic acid binding domain from bacteria to human. For example, the mammalian p50 protein is almost identical to mammalian Y-box transcription factors. It was found to be the most abundant protein in the cytoplasmic mRNPs in somatic cells (Ruzanov *et. al.*, 1999).This suggests a role for p50 in both transcription and translation. It also interacts with actin protein (Ruzanov *et. al.*, 1999) leading to more understanding of its role in coordinating cell functions.

In this study, we report that the phosphorylation state of mitochondrial proteins change their affinity to *orf256-coxI* RNA. *orf256-coxI* RNA binds mitochondrial proteins independent of the 5' or3' UTR regions. We used Biotin-labeled RNA for affinity purification of RNA binding proteins.

MATERIALS AND METHODS

Preparation of mitochondrial proteins

Wheat mitochondria and mitochondrial proteins were isolated using published procedures (El-Shehawi *et. al* 2003.; Song and Hedgcoth, 1994; Kemble *et. al.*, 1980) from 7-10 days old shoots. Mitochondrial protein extract was prepared by sonication according to Song and Hedgcoth (1994). Protein

concentration was estimated using Bradford Reagent (Bio-Rad) and a bovine serum albumin standard curve.

SDS-PAGE and electrotransfer of proteins to PVDF membrane

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Proteins were electrotransfered to a PVDF membrane (Immobilon-P, Millipore) from the SDS polyacylamide gel using a Bio-Rad semi-dry electroblotter (El-Shehawi *et. al.* 2003; Millipore).

Subcloning of *orf256-coxI* regions

orf256-coxI DNA template was used with PCR to amplify specific regions of the *orf256-coxI* gene. The sequences of primers used to amplify different regions of the *orf256-coxI* gene for subcloning are shown in Table (1). All amplified fragments were cloned in pGM-T vector (Promega).

Table (1): primers used in PCR for *orf256-coxI* gene region amplification.

Region	5' primer	3' primer
orfNT	**5:35**GCAGGTTTACTGCTTTC	**C:477**GGAACGAAGCGCTTCATCGA
orfCR	**5:35**GCAGGTTTACTGCTTTC	**C:785**TCAGAATTACTGAGCTAC
coxNT	**5:964H3**AGCTTGCAGGAGTGATGGGCACATG CTTC	**C:1469**GCTGTCACTAGAACGGACC
coxCR	**5:964H3**AGCTTGCAGGAGTGATGGGCACATG CTTC	**C:2450**GTCTGTTTTACCGCTGG
3'UTR	**5:2686**TAGGCGCACCTTTCATCTCTAACT	**C:3049**GCTTAGAAGGATTCTTTCAG AGCG

5: refers to forward primers on sense strand, C: refers to reverse primers on the complementary strand. Primer numbering is based on designating "1" for the first nucleotide of *orf256* sequence

Preparation of *orf256-coxI* 5' UTR DNA templates

The 5' UTR of *orf256-coxI* (Tt) is identical to that of *coxI* (Ta). To subclone this region, a plasmid preparation of pAH2-34AA was digested with SapI (Promega). The band containing the vector and T7 promoter preceding the 5' UTR was recovered from agarose gel. The purified DNA fragment, 2901 bp, was used for *in vitro* transcription for RNA probe synthesis using the T7 promoter.

Northwestern blots

Dig-RNA was synthesized using the RiboScribe *in vitro* transcription system (Epicentre, USA; El-Shehawi *et. al.,* 2003) various DNA templates and T7 polymerase following the manufacturer instructions. Northwestern blot procedures were done according to El-Shehawi *et. al.* (2003); Dooley et. al. (1992).

Dephosphorylation of mitochondrial protein extracts

Mitochondrial protein extract, 30 ىg, was incubated with 1 U of alkaline phosphatase attached to agarose beads (Sigma) in 25 ىL of reaction mixture for 30 min at room temperature. Alkaline phosphatase was removed by centrifugation. Dephosphorylated protein extracts, 20 ىg, were used for Northwestern blots.

Ammonium sulfate fractionation of mitochondrial proteins

Wheat embryos (Ta) were used for isolation of mitochondria using a method used by Hanic-Joyce *et al.* (1990). Final mitochondrial pellet was lysed and the S100 lysate was prepared. Ammonium sulfate fractionation of mitochondrial proteins was carried out using a published method (Hanic-Joyce *et al.*, 1990). S100 lysate was used to obtain 0 – 20%, 20 – 50%, and 50% supernatant fractions. The 0 to 20% and 20% to 50% $(NH_4)_2SO_4$ fractions, 0.5 mL of S100, and 2 mL of the 50% supernatant were dialyzed against 500 mL of buffer A in Slid-A-Lizer dialysis cassettes (Pierce). The protein concentrations of the fractions were estimated using the Bradford assay and stored at -85°C.

Affinity purification of RNA binding proteins

Labeling RNA with Biotin-21-UTP

The RiboScribe Kit (Epicentre) for *in vitro* transcription was used to generate a biotin-21-UTP-labeled RNA (Biot-RNA) probe from *orf256-coxI* DNA template. The same procedures used for synthesis of digoxigenin-11-UTP-labeled RNA probe were followed by replacing 87 µM digoxigenin-11-UTP with 25 µM biotin-21-UTP.

Binding of the Biot-RNA to streptavidin magnetic beads

Beads were incubated with Biot-RNA with occasional gentle mixing for 10 min at room temperature. Two samples were prepared, one serving as a control that received DEPC-treated water. Beads were washed and stored in Northwestern binding buffer (El-Shehawi *et. al.* 2003).

Protein binding and purification

Since the 42 and 39 kD proteins fractionate in the 0 – 20% ammonium sulphate fraction it was used to purify proteins binding to the *orf256-coxI* RNA probe. MICROCON columns (MWCO 10, Millipore) were used to concentrate and remove the dialysis buffer A from the protein fraction. The concentrated proteins were incubated for 30 min at room temperature with occasional gentle shaking at room temperature with the Biot-RNA-beads or beads (control). Beads were washed with binding buffer and bound proteins were eluted twice with elution buffer (2 M KCl; 0.75 mM $MgCl_2$; 5 mM Tris.HCl, pH 7.4). MICROCON columns (Millipore) were used to desalt and concentrate the eluted proteins through two rounds using PBS buffer, pH 7.2, for reconstitution. The preparation of affinity-purified proteins was stored at -20°C.

Analysis of the affinity purified proteins

Affinity-purified proteins were fractionated on SDS-PAGE and stained with Gel Code Reagent (Pierce) or transferred to PVDF membrane by electrotransfer. The membrane blot was stained with Gel Code Reagent (Pierce) and the visible bands were cut out individually and stored at 4°C. Some of the PVDF slices were used for N-terminus sequencing at the Biotechnology Core Facility (Department of Biochemistry, Kansas State University).

RESULTS AND DISCUSSION

Protein dephosphorylation effect on *orf256-coxI* RNA binding

Dephosphorylation reduced binding to *orf256-coxI* RNA in all lanes except the Tt lane (Fig. 1). Thus, phosphorylated mitochondrial proteins seem to have a higher affinity for RNA binding. The reduction in binding is very clear in the Ta lane for the 42, and 39 kD bands. In the case of the Tt extract, dephosphorylation appears to allow increased RNA binding. Getting different responses to dephosphorylation on probe binding in various extracts may support the possibility of proteomic differences, especially in Tt mitochondria. The effect of dephosphorylation on RNA binding raised another question: What is the original phosphorylation state of these proteins? Sodium vanidate is known as a phosphatase inhibitor (Wo *et al.*, 1998). It was used to inhibit alkaline phosphatase after dephosphorylation of DNA fragments preceding DNA ligation during cloning (Wo *et al.*, 1998). Mitochondrial protein extracts isolated in the presence of sodium vanidate (2 mM) were prepared and used for Northwestern blots. The most interesting effect of sodium vanidate is that it abolished RNA binding to the 42 and 39 kD bands in Ta extracts (Fig. 2). Although this result seems to contradict the results of dephosphorylation, it may highlight the complexity of mitochondrial protein binding to *orf256-coxI* RNA. It could reveal the possibility of involvement of a signal transduction pathway in the regulation of RNA binding to the 42 and 39 kD proteins, and may indicate a complexity of phosphorylated and dephosphorylated states. Results from this experiment show that the inhibited phosphatase activity is accompanied by the inability of the 42 and 39 kD proteins to bind to RNA. The target for this phosphatase activity could be the 42 and 39 kD proteins themselves. Dephosphorylation may be required to change these proteins from hyperphosphorylated to a lesser phosphorylated state that binds the RNA. Dephophorylation was shown to affect protein factors affinity to their nucleic acid ligands (Harter *et. al.*, 1994; Li and Zassenthaus 2000)

The 42 and 39 kD proteins may not bind to RNA because they have been hyperphosphorylated. This could be achieved by sodium vanidate inhibition of a phosphatase. Other protein factors could be involved. The inhibition of a phosphatase that works on those proteins could be indirect by implicating a signal transduction pathway in the regulation of 42 and 39 kD RNA binding activity. The protein factors are phosphorylated under normal conditions and they can bind RNA. When they are dephosphorylated by a phosphatase they still bind the RNA, but with lower affinity. Proteins can be restored to the normal state be a kinase. In the presence of sodium vanidate the phosphatase activity is inhibited. In this case, the 42 and 39 kD protein will be in a hyperphosphorylated state which can not bind the RNA. Hyperphosphorylation state can be achieved also be a kinase. A suggested model for the regulation of 42 and 39 kD affinity to the *orf256-coxI* RNA is shown in Fig (3).

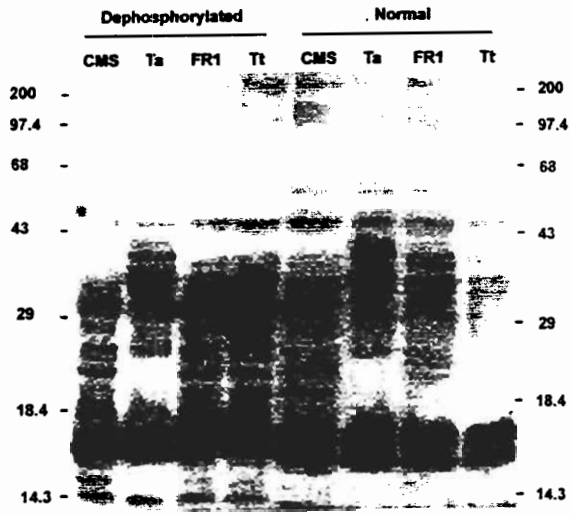

Figure (1): Northwestern blot of normal and dephosphorylated mitochondrial proteins probed with *orf256-coxI* Dig-RNA.

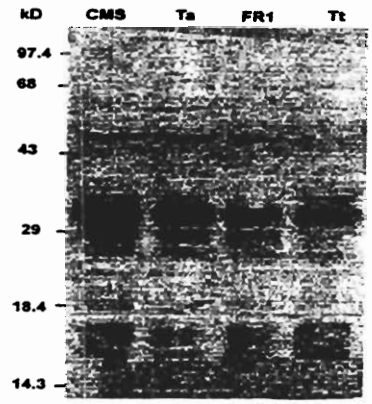

Figure (2): Northwestern blot of mitochondrial proteins isolated in the presence of 2 mM sodium vanidate and probed with *orf256-coxI* Dig-RNA.

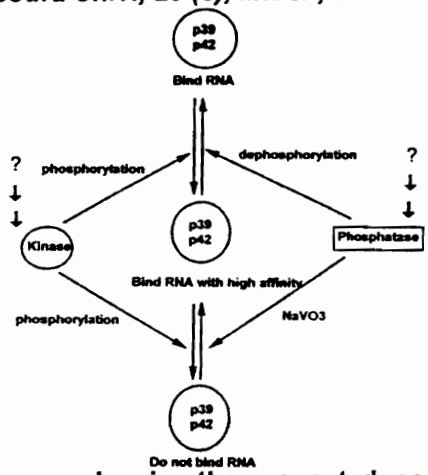

Figure (3): A Diagram showing the suggested model for the regulation of p39 and p42 affinity to *orf256-coxI* RNA.

Mapping of binding sites using different regions of *orf256-coxI* RNA probes

Different regions of the *orf256-coxI* gene were used as DNA template for RNA probe synthesis. These included *orf256* with its 5' UTR, *orf256* N-terminus, *orf256* coding region, *coxI* N-terminus, *coxI* coding region, *orf256-coxI* 5' UTR, *orf256-coxI* 3' UTR (Table 2).

Table 2: RNA probes used for Northwestern blots

RNA probe	Length, nt	Plasmid	RE used
orf256-coxI, sense	3059	pAH2-34	ClaI
orf256 N-terminus region	443	OrfNT	SalI
coxI N-terminus	505	CoxNT	SalI
orf256 coding region	751	orfCR	SalI
coxI coding region	1486	coxCR	SalI
orf256-coxI 3' UTR	364	3' UTR	SalI
orf256	1039	PAH2-34AA	ApaI
orf256-coxI 5' UTR	362	5' UTR	SapI

RE: Restriction enzyme used to prepare DNA template for in vitro transcription.

5' and 3' UTR probe show no binding to any extract proteins even after 10 hr of exposure of the Northwestern blot (data not shown). On the other hand, the shortest probe used that showed binding is the orfNT probe (443 nucleotides). This probe gives a response after 1 hr of exposure similar to a 30 min exposure for other probes. Therefore, negative results are best explained as the inability of these two probes (5' and 3' UTRs) to bind rather than that they are too short for detection in the usual detection time.

All other probes used (Fig. 4) gave the binding pattern as the whole *orf256-coxI* probe (El-Shehawi *et. al.*, 2003). This indicates that the coding regions of *orf256* and *coxI* bind these proteins independent of the 5' or 3' UTR. On the other hand, the 5' and 3' UTR do not show independent detectable binding. The similar results in binding in all regions studied, except

for the UTRs, as well as the whole *orf256-coxI* RNA could be due to the formation of similar stem-loops or dsRNA that may represent different targets binding by the different proteins. However, these secondary structures can not be established in the presence of 5' UTR and 3' UTR separated from the whole RNA molecule; therefore, they do not show binding to any of these proteins. In addition, some of the secondary structures in the coding regions might be essential for protein binding to the 5' and 3' UTR. Binding of the same proteins to different parts of the coding regions implies that these proteins could be involved in various mitochondrial functions. This could include their role in masking the mitochondrial RNA from translation, RNA transport, and RNA localization in mitochondria. Mammalian p50 protein is expected to have various roles in different cellular functions like gene expression, transcription and translation, and mRNA transport and localization (Ruzanov *et al.*, 1999). Pur-ᒍ protein is expressed in all eukaryotic cells and binds to all types of nucleic acids (dsDNA, ssDNA, RNA) as well as cellular proteins (Gallia *et al.*, 2000).

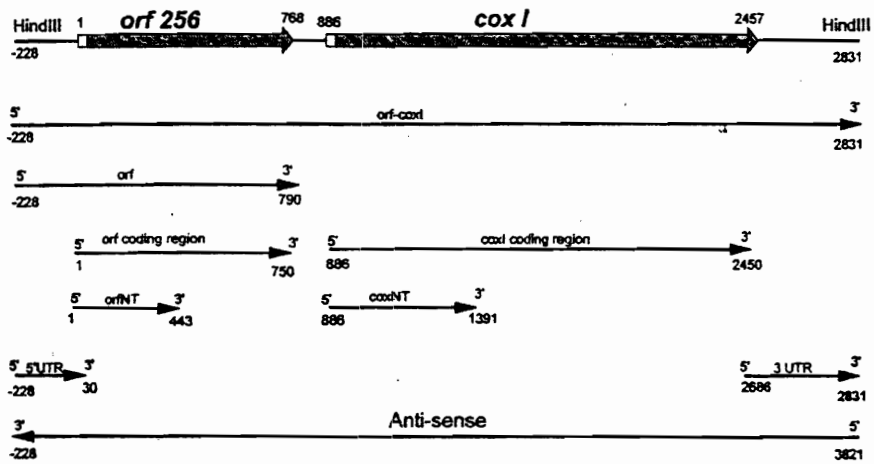

Figure (4): A diagram of the various RNA probes used in Northwestern blots. The *orf256-coxI* gene region is shown as dashed arrows. RNA probes are shown as arrows below their corresponding locations on the gene region. Number "1" is designated for the first nucleotide of *orf256* or *coxI* coding regions. Antisense *orf256-coxI* RNA is drawn in the reverse orientation of all other probes and gene regions.

Affinity purification of RNA binding proteins

Ammonium sulphate fractionation

Ammonium sulphate was used to fractionate a Ta mitochondrial extract and the fractions were assayed on Northwestern blots. These two interesting bands (42 and 39 kD) in the Ta extract fractionate in the 0-20%

ammonium sulphate fraction (Fig. 5). The mobility of the 42 and 39 kD bands appear little different in the 0-20% fraction because this fraction has many mitochondrial membrane components that may cause distortion in the mobility of this fraction on SDS-PAGE. In addition, these two bands are detected in the same location in the S100 fraction before ammonium sulphate fractionation. The 20-50% fraction shows little binding of probe on Northwestern blots, whereas the 50% supernatant shows no binding since it has very little protein remaining (Fig. 5).

Affinity purification

Since the 42 and 39 kD protein bands in Ta are fractionated in the 0-20% fraction and they are still able to bind RNA probe it is possible to purify them from this fraction. Biot-RNA was used to affinity purify these protein factors from the 0-20% fraction. Biot-RNA of *orf256-coxI* bound six proteins from the 0-20% fraction (97, 55, 46, 42, 35, and 33 kD), whereas a control sample which included only magnetic beads without RNA attached does not show binding of any proteins when assayed by coomassie blue staining (Fig. 6a). The band at 46 kD may represent a band detected in all extracts and only the 42 kD band may be the band found in Ta extracts.

Affinity-purified proteins were transferred to a PVDF membrane and all bands were cut out as membrane slices. The 42 kD band was used for N-terminus sequencing. Only 10 amino acid residues (KNTHAYQTAI) were obtained for the 42 kD band. A database search shows that the amino acids 5 to 10 of the N-terminus sequence of the 42 KD protein has 100% identity to the coatmer complex epsilon chain (ACC# 064748) from *Arabidopsis thaliana* (Fig. 6b).

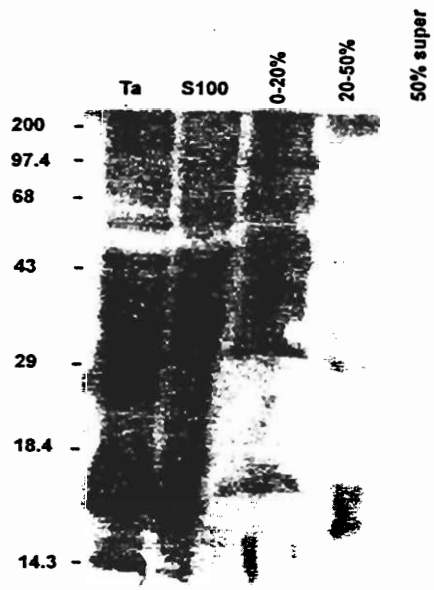

Figure (5): Northwestern blot of ammonium sulphate fractions of Ta mitochondrial protein extract probed with Dig-RNA of *orf256-coxI* gene region.

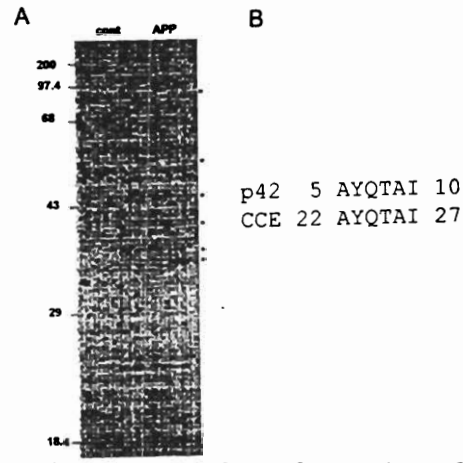

Figure (6): a: Commassie blue staining of proteins affinity purified using Biot-RNA and the 0-20% ammonium sulphate fraction. Cont: control; APP: affinity purified proteins. Detected protein bands are indicated by asterisks. b: Amino acid sequence alignment of the N-terminus sequence of the 42 kD protein and the Arabidopsis coatmer complex epsilon chain protein. p42: 42 kD protein; CCE: coatmer complex epsilon chain protein. The numbers following the sequences refer to the amino acid positions in the respective sequences.

The detected differences in RNA binding proteins among the four sources of mitochondrial proteins does not give a direct explanation for the presence of different transcripts in CMS, FR, and Tt mitochondria of the *orf256-coxI* gene region. The 42 and 39 kD proteins are present in Ta and are not detected in CMS, whereas FR and Tt show low levels of the 39 kD protein. The 42 kD protein is not detected in either FR or Tt. However, the presence of these two proteins together only in Ta extract, their absence in CMS, and the absence of the 42 kD protein only in FR and Tt is in accordance with the absence of *orf256* and its expression in Ta, the presence of *orf256* and its expression as RNA and protein in CMS, and the absence of *orf256* expression as protein in FR and Tt. During evolution, it seems Tt and Ta have developed unique systems to deal with the *orf256* problem through a specific molecular system at the transcription or translation levels. Mammalian p50 protein has a similar role in the regulation at the transcription and translation levels (Ruzanov *et. al.*, 1999). FR, like Tt, is fertile and it does not have the 42 kD protein but it has the 39 kD protein band. It has the *orf256* sequence and its transcripts, yet they can not express the *orf256* as 7 kD protein. Ta has both the 42 and 39 kD proteins, and it does not have the *orf256* sequence or its transcripts and consequently its protein. CMS does not have either 42 or 39 kD proteins, and it has the *orf256* sequence, its transcripts, and its 7 kD protein. This suggests that the 42 kD protein could be related to the presence of *orf256*. It is expressed when there is no *orf256* sequence upstream of the *coxI* gene as in the case of Ta. The presence of the 39 kD protein seems to be related to *orf256* translation. Its presence

might inhibit translation of *orf256* like the case of FR and Tt. The absence of both bands in CMS allows the presence, transcription, and translation of *orf256*. P50 and PurJ proteins may have similar roles in the regulation of transcription and translation as well as other cellular processes in eukaryotic cells (Ruzanov *et. al.,* 1999; Gallia *et. al.,* 2000)

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فصل بروتينات من ميتوكوندريا القمح *Triticum aestivum* التـــي ترتبـــط بالـــ RAN *orf256-coxI* بواسطة الانجذابية الجزيئية .

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صفة العقم الذكري السيتوبلازمي (cms) في القمح متلازمة مع التعبير الجيني للجين orf 256 في النباتات الهجين بين نوع القمح (Ta) *Triticum aestivum* والنـــوع *Triticum timopheevi*. (Tt) يقع الجين *orf256* قبل الجين coxI وينســـخ علـــى نفـــس جـــزئ الـــ mRNA فـــي النباتـــات *T.timeopheevi* والنباتات العقيمة سيتوبلازما (cms) وكذلك النباتات معـــاده الخصوبـــة Fertility restored (FR) ولكن يترجم الجين *orf-256* إلى بروتين ذو وزن جزيئي7 kD في النباتات العقيمة فقط cms. جزيئات الـــ RNA الناتجة من الجين *orf256-coxI* ترتبط بعديد من البروتينات من ميتوكوندريـــا القمح CMS,FR,Ta,Tt ولكنه يرتبط بالبروتينات ذات الوزن الجزيئي 42,39 kD فقط من ميتوكوندريـــا النباتات Ta .

في هذه الدراسة تم استخدام الـــ RNA من الجيـــن *orf256-coxI* لدراســـة تـــأثير الفســـفرة phoshorylation وكذلك مثبط أنزيمات الفسفرة phosphatase inhibitorعلـــى قـــدرة بروتينـــات الميتوكوندريا على الإرتباط بالـــ *orf256-coxI RNA*. تم أيضا استخدام الـــ *orf256-coxIRNA* لعزل وتنقية البروتينات التي ترتبط بالـــ RNA بواسطة الانجذابيـــة الجزيئيـــة affinity purification. أوضحت نتائج الدراسة أن ارتباط البروتينات بالـــ RNA لا يعتمد على وجود الأجـزاء ' 3 أو '5 الغـــير مترجمة. وجد أن الفسفرة تؤثر على ارتباط بروتينات الميتوكوندريا بالـــ RNA. البروتينـــات 42,39 kD تنفصل عند درجة تشبع 0-20% من كبريتات الأمونيوم. تم عزل 6 بروتينات بواسطة الانجذابية الجزيئيـــة من البروتينات المفصولة عند درجة تشبع 0-20% لكبريتات الأمونيوم وذلك بواسطة الـــ RNA المعلـــم بالـــ biotin تم قراءة تتابع الأحماض الأمينية للطرف الأميني للبروتين 42kD وأتضح أنه مشـــابه لأحـــد بروتينات نبات الـــ Arabidopsis.