Orf256-coxI RNA binds to wheat mitochondrial proteins

(Received: 20.12.2002; Accepted: 05.01.2003)

A.M. El-Shehawi*, M.M. Elseehy, and C. Hedgcoth
Department of Biochemistry, Kansas State University, Manhattan, Kansas 66506, USA
* Current address: Department of Genetics, Faculty of Agriculture, Alexandria University, Alexandria, Egypt

ABSTRACT

The chimeric gene orf256 is associated with cytoplasmic male sterility (CMS) in Triticum aestivum (Ta)/T. timopheevi (Tt) hybrids. It is upstream of coxI in T. timopheevi mitochondrial DNA (mtDNA) and is expressed with coxI in the same mRNA in T. timopheevi, fertility restored lines (FR), and CMS lines. The gene is not present in T. aestivum mtDNA. Expression as a 7 kDa protein occurs only in CMS lines. Both CMS and FR lines have full length (3.1 kb) transcripts as well as processed transcripts with 5' termini within the orf256 coding region. 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, precluding any significant expression of an orf256 protein product. The orf256-coxI mRNA was used to search for RNA binding proteins by Northwestern blotting to determine if differential binding occurs depending on the mitochondrial source. Northwestern blots showed that the RNA binds to more than 20 proteins in mitochondrial extracts. Two proteins of 42 and 39 kD are detected only in T. aestivum mitochondrial extracts. Control RNAs do not bind to mitochondrial proteins. Cytosolic proteins show no affinity for the RNA, whereas nuclear extract has many proteins binding the RNA. Addition of fresh protein extracts to the binding reaction changes the RNA binding to the various mitochondrial proteins. The results conclude that orf256-coxI RNA binds to the 42 and 39 kD proteins from Ta mitochondria. These proteins could have been involved in elimination of orf256 from Ta mtDNA.

Key words: *Wheat, cytoplasmic male sterility, fertility restored, mitochondria, RNA binding protein.*

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).

Mitochondrial transcripts from wheat lines that have *Tt* mitochondria consist of digenic transcripts with all of the *coxI* coding sequence and all or parts of orf256 RNA. There is no separate transcript for *orf256* in CMS, FR, or *Tt* lines. The full length transcript of 3.1 kb that contains the complete digenic *orf256* and *coxI* transcript was detected in CMS and fertility restored mtRNA. A shorter transcript of 2.7 kb is present in CMS and fertility restored lines with only trace amounts in *Tt* mitochondrial transcripts. Although the major transcript of *Tt* is about 2.3 kb and begins in the carboxyl terminal third of *orf256* at nucleotide 549 of the coding sequence, a residual amount of the full length transcript is detected by PCR (Zhan and Hedgcoth, unpublished data) but not by Northern blot analysis. A transcript of 2.0 kb was detected in *Ta* mitochondrial RNA. These differences in transcript populations for the *orf256-coxI* gene in various wheat lines, as well as the association of CMS with the expression of *orf256* as a 7 kD protein, could be attributed to post-transcriptional modification of the *orf256-coxI* RNA (Song and Hedgcoth, 1994).

RNA binding proteins represent a wide range of functions. They might be involved in regulation of RNA maturation, protection, translation, processing, transport, or localization, (Ruzanov *et al.*, 1999). RNA binding character was discovered as a new function of previously studied proteins (Soulard *et al.*, 1993; Shteiman-Kotler and Schuster, 2000; Pelletier *et al.*, 2000). This includes some well known enzymes. For example, NAD+-isocitrate dehydrogenase, Idh, from yeast (Saccharomyces pombe, S. cerevisiae) showed binding to the 5' UTR of cox2 mRNA (Elzinga *et al.*, 2000). Thymidylate synthase interacts with different regions of the 5' UTR and coding regions of its own mRNA and represses its translation in an autoregulatory fashion in vivo and in vitro. In addition, it binds to other cellular RNAs (Chu *et al.*, 1991; Chu and Allegra, 1996; Hentze, 1994; Lin *et al.*, 2000).

Because the presence of transcripts of different sizes for the *orf256-coxI* chimeric gene in mitochondria from different sources and a trace amount of the full length transcript was detected in *Tt* mitochondria, we used Northwestern blots to search for RNA binding proteins in protein extracts from different wheat mitochondrial sources that could be involved in: 1- processing the full length transcript or blocking translation of *orf256-coxI* RNA in *Tt* and FR to avoid *orf256* expression, 2- translation and/or protection of the full length transcript in CMS to produce the 7 kDa protein while other lines do not, or 3- elimination of *orf256* from *Ta* mitochondrial genome during evolution. These proteins would be absent in other mitochondrial sources and may be expressed, imported to, or active only in *Ta* mitochondria and have essential roles in mitochondrial biochemistry.

Here, we report the detection of 42 and 39 kDa RNA binding proteins that bind to *orf265-coxI* RNA in *Ta* mitochondria. These proteins are not detected in nuclear cytosolic proteins. Binding specificity was confirmed by using control RNAs and competition experiment.

MATERIALS AND METHODS

Preparation of mitochondrial proteins

Wheat lines described in Table 1 were used in this study. Wheat mitochondria were isolated using a modified procedure for maize mitochondrial isolation (Kemble *et al.*, 1980; Song and Hedgcoth, 1994) from 7-10 days old shoots. During the isolation of mitochondria, nuclei were isolated by centrifugation of the supernatant for 10 min at 3,000 g before the

centrifugation to pellet mitochondria. After centrifugation of mitochondria, the supernatant was used as the cytosolic fraction. Mitochondrial protein extract was prepared by sonication according to a published procedure (Song and Hedgcoth, 1994). Bradford Reagent (Bio-Rad) was used to determine protein concentration at 595 nm using 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) (Laemmli, 1970). Proteins were electrotransfered to a PVDF membrane (Immobilon-P, Millipore) from the SDS polyacylamide gel using a Bio-Rad semi-dry electroblotter (Millipore).

Synthesis of digoxigenin-11-UTP-labeled RNA (Dig-RNA) probe and unlabeled RNA

The RiboScribe *in vitro* transcription system (Epicentre, USA) was used to synthesize RNA probes labeled with digoxigenin-11-UTP using pAH2-34 plasmid cut with ClaI DNA templates and T7 polymerase following the manufacturer instructions. UTP was replaced with 164 μM UTP and 26 μM digoxigenin-11-UTP. Unlabeled RNA of *orf256-coxI* was synthesized using the *in vitro* transcription system RiboMax Kit (Promega, USA) using T7 RNA polymerase.

Northwestern blots

Northwestern blot procedures were done according to Dooley *et al.* (1992) with some modifications. After electrotransfer, the protein blot was kept in wash buffer (1X PBS: 58 mM Na_2HPO_4; 17 mM NaH_2PO_4; 68 mM NaCl; pH 7.2; 0.1% Tween 20; 0.02 % sodium azide) for 6 hr and transferred to renaturation buffer (25 mM HEPES-NaOH, pH 7.9; 25 mM NaCl; 5mM MgCl2; 0.5 mM DTT) overnight at 4 C. The blot was blocked in blocking buffer (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 10% glycerol; 2.5% Nonidet P-40; 0.1 mM DTT; 5% casein) and rinsed in binding buffer (10 mM Tris-HCl, pH 7.5; 40 mM NaCl; 1 mM EDTA; 8% glycerol; 1 mM DTT; 0.125% casein). The blot was incubated with gentle shaking for 1 to 2 hr at room temperature in binding reaction containing 80 U of RNase inhibitor, 27 μg/ml of yeast RNA (Fisher), and 10 ng/ml of Dig-RNA. The blot was washed in binding buffer and incubated in anti-digoxigenin-alkaline phosphatase conjugate (Boehringer Mannheim) solution, diluted in detection buffer (50 mM Tris-HCl, pH 7.5; 50 mM NaCl; 0.25% casein, USB), for 30 min. The blot was washed in detection buffer and rinsed in assay buffer (0.1 M diethanolamine, pH 10; 1 mM $MgCl_2$; 0.02% sodium azide) (Tropix). CDP-star was applied and the blot was exposed to X-ray film for 30 min to 1 hr.

RESULTS AND DISCUSSION

Northwestern blots were used to search for differences in mitochondrial RNA binding proteins interacting with *orf256-coxI* mRNA in an effort to explain the production of different transcripts from the *orf256-coxI* gene region (Song and Hedgcoth, 1994). The Dig-RNA probe of the whole *orf256-coxI* gene region of about 3059 nucleotides binds to more than twenty different protein bands in all mitochondrial sources with a range from 200 kD to 14 kD (Fig. 1). Although most of these protein bands are present in all mitochondrial sources, two interesting bands are detected consistently in only one mitochondrial protein source. A 42 kD band is detected only in *Ta* mitochondrial extracts, whereas it is not present in CMS, FR1, or *Tt* extracts. A 39 kD band in *Ta* mitochondrial extract is detected at

a low level in *Tt* mitochondrial extracts and is not found in CMS FR1 mitochondrial extracts (Fig. 1). This could represent binding to novel proteins or new binding character of known proteins or enzymes (Souiard *et al.*, 1993; Shteiman-Kotler and Schuster, 2000; Elzinga *et al.*, 2000; McGowan and Pekala, 1996).

Table (1): Wheat lines used in this study.

Wheat Line	Accession	Nucleus	Cytoplasm	Fertility	Source
CMS	A910	Ta	Tt	Sterile	Cargill, USA
Ta	Newton	Ta	Ta	Fertile	Niblett, KSU
FR1	WH102002	Ta/Tt*	Tt	Fertile	Cargill, USA
Tt	TA103	Tt	Tt	Fertile	Gill, KSU

Ta: *T. aestivum*; Tt: *T. timopheevi*; CMS: Cytoplasmic Male Sterile; FR1: Fertility restored line.
* FR1 is produced from CMS lines by introduction of nuclear genes from Tt.

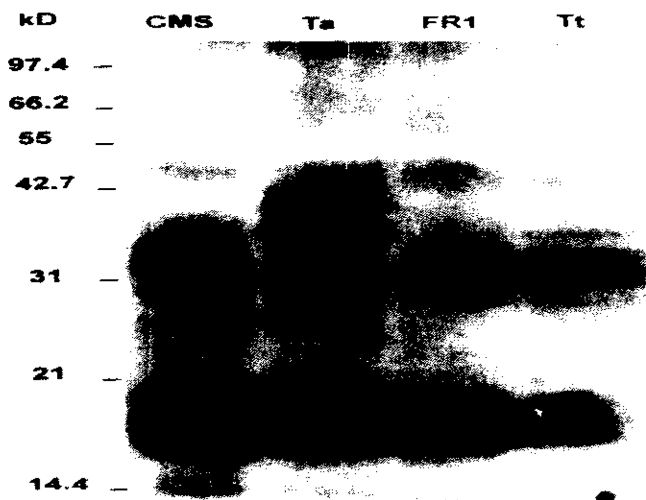

Fig. (1): Northwestern blot of mitochondrial proteins (CMS, Ta, FR1, and Tt) probed with orf256-coxI Dig-RNA. Exposure time is 30 min.

Affinity of orf256-coxI RNA probe for proteins in other subcellular extracts

Some of the proteins that show binding to orf256-*coxI* RNA on Northwestern blots might be cytosolic contaminants from the mitochondrial isolation procedure (Ruzanov *et al.*, 1999). To rule out this possibility, nuclear and cytosolic extracts were used on a Northwestern blot to compare the binding of the probe to proteins of the extracts of wheat lines under investigation (CMS, *Ta*, FR1, and *Tt*). The blot shown in Fig. (2) was washed with 1 M NaCl after the RNA binding step to test the specificity of binding. *Orf256-coxI* RNA probe shows high affinity to nuclear extracts, whereas it does not show any affinity to cytosolic proteins (Fig. 2). The high salt treatment of the blot in Fig. (2) reduced the intensity of many bands in mitochondrial extracts, yet the 42, 39, and 35 kD bands are still strongly detected only in *Ta* mitochondrial extract and not in the nuclear extracts (Fig. 2). A similar result was obtained for *Tt*.

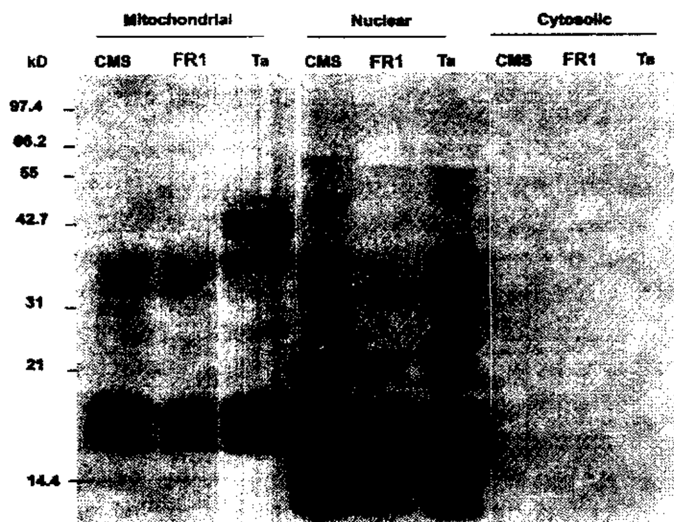

Fig. (2): Northwestern blot of mitochondrial, nuclear, and cytosolic proteins probed with orf256-coxI Dig-RNA. The blot represents the data after washing in 1M NaCl after RNA binding and before detection.

Affinity of other control RNA probes for proteins in mitochondrial extracts

Related and unrelated control RNA probes were assayed for their affinity for proteins in the mitochondrial extracts. Antisense *orf256-coxI* RNA (related control) and two other RNA probes that were prepared from commercial in vitro transcription Kits (unrelated controls) were used. A λ HindIII-EcoRI DNA fragment of about 1.3 kb (RiboScribe Kit, Epicentre) and *Xenopus* elongation factor 1 α (Xef1) DNA fragment of 1.7 kb (MegaScript Kit, Ambion) were used as control DNA templates to synthesize Dig-RNA. The three control RNAs show no affinity to any proteins of the mitochondrial extracts and resulted in a blank chemilumingram (data not shown). This strongly supports that *orf256-coxI* RNA binding to mitochondrial proteins is a mitochondrial-specific phenomenon. Some other proteins bind to various types of nucleic acids (Gallia *et al.*, 2000; Satoh *et al.*, 1999) with no specificity.

Extract addition during binding step

Protein extracts are separated on SDS-PAGE before they are transferred to PVDF membranes for Northwestern blots. This would cause dissociation of heterodimeric proteins to subunits and deposition at different locations on the blots (Philippe, 1994). Addition of extracts along with probe during the probe binding step may give rise to detection of some novel bands that can not be detected under normal conditions if some protein-protein interactions take place (Philippe, 1994). Effects of homologous and heterologous extract additions at the probe binding step were studied to determine whether more differences among the mitochondrial extracts might be found.

In homologous extract addition, an equal amount of protein extract, 20 μg, was added during binding step to a membrane slice that contains the same mitochondrial protein extract. Heterologous extract addition means addition of other extracts to membrane slices that have one specific extract. For example, with *Ta* proteins on membrane slices for heterologous extract addition, there was a

general reduction of probe binding to all bands in response to heterologous extracts (Fig. 3). Addition of *Tt* shows the least reduction in detection whereas CMS extract shows the highest; FR1 extract gave an intermediate response. The effect of addition of different extracts to *Ta* proteins is very clear especially on the 42 and 39 kD bands. CMS extract abolishes probe binding to most of the bands in region that includes the 42 kD and 39 kD bands (Fig. 3). The various combinations of extracts and their general effects are summarized in table (2). Data obtained from the extract additions (Fig. 3; table 2) show more variations among the mitochondrial protein contents (proteomes). This could be due to the variations in the nuclear-cytoplasmic relationships in these lines caused by different nuclear and mitochondrial genetic make up (Maan, 1979; Edwards, 1983).

Competition experiment

To test the specificity of *orf256-coxI* binding to mitochondrial proteins, unlabeled *orf256-coxI* RNA was used in increasing concentration to compete with the probe. This experiment was done only with *Ta* mitochondrial proteins since *Ta* proteins represent all of the bands detected on Northwestern blots. The unlabeled RNA appears to compete with the probe at 100 times of the probe concentration (Fig. 4). This result is further evidence that the binding of *orf256-coxI* RNA to mitochondrial proteins is specific (Fester and Schuster, 1995).

Table (2): Effects of homologous and heterologous extract addition combination on RNA binding to mitochondrial protein in Northwestern blots.

Protein on membrane	Extract added during binding			
	CMS	Ta	FR1	Tt
CMS	I	D	I	I
Ta	D	I	D	D
FR1	D	I	D	D
Tt	D	D	D	I

I: increase, when an extract enhances general RNA binding to specific proteins; D: decrease, when an extract reduces general RNA binding to specific proteins.

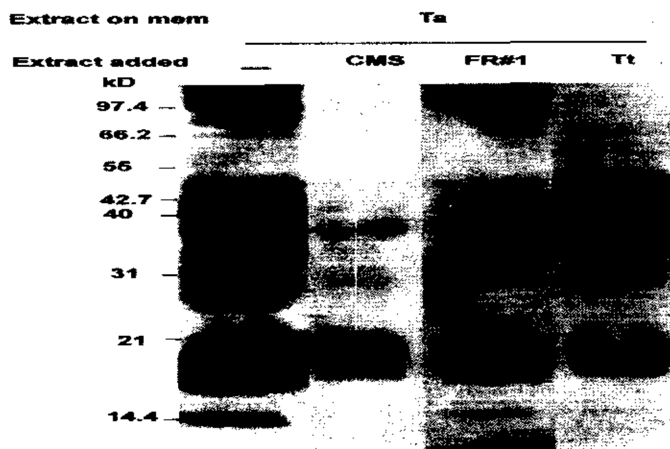

Fig. (3): Northwestern blot of Ta mitochondrial proteins probed with orf256-coxI Dig-RNA with the addition of CMS, FR1, and Tt heterologous extracts. The Ta membrane slices were treated during the RNA binding step with CMS, FR1, or Tt mitochondrial extracts containing 20 µg of protein.

Binding specificity of mitochondrial proteins to *orf256-coxI* RNA

Although *orf256-coxI* sense Dig-RNA binds to more than 20 proteins (Fig. 1), *orf256-coxI* antisense RNA probe as well as unrelated control RNAs shows no affinity for mitochondrial proteins. This indicates that even though the RNA recognizes many proteins there is specificity in the binding to the sense strand. In addition, the *orf256-coxI* sense probe does not show affinity to cytosolic proteins while it has affinity to many nuclear proteins (Fig. 2). This suggests that some of the mitochondrial proteins that bind to the RNA could be mitochondrial transcription factors that have common RNA binding activity resembling some cytosolic proteins (Ruzanov *et al.*, 1999). This also excludes these proteins from being similar to cytosolic translation factors since the RNA does not have affinity for any cytosolic proteins, which normally includes translation factors. Moreover, and more specifically, the 42 kD band is detected only in *Ta* extracts and the 39 kD band is absent in CMS extracts. The absence of these two bands in one or more mitochondrial extracts is a further evidence that the binding is specific. Also, the unlabeled orf256-coxI RNA competes with the probe for protein binding at 100 fold (Fig. 4) indicating that the RNA binds to these proteins in a specific manner. All together, the data are strong evidence that the *orf256-coxI* RNA binds to the mitochondrial proteins in a specific way. Mapping of binding sites for these proteins as well as their isolation could contribute significantly to uncover their biological functions that may strengthen our understanding of cytoplasmic male sterility in wheat at the molecular level.

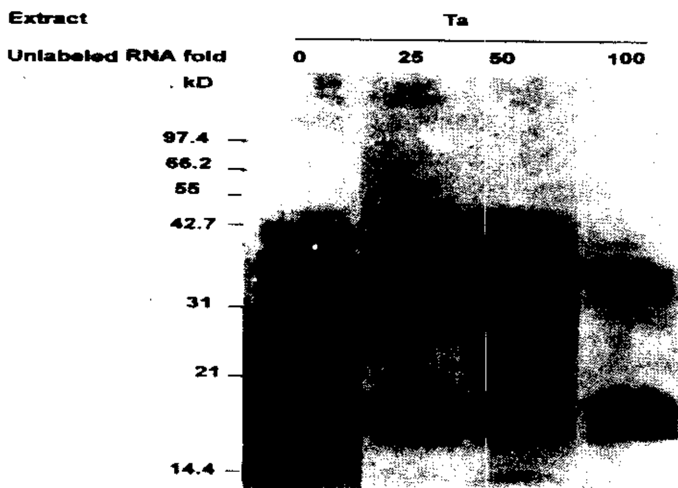

Fig. (4): Competition of binding of orf256-coxI Dig-RNA by unlabeled orf256-coxI RNA on Northwestern blots of Ta mitochondrial proteins.

REFERENCES

Chu, E. and Allegra, C.J. (1996). The role of thymidylate synthase as an RNA binding protein. BioEssays., 18: 191-198.

Chu, E., Koeller, D.M., Casey, J.L., Drake, J.C., Chabner, B.A., Elwood, P.C., Zinn, S. and Allegra, C.J. (1991). Autoregulation of human thymidylate synthase messenger RNA translation by thymidylate synthase.

Proc. Natl. Acad. Sci., 88: 8977-8981.

Dooley, S., Welter, C. and Blin, N. (1992). Nonradioactive Southwestern analysis using chemiluminescent detection. BioTechniques., 13: 540-543.

Edwards, I.B. (1983). An assesment of the role of cytoplasmic-genetic system in hybrid wheat production. Plant Mol. Biol. Rep. 1: 139-143.

Elzinga, S.D.J., Maat, K.V.O.C., Grivell, L.A. and Spek, H. V. D. (2000). Isolation and RNA-binding analysis of NAD^+-isocitrate dehydrogenase from Kluyveromyces lactis and Schizosaccharomyces pombe. Curr. Genet. 38: 87-94.

Fester, T. and Schuster, W. (1995). Potato mitochondrial manganese superoxide dismutase is an RNA-binding protein. Biochem and Mol. Biol. Inter. 36: 67-75.

Gallia, G., Johnson, E.M., and Khalili, K. (2000). Pur: a multifunctional single-stranded DNA and RNA-binding protein. Nucleic Acids Research. 28: 3197-3205.

Hedgcoth, C., El-Shehawi, A.M., Wei, P., Clardson, M. and Tamalis, D. (2002). A chimeric open reading frame associated with cytoplasmic male sterility in alloplasmic wheat with Triticum timopheevi mitochondria is present in several Triticum and Aegilops species, barley, and rye. Curr. Genet. 41: 357-365.

Hentze, M.W. (1994). Enzymes as RNA-binding proteins: a role for (di)nucleotide-binding domains. TIBS 19: 101-103.

Kemble, R.J., Gunn, R.E. and Flavell, R.B. (1980). Classification of normal and male-sterile cytoplasms in maize. II. Electrophoretic analysis of DNA species in mitochondria. Genet., 95 :451-458.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Lin, X., Parsels, L.A., Voeller, D.M., Allegra, C.J., Maley, G.F., Maley, F. and Chu, E. (2000). Characterization of a cis-acting regulatory element in the protein coding region of thymidylate synthase mRNA. Nucleic Acids Research, 28: 1382-1389.

Mann, S.S. (1979). Specificity of nucleo-cytoplasmic interactions in Triticum and Aegilops species (a review). Wheat Info. Serv. 50: 71-79.

McGowan, K. and Pekala, P.H. (1996). Dehydrogenase binding to the 3'-untranslated region of GLUT1 mRNA. Biochemical and Biophysical Research Communications. 221: 42-45.

Pelletier, M., Miller, M.M. and Read, L.K. (2000). RNA-binding properties of the mitochondrial Y-box protein RBP16. Nucleic Acids Research. 28: 1266-1275.

Philippe, J. (1994). The Southwestern assay. In: Protocols for gene analysis. Humana press Inc. Totowa, NJ., 349-361.

Rathburn, H. and Hedgcoth, C. (1991). A chimeric open reading frame in the 5' flanking region of coxI mitochondrial DNA from cytoplasmic male-sterile wheat. Plant Mol. Biol., 16 :909-912.

Rathburn, H., Song, J. and Hedgcoth, C. (1993). Cytoplasmic male sterility and fertility restoration in wheat are not associated with rearrangements of mitochondrial DNA in the gene regions for cob, coxII, or coxI. Plant Mol. Biol. 21: 195-201.

Ruzanov, P.V, Evdokimova, V.M., Korneeva, N.L., Heshey, J.W.P. and Ovchinnikov, L.P. (1999). Interaction of the universal mRNA-binding protein, p50, with actin: a possible link between mRNA and microfilaments. J. Cell Sci., 112 :3487-3496.

Satoh, M., Shaheen, V.M., Kao, P.N., Okano, T., Shaw, M., Yoshida, H., Richards, H.B. and Reeves, W.H. (1999). Autoantibodies define a family of proteins

with conserved double-stranded RNA-binding domains as well as DNA binding activity. J. Biol. Chem. 274: 43598-34604.

Shteiman-Kotler, A. and Schuster, G. (2000). RNA-binding characteristics of the chloroplast S1-like ribosomal protein CS1. Nucleic Acids Research. 28: 3310-3315.

Song, J. and Hedgcoth, C. (1994). A chimeric gene (orf256) is expressed as protein only in cytoplasmic male-sterile lines of wheat. Plant Mol. Biol., 26 :535-539.

Soulard, M., Valle, V.D., Siomi, M.C., Pinol-Roma, S., Codogno, P., Bauvy, C., Bellini, M., Lacroix, J., Monod, G., Drefuss, G. and Larsen. C. (1993). hnRNA G : Sequence and characterization of a glycosylated RNA-binding protein. Nucleic Acids Research 21: 4210-4217.

الملخص العربي

بروتينات ميتوكندريات القمح التي ترتبط بجزيئات orf256-coxI RNA

أحمد محمد الشهاوي ، منى محمد الصيحي ، تشارلز هيدجكوس
قسم الكيمياء الحيوية ، جامعة ولاية كانساس ، منهاتن ، كانساس ، أمريكا

الجيـن orf256 متلازم مع ظاهرة العقم الذكري السيتوبلازمي في هجين القمح بين الأنواع Triticum aestivum (*Ta*) / Triticum timopheevi (*Tt*) ويقـع هذا الجين قبل جين cytochrome oxidase I (*coxI*) في DNA ميتوكندريا القمح Tt . لا يوجد الجين في DNA ميتوكندريات القمح Tt ، يتم التعبير عن هذا الجين مع coxI في صورة جزئ واحد من الـ mRNA في نباتات *Tt* والنباتات معادة الخصوبة (FR) والنباتات العقيمة ذكرياً CMS . ينتج جين orf256 بروتيناً ذا وزن جزيئ 7 kD . يوجد جزيئ الـ orf256-coxI RNA الكامل (3.1 Kb) بالإضافة إلى نسخ تبدأ داخل المنطقة الكودية لـ orf256 في نباتات الـ FR , CMS . في نباتات *Tt* توجد كميات أثرية من جزيئات الـ RNA الكاملة بينما معظم الجزيئات تبدأ داخل المنطقة الكودية للـ orf256 وذلك بمنع تكوين أي بروتين من الـ *orf256* . تم استخدام تكنيك Northwestern للبحث عن جزيئات بروتين في ميتوكندريات القمح التي قد ترتبط بالـ *orf256 – coxI* RNA لدراسة ما إذا كان هناك اختلاف في الارتباط تبعاً لمصدر بروتين الميتوكندريات . وجد أن الـ *orf256-coxI* RNA يرتبط بأكثر من ٢٠ نوع بروتين مختلف من بروتينات الميتوكندريات من *Tt* , FR , *Ta* , CMS . وجد أن البروتينات ذات الوزن الجزيئي 42,39 kD توجد فقط في ميتوكندريات النباتات *Ta* . لا ترتبط جزيئات الـ control RNA ببروتينات الميتوكندريات . لا يرتبط الـ *orf256-coxI* RNA ببروتينات السيتوبلازم في حين يرتبط ببروتينات أنوية القمح . إضافة مستخلصات البروتينات إلى تفاعل ارتباط الـ RNA بالبروتين يؤثر في عملية الارتباط .