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Two Tcp-1-related but highly divergent gene families exist in oat encoding proteins of assumed chaperone function

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Tcp-1-related sequences have been isolated from a cDNA library of etiolated 6-day-old oat (*Avena sativa*) seedlings. This attempt was made to obtain cDNAs of a recently published 60 kDa plant chaperone that re-folds denatured phytochrome and which was biochemically characterised as a Tcp-1-related protein [(1993) Nature 363, 644–647]. The translation of the putative coding sequence from one full-length cDNA clone displays no specific homologies to amino acid sequences known from peptide sequencing of the oat 60 kDa chaperone. Antibodies raised against the 60 kDa chaperone and over-expressed protein from one full-length coding sequence for Tcp-1 from oat show no cross-reactivity, whereas a monoclonal antibody raised against mouse Tcp-1 protein recognizes both the 60 kDa protein purified from plant extracts and over-expressed protein from Tcp-1-related cDNA sequences.

cDNA sequence; Tcp-1-related gene family; Cytosolic 60 kDa chaperonin; *Avena sativa*

1. INTRODUCTION

The t-complex polypeptide-1 (Tcp-1) is constitutively expressed in almost all cells in mammals and up-regulated in the testes during spermatogenesis. It is a constituent of the Tcp-1 ring complex (TriC) that seems to be involved in tubulin biogenesis [2–4]. Tcp-1 proteins have been suggested to be the cytosolic hsp60 homologs of mitochondrial and plastidic 60 kDa chaperonins that show homologies to prokaryotic proteins such as GroEL in *E. coli* [5–8].

Structure and in vitro activities of TriC are distinct from the prokaryotic GroEL/ES chaperonins. The complex contains at least 6–7 different but related subunits of Tcp-1 proteins as can be concluded from microsequencing of peptides [2] and 2D gel-electrophoresis [3] and forms a double ring structure each composed of 8–9 subunits [2,3] of between 900–970 kDa [2,4]. The apparent molecular weight of the GroEL complex is about 800 kDa and consists essentially of the homomeric double 7-ring of the 60 kDa protein. This complex is associated with a 7-ring of GroES which is positioned at one end of the double-ring cylinder [9].

Tcp-1-related proteins have also been described in thermophilic bacteria such as *Sulfolobus shibatae* [10]. Mutants lacking these proteins are temperature sensitive, thus the Tcp-related peptide is known as thermophilic factor (TF55). TF55 forms rings of 8–9 subunits, that are assembled into a double doughnut structure, thereby ressembling the eukaryotic TriC structure. It was speculated whether TF55 also plays a role in the formation of cytoskeleton-like structures in the archaebacteria *Sulfolobus shibatae* [10].

Here we present the sequence of an additional Tcp-related protein from oat and discuss the relationship to a second possible 'chaperone machine' [11] from the same plant published recently [1].

2. MATERIALS AND METHODS

2.1. *Growth and treatment of plants*

Oat seedlings (*Avena sativa* cv Pewi) were grown and harvested as described [1].

2.2. *RNA extraction, polymerase chain reaction (PCR) and sequencing procedures*

Total RNA was extracted as described [12] and Poly(A) RNA prepared according to Aviv and Leder [13]. Two oligonucleotides were synthesized as fully degenerated primers corresponding to amino acid sequences TITNDGA and MPKRI respectively, that are consensus sequences in a series of Tcp-1-related sequences [10]. The MPKRI oligo was synthesized as a non-coding strand that was used for reverse transcription of oat Poly(A) RNA with AMV reverse transcriptase (Promega) at 42°C for 45 min. PCR was performed according to protocols for reverse transcription PCR [14]. After 30 cycles (cycle temperatures 92°C/47°C/72°C), the complete reaction mixture was separated on a 1% agarose gel and a DNA fragment of the appropriate size (520 bp) was excised and electro-eluted with a Biotrap (Schleicher & Schuell). The fragment was blunt-ended and cloned into pUC19 (Pharmacia) cut with *Sma*I according to standard protocols [15].

Sequencing was carried out by using a sequencing kit (USB) following the manufacturer's manual, analysing both strands of the PCR product obtained.

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The sequence appearing in this paper and a related sequence are stored under accesion numbers X75777 (ASTCP-K19, this paper) and X75778 (ASTCP-K36), respectively, at the EMBL databank, Heidelberg.

2.3. *Construction of cDNA library and screening procedures*

A cDNA/λZAP library from oat was established using a cDNA kit (Stratagene) following the suggestions of manufacturer's protocol supplied. 10^6 pfu were plated for the screening procedure according to the Stratagene protocol and the 520 bp PCR fragment used as a probe. 36 clones were isolated of which 30 were identified as Tcp-1-related by sequence analysis; 4 of them were full-length clones. 3 of the full-length cDNAs were sequenced in both directions using primers hybridizing with external plasmid and internal cDNA sequences. Subclones of cDNA fragments in pUC18, using a unique *Hin*dIII site in all three cDNAs, were also sequenced.

2.4. *Over-expression of PCR and cDNA fragments*

The 520 bp PCR fragment as well as full-length coding sequences synthesized by PCR from three cDNA clones were ligated into pQE plasmids (QIAGEN) for over-expression of proteins that possessed a His^6 tag at the N-termini. Purification of over-expressed peptides was performed by means of the His^6 tag/Ni^+–Agarose system following the manufacturer's manual (QIAGEN).

2.5. *Antibody production and Western blot analysis*

Purified 60 kDa chaperone from oat extracts [1] was taken to raise antibodies in mice. Antibodies against Tcp-1-related protein were raised in rabbit using over-expressed protein from the 520 bp PCR product containing Tcp-1-related sequences. Western blot analysis was performed following the procedure described by Hofmann et al. [16]

3. RESULTS AND DISCUSSION

We used a PCR-based approach to isolate Tcp-1-related sequences in order to screen for cDNAs from oat coding for a 60 kDa protein with a unique chaperone function possibly related to Tcp-1 [1]. From the four full-length cDNA sequences isolated, three of them (ASTCP-K1, ASTCP-K19, ASTCP-K20) encode the identical amino acid sequence with one clone (ASTCP-K19) showing minor differences in the 5′ and 3′ untranslated region of the transcript sequence. The fourth clone, ASTCP-K36, shows 98.7% similarity and 97.9% identity at the amino acid level compared to the other clones. The sequence of ASTCP-K19 (Fig. 1) encodes a protein consisting of 535 amino acids similar to a sequence from cucumber [17] with a calculated molecu-

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                                                CAGAACCACAGCGCCGCCTCACTCTCCCC   30

CCTTCAGATCAGCTCCCCGCGCGATCTGCCCTCCCAGGAGTTTCTTCAGGGGGGAGCAGCGCC   92

AGCGCTTCGGCGGCC ATG GCG CTC GCC TTC GAC GAG TAC GGG CGG CCC TTC   143
                 M  A  L  A  F  D  E  Y  G  R  P  F                12
ATC ATC CTC AGA GAG CAG GAG AAG AAG TCG CGG CTG CAG GGT CTC GAC   191
 I  I  L  R  E  Q  E  K  K  S  R  L  Q  G  L  D                  28
GCG CAC AAG GCC AAC ATC GCC GCC GCC AAG GCT ATC GCG CGC ATC CTC   239
 A  H  K  A  N  I  A  A  A  K  A  I  A  R  I  L                  44
CGC ACG TCC CTT GGG CCC AAG GGC ATG GAC AAG ATG CTC CAG TCC CCC   287
 R  T  S  L  G  P  K  G  M  D  K  M  L  Q  S  P                  60
GAC GGC GAC GTT ACC ATC ACT AAT GAC GGG GCG ACC ATC CTG GAG CTA   335
 D  G  D  V  T  I  T  N  D  G  A  T  I  L  E  L                  76
ATG GAC GTT GAC AAC CAG ATT GCA AAG CTG TTG GTG GAG TTG TCA CGC   383
 M  D  V  D  N  Q  I  A  K  L  L  V  E  L  S  R                  92
AGT CAA GAC TAT GAC ATT GGA GAT GGT ACC ACT GGA GTG GTG GTC ATG   431
 S  Q  D  Y  D  I  G  D  G  T  T  G  V  V  V  M                  108
GCT GGG GCT CTC CTA GAG CAG GCT GAG AAG CTA CTG GAA CGT GGT ATT   479
 A  G  A  L  L  E  Q  A  E  K  L  L  E  R  G  I                  124
CAC CCA ATA AGG GTT GCT GAA GGT TAT GAG ATG GCG TCA AGG ATA GCT   527
 H  P  I  R  V  A  E  G  Y  E  M  A  S  R  I  A                  140
GTT GAT CAC CTT GAA AGC ATC TCC ACG AAA TAT GAG TTC AGT GCC ACA   575
 V  D  H  L  E  S  I  S  T  K  Y  E  F  S  A  T                  156
GAC ATT GAG CCT TTG GTG CAG ACC TGC ATG ACA ACT TTG TCC TCA AAG   623
 D  I  E  P  L  V  Q  T  C  M  T  T  L  S  S  K                  172
ATT GTT AGC CGT TGT AAG CGG GCC CTA GCT GAG ATT TCT GTC AAA GCA   671
 I  V  S  R  C  K  R  A  L  A  E  I  S  V  K  A                  188
GTC CTT GCG GTT GCT GAT TTG GAA AGG AAG GAT GTA AAT CTG GAC TTG   719
 V  L  A  V  A  D  L  E  R  K  D  V  N  L  D  L                  204
ATT AAG GTG GAA GGC AAG GTT GGT GGG AAG CTA GAG GAT ACT GAG CTA   767
 I  K  V  E  G  K  V  G  G  K  L  E  D  T  E  L                  220
GTG GAA GGA ATC ATT GTT GAC AAA GAT ATG AGC CAC CCC CAA ATG CCA   815
 V  E  G  I  I  V  D  K  D  M  S  H  P  Q  M  P                  236
AAG AGA ATC TAT GAT GCT CAC ATT GCC ATT CTG ACC TGC CCA TTT GAG   863
 K  R  I  Y  D  A  H  I  A  I  L  T  C  P  F  E                  252
CCC CCG AAG CCC AAG ACC AAG CAT AAG GTT GAC ATT GAC ACT GTA GAG   911
 P  P  K  P  K  T  K  H  K  V  D  I  D  T  V  E                  268
AAA TTC CAG ACA CTG CGT GGG CAA GAG CAG AAA TAC TTC GAT GAG ATG   959
 K  F  Q  T  L  R  G  Q  E  Q  K  Y  F  D  E  M                  284

GTT CAG AAA TGC AAG GAT GTT GGT GCA ACC CTA GTT ATT TGT CAA TGG 1007
 V  Q  K  C  K  D  V  G  A  T  L  V  I  C  Q  W                  300
GGT TTT GAT GAT GAA GCC AAT CAT TTA CTA ATG CAA AGA GAA CTT CCT 1055
 G  F  D  D  E  A  N  H  L  L  M  Q  R  E  L  P                  316
GCT GTC AGA TGG GTT GGT GGT GTT GAA TTA GAA TTA ATT GCC ATT GCT 1103
 A  V  R  W  V  G  G  V  E  L  E  L  I  A  I  A                  332
ACA GGT GGG AGG ATT GTT CCG AGA TTC CAA GAG TTG AGT ACT GAA AAG 1151
 T  G  G  R  I  V  P  R  F  Q  E  L  S  T  E  K                  348
CTT GGG AAG GCT GGA TTA GTC AGA GAG AAG TCA TTT GGA ACA ACA AAA 1199
 L  G  K  A  G  L  V  R  E  K  S  F  G  T  T  K                  364
GAT CGG ATG CTT TAC ATC GAA AAG TGT GCC AAT TCC AAA GCT GTA ACT 1247
 D  R  M  L  Y  I  E  K  C  A  N  S  K  A  V  T                  380
ATT TTC ATC CGT GGA GGT AAC AAA ATG ATG ATT GAG GAG ACC AAG CGA 1295
 I  F  I  R  G  G  N  K  M  M  I  E  E  T  K  R                  396
AGT ATT CAT GAT GCT CTT TGT GTT GCA AGG AAT CTC ATC ATC AAC AAC 1343
 S  I  H  D  A  L  C  V  A  R  N  L  I  I  N  N                  412
TCA ATC GTG TAT GGT GGT GGT TCA GCA GAG ATA TCT TGC TCG ATT GCT 1391
 S  I  V  Y  G  G  G  S  A  E  I  S  C  S  I  A                  428
GTT GAA GCT GCT GCT GAT CGG CAT CCT GGA GTT GAG CAG TAT GCA ATC 1439
 V  E  A  A  A  D  R  H  P  G  V  E  Q  Y  A  I                  444
AGG GCA TTT GCT GAT GCT TTG GAT GCT ATT CCA CTG GCT TTA GCT GAA 1487
 R  A  F  A  D  A  L  D  A  I  P  L  A  L  A  E                  460
AAC AGT GGT TTG CCA CCT ATT GAT ACT CTG ACA GTG GTA AAA TCC CAA 1535
 N  S  G  L  P  P  I  D  T  L  T  V  V  K  S  Q                  476
CAT GTC AAG GAG AAC AAT TCC CGC TGC GGC ATT GAC TGC AAC GAT GTG 1583
 H  V  K  E  N  N  S  R  C  G  I  D  C  N  D  V                  492
GGT ACC AAT GAC ATG AAA GAG CAG AAT GTT TTT GAA ACT CTG ATT GGC 1631
 G  T  N  D  M  K  E  Q  N  V  F  E  T  L  I  G                  508
AAG CAG CAG CAG ATC TTG CTG GCA ACC CAG GTC GTC AAG ATG ATC CTC 1679
 K  Q  Q  Q  I  L  L  A  T  Q  V  V  K  M  I  L                  524
AAG ATC GAT GAT GTT ATC ACG CCT TCC GAA TAT TGA CGATGCCTGCAGAGC 1730
 K  I  D  D  V  I  T  P  S  E  Y  *                              535
ATGAATCGACTGTGCTAGGTGACATACGCTCCAGTAGGCAGCTGCATGGGTTGATTTGGATTT 1793

GCTCCTAGAATTCTGACACTGAGTGGTCGCTTTGTTTTGTTTGACGAGCTATATGGTTTTGTG 1856

CGCTCGCTTGATCATTATAACATTGGTGTTCACACTCTGTACAATAGGACAAATCCTGTCGAT 1919

ACCAGTTTAGTCCTTTGTTGTATTGTTTGGGATGCTTAATGCTATTGTCTGGATCATTATTTT 1982

GTCTGCTTTGAAGTAAGTGTTACATTTTTTTTAAAAAAAAAAAAAAAAAAAAAAAAA      2039
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Fig. 1. Nucleotide and deduced amino acid sequence of ASTCP-K19. Underlined amino acid sequence indicates a conserved motif within the putative nucleotide binding domain.

Table I

Amino acid sequence comparison of selected Tcp-1-related proteins

	Sulfolobus shibatae TF55	*Arabidopsis* Tcp-1-related protein	Yeast Tcp-1-related protein	Mouse Tcp-1 protein
Avena sativa ASTCP-K19 Tcp-1-related	60.6% Sim. 40.0% Id.	53.5% Sim. 33.1% Id.	54.1% Sim. 33.0% Id.	53.5% Sim. 33.2% Id.
Sulfolobus shibatae TF55		61.2% Sim. 37.4% Id.	59.6% Sim. 36.8% Id.	61.9% Sim. 39.5% Id.
Arabidopsis Tcp-1-related protein			78.4% Sim. 61.0% Id.	80.2% Sim. 65.8% Id.
Yeast Tcp-1-related protein				78.0% Sim. 62.3% Id.

Similarities include identical amino acids and conservative exchanges. The program was run at EMBL Heidelberg. Amino acids were taken from published nucleic acid sequences (ASTCP-K19, this paper; TF55 [10]; *Arabidopsis* [18]; yeast [21]; mouse [22]). Sim, similarity; Id, identity.

lar mass of 58.9 kDa. Another known Tcp-1-related cDNA sequence from a plant, *Arabidopsis thaliana* [18], predicts a deduced amino acid sequence of 545 amino acids.

Table I shows the homologies between selected Tcp-1-related amino acid sequences and ASTCP-K19. The values were calculated by the HUSAR program package at the DKFZ, Heidelberg, connected to the EMBL databank (Heidelberg). While mammalian sequences have more than 96% identities to each other and more than 60% identity with *Drosophila melanogaster* and yeast orthologues [3], the *Avena* sequence shows comparably low homologies, ranging from about 33% identitiy to mammalian, yeast and *Arabidopsis* sequences up to 40% in case of TF55. Therefore the sequence from oat seems to be a distinct type of Tcp-1 and is predominantly related to TF55.

All Tcp-1 sequences encode a putative nucleotide binding site in the N-terminal region, that shows striking homology to the cyclic AMP-dependent protein kinase (Fig. 1; [3,19,]). The hydrolysis of ATP and other nucleotides by TriC from bovine testes containing Tcp-1 has been demonstrated [2] and is a characteristic feature of molecular chaperones. A comparison of Tcp-1-related sequences to GroEL, mitochondrial hsp60 and rubisco-binding proteins – all chaperonins of about 60 kDa – displays this conserved nucleotide binding domain also in the organellar and bacterial amino acid sequences [2], which includes the absolutely conserved amino acid sequence motif, GDGTT (Fig. 1; [2]).

We compared the immunological relationship between the Tcp-1 protein from oat with a 60 kDa protein from the same plant that re-folds phytochrome [1]. Fig. 2 shows the Western blot of crude protein extract from oat seedlings, purified 60 kDa oat chaperone, over-expressed Tcp-1 full-length polypeptide and over-expressed GroEL protein. Antibodies raised against the 60 kDa chaperone does not cross-react with Tcp-1 and GroEL (Fig. 2A), while antibody against oat Tcp-1 protein does not recognize the 60 kDa protein or GroEL (Fig. 2B). In plant crude extracts, Tcp-1 protein is not detectable under the physiological conditions chosen (Fig. 2B), while the 60 kDa chaperone is abundant in etiolated oat seedlings (Fig. 2A). Fig. 2D shows the cross-reaction of monoclonal antibody 91A against mouse Tcp-1 [20] with both oat proteins being recognized, while no signal is detectable in the case of GroEL. The size of over-expressed oat Tcp-1 protein seems to be more than 60 kDa (Fig. 2C), but the lower migration rate might be due to the His^6 tag at the N-terminus of the polypeptide.

Obviously, the two oat proteins are related to each other, as shown by the cross-reaction with monoclonal antibody 91A (Fig. 2D). On the other hand, the amino acid sequences of the oat chaperone published by Mummert et al. [1] show no significant homologies to the Tcp-1-related sequence from the same plant. We assume that the Tcp-1-related protein is not part of the oligomer formed by the 60 kDa polypeptide, at least not in a stoichometric proportion as in mammalian extracts [2]. We suggest, therefore, that different Tcp-1-related cytosolic chaperone complexes exist in oat. Whether TriC-like heterooligomers appear in plants similar to mammalian species has still to be shown.

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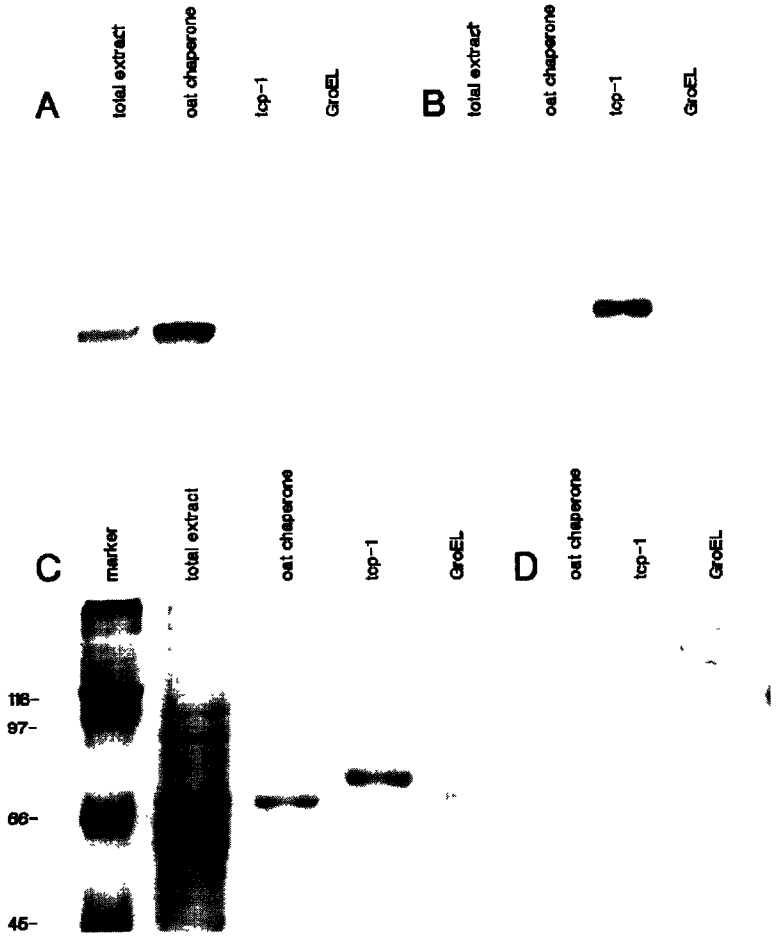

Fig. 2. Western blot analysis using antibodies against 60 kDa chaperone from oat, Tcp-1-related protein from oat and a monoclonal antibody against mouse Tcp-1 (91A; [20]). Gels were run with total protein extract from 6-day-old etiolated oat seedlings (total extract), purified oat chaperone (oat chaperone), over-expressed Tcp-1 protein (Tcp-1) and GroEL. (Fig. 2A–C, respectively; Fig. 2D was without total extract). Blotted filters were incubated with antibodies against oat chaperone (A), oat Tcp-1 protein (B) or monoclonal antibody 91A (D), respectively. Fig. 2C shows the Coomassie-stained gel including size marker (marker).

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