$CcmF_C$ involved in cytochrome *c* maturation is present in a large sized complex in wheat mitochondria

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Received 22 December 2003; revised 23 January 2004; accepted 10 March 2004

First published online 19 March 2004

Edited by Vladimir Skulachev

Abstract In land plant mitochondria, *c*-type cytochromes are assembled via a mechanism similar to that found in Gram-negative bacteria and different from that used by mitochondria from other eukaryotes. The wheat mitochondrial genome encodes CCM (for cytochrome *c* maturation) proteins, among them $CcmF_C$, a protein similar to the C-terminal part of the bacterial CcmF. The gene is transcribed into a 1.7 kb transcript at steady state. However, the 3′ termini of the transcript were found to occur upstream of the deduced gene termination codon. This discrepancy was solved by RNA editing that introduces a novel termination codon, thus shortening the reading frame by 27 codons. The processed transcript is translated into a protein integrated in the mitochondrial inner membrane. We also show that the protein is part of a large (700 kDa) protein complex, that possibly represents a cytochrome *c* assembly complex.

Key words: Plant mitochondrion; Cytochrome *c* biogenesis; Gene expression; RNA editing; Blue native

1. Introduction

c-type cytochromes are essential components of the mitochondrial electron transport chain that is responsible for the generation of cellular energy. These key proteins are present as soluble proteins, i.e. cytochrome *c*, in the mitochondrial intermembrane space but also as intrinsic proteins, i.e. cytochrome c_1, integrated into complex III, the cytochrome *c* reductase. Recently, interest in the much studied cytochrome *c* has been renewed with the discovery of its involvement in programmed cell death in animals e.g. [1] as well as in plants [2,3]. *c*-type cytochromes are distinguished from other cytochromes by the covalent attachment of heme to the apoprotein, via two thioether bonds between vinyl groups on the heme and conserved cysteines on the protein. There is an ongoing interest in understanding the assembly mechanism leading to the functional holoprotein. In yeast and animal mitochondria, a maturation process has evolved involving one or two cytochrome *c* heme lyases (CCHL) [4,5]. In bacteria two different systems were found for the assembly of *c*-type cytochromes: system I in Gram-negative bacteria and system II in Gram-positive bacteria [5,6]. Surprisingly, CCHL genes were not found in plant genomes, contrary to other investigated eukaryote genomes. It appears that plant mitochondria have retained an ancestral prokaryotic system similar to the bacterial system I [5]. Genes encoding proteins similar to bacterial cytochrome *c* maturation (CCM) proteins were found in both the nuclear and the mitochondrial genomes of plants, e.g. [7,8]. Together with ribosomal protein genes the presence of *ccm* genes differentiates the gene content of plant mitochondrial genomes from their animal counterparts [9]. Transcription and RNA editing [10] were observed for all *ccm* genes in *Arabidopsis* [11], in rapeseed [12] and in rice [13,14] and for some genes in various other plants. In addition translation of *ccm* genes was shown for some wheat proteins, i.e. for CcmB [15] and for $CcmF_N$ [16], a protein similar to the N-terminal part of the bacterial protein CcmF [17]. The protein described here, $CcmF_C$, is similar to the C-terminal region of CcmF. The proposed function of this protein would be to trigger the ligation of the heme to the apoprotein [18]. Here, we report the identification of $ccmF_C$, its transcription, the mapping of the transcript ends, RNA editing and the expression of its protein in wheat mitochondria. The protein is integrated in the mitochondrial inner membrane and found in a large sized protein complex.

2. Materials and methods

2.1. Wheat mitochondria extraction and fractionation

Mitochondria were extracted from 10 days old wheat (*Triticum aestivum*) etiolated leaves as described previously [19]. For mitoplast preparation, mitochondria were swollen in a solution containing 5 mM potassium phosphate buffer pH 7.2 with 1 mg of mitochondrial protein per 1 ml of buffer, on ice for 10 min. The suspension was loaded onto a step gradient composed of 60, 32 and 15% (w/v) sucrose steps in a buffer containing 10 mM MOPS pH 7.2 and 1 mM ethylenediamine tetraacetic acid (EDTA). Gradients were centrifuged at $92\,000\times g$ for 1 h at 4°C. Mitoplasts were collected at the 60/32% interphase and washed in a buffer containing 0.25 M sucrose, 50 mM Tris–HCl pH 7.5 and 3 mM EDTA and centrifuged at $18\,000\times g$ for 10 min at 4°C. The mitoplast pellet was resuspended in 0.1 M sodium phosphate buffer pH 7.5 at a concentration of 1 mg of protein per 1 ml of buffer. Mitoplasts were disrupted by three freeze/thaw cycles followed by three sonication bursts of 5 s at 300 W (Sonic Vibra cells). The lysate was centrifuged at $100\,000\times g$ for 30 min at 4°C. The pellet was kept as the inner membrane protein fraction and the supernatant as the matrix protein fraction.

2.2. Genomic and cDNA cloning and sequencing

An oligonucleotide specific from *Oenothera berteriana orf454* obtained from W. Schuster (Berlin, Germany) was used to screen a library of wheat *Sal*I mtDNA clones obtained from F. Quétier and

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Abbreviations: BN, blue native; CCM, cytochrome *c* maturation

0014-5793/04/$30.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(04)00291-1

B. Lejeune (Université Paris XI, France). A positive clone carrying *ccmF$_C$* was identified, subcloned and sequenced. The cDNA sequence covering *ccmF$_C$* coding region was amplified from wheat mitochondrial RNA by reverse transcription-polymerase chain reaction (RT-PCR) with the oligonucleotides O1 GAA CGGATCCAGCAGATGGT and O2 TTGAACCTTTTATGCAATTATGAACG. Sequencing was performed by the dideoxynucleotide chain termination method and analysed on an 'Applied Biosystems 373 DNA sequencer' (Perkin Elmer). *ccmF$_C$* genomic and cDNA sequences were deposited at GenBank under the accession numbers AY500223 and AY500224.

2.3. Transcript detection

RNA was extracted from wheat mitochondria as previously described [19]. Northern blot hybridisations were performed using standard methods [20]. Radiolabelled oligonucleotides O3 ACTTTCCCTATGGTTTTCGAACGTT and O4 GAGATGGGGAGACCC were used to probe Hybond-N membranes (Amersham) blotted with wheat mtRNA.

2.4. Transcript 3′ termini mapping

The transcript 3′ termini were determined by RNase protection. An RNA probe covering a region 202 nucleotides before and 50 nucleotides after the stop codon deduced from the genomic sequence was synthesised from a PCR product containing the T7 RNA polymerase promoter. 30 μg of mitochondrial RNA and 100 000 cpm of the RNA radiolabelled probe were ethanol precipitated and resuspended in 4 μl of hybridisation buffer containing 2 M NaCl, 50 mM PIPES–NaOH pH 6.4 and 16 μl formamide. The solution was denatured for 3 min at 75°C and hybridisation was carried out for 3 h at 50°C. 300 μl of a solution containing 10 mM Tris–HCl pH 7.5, 5 mM EDTA, 300 mM NaCl, 4 μg/ml RNase A and 480 U of RNase T1 was added. RNase digestion was performed for 1 h at 30°C. RNases were inactivated for 15 min at 37°C by adding 20 μl of sodium dodecyl sulphate (SDS) 10% and 4 μl of proteinase K (10 mg/ml). The remaining double strand RNA hybrid was extracted with phenol/chloroform, ethanol precipitated and analysed on a sequencing gel to determine its size.

2.5. Western blot immunoassays

Wheat CcmF$_C$ specific antibodies were raised against the peptide GGSRSLLRQLQKDKDKLHWN coupled with ovalbumin. Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon-N membranes (Millipore). Western blots were performed with rabbit antibodies against wheat CcmF$_C$, wheat Nad9 [21] and tobacco MnSOD [22] (obtained from F. van Breugesem, Gent, Belgium) at dilutions of 1/10 000. Goat anti-rabbit antibodies conjugated with horseradish peroxidase were used as secondary antibodies and revealed with enhanced chemiluminescent reagents.

2.6. Blue native (BN) PAGE

A mitochondrial membrane pellet was resuspended in ACA750 buffer containing 750 mM amino dicaproic acid, 50 mM bis-Tris pH 7.0 and 0.5 mM Na_2EDTA. Complexes were solubilised by adding *n*-dodecyl-maltoside (1 g detergent/1 g protein) and by pipetting for 30 min on ice. The resulting solution was centrifuged at 100 000 × *g* for 15 min at 4°C and 5% (v/v) Serva blue solution (750 mM ACA750 solution, 5% (w/v) Serva Blue G250) was added to the supernatant. For the first dimension, 500 μg of mitochondrial protein complexes were separated on 5–13% acrylamide gradient gels (in 0.5 M amino dicaproic acid, 50 mM bis-Tris pH 7.0). Electrophoresis was carried out for 9 h at 5 mA with 50 mM bis-Tris pH 7.0 anode buffer and 50 mM Tricine, 15 mM bis-Tris pH 7.0, 0.02% (w/v) Coomassie blue G250, cathode buffer. Gel lanes were excised and denatured for 1 h at room temperature in 50 mM Tris–HCl pH 6.8, 1% (w/v) SDS and 1% (v/v) β-mercaptoethanol. For the second dimension, subunits of the various complexes were separated by 12% SDS–PAGE.

3. Results and discussion

3.1. Structure of the ccmF$_C$ gene

Numerous genes involved in CCM have been characterised in higher plant mitochondrial genomes, e.g. [7]. In wheat particularly, three open reading frames of 206 (*ccmB*) [15], 240 (*ccmC*) [19] and 575 (*ccmF$_N$*) [16] codons were found. The latter gene encodes a protein homologous to the N-terminal part of the bacterial CcmF [17]. Therefore, a gene (*ccmF$_C$*) encoding the C-terminal part of CcmF was searched for. Such a gene, *orf454*, had been characterised for *Oenothera* [23]. An *orf454* specific oligonucleotide was used as a probe to identify clones covering the homologous sequence in a wheat mitochondria genomic library. A 11.7 kb *Sal*I fragment was found to contain the *ccmF$_C$* wheat gene. It is composed of two exons, 754 and 636 nucleotides long respectively, interrupted by a single intron, making a 464 codon open reading frame (Fig. 1A). The 1011 nucleotide long intron in *ccmF$_C$* belongs to the autocatalytic group IIb intron family [24]. The finding of an intron of this class is not surprising since all introns in higher plant mitochondria, with one notable exception [25], were found to be group II introns.

3.2. ccmF$_C$ is expressed as a 1.7 kb transcript at steady state

Northern blots of wheat total mitochondrial RNA were hybridised with various *ccmF$_C$* specific probes. Hybridisation of an oligonucleotide specific to the *ccmF$_C$* 5′ untranslated region (UTR) detected a major 1.7 kb transcript together with two transcripts of 1.9 and 2.2 kb (Fig. 1B). Since the hybridisation of another oligonucleotide specific to the intron sequence did not detect any signal, the transcripts previously detected are assumed to be spliced. The two minor bands could represent transcripts at different 5′ maturation stages. When considering the steady state 1.7 kb transcript, it was deduced that the 5′ and 3′ UTRs constitute approximately 300 nucleotides. In order to localise the transcript termini more precisely, 5′ and 3′ mapping experiments were performed.

3.3. Mapping of the transcript ends

The transcript 3′ end was determined by RNase protection experiments (Fig. 1C). An RNA probe of 280 nucleotides, overlapping the mRNA 3′ end was produced. The only fragments protected from RNase digestion had a size of 153 and 154 nucleotides. It was therefore calculated that the transcript termini are situated 45 and 46 nucleotides before the termination codon deduced directly from the DNA sequence. No other longer fragment, i.e. corresponding to 3′ UTRs extending beyond this codon was detected. In order to determine the transcript 5′ termini, an oligonucleotide complementary to the 5′ UTR was used in primer extension experiments. Several bands corresponding to 5′ termini were detected, the main signals represent positions 234, 252, 270 and 598 nucleotides before the initiation codon (data not shown). These would make transcripts with sizes varying from 1600 to 1900 nucleotides. It is likely, as already observed for plant mitochondria [26,27], that several transcript initiation sites as well as transcript 5′ maturation sites could exist for *ccmF$_C$*. The 3′ end mapping result was a strong indication that RNA editing would create a novel termination codon in the transcript.

3.4. RNA editing shortens the ccmF$_C$ reading frame

12 cDNA clones covering the full length of the *ccmF$_C$* coding region were produced by RT-PCR and analysed by DNA sequencing. Editing was only considered when the modifications were observed in at least two cDNA sequences. The two exons of *ccmF$_C$* were modified by 27 C to U conversions. 21 sites are present in all the cDNAs investigated, six therefore being partial editing sites. 7 sites were found to be silent due

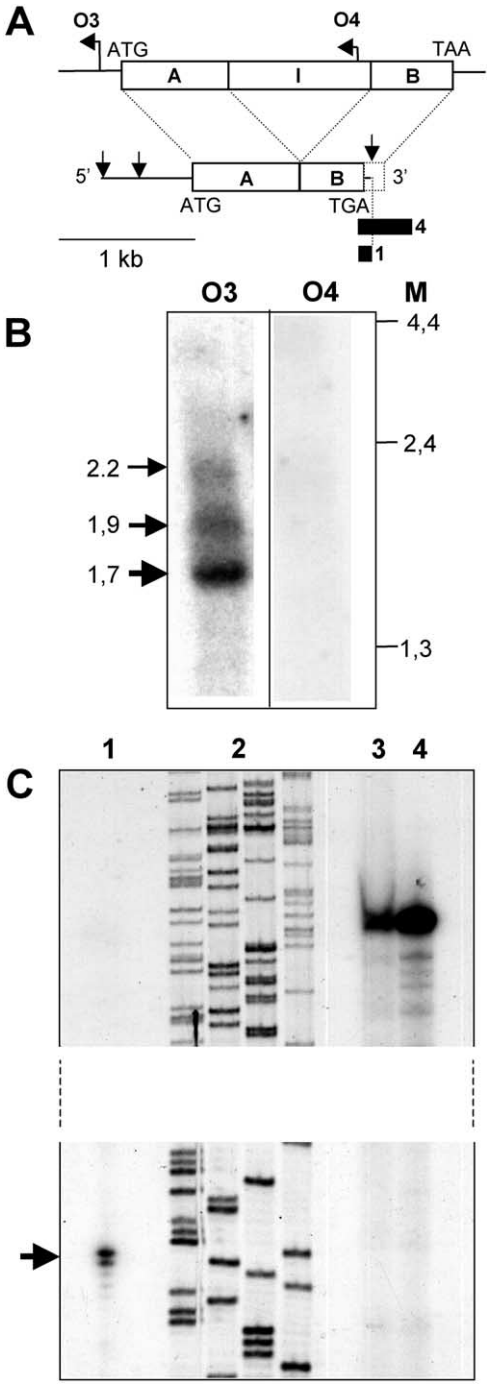

Fig. 1. *ccmF*$_C$ transcript and mapping of its ends. A shows the map of the gene and its transcript with the positions of exons A and B separated by a group II intron (I). Horizontal arrows in A represent the position and orientation of the oligonucleotides O3 and O4 used in Northern blot experiments (B), vertical arrows indicate the main signals observed in primer extension experiments (for 5′ end mapping) and the transcript termini found in 3′ by an RNase protection experiment (C). The black boxes give the positions of the RNA probe before (4) and after (1) RNase treatment in the RNase protection experiment. In B, horizontal arrows locate the main signals detected in Northern blot experiments. M gives the molecular weight marker in kb. In C, 1 shows the fragments protected from RNases, 2 is a DNA sequence used as a size standard, 3 is a control experiment done without RNases and 4 is the 280 nucleotides RNA probe. The arrow shows the 153 and 154 nucleotides long fragments protected from RNAses.

to modifications on the wobble position of the codons. RNA editing is accountable for the modification of 4.1% of the amino acids present in CcmF$_C$. The most notable consequence of editing in this case is the creation of a novel UGA termination codon (seen in all the cDNA clones analysed) thus shortening the protein by 27 amino acids in a way similar to *Oenothera* [23] and *Arabidopsis* [11]. These modifications highlight the importance of RNA editing to get a correct protein and to increase similarity between homologous proteins.

3.5. Comparison of CcmF$_C$ with homologous proteins

The protein sequence deduced from *ccmF*$_C$ edited cDNA is 437 amino acids long, with a calculated mass of 49.6 kDa. It encodes a very basic and hydrophobic protein with a p*I* of 10.23 and 36.8% hydrophobic residues. CcmF$_C$ presents high sequence similarities with other plant mitochondrial proteins such as *Arabidopsis* CcmF$_C$ (82% identity) (Fig. 2). CcmF$_C$ has also a similarity, albeit lower, to the C-terminal domain of several bacterial proteins. These include *Rhodobacter capsulatus* Ccl1 [28], *Escherichia coli* CcmF and NrfE [17] and *Bradyrhizobium japonicum* cycK [29]. In the bacteria *R. capsulatus*, CcmF topology has been investigated [30]. 11 transmembrane domains were found, the final six are present in the region homologous to plants CcmF$_C$. Protein topology models obtained with Toppred (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html) predict that mitochondrial CcmF$_C$ possess the six transmembrane domains (i.e. TM1–6). The alignment of plant mitochondrial and bacterial protein sequences has identified some conserved domains (Fig. 2), especially in the N- and C-terminal parts of the proteins, e.g. some strictly conserved amino acids are found in TM1, 2 and 6. The major sequence disparity resides in the presence in plants of large insertions between TM4 and 5 and TM5 and 6 oriented toward the matrix and the intermembrane space respectively. The nature of the strictly conserved residues especially 11 structurally important glycines and three prolines may be crucial for the structure and function of these proteins.

3.6. CcmF$_C$ is translated and located in the mitochondrial inner membrane

CcmF$_C$ specific antibodies detected two bands of apparent molecular weights of 55 and 60 kDa in mitochondria, mitoplasts and the inner membrane fractions (Fig. 3). The high hydrophobicity of CcmF$_C$ could be responsible for the discrepancy observed between the apparent and the calculated molecular weight. Since the editing site introducing the novel termination codon was found in all the cDNA clones investigated, it is very unlikely that the two bands could correspond to proteins translated from edited and partially edited transcripts. On the other hand, the upper band could be the CcmF$_C$ specific signal and the lower band a degradation product. It is, however, doubtful that degradation occurs during sample preparation since the lower band was not enriched in the final inner membrane fraction. The detection of two forms of a Ccm protein is not unique to CcmF$_C$; e.g. two forms of CcmE have also been observed [8]. The presence of both proteins in the mitoplast but not in the matrix fraction nevertheless clearly indicated that the two proteins detected were present in the mitochondrial inner membrane. To test the quality of the fractionation, blots were also probed with anti-

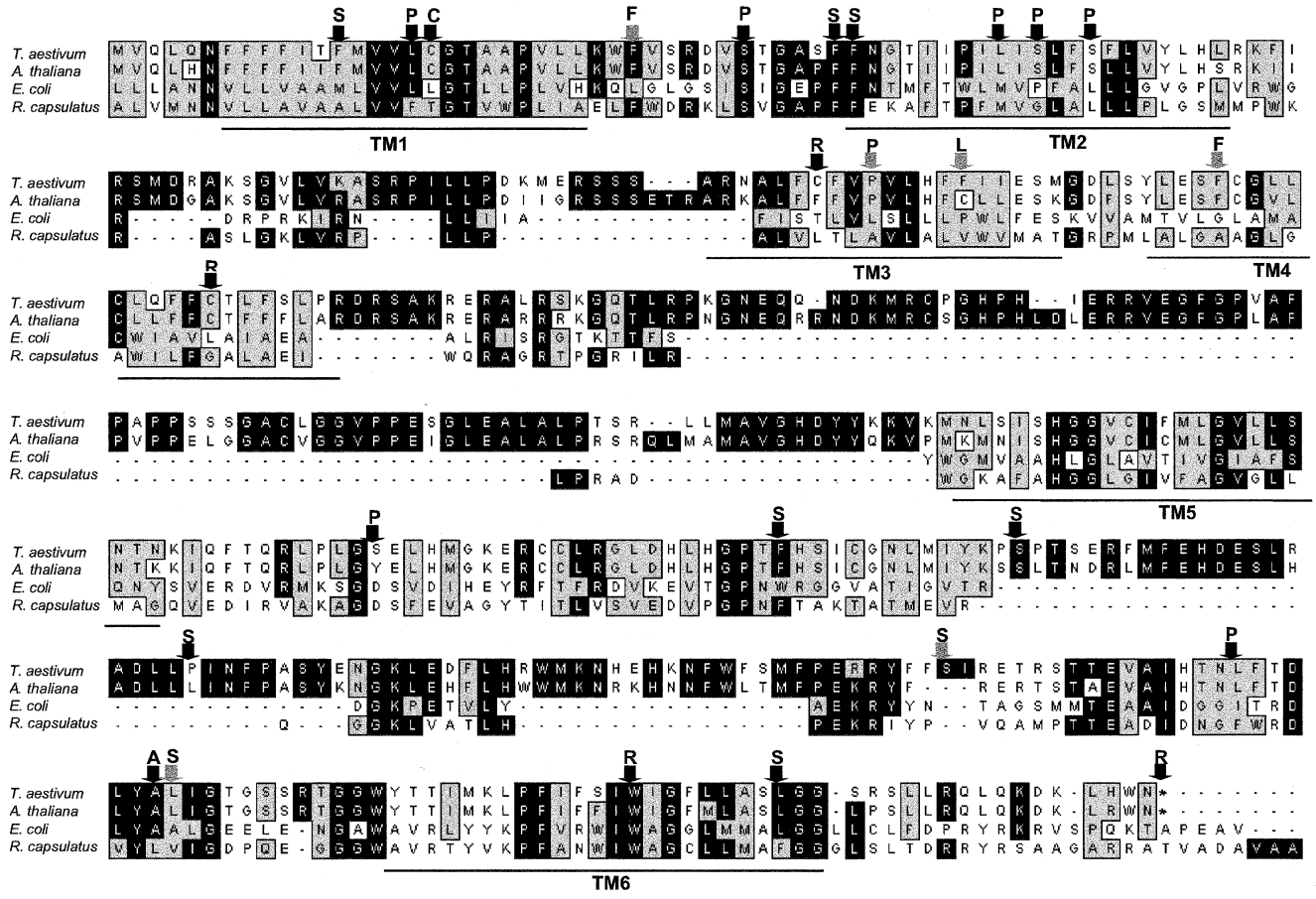

Fig. 2. Protein sequence alignment of *T. aestivum* and *Arabidopsis thaliana* $CcmF_C$, *E. coli* CcmF from residues 351 to 648 and *R. capsulatus* CcmF from residues 350 to 652. Identical amino acids in at least three out of four sequences are boxed in black whereas similar amino acids are boxed in grey. Black lines show the positions of the predicted transmembrane domains (TM1–6) for wheat $CcmF_C$. The black and grey arrows show fully edited and partially edited sites respectively for wheat with the induced amino acid transitions.

bodies raised against the mitochondrial inner membrane protein Nad9 [21] and against the matrix protein MnSOD [22] (Fig. 3).

3.7. $CcmF_C$ is part of a large sized protein complex

In order to get clues towards the understanding of the function of $CcmF_C$ and its position in mitochondria, its presence was investigated in protein complexes by BN PAGE [31–33]. BN gels and BN/SDS–PAGE two-dimensional gels were blotted and probed with $CcmF_C$ specific antibodies. For two-dimensional gels, the antibodies detected $CcmF_C$ bands of apparent molecular weights of 55 and 60 kDa belonging to a protein complex of an apparent size of 700 kDa (Fig. 4). The finding of both bands in a single complex is a good indication that the bands represent two forms of the same protein. The antibodies could not detect any signal from one-dimensional BN gels, indicating that the epitope representing the final 20 amino acids of $CcmF_C$ is hidden in the native complex. The precise nature and protein content of the 700 kDa complex remains unknown. A possible cytochrome *c* assembly complex taking into account a $A_2B_2CEF_NF_CH$ stoichiometry, with the calculated sizes of the identified CCM gene products in plant mitochondria, i.e. 28 kDa for CCMA (N. Rayapuram, unpublished data), 22 kDa for CcmB [15], 27 kDa for CcmC [19], 65 kDa for $CcmF_N$ [16], 50 kDa for $CcmF_C$, 27 kDa for CCME [8] and 20 kDa for

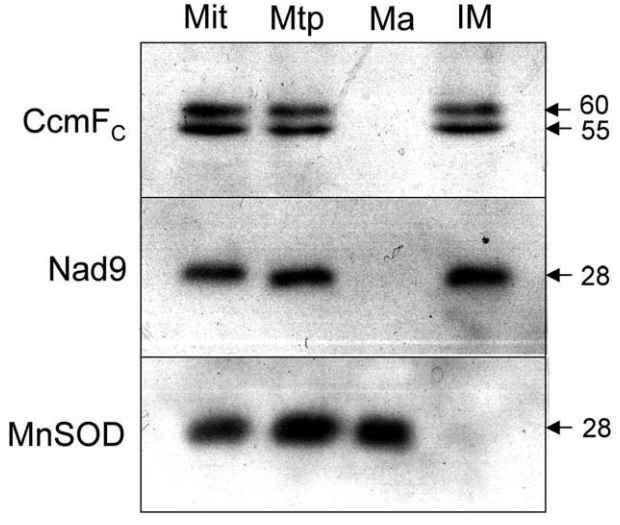

Fig. 3. Western blot immunodetections of $CcmF_C$, Nad9 and MnSOD on wheat mitochondria (Mit), mitoplasts (Mtp), matrix proteins (Ma) and inner membrane proteins (IM) fractions (20 μg). Arrows indicate the apparent molecular weights of the signals detected in kDa.

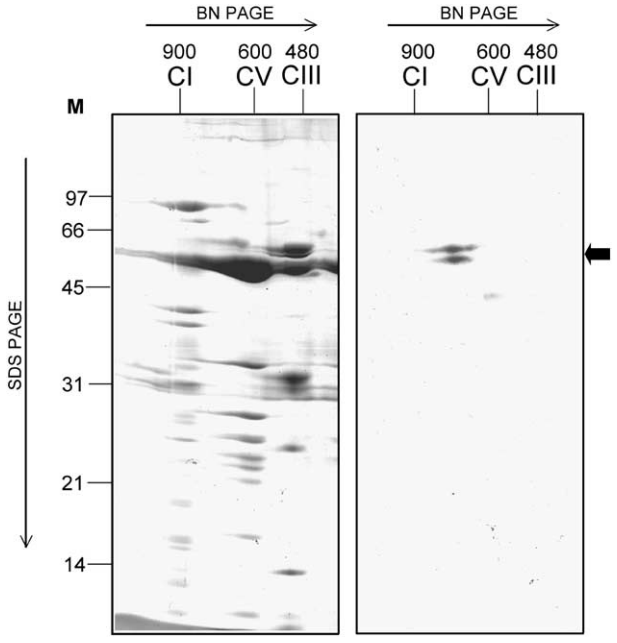

Fig. 4. BN/SDS–PAGE two-dimensional gels of wheat mitochondrial membrane proteins stained with colloidal Coomassie (on the left), blotted and immunodetected with $CcmF_C$ antibodies on the right. The black arrow shows the two bands detected. CI, CIII and CV indicate the respiratory chain complexes I, III and V with their approximate sizes as already identified in previous studies [33,36]. M gives the molecular weight marker in kDa.

CCMH (E. Meyer, unpublished data) would make a total of 289 kDa. This is still some way short of the observed complex of 700 kDa. This difference could be due to a different stoichiometry of the identified CCM proteins. Moreover, the homologues of other CCM proteins found in bacteria remain unidentified in plant mitochondria, i.e. CcmD and G [34,35]. Plants may also possess further plant specific CCM proteins. Alternatively, CCM proteins may be associated with other complexes such as respiratory chain complexes, thus potentially directly providing assembled *c*-type cytochromes to their site of activity.

Acknowledgements: This work was supported by the Centre National de la Recherche Scientifique. P.G. was supported by a MC-ERG grant from the European Union. N.R. was supported by CEFIPRA grant 2003-4. E.H.M. was supported by a fellowship from the French Ministère de la Recherche et de la Technologie. We thank A. Klein for technical assistance and Dr. L.J. Sweetlove for critical reading.

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