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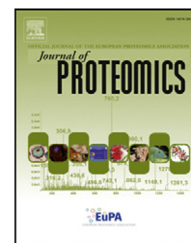

Analysis of the mitochondrial proteome in cytoplasmic male-sterile and male-fertile beets

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The reported analyses were aimed at identification of the mitochondrial proteome features which were associated with cytoplasmic male sterility (CMS) in beets. The set of analyzed accessions included CMS, maintainer and restored lines. Mitochondrial preparations were subjected to blue-native electrophoresis followed either by in-gel activity assays or separation in denaturing conditions. The CMS condition was associated with decreased activity of complex V and enhancement of additional complexes with the ATPase activity. This was accompanied by accumulation of heptamer HSP60, preSATP6 and an increase in the fraction of the free ATP9 oligomer (not bound to complex V). The ATP9 effect was reversed upon fertility restoration.

Biological significance
The reported work provides one of very few comprehensive comparisons of the mitochondrial proteomes from cytoplasmic male-sterile (CMS) and male-fertile plants. It shows that in beets the CMS trait is associated with altered functioning of the mitochondrial ATP synthase complex. The presence of CMS-specific ATP synthase subcomplexes resembles deficiencies of this enzyme reported for mammalian cells.
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1. Introduction

Cytoplasmic male sterility (CMS) refers to a maternally inherited failure to produce functional pollen. Since CMS is widely used for hybrid seed production, it has been extensively studied revealing that in both monocots and dicots its genetic determinants are located in the mitochondrial genome. These sequences are often chimeric and result from recombination events involving essential mitochondrial genes as well as non-coding sequences [1]. Interestingly, many of such sequence chimeras contain fragments of genes encoding subunits of ATP synthase and/or cytochrome c oxidase. CMS expression depends on the interaction between the mentioned mitochondrial determinants and specific nuclear genes which may either support or suppress the sterilizing action of the mitochondria [1–3].

The first description of CMS in beets was provided by Owen [4] who found male-sterile plants in sugar beet cultivar US-1. According to this author expression of sterility results from the interaction between the sterilizing cytoplasm (S-cytoplasm) and at least two recessive nuclear genes — *x* and *z*. Comparison of sugar beet CMS and fertile lines revealed differences in the

Abbreviations: BN-PAGE, blue-native polyacrylamide gel electrophoresis; 2DE, two-dimensional electrophoresis; CMS, cytoplasmic male sterility; DDM, n-dodecyl β-D-maltoside; ORF, open reading frame; ROS, reactive oxygen species.

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structure of several mitochondrial loci: *atp1*, *atp6*, *cob*, *cox1*, *cox2* and *rps3* [5–8]. From among these genes, *atp1*, *cob* and *cox1* exhibited altered transcription profiles upon fertility restoration [6,9]. Comparison of the fully sequenced mitochondrial genomes from the sterilizing (S) and normal (N) cytoplasm allowed identification of four transcribed open reading frames (ORFs) which were specific for the S-plasmotype: *Satp6*, *Scox2-2*, *Sorf324* and *Sorf119* [10]. Such ORFs may produce novel proteins which affect mitochondrial function in tissues which are responsible for pollen production. In sugar beet mitochondrial protein profiling revealed the presence of the 35 kDa polypeptide which was expressed specifically in plants with the S-cytoplasm but not in plants with the normal plasmotype. Detailed analyses showed that this protein is encoded by the *Satp6* presequence (*preSatp6*) — the S-cytoplasm-specific amino extension of the *atp6* ORF [10]. It was shown that preSATP6 assembled into the homogenous 200 kDa complex associated with the mitochondrial membrane fraction [11]. However, despite the S-cytoplasm specificity of *preSatp6*, accumulation of both its mRNA and protein was not influenced by the presence of the restorer genes [10,11].

This study was aimed at identification of differences between the mitochondrial proteomes from beets with the normal and Owen-type sterile cytoplasm. In addition to that, accumulation of mitochondrial proteins was examined in accessions with restored fertility. For these purposes mitochondrial lysates were first subjected to blue-native polyacrylamide gel electrophoresis (BN-PAGE), allowing separation of native protein complexes according to their molecular size. Next, the BN-PAGE separations were subjected either to in-gel activity assays or to electrophoresis in denaturing conditions (SDS-PAGE). With this approach it was possible to compare male-sterile and male-fertile beets both with respect to activity of the selected OXPHOS complexes as well as with respect to subunit composition of mitochondrial protein complexes in toto. Moreover, the proteins which either qualitatively or quantitatively differentiated the analyzed accessions were subjected to identification with the use of mass spectrometry. Thus this report provides one of quite few comprehensive comparisons of the mitochondrial proteome condition in cytoplasmic male-sterile and male-fertile plants [12–16].

2. Materials and methods

2.1. Plant material

Eleven lines of sugar beet (*Beta vulgaris* L.) were used in this study, including five male-sterile lines with the Owen S-cytoplasm (NS 06 11, NS 06 16, NS 06 34, NS 031 and SD 06 15A-83), four maintainer lines with the normal (N) cytoplasm (NO 06 11, NO 06 16, NO 06 34 and NO 031) and two S-cytoplasmic lines with restored fertility (RP 04 17-5 and RP 04 19-19). All this material was kindly provided by KHBC Straszków Ltd. (Poland). Additionally, one male-sterile table (red) beet line (Re1A) and the respective maintainer line (Re1B) were used to verify the protein accumulation differences found among the sugar beet accessions. Lines Re1A and Re1B originated from KHiNO Polan Ltd. (Kraków, Poland).

2.2. Isolation of mitochondria

For 2D electrophoresis mitochondria were isolated from 30 g of either storage roots or etiolated leaves using the method of Steinborn et al. [17] with the below-mentioned modifications. The organellar suspension was not treated with DNase I. The mitochondrial pellet obtained after centrifugation at 22 000 ×*g* was suspended in 400 μl of the homogenization buffer and loaded onto a three-step sucrose density gradient (50% — 7.5 ml, 40% — 7.5 ml, 25% — 6.8 ml in 50 mM Tris, 6 mM EDTA, pH 7.5) prepared in a 27 ml centrifuge tube. The gradient was centrifuged at 20 400 ×*g* in 4 °C for 60 min. After collecting the purified mitochondrial fraction, it was diluted six times with the homogenization buffer without BSA and centrifuged again at 20 400 ×*g* in 4 °C for 60 min. All centrifugations were performed using the 12139-H rotor from Sigma. The resulting mitochondrial pellet was resuspended in two volumes of the 3× ACA buffer (2.25 M aminocaproic acid, 150 mM Bis-Tris, 1.5 M EDTA, pH 7.0). Finally, the preparation was frozen in liquid nitrogen and stored at −80 °C. Protein concentration was determined with the method of Bradford [18].

For in-gel activity assays mitochondria were isolated from storage roots according to Mikami et al. [19] with minor modifications. Approximately 80 g of the chopped tissue was blended in 250 ml of the homogenization buffer. The organellar suspension was not treated with DNase I. The mitochondrial pellet obtained after centrifugation at 18 000 ×*g* (rotor F16/250, Sorvall) was suspended in 3 ml of the homogenization buffer and loaded onto a three-step sucrose density gradient (50% — 10 ml, 44.5% — 10 ml, 40% — 12 ml in 50 mM Tris, 2.5 mM EGTA, pH 7.5) prepared in a 50 ml centrifuge tube. The gradient was centrifuged at 87 000 ×*g* in 4 °C for 60 min (rotor F28/50, Sorvall). After collecting the purified mitochondrial fraction, it was treated as described in the previous paragraph. Calculations performed for the zymographic assays were based on three biological replicates from each analyzed accession. In the 2DE analysis male-sterile, maintainer and restored sugar beets were represented by nineteen, thirteen and six biological replicates, respectively. In case of both analyzed table (red) beet accessions this number was five.

2.3. Blue-native electrophoresis

The blue-native polyacrylamide gel electrophoresis (BN-PAGE) was performed according to Jänsch et al. [20] with some modifications. The frozen mitochondrial preparations (see above) were supplemented with 10% n-dodecyl β-D-maltoside (DDM) and 1× ACA buffer (0.75 M aminocaproic acid, 50 mM Bis-Tris, 0.5 mM EDTA, pH 7.0) to yield concentration of 0.75% and 1.3 μg/μl for DDM and protein, respectively. Solubilization was carried out in 4 °C for 15 min. After centrifugation at 20 000 ×*g* the supernatant was aliquoted into 14 μl portions and either stored at −80 °C or immediately used for BN-PAGE. Prior to electrophoresis the protein samples were supplemented with 4 μl of Coomassie blue solution (5% Serva Blue G-250 in 750 mM aminocaproic acid) and loaded onto the gel. Separation was carried out at 4 °C in the TV100YK unit from Scie-Plas using a linear gradient of polyacrylamide ranging

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