Heterogeneity of maize root mitochondria from plants grown in the presence of ammonium

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Abstract

Mitochondria isolated from root tissue of maize plants grown on a modified Knop solution containing 10.9 mM nitrate ± 7.2 mM ammonium were purified on the discontinuous Percoll density gradient with polyvinylpyrrolidone (PVP) added. The presence of PVP allowed separation of several mitochondrial fractions of a different density. Contrary to mitochondria isolated from plants grown in the presence of nitrate alone, revealing only two fractions, the mitochondria from NH_4^+/NO_3^--plants were distributed in four fractions. Total amount of mitochondria, as well as specific activities of some nitrogen metabolism enzymes and tricarboxylic acid (TCA) cycle enzymes of all mitochondrial fractions, and respiratory activities of two lower density fractions isolated from plants grown on mixed nitrogen were higher in comparison to mitochondria from nitrate-grown plants.

Additional key words: nitrogen form, respiration, *Zea mays* L.

Introduction

Our previous results (Hadži-Tašković Šukalović and Vuletić 1998) showed that mitochondria isolated from maize roots grown on mixed nitrogen sources, exhibited higher activities of glutamate dehydrogenase (GDH), alanine aminotransferase (GPT), NAD^+-isocitrate dehydrogenase (NAD^+-ICDH), succinate dehydrogenase (SDH) and malate dehydrogenase (MDH) and respiratory activities, as well as, protein content compared to mitochondria from nitrate-grown plants. Improving separation characteristics of the Percoll gradient by including PVP enabled us to obtain several mitochondrial fractions of different densities. In the present work, we analyzed a density distribution of mitochondrial populations and metabolic activities of obtained fractions, isolated from plants grown in media containing different nitrogen forms.

Materials and methods

Plants: Maize (*Zea mays* L.) inbred line VA35 was used. The seed, germinated on distilled water, was transferred after three days to plastic pots containing Knop solution, modified in nitrogen content. The first 7 d plants were grown on 1/4 strength nutrient solution and during the following 4 d on full strength solution, both being supplemented with different forms of nitrogen. Nitrogen was supplied as KNO_3, $Ca(NO_3)_2$ and $(NH_4)_2SO_4$ in two treatments, the concentration of NO_3^- and NH_4^+ in full strength solution being 10.9:0 and 10.9:7.2 [mM], respectively, *i.e.* the concentration of nitrogen in the form of nitrate was kept constant, while the presence of ammonium varied. The initial pH of the solution was adjusted to 5.6. Plants were kept in a growth

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Abbreviations: GDH - glutamate dehydrogenase, GPT - alanine aminotransferase, NAD^+-ICDH - NAD^+-isocitrate dehydrogenase, MDH - malate dehydrogenase, PVP - polyvinylpyrrolidone, SDH - succinate dehydrogenase, TCA - tricarboxylic acid, TPP - thiamine pyrophosphate.

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chamber under a 12-h photoperiod (irradiance of 190 μmol(photon) m^{-2} s^{-1}), day/night temperature of 22/18 °C and a relative humidity of about 70 %.

Isolation of mitochondria: Mitochondria were prepared by a modified procedure described by Schwitzguebel and Siegenthaler (1984). Roots were cut in 5 volumes of medium containing 0.4 M mannitol, 50 mM TES buffer (pH 7.5), 4 mM cysteine, 1 mM EDTA, 1 mM $MgCl_2$, 0.1 % (m/v) BSA and 1 % (m/v) soluble PVP 25 000, and ground with a mortar and pestle. The homogenate was filtered through 4 layers of muslin, centrifuged at 4 000 *g* for 5 min, and the obtained supernatant centrifuged at 39 000 *g* for 5 min. The resulting pellet was resuspended in a washing medium containing 50 mM TES buffer (pH 7.5), 0.4 M mannitol, 1 mM EDTA, 1 mM $MgCl_2$ and 0.1 % (m/v) BSA. This suspension was centrifuged at 10 000 *g* for 15 min, yielding a pellet of washed mitochondria.

Purification of mitochondria: Washed mitochondria were suspended in 3 - 4 cm^3 of the washing medium and layered on the top of a discontinuous Percoll gradient made of six layers prepared with 5 cm^3 60 % (v/v); 5 cm^3 50 %; 10 cm^3 45 %; 5 cm^3 27 %; 5 cm^3 20 % and 5 cm^3 13.5 % Percoll. Separation characteristics of the Percoll gradient were improved by including PVP which helped to narrow the distribution of the mitochondria. PVP was added to gradient layers of decreasing concentration in 1 % steps, from 6 % (m/v) to 1 % from the bottom to the top of the gradient. All Percoll/PVP solutions contained 0.35 M mannitol, 50 mM TES buffer (pH 7.4), I mM EDTA, 1 mM $MgCl_2$ and 0.1 % (m/v) BSA. Centrifugation was performed at 13 000 *g* for 30 min in a *Sorvall SS-34* (Newton, USA) fixed-angle rotor (Schwitzguebel and Siegenthaler 1984). The bands of mitochondria were collected with a Pasteur pipette and diluted approximately 10 times with the washing medium. Purified mitochondria were concentrated by centrifugation at 12 000 *g* for 15 min. Possible contaminations of mitochondria with peroxisomes was determined by analyzing activity of marker enzyme catalase (EC 1.11.1.6) (Aebi 1974).

Analysis of proteins: Protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. The content of BSA in the washing medium was subtracted from the total protein content in the case of mitochondrial protein calculation.

Enzyme activities: Enzyme activities were assayed spectrophotometrically at 30 °C using mitochondria solubilized in 0.04 % (v/v) Triton X-100. The activity of SDH was measured by the decrease of absorbance at 600 nm caused by reduction of 2,6-dichlorophenol-indophenol according to King (1967). GDH was assayed by monitoring the oxidation of NADH (aminating activity), or the reduction of NAD^+ (deaminating activity) at 340 nm (Bulen 1956). NAD^+-ICDH activity was determined according to Rasmusson and Møller (1990), by monitoring the increase of absorbance at 340 nm, due to NAD^+ reduction. GPT activity was determined using the colorimetric Thonazy method (Bergmeyer and Bernt 1974). MDH activity was determined as oxaloacetate reduction resulting in decrease of NADH absorbance at 340 nm, as described by Hayes *et al.* (1991). Catalase was assayed by measuring the loss of H_2O_2 (Aebi 1974). Succinate:cytochrome *c* oxidoreductase activity was assayed by measuring reduction of cytochrome *c*, absorbing at 550 nm, according to Douce *et al.* (1972).

Measurement of mitochondrial respiration rate. Oxygen consumption by mitochondria was measured at 25 °C using a Clark-type polarographic electrode (*Hansatech Ltd.*, King's Lynn, England). Mitochondria (0.2 - 0.4 mg of protein) were added to 1 cm^3 of the medium containing 10 mM KCl, 5 mM $MgCl_2$, 10 mM KH_2PO_4, 0.4 M mannitol, 1 mg cm^{-3} BSA and 10 mM MOPS buffer, adjusted to pH 7.5 with KOH. Oxygen consumption was measured with a cocktail of substrates containing malate, glutamate and succinate in the presence of thiamine pyrophosphate (TPP) to ensure maximum electron transport rates (Lambers *et al.* 1983). The transient stimulation of respiration (state 3) was triggered by the addition of 0.1 mM ADP and decreased upon ADP exhaustion (state 4). The respiratory control (the ratio of state 3 to state 4) and ADP/O value (the ratio of ADP added to oxygen consumed during state 3) were calculated according to Estabrook (1967).

Results and discussion

The isolation and purification of mitochondria on the discontinuous Percoll gradient allowed separation of several mitochondrial fractions. Separation characteristics of the Percoll gradient were improved by including PVP, which served to narrow the mitochondria bands due to interaction of PVP with the mitochondrial membranes increasing their density (Day *et al.* 1985). In our experiments the density distribution of mitochondria isolated from roots exhibited difference depending on available nitrogen source. High-density mitochondria at the interface 27/45 % (fraction F and F_1, from nitrate- and mixed nitrogen-grown plants, respectively) and low-

Table 1. The quality and purity of mitochondria, specific activities of mitochondrial enzymes, and respiration rate in fractions isolated from maize plants grown on different nitrogen sources (* - the ratio of respiration rate in the presence of added ADP to the rate obtained following ADP expenditure, ** - the number of moles of ADP added divided by the number of atoms of oxygen consumed during respiration in the presence of ADP). The results are mean ± SE of at least three independent experiments.

	10.9 mM NO_3^-	10.9 mM NO_3^- + 7.2 mM NH_4^+		
	F	F_1	F_2	F_3
Integrity of outer membrane [%]	80 - 84	78 - 81	80 - 88	80 - 88
Succinate:cyt *c* oxidoreductase [μmol mg^{-1}(protein) $s^{-1} \times 10^{-3}$]	0.250 ± 0.120	2.950 ± 0.830	2.600 ± 0.330	2.980 ± 0.170
Catalase [μmol mg^{-1}(protein) $s^{-1} \times 10^{-3}$]	1.770 ± 0.150	1.520 ± 0.070	0	2.080 ± 0.080
Mitochondrial protein content [mg g^{-1} (f. m.)]	0.094 ± 0.007	0.108 ± 0.005	0.042 ± 0.007	0.030 ± 0.005
NAD^+-ICDH [μmol mg^{-1}(protein) $s^{-1} \times 10^{-3}$]	0.58 ± 0.10	1.35 ± 0.07	1.75 ± 0.07	0.78 ± 0.03
SDH [μmol mg^{-1}(protein) $s^{-1} \times 10^{-3}$]	0.23 ± 0.08	1.17 ± 0.08	0.87 ± 0.05	0.35 ± 0.08
MDH-oxaloacetate reduction [μmol mg^{-1}(protein) $s^{-1} \times 10^{-3}$]	104.00 ± 3.00	269.00 ± 20.00	273.00 ± 27.00	289.00 ± 31.00
NADH-GDH [μmol mg^{-1}(protein) $s^{-1} \times 10^{-3}$]	2.57 ± 0.08	13.30 ± 1.00	13.10 ± 1.70	11.40 ± 3.70
GPT [μmol mg^{-1}(protein) $s^{-1} \times 10^{-3}$]	2.52 ± 0.08	14.90 ± 1.50	15.80 ± 7.20	13.80 ± 7.70
Oxygen uptake [μmol mg^{-1} (protein) s^{-1}]	0.645 ± 0.075	0.927 ± 0.105	1.000 ± 0.225	0.543 ± 0.193
Respiratory control*	1.280 ± 0.070	1.830 ± 0.080	1.420 ± 0.100	1.000
ADP/O**	1.330 ± 0.100	1.820 ± 0.200	2.480 ± 0.320	-

density mitochondria at the interface 20/27 % were observed in both cases, while mitochondria isolated from the plants grown in the presence of ammonia appeared additionally in another two bands at the interfaces 45/50 % (fraction F_2) and 50/60 % (fraction F_3).

By testing the bands collected from the Percoll gradient, using mitochondrial marker enzyme activities, succinate : cytochrome *c* oxidoreductase and SDH (Table 2), and respiratory activity (Table 1), we demonstrated that isolated fractions were enriched in mitochondria. The lowest density mitochondrial fraction, appearing in the gradient at interface 20/27 % exhibited a low outer membrane integrity, as tested by succinate : cytochrome *c* oxidoreductase activity (data not presented). This fraction probably consisted of mitochondria broken during isolation procedure, was discarded and was not taken in further considerations. On the other hand, all other fractions were of similar quality with respect to the outer membrane integrity, being approximately 80 % (Table 1). In order to rule out the possible contamination of higher density fractions with peroxisomes we tested isolated mitochondrial fractions for catalase activity (Table 1). The obtained results demonstrated that these fractions were not significantly contaminated with microbodies.

The purified mitochondria isolated from maize roots showed changes in their amount and properties depending on the nitrogen form used for plant growth. An increase of the yield of purified mitochondria, when expressed as the amount of total mitochondrial protein per root fresh mass unit, was evident when plants were cultured on NO_3^- + NH_4^+ (Table 1). Although the protein content of a mitochondrial fraction obtained at interface 27/45 % was higher only by 15 % in the plants grown in the presence of NH_4^+, significant increase (1.9 fold) of the total mitochondrial protein was due to the appearance of another two higher-density bands (F_2 and F_3) in such plants (Table 1). The heterogeneity of mitochondria with respect to the density distribution on the self-generating Percoll gradient was already reported for mitochondria isolated from both maize root during glucose starvation (Couée *et al.* 1992) and avocado during ripening (Saviani *et al.* 1998). In both cases some changes in metabolic activities were reported.

Metabolic activity of mitochondrial fractions was studied by analyzing the respiration rates and the activity of several key enzymes involved in carbon metabolism, NAD^+-ICDH, MDH and SDH, and enzymes of nitrogen metabolism, GDH and GPT. All mitochondrial fractions isolated from maize grown on NO_3^- + NH_4^+ showed higher specific activities of the investigated enzymes when compared to those obtained from NO_3^--grown plants (Table 1). Specific activities of TCA cycle enzymes, ICDH and SDH, were more than doubled, except in the F_3 fraction, while the activity of MDH was the highest in F_3 fraction. The differences were more pronounced for GDH and GPT activities, which were even about 5 fold increased in plants grown on NO_3^- + NH_4^+.

The rates of oxygen consumption were different in mitochondria isolated from roots of plants grown in the presence of different nitrogen forms (Table 1). Also, these mitochondria exhibited different respiratory properties upon ADP addition, expressed as respiratory control and ADP/O ratio. Since it can be assumed that oxygen uptake during this stimulated phase (phase 3) is proportional to the amount of ADP phosphorylated to ATP (Estabrook 1967), our results demonstrated different phosphorylating ability of isolated mitochondrial

fractions. The respiration rate, measured with a cocktail of substrates (malate, glutamate and succinate in the presence of TPP), as well as respiratory control and ADP/O ratios were higher in mitochondrial fractions F_1 and F_2 isolated from NO_3^- + NH_4^+-grown plants, in comparison to the mitochondria of plants grown in the presence of nitrate alone (Table 1). Respiratory control and ADP/O ratios of fractions F_1 and F_2 were of the same order of magnitude as those for higher-density mitochondria reported by Couée *et al.* (1992). The weak respiratory control shown in fraction F_3 is not the result of damage, since the outer membrane integrity was shown to be as high as in the other fractions. This result, as well as a lower rate of total mitochondrial respiration and different activities of some enzymes in this fraction point to altered characteristics of this fraction compared to the other two fractions obtained in the same preparation.

To conclude, the presence of NH_4^+ induces heterogeneity of mitochondrial population increasing the content of mitochondrial protein in maize roots. Enzyme activities of three density fractions and respiratory activities of the two lower density fractions obtained from such mitochondria were higher in comparison to mitochondria from nitrate grown plants.

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