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Functional characterization of mitochondria isolated from the ancient gymnosperm *Araucaria angustifolia*

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ABSTRACT

Mitochondria were isolated from embryogenic cell cultures of *Araucaria angustifolia*, an ancient gymnosperm. The mitochondria obtained oxidized NADH and succinate as respiratory substrates and were able to sustain a high transmembrane electrical potential. They were able to take up Ca^{2+} supported by substrate oxidation, sensitive to ruthenium red and dependent on membrane potential. The influx of the ion was Pi dependent and at least one efflux pathway was demonstrated by ruthenium red addition. The mitochondria also oxidized externally added NADH, and presented oxygen consumption insensitive to cyanide and sensitive to salicyl hydroxamic acid (SHAM) suggesting the presence of external NADH dehydrogenase and alternative oxidase (AOX), respectively. Besides, mitochondria were uncoupled, in the absence of BSA, by the addition of oleic acid suggesting the presence of the plant uncoupling mitochondrial protein.

1. Introduction

Araucaria angustifolia is an ancestral conifer of the Araucariaceae family that is found on the cold highlands of southern Brazil. Its metabolic properties and composition of distinct plant parts are now being studied [1,2]. Embryogenic cultures of *A. angustifolia* were induced from zygotic embryos excised from immature seeds and the establishment of in vitro conditions for its induction, stabilization and proliferation was determined [1,2]. Previously we reported a methodology to isolate mitochondria from embryogenic cell cultures of *A. angustifolia* and demonstrated the oxygen consumption by these organelles actively oxidizing succinate [3]. Our interest in studying mitochondria from this ancient gymnosperm is due to the fact that most former studies concerning important characteristics of plant mitochondria have been investigated preferentially in angiosperms.

Plant responses to the environmental conditions that induce stress have been reviewed by several authors [4–7]. Special attention has been directed to mitochondrial enzymes involved in stress regulation. Three of these enzyme systems of angiosperms have been the subject of many studies: the external NADH dehydrogenase, the alternative oxidase (AOX) and more recently the plant uncoupling proteins (PUMP), which are present in several isoforms [7–9]. These enzymes have been studied in monocot and dicot plants [10–12], however in gymnosperms scarce information about mitochondrial functions is available.

The purpose of this study was to characterize classical functions of mitochondria, such as oxygen consumption, the membrane potential and Ca^{2+} transport systems, and also to investigate the presence of some plant mitochondrial enzymes involved in stress. Some of the fundamental characteristics of mitochondrial functions that have been extensively submitted to investigation in plant mitochondria [13,14], and some functions in stress conditions are here being verified in Araucaria mitochondria.

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Abbreviations: AOX, alternative oxidase; PUMP, plant uncoupling mitochondrial protein; Hepes, [*N*-(2–hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)]; FCCP, carbonyl cyanide 4-trifluoromethoxyphenyl-hydrazone; BHAM, benzyl hydroxamic acid; SHAM, salicyl hydroxamic acid; DTT, dithiothreitol; EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; RR, ruthenium red.

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2. Material and methods

2.1. Plant materials

The source of mitochondria was an embryogenic cell culture of *A. angustifolia* [2], grown on BM culture medium [1] supplemented with 2 mg L^{-1} glycine, 0.5 mg L^{-1} pyridoxine·HCl, 0.5 mg L^{-1} nicotinic acid, 1 mg L^{-1} thiamine·HCl, 500 mg L^{-1} casein hydrolyzate, 100 mg L^{-1} *myo*-inositol, 1 g L^{-1} L-glutamine, 30 g L^{-1} sucrose, 7 g L^{-1} phytagar (Gibco®), 2 μM 2,4-dicholorophenoxyacetic acid, 0.5 μM benzylaminopurine and 0.5 μM kinetin. The pH of the culture medium was adjusted to 5.8 with KOH prior to autoclaving at 121 °C for 20 min. Casein hydrolyzate and L-glutamine solutions were then filter sterilized and added to the medium.

2.2. Preparation of mitochondria

Mitochondria were isolated by differential centrifugation, as previously described [3]. Briefly, the embryogenic cell cultures were smoothly homogenized in a van Potter–Elvehjem homogenizer and disrupted in a Turratec homogenizer by 4 s bursts in the presence of a cold extraction medium containing 0.25 M sucrose, 3 mM cystein, 2 mM EGTA, 0.2 g% BSA, 10 mM Hepes, pH 7.6 (35 g of fresh cells/200 mL medium). The homogenate was filtered through nylon cloth, and the pH was adjusted to 7.2. The filtrate was centrifuged for 10 min at 1000 × *g*. The supernatant was centrifuged for 10 min at 15,000 × *g* and each pellet was resuspended in wash medium (0.25 M sucrose, 0.25 mM EGTA, 0.2 g% BSA, 10 mM Na–Hepes, pH 7.2) and transferred to a single tube and centrifuged for 10 min at 1000 × *g*. The supernatant was centrifuged for 10 min at 15,000 × *g*. The pellet was resuspended in wash medium and the resultant mitochondrial suspension (~10 mg protein mL^{-1}) was kept on ice until use.

2.3. Oxygen uptake

Oxygen consumption by isolated mitochondria was measured using a Clark-type electrode (Yellow Springs Instruments) connected to a Gilson oxygraph using 1.2 mL of a standard reaction medium containing 0.25 M sucrose, 10 mM Hepes, pH 7.2, 2 mM KCl, 2 mM Pi at 28 °C. Other additions are specified in the figure legends. Mitochondrial preparations with respiratory control above 2.5 were used (data not shown). The respiratory rates are expressed in ng atom O min^{-1} mg^{-1}; oxygen solubility in water at 28 °C and 1 atm was taken as 233 μM [15].

2.4. Determination of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\psi$) was measured fluorometrically with a Shimadzu RF-5301 PC spectrofluorophot-

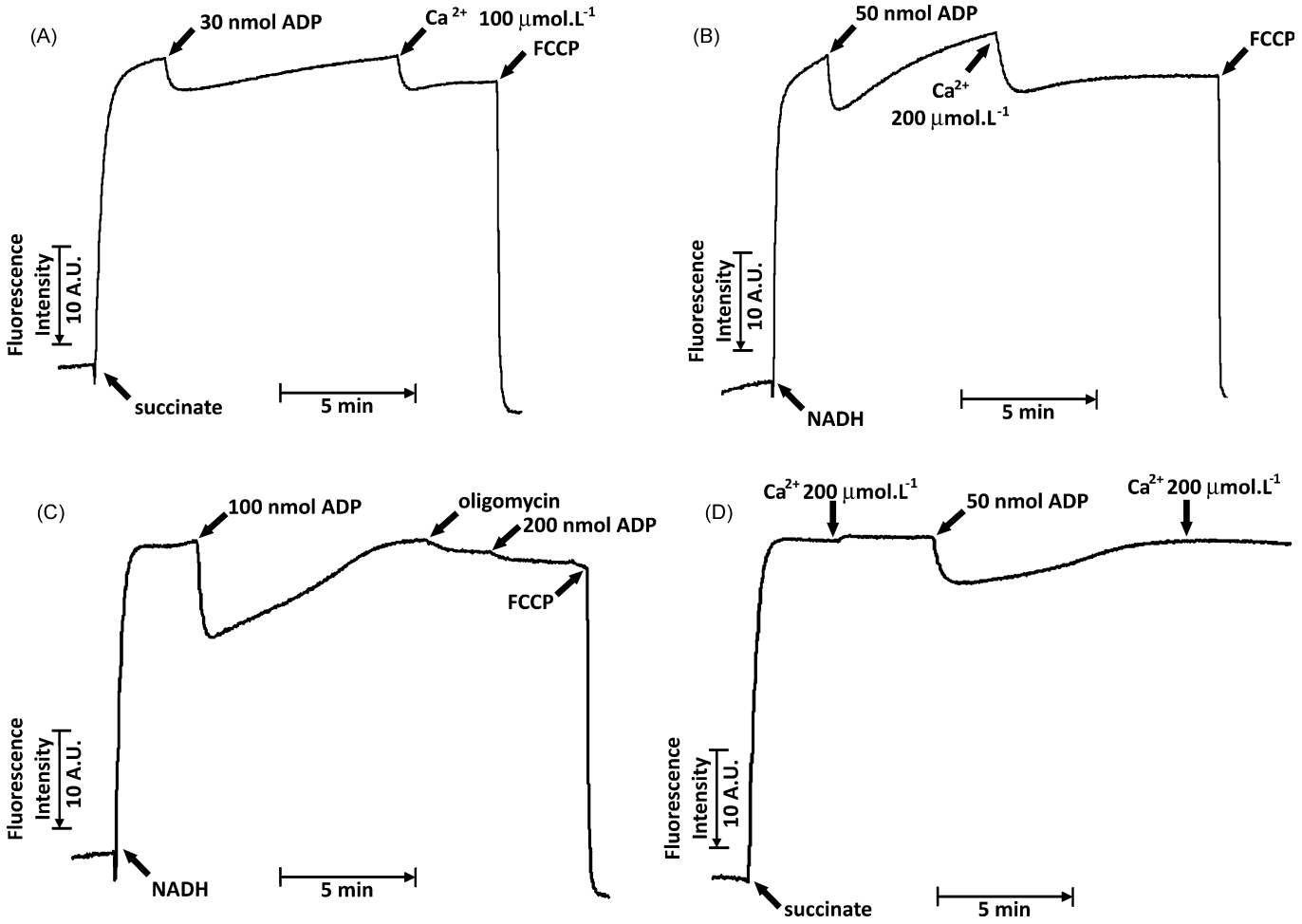

Fig. 1. Membrane potential determination. Mitochondria (300 μg mL^{-1}) were added to 2 mL of reaction medium as described. Addition of succinate (A and D) or NADH (B and C), ADP (30, 50 or 100 nmol), Ca^{2+} (100 or 200 μM), 2.5 μg mg^{-1} oligomycin O and 1 μM FCCP, was as indicated. (D) Mitochondria were preincubated for 2 min with 5 μM RR. The figure is representative of three independent experiments.