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Biogenesis of mitochondria in cauliflower (*Brassica oleracea* var. *botrytis*) curds subjected to temperature stress and recovery involves regulation of the complexome, respiratory chain activity, organellar translation and ultrastructure

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ABSTRACT

The biogenesis of the cauliflower curd mitochondrial proteome was investigated under cold, heat and the recovery. For the first time, two dimensional fluorescence difference gel electrophoresis was used to study the plant mitochondrial complexome in heat and heat recovery. Particularly, changes in the complex I and complex III subunits and import proteins, and the partial disintegration of matrix complexes were observed. The presence of unassembled subunits of ATP synthase was accompanied by impairment in mitochondrial translation of its subunit. In cold and heat, the transcription profiles of mitochondrial genes were uncorrelated. The *in-gel* activities of respiratory complexes were particularly affected after stress recovery. Despite a general stability of respiratory chain complexes in heat, functional studies showed that their activity and the ATP synthesis yield were affected. Contrary to cold stress, heat stress resulted in a reduced efficiency of oxidative phosphorylation likely due to changes in alternative oxidase (AOX) activity. Stress and stress recovery differently modulated the protein level and activity of AOX. Heat stress induced an increase in AOX activity and protein level, and *AOX1a* and *AOX1d* transcript level, while heat recovery reversed the AOX protein and activity changes. Conversely, cold stress led to a decrease in AOX activity (and protein level), which was reversed after cold recovery. Thus, cauliflower AOX is only induced by heat stress. In heat, contrary to the AOX activity, the activity of rotenone-insensitive internal NADH dehydrogenase was diminished. The relevance of various steps of plant mitochondrial biogenesis to temperature stress response and recovery is discussed.

1. Introduction

The plant mitochondrial proteome, which is estimated to include at least 1500 proteins [91], dynamically responds to diverse genetic, environmental and developmental signals [60]. Contrary to the structural variability, the plant mitochondrial genomes encode a limited number of proteins [1]. Taylor et al. [92] estimated that 22% of the stress-responsive organellar proteins in Arabidopsis are localized in mitochondria, but the number of mitochondrial proteins involved in the response to abiotic stress conditions is underestimated. Little is known about the crucial steps required for proper mitochondrial biogenesis and how the balance between coordinated expression of mitochondrial and nuclear genes is altered in the assembly of large supramolecular structures during stress conditions [27,40]. Complex studies integrating proteomic, transcriptomic, metabolomic and other approaches for the elucidation of the biological relevance of plant mitochondrial responses to stress and stress recovery in particular are still limited.

Diverse thermal treatments, which are often accompanied by oxidative stress [66], modulate the expression, activity and interactions of many mitochondrial proteins [82]. These proteins include classical components of the stress response, for instance heat shock proteins (HSPs), including low molecular weight HSPs (15–30 kDa) that protect some respiratory complexes (Cs) from degradation [7,20]; nucleoside

Abbreviations: ACO, aconitase; AOX, alternative oxidase; ATP1, ATP2, ATP6, ATP synthase subunits; BN, blue native; CBB, Coomassie Brilliant Blue; Cs, protein complexes; CI, CII, CIII, CIV, respiratory chain complexes; COX, cytochrome *c* oxidase (complex IV); DIGE, fluorescence difference gel electrophoresis; DSP, dithiobis[succinimidyl propionate]; FtSH, plant metalloprotease; HSP(s), heat shock proteins(s); IDH, isocitrate dehydrogenase; LC-ESI-Q-TOF-MS, liquid chromatography-electrospray ionization-quadrupole-time of flight-mass spectrometry; MPP, mitochondrial processing peptidase; NAD, complex I subunits (mitochondrially encoded); NDH, rotenone-insensitive alternative internal/external NADH dehydrogenase; OXPHOS, oxidative phosphorylation; RCR, respiratory control ratio; QCR, quinol:cytochrome *c* reductase; q-RT-PCR, quantitative reverse transcription PCR; SCs, supercomplexes; SDH, succinate dehydrogenase; State 3, phosphorylating respiration; State 4, non-phosphorylating respiration; TIM/TOM, translocase of the inner/outer mitochondrial membrane; TMPD, *N,N,N′N′*-tetramethyl-*p*-phenylenediamine; VDAC, voltage-dependent anion channel

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diphosphate kinase [22]; glycine decarboxylase H-protein and other matrix enzymes to be degraded also in oxidative stress [87,89,90]; alternative NAD(P)H dehydrogenases and dehydrin-like proteins [81]. Energy-dissipating components, including alternative oxidase (AOX) and uncoupling proteins (UCP), play a key role in the stress response in plant mitochondria [11,77]. So far, only a few exhaustive studies on the plant mitochondrial proteome under cold and heat stress have been carried out [74,88,90,104].

This work was undertaken to gain a comprehensive view of the influence of temperature stress, including cold and heat, and the recovery from cold and heat, on the biogenesis of cauliflower (*Brassica oleracea* var. *botrytis*) curd mitochondria using proteomic, complexomic, transcriptomic and functional studies. Cauliflower is one of the most important vegetable crops worldwide [32], thus, it is valuable to analyze organellar proteomes of this plant species [8,35,84]. Notably, in our extensive report, we used two dimensional fluorescence difference gel electrophoresis (2D-DIGE) to analyze, for the first time, the detailed responses of the mitochondrial proteome and complexome of a vegetable plant species to temperature stress and stress recovery. Specifically, we studied: (i) the stability of the oxidative phosphorylation (OXPHOS) system and the abundance of OXPHOS proteins; (ii) the transient interactions between respiratory chain Cs; (iii) the *in-gel* activity of respiratory Cs, the activity of the cytochrome and alternative (AOX-mediated) pathways in isolated mitochondria; (iv) respiratory parameters in cauliflower leaves to evaluate the plant physiological status; (v) alterations in the accumulation of AOX isoforms at the proteomic and transcriptomic levels; (vi) changes in the *de novo* synthesis of mitochondrial proteins, and (vii) variations in mitochondrial morphology. On the whole, we investigated the complex nature of the temperature stress responses which allowed us to estimate the role of mitochondria in a higher plant during cold/heat stress and after recovery.

2. Material and methods

2.1. Growth of plant material, temperature stress application and analyses of physiological responses

Seeds of cauliflower (*B. oleracea* var. *botrytis*) cultivar 'Diadom' were purchased from Bejo Zaden (Poland). The plants were grown for 3 months in cultivation chambers at a local breeding station (University of Life Sciences, Poznan, Poland) at 23/19°C (day/night) and 70% relative humidity under photon flux density 200 μmol m^{-2} s^{-1} (16 h of light/8 h of dark). Stress conditions were applied to the plants with young curds (approximately 10 cm-diameter curds). Cold stress (8°C) and heat stress (40°C) was applied to growing plants for 10 days and 4 h, respectively. After termination of the given stress treatment, some of the cauliflower plants were transferred to standard growth conditions for 48 h (stress recovery). The curds were harvested either immediately after the termination of the stress treatment or after the completion of stress recovery.

Physiological analyses were conducted on well-developed cauliflower leaves using the LI-6400 XT infrared gas analyzer (Li-Cor). The rate of leaf respiration in the light (day respiration) was determined according to the Laisk method [51]. CO_2 assimilation rate representing a given total respiration rate was recorded during intercellular CO_2 concentration (C_i) decreased to 0 ppm at 22°C and 50% relative humidity. For each value of photon flux density at 200, 400 and 600 μmol m^{-2} s^{-1}, 1, the linear regression of CO_2 assimilation (A) versus C_i was calculated and the photorespiration rate was determined as difference between total and day respiration (the latter one expressed as a given CO_2 evolution rate at the crossing point of all A/C_i curves). Duration of the heat stress was estimated on the basis of temperature measurements of cauliflower curds as well as the leaf surface. When heat stress reached up to 40°C, plant surface temperature increased and remained stable for 2 h. After the completion of this period, plants were heat-stressed for further 2 h (4 h in total). Such treatment appeared necessary for the visible increase in the respiration and photorespiration rates (Supplementary Fig. 1). However, when heat stress exceeded 4 h, the leaf turgor decreased and was accompanied by visible rising lesion symptoms, including leaf yellowing and the necrosis during further cultivation days. At 8°C, physiological responses of cauliflower plants were evident after 8 h of cold stress (Supplementary Fig. 1). At this temperature, only physiological parameters, including day and total respiration, and photorespiration rates were significantly decreased. Exposure to cold for 10 days was necessary to observe proteomic and functional alterations. Similarly, the duration of the stress recovery was also optimized in order to detect the significant proteomic and functional changes.

2.2. Isolation of mitochondria, purity assays and protein determination

Mitochondria from the topmost 5-mm-thick layer of the cauliflower curds were isolated by differential centrifugation and purified in Percoll gradients according to Pawlowski et al. [69]. During isolation, the Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche) was added. Purity assays of the isolated mitochondria were conducted according to Rurek [81] and Pawlowski et al. [69]. The protein content was determined using the BioRad Protein Assay, using bovine serum albumin as a standard curve calibrator. The efficiency of the mitochondria preparation was 4–6 mg of mitochondrial proteins per 100 g of cauliflower curds.

2.3. Crosslinking assay

Mitochondria (100 μg) were suspended in a washing medium without bovine serum albumin [81] to a final protein concentration of 1 mg ml^{-1}. Dithiobis[succinimidyl propionate] (DSP, Pierce) that was freshly dissolved in *N*,*N*-dimethylformamide (DMF; Sigma) was added to final concentrations of 0.125, 0.25 and 0.5 mM to act as a crosslinker. Crosslinking was carried out for 2 h at 4°C, and then 10 mM Tris–HCl (pH 7.5) was added. After a subsequent incubation (4°C, for 15 min), the mitochondria were pelleted and the respiratory Cs were analyzed by blue native polyacrylamide gel electrophoresis (BN-PAGE).

2.4. BN-PAGE

Pelleted mitochondria (50–100 μg) were suspended in a 6-aminocaproic acid (ACA) solution [28]. For solubilization (4°C, for 30 min), digitonin (Fluka) was used (4 g of detergent per 1 g of proteins). After centrifuging to separate the non-solubilized material (at 18,300 ×*g*, for 20 min, 4°C), Blue G (Serva) was added to the obtained supernatant. BN-PAGE was carried out according to a modified protocol from Giegé et al. [28] using a mini-gel system from Biometra (5–13% polyacrylamide 10 × 10 cm, 1.5 mm-thick gels). The gels were run at 60 V for 45 min and then at 250 V for 3 h with the current limited to 25 mA (all steps at 4 °C).

2.5. In-gel activity assays

NADH dehydrogenase activity, including the activity of complex I (CI), was detected using a method modified from Zerbetto et al. [105] in a presence of 0.14 mM NADH, 1 mg ml^{-1} nitroblue tetrazolium, 0.1 M Tris–HCl (pH 7.4). Complex II (CII) activity was detected in reaction medium (50 mM phosphate buffer, pH 7.4, 84 mM succinate, 0.2 mM phenazine methosulfate, 2 mg ml^{-1} nitroblue tetrazolium, 4.5 mM EDTA, 10 mM cyanide) according to Jung et al. [44]. Complex IV (CIV) activity (also modified from [105]) was detected in a presence of 10 mM phosphate buffer (pH 7.4), 1 mg ml^{-1} diaminobenzidine, 75 mg/ml sucrose, 19 U ml^{-1} catalase and 16 mM cytochrome *c*. F_0F_1 ATP synthase activity [95] was determined overnight in 50 mM HEPES, pH 8.0, 10 mM ATP and 30 mM $CaCl_2$. All assays were