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Article

Cold and Heat Stress Diversely Alters Both Cauliflower Respiration and Distinct Mitochondrial Proteins Including OXPHOS Components and Matrix Enzymes

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Abstract: Complex proteomic and physiological approaches to study cold and heat stress responses in plant mitochondria are still limited. Variations in the mitochondrial proteome of cauliflower (*Brassica oleracea* var. *botrytis*) curds after cold and heat and after stress recovery were assayed by 2D PAGE in relation to respiratory parameters. Quantitative analysis of the mitochondrial proteome revealed numerous stress-affected protein spots. In cold alternative oxidase isoforms were extensively upregulated; major downregulations in the level of photorespiratory enzymes, porine isoforms, oxidative phosphorylation (OXPHOS) and some low-abundant proteins were observed. On the contrary, distinct proteins, including carbohydrate metabolism enzymes, heat-shock proteins, translation, protein import, and OXPHOS components were involved in heat response and recovery. Few metabolic regulations were suggested. Cauliflower plants appeared less susceptible to heat; closed stomata in heat stress resulted in moderate photosynthetic, but only minor respiratory impairments, however photosystem II performance was unaffected. Decreased photorespiration corresponded with proteomic alterations in cold. Our results show that cold and heat stress not only operate in diverse mode (exemplified by cold-specific accumulation of some heat shock proteins), but exert some associations on molecular and physiological levels. This implies more complex model of action of investigated stresses on plant mitochondria.

Keywords: cold stress; heat stress; stress recovery; mitochondria; proteomics; respiration; *Brassica*; angiosperms

1. Introduction

Abiotic stress, including excessive cold or heat, cause the failure in the cultivation of many plant species. Such conditions may significantly reduce yield of most major crops. In the course of evolution, plants acquired various physiological and metabolic responsive mechanisms, which act within the complex network to avoid the harmfulness of unfavorable environmental stimuli [1-3]. Understanding of these mechanisms improves our knowledge about stress resistance allowing to breed adequate plant varieties.

Numerous aspects of plant responses to cold and heat have been studied till today. They may contrast between plant species [4-6]. Low and high temperature could decrease chlorophyll biosynthesis, significantly impede chloroplast development and may result in PSII damage [7-11]. Cold-grown plants generate a vast number of reactive oxygen species (ROS) [12]. Armstrong et al. [13] who analyzed temperature-dependent sensitivity of leaf respiration in Arabidopsis during cold acclimation suggested the importance of alternative oxidation pathway in this process. Moreover, Talts et al. [14] observed that cold treated plants often displays higher rates of respiration. However, heat stress (depending on its intensity and duration) can exert particularly diverse effects on the photosynthetic apparatus [15], including the increase of the cyclic electron flow around PSI [9,16-19].

Despite reports, concerning evident alterations in plant physiological parameters during stress response, data on the correlation of those changes with mitochondrial proteomes are quite limited. Organellar proteomic analyses, including mitochondrial ones, may help to reveal the intrinsic mechanisms of stress response by elucidating the relationship between protein variations and the general plant tolerance to environmental factors [20]. Nowadays characterization of total proteomes or sub-proteomes of important crop and vegetable plants, including cauliflower (*Brassica oleracea* var. *botrytis*) one, appears to be very important [21-26].

Plant mitochondrial proteome is a very dynamic entity which can be remodeled in plethora of environmental conditions and developmental signals [27,28]. It is known that dozens nuclear genes encoding mitochondrial proteins responding to stress conditions form a functional network [29]. Using the integrative approach, Cui et al. [30] found 503 Arabidopsis mitochondrial proteins participating in stress protein interaction network. This suggests the general dependence of plant mitochondria on other plant cell compartments during stress response. Furthermore, Taylor et al. [31] estimated that only 22% of total Arabidopsis organellar proteins that are stress-responsive, comprise mitochondrial proteins. It seems that the amount of mitochondrial proteins involved in stress response is still underestimated, because of limited complexity of some reports and the fact that the significant number of results came from analyses of total plant proteomes and main metabolic pathways only [25,32-34]. It has to be mentioned that the number of low-abundant mitochondrial proteins responsive to temperature stress is still far from understanding [31]. Recently, these issues were improved by the application of isobaric tags for the absolute quantification (iTRAQ) or label-free peptide counting coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) [35-38]. Using *gel-free* approach Tan et al. [39] found that cold stress made concerted decrease in respiratory protein level, accompanied by the increase in abundance of some import/ export protein machinery components. However, the overall amount of cold-responsive proteins was smaller, when compared to other suboptimal stimuli.

Although it is known that temperature stress modulates mitochondrial proteins activity, level, biogenesis and interactions [40-42], crucial steps of achieving the proper coordination during mitochondrial biogenesis in stress need to be further investigated. For instance, Giegé et al. [43] showed that regulation of mitochondrial biogenesis in Arabidopsis cell cultures during sugar starvation seems to be rather coordinated at the complex assembly. Approaches linking molecular and physiological data dealing with temperature stress impact on mitochondria are still welcomed. Some mitochondrial proteins, e.g. alternative oxidase (AOX) are 'classical' modulators of stress response among plants [44-46]. Regulation of diverse *AOX* genes varies between monocots and dicots. In a number of plant species the alterations of AOX protein are less pronounced [47,48]. In

addition, AOX may not to be increased in abundance by certain stress treatments, for example chilling [49,50]. The latter phenomenon was also confirmed in our previous study [41]; we reported significant decline in AOX level in cauliflower mitochondria under cold stress and recovery. Overall AOX gene family responses on proteomic and transcriptomic levels were only partially associated and AOX was a suggested target of translational regulation in diverse temperature treatments. In tobacco (*Nicotiana tabacum*) leaves abundance of this protein reached maximum after 48 h of cold stress and slowly decreased afterwards [51]. This highlights the importance of the length of cold stress treatment to gain the plant acclimation, presumably by the induction of regular changes in the transcriptome first [52].

Assuming limitations of the deposited data, this work was undertaken to gain the comprehensive view about the influence of cold and heat treatment (as well as cold and heat recovery) on cauliflower mitochondrial proteome in relation to leaf transpiration and respiration rate, stomatal conductance, the rate of leaf photosynthesis, photorespiration as well as chlorophyll content and fluorescence. The current study extends our previous complexomic and functional data [41]. To determine mitochondrial proteome response in relation to plant respiration, we aimed to (1) investigate the dynamic nature of mitochondrial proteome under cold and heat treatment and stress recovery; (2) identify the most variable proteins in cauliflower inflorescence mitochondrial extracts; and (3) link proteomic and discussed metabolic/functional aspects with alterations of analyzed physiological parameters. On the whole, the broaden set of identified proteins responding to cold/heat stress and after stress recovery, which correlate with alterations in plant respiration and some general metabolic demands, was about to be characterized in cauliflower mitochondria.

2. Results

2.1. Proteome Maps of Cauliflower Mitochondria Under Stress Conditions

Mitochondrial proteins isolated from curds of control plants and from plants submitted to cold or heat treatment were resolved by the two-dimensional gel electrophoresis (2D PAGE). We also examined the mitochondrial proteomes from curds of stress recovered cauliflower plants with the idea to study impact of stress on the mitochondrial proteome under stress recovery. 2D gels for 5 different variants, including control, were run in triplicate. In order to create master gel, we chose the image of control variant as the reference and then we added the specific spots detected on the gels of remaining variants. The number of spots on silver-stained 2D gels varied from 347 to 511 between all analyzed variants including also the control one. It made 694 different spots that were taken into account for the building of synthetic silver-stained master gel (Figure 1). Contrary to silver-stained gels, the number of protein spots on colloidal Coomassie Brilliant Blue (CBB)-stained 2D gels was lower. Finally, for the analysis of spot variation, only 413 spots representing highly-abundant proteins from silver-stained gels were taken into account.

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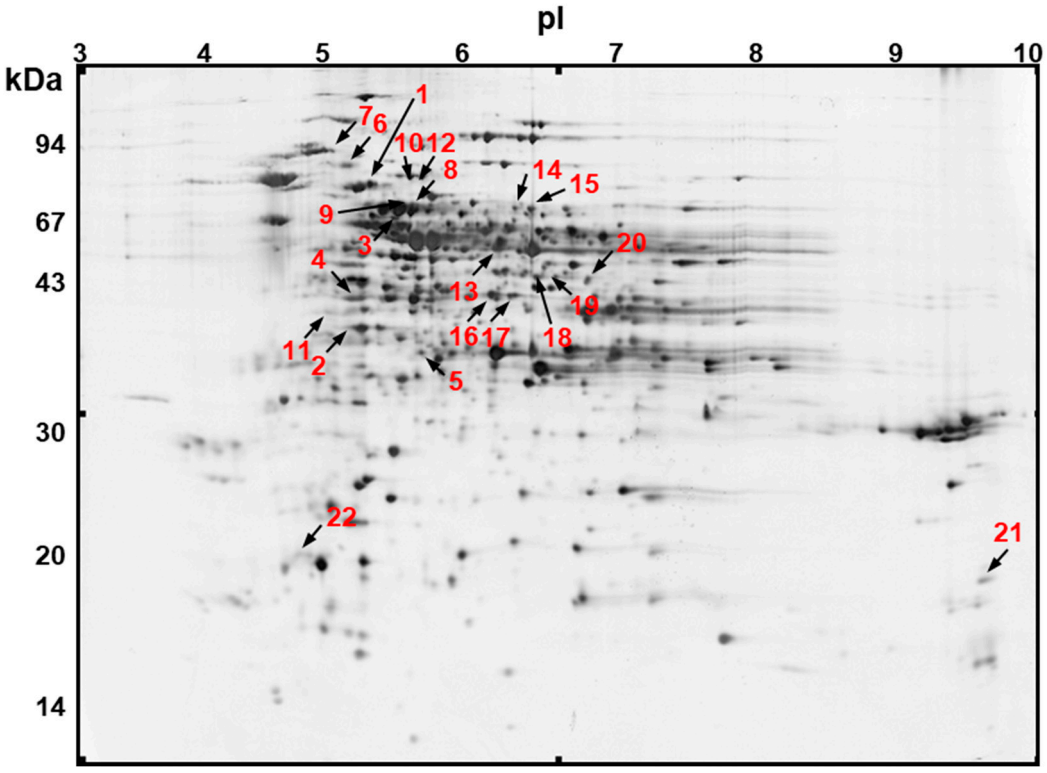

Figure 1. Position of varying spots on 2D silver-stained master gel of cauliflower curd mitochondrial proteome (in total 100 µg of mitochondrial proteins pooled from all experimental variants), including 694 repeatable spots. 24-cm immobilised pH gradient strips (linear pH 3-10) for the first dimension and precasted Ettan DALT 12.5% SDS- polyacrylamide gels for the second dimension were used. It shows the position of the 22 variable spots that were mapped and identified (they appear also in Figure S1 and Table S1). Protein molecular mass standards (Thermo Fisher Scientific) sizes are given in kilodaltons (kDa); pI- isoelectric point. Further experimental details in 'Materials and Methods'.

2.2. *Identification of Variable Protein Spots*

Twenty two spots (3.2 % on the silver-stained master gel) were significantly variable (verified by the analysis of variance [ANOVA] and Tukey's honest significant difference [HSD] test) as detected by using Image Master 7 Platinum software. Spot positions depicted on Figures 1 and S1 were calculated from three biological replicates. All spots were successfully identified by LC-MS/MS. The obtained data, were used for searching Mascot against National Center for Biotechnology Information (NCBI) database (version 20100203). Due to the limitations of protein matching to species-specific database (lack of completed cauliflower nuclear genome and total proteome, despite deposited *Brassica oleracea* mitochondrial genome [53]), cauliflower stress-responsive mitochondrial proteins were identified by using *Viridiplantae* section of the database. To avoid possible misidentifications resulted from large datasets, as pointed out by Schmidt et al. [24], we were able to set the threshold of false positive rate to 5%.

Identifications of protein spots are presented in Table S1 and properties of individual peptides for each protein spot are given in Table S2. As it was shown, all 22 spots represented 16 non-redundant stress-responsive proteins. Unambiguous results were obtained from MS analyses; the percentage of sequence coverage ranged from 14 to 42% and the total number of identified

peptides varied from 12 to 301. Among all spots, mitochondrial proteins in all but one spots (spot no. 13 to *Brassica napus* protein sequence) were identified basing on their high similarity to Arabidopsis sequences. The experimental molecular mass corresponded roughly to the theoretical value for the majority of spots. Some proteins including phosphoglycerate kinase isoform 1 (PGK1; spots no. 4, 10), mitochondrial elongation factor Tu (mtEF-Tu; spots no. 16, 17), isocitrate dehydrogenase (IDH; spot no. 18) and citrate synthase (CS; spots no. 19, 20) showed a few kDa decrease in molecular mass between theoretical and gel values. We are rather convinced that this did not resulted from the excessive proteolysis in cauliflower mitochondria.

Seven spots (about 1% of all) displayed significant variations in their abundance after cold stress and cold recovery. According to Tukey's HSD test, 4 proteins, including 3 members of heat shock protein (HSP) family (spots no. 1, 6, 7) and 3-phosphoglycerate dehydrogenase (PGDH)- like protein (spot no. 3) were increased in abundance during cold treatment. After cold recovery another 3 proteins were significantly increased in abundance, namely pyruvate dehydrogenase β-subunit (PDHβ; spot no. 2), PGK1 (spot no. 4) as well as NAD^+-dependent malate dehydrogenase (MDH; spot no. 5; Table S1).

After heat stress and heat recovery, 15 responsive spots (representing 11 non-redundant proteins) were identified (about 2.2% of all spots; Table S1). It happened that 4 proteins: PGDH-like protein, δ-1-pyrroline-5-carboxylate dehydrogenase (P5CDH), mtEF-Tu as well as CS were represented by double spots (no. 8/9, 14/15, 16/17 and 19/20, respectively) displaying slightly different molecular mass and pI values. According to Tukey's HSD test, it appeared that 3 proteins significantly raised their level during heat treatment: PGDH- like protein (spots no. 8), mtEF-Tu (spots no. 17), as well as d-subunit of the mitochondrial ATP synthase (ATPQ; spot no. 22), but 2 proteins: P5CDH (spot no. 15) and CS (spots no. 19/20) were decreased in abundance.

Some variations were also observed after heat recovery. Here, we detected a more intense accumulation of the broaden set of proteins, namely PGDH- like protein (spots no. 8, 9), PGK1 (spot no. 10), β-subunit of succinyl-CoA ligase (SCLβ; spot no. 11), chaperonin 10 (CPN10; spot no. 21), and ATPQ (spot no. 22). Notably, CPN10 extensively increased in abundance. In those conditions, we also noticed the significant decline of the ATP synthase α-subunit (ATP1; spot no. 13), mitochondrial processing peptidase subunit-β (MPPβ; spot no. 12), P5CDH (spots no. 14, 15), mtEF-Tu (spots no. 16/17), IDH (spot no. 18) and CS level (spots no. 19/20; Table S1). In addition, with a help of polyclonal antibodies, we verified the abundance of ATP1 after heat recovery on 2D Western blot. As it was shown on Figure 2, the respective variations of this protein assayed by Western immunodetection roughly followed the protein variations on the silver stained 2D gels. Notably, 4 proteins that were identified as double spots, showed very similar response after heat and heat recovery, which is in favor for the correctness of their assignments (Table S1).

In order to check whether the presence of unknown posttranslational protein modifications (PTMs) could be associated with protein multi-spotting, we have estimated both number of modified residues from spectra of tryptic peptides representing all double spotted stress-responsive proteins as well as expected molecular mass difference from the extent of given PTM between compared spots. Full peptide data are presented in Table S3a and the summary of PTMs in Table S3b. We focused mostly on phosphorylations, deamidations, methylations, formylations and ethylations. As no phosphoprotein enrichment was performed, we were unable to characterize further phosphoproteome in cauliflower mitochondria, despite phosphorylated residues accounted

mostly for total theoretical molecular mass difference between double spots. However, only limited correlation was found between this value and the experimental molecular mass difference for those spots. Therefore multi-spotting of some analyzed proteins came from not investigated modifications and/or from the expression of gene family members.

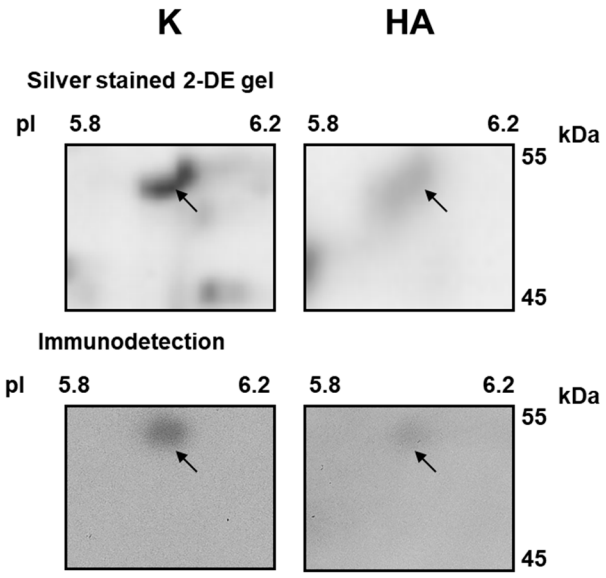

Figure 2. Western immunodetection of ATP1 on 2D blots containing cauliflower curd mitochondrial proteins from control grown plants (**K**) and from heat recovered plants (**HA**). 100 µg of mitochondrial proteins were loaded onto all gels. 24- cm immobilized pH gradient strips (linear pH 3-10) for the first dimension and precasted Ettan DALT 12.5% SDS-polyacrylamide gels for the second dimension were used. For protein transfer onto Immobilone membrane, semidry system was applied. Blots were probed with polyclonal antibodies raised against mitochondrial ATP synthase subunit α (ATP1). Detection was carried with chemiluminescence assays after incubation with HRP-conjugated secondary antibody. Representative results (from triplicates) are shown. For the comparison, panels showing fragments of silver stained 2D gels that contains spots for ATP1 are displayed. Arrows (indicated in each blot) show position of ATP1 on immunoblots and 2D gels. Protein molecular mass standards (Thermo Fisher Scientific) sizes are given in kilodaltons (kDa); pI-isoelectric point. Further experimental details in 'Materials and Methods'.

2.3. *Functional Categorization of Identified Proteins*

Based on Arabidopsis protein orthologs, we used functional categorization (FunCat) scheme at Munich Information Center for Protein Sequences database (http://mips.gsf.de) for clustering of stress-responsive proteins resolved on 2D gels into five functional categories (Figure 3 and Table S1). Counting the number of spots within each category (Figure 3, panel: protein spots by number), it appeared that the majority of cauliflower mitochondrial protein spots responsive to cold and heat stress, belonged to the class participating in carbohydrate metabolism, including tricarboxylic acid (TCA) cycle components (about 36% spots) as well as amino acid metabolism and protein fate (each of ca. 23%). The next ones were represented by respiratory chain (RC) components and protein synthesis apparatus (each of ca. 9%). Interestingly, eight spots (36%) representing 6 proteins were already annotated as stress responsive in MIPS database.

Spots linking with RC components increased in abundance after heat stress, as well as after heat recovery; however the ones linked with amino acid metabolism were upregulated after cold and heat stress. On the contrary, spots linked with carbohydrate metabolism decreased in abundance after cold and heat, but markedly upregulated in cold and heat recovered plants (Figure 3, panel: protein spots by abundance), Interestingly, the total abundance of spots related with the protein fate showed some increase after cold, but neither after heat stress (where it was decreased), nor after recovery phase. It seems that the majority of identified protein spots that belonged to

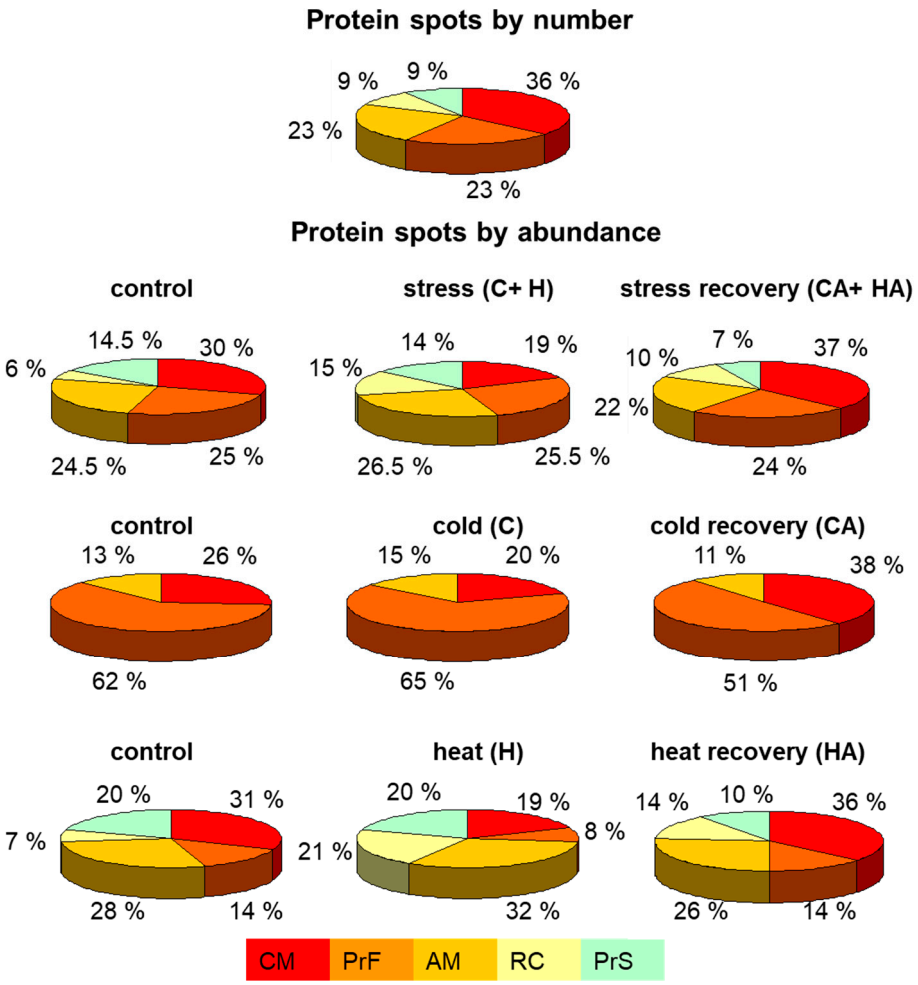

Figure 3. Functional categorization of cauliflower stress-responsive protein spots, analyzed by 2D PAGE. Spots were analyzed both according to their number (upper) as well as densitometrical volume (abundance; below) calculated for the each of functional groups in **control** plants, cold or heat stressed plants (**C** or **H**, respectively) and cold- or heat-recovered plants (**CA** or **HA**, respectively). Bar legend of categories: CM- carbohydrate metabolism, PrF- protein fate, AM- amino acid metabolism, RC- respiration (respiratory chain components), PrS- protein synthesis.

protein fate class appeared responsive in cold stress and cold recovery, which indicates for its overall importance in low temperature response in cauliflower mitochondria. Many protein functional classes, however, were regulated by heat and heat recovery (Figure 3, panel: protein spots by abundance, at the bottom).

2.4. The Effect of Cold Stress on the Abundance of Additional Mitochondrial Proteins

Due to the fact, that the number of cold-regulated proteins was lower from those regulated by the heat stress in cauliflower mitochondria, we decided to verify our analyses by additional Western immunoassays (Figure 4). The level of selected proteins was monitored in mitochondria isolated from curds of cauliflower plants grown either in control conditions or submitted to cold, or from cold recovered plants. To verify protein loading, Western blots were Coomassie-stained.

We assayed the level of glycine decarboxylase subunit-H (GDC-H), serine hydroxymethylransferase 1 (SHMT), mitochondrial porine isoform 1 (VDAC-1), some OXPHOS proteins, including complex I (CI) NAD9 subunit, cytochrome c_1 and complex IV (CIV) COXII subunit as well as proteins engaged in cytochrome *c* (cyt. *c*) maturation in plant mitochondria, particularly ABC transporter I family member 1 (CCMA) and CcmF N-terminal-like mitochondrial proteins 1 and 2 ($CcmF_{N1}$ and $CcmF_{N2}$, respectively). With the application of specific antibodies, we also investigated the level of cytoplasmic small Hsp17.6 of class I (sHsp17.6C-CI), that interacts with mitochondria under temperature stress [54]. It appeared, that the level of Hsp17.6C-CI associated with mitochondrial membranes raised up extensively after cold stress and remained quite high after cold recovery (Figure 4). The abundance of GDC-H showed almost three-fold change decrease after cold recovery, but only slightly after cold stress. Similar decrease in the level of $CcmF_{N1}$ and $CcmF_{N2}$ proteins was observed in cold and cold recovery conditions (up to two- and three-fold change, respectively). On the contrary, the accumulation of the CCMA transporter protein was not affected by cold, however it was decreased (by almost 50%) after cold recovery. In the tested conditions, the relative abundance of SHMT and VDAC-1 was also decreased. Regarding RC proteins, we detected small upregulation of NAD9 subunit of CI after cold stress and the subsequent major decline under cold recovery as well as the small, but significant downregulation of CIV COXII subunit and CIII cyt. c_1 in stress (Figure 4).

2.5. Cauliflower Physiological Responses To Cold and Heat Stress, and After Stress Recovery

Besides analyses of cauliflower mitochondrial proteome, we studied how leaf respiration was affected after cessation of cold and heat treatment as well as after post-stress plant recovery. By using appropriate assay [55], we determined mitochondrial respiration in the light (non-photorespiratory intracellular decarboxylation; R_d) in gas phase as the rate of CO_2 release. In addition, we also measured the rate of respiration of darkened leaves (R_n). It appeared, that the respiratory production of CO_2 in illuminated leaves was lowered in cold-stressed plants; however, under cold recovery, a significant burst of R_d was observed. R_n rate was also lowered after cold treatment and remained so after cold recovery. On the contrary, both R_d and R_n rates significantly increased in heat stress and decreased almost to control stage values after heat recovery (Figure 5).

To gain more complete view of cauliflower plant physiological status, we assayed also the impact of stress conditions and stress recovery onto leaf transpiration (E) rate, stomatal conductance (g_s) as well as essential photosynthetic parameters. We detected the decrease in E rate as well as lower g_s value under cold and heat stress, but not after cold recovery. However, after heat recovery leaf transpiration was slightly elevated (Figure 6).

To investigate whether all those responses were also accompanied by impaired photosynthetic performance, we also measured the rate of net CO_2 assimilation at three photosynthetic photon flux

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densities (PPFDs)- 200, 400 and 600 µmol m^{-2} s^{-1}. Here, the net photosynthesis intensity was presented only for 400 µmol m^{-2} s^{-1} (A_{n400}), which appeared the most optimal PPFD; the respective net

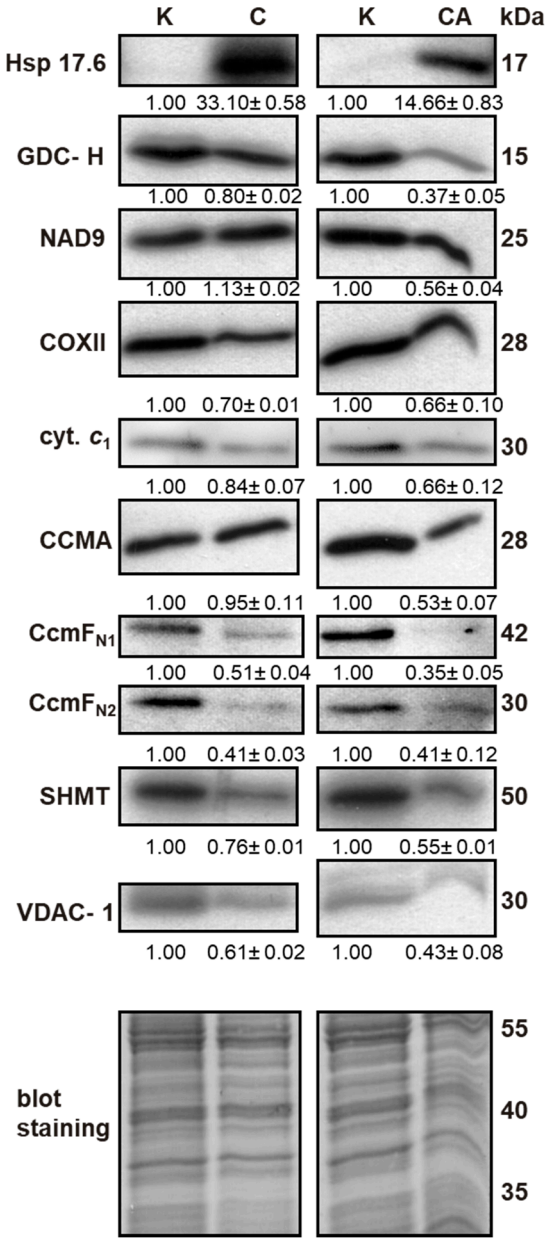

Figure 4. Western immunodetection of proteins from control grown plants (**K**), cold stressed plants (**C**) and from cold recovered plants (**CA**). About 10 µg of mitochondrial proteins from cauliflower curds were loaded onto SDS-polyacrylamide gels. Proteins were transferred onto Immobilone membrane using semidry system. All assays were performed using specific primary antibodies raised against heat shock protein 17.6C class I (Hsp17.6), glycine decarboxylase subunit H (GDC-H), NADH dehydrogenase (CI) subunit 9 (NAD9), cyt. c oxidase (CIV) subunit 2 (COXII), cyt. c_1, cyt. *c* maturation proteins (CCMA, $CcmF_{N1}$, $CcmF_{N2}$), serine hydroxymethyltransferase 1 (SHMT) and voltage-dependent anion channel 1 (VDAC-1). Detection was carried with chemiluminescence assays after incubation with HRP-conjugated secondary antibody. Representative results from triplicates are shown. The relative abundance of bands is given below each panel. The abundance in stress conditions (value ± SD) is standarized to 1.00 in control variants. For the loading control, blot

staining with Commassie Brilliant Blue is additionally shown. Protein molecular mass standards (Thermo Fisher Scientific) sizes are given in kilodaltons (kDa). Further experimental details in 'Materials and Methods'.

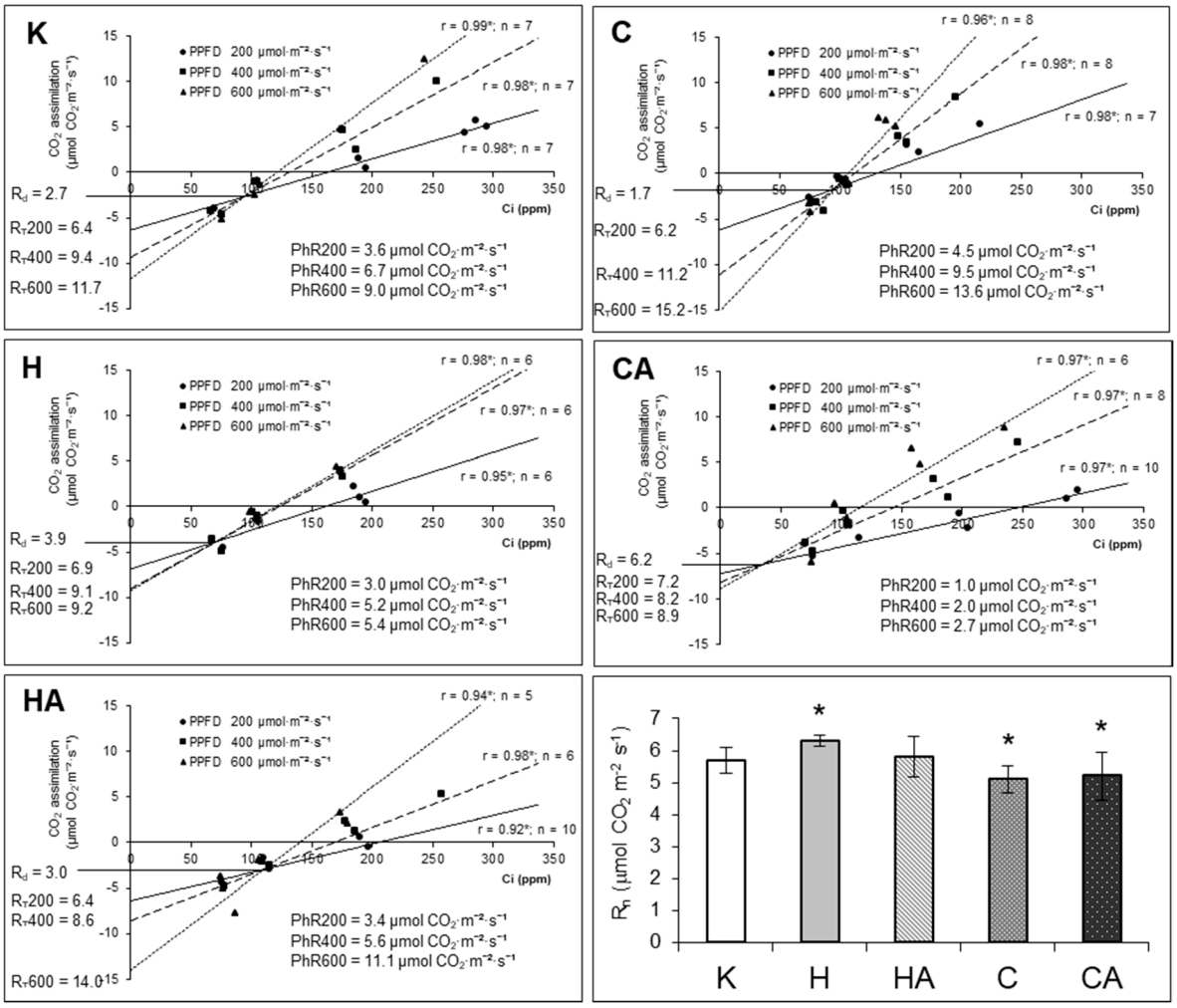

Figure 5. Changes in cauliflower leaf light (R_d), dark (R_n) respiration as well as total light (R_T) respiration and (PhR) photorespiration (all expressed in mmol CO_2 m^{-2} s^{-1}) at 200 (R_T200 and PhR200), 400 (R_T400 and PhR400) and 600 (R_T600 and PhR600) mmol m^{-2} s^{-1} illumination rate in control grown (**K**), heat stressed (**H**), heat recovered (**HA**), cold stressed (**C**) and cold recovered (**CA**) plants. All parameters were measured on 3-month-old plants with fully developed leaves with the application of the infrared gas analyzer. The data was recorded after at least 2 h of illumination. During experiment, each of the analyzed leaves were placed into a 6-cm^2 chamber of the analyzer. Results were recorded after initial leaf acclimation to the desired light and CO_2 concentration, relative humidity and temperature. The R_d rate was determined according to Laisk [55] method. The photorespiration rate for each PPFD value was determined as difference between R_T and R_d values. Asterisks indicate significantly different curves at $P = 0.05$ (Student's t-test). Further experimental details in 'Materials and Methods'.

CO_2 assimilation rate values at the remaining photon flux densities (A_{n200}, A_{n600}) followed similar to A_{n400} trends in stress response. The rate of A_{n400} was markedly decreased after cold, heat and also after

heat recovery and generally it accompanied similar variations in stomatal closure and leaf transpiration (Figure 6).

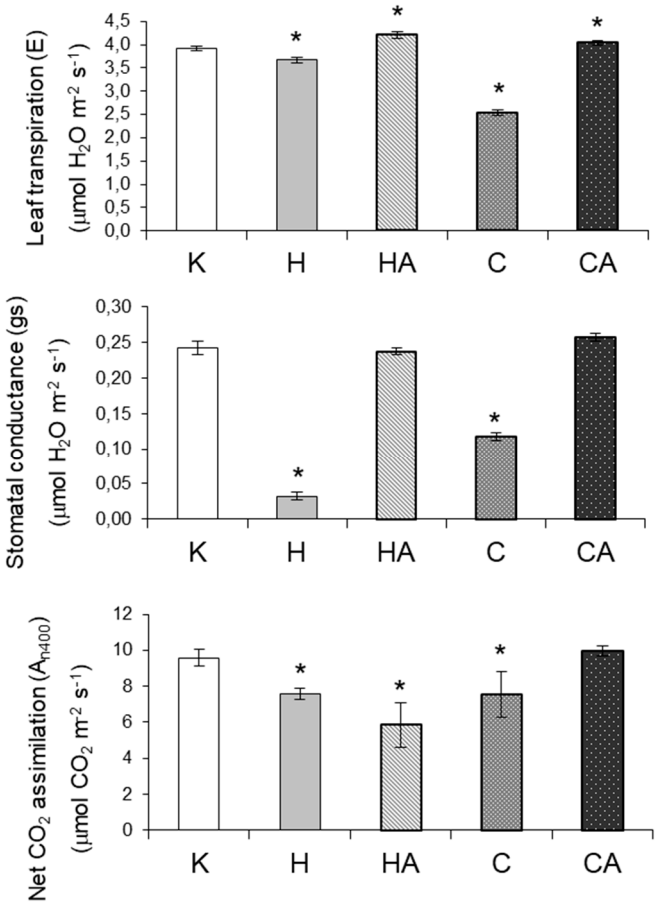

Figure 6. Changes in cauliflower leaf transpiration, stomatal conductance to water vapor and net CO2 assimilation rate at 400 mmol m^{-2} s^{-1} illumination in control grown (**K**), cold stressed (**C**), heat stressed (**H**), cold recovered (**CA**) and heat recovered (**HA**) plants. All parameters were measured on 3-month-old plants with fully developed leaves with the application of the infrared gas analyzer. The data was recorded after at least 2 h of illumination. During experiment, each of the analyzed leaves were placed into a 6-cm^2 chamber of the analyzer. Results were recorded after initial leaf acclimation to the desired light and CO_2 concentration, relative humidity and temperature. Bars are means ± SD (n> 3) and asterisks indicate significant differences (P< 0.05; Student's t-test) from the control (**K**). Further experimental details in 'Materials and Methods'.

Notably, all those parameters did not correlate with alterations in variable (F_v) to maximal (F_m) chlorophyll fluorescence ratio, which appeared relatively constant for all investigated stress conditions. However, F_v and F_m significanly decreased both after heat and cold stress as well as after heat recovery. The relative chlorophyll content (assayed by chlorophyll meter) was affected only after cold stress and cold recovery (Figure 7). Due to the fact, that in cauliflower curds, which are not involved in CO_2 assimilation, the decrease in abundance of two main photorespiratory enzymes (GDC and SHMT) was noticed (Figure 4), we aimed also to investigate photorespiration (PhR) in

photosynthetically active organs- fully expanded leaves. Using Laisk [55] method we determined the ratio of photosynthetic rate under three investigated PPFDs between ambient and low CO_2 concentration. It appeared, that PhR at all PPFDs markedly increased in cold stressed plants; however after cold recovery it was severily impaired. On the contrary, heat stress and heat recovery resulted only in the slight decline of PhR200 and PhR400 values, whereas PhR600 was more affected at heat stress, but it was recovered after heat recovery (Figure 5).

Overall, we showed that cauliflower plants, besides mitochondrial proteome plasticity, at the physiological level display only partial, but diverse alterations in various photosynthetic and respiratory parameters.

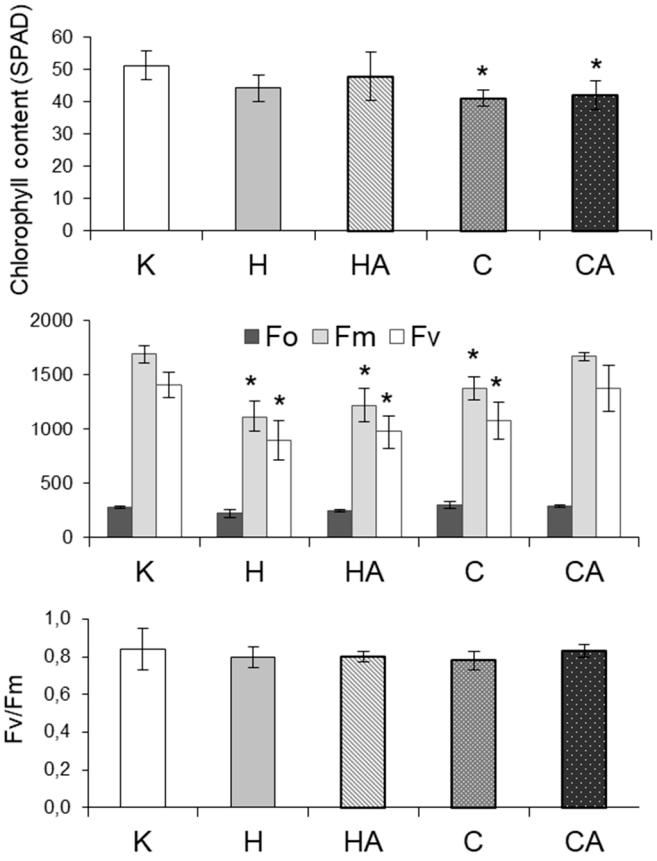

Figure 7. Changes in cauliflower leaf chlorophyll content minimal (Fo), maximal (Fm) and variable (Fv) fluorescence and Fv/Fm ratio in control grown (**K**), cold stressed (**C**), heat stressed (**H**), cold recovered (**CA**) and heat recovered (**HA**) plants. The chlorophyll content measured with the chlorophyll meter was expressed in relative units. Chlorophyll fluorescence was measured using the portable fluorometer. Before measurement, leaves were dark adapted for 30 min. Photochemical efficiency of PSII could be estimated from the Fv/Fm ratio, where Fv is the difference between Fm and Fo. Bars are means ± SD (n> 3) and asterisks indicate significant differences (P< 0.05; Student's t-test) from the control (**K**). Further experimental details in 'Materials and Methods'.

3. Discussion

3.1. Identification of Cauliflower Stress-Responsive Proteins by MS Analysis

In order to get more general view of the impact of cold and heat stress to the functioning of cauliflower mitochondria, we have started our study from the analysis of their proteome. Using 2D PAGE, twenty two stress-responsive spots representing 19 non-redundant proteins were selected. Although some proteins belong to the general components of the abiotic stress response [40], the set of cauliflower mitochondrial proteins responsive to temperature stress was in this study broadened by stress recovery data showing new candidates for stress response (Table S1). Our previous analyses [41,56] suggested that stress recovery is associated with the possible acquiring of stress tolerance by cauliflower displaying some alterations within the mitochondrial OXPHOS and dehydrin-like proteins. Nowadays we would like to complement study of mitochondrial complexome by extended physiological and proteomic (2D PAGE) analyses and to follow the importance of stress recovery conditions in such assays [57].

Cauliflower is closely genetically related with other *Brassica* species and the identification of mitochondrial proteins was conducted basing on protein sequence similarity between *Brassicaceae* members. Schmidt et al. [24] and Zhu et al. [36] have identified some proteins (e.g. ATPQ, CPN10, MDH, PDHβ and HSP81-1) that appeared stress-responsive in our study. The presence of protein spots containing glycolytic enzymes (for instance PGK1) (Table S1) was not curious, because this enzyme was reported to be associated with outer mitochondrial membrane [58,59]. Such finding was concluded mainly from the measurements of its enzymatic activity in mitochondrial extracts, however the cytosolic member of this enzyme family (At1g79550), distinct to the Arabidopsis homolog (At3g12780) of cauliflower protein, was also identified in large protein complex associated with mitochondrial membranes [58,60]. Interestingly, the Arabidopsis PGK1 ortholog from plastid proteome showed cold response [61], whereas cauliflower mitochondrial protein was affected after heat recovery. Cauliflower PGK1 displayed high similarity of its sequence to cytoplasmic isoform, but not to plastid one (data not shown). Therefore their responses can be different.

Four cauliflower mitochondrial proteins (3-PGDH, P5CDH, mtEF-Tu and CS) were represented as double spots. The presence of multiple spots on 2D gels was reported in numerous proteomic analyses, including also proteins analyzed in this study (Figure S1) [21,22,32,50,62-67]. Consequently, we checked, in how extent posttranslational modifications might be responsible for the presence of multiple spots for the investigated proteins. Due to the lack of quantitative analysis including laborious enrichment of protein extracts in modified proteins and technical limitations of our protein separation methods we weren't able to analyse accurately most of PTMs. Instead, we focused on few selected modifications only (Tables S3a and S3b). However, various algorithms used for the PTM prediction among Arabidopsis emphasize that our data is largely novel and also significantly broaden deposited records. Among investigated modifications, many phosphorylated and methylated peptides were detected. Phosphorylation (together with the oxidation) belongs to the most important PTMs, regulating the activity of many stress-responsive proteins; in plant mitochondria phosphorylation was especially studied in various detail [34,68-71]. Energy and transport proteins, HSPs and even RC components were identified as potent phosphorylation targets [71]. Among proteins that were present in multiple spots in our study, it was shown that rice (*Oryza sativa*) CS can be phosphorylated [72] and mtEF-Tu was subjected to oxidation [73]. Overall,

multi-spotting of cauliflower mitochondrial protein may depends not only on the presence of different PTMs, but largely on multigenic families coding novel protein isoforms, which resulted from the complex evolution of *Brassica* nuclear genomes as they underwent numerous chromosomal doublings, hybridizations and rearrangements [74]. More sensitive and quantitative proteomic assays should be implemented in the future for the better characterization of PTMs in cauliflower mitochondrial proteome.

We noticed also minor differences in molecular mass between nominal and observed values of some cauliflower mitochondrial proteins (Table S1). However, such discrepancies may be even more evident due to the protein degradation [75]. Taylor et al. [50] and Imin et al. [76] have shown that abiotic stress could induce accumulation of protein degradation products. We routinely used protease inhibitors for the preparation of mitochondria, therefore we think that the extensive proteolysis could not account for major molecular mass discrepancies. We also excluded protein hydrophobicity, as no correlation was observed between the grand average of hydropathy index and the differences between nominal and observed molecular mass of protein spots (data not shown). Overall, despite the general similarity of 2D maps, it seems that numerous mitochondrial proteins may slightly differs in some physicochemical properties between Arabidopsis and cauliflower. We expected this from our previous analyses [23].

3.2. *Variations in the Pattern of Cauliflower Mitochondrial Proteome in Stress and Stress Recovery, and Their Metabolic Relevance*

From the identified 16 stress-responsive proteins, at least ca. two-fold change variations in protein abundance were shown for most of them (Table S1). Under heat stress and heat recovery more proteins which varied in abundance were identified, comparing to cold/cold recovery.

Some proteins detected in our study were previosly shown to vary under diverse abiotic stress conditions. PDH participates in regulation of carbon flux from glycolysis to TCA cycle. In the published data upregulations of PDH subunits prevail; in pea (*Pisum sativum*) mitochondria, PDHβ proteolytic products accumulate [50,77,78]. In rice leaves, however, contrasting PDHα responses (similarly to HSP90, see below) were noted under diverse cold conditions [37]. It is known, that also other components of PDH complex including dehydrolipoamide dehydrogenase, may be upregulated during heat stress [79]. On the contrary, we showed extensive accumulation of PDHβ after cold recovery, but not after heat treatment. In rice, PDHβ was downregulated during hypoxia [80], however subunit-α of this enzyme increased during heat treatment and decreased in abundance after stress cessation [81]. Our results suggest that despite the overall number of major cold-responsive mitochondrial proteins was lower than those regulated by heat, it seems that carbon transfer from glycolysis to TCA cycle is increased in cauliflower cold response. Nonetheless, stress can regulate plant energetic and metabolic demands, including ATP/ADP intracellular and intramitochondrial ratio and the need for carbon skeletons [82].

Mitochondrial NAD^+-dependent MDH, which was increased in abundance after cold recovery in cauliflower mitochondria, in Arabidopsis was accumulated in response to different environmental stimuli including cold de-acclimation (but not cold acclimation) [32,33,83,84]. Arabidopsis MDH1 was suggested to belong to translational regulation targets [85]. The level of this enzyme (together with CS) was diversely modulated by various chilling conditions; generally, MDH abundance increased in cold-sensitive plant species [26,37,50,78,86,87]. Dumont et al. [88]

investigating alterations in MDH abundance in diverse pea genotypes submitted to the combined cold and frost action and obtained contrasting results depending on the stress treatment and duration, similarly to Yin et al. [89] and Cheng et al. [90] studies on MDH1 level in soybean (*Glycine max*) embryonic axes. Interestingly, CS responses depend on the severity of the temperature treatment, e.g. in the severe chilling the abundance of this enzyme declined, whereas under moderate treatment it increased [91]. During 2-day-long heat stress MDH was also diversely downregulated in two *Agrostis* species depending on their thermotolerance [92]. Such decrease in abundance was reported also for soybean MDH [89]. The significant up-regulation of cauliflower MDH only to cold recovery suggests that it may be the cold recovery marker [93]. However, heat recovery appeared detrimental for the level of this enzyme in cauliflower mitochondria [41]; cauliflower IDH and CS markedly declined after 2-day-long heat recovery. Similar changes were reported for CS in heat adapted *Populus euphratica* [79]. In general, heat (which may lead to the intramitochondrial oxidative damage) results in TCA enzymes, mitochondrial NADH pool and ATP synthesis impairments [94] and that the cold stress results in general stimulation of respiratory metabolism.

It appeared that cold stress causes the increase in the level of cauliflower HSPs; interestingly, in our study HSP70 and HSP90 increased more than in pea (*Pea sativum*) and rice leaves and peach (*Prunus persica*) barks [38,50,95]. Similar trend was observed in rice during salinity [96] and heat action in Arabidopsis [97]. However, mitochondrial HSP70 declined in the abundance in the stored or detached peach fruits submitted to the prolonged cold [77,98]. Van Aken et al. [29] reported that Arabidopsis mitochondrial heat shock proteins responded only slightly to some forms of abiotic stress, as HSP70 in case of cadmium treatment [83]. Another protein, HSP81-2, appeared to be cold responsive in cauliflower mitochondria, contrary to the Arabidopsis ortholog, which was regulated by heat [31,97]. Notably, the regulation of HSP90 level in rice leaves depended on the cold duration [38]. CPN10 remained unaffected after cold stress in pea [50], but in cauliflower mitochondria this protein accumulated very extensively under heat recovery. Also mitochondrial sHsp22 was induced preferentially by heat (but not by cold) in soybean seedlings [25]. Together with FunCat data all those findings suggest that the accumulation of some HSPs in cauliflower mitochondria may be specific for the preferential temperature stress conditions. Some HSPs can also diversely participate in various stress conditions leading to the distinct stress responses. It should be also noted, that the expression of two proteins (HSP70 and MDH1) regulated by low-temperature treatment, as well as additional proteins (mtEF-Tu, CS) responded to heat/heat recovery in our study is known to be modulated by the specific glycine-rich protein (displaying RNA chaperone activity) under cold adaptation in Arabidopsis plants [99].

Cauliflower mtEF-Tu increased in abundance mainly after heat stress and did not last after heat recovery; overall this may imply that the mitochondrial translation apparatus is impaired after heat cessation and rapid shift to control growth conditions of cauliflower, which was observed also for instance in case of chilled soybean embryo axes [89]. mtEF-Tu together with β-subunit of succinyl-CoA ligase increased in abundance in drought and partially in flood and MPP and ATP1 by salinity in Arabidopsis [32,84]. Curiously, β-subunit of succinyl-CoA ligase showed heat duration-dependent responses in soybean roots and rice leaves [25,37]. The major downregulation of succinyl-CoA ligase β-subunit in cauliflower mitochondria followed alterations of other TCA cycle components (IDH, CS) after heat stress [37,79], but not after heat recovery. Therefore, we can

speculate that succinyl-CoA ligase may be preferentially accumulated in cauliflower during heat recovery in order to adjust the mitochondrial metabolism to control conditions.

ATP1 belongs to the proteins with level alterations dependent on the given species as well as stress intensity and duration [42]. In our study, ATP1 was greatly declined in abundance after heat recovery. Similar trends were noted for pea, Arabidopsis, and *Zea mays* in a course of chilling, prolonged heat, CuCl or H_2O_2 treatment [100,39,50,101]. On the contrary, cauliflower ATP1 abundance slightly increased in heat, similarly to the unassembled subunit *b* of ATP synthase [41]. We also found that heat caused the vast increase in abundance of ATP synthase d-subunit, contrary to its major downregulation reported by Gammulla et al. [37] and Tan et al. [39] for cold-stressed Arabidopsis cell cultures and heat-treated rice leaves, respectively. During oat (*Avena sativa*) seed storage, ATP1 level was consistently declined as the temperature increased from 35 to 50°C, whereas subunit d of ATP synthase initially increased and then decreased in abundance under the same treatment; notably, subunits d and α were differentially accumulated at 10 and 16% moisture content, respectively [102]. Overall, those findings suggest that demand for ATP synthesis during heat treatment increases and the excess of *de novo* synthesized diverse ATP synthase subunits, e.g, mitochondrially-encoded ATP1 or nuclear-encoded ATP7 proteins is likely to be assembled into novel ATP synthase holocomplexes, labile in heat recovery [41].

As concerns the decrease in the level of MPPβ after heat recovery, we think that this may reflect the impairment of import machinery, which may not be fully restored after stress recovery: according to our previous study [41], another subunit- MPPα appeared also to be down-regulated in heat recovery. Gammulla et al. [37] and Neilson et al. [38] noticed contrasting changes in the level of MPP subunits in rice leaves under low temperature and the overall downregulations under heat. The level of MPP subunits underwent major changes in flood, indicating for mitochondrial damage [84]. The influence of abiotic stresses on the efficiency of protein import into plant mitochondria was investigated, inter alia, by Taylor et al. [103] and Giegé et al. [43]. Taylor et al. [103] observed import inhibition of all tested pre-proteins into pea mitochondria during thermal stress. In turn, Giegé et al. [43] reported that the capacity for *in vitro* mitochondrial protein import is not affected after sucrose starvation in Arabidopsis cell cultures. Owing our present and previous results [41] the pattern of protein import into cauliflower mitochondria under temperature stress should be investigated.

Another down-regulated cauliflower mitochondrial protein in heat was P5CDH, an enzyme involved in proline degradation pathway of Pro/P5C cycle [104]. Enzymes of this cycle, including P5C synthetase and proline dehydrogenase (ProDH) could be reciprocally expressed under stress. Moreover, ProDH closely associates with OXPHOS system [42,105]; it was suggested that P5CDH prevents oxidative stress and electron run-off within mitochondrial respiratory chain during Pro metabolism [106]. Free Pro accumulated in leaves of cold treated cauliflower of wild type and mutant clones selected on hydroxyproline containing medium, however after salinity stress- in mutated populations [107,108]. Interestingly, the level of P5CDH messengers significantly decreased in Arabidopsis plants expressing ectopically P5C synthetase 1 in response to heat stress. Pro accumulation impeded Arabidopsis seedlings growth in heat stress and may not serve as protective osmolyte [109]. Therefore it would be important to determine whether the decrease in abundance of P5CDH in cauliflower curds is associated with the increased Pro level after heat stress and heat recovery.

To extend our knowledge about cauliflower cold-responsive proteins, we carried out Western immunoassays using antisera against dedicated proteins (Figure 3). We observed vast accumulation of cytosolic Hsp17.6C-CI after cold stress and recovery indicating for the interaction of small HSPs with cauliflower mitochondrial membranes under prolonged cold treatment (as it was speculated by Rikhvanov et al. [54] for heat-stressed Arabidopsis cell cultures) and the importance of stress recovery phase in gaining of stress resistance. Overall, HSPs are known to form oligomeric complexes with stress-affected proteins [110]. Important photorespiratory enzymes, GDC and SHMT, were decreased in abundance after cold recovery, similarly to *Agrostis scabra*, *A. stolonifera*, Arabidopsis, pea, *P. cathayana*, rice and wheat (*Triticum aestivum*) proteins in cold, heat and drought [37,38,50,92,111-113]. This is along with the reported declined level of those enzymes in plant mitochondria under unfavorable conditions, leading to photorespiratory impairments [111]. However, under microspore development in rice plants submitted to cold, GDC-H was upregulated [114]. Interestingly, such up-regulation of GDC-H was also reported in in pea leaves under frost and independently to cold tolerance and in case of SHMT- in cold and salinity [37,88,115]. GDC-H slightly increases in abundance also in the early stages of low temperature action [116]. With accordance to that, as a part of protective mechanisms, vast accumulation of GDC-H transcripts in Arabidopsis leaves in the response to short cold treatment was also reported [117].

We also checked the variations in the level of some proteins engaged in maturation of cyt. *c*. Interestingly, major level downregulations of $CcmF_{N1}$ and $CcmF_{N2}$ proteins suggest that components of cyt. *c* maturation apparatus, including putative heme lyase components, may be sensitive to temperature stress. Generally, evidences for alterations of the level of those proteins in plant mitochondria during stress conditions are quite scarce. However, Naydenov et al. [118] found that *ccm*F_N messengers responded during 3-day-long cold in maize embryos.

Regarding the level of other mitochondrial proteins during cold stress and cold recovery, VDAC-1 was downregulated in cauliflower mitochondria, which is generally in favor to the previously published results [37,77,94,119]. Interestingly, according to our previous study [41] we found the affected level of another VDAC isoform (VDAC-2) under heat recovery only. In addition, we detected some level regulations of selected RC components, e.g. NAD9, COXII and cyt. c_1. Despite Tan et al. [39] found cyt. c_1 abundance alterations (roughly followed by our data) among number Arabidopsis proteins declined in cold, they did not identify COXII among them. However, those authors reported also the increased level of NAD9 protein in chilled Arabidopsis cell cultures. Longer cold acclimation led to the downregulation of this protein in wheat crowns, similarly to our data [112], but 72 h-long cold stress resulted in NAD9 increase [37]. Selected CIV subunits (e.g. 6b-1 in chickpea [*Cicer arietinum*]) could be also declined in abundance in cold, which suggests overall respiratory activity decreased [120]. In most of the investigated plants, the level of COXII increased under low temperature and the overall changes in NAD9 abundance seems to be species-specific under temperature stress [5,121,122]. OXPHOS components are heat action sites in cauliflower [41]. It should be underlined that cold/cold recovery responses in cauliflower mitochondria resulted also in few protein upregulations as it was evident from 2D PAGE.

Finally, Western immunoassays extended the 2D PAGE data for cold-regulated proteins and the current study have also broaden the knowledge on temperature stress responsive mitochondrial proteins, comparing to our previous complexomic data [41]. Obtained results indicate for the variations of the same mitochondrial proteins in analyzed stress conditions between cauliflower and

other plant species. Comparing to existing data, few novel proteins, representing various pathways of mitochondrial metabolism were discovered as responsive ones in thermal stress in cauliflower mitochondria. Therefore, one could speculate that numerous signaling pathways may be induced during action of cold or heat stress, allowing to alter partially the pattern of mitochondrial proteome. However, from our proteomic and FunCat data (which is partially distinct when compared to the literature), we can conclude that those pathways may be distinct for cold and heat treatment. Various metabolic pathways (e.g. TCA cycle) may diversely participate in the particular stress response, what result in plethora of various proteomic effects for cold, heat stress conditions and for stress recovery.

3.3. Cauliflower Leaf Respiratory Responses To Cold and Heat Stress

Temperature belongs to the critical factors controlling plant growth and development. Understanding both molecular, physiological as well as metabolic responses of crop and vegetable species to teperature stress in order to improve their tolerance and sustain the high field yield is crucial [123]. However, the data regarding physiological functioning of *Brassica* species, including cauliflower, in cold and heat treatment [15,124] is still insufficient, contrary to some other environmental conditions, e.g. salinity or cadmium treatment [125-129].

Cauliflower is one of the most agriculturally important vegetable crops worldwide [107]. Notably, cauliflower and kale (*B. oleracea* var. *acephala*) belong to species better cold- and frost-adapted than Arabidopsis [107,130]. In our study, cold or heat stress was applied to cauliflower plants at the early stage of curd development, which enabled us to study the stress response of plants both on the molecular and physiological level. Previously we used polarographic assays for investigating of physiological properties and the activity of alternative pathway under temperature stress and recovery in isolated cauliflower mitochondria [41]. For physiological measurements in the current report, fully developed cauliflower leaves instead of curds were chosen; leaves, contrary to other plant organs, appeared more cold sensitive, what make them most suitable for physiological assays [131]. We determined leaf respiration rate (by gas-exchange measurements on illuminated and darkened leaves), transpiration, stomatal opening, net CO_2 assimilation rate as well as chlorophyll level and fluorescence parameters which appeared were affected by the same treatments at various extent (Figures 5-7), complementing our previous data.

In our study, the increase of R_n and R_d after heat stress and their subsequent decrease to the level of control variant during heat recovery suggest that adaptative forces of respiratory metabolism of cauliflower leaves to thermal treatment depends on the stress duration. The increase of respiration after heat stress was also assayed in a number of plants, e.g. pepper (*Capsicum annuum*) leaves, which is a thermotolerant species with effective energy dissipation and ROS scavenging systems [132]. Due to the fact, that cold stress acted for longer period and appeared even more detrimental than heat, we did not observed R_n return to the level of control variant after cold recovery. This indicates for some irreversible effects in the cold, contrary to heat response (Figure 5). Such temperature recovery is expected to control energetic needs during acclimation, because of larger maintenance costs due to the increased activity of number enzymes [14].

The rate of respiration belongs to the first processes affected in plants subjected also to the low temperature treatments [27]. In the illuminated cauliflower leaves, R_d burst after cold recovery was evident; also for cold-acclimated Arabidopsis plants light respiration increased [14Talts et al., 2004].

In some plants, however, the increase in respiration rate is visible at the early stage of cold treatment and it declines afterwards [42]. Overall, the importance of R_d in thermal adaptation is suggested. In addition, Talts et al. [14] showed that R_n of Arabidopsis leaves was more sensitive to cold stress than R_d. We noticed more evident decrease in R_d rather than in R_n after cold treatment (Figure 5). However, in various winter and spring wheat and rye (*Secale cereale*) cultivars, chilling resulted also in the small R_n increase [133]. Apart from the known various temperature treatments, species-specific respiratory responses are suggested between various plant species.

In our study, cold, heat and heat recovery resulted in significant decrease of net photosynthesis rate, which partially accompanied by the decreased stomatal conductance [14,132,134,135]. Similarly to cauliflower data, the post-cold plant acclimation resulted in recovery of photosynthesis [134]. Copolovici et al. [135] pointed out the relevance of various cold/ heat treatments for different photosynthesis and stomatal conductance decrease in tomato (*Solanum lycopersicum*) leaves, which is also important in our case (Figure 6). Dahal et al. [133] showed that in some wheat and rye cultivars, cold resulted in the decrease both net CO_2 assimilation and as well as leaf transpiration and stomatal conductance. Leaf transpiration, stomatal conductance, chlorophyll content and photosynthesis also responded to cold in *P. cathayana* [136]. The decreased photosynthetic CO_2 assimilation rate in cauliflower leaves also correlated with apparent decrease in stomatal conductance and transpiration rate after cold stress, however, in heat stress, stomata were closed even in more extent (Figure 6). Similarly, heat stress affected photosynthetic parameters and decreased stomatal conductance in grapevine (*Vitis amurensis*) and tobacco leaves [137,138]; in cauliflower leaves after heat recovery, despite quick stomatal opening, the net photosynthetic rate remained decreased (Figure 6).

Under the temperature stress, chlorophyll level and fluorescence as well as PS performance could be affected at various extent [10,124,137,139]. In our case, the decrease of F_m and F_v was accompanied by the lower amounts of chlorophyll in cauliflower leaves only after cold treatment and chlorophyll fluorescence parameters were not restored only after heat recovery. The lower chlorophyll content in leaves of cold stressed may suggest some damages in photosynthetic apparatus as photosynthetic rate was decreased. Generally, heat stress may result in the decrease of F_v/F_m [132]. However, in our study, PSII performance was largely unaffected due to the overall stable F_v/F_m ratio in all stress conditions investigated (Figure 7). It is known that the heat damage of PSII, accompanied by the decrease of CO_2 assimilation rate, occurs when severe stress conditions (exceeded 42°C) were applied on illuminated leaves; however, this damage could be restored either in cases when 'point of no return' is not exceeded or when exogenous Ca is applied for stomata opening [138,140,141]. We conclude that despite our heat treatment conditions bordered with this treshold between mild and severe conditions, closed stomata in heat stress resulted in the overall photosynthetic, but not respiratory decrease (Figures 5 and 6) and the overall less susceptibility of cauliflower to heat than cold treatment.

We also noticed association between decreased photorespiration rate and GDC-H and SHMT levels in cold recovery (Figures 4 and 5). Photorespiratory decline under temperature stress may results from GDC and SHMT downregulations in abundance and/or activity [50,92,111,117]. In our study the photorespiratory impairment was noticed after heat treatment and heat recovery. Here decreased photorespiration corresponded with the decreased photosynthetic activity (due to the over-reduction of the photosynthetic chain) and appeared irreparable after heat recovery. Also in pepper leaves heat treatment decreased both net photosynthetic as well photorespiratory rate [132].

Interestingly, in cauliflower mitochondrial proteome, the increased level of MDH was associated with GDC-H and SHMT downregulation also in cold stress and cold recovery. Mitochondrial MDH, which assists in metabolic flux through TCA cycle, could operate in reverse way, by reducing oxaloacetate to malate, providing NAD^+ for photorespiratory glycine decarboxylation [142]. Regarding our study, mitochondrial MDH did not responded to heat treatment and heat recovery. Despite distinct tissues (curds and leaves) were chosen for proteomic and physiological experiments, still we may speculate whether cauliflower NAD^+- dependent MDH is engaged rather in the increase of NADH pool inside mitochondria by acting within TCA cycle, and not in NAD^+ regeneration necessary for photorespiration in cold stress, because the level of MDH and GDC-H were regulated conversely. Further experimental attempts are necessary to elucidate this issue.

In general, we suggest that distinct cold and heat stress response pathways act variously not only on cauliflower mitochondrial proteome, but on investigated physiological parameters with limited association. Numerous differences in physiological responses to cold and heat stress between cauliflower and other plant species were easily observed.

4. Materials and Methods

4.1. Plant Material, Growth Conditions and Stress Treatment

Cauliflower (*Brassica oleracea var. botrytis* subvar. *cauliflora* DC cv. 'Diadom') seeds were purchased from Bejo Zaden (Poland). Cauliflower seedlings were produced in 0.09 dm^3 pots filled with peat substrate for growing cruciferous vegetables (Kronean-Clasmann). Seedlings with three-four leaves were transferred to larger containers (5 dm^3 in volume). Plants were grown for 3 months in cultivation chambers at a local breeding station (Poznan University of Life Sciences, Poland) at 23/19°C (D/N) and 70% relative humidity under photon flux density 200 µmol m^{-2} s^{-1} (16 h of light/8 h of dark). After 3 months of growth corresponding to the young inflorescence (10 cm in diameter) stage, plants were divided into few sets for stress treatment and the parallel control variants (plants grown in conditions described above).

Two stress variants were tested in this study: the direct stress treatment- heat or cold and post-stress plant cultivation (stress recovery). For the application of cold stress, plants before the isolation of mitochondria were transferred for 10 days to 8^0C. Heat treatment (40^0C) was applied to growing plants for 4 h before the isolation of mitochondria. After stopping the stress treatment, part of cauliflower plants were transferred to the standard growth conditions for 48 h for the stress recovery. Curds (5 mm topmost layer) were directly harvested either after stopping the stress treatment or after stress recovery.

4.2. Gas Exchange Measurements

All analyses have been carried out on at least three fully developed leaves from three 3-month-old plants. Leaves were taken from each plant representing all experimental variants (control versus stress- treated or control versus stress recovered plants). At least three biological replicas were concerned. All parameters (the rate of total CO_2 assimilation [A_g], A_n, total respiration rate [R_T], R_d, R_n, E, and g_s) were measured using LI-6400 XT infrared gas analyzer (Li-Cor) and adjusted to the enclosed leaf area determined by LI-300 leaf meter (Li-Cor). Data was recorded after at least 2 h-long illumination. During experiment, each of the analyzed leaves were placed into a

687 6-cm^2 chamber of the analyzer. Results were recorded after initial leaf acclimation to the desired light
688 and CO_2 concentration, relative humidity and temperature. Gas-exchange parameters were recorded
689 after leaf acclimated in the gas exchange chamber under following conditions: PPFD of 400 µmol m^{-2}
690 s^{-1}, 50% of the relative humidity (RH), 22^{o}C, 350 ppm of CO_2. CO_2 assimilation rate was also
691 determined at two additional PPFD values (200 and 600 µmol m^{-2} s^{-1}).
692 R_d rate was determined according to Laisk [55]. For each leaf, CO_2 assimilation rate representing
693 a given R_T was recorded during decreasing intercellular CO_2 concentration (C_i) to 0 ppm at 22^{0}C and
694 50% RH, and for each of the three different PPFD values (200, 400 and 600 µmol m^{-2} s^{-1}). For each
695 PPFD, the linear regression of CO_2 assimilation (A) versus C_i was calculated (A/C_i curve) and the
696 photorespiration rate (PhR for each PPFD value, denoted as PhR200, PhR400 and PhR600) was
697 determined as difference between R_T and R_d values (the last one expressed as a given CO_2 evolution
698 rate at the point of crossing of all A/C_i curves). The R_n rate was extrapolated from A value during
699 decreased PPFD to 0 µmol m^{-2} s^{-1} from A/PPFD curve.

700 *4.3. Chlorophyll Content and Fluorescence Measurements*

701 Chlorophyll content was measured with SPAD-502 chlorophyll meter (Minolta) and expressed
702 in relative units. Chlorophyll fluorescence was determined using the portable fluorometer
703 (PAM-2000; Walz) in a dark room with stable conditions. Before measurement, leaves were dark
704 adapted for 30 min. Minimal fluorescence (F_o) was measured under 650 nm wavelength with a very
705 low intensity (0.8 µmol m^{-2} s^{-1}). F_m was estimated after 1 s application of the saturating pulse of white
706 light (3000 µmol m^{-2} s^{-1}). PSII photochemical efficiency was estimated from the F_v/F_m ratio, where F_v
707 stands for the difference between F_m and F_o.

708 *4.4. Preparation of Mitochondria*

709 Mitochondria from 100 to 500 g of 5 mm-thick apical layer of cauliflower curds were isolated
710 using modified protocol of Boutry et al. [143], as decribed by Pawlowski et al. [23]. During isolation,
711 the Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche) was added. Protein concentration
712 was determined by the Bradford [144] method, using BSA as a calibrator.

713 *4.5. Control Assays*

714 Purity assays of isolated mitochondria (measurement of activities of mitochondrial cyt. *c*
715 oxidase, peroxysomal catalase, plastid alkaline pyrophosphatase and cytoplasmic alcohol
716 dehydrogenase) were conducted according to Pawlowski et al. [23]. Additionally, the purity of
717 isolated mitochondria was verified by transmission electron microscopy (JEOL 1200EXII, Jeol; [56]).

718 *4.6. Preparation of Samples for Two-Dimensional Electrophoresis (2D SDS-PAGE)*

719 Freshly isolated samples of cauliflower mitochondria were precipitated with trichloroacetic
720 acid at –20^{o}C overnight [145,146]. After centrifugation for 5 min (16,000*g*, 4^{0}C), pellets were washed
721 once with 1 ml of acetone supplemented with 20 mM dithiothreitol (DTT) and re-centrifuged as
722 described above. After vacuum drying, pellets were resuspended in the lysis buffer (7 M urea, 2 M
723 thiourea, 0.5% [*w*/*v*] 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate [CHAPS], 1.5%

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[*w*/*v*] DTT, 0.5% [*v*/*v*] pharmalyte, pH 3-10) and protein concentration was determined either with the modified Bradford assay [147] or using 2D Quant Kit (Amersham Biosciences).

4.7. 2D SDS-PAGE

All analyses were conducted at 15^{0}C; at least three biological replicas were concerned. Mitochondrial proteins (100 µg for silver nitrate staining or 500 µg for colloidal CBB) were first separated according to their charge on rehydrated Immobiline dry strips (24 cm, containing linear gradient of pH 3-10) with the rehydration buffer (8 M urea, 2% [*w*/*v*] CHAPS, 0.3% [w/v] DTT, 2% [*v*/*v*] pharmalyte, pH 3 to10) on IPGphor apparatus (GE Healthcare, Uppsala, Sweden). Conditions for isoelectrofocusing (IEF) were as follows: 1 h at 500 V (step), 1 h at 1000 V (gradient), 3 h at 8,000 V (gradient) and finally 5.5 h at 8,000 V (step). The strips were either stored at –80^{o}C or they were directly treated for 10 min with solution A (6 M urea, 50 m◎ Tris-HCl, pH 6.8, 30% [*v*/*v*] glycerol, 2% [*w*/*v*] SDS, 0.25% [*w*/*v*] DTT) and for the same time with solution B (solution A supplemented with 4.5% [*w*/*v*] iodoacetamide without DTT) and subjected for the second dimension run (SDS-PAGE).

For SDS-PAGE precasted Ettan DALT 12.5 % (*w*/*v*) polyacrylamide gels (GE Healthcare) and the Ettan Dalt Six electrophoretical chamber (for six gels) were used. Conditions for run were as follows: 45 min at 80 V and 15 h at 120 V. After electrophoresis, proteins on gel triplicates were either silver stained [148] for protein variation analysis or stained with colloidal CBB, according to Neuhoff et al. [149] for MS analyses. 2D gels were scanned, analyzed using 2D Image Master 7 Platinum software (GE Healthcare) and the normalized quantitative volume of protein spots was determined.

4.8. Statistical Analysis of 2D Protein Pattern Variations

Protein spots showing variations in abundance were submitted to ANOVA to select spots for which stress treatment of post-stress plant cultivation had a significant effect ($P < 0.05$) on their volume. Additionally, the most variable proteins were also checked using Tukey's HSD test (JMP Software, SAS Institute, Cary, USA). These variable proteins were further identified by MS.

4.9. Protein Identification by MS

For MS analysis, gel spots were subjected to standard '*in-gel* digestion' procedure during which proteins were reduced with 100 mM [*w*/*v*] DTT (for 30 min at 56^{0}C), alkylated with iodoacetamide (45 min at room temperature in dark) and digested overnight with trypsin (sequencing Grade Modified Trypsin - Promega V5111). Resulting peptides were eluted from gel with 0,1% [*v*/*v*] trifluoroacetic acid, 2% [*v*/*v*] acetonitrile.

Peptide mixtures were separated by liquid chromatography prior to molecular mass measurements (LC coupled to an linear ion trap- Fourier transform ion cyclotron resonance [LTQ-FTICR] mass spectrometer) on Orbitrap Velos mass spectrometer (Thermo Electron Corporation, San Jose, CA) at the Mass Spectrometry Laboratory (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland). Peptide mixture was applied to RP-18 precolumn (nanoACQUITY Symmetry® C18 – Waters 186003514) using water containing 0,1% [*v*/*v*] trifluoroacetic acid as mobile phase and then transferred to nano-HPLC RP-18 column (nanoACQUITY BEH C18 – Waters 186003545) using the acetonitrile gradient (0 to 60 % [*v*/*v*] acetonitrile for 120 min) in the presence of 0.05% [*v*/*v*] formic acid with the flow rate of 150 nl min^{-1}.

Column outlet was directly coupled to the ion source of the spectrometer working in the regime of data dependent MS to MS/MS switch. A blank run ensuring lack of cross contamination from previous samples preceded each analysis.

Acquired raw data were processed by Mascot Distiller followed by Mascot search (Matrix Science, London, UK, 8-processor on-site license) against NCBInr (version 20100203) with taxonomy restricted to *Viridiplantae*. Search parameters for precursor and product ions mass tolerance were 40 ppm and 0.8 Da, respectively, with allowance made for one missed trypsin cleavage, the following fixed modifications: cysteine carbamidomethylation and allowed variable modifications: lysine carbamidomethylation and methionine oxidation, serine, threonine and tyrosine phosphorylation as well as deamidations, methylations, formylations and ethylations. Peptides with Mascot Score exceeding the threshold value corresponding to < 5% False Positive Rate, calculated by Mascot procedure, were considered to be positively identified. Phosphorylation sites were predicted by PhosPhAt v4.0 (www.phosphat.uni-hohenheim.de; [150]), NetPhos v2.0 (www.cbs.dtu.dk/services/NetPhos; [151]) and MUsite v1.0 (www.musite.net; [152]). Methylation sites were predicted by PMes (http://bioinfo.ncu.edu.cn/inquiries_PMeS.aspx; [153]). The data was compared with Arabidopsis data at PPDB (http://ppdb.tc.cornell.edu/dbsearch/searchmod.aspx). PPDB experimental sources concerned Zybailov et al. [154] as well as Kim et al. [155] data. Additional modified residues were predicted by FindMod (http://web.expasy.org/findmod; [156]).

4.10. SDS-PAGE and Western Blotting

Aliquots containing 20 µg of mitochondrial proteins were separated by 12% (*w*/*v*) SDS-PAGE [157]. For immunoassays, proteins were electroblotted from 1D (SDS-PAGE) or 2D (IEF/SDS-PAGE) gels onto polyvinylidene difluoride Immobilon-P membranes (Millipore), using Sedryt semidry blotting apparatus (Kucharczyk). Membranes were CBB-stained to ensure that equal amounts of proteins were transferred. After destaining and the subsequent blocking of the membrane, they were incubated overnight with antibodies. Indicated antibodies (Table S4) were purchased from Agrisera (Vännäs, Sweden). Antibodies against cyt. c_1 and ATP1 were kindly donated by Prof. Gottfried Schatz (University of Basel). Hsp17.6 antisera were a generous gift from Prof. Elisabeth Vierling (University of Massachusetts). Antibodies against NAD9 and CCMA were produced by [158] and [159], respectively. $CcmF_{N1}$ and $CcmF_{N2}$ antisera were generated by [160]. Bound sera were detected using an anti-rabbit immunoglobulin G horseradish peroxidase or alkaline phosphatase conjugate diluted 1/10000 (BioRad) and visualized with enhanced chemiluminescent reagents (GE Healthcare) or with Lumi-Phos WB Chemiluminescent Substrate (Pierce). Western blot images in triplicates were analyzed by Multi Gauge (v.2.2) software and the representative pattern was presented. Western blot band intensities were calibrated to the protein loading in the linear relationship (the control denoted as 1.00); the other bands were calculated relatively to this value.

5. Conclusions

Our approach comprises general data about variations regarding cold and heat stress responses in the mitochondrial proteome of cauliflower and in the physiological parameters, related particularly to plant respiration. It appeared that the set of cauliflower mitochondrial proteins responded to temperature stress conditions as well as to the stress recovery varied from the

previously described ones. It significantly extends the deposited data also by means of investigated quantitative alterations. However, proteomic and respiratory physiological responses related with the functioning of cauliflower mitochondria in stress were not fully correlated. For instance, the rate of respiration in illuminated leaves together with leaf transpiration and photorespiration was greatly affected by cold and/or cold recovery, despite more proteins of various functional classes were involved by heat/heat recovery. We would like to emphasize that heat regulated proteins were distinct (with minor exceptions) from the ones regulated by cold/cold recovery. Overall, we (1) noticed the impaired photorespiration rate which was followed by alterations in photorespiratory enzymes after cold recovery; (2) suggested possible metabolic impairments in various TCA components and Pro catabolism, and also in protein import apparatus; (3) observed the elevated demand for ATP synthesis after heat/heat recovery (e.g. ATP1 and ATPQ level); (4) noticed the evident downregulation of some RC subunits (e.g. ATP1, NAD9, COXII) and (5) studied the sensitivity of *c*-type cytochrome biogenesis apparatus to cold stress and cold recovery. Our data show that selected proteomic alterations cannot be fully restored after temperature recovery. All those results imply the necessity (1) to go deeper in the quantitative analysis of protein posttranslational modifications and (2) to study further tissue- specific proteomic and physiological alterations in order to build up the full model of plant mitochondrial biogenesis under temperature stress.

Supplementary Materials: A list of the following supplemental materials that are available in the online version of this article is presented below. Figure S1 and Table S1 are Word-based files. The remaining Tables S2-S3 represent Excel files.

Figure S1. Representative silver-stained 2D gels of cauliflower mitochondrial proteome variations from control (K) grown plants, cold (C) and heat (H) stressed plants and from cold or heat recovered plants (CA and HA, respectively).

Table S1. List of cauliflower mitochondrial proteins whose level varied during stress treatment.

Table S2. List of all peptides for each protein spots.

Table S3a. Full data for parental peptides representing double spots after Mascot error tolerant search.

Table S3b. Summary of estimated peptides with modified residues from analyzed 2D gel double spots.

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Author Contributions: M.R., T.P. and W.K. conceived and designed the experiments; M.R. was principle investigator, who designed this study, performed extraction of mitochondria, control assays, carried out SDS-PAGE and Western immunoassays, analyzed proteomic results (together with FunCat), prepare and wrote the paper; M.Cz. assisted in isolation of mitochondria, prepared protein samples for Western analyses, analyzed MS data and participated in writing of the paper; T.P. prepared protein samples for 2D PAGE, performed 2D PAGE and the statistical analysis of spot variations, selected stress-responsive protein spots, submitted protein spots for MS analyses and participated in writing of the paper; W.K. cultivated plant material, and conducted all physiological analyses (gas exchange measurements and fluorescence assays), analyzed their results and participated in paper preparation; T.S. assisted in cultivation and maintenance of the plant material in control conditions and after stress dosage, prepared nutrient media, subjected plants to stress conditions and participated in physiological analyses. All Authors have approved the submitted version and agreed to be personally accountable for the Author's own contributions and for ensuring that questions related

to the accuracy or integrity of any part of the work, even those in which the Author was not personally involved, are appropriately investigated, resolved, and documented in the literature.

Conflicts of Interest: The authors declare no conflicts of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Abbreviations

A_n	net CO_2 assimilation rate
A_g	total CO_2 assimilation rate
ANOVA	analysis of variance
AOX	alternative oxidase
BSA	bovine serum albumin
CBB	Coomassie Brilliant Blue
Ccm/CCM	cytochrome *c* maturation
CAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
C_i	intercellular CO_2 concentration
CPN	chaperonin
CS	citrate synthase
2D PAGE	two-dimensional gel electrophoresis
DTT	dithiothreitol
E	transpiration
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EF	elongation factor
F_m	maximal fluorescence
F_o	minimal fluorescence
FunCat	functional categorization
F_v	variable fluorescence
GDC	glycine decarboxylase
g_s	stomatal conductance
HSD	honest significant difference
HSP	heat shock protein
IDH	isocitrate dehydrogenase
IEF	isoelectrofocusing
iTRAQ	isobaric tags for the absolute quantification
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MDH	malate dehydrogenase
MIPS	Munich Information Center for Protein Sequences
MPP	mitochondrial processing peptidase
NAD	complex I subunit (mitochondrially-encoded)
NCBI	National Center for Biotechnology Information
OXPHOS	oxidative phosphorylation
P5CDH	δ-1-pyrroline-5-carboxylate dehydrogenase
PDH	pyruvate dehydrogenase
PGDH	3-phosphoglycerate dehydrogenase
PGK	phosphoglycerate kinase
PhR	photorespiration rate
PPFD	photosynthetic photon flux density
ProDH	proline dehydrogenase
PS	photosystem
PTM	posttranslational protein modification
RC	respiratory chain
R_d	respiration in the light (day respiration) rate
RH	relative humidity

R_n	respiration in the dark (night respiration) rate
ROS	reactive oxygen species
R_T	total respiration rate
SCL	succinyl-CoA ligase
SHMT	serine hydroxy-methyl aminotransferase
TCA	tricarboxylic acid
VDAC	voltage-dependent anion channel

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