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Article

Mitochondrial Biogenesis in Diverse Cauliflower Cultivars under Mild and Severe Drought Involves Impaired Coordination of Transcriptomic and Proteomic Response and Regulation of Various Multifunctional Proteins

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Abstract: The early generative phase of cauliflower (*Brassica oleracea* var. *botrytis*) curd ripening is sensitive to the water deficit. Mitochondrial responses under drought within *Brassica* genus are poorly understood. The main goal of this study was to investigate the mitochondrial biogenesis of three cauliflower cultivars varying with drought tolerance. Diverse quantitative changes (down-regulations mostly) in the mitochondrial proteome were assayed by 2D PAGE coupled with LC-MS/MS. Respiratory (e.g. CII, CIV and ATP synthase subunits), transporter (including diverse porin isoforms) and matrix multifunctional proteins (e.g. components of RNA editing machinery) appeared diversely affected in their abundance under two drought levels. Western immunoassays showed also cultivar-specific responses of selected mitochondrial proteins. Dehydrin-related tryptic peptides found in few 2D spots that appeared immunopositive with dehydrin-specific antisera highlighted the relevance of mitochondrial dehydrin-like proteins for the drought response. The level of selected messengers participating in drought response was also determined. We conclude that the mitochondrial biogenesis was strongly, but diversely affected in various cauliflower cultivars and associated with drought tolerance on the proteomic and functional levels. However, transcriptomic and proteomic regulations were largely uncoordinated due to the suggested altered availability of messengers for translation, mRNA/ribosome interactions and/or miRNA impact on transcript abundance and translation.

Keywords: dehydrins; 2D PAGE; drought; mitochondrial biogenesis; mitochondrial proteome; plant transcriptome

1. Introduction

Under drought, plants respond by numerous physiological and molecular mechanisms [1]. Each plant species possess an unique drought resistance level, which is accompanied by the diverse sensitivity of selected growth and metabolic processes to the progressing stress conditions [2]. The balance between water uptake and the transpiration is controlled by the water potential. The leaf

surface controls the respiration rate as well as CO_2 assimilation and the photosynthesis rate. Excessive transpiration may decrease the water potential in plant, resulting in growth cessation [3]. Initially, drought results in stomatal closure and the declined transpiration to prevent further water losses in drought-sensitive species, osmolyte synthesis and consequently in the inhibition of leaf cell growth [4-7]. In general, drought regulates leaf respiration at various directions, however those alterations may enable the prompt stress recovery [8].

The mitochondrial proteome is the highly dynamic entity containing at least 1500 diverse proteins (1060 ones experimentally identified in potato [*Solanum tuberosum*] mitochondria by Salvato et al. [9]) actively responding to the environmental conditions. Since the past 15 years, a significant progress has been made on the elucidation of the key steps of mitochondrial biogenesis which implies the finely coordinated expression of mitochondrial and nuclear genes that can be disrupted under the stress action [10-13]. Taylor et al. [14] estimated 22% of the stress-responsive organellar proteins in Arabidopsis to be targeted to mitochondria, but the number of mitochondrial proteins involved in the response to diverse stress treatments remains still underestimated. More complex studies integrating numerous physiological, structural and molecular approaches for the better understanding of the biological relevance of such replies are thus welcomed.

Drought also results in the dynamic alterations within the cellular transcriptome and proteome [7,15-17], including also mitochondrial proteome [18,19]. Drought response is often connected with the increase or induction of diverse protective proteins, including drought-induced proteins, e.g. dehydrins [20]. Mitochondrial proteins may be directly involved in developing of drought tolerance as well, including important enzymes, e.g. pyruvate dehydrogenase (PDH), glycine decarboxylase (GDC), and in less extent - malate dehydrogenase (MDH); under drought some novel protein isoforms may be also induced, however the proteolysis of key mitochondrial proteins was also reported [19,21-24]. Numerous mitochondrial heat shock-proteins (HSPs), including small HSPs (sHSPs) and diverse Mn-superoxide dismutase (Mn-SOD) isoforms are also active players in drought response and plant development [25-27]. Variations in abundance of numerous mitochondrial proteins, however, may not easily correspond with the drought intensity.

Brassica genus contains important plant species for the worldwide agriculture. Total cellular proteomic and transcriptomic responses of *Brassica* species in drought were already investigated, however without deepened attention towards elucidation of the particular aspects of mitochondrial biogenesis [28-32]. Interestingly, drought response of close *Brassica* relatives, including *Thellungiella* are distinct from the Arabidopsis one [33]. Even though, the reports comparing responses of Brassica cultivars with contrasting drought tolerance are still limited [34-38], contrary to the other species data [6,39-48]. Moreover, search for protein markers in order to develop drought-tolerant plant accessions belongs to the current goals of proteomic analyses [7].

Owing the recent research trends, this work was undertaken to gain a comprehensive view of the influence of the middle and severe water deficiency conditions on the mitochondrial biogenesis of three cauliflower (*Brassica oleracea* var. *botrytis*) cultivars displaying diverse drought tolerance. Previously, we studied cauliflower mitochondrial biogenesis under temperature stress and the recovery [13]. Cauliflower belongs to vegetables with the major cultivation yield in the Middle Europe. The early generative phase of curd ripening belong to the key developmental stages with some physiological demands. In addition, due to the size of its vegetative organs it is sensitive to the low water level in the soil. To determine mitochondrial responses in relation to plant respiration, we

aimed (1) to investigate the dynamic nature of mitochondrial proteome; (2) to identify the most variable proteins in mitochondria of cauliflower curds; (3) to check the abundance of selected mitochondrial proteins, including previously investigated dehydrin-like proteins [49]; (4) to analyze the relevant proteomic and transcriptomic regulations and (5) to link them with the physiological level (respiratory and photorespiratory alterations) for the discussion of the general responses to mild and severe water deficit. This is the first comprehensive study of the mitochondrial proteome of the Brassica genus member which allowed to characterize a broaden set of drought-responsive mitochondrial proteins in the cultivar context. On the whole, it highlights the participation not only oxidative phosphorylation (OXPHOS) proteins, but a number of multifunctional mitochondrial proteins (including RNA editing factors and dehydrin-like proteins) in drought response.

2. Results and Discussion

2.1. Respiration and Photorespiration Pattern in Cauliflower Leaves

In order to study mitochondrial response on physiological and molecular levels in '*Adelanto*' ('*A*') and '*Casper*' ('*C*') drought-sensitive cultivars as well as in the '*Pionier*' ('*P*') drought-tolerant cultivar, the total respiration, respiration in the light (day respiration), night respiration as well as the photorespiration rates were determined. Previously we showed that the respiratory rate exceeded the photosynthetic one in cauliflower leaves under the severe (but not moderate) water deficiency [50]. This emphasizes the importance of the adequate respiratory adaptations in this species under the mentioned unfavorable conditions. Drought limits crop yield and alters physiological parameters necessary for keeping the cultivation profitability high. For instance, the internal leaf CO_2 concentration may be unstable and significantly decreased in the initial phase of drought, so that photosynthetic rate markedly declines under the water deficit; however, this effect can be alleviated under the drought recovery or after the addition of exogenous factors or the overexpression of specific proteins [51,52]. Often respiratory impairments are accompanied by visible phenotypic effects, e.g. the decreased length and weight of diverse plant organs [29,31,35]. Respiration and photorespiration become a part of the complex network response under the water deficiency and the photorespiration participates in the oxidative damage avoidance while optimizing the photosynthesis [53].

Generally, R_T value slightly increased under the mild drought, but markedly decreased in the severe treatment in '*A*' and in special extent in '*C*. On the contrary, '*P*' showed significant increase of R_T value in the mild drought, however it was only slightly decreased under the severe treatment (Figure S1). The R_d (the average from all illumination conditions) as well as R_n rates increased progressively in the drought sensitive cultivars under all investigated treatments. However, R_d rate was markedly decreased in '*P*' in the mild drought and the highest increase of R_n was noted in '*A*' cultivar under the severe treatment. The highest increase of R_d rate was visible in the same conditions (especially in '*A*' and also in '*P*'; Figure S1). Interestingly, the respiratory decline under prolonged water deficit is a well-known phenomenon (also for *Brassica* species) and R_n rate is affected much under fast drying [19,30,54]. Mitochondrial R_d can be also inhibited by drought [55]. In some plants, it markedly increases in the prolonged drought (while R_d exceeds the R_n rate), but declines under short water deficit [56,57]. In our case the effect depended on cultivar. In '*P*' R_d was higher than R_n, both in control conditions of the growth and under the severe drought treatment,

while in '*A*' cultivar the R_d rate exceeded R_n only under the severe stress. In '*C*' R_d was always lower than R_n regardless the level of the drought stress. Interestingly, *in-planta* overexpression of some uncoupling proteins influences such physiological effects [58]. Drought-sensitive crop cultivars exhibits larger R_T decrease, than the sensitive ones; however, both effects could be reversed under drought recovery [59].

In '*A*' and '*C*' cultivars the PhR rate showed slight decrease under the mild stress, however severe drought conditions decreased it in more extent, especially in '*A*'. On the contrary, a slight increase of the PhR rate was observed after the mild drought in '*P*'. Interestingly, the increase of the photorespiratory to the gross CO_2 assimilation ratio under drought is often expected in order to protect the photosynthetic machinery against photoinhibition [40,55]. In field-grown *Gossypium hirsutum* drought resulted in affected stomatal conductance and elevated respiratory and PhR rates, while the photosynthetic electron transport was not affected [60]. Overall, in *A*' and '*C*' PhR rate is altered progressively along the stress duration. This is in line with Liu et al. [61] data, suggesting that the PhR in drought-sensitive cultivars cannot be a major energy dissipation strategy, as it was reported for some Asiatic and Mediterranean-originated plant species. In opposite to that findings, the drought-tolerant cultivar ('*P*') showed the reversed responses- the visible increase of PhR rate in the mild treatment (known among drought-tolerant species; [62]) and its notable decrease in severe drought, however in less extent than the discussed down-regulated rates for '*A*' and '*C*' (Figure S1). Those general trend in PhR response could be reversed at the early vegetative stage in some species varying with drought tolerance [63]. In drought-tolerant and drought-sensitive cultivar of *Malus domestica* even moderate drought resulted in the major PhR decline [64].

In general, in the course of the drought progression, both in '*A*' and (in less extent) in '*C*' cultivar the rapid increase of the contribution of the R_d and the decrease of the contribution of PhR in the R_T value was noticed. On the contrary, in '*P*' leaves the R_d contributes to the R_T rate in less extent manner; consequently, the PhR contributes in greater extent to R_T under the mild drought (Figure S1). Overall, observed regulations in the respiratory parameters coincide with the level of drought tolerance among investigated cauliflower cultivars and suggest the distinct regulations of drought physiological responses in '*P*'. Next, we aimed to study whether the diverse pools of drought-responsive mitochondrial proteins accompany the studied respiratory alterations in stress-sensitive and stress-tolerant cultivars. Therefore the dynamic nature of cauliflower mitochondrial proteome in those cultivars under water deficit was about to be investigated.

2.2. *The Specificity of Mitochondrial Proteome Alterations under Drought in Diverse Cauliflower Cultivars*

Mitochondrial proteins isolated from curds of cauliflower plants grown in control conditions (0) as well as in the mild (1) and severe (2) water deficit were resolved by 2D PAGE. In order to study mitochondrial proteome alterations according to the drought tolerance, we used three mentioned cultivars (section *2.1*). Experimental variants were analyzed in pairs as follows: '*A1*' vs '*A0*', '*A2*' vs. '*A0*', '*C1*' vs. '*C0*', '*C2*' vs. '*C0*', '*P1*' vs. '*P0*', '*P2*' vs. '*P0*'. In addition, 2D gels for 9 different variants, including control, were run in triplicate. The CBB- stained master gel (a fused image) was created basing on the pooled samples containing the equal amounts of mitochondrial proteins from the all experimental variants, giving in a final number of 370 different spots. The number of spots on particular 2D gels varied from 231 to 370 between all analyzed variants (including also controls) for each cultivar (Figures 1 and S2).

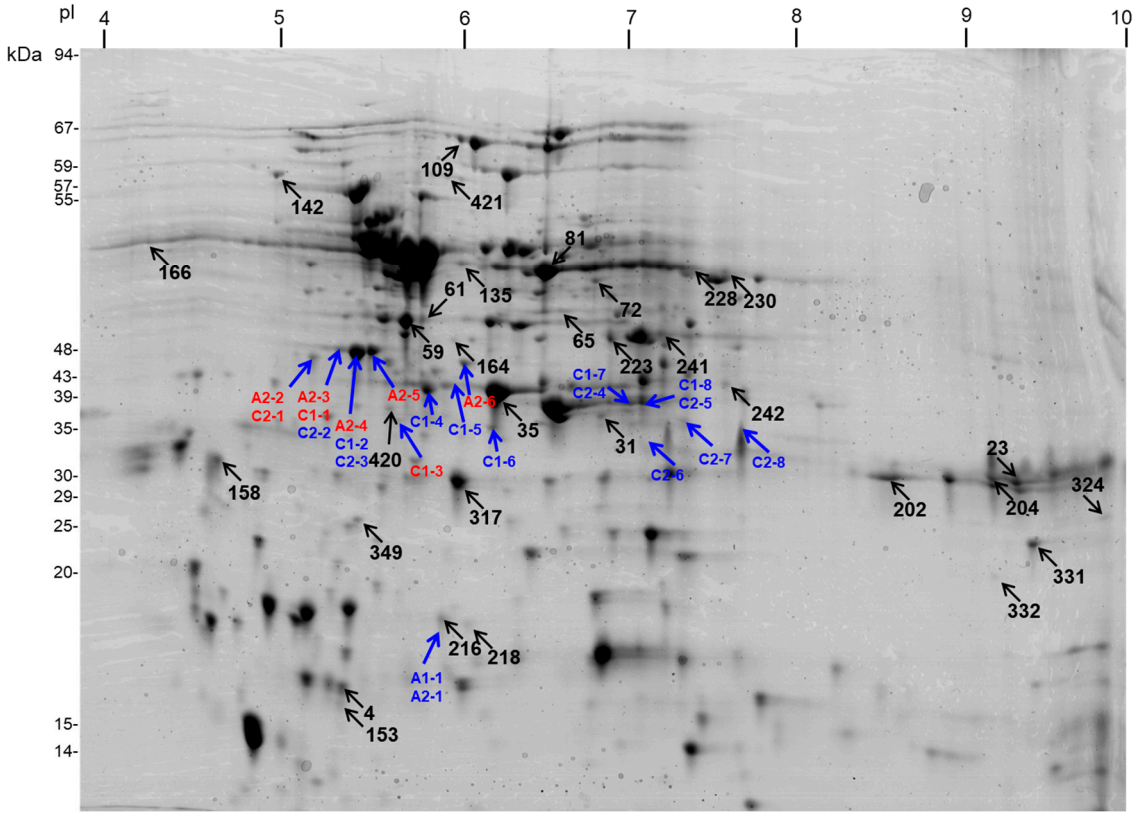

Figure 1. Position of thirty two drought-responsive spots (*black arrows*) on CBB-stained 2D master gel with separated cauliflower mitochondrial proteins (the proposed identities of all protein spots are shown in Table S1 and the peptide data in Table S2). This map shows also the position of the additional protein spots (*blue arrows*) from 2D gels containing resolved mitochondrial proteins of '*Adelanto*' (A) and '*Casper*' (C), that were cut out and used for the identification of tryptic peptides specific to the dehydrin-like proteins (the proposed identity for those spots appears in Table S4). Identifiers for spots containing the mentioned peptides are marked *in red* (the remaining labels - *in blue*). For the molecular mass calibration (kDa) of protein spots, the PageRuler Prestained Protein Ladder (Thermo Scientific) and LMW-SDS Marker Kit (GE Healthcare) were used. For the calibration of the spot isoelectric point (pI), Broad pI Kit (GE Healthcare) was used. Further data in the text.

Thirty two spots (8.65 % of all spots from the master gel) from all cultivars appeared significantly variable and their positions (Figures 1 and S2) were assessed from three biological replicates. Data for the responsive protein spots, including their abundance are shown in Table S1 and on Figures 2 and S3. Under the mild water deficiency, 13 (in each of '*A*' and '*C*') and 11 spots (in '*P*') were down-regulated. In the same stress conditions we noticed up-regulations for 6 (in '*A*') and 3 (in '*C*') and 2 spots (in '*P*'). However, in the severe drought, 15 (in '*A*') and 9 spots (in each of '*C*' and '*P*'), were down-regulated and only 2 spots became up-regulated within each investigated cultivar (Figure S3).

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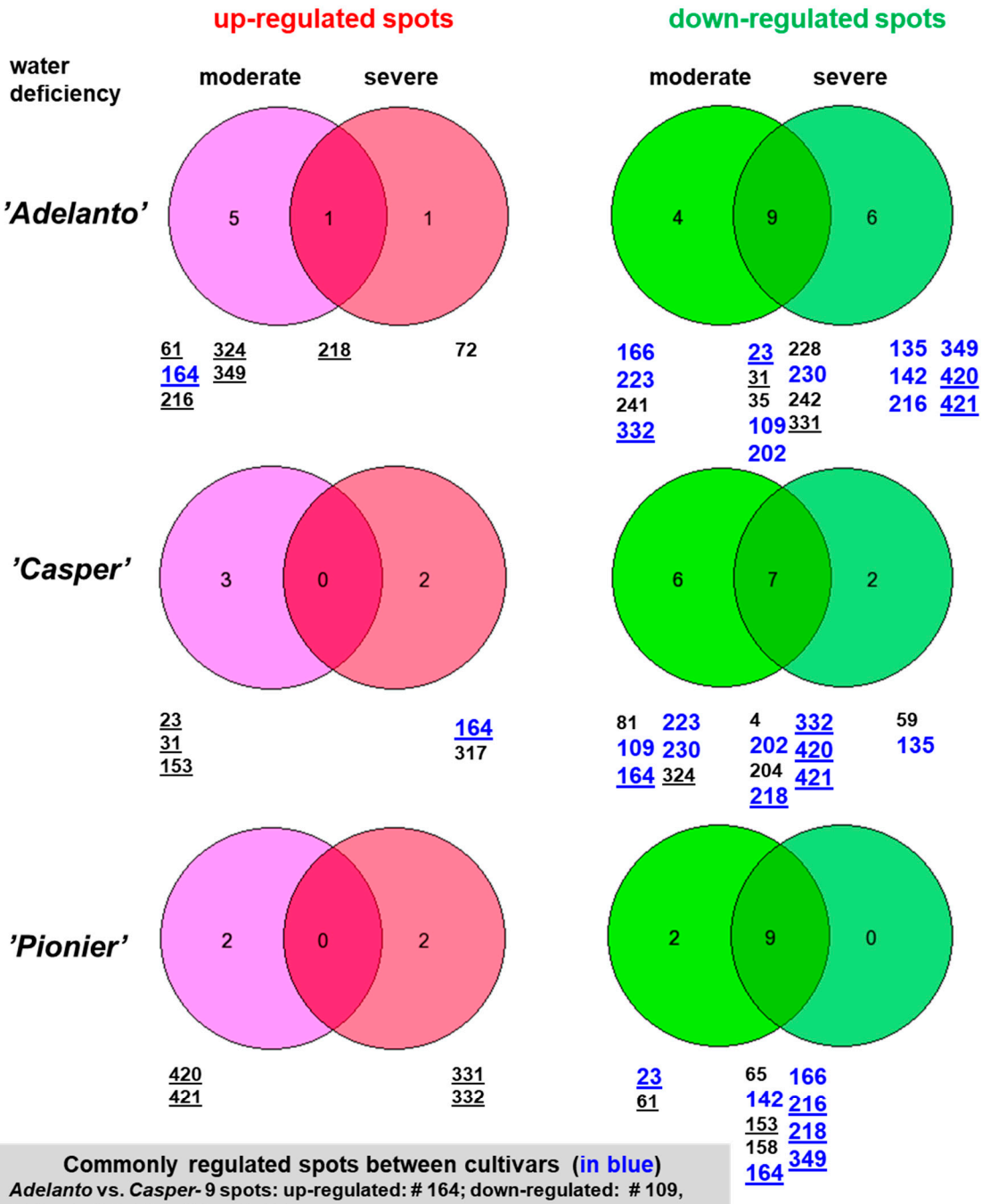

Figure 2. Venn-diagrams showing the distribution of the up-regulated and down-regulated specific/common protein spots to the mild and severe drought conditions across the investigated cultivars. Numbers refer to the protein spot identifiers. Commonly and diversely regulated spots between cultivars were marked *in blue* (a larger font) and underlined, respectively; they were listed below diagrams. Further data in the text.

Regarding the down-regulated spots, 9 spots (for the each of '*A*' and '*P*' cultivars) and 7 spots (in '*C*') overlapped between the mild and severe water deficiency. Among the up-regulated spots, the only single spot overlapped in '*A*' for the two tested stress conditions. This means, that under the mild water deficiency, 4 (in '*A*'), 6 (in '*C*'), and 2 spots (in '*P*') were specifically down-regulated. In the same stress conditions, we noticed specific up-regulations for 5, 3 and 2 spots in those cultivars, respectively. However under the severe drought, 6 (in '*A*'), 2 (in '*C*'), and no spots (in '*P*') were specifically down-regulated and only 1, 2 and 2 spots appeared specifically up-regulated in those cultivars, respectively (Figures 2 and S3). Thus mitochondrial proteomes significantly varied among all cultivars; in case of up-regulated spots, the ones specific to the given drought level dominated over the spots common for both treatments. The number of diversely regulated spots in abundance between the investigated drought-sensitive cultivars and the drought-tolerant '*P*' cultivar exceeded the number of such spots within drought sensitive cultivars. Conversely, the commonly regulated spots increased in number between drought-sensitive cultivars (Figure 2). The drought responses for the drought-tolerant cultivar ('*P*') were particularly specific. The severe drought resulted in regulation of abundance of the distinct set of protein spots when compared to the impact of the mild stress. Overall, the massively down-regulated spots exceeded up-regulated ones. Interestingly, we did not observed any biases towards up-regulated protein spots in drought-tolerant cultivar, which is generally contrary to the Mohammadi et al. [35] data on rapeseed. This suggest distinct regulations within cauliflower proteome which are potentially associated with drought tolerance (section *2.3*).

2.3. Mitochondrial Response to Drought Involves Diverse Multifunctional OXPHOS, Transporter and Matrix Proteins in Various Cauliflower Cultivars

Proteins from spots collected from the 2D master gel were identified by LC-MS/MS. The obtained data were used for searching Mascot against NCBInr database (version 20160525 containing 88005140 protein sequences). Due to the limitations of protein matching to species-specific database (lack of completed cauliflower nuclear genome sequence and availability of the mitochondrial genome sequence of *B. oleracea* var. *oleracea* as the close variety; [65]), mitochondrial proteins were identified by using *Viridiplantae* section of the database. In addition, the Gelmap tool (https://gelmap.de/projects-arabidopsis/) was used to compare the cauliflower and Arabidopsis proteomic maps and to validate further MS identifications. Because we investigated the mitochondrial proteome from the non-green apical part of cauliflower curds, the use of IEF/SDS-PAGE reference map of Arabidopsis cell culture mitochondrial proteome was particularly advisable. In some cases, however, we also used the map representing Arabidopsis mitochondrial proteome of the green tissues [13].

The identifications of protein spots are presented in Table S1; the individual peptides for each protein spot are also listed in Table S2. Almost all analyzed spots contained more than a single protein or protein isoform. Together, all spots represented 91 non- redundant protein within records that fit to the experimental data. Of this number, 69 non-redundant proteins with the highest probability of identification were found; see bolded records in Table S1). The unambiguous results were obtained from the MS analyses; the percentage of sequence coverage ranged 11-73% and the numbers of the total identified peptides and the unique peptides were up to 260 and 38, respectively. Proteins were identified basing mostly on the high similarity to sequences of diverse cruciferous

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species. Generally, for the majority of spots, the experimental molecular mass corresponded roughly to the theoretical one. Many proteins, however showed some shift in molecular mass between theoretical and gel values. Contrary to Taylor et al. [19], we are rather convinced that investigated stress conditions did not resulted in excessive proteolysis in cauliflower mitochondria. Overall, various respiratory (e.g. ATP synthase, proteins for CII and CIV biogenesis, mostly down-regulated), transporter (ex. diverse VDAC isoforms and dicarboxylate antiporters) and matrix proteins (ex. HSPs, DNA-binding proteins, RNA editing and translation factors, mitochondrial thioredoxins, diverse multifunctional enzymes for amino acid, carbohydrate, lipid and nucleotide metabolism and some novel proteins) appeared drought-responsive. Some proteins identified in double spots (e.g. no. 228, 230) showed quite similar responses, which is in favor for the correctness of their assignments (Table S1). Such protein multi-spotting may have resulted both from post-translational modifications (PTMs) and/or rather from the expression of diverse gene family members.

Based on Arabidopsis protein orthologs, next we used the functional classification by the Munich Information Center for Protein Sequences (MIPS) at VirtualPlant 1.3 (http://virtualplant.bio.nyu.edu; [66]) for the clustering of the drought-responsive proteins resolved on 2D gels into the functional categories (Table S3). Notably, in the studies dealing with the impact of water deficiency to the alterations within plant total proteomes, some of those functional classes (e.g. defense, amino acid metabolism and OXPHOS proteins) are often under-represented, highlighting the relevance of the organelle-specific studies [67].

The majority of proteins within responsive spots belonged to the class participating in various metabolic routes (ca. 44%). Metabolism-related proteins are often overrepresented in drought response [68] and regulated in diverse manner among drought-sensitive and tolerant cultivars [45], similarly to our study (Table S1). These were C compounds and carbohydrate metabolism (23.1%) as well as amino acid metabolism proteins (18.7%). The next classes were represented by proteins participating in cell rescue, defense and virulence (16.5%), energy conversions (12,1%) as well as in N and S metabolism (7.7%; Table S3). Notably, carbohydrate and amino acid metabolism proteins often respond to drought [7,69] and their accumulation profiles assayed by time kinetics-coupled proteomic approaches are diversely modulated [48].

Interestingly, MDH, succinyl-CoA ligase subunit and aconitate hydratase 2 (aconitase 2 [ACO2]; corresponding to spots no. 35, 59, 109) belonging to the key enzymes for carbohydrate metabolism, were down-regulated in drought-sensitive cauliflower cultivars (the present study), in the roots of the drought-sensitive rapeseed (*B. napus*) genotype [35] as well as in other reports elucidating the impact of the prolonged water deficit on the plant proteome [46,48]. Regarding the mentioned above proteins, Ndimba et al. [18] in their study on the sorbitol-induced drought obtained results contrary to ours. In the leaf proteome of rapeseed cultivars MDH can be also upregulated in order to cope with the increased cellular demands for NADH [38]. Notably, ACO2 is predominantly localized in plant mitochondria and aconitase-containing complexes were shown to be unstable in stress [13,70]. However, in poplar *Populus* x *euramericana* plants the level of succinyl-CoA was decreased and aconitase increased in abundance [40]. Two-week-long drought resulted in metabolic re-programming in rapeseed seedlings by increase of key stress-related proteins and in the decrease of proteins related with metabolism, protein folding and degradation [30].

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Regarding other functional classes, the participation of electron transport (7.7%), complex cofactor binding proteins (6.6%) and folding proteins (4.4%) in drought response in cauliflower mitochondria was also distinctive (Table S3). The energetic and carbohydrate metabolism proteins play a significant role in drought response [44,45]. Recent studies reported on the up-regulation of distinct VDAC isoforms (irrespectively of the drought tolerance), also among inbred line of *Brassica rapa* subjected to 48 h-long drought [32,41,48,69], which is contrary to the massive down-regulation of VDAC1 visible mostly in drought-sensitive cultivars (spots no. 23, 202, 204; Table S1); other cauliflower VDAC isoforms (including porin 2-like) were also decreased in abundance, which is in line with the data for VDAC2 in rice roots under drought and salt stress [71]. Subunits of OXPHOS complexes (e.g. CI and CII) appeared down-regulated in cauliflower mitochondria; the general trend of their level alterations was similar to Taylor et al. [19] study. Strikingly, CIV subunits were unaffected in our case, contrary to Zhang et al. [31] and Budak et al. [44]. Regulations of the succinate dehydrogenase (SDH; CII) subunit 5 (SDH5; spot no. 4) level were also distinct from the Vítámvás et al. report [47] on barley (*Hordeum vulgare*) responses in drought; in our case we observed massive down-regulation of this protein, together with SDH subunit 1 (SDH1; spots no. 23, 204; Table S1). The expression of *SDH1* gene was repressed in rice leaves and roots under the combined drought and salinity treatment [71]. Interestingly, the level of CII subunits was increased after re-watering of *Populus euphratica* plants [2].

One of the expected down-regulated mitochondrial enzymes in cauliflower mitochondria (according to our previous findings; [13]) was also ATP synthase. The assembly of ATP synthase may be affected as diverse cellular energy demands rise under stress, and the regulation of ATP synthase subunits is often related to the general stress responses and stress adaptation [67,72,73]. A strong decrease in the abundance of ATP synthase subunits under the water deficit was reported by a number of studies [16,39] contrary to the other ones [43]. Accordingly to our data (spots no. 135, 166), the smaller decrease in ATP synthase subunit β abundance was noted by Mohammadi et al. [35] in drought-sensitive rapeseed line. However, we observed the increased abundance of another, 24 kDa subunit of ATP synthase in '*C*' cultivar under the severe drought (spot no. 317; Table S1).

The alterations of the *Hippophae rhamnoides* PDH E1 subunit α level in drought [81] contrasts to our data (we noticed down-regulation of PDH subunit in spots no. 223, 241 for '*A*' and '*C*' cultivars; Table S1). Comparing to other abiotic stress conditions, water deficit results in the highest decrease in abundance of the two PDH subunits [19]. Interestingly, *NRGA1* messengers, encoding for a mitochondrial pyruvate carrier were reported to be up-regulated in drought, according to pyruvate demands; moreover, *NRGA1* gene is co-expressed with the gene for mitochondrial carrier MPC1 in order to enhance pyruvate import to mitochondria [74,75]. Generally, genes coding for mitochondrial dicarboxylate carriers may be induced in water deficit [71]. However, in our case, the down-regulation of PDH did not associated with the co-regulation of the expression level of any of the specific substrate carrier among drought-regulated proteins. Instead, the level of dicarboxylate/tricarboxylate mitochondrial transporters declined in drought-sensitive cultivars only (spot no. 202, 204; Table S1).

Owing the protein relevance in drought response, some protein regulations within the particular spots were quite surprising. Two spots (no. 223, 241; Table S1) in '*A*' and '*C*' which were strongly decreased in abundance even under mild drought contained, inter alia, formate dehydrogenase (FDH). Interestingly, the relationship between FDH abundance and the proline

accumulation was suggested [76]. The massive decrease in abundance of FDH in two *Phaseolus vulgaris* cultivars differing with drought tolerance was noticed [23]. However, the level of this enzyme is often increased in the vegetative tissues in the prolonged water deficiency and during initial stages of grain development [32,42,76], which was not the case in our study. Both in '*A*' (under the mild stress) and '*P*' (in all treatments) we observed decrease in abundance of protein spot no. 166 containing mitochondrial processing peptidase subunit β (MPPβ); in addition, in '*A*' cultivar the abundance of spot no. 230 containing translocase of the inner mitochondrial membrane subunit (TIM44-2-like) was also massively declined (Table S1). In some cases, the induction of gene coding for MPPβ was reported [71] and the level of the mentioned protein increased also under sorbitol-affected drought [14,18]. In diverse genotypes of poplar *P.* x *euramericana* and in two citrus rootstocks: *Citrus limonia* and *C. sunki* variations of MPP subunit α were reported [40,46]. Results of our study may thus indicate for some perturbations in the general import pathway. It is nonetheless known that drought may alter protein import into plant mitochondria [77]. Notably, MPP together with MDH were considered to form the potent hubs within the interactome network [19,46].

Some spots, e.g. spot no. 23 (containing porin 2-like and adenosine nucleotide translocator protein) as well as spot no. 31 (containing hydrolase domain-containing protein and carbonic anhydrase) showed the inconsistent regulation among the drought-sensitive cultivars [67]. Notably, spots containing porin 2-like appeared upregulated in '*C*' (but not in '*A*') under the mild drought and isoforms 1 and 3 (spots no. 203 and 204) were strongly downregulated in those cultivars. Overall the decrease in porin isoforms have dominated the pattern (Table S1). Contrary to that, some porin isoforms were increased in abundance especially in drought-tolerant wheat cultivars [41]. Also 48h-long drought in *Brassica rapa* increases notably the level of porin isoforms [32]. On the opposite, some other spots (no. 59, 61, 218, 331, 332) with ssDNA-binding proteins as well as proteins related with RNA metabolism and translation (e.g. RNA editing factors 1 and 6, elongation factor EF-Tu) were regulated in the opposite direction between drought-sensitive and tolerant cauliflower cultivars. Interestingly, the EF-Tu up-regulation was more pronounced in our study than in Taylor et al. [14] and Ndimba et al. [18] reports. The similar trend was also seen in case of spots no. 153 (with SWIB/MDM2 domain superfamily protein), 164 (containing similar to calcineurin-like metallo-phosphoesterase superfamily and sucrose/ferredoxin-like proteins), 349 (containing chaperonin and mitoribosomal protein L21), 420 (diverse enzymes) and 421 (representing HSP70 isoforms, Table S1). Mitochondrial DNA/RNA-binding proteins, chaperonins, heat-shock proteins as well as a number of enzymes for mitochondrial metabolism may thus differentiate cauliflower cultivars varying with drought tolerance.

For the drought-tolerant '*P*' cultivar we noticed some differences in the abundance of functional categories across proteins present in stress-responsive spots comparing to the data obtained for all cultivars (discussed above; Table S3). C- compound and carbohydrate metabolism proteins as well as proteins related with energy conversions contributed in less (18.6% and 9.3%, respectively), however, N- and S-metabolism proteins as well as folding proteins- in more extent (9.3% and 7%, respectively). Stress response proteins within cell rescue, defense and virulence category were significantly enriched in drought-tolerant cultivar (16.3%). Interestingly, the protein enrichment in the discussed categories was reported across plant tissues and drought-tolerant genotypes, contrary to the sensitive ones under diverse treatments [6,46,48].

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Overall, the prevalent down-regulations after the mild drought treatment and more specific alterations in the second level of drought likely represents specific adaptations in the cauliflower mitochondrial proteome to diverse intensity of the water deficiency. However, the functional implications of the observed stress-affected changes within the mitochondrial proteome require further exploration.

2.4. The Level of Key Matrix Proteins, cyt. c and the Components of Dissipating Energy Systems is Diversely Affected in Two Drought Levels among Cauliflower Cultivars

In stress the activity of mitochondrial matrix proteins is under the tight regulation [78]. We performed Western immunoassays (Figure 3), which accompanied our proteomic analyses (sections *2.2* and *2.3*) to evaluate the level of additional, drought-responsive mitochondrial proteins (including key matrix components). In addition, the level selected proteins identified in 2D spots affected in abundance under the water deficit (section *2.3*) was also validated. For this step we used specific antisera dedicated against investigated proteins.

The accumulation profile of those proteins varied depending on the cultivar and the stress intensity. Under the mild drought, the significant decrease in GDC-H abundance was visible only in '*A*' and '*C*' mitochondria, however, in the severe stress such decrease was detected only in '*C*'; in other stress variants (especially in '*P*') GDC-H level increased (Figure 3). GDC participates in the glyoxylate cycle and in the amino acid synthesis by transaminase activity [7]. It should be underlined that the massive decrease in the abundance of GDC subunits in plethora of stress conditions (including drought) is a well-known phenomenon [19,40,45,67]. Ford et al. [41] reported the slight down-regulation of GDC subunits as well as SHMT in drought-tolerant wheat cultivars, whereas the drought-intolerant cultivar exhibited enhanced accumulation of those proteins. However, barley GDC subunits were down-regulated irrespectively to the drought-sensitivity across cultivars [45]. In our study the changes of GDC-H abundance roughly corresponded to the decline in the photorespiration rate in drought-sensitive cultivars under both mild and severe drought (section *2.1*; Figure S1). Strikingly, the level of another important photorespiratory enzyme-SHMT- did not correlated with GDC-H alterations (Figure 3) as it decreased in abundance in '*C*' under mild drought only and remained stable in other experimental variants, similarly to pea (*Pisum sativum*) mitochondria [19], but contrary to SHMT from rice (*Oryza sativa*) leaves [15]. Similarly to GDC subunits, SHMT is often downregulated [30]. Notably, changes in GDC-H and SHMT level detected by Western immunoassays were not fully associated with the alterations of down-regulated protein spots no. 158, 228, 230 and 241 (Table S1).

The level of HSP70 decreased in '*A*' and '*P*' (especially in severe drought) and slightly increased in '*C*' under mild stress; therefore, those alterations were not associated with drought tolerance (Figure 3). Changes in HSP70 abundance assayed by immunoassay roughly agree with 2D PAGE data (spot no. 421; Table S1). HSP70 regulation under drought has been already reported. In such conditions, interestingly, the more evident HSP70 up-regulation was noted in pea mitochondria [19], however Vincent et al. [39] reported the declined abundance of this protein among *Vitis* plants.

The accumulation of isocitrate dehydrogenase (IDH) raised up only in the severe drought in '*A*' (Figure 3; spots no. 223 and 241 containing this protein under the mild drought were, however, down-regulated in the same cultivar; Table S1). Overall, the general HSP70 down- and IDH

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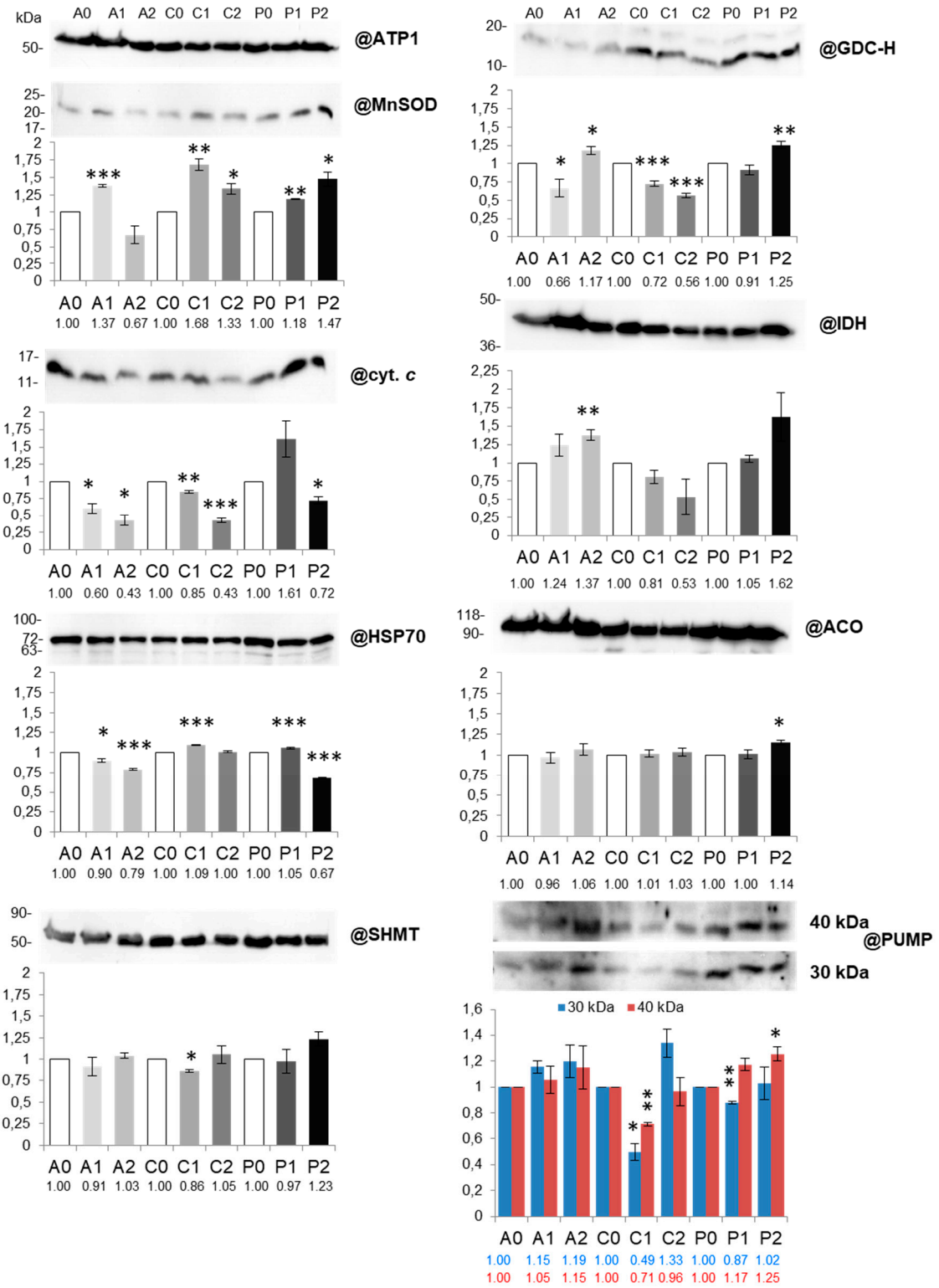

Figure 3. The abundance of Mn-superoxide dismutase (@MnSOD), cytochrome *c* (@cyt. *c*), heat shock protein 70 (@HSP70), serine hydroxymethyl dehydrogenase (@SHMT), glycine decarboxylase, subunit H (@GDC-H), isocitrate dehydrogenase (@IDH), aconitase (@ACO) and 40 and 30kDa immunoreactive uncoupling proteins (@PUMP) in mitochondria isolated from control grown '*Adelanto*', '*Casper*' and '*Pionier*' plants (A0, C0, P0), plants grown in the moderate (A1, C1, P1) and in the severe water deficiency (A2, C2, P2, respectively). The protein level on the representative SDS-PAGE blots was analyzed using specific antibodies. For the loading control, the antibody

against subunit-α of mitochondrial ATP synthase (@ATP1) was used. For the molecular mass calibration, the PageRuler Prestained Protein Ladder (Thermo Scientific) was used. The protein molecular mass is indicated in kDa. All results of are presented as the mean values (± S.E.) from triplicate detection. The significant alterations are marked with asterisks: ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ versus control values for each cultivar. Further data in the text.

up-regulation are in line with the typical variations of those proteins in stress [7,18,41]. The aconitase abundance (the applied antibodies can cross-react both with ACO2 and ACO3 isoforms) appeared relatively stable and it was up-regulated only in '*P*' under the severe treatment; the ACO2 down-regulation n drought-sensitive cultivars were detected by 2D PAGE (spot no. 109; Table S1, Figure 3). Diverse variations in the level of the mentioned enzymes may be a part of the adaptive response of Krebs cycle, due to the altered NADH and carbon skeleton demands in drought. In some cases, however, enzymes of Krebs cycle are massively down-regulated in water deficiency [79].

The overproduction of reactive oxygen species (ROS) in plant mitochondria under drought is inevitable and frequently accompanied by the increase in SODs abundance and the extensive protein carbonylation [7,80,81]. Drought stress is often combined with the oxidative damage [32]. Under Mn-SOD *in planta* overexpression, ROS level is decreased and the osmolytes accumulation is seen [82]. The activity of the diverse antioxidative enzymes (including SODs) in drought-treated cauliflower (contrary to rapeseed) seedlings was increased; however, in drought recovery their level decreased to control amount [30,52]. In our study, the level of Mn-SOD increased in almost all investigated stress conditions, except the severe drought treatment in '*A*'. The maximal increase of abundance of this protein was observed in one of the drought-sensitive cultivars ('*C*'; Figure 3). With accordance to that we noticed regulation of the abundance of spot no. 4 containing mitochondrial-like glutaredoxin in this cultivar (Table S1). This implies that in our case the redox regulation likely accompanies drought response [2]. Mn-SOD displays diverse expression pattern between cultivars with the variable drought sensitivity, often by the increase in abundance [14,41,44] even after 24h-long-dehydratation [32]. Interestingly, Mn-SOD down-regulation was also reported [23], which suggests that this enzyme may not participate in the oxidative response at least in some species.

Intra-mitochondrial pool of cytochrome *c* (cyt. *c*) was also decreased in the severe drought in all investigated cauliflower cultivars, but in the mild stress- only in case of drought-sensitive ones ('*A*' and '*C*'; Figure 3). This suggests that cyt. *c* can be released from cauliflower mitochondria even in the first level of water deficiency. Cyt. *c* release often accompanies programmed cell death (PCD), which was suggested by us to participate in temperature stress response [13]. However in some plant species, e.g. in pea mitochondria the drought did not resulted in PCD appearance [19].

In plant mitochondria the energy dissipating systems including uncoupling proteins (PUMPs), the alternative oxidase (AOX) and membrane channels were identified to take play in the bioenergetical adaptations under water deficiency. They can improve the drought tolerance by reducing ROS imbalance while overexpressed [51,83] and functional modulation of the drought response by influencing key signaling pathways [8]. We used antisera against potato PUMPs and were able to immunodetect proteins of 30 and 45 kDa in cauliflower mitochondria. In our study, the level of both polypeptides representing isoforms of uncoupling proteins were decreased significantly in '*C*' and in less extent in '*P*' under mild drought. However, in severe drought we

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noticed visible up-regulation of 40 kDa PUMP in '*P*' (Figure 3). Interestingly, in pea mitochondria, the abundance of the only selected PUMP isoforms (e.g. 30 kDa) was increased in drought [19].

To extend our proteomic data on the further components of energy dissipating systems we characterized also the level of the alternative oxidase (AOX) protein. The alternative oxidase is an active modulator of the mitochondrial stress response in plant cells [22]. It also participates in drought stress and controls the respiration rate and the photosynthetic efficiency under the moderate water deficit [84]. Standard monoclonal antibodies raised against the *Sauromatum guttatum* enzyme cross-reacted with three polypeptides of 29-36 kDa in cauliflower mitochondria [13], which likely represent AOX isoforms resulting from the expression of *AOX* gene family and diverse posttranslational modifications of this protein (Figure 4). Strikingly, the polypeptides of 29 and 33 kDa were significantly decreased in their abundance in all investigated cauliflower cultivars, however, in '*P*' such trend was less pronounced than among drought-sensitive cultivars. Those results contrast with Taylor et al. [19] data showing the drought-affected induction of the similarly sized 31 kDa isoform in pea mitochondria. However, a very high accumulation of 36 kDa immunoreactive band after severe drought treatment of '*A*'. AOX often increases in abundance in severe water deficit and during re-watering on the protein level [85]. Overall, our results (Figure 4) are generally in line with finding that the abundance of AOX polypeptides could be substantially increased among drought-sensitive cultivars; regarding the functional level, the cytochrome pathway activity in drought-tolerant cultivars responds in the less extent manner than in drought-sensitive ones [59].

2.5. The Pattern of Dehydrin-Like Proteins (Dlps) in Cauliflower Mitochondria is Regulated By Drought

Under unfavorable conditions, late embryogenesis abundant (LEA) proteins preserve the stability of membrane proteins and adjust intracellular osmotic pressure to prevent protein denaturation; they often display steadily increased abundance under the progressing drought [32,86]. Using immunochemistry and Western immunoassays, some LEA proteins, including proteins related to dehydrins (dlps) appeared to be mitochondrially-localized in a number of crop species [87-90]. Heat-stable dehydrins contain conserved amino acid motifs (e.g. K-motif; [91]). Our previous report showed, that the profile of accumulation of mitochondrial dlps was altered in abundance under temperature stress and after post-stress plant recovery [49]. In the present study, we investigated the pattern of dlps in mitochondria of three cultivars of cauliflower in the mild and severe drought (Figure 5). Accordingly with our previous immunoassays, three independent dehydrin-specific antisera were used. Two of them were directed against dehydrin K-segment; the third one recognize dehydrins containing SK_3- segments (section *3.8*), which were also reported among *Brassicaceae* [20].

Results obtained in our study indicate that the low-molecular weight dlps (18-80 kDa) respond in abundance as a part of mitochondrial adaptations to the water deficiency. Stressgen antibodies allowed for detecting of small, but significant up-regulation of dlps level in '*C*' cultivar. On the whole, the most evident increase in dlp abundance was noticed in two tested stress conditions in mitochondria of '*A*' and '*C*' for 18 and 27 kDa dlps and in less extent- in '*A*' for 37 kDa protein. Interestingly, the highest increase of 18 and 27 kDa dlps was seen in '*A*' in the mild drought and in '*C*' in the severe treatment. The level of large-sized dlps (ca. 80 kDa), which was showed

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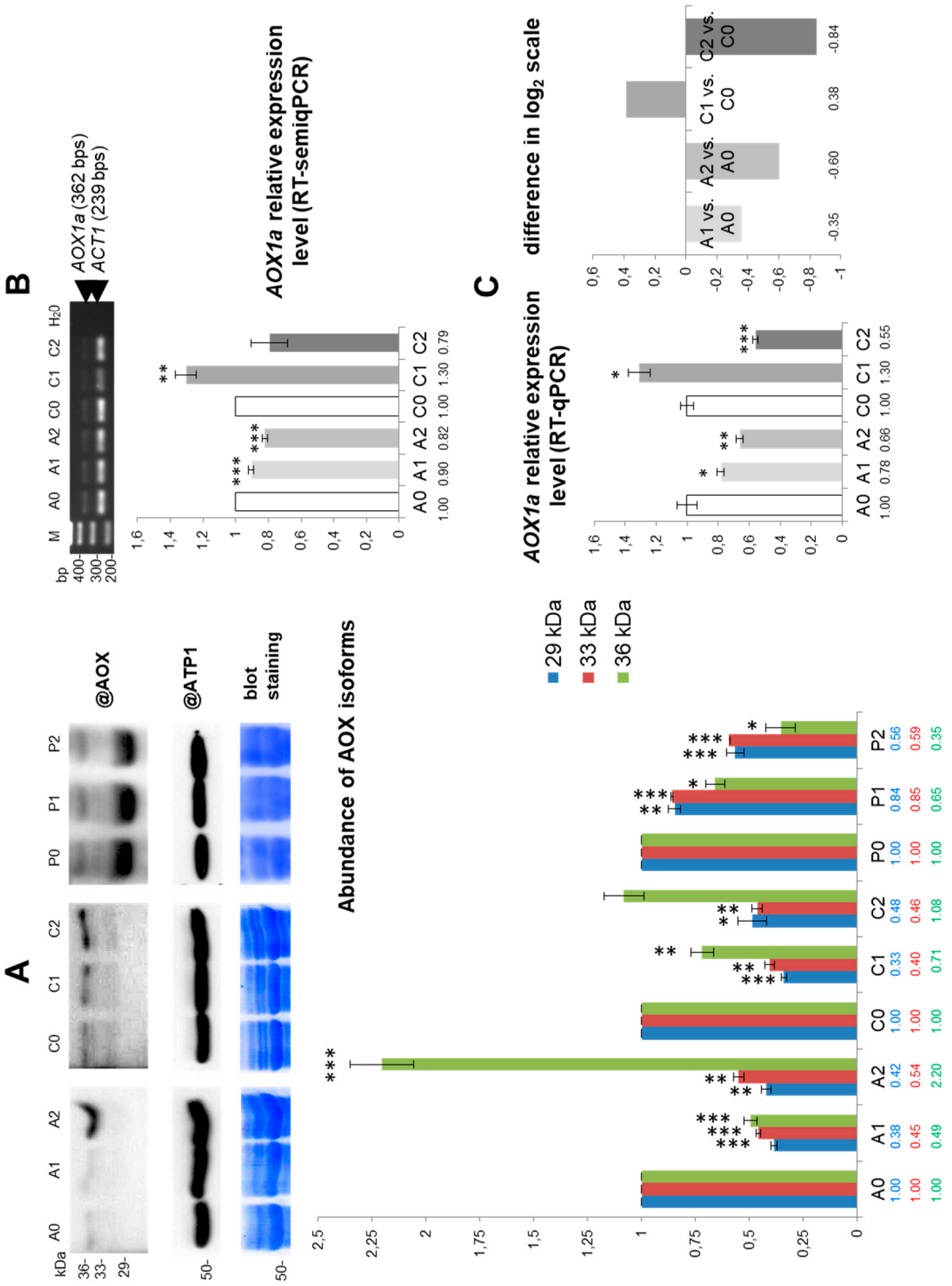

Figure 4. The abundance of the alternative oxidase (AOX) at the protein and transcript level in mitochondria isolated from control grown '*Adelanto*', '*Casper*' and '*Pionier*' plants (A0, C0, P0), plants grown in the moderate (A1, C1, P1) and in the severe water deficiency (A2, C2, P2, respectively). (A) Analysis of the immunoreactive polypeptides (29, 33, 36 kDa) of AOX on representative SDS-PAGE blots using respective antibodies (@AOX). For the loading control, the antibody against subunit-α of mitochondrial ATP synthase (@ATP1) was used. Equal protein loading was also shown by CBB staining of Western blots. For the molecular mass calibration, the PageRuler Prestained Protein Ladder (Thermo Scientific) was used. The protein molecular mass is indicated in kDa. (B)

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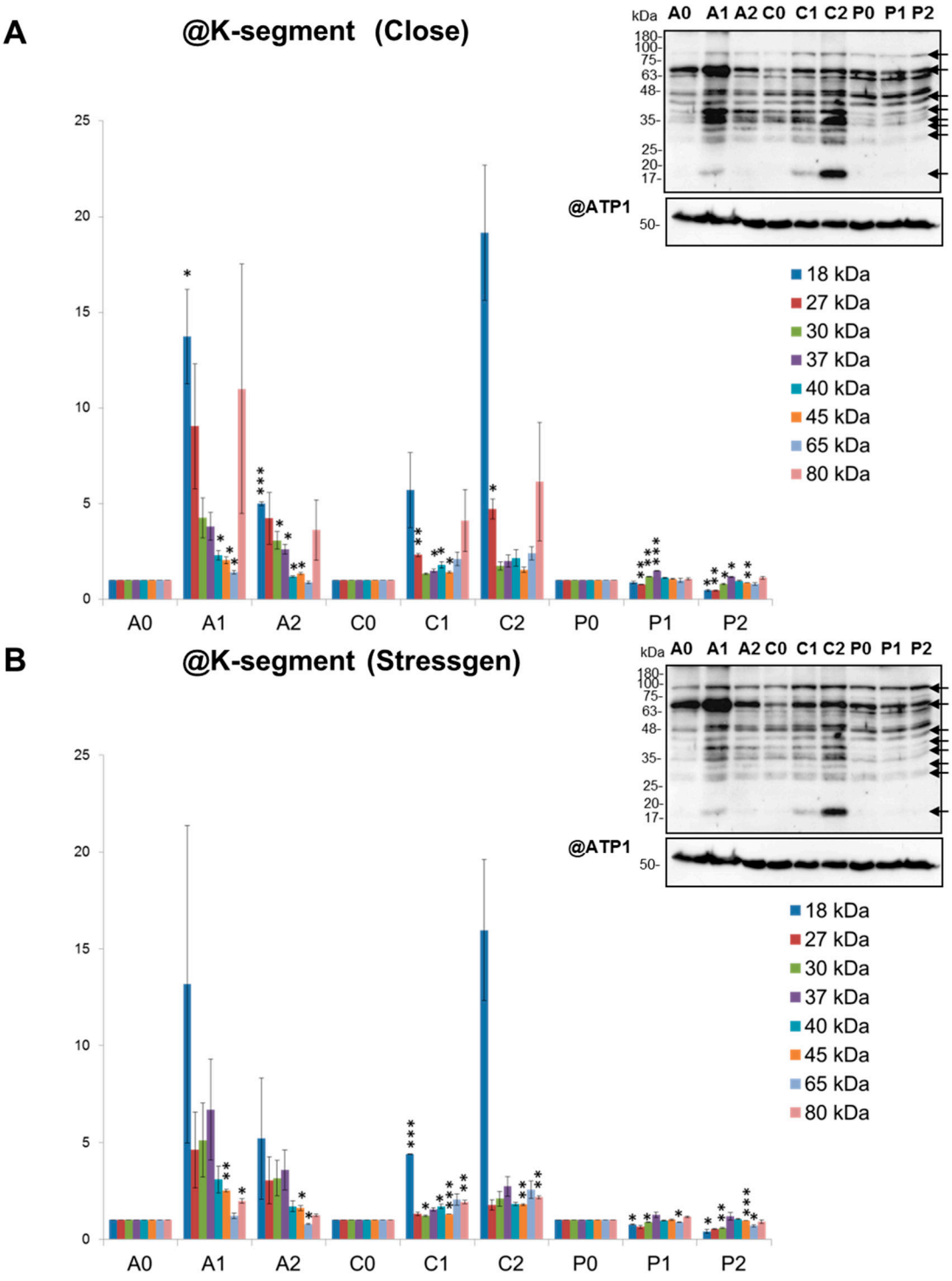
RT-semiqPCR analysis of the relative level of cauliflower *AOX1a* transcripts. For normalization, cauliflower actin1 (*ACT1*) was used. The representative 2% agarose gel is shown. (C) The relative expression of *AOX1a* determined by RT-qPCR. Graph *at the left*, relative expression level normalized to the average level (mean log expression = 1). Graph *at the right*, differences in $\log_2$ scale between treated and control variants are shown. For normalization, *ACT1* was used. The results of the densitometric analysis in (A), (B) and (C) are presented as the mean values (± S.E.) from triplicate detection. These values were standardized relative to the control (set to 1.00). The significant alterations are marked with asterisks: ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ versus control values for each cultivar. Further data in the text.



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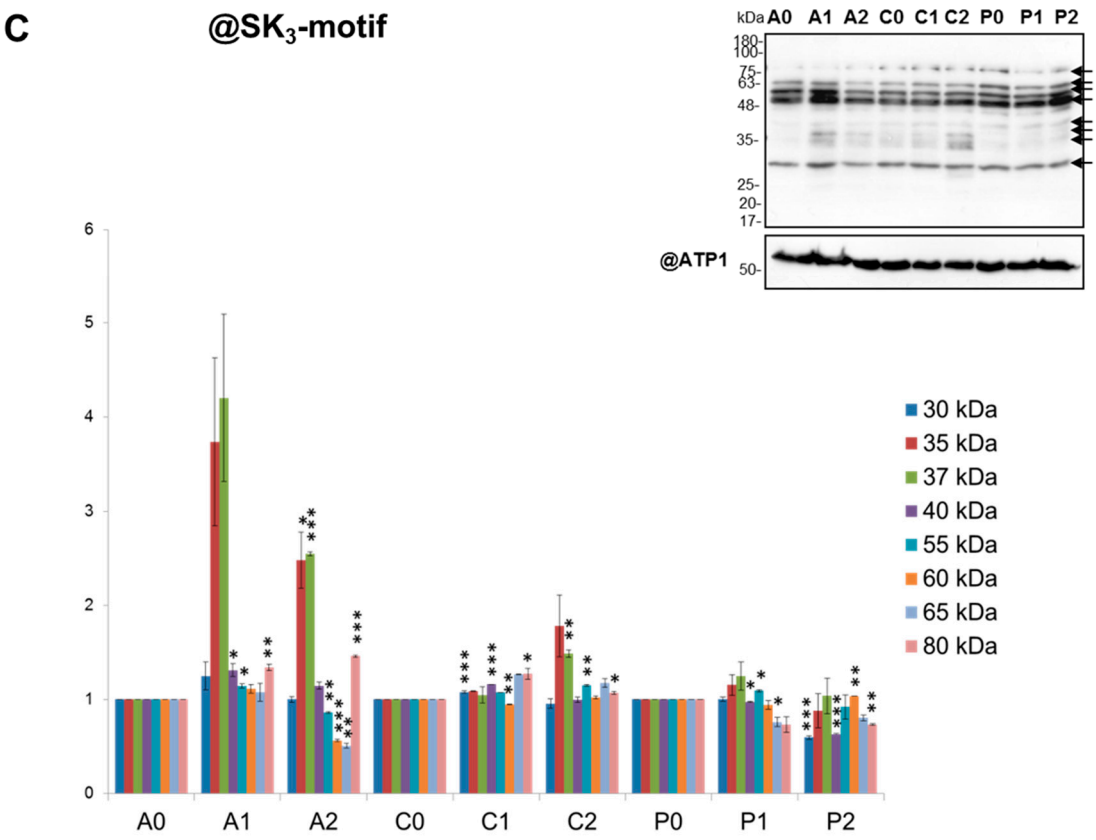

Figure 5. (pp. 16 and 17) Dehydrin-like proteins (dlps) abundance in mitochondria extracted from control grown '*Adelanto*', '*Casper*' and '*Pionier*' plants (A0, C0, P1), plants grown in the moderate (A1, C1, P1) and in the severe water deficiency (A2, C2, P2, respectively). Analysis of immunoreactive dlps of various size (indicated in kDa) on representative SDS-PAGE blots was carried out using antibodies directed against dehydrin K-segment either from Close [105] [@K-segment (Close)] or from Stressgen [@K-segment (Stressgen)], or antibodies recognizing dehydrin SK_3 motif [@SK_3]. For the loading control, the antibody against subunit-α of mitochondrial ATP synthase (@ATP1) was used. For the molecular mass calibration, the PageRuler Prestained Protein Ladder (Thermo Scientific) was used. The protein molecular mass is indicated in kDa. Investigated dlps were marked by arrows on gel blots. The significant alterations from triplicate detection are marked with asterisks: ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ versus control values for each cultivar. Further data in the text.

previously to interact with the mitochondrial membranes [49], also markedly increased in drought-sensitive cultivars, however the highest increase in level of those proteins was noted under the mild stress in '*A*' mitochondria. Notably, the middle-sized dlps (40-65 kDa) responded also in abundance (Figure 5).

The antisera against dehydrin SK_3-motif recognized smaller, but the significant increase in the abundance of ca. 30/35 kDa dlps under both drought conditions in '*A*' and under the severe drought in '*C*' (Figure 5). The alterations in the level of middle and large-sized dlps were less visible. It seems that the accumulation of dlps ranging 55-65 kDa in size slightly decreased in the severe drought in '*A*' cultivar. The similar phenomenon was observed for 30 and 40 kDa dlps in '*P*'. Overall, the abundance of immunodetected dlps in '*P*' mitochondria (irrespectively to the antisera used)

appeared to be relatively stable in the analyzed adverse conditions, which agrees with the elevated drought tolerance of this cultivar at the physiological level (section *2.1*) and with the relatively lower increase in dehydrin mRNA abundance (e.g. DHN8) under progressing drought among plant genotypes enabling high adaptability to such stress conditions [36].

Among of a plethora of stress conditions, the water deficit belong to the widely known conditions regulating dehydrin abundance by inducing or enhancing their expression level [92]. In general, the accumulation pattern of mitochondrial dlps in drought is distinct from the one related with the impact of temperature stress on the level of those proteins [49]. Presently, we noticed even more significant alterations in the dlps abundance as well as the induction of other dlps (Figure 5). This substantially extends the whole data on mitochondrial proteins related to dehydrins in *Brassicaceae*.

2.6. Identification of drought-responsive spots containing putative dehydrin-like proteins

The most notable changes in the accumulation of dlps were noticed in '*A*' and '*C*' cultivars under all analyzed drought conditions. In order to further characterize the drought-responsive cauliflower dlps, we prepared 2D (IEF-SDS/PAGE) blots with separated whole mitochondrial proteins from '*A*' and '*C*' cultivars submitted to the mild as well as to the severe drought (dlps from '*P*' cultivar appeared largely unaffected by drought; section *2.5*) and we use such blots to immunodetect dlps within the particular protein spots. For 2D immunoassays, antisera against dehydrin K-segment (section *3.8*) were used. Results of the dlp immunodetection on 2D spots were shown on Figure 6. Due to the assay sensitivity, we were able to detect few spots per investigated cultivar/treatment within the pI range of ca. 5-8 and the molecular weight of 18-48 kDa (35-48 kDa mostly) regulated in abundance by the water deficit (section *2.5*). However, in '*A*' cultivar under mild drought, the only single spot was immunodetected. Interestingly, some spots (C1-7 and C1-8, C2-4 to C2-6) appeared to migrate in more neutral pI values, whereas the others (C2-7 and C2-8) represented basic proteins. Basing on immunoassay results, we cut out all the immunodetected protein spots from 2D gels, and proteins were identified by tandem mass spectrometry coupled with liquid chromatography (LC-MS/MS). The position of all those spots from Western blots were superimposed on the spot pattern within the master gel image (Figure 1) and the results of protein identification within individual spots were shown in Table S4.

Notably, only selected spots contained dehydrin-related tryptic peptides that showed high similarity of their protein sequences to the selected *Brassica* dehydrins. These were: 5 spots (A2-2 to A2-6) in '*A*' cultivar under the severe drought, 2 spots (C1-1 and C1-3) in '*C*' under the mild stress and the single spot (C2-1, all marked in red on Figure 6) in the latter cultivar under severe water deficit. The detected tryptic peptides appeared highly similar to the ERD14 and ERD14-like dehydrins from various *Brassica* species, especially *B. oleracea* var. *oleracea* and *B. rapa* (GenBank accession.version identifiers XP_013592580.1 and XP_009128158.1, respectively; Table S4). After the careful inspection of the peptide data for each spot, it appeared, however, that selected protein spots contain highly-abundant mitochondrial proteins including, inter alia, organellar editing factors, mercapto-pyruvate sulfurtransferase, PDH subunit β, ascorbate peroxidase, cysteine synthase, MDH1, γ carbonic anhydrase 2 and the subunits of succinyl-CoA ligase (all proteins listed in Table S4). This result justified the choice of the additional experimental strategy for the analysis of dlp identity. In fact, we could not employ protein micro-sequencing for the identification of the chosen

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dlps, which would allow us for the subsequent amplification and cloning full-length cDNAs due to the fact that highly abundant proteins in spots would mask dlps. Therefore, we were not able to determine N-terminal sequence for given dlps. On the other hand, the depletion of any abundant proteins would enhance the risk of sample cross-contamination and hamper dlp identification. Instead, we applied tandem MS to identify the tryptic peptides for the putative candidates of dlps. Finally, we focused on the comparison of proteomic and transcriptomic responses important for the cauliflower mitochondrial biogenesis in drought, including profiling the level of messengers coding for the identified dehydrins.

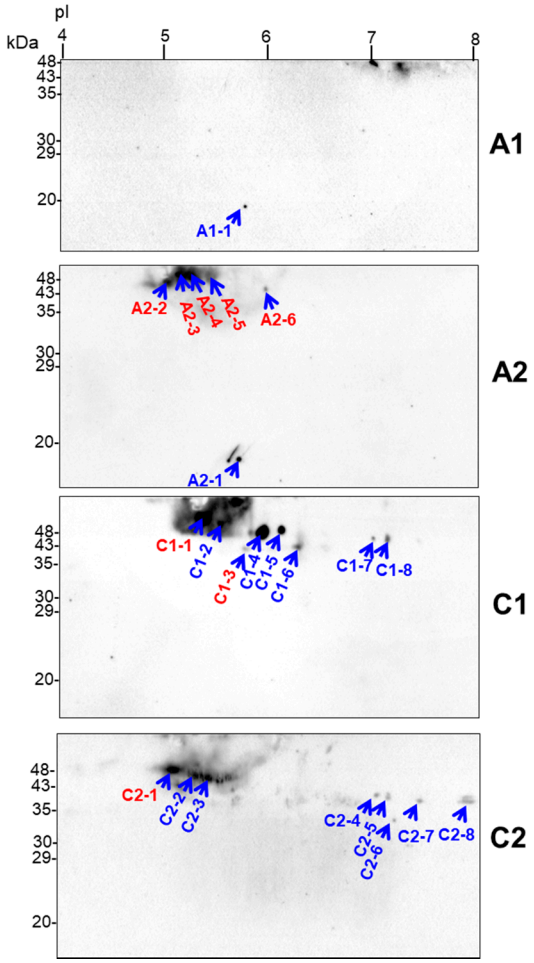

Figure 6. The representative pattern of dehydrin-like proteins of '*Adelanto*' and '*Casper*' plants grown in the moderate (A1, C1) and in the severe water deficiency (A2, C2, respectively) immunodetected with antibodies directed against dehydrin K-segment (Close, 1997) on Western 2D (IEF/SDS-PAGE) blots. Protein spots referring to the immunodetection signals that were cut out from the respective 2D gels for protein identification by LC-MS/MS (*blue arrows*) appeared also on Figure 1 (denoted *in blue* and *red*). The proposed identity for those spots and all tryptic peptide data appear in Table S4. Identifiers of spots which appeared to contain tryptic peptides specific to dehydrins were marked *in red* (the remaining labels are shown *in blue*). For the molecular mass calibration (kDa) of the protein spots, the PageRuler Prestained Protein Ladder (Thermo Scientific) and LMW-SDS Marker Kit (GE Healthcare) were used. For the calibration of spot isoelectric point (pI), Broad pI Kit (GE Healthcare) was used. Further data in the text.

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2.7. The Transcriptomic Responses and the Coordination of the Mitochondrial Biogenesis in Drought

We also studied transcriptomic responses to drought connected with the profiling of the level of selected nuclear and mitochondrial transcripts affecting mitochondrial biogenesis in those conditions. We focused on drought-sensitive cauliflower cultivars. During assessing transcript abundance results obtained by RT-semiqPCR assays were validated by RT-qPCR with the application of primers specific for the dedicated cDNAs fragments (Table S5). As internal calibrator of gene expression, cauliflower *ACT1* messengers were applied (the partial sequence was previously cloned and deposited in GenBank under accession.version KC631780.1; [13]). We noticed minor, but significant down-regulations of *atp1* (mitochondrial transcripts for the subunit 1 of ATP synthase) and *HSP70* (nuclear transcripts coding for the mitochondrial HSP70) messengers only in '*C*' under mild water deficit, which did not corresponded with protein variations, because the level of ATP1 protein was almost untouched. In addition, the level of HSP70 protein increased in the mentioned experimental variant, however, the abundance of the mature *HSP70* messengers appeared decreased in the mild drought in '*C*' (Figures 3 and 7). The abundance of both *atp1* and *HSP70* mRNAs in the remaining stress conditions in two drought-sensitive cultivars appeared quite stable (Figure 7).

We checked whether the studied transcriptomic regulations in the abundance of *AOX* messengers accompanied the respective protein alterations. Cauliflower *AOX1a* partial sequence was previously cloned and deposited in the GenBank (accession.version KC631778.1; [13]). The accumulation of *AOX1a* transcripts is often affected by the abiotic stress [93]. Other members of AOX multigenic family, e.g. *AOX1d,* can be up-regulated in drought as well [31]. The choice of *AOX1a* for the present study can be justified by its crucial role in the determination of the redox balance in a number of suboptimal stimuli, including combined light and drought [94]. The accumulation of the *AOX1a* mRNAs was significantly increased under the mild drought in '*C*' and it was strongly down-regulated under the mild and severe drought in '*A*' and in the severe drought in '*C*' (Figure 4). Overall, the most severe drought, the most intense *AOX1a* decrease was observed. Comparing expression pattern of *AOX1a* gene on the protein and transcript level, surprisingly a major imbalance between the protein and mRNA abundance in '*C*' and '*A*' cultivars was noticed. In the latter cultivar's mitochondria two immunoreactive AOX protein bands appeared decreased under the mild and severe stress (which is in line with *AOX1a* transcript level in the same conditions), whereas the third band (36 kDa) notably increased in abundance in the severe treatment (section *2.4*; Figure 4). Conversely, in '*C*' no significant up-regulation was detected on AOX protein level, contrary to the messengers abundance variations in the mild drought. The lack of the coordination between proteomic and transcriptomic responses in this case may resulted from the synthesis of investigated immunoreactive polypeptides from transcripts coding the distinct AOX isoforms (especially 33 kDa protein). In '*A*', under severe drought, the lowered amount of *AOX1a* messengers contrasted with the enhanced accumulation of 36 kDa AOX isoform (Figure 4).

Overall, under drought conditions, mRNA compensatory up-regulations were accompanied by the down-regulations in the protein level and vice versa (*AOX1a* in mild drought in 'C' and in severe drought in 'A', *HSP70* in mild drought in 'C'; Figures 3 and 7); additionally, in some cases the decreased amount of transcripts (e.g. *atp1* in mild drought in 'C'; Figure 7) accompanied the constitutive pattern of expression at the protein level. We can speculate that the regulation of transcriptomic responses in case of *AOX*, *atp* and *HSP* mature messengers may depends on the

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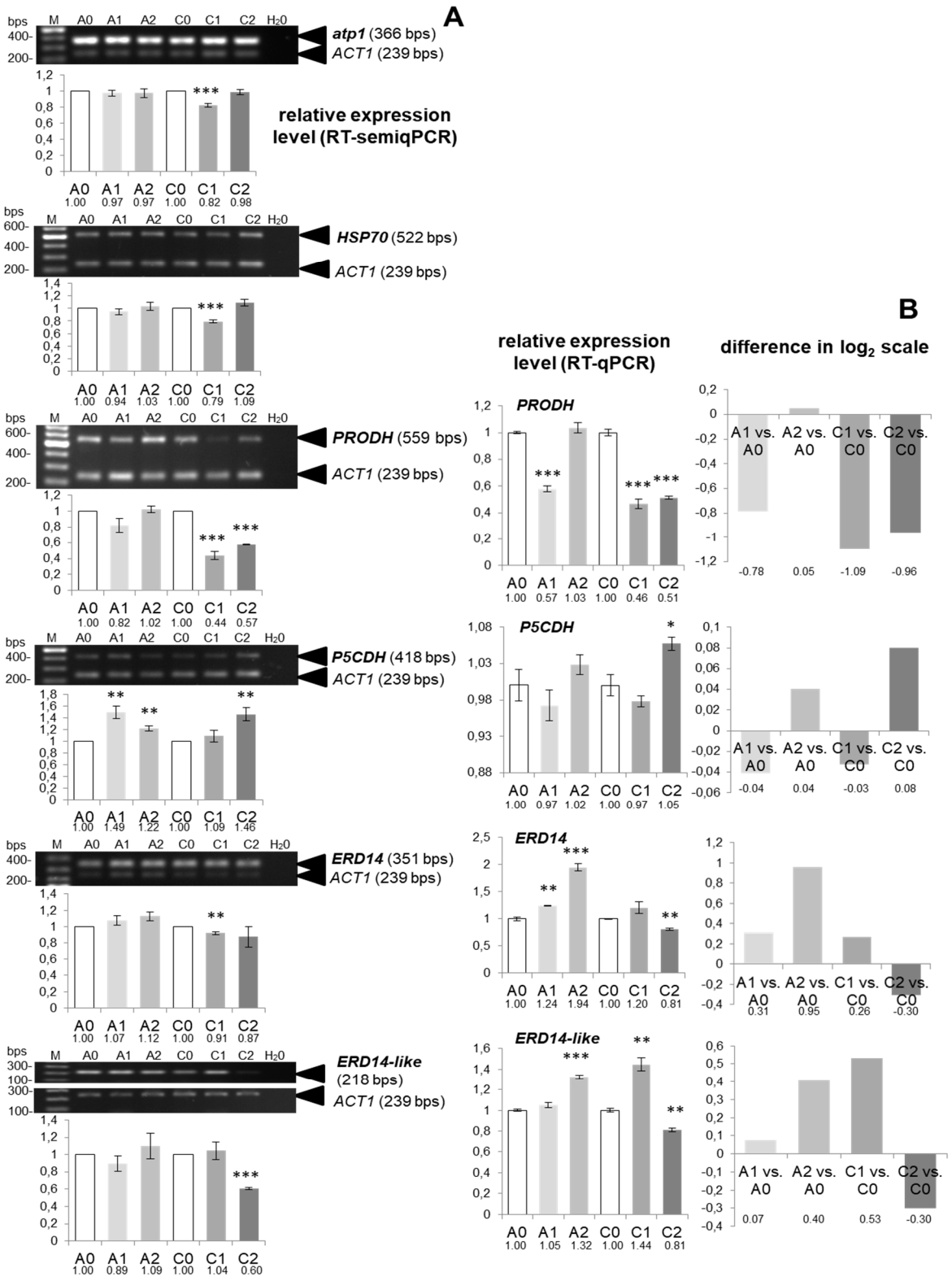

Figure 7. The relative level of transcripts for cauliflower subunit-α of mitochondrial ATP synthase (*atp1*), mitochondrial heat shock protein 70 (*HSP70*), proline dehydrogenase (*PRODH*), Δ-1-pyrroline-5-carboxylate dehydrogenase (*P5CDH*), ERD14 dehydrin (*ERD14*) and ERD14-like dehydrin (*ERD14-like*) in control grown '*Adelanto*', '*Casper*' and '*Pionier*' plants (A0, C0, P0), plants

grown in the moderate (A1, C1, P1) and in the severe water deficiency (A2, C2, P2, respectively). (A) RT-semiqPCR assays. The representative 2% agarose gels were shown. The results of the densitometric analysis are presented as the mean values (± S.E.) from triplicate detection. These values were standardized relative to the control (set to 1.00). (B) The relative accumulation of mRNAs determined by RT-qPCR. Graphs *at the left*, relative expression level normalized to the average level (mean log expression = 1). Graphs *at the right*, differences in $\log_2$ scale between treated and control variants are shown. In all cases, for normalization, *ACT1* was used. The significant alterations are marked with asterisks: ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ versus control values for each cultivar. Further data in the text.

changes in selection of transcripts for the translation, diverse mRNA/ribosome interactions and/or affected protein synthesis [7]. The decreased pool of those transcripts in some drought conditions/ cultivars had to enable for their efficient translation leading to the protein excess and thus those mRNAs seems to be fully translatable. In general, positive transcriptomic response of *AOX1a* gene to the analyzed drought conditions involves only selected cauliflower cultivars ('*C*') as well as drought treatments (e.g. the mild water deficit, Figure 4). Nevertheless, the participation of non-coding RNAs, including mitochondrial regulatory RNAs and microRNAs (miRNAs), in the regulation of the level of investigated transcripts or their translational efficiency should be also concerned. For instance, 3'flanking region in Arabidopsis *atp1* transcripts is targeted by the intermediate-sized non-coding RNA (nc4460) of 111 bps [95,96]. Moreover, the presence of miRNAs (nuclear-encoded and processed before import to mitochondria) or components of miRNA biogenesis machinery present within the mitochondrial compartment was supported by some evidences [97-99]. Regarding nuclear-encoded transcripts, we studied *in silico* putative miRNA candidates that may putatively target *B. oleracea AOX1a* and *HSP70-9* mRNAs (primers specific to those sequences were used in RT-PCR) by psRNATarget (http://plantgrn.noble.org/psRNATarget/analysis?function=2; [100]) search (Table S5). We focused on Arabidopsis and *Brassica* miRNAs, because of the relative high similarity of *AOX1a* and *HSP70-9* nucleotide sequence (accessions.versions KC631778.1 and XM_013773775.1, respectively) between those genera. According to our data, most of the predicted miRNAs resulted rather in messenger degradation than in affected translation. This does not obviously exclude, however, the possibility that the multiple routes of non-coding RNA action could be expected on the steady-state level of investigated transcripts and protein abundance.

Proline, the potent osmoprotectant, can be over-accumulated under drought with slight differences among plethora of plant species (including *Brassica* members); it can be down-regulated under drought recovery [7,29,30,52]. However, proline content variations may not simply correspond to the level of the drought adaptation [36]. During under-expression of formate dehydrogenase, proline accumulation was also noticed [76]. Variations in the abundance of the enzymes for the proline metabolism accompany plant stress response [7,30]; previously, we showed cauliflower Δ-1-pyrroline-5-carboxylate dehydrogenase (P5CDH) upregulated both in heat and heat recovery [13]. Basing on our results, we postulate the reciprocal regulation of the level of proline dehydrogenase (*ProDH*) as well as *P5CDH* transcripts under drought. Generally, the abundance of *ProDH* messengers decreased in '*C*' in both drought treatments; however the most severe impact was visible under the mild drought. In '*A*' cultivar the significant decrease of messenger abundance was noted only in the mild stress. On the opposite, the level of *P5CDH* transcripts was markedly elevated only in '*C*' under the severe drought (Figure 7). Those findings suggests the elevated

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proline content in cauliflower curds. However, the down-regulation of messengers coding for proline catabolism enzymes was not equal between the investigated cultivars indicating for the diverse osmolyte accumulation in those cultivars [37].

As the respective proteomic responses related with dlps profile appeared very evident for drought-sensitive cultivars (section *2.5*), we investigated the level of mRNAs coding for ERD14 and ERD14-like dehydrins as tryptic peptides with high sequence similarity to those proteins were found in the immunoreactive with dehydrin K-segment antisera protein spots on 2D blots (section *2.6*; Figure 6, Table S4). The highest increase in both transcript level was noticed in '*A*' cultivar in the mild and severe drought, which is in line with the induction of *ERD14* gene expression within the short dehydration period as well as with the presence of dehydrin-related tryptic peptides in analyzed protein spots. Some *ERD* messengers display maximal accumulation at the late stage of drought treatment [68]. *ERD14* is also induced by ABA, salinity and low temperature treatment [101-102]. Notably, *ERD14* and *ERD14-like* messengers were significantly decreased in the second investigated cultivar under severe water deficit (Figure 7). All that suggests that the described strong up-regulation of dlps in '*A*' cultivar mitochondria (Figure 5) coincides with the positive transcriptomic response.

3. Materials and Methods

3.1. Growth of Plant Material and Stress Application

Seeds of three analyzed cauliflower (*Brassica oleracea var. botrytis* subvar. *cauliflora* DC cv. 'Diadom') cultivars ('*Adelanto*', '*Casper*' and '*Pionier*') were obtained from Bejo Zaden (Poland). Cauliflower seedlings were produced in 0.09 dm3 pots filled with peat substrate (Kronean-Clasmann). Seedlings with three-four leaves were transferred to 5 dm^3 in-volume containers. Plants were grown for 3 months in cultivation chambers at a local breeding station (Poznan University of Life Sciences, 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 developing curds up to the curd diameter of 7-10 cm. The water capacity of the substrate was 40% [*v*/*v*] and under drought stress water content decreased to 22% [*v*/*v*] (mild drought stress) and to 15% [*v*/*v*] (severe drought stress). After the occurrence of the assumed level of drought stress, plants were irrigated to 40% [*v*/*v*] and then the drought stress was applied again. This treatment was repeated for ca. 2-3 weeks. Curds were harvested immediately after the stress treatment cessation. Duration of the drought stress was estimated on the basis of relative water content (RWC) in the soil and in plant leaves and curds. The RWC in developed, mature cauliflower leaves in the mild drought conditions (the first level of drought) were achieved at the RWC of 94%, 92% and 95% for '*A*', '*C*' and '*P*' cultivars, respectively. In case of the severe drought treatment (the second level of drought), the RWC values lasted 69%, 74% and 73% for the mentioned three cultivars, respectively. The RWC of cauliflower leaves under the severe drought referred to the third and fourth day of the drought treatment.

3.2. Physiological Analyses

Physiological analyses were conducted on well-developed cauliflower leaves with the LCpro+ infrared gas analyzer (ADC). To obtain more reliable results, extra experimental replicas (n=8) were

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used. The rate of leaf respiration in the light (day respiration, R_d) was determined according to the Laisk [103] method. CO_2 assimilation rate representing a given total respiration rate (R_T) was recorded during intercellular CO_2 concentration (C_i) decreased to 0 ppm at 22°C and 50% relative humidity. For the each value of photosynthetic photon flux density (PPFD) at 200, 400 and 600 µmol m^{-2} s^{-1}, the linear regression of CO_2 assimilation (A) versus C_i was calculated. The intersection of three regression lines was determined by minimizing the sum of the squares of errors between the measured values and calculated for each PPFD level while minimizing the standard deviation for the intersection. The photorespiration (PhR) rate was determined as difference between R_T and R_d values at Ci 0 ppm (the latter one expressed as a given CO_2 evolution rate at the crossing point of all A/C_i curves). The rate of mitochondrial respiration in the dark (R_n) was measured after 30 min of the adaptation to the dark. The applied drought treatments were necessary for the visible alterations in the respiratory and photorespiratory rates (Figure S1). The given drought exposures (section *3.1*) were necessary to observe the observed proteomic and physiological alterations.

3.3. Isolation of Mitochondria, Purity Assays and Protein Determination

Mitochondria from the topmost 5-mm-thick layer of the cauliflower curds were extracted by differential centrifugation and purified in Percoll gradients according to Pawlowski et al. [104]. During isolation, the Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche) was used. Purity assays of the isolated mitochondria were conducted according to the previous reports [49,104]. The protein content was determined using the BioRad Protein Assay, using bovine serum albumin as a standard curve calibrator. The efficiency of the organellar preparations (proteins per 100 g of cauliflower curds) varied from 0,9-3,5 mg for '*C*' and '*P*' and 0,1-1,9 mg for '*A*' cultivar.

3.4. Sample Preparation for the Two Dimensional Isoelectric Focusing/ SDS Polyacrylamide Gel Electrophoresis (2D IEF/ SDS-PAGE)

Proteins were extracted and precipitated overnight at −20 °C in a 10% solution of trichloroacetic acid in acetone containing 20 mM dithiothreitol (DTT) by method of Staszak and Pawłowski [105]. After centrifugation (16,000 *g* for 5 min at 4 °C), the resulting pellets were washed three times with 1 mL of acetone supplemented with 20 mM DTT. Samples were re-centrifuged after each washing and the resulting pellets were vacuum dried and then resuspended in the lysis buffer (7M urea, 2M thiourea, 2% CHAPS, 1.5% DTT, 0.5% IPG buffer pH 4–7), and supplemented with a Protease Inhibitor Cocktail (Roche, Basel, Switzerland) according to the manufacturer's suggestions. Protein concentration in the processed samples was determined using the Bradford assay [106].

3.5. 2D IEF/ SDS-PAGE

All analyses were conducted at 25°C with at least three biological replicas. Proteins (500 µg for Coomassie Brilliant Blue [CBB]) were first separated according to their charge on rehydrated Immobiline DryStrips (24 cm in length, containing linear gradient of pH 3-10) with the rehydration buffer (6 M urea, 2 M thiourea, 2% CHAPS, 20 mM DTT, and 0.5% Pharmalyte, pH 4-7) on Ettan IPGphor 3 IEF System (GE Healthcare Life Science). The program for isoelectric focusing was according to the manufacturer's suggestions. The strips were either stored at –80°C or they were directly treated for 10 min with the equilibration solution I (6 M urea, 1.5 M Tris-HCl, pH 8.8, 30%

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glycerol, 2% SDS, and 1% DTT) and for the same time with equilibration in the solution II (solution I supplemented with 2.5% 2-iodoacetamide, without DTT) and subjected for the second dimension run (SDS-PAGE).

For SDS-PAGE, Ettan DALT 12.5% Precast Polyacrylamide Gels and the Ettan DALTsix Electrophoresis System (both from GE Healthcare) were used. Conditions for the run were as follows: 1 h at 80 V and 5 h at 500 V. Broad pI Kit, pH 3-10 (GE Healthcare) for protein spot pI calibration within 3.5-9.3 as well as PageRuler Prestained Protein Ladder (Thermo Fisher Scientific) and LMW-SDS Marker Kit (GE Healthcare) for protein spot MW calibration within 14.4–94.0 kDa were used. After electrophoresis, proteins on gels were stained with colloidal CBB, which, in addition to visualization and quantification, also allowed for the downstream MS analysis [107].

3.6. Proteome analysis

The gels were scanned and evaluated using ImageMaster 2D Platinum v7.0 software (GE Healthcare). After spot detection, 2D gels (three gels from three independent biological samples) were aligned and matched, and the normalized spot volumes were determined quantitatively. For each matched spot, the per cent volume was calculated as the volume divided by the total volume of matched spots. Spots with variations in abundance were subjected to ANOVA and the Tukey-Kramer HSD test (JMP software, SAS Institute, Cary, USA) to preliminary select spots that significantly varied in abundance for the drought intensity vs. control variants within each of the analyzed cultivars. Variations of selected spots were also tested within the Student's test (section *3.9*). Proteins were subsequently identified by MS from spots that significantly varied in abundance.

3.7. Protein identification by mass spectrometry (MS)

The gel spots were subjected to a standard '*in-gel* digestion' procedure in which proteins were reduced with 10 mM DTT (for 30 min at 56°C), alkylated with 55 mM 2-iodoacetamide (45 min in the dark at room temperature) and digested overnight with trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate. The resulting peptides were eluted from the gel matrix with 0.1% trifluoroacetic acid in 2% acetonitrile.

Peptide mixtures were analyzed by liquid chromatography coupled to the mass spectrometer in the Laboratory of Mass Spectrometry in the Institute of Biochemistry and Biophysics at the Polish Academy of Sciences (Warsaw, Poland). Samples were concentrated and desalted on a RP-C18 pre-column (nanoACQUITY Symmetry® C18, Waters, Milford, MA, USA), and further peptide separation was achieved on a nano-Ultra Performance Liquid Chromatography (UPLC) RP-C18 column (Waters, BEH130 C18 column, 75 µm id, 250 mm long) of a nanoACQUITY UPLC system, using a linear 0–60% CAN gradient for 120 min in the presence of 0.05% formic acid with a flow rate of 150 nl min^{-1}. The column outlet was directly coupled to the electrospray ionization (ESI) ion source of the Orbitrap Velos type mass spectrometer (Thermo Electron Corp., San Jose, CA, USA), working in the regime of data dependent MS to MS/MS switch. An electrospray voltage of 1.5 kV was used. A blank run preventing cross contamination from previous samples preceded each analysis.

Proteins were identified using the Mascot search algorithm (www.matrixscience.com) against the NCBInr (www.ncbi.nig.gov) databases. Protein identification was performed using the Mascot search probability-based molecular weight search (MOWSE) score. The ion score was $-10 \times \log(P)$,

where *P* was the probability that the observed match was a random event. To avoid possible misidentifications resulted from the implementation of large datasets, as pointed out by Schmidt et al. [108], we were able to set the threshold of false positive rate. Peptides with a Mascot score exceeding the threshold value, corresponding to a <5% false positive rate as calculated by the Mascot procedure, were considered to be positively identified.

3.8. SDS-PAGE, Western blotting and immunodetection of proteins

Proteins resolved by one-dimensional SDS polyacrylamide gel electrophoresis (12% SDS-PAGE; [49]) were electroblotted in semidry conditions onto Immobilon-P membranes (Millipore), using a TE77 PWR ECL Semi-Dry blotting apparatus (GE Healthcare Life Science) and the standard transfer buffer (20% methanol, 48 mM Tris, 39 mM glycine, 0.0375% SDS). Proteins resolved on two-dimensional gels were electroblotted in semidry conditions using the same apparatus and the alternative transfer buffer (10% methanol, 10 mM CAPS pH 11.0). The protein immunodetection was carried out with rabbit polyclonal antibodies directed against the Mn-SOD (product. no. AS09 524, 1:5000 dilution), cyt. *c* (product no. AS08 343A, 1:5000), mitochondrial HSP70 (product no. AS08 347, 1:4000), SHMT1 (product. no. AS05 075, 1:10000), GDC-H (product no. AS05 074, 1:4000), IDH (product no. AS06 203A, 1:4000), aconitase (product no. AS09 521, 1:5000; all antisera listed above from Agrisera, Vännäs, Sweden), PUMP (1:1000; [109,110]), dehydrin K-segment (1:1000, a gift of T.J. Close, University of California at Riverside, USA; [111]), dehydrin K-segment with N terminal cysteine on the synthetic peptide (product no. PLA-100, 1:1400; Stressgen), SK_3-motif of *Solanum sogarandinum* DHN24 dehydrin (1:500, a gift of T. Rorat, Institute of Plant Genetics, Polish Academy of Sciences, Poznań, Poland; [112]) and mouse monoclonal antibodies directed against AOX (1:1000; [113]), and ATP1 (1:200; [114]; both antisera donated by T. Elthon, University of Lincoln, Nebraska, USA). Details of the immunoassays were described previously [13,49]. Enhanced chemiluminescence (ECL) signals were quantified with Multi Gauge (v2.2).

3.9. RNA isolation and RT-PCR assays

Total RNA from cauliflower curds was extracted using EZ-10 Spin Column Plant RNA Mini-Preps Kit (BioBasic, Canada) according to the manufacturer's protocol. Genomic DNA contaminants were removed by RQ1 DNase I free of RNase (Promega). cDNA was synthesized using 1 µg of RNA, 0.2 µg of random hexamers mixture from HexaLabel DNA Labeling Kit (Fermentas) and 200 units of M-MLV reverse transcriptase (Promega) in a 20 µl total volume for 1 h at 37°C. After first strand synthesis, the reaction mixture was diluted with 10 mM Tris-HCl, pH 8.0 three or six times for RT-semiquantitative multiplex PCR (RT-semiqPCR) or RT-quantitativePCR (RT-qPCR), respectively, and after normalization, aliquots of 1-2 µl were subjected to RT-semiqPCR in a 15 µl total volume or RT-qPCR using the Thermo Fisher Scientific Luminaris Color HiGreen High ROX qPCR Master Mix kit on an Applied Biosystems StepOnePlus Real-Time PCR System. For RT-qPCR assays the following profile was used: 5 min at 95°C followed by 40 cycles of 20 s at 95°C, 1 min at 60°C and finally melting step. The quality of the q-RT-PCR assays was verified by LinRegPCR (v.2012.3). Outliers were manually removed.

RT-semiqPCR was performed in an Applied Biosystems 2720 thermal cycler with the following profile: 3 min at 95°C followed by 25-26 cycles depending of amplicon of 20 s at 95°C, 30 s

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at 55°C and 30 s at 72°C, and with a final incubation for 5 min at 72°C. Amplification of *AOX1b* and *AOX2* cDNAs was conducted with the following profile: 2 min at 98°C followed by 30 cycles of 20 s at 95°C, 30 s at 53°C, and 45 s at 72°C, and with a final incubation for 10 min at 72°C. The PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. The gels were documented using a GBOX XL1.4 (Syngene) imaging system and quantified with Multi Gauge (v.2.2). For RT-PCR assays two biological and at least three technical replicates were included.

Cauliflower cDNA fragments for selected mitochondrial proteins were amplified using specific primers (Table S6); a 239-bp fragment of cauliflower actin1 (*ACT1*) cDNA was used as an internal standard. The amplicons were directly sequenced bi-directionally (Big Dye Terminator v.3.1 Cycle Sequencing kit, Applied Biosystems) on an ABI Prism 31-30 XL system (Applied Biosystems) for the sequence identity verification. In case of *AOX1a* and *ACT1* the amplicons were additionally cloned to pGEM T-Easy vector with pGEM T-Easy Ligation System II (Promega) before sequencing.

3.9. Statistical analysis

All experiments were conducted in triplicate, unless otherwise indicated. The results of the densitometric analyses (sections *2.4*, *2.5* and *2.7*), and the 2D PAGE spot pattern alterations based on the spot volume (sections *2.2* and *2.3*) are presented as the means ± S.E. An unpaired two-tailed Student's t-test was used to identify significant differences; in particular, differences were considered to be statistically significant if $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.001$ (***).

4. Conclusions

Results of our study that significantly broaden *Brassica* data suggest that plant mitochondria (across the distinct cultivars) are actively engaged in the response to the water deficit. Drought often induces numerous defense strategies and may induce some proteolytic events in plant mitochondria [19]. We did not observe enhanced proteolysis in cauliflower mitochondria under drought. However, in such stress conditions mitochondrial proteomes of three investigated cauliflower cultivars significantly varied by the major downregulated spots. Some spots were also specific to the intensity of the water deficit. Overall, multifunctional proteins which responded to drought suggested distinct regulations within the mitochondrial metabolism in the mild and severe drought. The next step in proteomic analyses should encompass the study of the dynamic pattern of PTMs of those proteins across cultivars and drought treatments; for that we will use the spectral data obtained from MS/MS peptide sequencing. Clearly, drought-responding proteins are subjected to diverse PTMs, including oxidation and phosphorylation; this pattern is dynamically altered as stress progresses [30]. Owing the relevance of protein phosphorylation in the regulation of activity of mitochondrial proteins and stress-signaling pathways and the fact that a number of mitochondrial proteins identified in this study involved in energy conversions, mitochondrial metabolism, protein fate and protein transport are extensively phosphorylated [115], we will pay a special attention to such PTMs in our future analyses. In the present study, distinct accumulation profiles among analyzed cultivars and treatments were also shown for additional mitochondrial proteins by Western immunoassays. In addition, low-molecular-weight dlps were mainly upregulated in drought-sensitive cultivars. The identification of dehydrin-specific tryptic peptides in few spots from 2D gels highlights the importance of the participation of such proteins in the cauliflower drought response.

Under various drought treatments, the biogenesis of cauliflower mitochondrial proteome was strongly and in diverse manner affected in distinct cultivars differing with drought tolerance. The investigated proteomic variations coincided roughly with drought tolerance. However the general transcriptomic alterations (particularly for messengers coding for the main stress-affected AOX

isoform) were largely unassociated with the proteomic ones, which is contrary to the findings obtained from our previous study of plant mitochondrial biogenesis in temperature stress and the thermal recovery [13]. Because during our transcriptomic analyses the abundance of mature messengers was investigated only, our results suggest that the enhanced availability of nuclear and mitochondrial mRNAs (encoding selected mitochondrial proteins) for translation may be an important regulatory point for the drought response of cauliflower mitochondria. Thus results of our study could be at least partially explained by Nakaminami et al. [116] findings on the imbalanced mRNA/protein pools and the altered pattern of translation initiation through stress acclimation and de-acclimation. Further studies are expected to elucidate the impact of drought to the transcript binding to ribosomes, to investigate of mechanisms of the increased selection of messengers for the protein biosynthesis and the participation of non-coding RNAs in mitochondrial biogenesis in drought. Particularly, cauliflower microtranscriptome targeting investigated messengers have to be characterized. Investigation of the mitochondrial translation pattern (notably affected in cold and heat [13]) in those conditions should be, nevertheless, valuable.

Supplementary Materials: A list of the following supplemental materials that are available in the online version of this article is presented below. Figures S1-S3 are PowerPoint files. Tables S1 and S5 are Word files. Tables S2-S4 are Excel files.

Figure S1. Changes in the cauliflower mature leaf (A) day respiration (R_d), total light respiration (R_T) and photorespiration (PhR) rates at 200 (R_T200 and PhR200), 400 (R_T400 and PhR400) and 600 (R_T600 and PhR600) µmol m^{-2} s^{-1} illumination as well as (B) night respiration (R_n) rates, all expressed in µmol CO_2 m^{-2} s^{-1}.

Figure S2. The representative 2D (IEF/SDS-PAGE) maps of the mitochondrial proteome of '*Adelanto*', '*Casper*' and '*Pionier*' control grown plants (A0, C0, P0), plants grown in the moderate (A1, C1, P1) and in the severe water deficiency (A2, C2, P2, respectively).

Figure S3. Drought-responsive protein spot accumulation in all experimental variants.

Table S1. Variations in abundance of cauliflower mitochondrial protein spots and their proposed identities.

Table S2. The detailed list of all tryptic peptides covering records of protein spots listed in Table S1.

Table S3. The functional classification of Arabidopsis proteins orthologous to the identified cauliflower drought-responsive proteins (see Table S1) by the Munich Information Center for Protein Sequences (MIPS) at VirtualPlant 1.3 tool (http://virtualplant.bio.nyu.edu).

Table S4. The identification of the additional protein spots from 2D gels that appeared immunoreactive with antisera against dehydrin K-segment.

Table S5. The list of miRNAs candidates (representing *Brassicaceae* family data) putatively targeting *Brassica oleracea* *AOX1a* and *HSP70-9* transcripts by psRNA Target server (http://plantgrn.noble.org/psRNATarget/analysis?function=2; Dai *and* Zhao, 2011) search (2017 updated version).

Table S6. Primers used for RT-PCR analyses.

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Author Contributions: M.R. was principle investigator, who designed this study, performed extraction of mitochondrial proteins, carried out all immunoassays and partially RT-semiq-PCRs, analyzed proteomic results, prepared and wrote the paper; M.Cz. performed isolation of total and mitochondrial RNA, performed most of RT-q-PCR assays and partially RT-semiq-PCRs and participated in writing of the paper; T.P. prepared protein samples for 2D PAGE, participated partially in 2D PAGE and the statistical analysis of spot variations, selected stress-responsive protein spots, submitted protein spots for MS analyses, participated in preparation of 2D blots and substantially in manuscript writing; A.M.S. participated partially in 2D PAGE, statistical analysis of spot variations and in paper writing; W.N. optimized RT-q-PCR assays and designed primers to them, sequenced amplicons, participated in RT-q-PCRs and in writing of the paper; W.K. cultivated plant

material, conducted all physiological assays, analyzed their results and participated in paper writing; 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

ACO	aconitase
AOX	alternative oxidase
ATP1	ATP synthase subunit 1
BSA	bovine serum albumin
CBB	Coomassie Brilliant Blue
CI, CII, CIV	respiratory complexes
CAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate
2D PAGE	two-dimensional gel electrophoresis
DHN	dehydrin
dlp	dehydrin-like protein
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EF	elongation factor
ERD	early response to dehydration
FDH	formate dehydrogenase
GDC	glycine decarboxylase
HSP	heat shock protein
IDH	isocitrate dehydrogenase
IEF	isoelectrofocusing
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LEA	late embryogenesis abundant
MDH	malate dehydrogenase
miRNA	microRNA
MPP	mitochondrial processing peptidase
OXPHOS	oxidative phosphorylation
P5CDH	δ-1-pyrroline-5-carboxylate dehydrogenase
PDH	pyruvate dehydrogenase
PhR	photorespiration rate
PPFD	photosynthetic photon flux density
ProDH	proline dehydrogenase
PTMs	posttranslational protein modifications
PUMP	plant-uncoupling mitochondrial protein
R_d	respiration in the light (day respiration) rate
R_n	respiration in the dark (night respiration) rate
ROS	reactive oxygen species
R_T	total respiration rate
RT-semiq(q)PCR	reverse transcription semiquantitative (quantitative) PCR
RWC	relative water content
SDH	succinate dehydrogenase (complex II)
SHMT	serine hydroxy-methyl aminotransferase
SOD	superoxide dismutase

UPLC	nano-ultra performance liquid chromatography
VDAC	voltage-dependent anion channel

References

1. Zhang, S.; Chen, F.; Peng, S.; Ma, W.; Korpelainen, H.; Li, C. Comparative physiological, ultrastructural and proteomic analyses reveal sexual differences in the responses of *Populus cathayana* under drought stress. *Proteomics* **2010**, *10*, 2661–2677, DOI: 10.1002/pmic.200900650.
2. Bogeat-Triboulot, M.-B.; Brosché, M.; Renaut, J.; Jouve, L.; Le Thiec, D.; Fayyaz, P.; Vinocur, B.; Witters, E.; Laukens, K.; Teichmann, T.; Altman, A.; Hausman, J.-F.; Polle, A.; Kangasjärvi, J.; Dreyer, E. Gradual Soil Water Depletion Results in Reversible Changes of Gene Expression, Protein Profiles, Ecophysiology, and Growth Performance in *Populus euphratica*, a Poplar Growing in Arid Regions. *Plant Physiol.* **2007**, *143*, 876–892, DOI: 10.1104/pp.106.088708.
3. Li, Y. L.; Stanghellini, C. Analysis of the effect of EC and potential transpiration on vegetative growth of tomato. *Sci. Hort.* **2001**, *89*, 9–21, DOI: 10.1016/S0304-4238(00)00219-3.
4. Chaves, M. M. How Plants Cope with Water Stress in the Field? Photosynthesis and Growth. *Ann. Bot.* **2002**, *89*, 907–916, DOI: 10.1093/aob/mcf105.
5. Flexas, J., Medrano, H. Drought-inhibition of Photosynthesis in C3 Plants: Stomatal and Non-stomatal Limitations Revisited. *Ann. Bot.* **2002**, *89*, 183–189, DOI: 10.1093/aob/mcf027.
6. Alvarez, S.; Roy Choudhury, S.; Pandey, S. Comparative Quantitative Proteomics Analysis of the ABA Response of Roots of Drought-Sensitive and Drought-Tolerant Wheat Varieties Identifies Proteomic Signatures of Drought Adaptability. *J. Proteome Res.* **2014**, *13*, 1688–1701, DOI: 10.1021/pr401165b.
7. Johnová, P.; Skalák, J.; Saiz-Fernández, I.; Brzobohatý, B. Plant responses to ambient temperature fluctuations and water-limiting conditions: A proteome-wide perspective. *Biochim. Biophys. Acta* **2016**, *1864*, 916–931, DOI: 10.1016/j.bbapap.2016.02.007.
8. Atkin, O. K.; Macherel, D. The crucial role of plant mitochondria in orchestrating drought tolerance. *Ann. Bot.* **2008**, *103*, 581–597, DOI: 10.1093/aob/mcn094.
9. Salvato, F.; Havelund, J. F.; Chen, M.; Rao, R. S. P.; Rogowska-Wrzesinska, A.; Jensen, O. N.; Gang, D. R.; Thelen, J. J.; Møller, I. M. The Potato Tuber Mitochondrial Proteome. *Plant Physiol.* **2014**, *164*, 637–653, DOI: 10.1104/pp.113.229054.
10. Møller, I. M. What is hot in plant mitochondria? *Physiol. Plant.* **2016**, *157*, 256–263, DOI: 10.1111/ppl.12456.
11. Giegé, P.; Sweetlove, L. J.; Cognat, V.; Leaver, C. J. Coordination of Nuclear and Mitochondrial Genome Expression during Mitochondrial Biogenesis in Arabidopsis. *Plant Cell* **2005**, *17*, 1497–1512, DOI: 10.1105/tpc.104.030254.
12. Howell, K. A.; Cheng, K.; Murcha, M. W.; Jenkin, L. E.; Millar, A. H.; Whelan, J. Oxygen Initiation of Respiration and Mitochondrial Biogenesis in Rice. *J. Biol. Chem.* **2007**, *282*, 15619–15631, DOI: 10.1074/jbc.M609866200.
13. Rurek, M.; Woyda-Ploszczyca, A. M.; Jarmuszkiewicz, W. 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. *BBA-Bioenergetics* **2015**, *1847*, 399–417, DOI: 10.1016/j.bbabio.2015.01.005.
14. Taylor, N. L.; Tan, Y.-F.; Jacoby, R. P.; Millar, A. H. Abiotic environmental stress induced changes in the *Arabidopsis thaliana* chloroplast, mitochondria and peroxisome proteomes. *J. Proteomics* **2009**, *72*, 367–378, DOI: 10.1016/j.jprot.2008.11.006.

15. Ali, G. M.; Komatsu, S. Proteomic Analysis of Rice Leaf Sheath during Drought Stress. *J. Proteome Res.* **2006**, *5*, 396–403, DOI: 10.1021/pr050291g.
16. Aranjuelo, I.; Molero, G.; Erice, G.; Avice, J. C.; Nogués, S. Plant physiology and proteomics reveals the leaf response to drought in alfalfa (*Medicago sativa* L.). *J. Exp. Bot.* **2011**, *62*, 111–123, DOI: 10.1093/jxb/erq249.
17. Kosová, K.; Vítámvás, P.; Urban, M. O.; Klíma, M.; Roy, A.; Prášil, I. T. Biological Networks Underlying Abiotic Stress Tolerance in Temperate Crops--A Proteomic Perspective. *Int. J. Mol. Sci.* **2015**, *16*, 20913–20942, DOI: 10.3390/ijms160920913.
18. Ndimba, B. K.; Chivasa, S.; Simon, W. J.; Slabas, A. R. Identification of *Arabidopsis* salt and osmotic stress responsive proteins using two-dimensional difference gel electrophoresis and mass spectrometry. *Proteomics* **2005**, *5*, 4185–4196, DOI: 10.1002/pmic.200401282.
19. Taylor, N. L.; Heazlewood, J. L.; Day, D. A.; Millar, A. H. Differential Impact of Environmental Stresses on the Pea Mitochondrial Proteome. *Mol. Cell Proteomics* **2005**, *4*, 1122–1133, DOI: 10.1074/mcp.M400210-MCP200.
20. Bies-Ethève, N.; Gaubier-Comella, P.; Debures, A.; Lasserre, E.; Jobet, E.; Raynal, M.; Cooke, R.; Delseny, M. Inventory, evolution and expression profiling diversity of the LEA (late embryogenesis abundant) protein gene family in *Arabidopsis thaliana*. *Plant Mol. Biol.* **2008**, *67*, 107–124, DOI: 10.1007/s11103-008-9304-x.
21. Taylor, N. L.; Day, D. A.; Millar, A. H. Environmental Stress Causes Oxidative Damage to Plant Mitochondria Leading to Inhibition of Glycine Decarboxylase. *J. Biol. Chem.* **2002**, *277*, 42663–42668, DOI: 10.1074/jbc.M204761200.
22. Vanlerberghe, G. C. Alternative Oxidase: a Mitochondrial Respiratory Pathway to Maintain Metabolic and Signaling Homeostasis During Abiotic and Biotic Stress in Plants. *Int. J. Mol. Sci.* **2013**, *14*, 6805–6847, DOI: 10.3390/ijms14046805.
23. Zadražnik, T.; Hollung, K.; Egge-Jacobsen, W.; Meglič, V.; Šuštar-Vozlič, J. Differential proteomic analysis of drought stress response in leaves of common bean (*Phaseolus vulgaris* L.). *J. Proteomics* **2013**, *78*, 254–272, DOI: 10.1016/j.jprot.2012.09.021.
24. Yu, C.; Wang, L.; Xu, S.; Zeng, Y.; He, C.; Chen, C.; Huang, W.; Zhu, Y.; Hu, J. Mitochondrial ORFH79 is Essential for Drought and Salt Tolerance in Rice. *Plant Cell Physiol.* **2015**, *56*, 2248–2258, DOI: 10.1093/pcp/pcv137.
25. Reddy, P. S.; Kavi Kishor, P. B.; Seiler, C.; Kuhlmann, M.; Eschen-Lippold, L.; Lee, J.; Reddy, M. K.; Sreenivasulu, N. Unraveling Regulation of the Small Heat Shock Proteins by the Heat Shock Factor HvHsfB2c in Barley: Its Implications in Drought Stress Response and Seed Development. *PLoS ONE* **2014**, *9*, e89125, DOI: 10.1371/journal.pone.0089125.
26. Wu, G.; Wilen, R. W.; Robertson, A. J.; Gusta, L. V. Isolation, Chromosomal Localization, and Differential Expression of Mitochondrial Manganese Superoxide Dismutase and Chloroplastic Copper/Zinc Superoxide Dismutase Genes in Wheat. *Plant Physiol.* **1999**, *120*, 513–520.
27. Huseynova, I. M.; Aliyeva, D. R.; Aliyev, J. A. Subcellular localization and responses of superoxide dismutase isoforms in local wheat varieties subjected to continuous soil drought. *Plant Physiol. Biochem.* **2014**, *81*, 54–60, DOI: 10.1016/j.plaphy.2014.01.018.
28. Dalal, M.; Tayal, D.; Chinnusamy, V.; Bansal, K. C. Abiotic stress and ABA-inducible Group 4 LEA from *Brassica napus* plays a key role in salt and drought tolerance. *J. Biotechnol.* **2009**, *139*, 137–145, doi:10.1016/j.jbiotec.2008.09.014.

29. Efeoğlu, B.; Ekmekçi, Y.; Çiçek, N. Physiological responses of three maize cultivars to drought stress and recovery. *South African J. Bot.* **2009**, *75*, 34–42, DOI: 10.1016/j.sajb.2008.06.005.
30. Koh, J.; Chen, G.; Yoo, M.-J.; Zhu, N.; Dufresne, D.; Erickson, J. E.; Shao, H.; Chen, S. Comparative Proteomic Analysis of *Brassica napus* in Response to Drought Stress. *J. Proteome Res.* **2015**, *14*, 3068–3081, DOI: 10.1021/pr501323d.
31. Zhang, J.; Mason, A. S.; Wu, J.; Liu, S.; Zhang, X.; Luo, T.; Redden, R.; Batley, J.; Hu, L.; Yan, G. Identification of Putative Candidate Genes for Water Stress Tolerance in Canola (*Brassica napus*). *Front. Plant Sci.* **2015**, *6*, DOI: 10.3389/fpls.2015.01058.
32. Kwon, S.-W.; Kim, M.; Kim, H.; Lee, J. Shotgun Quantitative Proteomic Analysis of Proteins Responding to Drought Stress in *Brassica rapa* L. (Inbred Line "Chiifu"). *Int. J. Genomics* **2016**, *2016*, 4235808, DOI: 10.1155/2016/4235808.
33. Wong, C. E.; Li, Y.; Whitty, B. R.; Díaz-Camino, C.; Akhter, S. R.; Brandle, J. E.; Golding, G. B.; Weretilnyk, E. A.; Moffatt, B. A.; Griffith, M. Expressed sequence tags from the Yukon ecotype of *Thellungiella* reveal that gene expression in response to cold, drought and salinity shows little overlap. *Plant Mol. Biol.* **2005**, *58*, 561–574, DOI: 10.1007/s11103-005-6163-6.
34. Li, Z.; Zhao, L.; Kai, G.; Yu, S.; Cao, Y.; Pang, Y.; Sun, X.; Tang, K. Cloning and expression analysis of a water stress-induced gene from *Brassica oleracea*. *Plant Physiol. Biochem.* **2004**, *42*, 789–794, DOI: 10.1016/j.plaphy.2004.09.001.
35. Mohammadi, P. P.; Moieni, A.; Komatsu, S. Comparative proteome analysis of drought-sensitive and drought-tolerant rapeseed roots and their hybrid F1 line under drought stress. *Amino Acids* **2012**, *43*, 2137–2152, DOI: 10.1007/s00726-012-1299-6.
36. De Mezer, M.; Turska-Taraska, A.; Kaczmarek, Z.; Glowacka, K.; Swarcewicz, B.; Rorat, T. Differential physiological and molecular response of barley genotypes to water deficit. *Plant Physiol. Biochem.* **2014**, *80*, 234–248, DOI: 10.1016/j.plaphy.2014.03.025.
37. Das, A.; Mukhopadhyay, M.; Sarkar, B.; Saha, D.; Mondal, T. K. Influence of drought stress on cellular ultrastructure and antioxidant system in tea cultivars with different drought sensitivities. *J. Environ. Biol.* **2015**, *36*, 875–882.
38. Urban, M. O.; Vašek, J.; Klíma, M.; Krtková, J.; Kosová, K.; Prášil, I. T.; Vítámvás, P. Proteomic and physiological approach reveals drought-induced changes in rapeseeds: Water-saver and water-spender strategy. *J Proteomics* **2017**, *152*, 188–205, DOI: 10.1016/j.jprot.2016.11.004.
39. Vincent, D.; Ergül, A.; Bohlman, M. C.; Tattersall, E. A. R.; Tillett, R. L.; Wheatley, M. D.; Woolsey, R.; Quilici, D. R.; Joets, J.; Schlauch, K.; Schooley, D. A.; Cushman, J. C.; Cramer, G. R. Proteomic analysis reveals differences between *Vitis vinifera* L. cv. Chardonnay and cv. Cabernet Sauvignon and their responses to water deficit and salinity. *J. Exp. Bot.* **2007**, *58*, 1873–1892, DOI: 10.1093/jxb/erm012.
40. Bonhomme, L.; Monclus, R.; Vincent, D.; Carpin, S.; Lomenech, A.-M.; Plomion, C.; Brignolas, F.; Morabito, D. Leaf proteome analysis of eight *Populus xeuramericana* genotypes: Genetic variation in drought response and in water-use efficiency involves photosynthesis-related proteins. *Proteomics* **2009**, *9*, 4121–4142, DOI: 10.1002/pmic.200900047.
41. Ford, K. L.; Cassin, A.; Bacic, A. Quantitative Proteomic Analysis of Wheat Cultivars with Differing Drought Stress Tolerance. *Front. Plant Sci.* **2011**, *2*, 44, DOI: 10.3389/fpls.2011.00044.
42. Ge, P.; Ma, C.; Wang, S.; Gao, L.; Li, X.; Guo, G.; Ma, W.; Yan, Y. Comparative proteomic analysis of grain development in two spring wheat varieties under drought stress. *Anal. Bioanal. Chem.* **2012**, *402*, 1297–1313, DOI: 10.1007/s00216-011-5532-z.

43. Ashoub, A.; Beckhaus, T.; Berberich, T.; Karas, M.; Brüggemann, W. Comparative analysis of barley leaf proteome as affected by drought stress. *Planta* **2013**, *237*, 771–781, DOI: 10.1007/s00425-012-1798-4.
44. Budak, H.; Akpinar, B. A.; Unver, T.; Turktas, M. Proteome changes in wild and modern wheat leaves upon drought stress by two-dimensional electrophoresis and nanoLC-ESI-MS/MS. *Plant Mol. Biol.* **2013**, *83*, 89–103, DOI: 10.1007/s11103-013-0024-5.
45. Kausar, R.; Arshad, M.; Shahzad, A.; Komatsu, S. Proteomics analysis of sensitive and tolerant barley genotypes under drought stress. *Amino Acids* **2013**, *44*, 345–359, DOI: 10.1007/s00726-012-1338-3.
46. Oliveira, T. M.; da Silva, F. R.; Bonatto, D.; Neves, D. M.; Morillon, R.; Maserti, B. E.; Filho, M. A. C.; Costa, M.; Pirovani, C. P.; Gesteira, A. S. Comparative study of the protein profiles of Sunki mandarin and Rangpur lime plants in response to water deficit. *BMC Plant Biol.* **2015**, *15*, 69, DOI: 10.1186/s12870-015-0416-6.
47. Vítámvás, P.; Urban, M. O.; Škodáček, Z.; Kosová, K.; Pitelková, I.; Vítámvás, J.; Renaut, J.; Prášil, I. T. Quantitative analysis of proteome extracted from barley crowns grown under different drought conditions. *Front. Plant Sci.* **2015**, *6*, 479, DOI: 10.3389/fpls.2015.00479.
48. Cheng, L.; Wang, Y.; He, Q.; Li, H.; Zhang, X.; Zhang, F. Comparative proteomics illustrates the complexity of drought resistance mechanisms in two wheat (*Triticum aestivum* L.) cultivars under dehydration and rehydration. *BMC Plant Biol.* **2016**, *16*, 188, DOI: 10.1186/s12870-016-0871-8.
49. Rurek, M. Diverse accumulation of several dehydrin-like proteins in cauliflower (*Brassica oleracea* var. *botrytis*), *Arabidopsis thaliana* and yellow lupin (*Lupinus luteus*) mitochondria under cold and heat stress. *BMC Plant Biol.* **2010**, *10*, 181, DOI: 10.1186/1471-2229-10-181.
50. Krzesiński, W.; Kałużewicz, A.; Frąszczak, B.; Zaworska, A.; Lisiecka, J. Cauliflower's response to drought stress. *Nauka Przyr. Technol.* **2016**, *10*, DOI: 10.17306/J.NPT.2016.4.44.
51. Begcy, K.; Mariano, E. D.; Mattiello, L.; Nunes, A. V.; Mazzafera, P.; Maia, I. G.; Menossi, M. An *Arabidopsis* Mitochondrial Uncoupling Protein Confers Tolerance to Drought and Salt Stress in Transgenic Tobacco Plants. *PLoS ONE* **2011**, *6*, e23776, DOI: 10.1371/journal.pone.0023776.
52. Wu, H.; Wu, X.; Li, Z.; Duan, L.; Zhang, M. Physiological Evaluation of Drought Stress Tolerance and Recovery in Cauliflower (*Brassica oleracea* L.) Seedlings Treated with Methyl Jasmonate and Coronatine. *J. Plant Growth Regul.* **2012**, *31*, 113–123, DOI: 10.1007/s00344-011-9224-x.
53. Voss, I.; Sunil, B.; Scheibe, R.; Raghavendra, A. S. Emerging concept for the role of photorespiration as an important part of abiotic stress response. *Plant Biol. (Stuttg.)* **2013**, *15*, 713–722, DOI: 10.1111/j.1438-8677.2012.00710.x.
54. Kim, J.; van Iersel, M. W. Slowly developing drought stress increases photosynthetic acclimation of *Catharanthus roseus*. *Physiol. Plant.* **2011**, *143*, 166–177, DOI: 10.1111/j.1399-3054.2011.01493.x.
55. Haupt-Herting, S.; Klug, K.; Fock, H. P. A New Approach to Measure Gross CO_2 Fluxes in Leaves. Gross CO_2 Assimilation, Photorespiration, and Mitochondrial Respiration in the Light in Tomato under Drought Stress. *Plant Physiol.* **2001**, *126*, 388–396.
56. Campos, H.; Trejo, C.; Peña-Valdivia, C. B.; García-Nava, R.; Conde-Martínez, F. V.; Cruz-Ortega, M. R. Stomatal and non-stomatal limitations of bell pepper (*Capsicum annuum* L.) plants under water stress and re-watering: Delayed restoration of photosynthesis during recovery. *Environ. Exp. Bot.* **2014**, *98*, 56–64, DOI: 10.1016/j.envexpbot.2013.10.015.
57. Sperlich, D.; Barbeta, A.; Ogaya, R.; Sabaté, S.; Peñuelas, J. Balance between carbon gain and loss under long-term drought: impacts on foliar respiration and photosynthesis in *Quercus ilex* L. *J. Exp. Bot.* **2016**, *67*, 821–833, DOI: 10.1093/jxb/erv492.

58. Begcy, K.; Mariano, E. D.; Gentile, A.; Lembke, C. G.; Zingaretti, S. M.; Souza, G. M.; Menossi, M. A Novel Stress-Induced Sugarcane Gene Confers Tolerance to Drought, Salt and Oxidative Stress in Transgenic Tobacco Plants. *PLoS ONE* **2012**, *7*, e44697, DOI: 10.1371/journal.pone.0044697.
59. Vassileva, V.; Simova-Stoilova, L.; Demirevska, K.; Feller, U. Variety-specific response of wheat (*Triticum aestivum* L.) leaf mitochondria to drought stress. *J. Plant Res.* **2009**, *122*, 445–454, DOI: 10.1007/s10265-009-0225-9.
60. Chastain, D. R.; Snider, J. L.; Collins, G. D.; Perry, C. D.; Whitaker, J.; Byrd, S. A. Water deficit in field-grown *Gossypium hirsutum* primarily limits net photosynthesis by decreasing stomatal conductance, increasing photorespiration, and increasing the ratio of dark respiration to gross photosynthesis. *J. Plant Physiol.* **2014**, *171*, 1576–1585, DOI: 10.1016/j.jplph.2014.07.014.
61. Liu, C.; Wang, Y.; Pan, K.; Wang, Q.; Liang, J.; Jin, Y.; Tariq, A. The Synergistic Responses of Different Photoprotective Pathways in Dwarf Bamboo (*Fargesia rufa*) to Drought and Subsequent Rewatering. *Front. Plant Sci.* **2017**, *8*, 489, DOI: 10.3389/fpls.2017.00489.
62. Abogadallah, G. M. Differential regulation of photorespiratory gene expression by moderate and severe salt and drought stress in relation to oxidative stress. *Plant Sci.* **2011**, *180*, 540–547, DOI: 10.1016/j.plantsci.2010.12.004.
63. Lima Neto, M. C.; Cerqueira, J. V. A.; Cunha, J. R. da; Ribeiro, R. V.; Silveira, J. A. G. Cyclic electron flow, NPQ and photorespiration are crucial for the establishment of young plants of *Ricinus communis* and *Jatropha curcas* exposed to drought. *Plant Biol. (Stuttg.)* **2017**, *19*, 650-659, DOI: 10.1111/plb.12573.
64. Zhou, S.; Li, M.; Guan, Q.; Liu, F.; Zhang, S.; Chen, W.; Yin, L.; Qin, Y.; Ma, F. Physiological and proteome analysis suggest critical roles for the photosynthetic system for high water-use efficiency under drought stress in *Malus*. *Plant Sci.* **2015**, *236*, 44–60, DOI: 10.1016/j.plantsci.2015.03.017.
65. Chang, S.; Yang, T.; Du, T.; Huang, Y.; Chen, J.; Yan, J.; He, J.; Guan, R. Mitochondrial genome sequencing helps show the evolutionary mechanism of mitochondrial genome formation in *Brassica*. *BMC Genomics* **2011**, *12*, 497, DOI: 10.1186/1471-2164-12-497.
66. Katari, M. S.; Nowicki, S. D.; Aceituno, F. F.; Nero, D.; Kelfer, J.; Thompson, L. P.; Cabello, J. M.; Davidson, R. S.; Goldberg, A. P.; Shasha, D. E.; Coruzzi, G. M.; Gutiérrez, R. A. VirtualPlant: a software platform to support systems biology research. *Plant Physiol.* **2010**, *152*, 500–515, DOI: 10.1104/pp.109.147025.
67. Caruso, G.; Cavaliere, C.; Foglia, P.; Gubbiotti, R.; Samperi, R.; Laganà, A. Analysis of drought responsive proteins in wheat (*Triticum durum*) by 2D-PAGE and MALDI-TOF mass spectrometry. *Plant Sci.* **2009**, *177*, 570–576, DOI: 10.1016/j.plantsci.2009.08.007.
68. Chen, L.; Ren, F.; Zhong, H.; Feng, Y.; Jiang, W.; Li, X. Identification and expression analysis of genes in response to high-salinity and drought stresses in *Brassica napus*. *Acta Biochim. Biophys. Sin. (Shanghai)* **2010**, *42*, 154–164, DOI: 10.1093/abbs/gmp113.
69. He, C. Y.; Zhang, G. Y.; Zhang, J. G.; Duan, A. G.; Luo, H. M. Physiological, biochemical, and proteome profiling reveals key pathways underlying the drought stress responses of *Hippophae rhamnoides*. *Proteomics* **2016**, *16*, 2688–2697, DOI: 10.1002/pmic.201600160.
70. Bernard, D. G.; Cheng, Y.; Zhao, Y.; Balk, J. An Allelic Mutant Series Of *ATM3* Reveals Its Key Role in the Biogenesis of Cytosolic Iron-Sulfur Proteins in Arabidopsis. *Plant Physiol.* **2009**, *151*, 590–602, DOI: 10.1104/pp.109.143651.
71. Landi, S.; Hausman, J.-F.; Guerriero, G.; Esposito, S. *Poaceae* vs. Abiotic Stress: Focus on Drought and Salt Stress, Recent Insights and Perspectives. *Front. Plant Sci.* **2017**, *8*, 1214, DOI: 10.3389/fpls.2017.01214.

72. Hamilton, C. A.; Good, A. G.; Taylor, G. J. Induction Of Vacuolar ATPase and Mitochondrial ATP Synthase By Aluminum in an Aluminum-Resistant Cultivar of Wheat. *Plant Physiol.* **2001**, *125*, 2068–2077.

73. Moghadam, A. A.; Taghavi, S. M.; Niazi, A.; Djavaheri, M.; Ebrahimie, E. Isolation and *in silico* functional analysis of *MtATP6*, a 6-kDa subunit of mitochondrial F_1F_0-ATP synthase, in response to abiotic stress. *Genet. Mol. Res.* **2012**, *11*, 3547–3567, DOI: 10.4238/2012.October.4.3.

74. Li, C.-L.; Wang, M.; Ma, X.-Y.; Zhang, W. NRGA1, a Putative Mitochondrial Pyruvate Carrier, Mediates ABA Regulation of Guard Cell Ion Channels and Drought Stress Responses in *Arabidopsis*. *Mol. Plant* **2014**, *7*, 1508–1521, DOI: 10.1093/mp/ssu061.

75. Wang, M.; Ma, X.; Shen, J.; Li, C.; Zhang, W. The ongoing story: the mitochondria pyruvate carrier 1 in plant stress response in Arabidopsis. *Plant Signal. Behav.* **2014**, *9*, e973810, DOI: 10.4161/15592324.2014.973810.

76. Ambard-Bretteville, F.; Sorin, C.; Rébeillé, F.; Hourton-Cabassa, C.; Colas des Francs-Small, C. Repression of formate dehydrogenase in *Solanum tuberosum* increases steady-state levels of formate and accelerates the accumulation of proline in response to osmotic stress. *Plant Mol. Biol.* **2003**, *52*, 1153–1168, DOI: 10.1023/B:PLAN.0000.

77. Taylor, N. L.; Rudhe, C.; Hulett, J. M.; Lithgow, T.; Glaser, E.; Day, D. A.; Millar, A. H.; Whelan, J. Environmental stresses inhibit and stimulate different protein import pathways in plant mitochondria. *FEBS Lett.* **2003**, *547*, 125–130, DOI: 10.1016/S0014-5793(03)00691-4.

78. Obata, T.; Matthes, A.; Koszior, S.; Lehmann, M.; Araújo, W. L.; Bock, R.; Sweetlove, L. J.; Fernie, A. R. Alteration of mitochondrial protein complexes in relation to metabolic regulation under short-term oxidative stress in Arabidopsis seedlings. *Phytochemistry* **2011**, *72*, 1081–1091, DOI: 10.1016/j.phytochem.2010.11.003.

79. Riccardi, F.; Gazeau, P.; de Vienne, D.; Zivy, M. Protein Changes in Response to Progressive Water Deficit in Maize: Quantitative Variation and Polypeptide Identification. *Plant Physiol.* **1998**, *117*, 1253–1263, DOI: 10.1104/pp.117.4.1253.

80. Bartoli, C. G.; Gómez, F.; Martínez, D. E.; Guiamet, J. J. Mitochondria are the main target for oxidative damage in leaves of wheat (*Triticum aestivum* L.). *J. Exp. Bot.* **2004**, *55*, 1663–1669, DOI: 10.1093/jxb/erh199.

81. Cruz de Carvalho, M. H. Drought stress and reactive oxygen species: Production, scavenging and signaling. *Plant Signal. Behav.* **2008**, *3*, 156–165.

82. Kaouthar, F.; Ameny, F.-K.; Yosra, K.; Walid, S.; Ali, G.; Faiçal, B. Responses of transgenic *Arabidopsis* plants and recombinant yeast cells expressing a novel durum wheat manganese superoxide dismutase *TdMnSOD* to various abiotic stresses. *J. Plant Physiol.* **2016**, *198*, 56–68, DOI: 10.1016/j.jplph.2016.03.019.

83. Pastore, D.; Trono, D.; Laus, M. N.; Di Fonzo, N.; Flagella, Z. Possible plant mitochondria involvement in cell adaptation to drought stress: A case study: durum wheat mitochondria. *J. Exp. Bot.* **2006**, *58*, 195–210, DOI: 10.1093/jxb/erl273.

84. Dahal, K.; Wang, J.; Martyn, G. D.; Rahimy, F.; Vanlerberghe, G. C. Mitochondrial Alternative Oxidase Maintains Respiration and Preserves Photosynthetic Capacity during Moderate Drought in *Nicotiana tabacum*. *Plant Physiol.* **2014**, *166*, 1560–1574, DOI: 10.1104/pp.114.247866.

85. Galle, A.; Florez-Sarasa, I.; Thameur, A.; de Paepe, R.; Flexas, J.; Ribas-Carbo, M. Effects of drought stress and subsequent rewatering on photosynthetic and respiratory pathways in *Nicotiana sylvestris* wild type and the mitochondrial complex I-deficient CMSII mutant. *J. Exp. Bot.* **2010**, *61*, 765–775, DOI: 10.1093/jxb/erp344.

86. Hincha, D. K.; Thalhammer, A. LEA proteins: IDPs with versatile functions in cellular dehydration tolerance. *Biochem. Soc. Trans.* **2012**, *40*, 1000–1003, DOI: 10.1042/BST20120109.
87. Borovskii, G. B.; Stupnikova, I. V.; Antipina, A. I.; Vladimirova, S. V.; Voinikov, V. K. Accumulation of dehydrin-like proteins in the mitochondria of cereals in response to cold, freezing, drought and ABA treatment. *BMC Plant Biol.* **2002**, *2*, 5, DOI: 10.1186/1471-2229-2-5.
88. Grelet, J.; Benamar, A.; Teyssier, E.; Avelange-Macherel, M.-H.; Grunwald, D.; Macherel, D. Identification in Pea Seed Mitochondria of a Late-Embryogenesis Abundant Protein Able to Protect Enzymes from Drying. *Plant Physiol.* **2005**, *137*, 157–167, DOI: 10.1104/pp.104.052480.
89. Boswell, L. C.; Moore, D. S.; Hand, S. C. Quantification of cellular protein expression and molecular features of group 3 LEA proteins from embryos of *Artemia franciscana*. *Cell Stress Chaperon.* **2014**, *19*, 329–341, DOI: 10.1007/s12192-013-0458-3.
90. Boswell, L. C.; Hand, S. C. Intracellular localization of group 3 LEA proteins in embryos of *Artemia franciscana*. *Tissue Cell* **2014**, *46*, 514–519, DOI: 10.1016/j.tice.2014.09.004.
91. Close, T. J. Dehydrins: A commonalty in the response of plants to dehydration and low temperature. *Physiol. Plant.* **1997**, *100*, 291–296, DOI: 10.1111/j.1399-3054.1997.tb04785.x.
92. Hanin, M.; Brini, F.; Ebel, C.; Toda, Y.; Takeda, S.; Masmoudi, K. Plant dehydrins and stress tolerance: versatile proteins for complex mechanisms. *Plant Signal. Behav.* **2011**, *6*, 1503–1509, DOI: 10.4161/psb.6.10.17088.
93. Clifton, R.; Millar, A. H.; Whelan, J. Alternative oxidases in Arabidopsis: A comparative analysis of differential expression in the gene family provides new insights into function of non-phosphorylating bypasses. *Biochim. Biophys. Acta* **2006**, *1757*, 730–741, DOI: 10.1016/j.bbabio.2006.03.009.
94. Giraud, E.; Ho, L. H. M.; Clifton, R.; Carroll, A.; Estavillo, G.; Tan, Y.-F.; Howell, K. A.; Ivanova, A.; Pogson, B. J.; Millar, A. H.; Whelan, J. The Absence of ALTERNATIVE OXIDASE1a in Arabidopsis Results in Acute Sensitivity to Combined Light and Drought Stress. *Plant Physiol.* **2008**, *147*, 595–610, DOI: 10.1104/pp.107.115121.
95. Wang, Y.; Wang, X.; Deng, W.; Fan, X.; Liu, T.-T.; He, G.; Chen, R.; Terzaghi, W.; Zhu, D.; Deng, X. W. Genomic Features and Regulatory Roles of Intermediate-Sized Non-Coding RNAs in *Arabidopsis*. *Mol. Plant* **2014**, *7*, 514–527, DOI: 10.1093/mp/sst177.
96. Rurek, M. Participation of non-coding RNAs in plant organelle biogenesis. *Acta Biochim. Pol*. **2016**, *63*, 653–663, DOI: 10.18388/abp.2016_1346.
97. Das, S.; Ferlito, M.; Kent, O. A.; Fox-Talbot, K.; Wang, R.; Liu, D.; Raghavachari, N.; Yang, Y.; Wheelan, S. J.; Murphy, E.; Steenbergen, C. Nuclear miRNA Regulates the Mitochondrial Genome in the Heart. *Circ. Res.* **2012**, *110*, 1596–1603, DOI: 10.1161/CIRCRESAHA.112.267732.
98. Leung, A. K. L. The Whereabouts of microRNA Actions: Cytoplasm and Beyond. *Trends Cell Biol*. **2015**, *25*, 601–610, DOI: 10.1016/j.tcb.2015.07.005.
99. Ro, S.; Ma, H.-Y.; Park, C.; Ortogero, N.; Song, R.; Hennig, G. W.; Zheng, H.; Lin, Y.-M.; Moro, L.; Hsieh, J.-T.; Yan, W. The mitochondrial genome encodes abundant small noncoding RNAs. *Cell Res.* 2013, 23, 759–774, DOI: 10.1038/cr.2013.37.
100. Dai, X.; Zhao, P. X. psRNATarget: a plant small RNA target analysis server. *Nucl. Acids Res*. **2011**, *39*, W155–W159, DOI: 10.1093/nar/gkr319.
101. Kiyosue, T.; Yamaguchi-Shinozaki, K.; Shinozaki, K. Characterization of two cDNAs (ERD10 and ERD14) corresponding to genes that respond rapidly to dehydration stress in Arabidopsis thaliana. *Plant Cell Physiol.* **1994**, *35*, 225–231.

102. Nylander, M.; Svensson, J.; Palva, E. T.; Welin, B. V. Stress-induced accumulation and tissue-specific localization of dehydrins in Arabidopsis thaliana. *Plant Mol. Biol.* **2001**, *45*, 263–279, DOI: 10.1023/A:100646912.
103. Laisk, A. *Kinetics of Photosynthesis and Photorespiration in C3-Plants*; Nauka: Moscow, 1977.
104. Pawlowski, T.; Rurek, M.; Janicka, S.; Raczynska, K. D.; Augustyniak, H. Preliminary analysis of the cauliflower mitochondrial proteome. *Acta Physiol. Plant.* **2005**, *27*, 275–281, DOI: 10.1007/s11738-005-0003-9.
105. Staszak, A.; Pawłowski, T. Proteomic Analysis of Embryogenesis and the Acquisition of Seed Dormancy in Norway Maple (*Acer platanoides* L.). *Int. J. Mol. Sci.* **2014**, *15*, 10868–10891, DOI: 10.3390/ijms150610868.
106. Ramagli, L. S.; Rodriguez, L. V. Quantitation of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. *Electrophoresis* **1985**, *6*, 559–563, DOI: 10.1002/elps.1150061109.
107. Neuhoff, V.; Arold, N.; Taube, D.; Ehrhardt, W. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* **1988**, *9*, 255–262, DOI: 10.1002/elps.1150090603.
108. Schmidt, U. G.; Endler, A.; Schelbert, S.; Brunner, A.; Schnell, M.; Neuhaus, H. E.; Marty-Mazars, D.; Marty, F.; Baginsky, S.; Martinoia, E. Novel Tonoplast Transporters Identified Using a Proteomic Approach with Vacuoles Isolated from Cauliflower Buds. *Plant Physiol.* **2007**, *145*, 216–229, DOI: 10.1104/pp.107.096917.
109. Nantes, I. L.; Fagian, M. M.; Catisti, R.; Arruda, P.; Maia, I. G.; Vercesi, A. E. Low temperature and aging-promoted expression of PUMP in potato tuber mitochondria. *FEBS Lett.* **1999**, *457*, 103–106, DOI: 10.1016/S0014-5793(99)01017-0.
110. Ježek, P.; Žáčková, M.; Košařová, J.; Rodrigues, E. T.; Madeira, V. M.; Vicente, J. A. Occurrence of plant-uncoupling mitochondrial protein (PUMP) in diverse organs and tissues of several plants. *J. Bioenerg. Biomembr.* **2000**, *32*, 549–561.
111. Close, T. J.; Fenton, R. D.; Moonan, F. A view of plant dehydrins using antibodies specific to the carboxy terminal peptide. *Plant Mol. Biol.* **1993**, *23*, 279–286.
112. Rorat, T.; Szabala, B. M.; Grygorowicz, W. J.; Wojtowicz, B.; Yin, Z.; Rey, P. Expression of SK3-type dehydrin in transporting organs is associated with cold acclimation in *Solanum* species. *Planta* **2006**, *224*, 205–221, DOI: 10.1007/s00425-005-0200-1.
113. Elthon, T. E.; Nickels, R. L.; McIntosh, L. Monoclonal Antibodies to the Alternative Oxidase of Higher Plant Mitochondria. *Plant Physiol.* **1989**, *89*, 1311–1317.
114. Luethy, M. H.; Horak, A.; Elthon, T. E. Monoclonal Antibodies to the α- and β-Subunits of the Plant Mitochondrial F1-ATPase. *Plant Physiol.* **1993**, *101*, 931–937.
115. Havelund, J. F.; Thelen, J. J.; Møller, I. M. Biochemistry, proteomics, and phosphoproteomics of plant mitochondria from non-photosynthetic cells. *Front. Plant Sci.* **2013**, *4*, 51, DOI: 10.3389/fpls.2013.00051.
116. Nakaminami, K.; Matsui, A.; Nakagami, H.; Minami, A.; Nomura, Y.; Tanaka, M.; Morosawa, T.; Ishida, J.; Takahashi, S.; Uemura, M.; Shirasu, K.; Seki, M. Analysis of Differential Expression Patterns of mRNA and Protein During Cold-Acclimation and De-Acclimation in *Arabidopsis*. *Mol. Cell. Proteomics* **2014**, *13*, 3602–3611, DOI: 10.1074/mcp.M114.039081.