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Ph.D. Thesis



**The physiological and proteomic characterisation of winter oilseed
rape upon abiotic stress**
**Fyziologická a proteomická charakterizace vlivu abiotických stresů
na ozimou formu brukve řepky olejky**

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Ph.D. thesis author declaration

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Prague, 15th February 2017

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In the name of other co-authors, I agree with the fact that selected articles are used in this Ph.D. thesis and for thesis defence.

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Abstract

In some years, the agricultural production of oilseed rape, an important crop in the Czech Republic, is - besides biotic stress - facing the problem of damage caused by frost or drought. Together with special attention paid to proteins revealing responses between crop genotypes with differential abiotic stress tolerance levels we reviewed possible applications of proteomic results in crop breeding programs aimed at an improvement of crop stress tolerance (paper 1).

For first original result, cold temperature was imposed upon non-vernalized plants in the stage of leaf rosette. The article (paper 2) shows a significant correlation between frost tolerance (FT), dehydrin (DHN) accumulation, and photosynthetic acclimation in five cultivars (cvs). Newly, the specific DHN D97 was shown to accumulate and other DHNs were shown to have qualitative differences in accumulation. These results imply that proper FT assessment is based on rapid photosynthetic acclimation together with higher accumulation of protective compounds. Drought stress (paper 3) was imposed in the water-demanding stem prolongation phase before flowering, because late-spring drought before and during flowering decreases the yield and seed quality significantly. This paper newly describes two water-uptake strategies in detail in four cvs. Surprisingly - in this developmental stage - both groups contain drought adaptable cvs. These data suggest that relevant “drought adaptation rate” of rapeseeds in stem-prolongation phase can be predicted only if gasometric, biochemical and proteomic data (based on 2-D DIGE) are taken and understood together. Osmotic stress (PEG-influenced media; paper 4) was imposed *in vitro* in microspore-derived embryos (MDE) of two cvs to estimate the power and limitations of MDE technique for early cvs selection. The differentially accumulated proteins were detected by 2-D DIGE and the expression profiles of several genes were evaluated by qRT-PCR. Cv Cadeli (D) showed tolerance strategy thanks to the accumulation of biomass. In D proteins of energy metabolism, redox homeostasis, protein targeting, and signalling functional groups were accumulated. On the contrary, sensitive cv Viking (V) shows a high need for ATP and nutrients with a significant number of stress-related proteins and cell structure changes.

Based on obtained results we have proposed some traits (biomass accumulation, WUE, TE, water-saving strategy, photosynthetic acclimation upon cold and during drought, etc.) and techniques (DHN accumulation) for oilseed rape phenotyping against frost and drought stress. In addition, some candidate proteins and genes were selected to have the ability to reveal adaptability of cvs to abiotic stress. These results can be used in oilseed rape breeding programmes focused on adaptable component cvs or pre-selection of new breeding materials.

Key words: *Brassica napus*, Proteomics, Dehydrins, Drought, Frost tolerance, Water-savers, Water-spenders, Microspore cultures, Adaptability

Abstract in Czech language

V některých letech je zemědělská produkce brukve řepky olejky – významné plodiny České republiky – ohrožena (kromě biotických stresů) mrazem či suchem. V prvním přehledovém článku jsme proto věnovali pozornost proteinům, které byly nalezeny ve studiích porovnávajících genotypy s různou mírou tolerance k abiotickým stresům. V tomto článku jsme se také zaměřili na aplikaci výsledků proteomických studií ve šlechtitelských programech a při zvyšování odolnosti plodin ke stresům obecně.

Vlastní výsledky jsme pozorovali u chladově aklimovaných, nejarovizovaných rostlin řepky ve fázi listové růžice. V druhém článku jsme prokázali signifikantní vztah mezi mrazuvzdorností, akumulací dehydrinů a aklimací fotosyntetického aparátu u pěti odrůd řepky. Při porovnání s dřívějšími výsledky jsme pozorovali nejen specifickou akumulaci dehydrinu D97, ale i kvalitativní změny dalších dehydrinů. Tyto výsledky naznačují, že úroveň získané mrazuvzdornosti je závislá nejen na aklimaci fotosyntézy, ale i na vyšší akumulaci ochranných složek. Stres sucha (článek č. 3) byl navozen ve fázi prodlužování stonku před kvetením, což je fáze, ve které rostlina musí přijmout větší množství vody. Pozdní jarní přísušky před nebo během kvetení výrazně snižují výnos a kvalitu produkce. Tento článek poprvé detailně popsal dvě strategie hospodaření s vodou u čtyř odrůd řepky. Překvapením bylo zjištění, že obě strategie v této fázi vývoje mohou obsahovat adaptabilní (adaptované) odrůdy na suchu. Tyto výsledky naznačují, že relevantní informace o míře adaptability k suchu v době prodlužování stonku mohou být získány pouze v případě, pokud sledujeme a společně vyhodnotíme výsledky gazometrické, biochemické a proteomické. Osmotický stres (v PEG-em aktivizovaném médiu, článek č. 4) byl aplikován v in vitro kultuře mikrosporových embryí u dvou odrůd řepky proto, abychom vyhodnotili možnosti a limity této metody pro časnou selekci šlechtitelských materiálů. Diferenčně akumulované proteiny byly detekovány pomocí 2-D DIGE a u vybraných genů byly pomocí qRT-PCR stanoveny jejich expresní profily. Odrůda Cadeli (D) vykazovala znaky tolerance díky akumulaci sušiny a akumulovaly se v ní proteiny energetického metabolismu, REDOX, targetingu a signalizace. Na druhou stranu, citlivá odrůda Viking (V) více akumulovala proteiny spojené s přeměnami ATP a využíváním živin, spolu s proteiny odpovídajícími na stres a ovlivňujícími buněčnou strukturu.

Na základě těchto výsledků doporučujeme některé znaky (akumulace biomasy, WUE, TE, fotosyntetická aklimace v chladu a během sucha) a techniky (akumulace DHN), které umožní fenotypování řepky olejky k odolnosti na mráz a suchu. Navíc jsme vybrali kandidátní proteiny a geny, které mohou selektovat adaptabilní genotypy k abiotickým stresům. Tyto výsledky mohou být použity ve šlechtitelských programech, zaměřených na výběr adaptabilních komponentních odrůd či pre-selekci nových šlechtitelských materiálů.

Klíčová slova: *Brassica napus*, proteomika, dehydriny, sucho, mrazuvzdornost, genotypy šetřící vodou, genotypy vodou plýtvající, mikrosporové kultury, adaptabilita

Abbreviations

1D SDS-PAGE - one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis
ABA - abscisic acid
AFLP - amplified fragment length polymorphism
BEN - cultivar Benefit
C or CAL - Californium
CA - cold acclimation
Car - carotenoids
CBF - C-REPEAT BINDING FACTOR (gene)
Chl - chlorophyll
 C_i/C_a - ratio of leaf intercellular $[CO_2]$ to ambient air $[CO_2]$
Cor - Cold-regulated (gene)
COR – Cortes
cv(s) - cultivar(s)
D or CAD - Cadeli
DAS - day(s) after beginning of treatment
DAP - differentially accumulated proteins
DH - doubled haploids
DHN - dehydrin
DRE - DROUGHT-RESPONSIVE ELEMENT (promoter regulatory element)
DREB - DROUGHT-RESPONSIVE ELEMENT BINDING FACTOR (gene)
DIGE - fluorescent differential gel electrophoresis
DTT - dithiothreitol
DW - dry weight
E - leaf transpiration rate
ET - evapotranspiration change
EUW - effective use of water
FLC - FLOWERING LOCUS C (gene in *Arabidopsis thaliana*)
Fr - Frost resistance locus
FT - frost tolerance
FTSW - fraction of transpirable soil water
FW - fresh weight
GxExM – genotype by environment by management interactions
GO - Gene Ontology
GS or gs - stomatal conductance
GSTF2- glutathione-S-transferase F2
IUPs - intrinsically unstructured proteins
IPG - immobilised pH gradient
LD - long-day (photoperiod)
Lea - Late embryogenesis abundant (gene)
LT - low temperature (cold - temperatures above freezing point in the range of cca +12 - +10 °C - 0 °C; frost - temperatures below freezing point)
LT50 - lethal temperature when 50 % of the sample die (in frost tests)
LTI30 - dehydring family protein
MAS - marker-assisted selection
MALDI-TOF, Matrix-assisted laser desorption/ionization - Time of Flight
mb Diff - differential H_2O concentration in mbar
mb Ref - reference H_2O concentration in mbar
MDE - microspore-derived embryos
MD - microspore-derived
N or NAV - Navajo

NET - normalized evapotranspiration
NMMDS - non-metric multidimensional scaling
OP - osmotic potential
PCA - principal component analysis
PEG - polyethylene-glycol
Pn - net CO₂ assimilation (net photosynthesis)
Ppd - photoperiod (locus)
PPI - protein-protein interaction network
PRO - proline
qPCR - quantitative polymerase chain reaction (real-time PCR)
QTL - quantitative trait loci
RAPD - random amplified polymorphic DNA
RFLP - restriction fragment length polymorphism
RWC - relative water content
ROS - reactive oxygen species
SD - short-day (photoperiod)
SDS - sodium dodecyl sulfate
SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis
SI - supplementary information
SSR - simple sequence repeat
STS - sequence-tagged site
SWC - soil water content
TCA - trichloroacetic acid
TE - transpiration efficiency
TF - transcription factor
TPI - triosephosphate isomerase
TTSW - total transpirable soil water
UV - ultra-violet light ($\lambda = 190 - 380 \text{ nm}$)
ÚKZÚZ - Central Institute for Supervising and Testing in Agriculture
UPGMA - unweighted pair group method with arithmetic mean
V - Viking
WUE - water-use efficiency (Pn/E)
WUEi - intrinsic water-use efficiency (Pn/GS)

Prologue

Crop production has to double by 2050 to meet the predicted demands of the global population. On the production side (besides processing, transport etc.), the main constraint to fulfil this goal is believed to be losses of yield or quality due to abiotic or biotic stress. The rapid, cheap and high-throughput methods to select sufficiently adaptable genotypes (negative selection of unfit new breed materials), especially in smaller breeding companies is needed. The production of oilseed rape is facing the problem of damage caused by slow adaptation to frost and/or insufficient oil quality upon drought period.

The first part of the dissertation is a broader introduction (chapter 1) into each individual stress (based on a review – paper 1). Besides of general introduction, the introduction includes the knowledge of stresses with a special focus on rapeseed. In addition, a thesis design and hypotheses are mentioned in this part. In a discussion (chapter 4), two papers (paper 2 and 3), and manuscript (paper 4) are discussed in detail, according to a similar scheme: scientific novelty, synthesis, research limitations, and future implications of results.

This thesis is focused on winter oilseed rapes responses to abiotic stress. Different stages of development (microspores, plants just undergoing vernalization period and vernalized plants before flowering in stem prolongation phase) and three main stresses (osmotic, cold, drought) were chosen to somehow cover the lag in recent knowledge and research. The vision for a future is to combine different approaches (physiology-based understanding, omics-techniques, QTL mapping, epigenetic breeding, and other tools) to improve the abiotic stress tolerance of crops in field conditions, particularly to drought and heat.

All data in the thesis are based on understanding the physiological and biochemical parameters, accompanied by the proteomic-based approach. For FT evaluation, both, the “direct” methods (conductivity measurement of leaf samples ion leakage after stress, frost tests) and “indirect” methods (physiological, biochemical, molecular – dehydrin accumulation - DHN) were used. These differentially accumulated proteins were selected based on 2-D difference in gel electrophoresis (2-D DIGE), identified by MALDI-TOF/TOF and processed by bioinformatics. Also, some optimizing visualization procedures for dehydrins were used, because we discovered almost 100 x lower accumulation of DHN in rapeseed in contrast to wheat and barley. All results were put into a broad picture to understand the problem and recommend some advice for further oilseed rape breeding.

At the world level, 10 percent of total calories available for human consumption come from oil-bearing crops. Those percentages are 2-3 times higher than those observed 40 years

ago (FAOSTAT). Rapeseed (*Brassica napus* L.) is the most important oil-bearing crop in Czech Republic (CZ) and third largest oil source in the world. For example, in 2016 85 % of oil-bearing fields in CZ were sown by oilseed rape.

Brassica napus is a “golden plant” for farmers not only because this is an early and valuable “cash crop” (2015 price was 9672,-- CZK per ton) but also because of a unique oil content and health influence comparable to olive oil. Thanks to many scientists in the past (prof. Fábry, prof. Vašák, etc.) the Czech agronomical and technological systems used for oilseed rapeseed production are one of the most sophisticated in the world. Winter oilseed rape in Czech Republic was cultivated in 2016 on 392 990 ha with average yield of 3,57 t/ha which leads Czech agriculture to the third position in yield in the EU. No surprise that there is an enormous interest in oilseed rapeseed although the environment and abiotic/biotic stresses can damage the whole harvest. Even though the ÚKZÚZ (Central Institute for Supervising and Testing in Agriculture) recommends every year some older and new cultivars on the basis of overwintering ability, yields, disease resistance, etc., there is no simple technique generally used by breeders in negative selection of new breeds of oilseed rapeseed. Only the data from fields are used for this selection – however, these data are influenced by specific year climate conditions and cannot be used without normalization.

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During my Ph.D study, I also investigated wheat, barley, sorghum, and maize. However, rapeseed becomes the main part of my study and only selected data are presented in this thesis. I also started a fruitful collaboration with prof. Van Volkenburgh (University of Washington, USA), Dr. Dana Holá (Charles University, CZ), the team of prof. Čurn (University of South Bohemia in České Budějovice, CZ), Dr. Jenny Renaut (Luxembourg

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In the prologue, I think it is a good idea to introduce some terms used in the thesis. This semantics is necessary especially if some thesis results or opinions will be compared with other theses or research:

Adaptation – Trait with a current functional role in the life of an organism that is maintained and evolved by means of natural selection. Adaptation refers to both the current state of being adapted and to the dynamic evolutionary process that leads to the adaptation. Adaptations enhance the fitness and survival of individuals. All adaptations help organisms survive in their ecological niches. These adaptive traits may be molecular, structural or behavioural.

Acclimation – Is a part of adaptive traits in which an individual organism adjusts to a change in its environment (such as a change in altitude, temperature, humidity, photoperiod, or pH), allowing it to maintain performance across a range of environmental conditions.

Plasticity - Highly plastic cultivars should possess regulated stress-response ability, without influencing crop performance when stress is absent [1, 2]. *Plasticity* means the phenotypic (genotypic + morphological + developmental) manifestation of the ability to respond to the environment. In this point of view, this is more “elasticity” (reversible acclimation) than “plasticity” (irreversible acclimation). The developmental norm of reaction for any given trait is essential to the correction of plasticity as it affords a kind of biological insurance or resilience to varying environments.

Stress-adaptable – Adaptable cultivars are believed here to be as cultivars able to respond to stress by various acclimation strategies including phenotypic plasticity [3], avoidance and tolerance, characterized by significantly visible or measurable traits (transpiration, transpiration rate, growth rate, yield, etc.). These traits should be different (higher or lower) from traits of sensitive varieties upon stress.

Stress-sensitive – These cultivars are believed to respond to stress in a different manner and with lower efficiency in contrast to the tolerant ones. The “sensitivity” can be measured and quantified by a combination of different approaches (physiological, biochemical, molecular, etc.). The main differences between tolerant vs. sensitive can be measured e.g. as a growth rate and yield depression upon stress. From this point of view, we can postulate sensitive varieties as non-plastic cultivars.

Water-saver – Cultivars saving field/pot water by increasing of TE (biomass produced/water transpired), especially useful in conditions of terminal drought or when limited water sources are available in the soil profile. Water-savers (controls and/or treated) usually produce lower biomass than water-spenders.

Water-spender – Spending water means a feature (genetic, morphological, biochemical) that allows a plant to transpire. In the first phase when stress can occur this group can grow more rapid than savers. Under more severe stress (if there is no precipitation probability, or water is not present in the soil already), this group will wilt earlier and can be severely damaged (e.g., root hydraulic conductance).

Sometimes, both groups behaviour (savers, spenders) can go across tolerance/sensitivity. In that case, quick and/or less-energy consuming acclimation can increase fitness and survival.

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1 Introduction

1.1 General introduction

The family *Brassicaceae*, is one of the major groups of the plant kingdom and comprises diverse species of great economic, agronomic and scientific importance, including the model plant *Arabidopsis*. Oilseed rape (*Brassica napus* L.) – the winter form – is considered one of the most valuable oilseeds [4], and thus breeders are continuously engaged in improving its yield and quality parameters. In the conditions of Czech agriculture, oilseed rapeseed is the first and highly priced cash crop (approx. 10 thousand CZK/t of seed \approx 400 USD \$ in 2015). No surprise, that development of the oilseed rape cultivars has become a highly competitive business, in which breeders focus on the use of elite lines in their crossing programmes to get results in a short time period [5]. However, the long and intensive breeding of this oilseed, acting in concert with its limited geographic range, has led to a restricted gene pool from which new (broader) genetic variability is limited [6, 7]. In oilseed rape, unlike several other important crops, sources of genetic variation from natural populations cannot be further used mainly because of bad seed quality (glucosinolates, erucic acid etc.; [8, 9]). Winter oilseed rape have high genetic diversity between cultivars [10] and show variability physiological acclimation related traits [11, 12]. Furthermore, pathways triggered by plant-environment interactions (such as nitrogen resources), environment-related plant architecture, and, therefore, sink-source relations *in planta*, seem to be globally conserved between the model plant *Arabidopsis* and *B. napus* [13]. These findings encourage the transfer of knowledge from *Arabidopsis* to the crop *B. napus*.

The first stage of crop-related research is to observe the world around us and to ask questions about why things are happening. The same is true for stress-related signs, traits, and behaviour. This question can be asked if the “stress” and subsequent *in-planta* “strain” is necessary for “normal” plant life, connected clearly to adaptation and evolution. No surprise that effects of plant growth, genes and protein expression in response to stress are highly dose responsive, suggesting the existence of very sensitive machinery assessing the stress level and fine-tuning of molecular responses [14]. For example environmental water deficiency triggers an osmotic stress-signalling cascade which induces short-term cellular responses to reduce water loss and long-term responses to remodel the transcriptional network, physiological and developmental processes [15]. Moreover, stress factors usually do not affect plants independently but in various combinations under field conditions and the effect of joint stress factor action (for example drought and heat) does not equate the sum of separate stress factor effects [16, 17].

Some “new approaches” has been revealed through past 5 years in wider understanding of how plants sense, react, and adapt the stress stimulations:

1) Pathogens, sucking insects, temperature extremes, salinity, heavy metals, water or nutrient deficiency have been observed to cause genome-wide cytosine hypomethylation. Interestingly, the stress-primed DNA hypomethylation and so induced stress tolerant phenotype(s) are transmitted to subsequent generation(s) [18].

2) A different response to a similar stress represents the concept of 'stress memory'. A coordinated reaction at the organism, cellular, and gene/genome levels is thought to increase survival chances by improving the plant's adaptability. Ultimately, stress memory may provide a mechanism for acclimation and adaptation. At the molecular level, the concept of stress memory indicates that the mechanisms responsible for memory-type transcription during repeated stresses are not based on repetitive activation of the same response pathways activated by the first stress [19, 20].

3) Recent evidence that circadian clock contributes to plants' ability to tolerate different types of environmental stress, and to acclimate to them. The clock controls expression of a large fraction of abiotic stress-responsive genes, as well as biosynthesis and signaling downstream of stress response hormones. Conversely, abiotic stress results in altered expression and differential splicing of the clock genes, leading to altered oscillations of downstream stress-response pathways [21].

4) The majority of plants live in close collaboration with a diversity of soil organisms among which arbuscular mycorrhizal fungi play an essential role. Mycorrhizal symbioses contribute to plant growth and plant protection against various environmental stresses [22]. In addition, the endophytic bacteria [23, 24] or fungal endophytes [25-27] with mutualistic symbiosis effect have been described helping the plant to survive. The great expectations are also in mycoviruses [28, 29].

5) Altered expressions of miRNAs implicated in plant growth and development have been reported in several plant species subjected to abiotic stress conditions such as drought, salinity, extreme temperatures, nutrient deprivation, and heavy metals. These findings indicate that miRNAs may hold the key as potential targets for genetic manipulations to engineer abiotic stress tolerance in crop plants [30]. Recently, great progress has been achieved also on epigenetic regulation of cold/heat responses which regulate the expression of heat-responsive genes and function to prevent heat-related damages [31].

6) Reversible phosphorylation of protein plays a crucial role in signalling as well as protein chaperone and enzymatic activities. The observed differences at proteome level regarding not only changes in protein relative abundance, but also changes in protein

interactions and protein activity significantly determine differences observed at metabolite and physiological (functional) levels. Plant acclimation and tolerance to an abiotic stress are always associated with significant changes in PTMs of specific proteins. Understanding of plant responses to abiotic stress at the PTMs level will be also important to crop improvement phenotyping [32].

An efficient active response to each kind of stress is associated with specific physiological and molecular adaptations depending on timing and severity of drought stress. For example high-yielding drought tolerant cultivars are believed to respond to stress by various acclimation strategies including phenotypic plasticity [3], because adaptation to stress has metabolic and energy costs. An active plant stress acclimation represents an energy-demanding process. However, alterations in plant cellular environments cause an enhanced risk of imbalances between the individual reactions resulting in an enhanced ROS formation.

The dynamic change in protein abundance is an important part of plants response to stress [17, 33]. Therefore, in plant abiotic stress proteome studies, it is common to analyse stressed plants in contrast to the control ones, attempting to correlate changes in protein accumulation with the plant phenotypic response [1, 34]. Additionally, comparisons between genotypes with different sensitivity to stress are crucial to understanding the putative influence of differentially abundant proteins in tolerant genotypes.

Crop production has to double by 2050 to meet the predicted demands of the global population and to achieve crop yields increases at the rate of 2.4% per year (now 1.3%) [35-37]. A combination of different approaches (physiology-based understanding, *omics*-techniques, QTL mapping, epigenetic breeding, genome editing and other tools) will likely be needed to significantly improve the abiotic stress tolerance of crops in field conditions, particularly to drought [15].

1.2 Cold stress

Even though, the oilseed rape is the third most important oil-producing crops in the world, its potential for global cultivation is limited due to its sensitivity to cold and overwintering success.

The fact that temperature changes can induce cellular responses indicates that temperature is sensed and that the temperature signal is transferred into the cell. While the signalling pathways triggered temperature changes are well described, the way plants sense temperature is often considered as elusive [38]. Plants have no internal thermometer as such,

but the very alterations in cellular equilibrium triggered by temperature changes act as networked thermostats to sense heat and cold.

The life of plants growing in cold extreme environments has been well investigated in terms of morphological, anatomical, and eco-physiological adaptations. In contrast, long-term cellular or metabolic studies have been performed by only a few groups. The interplay between plastids, mitochondria, and peroxisomes, known as photorespiration, seems to be more intense in highly FT plants [39]. Therefore, to better understand the process of cold tolerance and develop strategies to improve adaptability, it was found that the genomic approaches need to be complemented by qualitative and quantitative analyses of the plant at several levels including the transcriptome, proteome, and metabolome.

The ability of plants to cope with cold stress is a very complex trait; during cold acclimation (CA) many physiological, biochemical, and molecular changes occur [40, 41]. CA is believed to result from the long-term application of low temperatures above zero. The main evolutionary goal of CA is frost tolerance (FT), which seems to be the main factor influencing the winter survival of winter oilseed rape. During low temperature exposure, oilseed rapes increase in freezing tolerance in a process termed cold acclimation. If the CA is complete and vernalization requirements are fulfilled, the plant induces the shift from vegetative to generative stage (usually in early spring). However, the correct timing and rate of deacclimation, resulting in loss of freezing tolerance and initiation of generative growth is equally important for plant fitness and survival. In addition, it is worth to select-out cvs with very rapid deacclimation period, as this can be very crucial if late frosts come to the field.

FT is the result of a wide range of physical and biochemical processes, such as the induction of antifreeze proteins, changes in membrane composition, the accumulation of osmoprotectants, and changes in the redox status, which allow plants to function at low temperatures. Even in frost-tolerant species, a certain period of growth at low but non-freezing temperatures, known as frost or cold acclimation (hardening), is required for the development of a high level of frost hardiness. The research on FT has recently been focused on the characterization of genes that are up/down-regulated and are important for the capacity of each genotype to develop higher FT [42].

However, the already acquired FT can be dynamically changed by environmental conditions; thus, it is not easily predictable [43]. FT is a highly dynamic stress-response phenomenon and involves complex cross-talk between different regulatory levels. Proteins are the major players in most cellular events and are directly involved in plant LT response; thereby proteome analysis could help uncover additional novel proteins associated with LT tolerance. Generally, LT stress down-regulates many photosynthesis-related proteins [44]. On

the contrary, pathways/protein sets that are up-regulated by LT include carbohydrate metabolism (ATP formation), ROS scavenging, redox adjustment, cell wall remodelling, cytoskeletal rearrangements, cryoprotection, defence/detoxification. These modifications are common adaptation reactions observed in the plant model *Arabidopsis*, thus representing key potential biomarkers and critical intervention points for improving LT tolerance of crop plants in cold regions with short summers.

Past studies have found that several metabolites that could functionally contribute to induced stress tolerance have been associated with stress responses. Recent metabolite-profiling studies have refocused attention on these and other potentially important components found in the 'temperature-stress metabolome' [45]. These metabolomic studies have demonstrated that active reconfiguration of the metabolome is regulated in part by changes in gene expression initiated by temperature-stress-activated signaling and stress-related transcription factors. One aspect of metabolism that is consistent across all of the temperature-stress metabolomic studies to date is the prominent role of central carbohydrate metabolism, which seems to be a major feature of the reprogramming of the metabolome during temperature stress.

FT has several components, e.g., desiccation tolerance, due to the possibility of ice crystal nuclei formation within intercellular spaces, which are accompanied by cellular dehydration. The quick method to quantify the FT level is an evaluation of the lethal temperature of leaf segments using the frost test [46, 47]. However, a more reliable pre-screening method as well as a better understanding of FT in *Brassica napus* L. cultivars are clearly needed [48].

Decreasing water availability in the cold initially leads to an inhibition of photosynthesis, because limited water supply changes transpiration, stomatal conductance, chlorophyll contents, inhibits photochemical activities, and decreases the activities of enzymes [49-51]. Photosynthesis provides the energy necessary for the maintenance of cold acclimation; therefore, maximized frost tolerance in plants. Thus, any negative impact on photosynthesis may influence FT [52].

It has long been known that frost hardening at low temperature under low light intensity is much less effective than under normal light conditions; it has also been shown that elevated light intensity at normal temperatures may partly replace the cold-acclimation period [53]. Earlier results indicated that cold acclimation reflects a response to a chloroplastic redox signal while the effects of excitation pressure extend beyond photosynthetic acclimation, influencing plant morphology and the expression of certain nuclear genes involved in cold acclimation. Recent results have shown that not only are

parameters closely linked to the photosynthetic electron transport processes affected by light during hardening at low temperature. The light may also have an influence on the expression level of several other cold-related genes and several cold-acclimation processes can function efficiently only in the presence of light [43, 54-62].

Plants have evolved sophisticated signalling cascades that enable them to withstand chilling or even freezing temperatures. Different families of proteins are known to be associated with a plant's response to cold stress by being newly synthesized, accumulating or decreasing. In several studies [11, 63-66] it has been shown that CA timing, dehydrin (DHN) expression, and relative DHN protein accumulation *in planta* reveals a significant relationship with plant-acquired frost tolerance. DHNs represent a unique group in the family of COR/LEA proteins [67] DHN proteins are present in all higher plants, mostly in young plant organs in the sub-epidermal tissues, because they are the first influenced by dehydration stress [65, 68]. Due to their ability to bind water with a minimum of intracellular hydrogen bonds (thanks to their intrinsically unstructured character), DHNs exhibit many regulatory and defence functions in *Brassica* spp. to cold [69-72]. DHNs protect membranes and other proteins against loss of the water envelope, which could lead to their denaturation. In the *Brassicaceae* family, DHNs could be considered possible indicators of FT on the basis of the content of dehydrin proteins in the leaves of cold-treated plants [73]. Other papers have been focused on expression profiles and/or function of DHNs (*BjDHN1, 2 and 3, Bndhn ERD 10*) in *Brassica* spp [69-72]. Gallardo-Cerda et al. [74] also observed a new role of dehydrins. In a very low and freezing temperature the *Nothofagus domeyi*'s hydraulic conductivity when dehydrins are present is similar to non-freezing control individuals.

In comparison to CA wheat, the DHN accumulation in CA winter oilseed rape is about 100 times lower [11]. No surprise, that within the *Brassica* species, DHN proteins have previously been visualized only in the experiments of Rurek [72] (mitochondrial fraction of *B. oleracea*), and as the result of cold stress by Klíma et al. [73] (fraction of proteins soluble upon boiling of *B. napus* and *B. carinata* cvs) also. However, up to our results in paper 2 in 2013, the qualitative differences in DHNs accumulation between *Brassica* species genotypes was not described in literature. However, it should be taken into consideration that the overexpression of DHN genes alone generally does not result in an increased plant stress tolerance and support idea that FT is a polygenic trait. CA is a quantitative trait involving the action of many genes and current evidence suggests that multiple mechanisms are involved in activating the cold-acclimation response [75, 76]. The process of CA encompasses biological modification on many levels, e.g. modulation of gene expression, accumulation and degradation of proteins, changes in sugar content and changes in the photosynthetic

machinery [75].

Increasing light energy consumption by increasing photosynthetic capacity or by dissipation through xanthophyll cycle is the first step of photosynthetic apparatus to acclimation in over-wintering plants to relative excess of light. The second step to protect photosynthesis can be the production and/or accumulation of protective non-photochemical mechanisms. However, it is hard to decide which way is more important, because in some articles plants which were not able to increase adaptability to photoinhibition were also not able to increase FT [52, 77-79].

The connexion of starch and sucrose metabolism is influenced by photosynthesis (generate osmotic adjustment, available energy source etc.) and DHNs are believed to be another, but synergistic part of adaptability to cold. Furthermore, DHNs, osmotic processes and physiological changes can be also cross-linked between drought and cold adaptability [80]. The main operator in this cross-link can be ABA-dependent and -independent pathways. Generally, higher photosynthesis increases the inflow of energy under drought or CA; however, only this is not sufficient for good FT. As also Rapacz [81] postulated on vernalization requirements: *“Frost tolerance is induced only by low temperature, but the development of frost tolerance is dependent upon both irradiance, which affects the amount of photoassimilates available, and the day length, which may affect the partitioning of photoassimilates between growth and frost tolerance.”* It is well known, that cold-acclimation of oilseed winter cvs comprises also the enzymatic apparatus (in contrast to oilseed spring cvs) to functioning at lower temperatures and even under higher amount of soluble carbohydrates. Spring cvs have higher FT in very early phase of development. However, they are not able to keep this high level for a longer time also because of low (or zero) vernalization requirements and consequently continuing development.

1.3 Drought and osmotic stress

Drought remains the most severe abiotic stress factor for a global crop production in the 21st century. Drought adaptability represents a polygenic trait with multiple components associated with plant water status, cellular metabolism, growth and developmental characteristics affecting the final crop yield whose values depend on many interacting genetic and environmental factors.

There are several definitions of drought depending on different viewpoints, such as meteorological drought (a deficit in rainfall with respect to average values at a given time in a given area), hydrological drought (water deficit in surface and subsurface water reservoirs),

socioeconomic drought (a reduction in water consumption recommended by local authorities), physiological drought (an excessive water release by shoot with respect to water uptake by root resulting in plant wilting), agronomical drought (a water deficit period leading to reduced yield), etc.

Water deficit imposed by either drought or salinity brings about severe growth retardation and yield loss of crops. Since Brassica crops are important contributors to total oilseed production, it is urgently needed to develop tolerant cultivars to ensure yields under such adverse conditions. There are various physiochemical mechanisms for dealing with drought and salinity in plants at different developmental stages. Accordingly, different indicators of tolerance to drought or salinity at the germination, seedling, flowering and mature stages have been developed and used for germplasm screening and selection in breeding practices. Classical genetic and modern genomic approaches coupled with precise phenotyping have boosted the unravelling of genes and metabolic pathways conferring drought or salt tolerance in crops. QTL mapping of drought and salt tolerance has provided several dozen target QTLs in Brassica and the closely related Arabidopsis. Many drought- or salt-tolerant genes have also been isolated, some of which have been confirmed to have great potential for genetic improvement of plant tolerance. It has been suggested that molecular breeding approaches, such as marker-assisted selection and gene transformation, that will enhance oil product security under a changing climate be integrated in the development of drought- and salt-tolerant Brassica crops.[82]

Alternative concepts of water-saving strategies have been outlined in recent years; for example, Blum [83] proposed a concept of „effective use of water (EUW)“ which means that a plant tries to maximize water uptake from soil and to minimize water release from shoot by all other ways except for stomatal transpiration (the difference from WUE). The „trade-off“ between stomatal transpiration and photosynthesis represents a crucial problem in breeding of C3 crops for improved drought tolerance. The crucial question is which of the alternative plant strategies results in higher final crop yield: The conservative, water-saving strategy (in paper 3 characterized as water-savers) based on minimization of water loss via transpiration under water deficit conditions resulting in stomatal closure, or a water-consuming strategy (in paper 3 characterized as water-spenders) based on open stomata and maintenance of high photosynthesis rates under water deficit conditions? The answer on this crucial query lies in ambient environmental conditions, especially timing and severity of drought stress, and management practices used in the given area including costs and benefits.

Moreover, under field conditions, plants are usually exposed to combined effects of multiple abiotic together with biotic stresses [15-17]. It also has to be kept in mind that the

same ambient conditions reveal different effects on different genotypes and plant growth stages indicating that plant-associated parameters such as tissue relative water content (RWC) are more relevant parameters of stress than just soil water content. Therefore, no universal breeding strategy to enhance drought tolerance in oilseed rapeseed can be outlined. However, it can be summarised that the crucial factors affecting final seed yield in drought-prone environments include mechanisms associated with plant water uptake from soil (architecture of plant root system), leaf photoprotection (photosynthetic pigments, leaf rolling, waxy cuticle), stomatal openness and transpiration efficiency, delayed leaf senescence („*stay-green*“ phenotype) and assimilate partitioning between seeds and other parts of the plant, i.e., characteristics associated with plant harvest index, early maturity etc. In particular, the alpha and beta subunits of protein farnesyl transferase have been identified as negative regulators of ABA-mediated stomatal responses, and their effectiveness as the targets for engineering drought tolerance and yield protection has been confirmed in canola in the field [84].

Rapeseed is classified as salinity-tolerant [85], however, it is a drought-sensitive crop. Of the major crops, rapeseed generally exhibits a middle salt tolerance with respect to other cereal crops, such as wheat, rice, and maize. The higher salt tolerance to drought tolerance in oilseed rape means, that sufficient content of soil water (despite salt contamination and lower osmotic potential) is more important than the lack of the water at all (drought) for maintenance of adaptability to stress.

Oilseed rape sensitivity depends on and varies with the developmental stage [86]: during the seedling stage [87], in the vegetative (stem prolongation) [88] and the reproductive stage (flowering) [89-92]. The seeds filling stage is a less perceptive stage to moderate drought stress due to a sufficient and rapid translocation of assimilates from the stem [93]. However, this stage is connected to sufficient stem prolongation period because stem prolongation developmental stage is a highly water-demanding stage.

Furthermore, in the warm, humid continental climate of The Czech Republic (Cfb; Koeppen-Geiger classification [94]), opposing water-use patterns can be useful for drought adaptation of crops, as it is postulated for other similar regions [95-103]. The so-called water-saver plants can maintain satisfactory yield in long-term drought conditions. On the other hand, in a quick drought, the second group - water-spenders - showed delayed response to drought (so they seem to be less perceptive to actual water content in the soil profile) [102, 104-106]. Water-spenders keep stomata open and thus can sustain higher net assimilation and growth. Furthermore, genotypes that keep growing throughout the decreasing water supply (water-spenders or spenders) perform well in the field whenever there is adequate water in the subsoil and a prospect of rain [102], and thus can possess future productive advantage

[100].

Among the proteomics studies and reviews published on *Brassica* spp. to date [107-120], few differential proteomic studies are focused on *B. napus* drought stress response (comparative proteomics on seedling roots [121] and 6 weeks old plant leaves [122]). Only two papers were published to cover the question of adaptability of oilseed rapes to drought on the proteomic level. In our study (published in paper 3), we then screened for differentially regulated proteins in four vernalized winter oilseed rapes under long-term drought stress. Proteomics was carried out by modification of 2DE, the two-dimensional difference in gel electrophoresis (2D-DIGE) [123]. This was the first proteomic study on drought using mature plants for a research.

Plants reveal significant differences in their abilities to cope with drought. However, the differences observed at proteome level represent only a part of plant complex adaptation mechanisms to drought. It is becoming evident that the differences at proteome level are determined and underlined by differences at genome and transcriptome levels. Similarly, differences at proteome level affect also plant adaptation at metabolic and functional (physiological) level. Proteins thus represent an important means of transformation of the differences encoded at genome level into differential cell, organ and whole plant function. Already published proteomic and other data point to the fact that susceptible plants can be characterised by mobilization of their energy reserves, consumption of energy reserves and enhanced protein degradation under stress while tolerant plants are able to cope with the stress due to enhancement (protecting) of photosynthetic assimilation and protein biosynthesis. Thus, increased demands on energy and novel proteins because of an enhanced risk of protein damage can be satisfied in tolerant plants. Upon a low CO₂ internal concentration (C_i) in leaf mesophyll due to stomatal closure, photosynthesis becomes limited and, moreover, competed by a photorespiration due to a dual carboxylase/oxygenase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). A reduced RuBisCO carboxylation activity leads to a disbalance between the rate of photosynthetic electron transport processes and carbon assimilation resulting in a photo-oxidative stress, which represents a major concomitant stress to drought in natural environments [124]. For detailed information about physiological mechanisms involved in drought adaptability, please see our publications [33, 125-128].

The major aims in breeding for an enhanced drought adaptability and yield under drought-stressed conditions lie first in the knowledge of the target environment, i.e., soil characteristics with respect to water regime (soil depth, particle size, chemical composition, and retention and infiltration capacity), timing, and severity of drought stress with respect to

crop life cycle. The knowledge of the target environment is necessary to construct crop ideotypes and to design the optimum breeding strategy for maximizing drought adaptability and the final yield. Then, identification of crucial phenotypic traits and their quantitative values is necessary. Selection of the most suitable genetic resources and identification of QTLs underlying desirable phenotypic traits should follow as a next step. Transfer of the proposed QTL underlying drought adaptability-associated traits has to be followed by evaluation of the effect of the transferred QTL on plant phenotype. Genetic interactions with other QTLs and G x E interactions have to be considered [129].

Plant developmental phase when drought stress occurs also affects the target traits for breeding programmes. When drought affects young plants, breeding for an early vigour leading to an early canopy closure and an elimination of soil water evaporation represents an efficient strategy leading to drought avoidance. On the other hand, when drought affects plants during a grain-filling period, a „stay-green“ phenotype resulting in a delay of leaves senescence represents an efficient strategy leading to an increase in cumulative photosynthesis and the final yield. Therefore, we focus not only on mature plants upon drought, but also on *in vitro* MDE obtained from microspore system (paper 4).

Breeding for improved drought adaptability still remains a great challenge due to a complex nature of a trait. Selection of the most suitable breeding strategies depends on both the plant developmental phase and the severity of environmental conditions. Strategies based on maximizing EUW, i.e., maximizing plant water uptake without reducing water release via stomatal transpiration as observed in high $\Delta^{13}\text{C}$ cereals, seem to represent a more efficient way with respect to the final yield than conservative strategies based on maximizing WUE. However, total water use and cumulative photosynthesis (seen as harvest index) over the whole crop life cycle seem to represent more relevant criteria with respect to the final yield than WUE or TE [2, 130].

1.4 Thesis design, the lack in the knowledge, hypotheses

The thesis is focused on oilseed rape and comes out with the idea of comparison of all main abiotic stresses, which can severely decrease yield or quality of oilseed production. In thesis, the cold during vernalization period is studied on the base of physiological and biochemical characterization. Drought experiments were done in stem prolongation period. This developmental stage has been recognized as water-demanding stage really influencing flowering and then yield and its quality. Osmotic stress (PEG infused solid media) was studied during MDE development from microspores. This stage was chosen because of

higher biomass accumulation and progressive growth. PEG was used only as a osmotic activator of solid media water potential. In the present study, we described the application of MDE of *B. napus* as a system for studying drought-related protein abundances between water-saver and water-spender genotype.

The main aim of our first study was to observe whether there is a relationship between frost tolerance (expressed as LT_{50} values), accumulation of dehydrins, and other selected physiological characteristics in five winter oilseed rape cultivars. The results are discussed with respect to the possible role of the above-mentioned parameters in the development of cold acclimation. To maintain this goal we had to improve the visualization of dehydrin accumulation, which is 100 x lower for oilseed rapes than for cereals. These data were missing so far.

The main aim of the drought-related study was the analysis of gasometric, biochemical and water-related characteristics with special interest in how proteome modulates and/or is modulated during response to gradual drought stress. Detailed comparison of these changes enabled genotype separation according to their actual behaviour based on soil water content. To reach these aims, we optimize the drought onset, proline detection, chlorophyll measurements, and others. For proteome analysis, the 2-D DIGE method was used. A methodology to label proteins from pools to be compared with different dyes was originally introduced by Unlu et al. [131] and has been further developed and is now termed DIGE standing for differential in gel electrophoresis. The separate pools of proteins are covalently labelled with N-hydroxysuccinimidyl derivatives of the fluorescent cyanine dyes (Cy2, Cy3, and Cy5). These fluorescent dyes are designed to modify the ϵ -amino group of lysine residues in proteins. The technique allows the analysis of up to three pools of protein samples simultaneously on a single 2-D gel, thereby minimizing the problem of gel-to-gel variability. In a standard protocol, two of the dyes (Cy3, Cy5) are used to label two protein samples to be compared, and the third dye (Cy2) is used to label an internal standard that consists of equal amounts of all the samples to be analysed within the overall experiment. The inclusion of such an internal standard allows experimental errors to be corrected and therefore improves the quantitative comparison of protein expression. These data were missing so far.

The main aim of the MDE study was to screen for proteins in winter oilseed rapes embryos. Only proteins that are differentially regulated under osmotic stress are explored in details to answer the molecular basis of the response to an osmotic stress during the MDE development. Moreover, we continue in MDE research by comparing the transcriptome level (associative transcriptomics, for details see [132, 133]), however, together with

phytohormonal profiling, these data are not present in the manuscript (paper 4) and are not a part of this thesis. To author best knowledge no similar comparative analyses of MDE was published so far.

This introduction contains many ideas and particular research data, which allow us to express some tested hypotheses:

1) The lower rate of photosynthesis acclimation and lower accumulation of protective molecular components during cold acclimation will cause lower oilseed rapes frost tolerance.

2) The responsiveness of oilseed rape to drought (sensitivity or adaptation) is mainly based on different water-uptake strategies: water-savers will save water in the soil because of slower growth (and lower biomass accumulation). On the other hand, water-spender will have high growth rate and high water uptake. The highly energy-demanding homeostatic equilibrium will be more detrimental in water-spender, as they will deplete soil water more quickly and can undergo severe stress.

3) MDE technique is a reliable source of information about distinct cultivars. On the base of MDE-related proteomic results, there is a possibility to distinguish between cultivars if non-lethal osmotic stress is applied.

The procedures for Ph.D. thesis aims formulation is based on the information already described in the introduction, summary ideas and hypotheses described in this chapter.

2 Aims of the study

The main aim of the research is to describe the physiological, biochemical and proteomic responses of selected genotypes of winter oilseed rapes to 3 types of abiotic stresses – drought, cold, osmotic stress, in different developmental stages and recommend traits to be used in new breeds selection process.

To resolve this question, I postulated specific aims:

- 1) Firstly, to get knowledge about the contribution of proteomic studies in understanding the complexity of crop response to abiotic stresses and to explore possibilities to identify and utilize protein markers in crop breeding processes (paper 1).
- 2) To investigate the effect of cold acclimation on winter oilseed rape cultivars (paper 2) with special interest in:
 - a. the role of photosynthesis acclimation on the level of frost tolerance,
 - b. dehydrins content, and the evaluation of dehydrin accumulations as suitable “markers” for frost tolerance,
- 3) To clarify the effect of progressive drought in stem-prolongation phase on selected oilseed rapes belonging to different geographic origin. To analyze comprehensively the gasometric, biochemical, and proteomic data and to outline cultivar-based water consumption peculiarities (paper 3).
- 4) To analyse differential proteome profile of osmotically treated MDE and:
 - a. find possible protein markers suitable for early microspore selection of contrasting cultivars,
 - b. verify the selected proteomic data with the transcriptomic data (paper 4).

3 Article summary of obtained results

Summary of the published results mirrored the main goals of this thesis to be fulfilled and covered.

Results are mainly summarized in three already published articles with IF (1 x review, 2 x original first-author articles) and in one prepared first-author manuscript. Only these 4 papers are present in the attachments.

As additional sources of some data, the list of books, articles in “Úroda” and in other booklets and proceedings was added. However, only the articles with IF are supplemented in attachments. If data from different sources than from these 4 papers are presented in the text of thesis, they are properly cited.

Publications included in Ph.D. thesis (however, the complete author publications detailed in CV):

Original review and 3 articles

1) Kosová, K., Vítámvás, P., Urban, M. O., Klíma, M., Roy, A., Prášil, I. Biological Networks Underlying Abiotic Stress Tolerance in Temperate Crops—A Proteomic Perspective. *International Journal of Molecular Sciences*, 2015, 16 (9): 20913 – 20942; IF₂₀₁₅ = 3.257; Q1.

Short description: The major focus of the review is on contribution of proteomic studies to elucidation of biological mechanisms underlying stress response in temperate crops, with special attention paid to proteins revealing differential responses between crop genotypes with differential stress tolerance levels as well as proteomic studies dealing with the effects of multiple stress factors. In conclusion, future challenges in proteomic studies focused on elucidation of protein roles under stress are discussed and possible applications of proteomic results in crop breeding programs aimed at an improvement of crop stress tolerance are suggested.

Author contributions: Kosová has outlined the manuscript idea and prepared the manuscript text. Vítámvás, Milan Oldřich Urban (MOU), Klíma, Roy and Prášil have searched for relevant literature, prepared literature outline in the form of Supplementary Table and prepared the graphics. MOU also prepared the reference list using EndNote programme.

2) Urban, M O., Klíma M., Vítámvás P., Vašek J., Kučera V. Significant relationship between frost tolerance and net photosynthetic rate, water use efficiency and dehydrin accumulation in cold-treated winter oilseed rapes. *Journal of Plant Physiology*, 10.1016/j.jplph.2013.07.012, 2013. 170 (18): 1600 – 1608; IF₂₀₁₅ = 2.971, Q2.

Short description: For the full development of FT, it seems to be necessary to have a high rate of assimilation (available energy) as well as the synergistic and/or protective effect of the DHNs. The present study indicates that, during acclimation of winter rape to cold, the

complex interaction of acclimated photosynthesis (here in terms of Pn) and DHN production and accumulation occurs.

Author contribution: All physiological and proteomic data were obtained by MOU. Part of the statistical analysis (PCA) was obtained by MOU. MOU also wrote the whole manuscript. MOU with Klíma prepared the DHN extraction. Vašek and Klíma obtained a second part of the statistical analysis (ANOVA) and with Vítámvás and Kučera they added some literature sources and edited the final version of manuscript. Vítámvás supervised the dehydrin visualization and accumulation evaluation.

3) Urban MO, Vašek J, Klíma M, Krtková J, Kosová K, Prášil IT and Vítámvás P. Proteomic and physiological approach reveals drought-induced changes in rapeseeds: Water-saver and water-spender strategy. *Journal of Proteomics* 152 (2017) pp. 188-205, DOI information: 10.1016/j.jprot.2016.11.004; IF₂₀₁₅ = 3.867, Q1.

Short description: The data in this study demonstrates for the first time that in stem-prolongation phase cultivars respond to progressive drought in different ways and at different levels. Analysis of physiological and proteomic data showed two different water regime-related strategies: water-savers and spenders. Water-savers showed efficient nitrogen metabolism, higher accumulation of ATP conversion proteins, higher ROS levels, higher accumulation of signalling and stress-related proteins. Water-spenders down-accumulated proteins of carbohydrate and energy metabolism and photosynthesis. However, not only water uptake rate itself, but also individual protein abundances, gasometric and biochemical parameters together with final biomass accumulation after stress explained genotype-based responses and found tolerant cultivars in both groups.

Author contribution: MOU obtained almost all measured data (otherwise specified below). MOU with Vítámvás extracted samples and prepared DIGE. Vítámvás supervised the DIGE procedure. Vašek prepared statistical analysis of the data. Krtková arranged the data into tables and figures. MOU outlined the manuscript idea and prepared the manuscript text. Krtková, Klíma, Kosová and Vítámvás significantly edited the manuscript text and added some explanation to the data.

4) Urban MO, Jelínková I, Klíma M, Renaut J, Planchon S, and Vítámvás P. Proteomic analysis of two drought-tolerance contrasting oilseed rape microspore-derived embryos showed different profile after drought-simulation via infusion of polyethylene-glycol into cultivation media. *Prepared manuscript*

Short description: The microspore-derived embryos (MDE) from two drought contrasting genotypes of winter oilseed rapes Cadeli (D) and Viking (V) were exposed to polyethylen-glycol infusion activated cultivation media for 1 and 7 days. Taking findings together cv D showed better adaptation to osmotically activated PEG-infused cultivation media by higher numbers of accumulated proteins especially in protein and energy metabolism functional groups. While cv V showed high numbers of down-accumulated proteins. Selected proteins after transcriptomic verification can be used as a protein markers for further breeding. The comparison of MDE and drought-influenced leaf proteome is discussed.

Author contribution: MOU and Klíma designed the experiment. MOU performed the whole experiment and with Vítámvás prepared 2-D DIGE and excised of spots from the gels.

Vítámvás supervised the DIGE procedure. Planchon and Renaut identified the proteins. Jelínková analyzed to transcriptomic data. MOU analyzed the data, wrote the whole manuscript (except for a part of transcriptomics materials and methods) and finished the manuscript. All authors (except for Planchon) also edited the manuscript.

Chapters in Books (in Czech language)

Urban M. O. Metody vhodné pro detekci odolnosti a citlivosti plodin vůči abiotickým stresům sucha, zasolení a teplotnímu diskomfortu. In: Rostliny v podmínkách stresu - Abiotické stresory, Publisher: Česká zemědělská univerzita v Praze, Editors: Hnilička F., Středa T., pp.164-207, ISBN 978-80-213-2680-4

This chapter “The methods suitable for detection of sensibility and tolerance of crops against abiotic stress: drought, salinity and temperature imbalances” is intended for everybody dealing with different stress-related instruments. The chapter is divided into four parts: biochemical, molecular, physiological and phenotype-based methods. More than 14 chosen methods are described in details with special focus on methodological problems.

Journal “Úroda” (= Crop Yield; J Rec – peer reviewed articles;

<http://www.periodik.cz/predplatne/casopis.php?akce=archiv&titul=3705>

Urban, M. O., Holá, D., Klíma, M., Vítámvás, P., Kosová, K., Hilgert-Delgado, A., Prášil, I. Vliv chladové aklimace na biochemické a molekulární parametry šesti vybraných genotypů řepky ozimé ve vztahu k parametrům fluorescence chlorofylu. *Úroda*, 2015, 63(vědecká příloha): 33 - 40

Jelínková, I., Chikkaputtaiah, C., Havlíčková, L., Vítámvás, P., Urban, M. O. Expres genů/proteinů indukovaných v podmínkách stresu suchem u řepky olejky. *Úroda*, 2014, 62(vědecká příloha): 187 - 190

Havlíčková, L., Jelínková, I., Chikkaputtaiah, C., Prášil, I., Urban, M. O. Studium exprese genů spojených s abiotickým stresem u řepky *Úroda*, 2013, 61 (vědecká příloha): 142 – 145

Urban, M. O., Vítámvás, P., Klíma, M., Prášil, I. Vliv nedostatku vody na vybrané fyziologické parametry u řepky olejky. *Úroda*, 2013, 61 (vědecká příloha): 182 – 185

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4 Discussion

In this part, only original manuscripts (not a review) are discussed in terms of their wider context, research limitations and outlined possible direction of further research. Please, for every detailed result (data, figures, tables mentioned as Fig., Table...etc.), see the original articles and manuscript (chapter 9).

To fulfil the thesis aims, I have chosen different genetic material (cultivars), based on previous observations. For drought-related study, I choose 4 cvs with different biogeographic origin. For cold/frost-related study, I have chosen cultivars with significantly different frost tolerance in the field conditions. Surprisingly, one cultivar (Benefit) showed qualitatively different accumulation of dehydrins. Finally, for MDE study, I have used data from drought-related experiments, unfortunately, only few cultivars proved sufficient embryogenic abilities.

4.1 The frost tolerance of winter oilseed rapes is a combination of the rate of photosynthetic acclimation and accumulation of novel cold-induced stress proteins

Notice: All figure and table numbers refer to original article included in 9.2. chapter.

4.1.1 Scientific novelty and theoretical significance of the results

The aim of the first study was to evaluate modern currently used cultivars of winter rapeseed oil in terms to FT. Our results are new in a way of biochemical (DHN) and physiological photosynthetic parameters) interconnection of data. Some other studies [12, 72, 79, 81, 134-136] are very deep and comprehensive from the morphological or physiological (chlorophyll fluorescence) point of view, however, none of winter rapeseed oriented studies have ever revealed DHN accumulation (and found DHN 97kDa) in direct relation to FT. The focus of our first work was to study whether selected physiological parameters are good indicators of FT for chosen winter oilseed rape cvs when subjected to cold temperatures.

This paper significantly proved the relationship between FT, DHN accumulation and gasometric acclimation in selected winter oilseed rape cultivars under the cultivation conditions mentioned in details the paper 2.

For the first time, the specific DHN D97 (dehydrin around 97kDa) was shown accumulated in rapeseed and other DHNs was shown having “dual” nature (very close size of two DHNs – 47+45 and 37+35) which were not visible in our previous study Klíma et al. [73]. This is evident from a comparison of the DHN spectrum accumulated in the CAL, used in both experiments. This result was achieved by optimizing visualization procedures;

specifically, more concentrated DHN samples, increasing the amount of loaded samples, using a gel with a higher concentration of acrylamide, and longer incubation with the primary antibody.

The presented study indicates that during acclimation of winter rapeseeds to cold the complex interaction of Pn and DHN accumulation occurs. All genotypes which showed higher FT (COR, NAV) also showed higher net photosynthesis after CA (see). This increase is connected - besides other things - with a higher amount of DHN's present in such tissues contrary to others with lower FT (correlation of DHN to FT, $r = 0.815$). We do not believe that the observed correlation proves any direct mechanistic link between Pn and FT (and this confirmation wasn't an aim of the study). As some authors postulate [51, 52, 60, 62, 77, 137, 138], we rather favour the view that photosynthesis provides rapeseed the energy necessary for the cellular changes (DHNs and others) required for higher FT. These cellular changes may be covered here by DHNs accumulation. Furthermore, in some cases, correlations are linked with other hidden developmental strategies realised among winter cultivars. That implies the lack of photosynthesis acclimation caused by reducing the amount of energy available for plant acclimation can cause difficulty in this very proper assessment of FT.

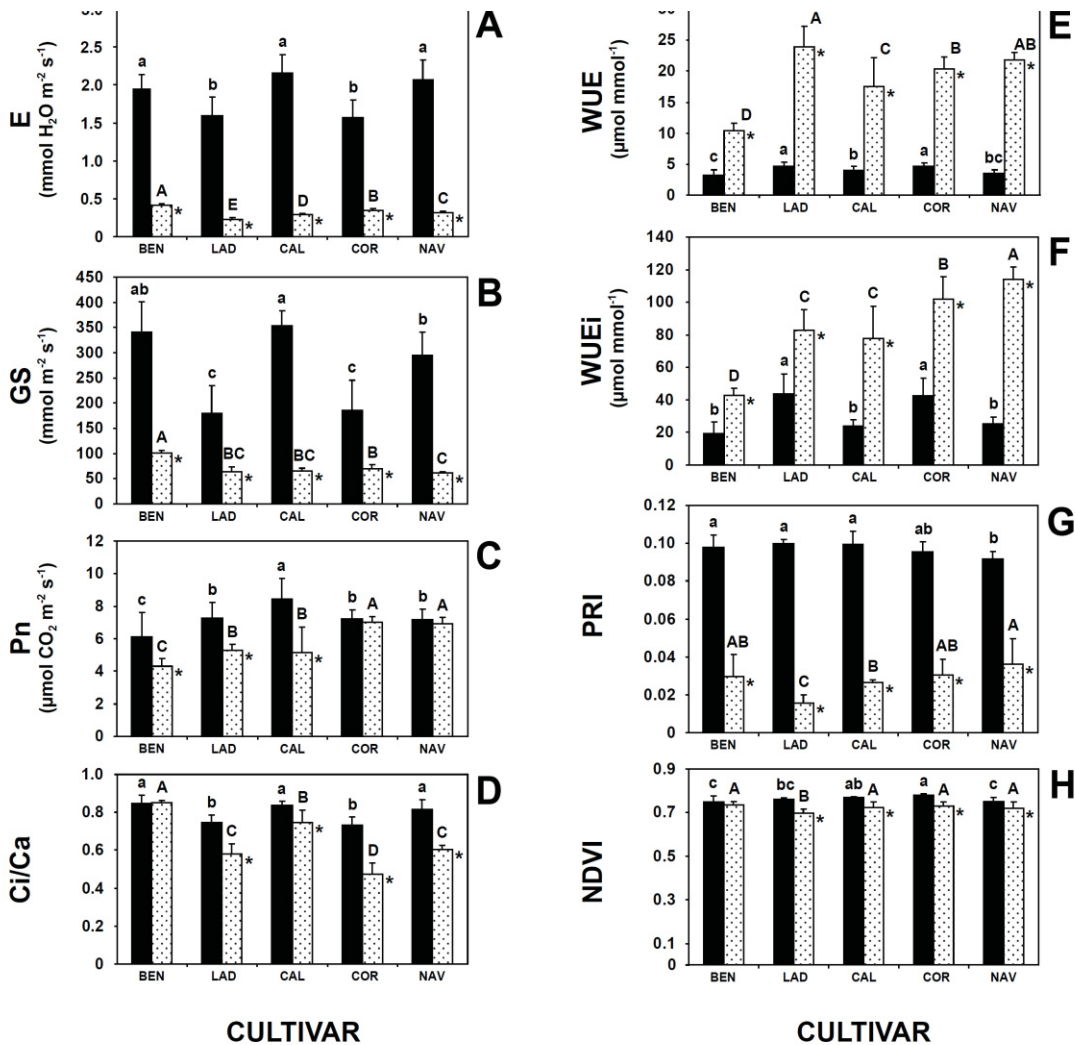
4.1.2 Discussion and synthesis into wider context

The main aim of this study was to observe whether there is a relationship between FT (expressed as LT_{50} values), accumulation of dehydrins, and other selected physiological characteristics in chosen winter oilseed rape cvs.

All detailed results are shown in paper 2. Shortly: Analysis of variance confirmed the significant impact of genotype on the degree of FT. Regarding the effect of genotype, the largest differences upon cold were recorded in E values, followed by Ci/Ca, WUE, and PRI (Fig. 1A, D, E and G in Paper 2); the other parameters of the cold treatment exhibited smaller differences between cvs, according to homogeneous groups, derived from multiple comparisons among the means. DHNs of different molecular masses, extracted from the leaves of cold-treated plants, were detected in all cvs (Fig. 2B in Paper 2). Both the qualitative and quantitative changes were observed. Among the highly significant correlations, there are some with a high physiological importance. Significant correlation between LT_{50} and other characteristics was observed in the case of DHN accumulation ($r = -0.815$), followed by WUE_i ($r = -0.643$), Pn ($r = -0.628$), GS ($r = 0.511$) and Ci/Ca ($r = 0.505$). On the other hand, no significant correlation was observed between LT_{50} and E, PRI, or NDVI.

Legend to Fig. 1: Photosynthetic parameters, water use characteristics and reflectance indices of winter oilseed rape leaves. The transpiration rate (E; A), stomatal conductance (GS; B), net photosynthetic rate (Pn; C), intracellular/intercellular CO₂ concentration (Ci/Ca; D), water use efficiency - calculated as Pn/E (WUE; E), intrinsic water use efficiency - calculated as Pn/GS (WUEi; F), photochemical reflectance index (PRI; G), and normalized difference vegetation index (NDVI; H). Five cultivars of winter oilseed rape Benefit (BEN), Ladoga (LAD), Californium (CAL), Cortes (COR), and Navajo (NAV) were subjected to 30 days of cold (dotted bars) and control conditions (solid bars). The means ± SD are shown. The small letters denote the statistical significance (as determined by the Tukey HSD

Fig. 1 Gasometric and other characteristics of cold acclimated rapeseeds



test) of mean differences between control treatments; capitals denote cold treatment mean differences between genotypes. Only those marked with different letters differ significantly at $p < 0.05$. Asterisks denote significant differences (at $p < 0.05$) between control and cold treatments within a particular genotype.

During the reduced availability of water in the plant upon becoming cold, other metabolic processes including photosynthesis, may be limited until the plants are fully CA. Similar results to ours were obtained in the experiments of Hall et al. [139] in drought-stressed *Brassica* ssp.; by Hurry et al. [77] in cold-stressed *Brassica* ssp.; and in *A. thaliana* [140].

Interestingly, the Pn rate in high FT cultivars (COR and NAV) seem not to be reacting to lower E or GS values after full CA. This decrease sensitivity of Pn to variations in transpiration and stomatal openness generally increase at warmer (not colder) temperatures [141]. CA can be then metabolically and by signalling linked with increased O₂ and CO₂ availability at low temperatures, indicating a disproportional enhancement of the inorganic

phosphorus regeneration capacity [141]. Such a capacity can *B. napus* increase through the expression of the enzymes via starch and sucrose synthesis [77]. Pons [140] also observed higher Pn and carboxylation capacity in frost tolerant *A. thaliana* (the same family as *B. napus*) accession Hel-1 even in lower Ci in contrary to frost susceptible accession CVI-0. Both had similar GS, leaf mass per unit area, RuBisCO and chlorophyll content. The capacity to adjust Pn rate to the rate of controls still appeared to be associated with limited biochemical and physiological limitation (triose phosphate, ATP/ADP ratio etc.), but no restriction at the level of RuBisCO compared to controls were observed in wheat [78]. Also the GS level in 5 °C was observed in winter wheat to be lower than in spring cultivar (24 mol/m²/s to 33), despite the rate of Pn was in a contrary (11.1 to 6.7). This fact supports again the idea of low correlation of GS to Pn and also the necessity of progressive CA of photosynthetic apparatus.

Changes in cold conditions are believed to also have primary relationships to receptors of turgor pressure changes in cells [142] and thus influence the GS. In drought, osmotic adjustment (OA) is proved as a positively correlated to yield [143] and accumulation of osmotic compounds may be similar biochemical crosstalk in both, cold and drought acclimation. Genotypes more resistant to (cold-related) dehydration prevented such a state by a large portion of the stomata closing (Fig. 1B in paper 2). This is in agreement with Aroca et al. [142] who observed that the stomata of the cold sensitive maize remain open, while those of the tolerant plants close more rapidly. Stomata can be more closed (and WUE_i rises) if the inner CO₂ concentration (Ci) is sufficient enough to saturate carboxylation, contrary to other cultivars. Interestingly, transgenic Arabidopsis that overexpressed *MtCAS31* (*Medicago truncatula* cold-acclimation specific protein 31) was used to determine the function of this dehydrin-like protein [144]. *MtCAS31* overexpression dramatically reduced stomatal density and markedly enhanced the drought tolerance of transgenic Arabidopsis and plays a role in stomatal development.

Additionally, GS is controlled by the phytohormone ABA; and, according to our DHN data, there may be a hypothetical crosslink to ABA-dependent and ABA-independent COR/LEA protein (DHNs) expression as the final products of signalling cascades. It is proven that the low temperature brings about an increase in ABA [75]. Thus, with higher Pn and lower water diffusion from the leaves, our experiments showed both water use efficiencies increased (WUE and WUE_i; Fig. 1E, F). If the plant is able to effectively managed water deficit, high energy-consuming metabolic processes (e.g., cryo-protective) need not be significantly affected. Therefore, it seems that the observed positive relationship between Pn and WUE_i ($r = 0.878$) may also be directly related to FT because WUE_i also has

a connection to DHN ($r = 0.844$; see also Fig. 4 in paper 2). Thus, the higher the WUE_i is, the better the ability to face the dehydration of cells. Energy-consuming metabolic processes are significantly influenced by sufficient water availability, which implies that higher WUE_i is a beneficial indicator of resistant (highly adapted) cultivars even in cold.

Pons [140] observed that the low growth temperature requires a large investment in the photochemical apparatus to compensate for the reduced enzyme activity. To explain more changes in the physiological response at the individual cvs level the spectral indices (PRI; NDVI) were used. Our results on PRI showed that in all cvs, the photoprotective mechanisms increased their activity significantly during CA () as PRI value decreased. In the experiments of Gamon et al. [145], PRI was correlated with P_n under field conditions ($r = 0.54$). In agreement with our results, Savitch et al. [146] confirmed an increase of xanthophyll cycle pigments deepoxidation (which corresponds to PRI decrease) in *B. napus* *BNCBF/DREB1* after 2 to 4 weeks at 4 °C. *BNCBF/DREB1* overexpression in *Brassica* not only resulted in increased constitutive freezing tolerance but also partially regulated chloroplast development to increase photochemical efficiency and even photosynthetic capacity. The lower PRI indicated the increase of the xanthophyll cycle and carotenoid pigments were observed in cvs with lower DHN and LT₅₀ which means lower FT plants (LAD, CAL) suffered under photosynthetic stress more than acclimated cvs. Even so; the reduction of chlorophyll content in the leaves upon cold has been recognized as a general phenomenon [136]. NDVI values in our experiments showed only small decrease in relation to controls which means there was only small reduction of chlorophyll content in leaves as well. However, the NDVI values may indicate that the deficiency of RuBisCO in low FT cultivars is not the cause. Nevertheless, we assume neither the NDVI nor the PRI values are directly suitable to distinguish among cvs in terms of FT. Similar results were obtained in field conditions by Chytky et al. [147] in 11 spring wheat cvs.

The sum of D45 and D47 DHNs were correlated the same way as the sum total of the DHNs accumulation. Consequently, we assume that the absence of some DHNs (especially D47, which was observed as the most abundant in the other cvs; Fig 2A in paper 2) is associated with lower FT in BEN. The strong correlation between the LT₅₀ and the accumulation of DHNs (i.e., the highest value observed in our experiments; $r = -0.815$) has already been confirmed in a number of papers for other plants than rapeseeds [63-65]. Additionally, the accumulation of DHNs in wheat has been considered as a reliable indicator of FT, where even reduced contrast genotypes in FT can be distinguished among from each other and even in controlled conditions (20° C) [148, 149]. Hence, the different accumulation of DHNs in cultivars could indicate that a stress-regulated pathway leading to the

accumulation of DHNs was fully functional but the pathway had different levels of regulation in cultivars with different FT. The accumulation of DHNs is only a small part of the very complex CA process (e.g., [41]). Therefore, other components of the CA process should be studied to have a higher probability for the phenotyping of crop FT. Consequently, the close relationships of LT₅₀ with other easily measurable and high hereditary physiological parameters found are important.

In some cases more interesting than simple observation of control and stress values is to compare their ratios stress/control values (S/C; Tab S1). In many cases, the statistically similar decrease of absolute values can be diminished after comparison of these ratios.

Table 1 Stress/control ratios of measured parameters in cold acclimated rapeseeds. Photosynthetic parameters, water use characteristics and reflectance indices of winter oilseed rape leaves in control conditions. The transpiration rate (E), stomatal conductance (GS), net photosynthetic rate (Pn), intracellular/intercellular CO₂ concentration (Ci/Ca), water use efficiency - calculated as Pn/E (WUE), intrinsic water use efficiency - calculated as Pn/GS (WUEi), photochemical reflectance index (PRI), and normalized difference vegetation index (NDVI). The control values of five cultivars of winter oilseed rape Benefit (BEN), Ladoga (LAD), Californium (CAL), Cortes (COR), and Navajo (NAV). The means ± SE are shown. The letters denote the statistical significance (as determined by the Tukey HSD test) of mean differences between control treatments. Only those marked with different letters differ significantly at p < 0.05. S/C is a ratio of stress to control values for each genotype and parameter (compare to Fig. 1). Asterisks denote significant differences (at p < 0.05) between control and cold treatments within a particular genotype.

Cultivar	E	S/C	GS	S/C	Pn	S/C	Ci/Ca	S/C
BEN	1.971 ± 0.04a	0.21*	340.417 ± 15.68ab	0.30*	6.142 ± 0.37c	0.70*	0.847 ± 0.011a	1.00
LAD	1.593 ± 0.06b	0.14*	179.500 ± 14.04c	0.36*	7.281 ± 0.24b	0.72*	0.747 ± 0.010b	0.78*
CAL	2.156 ± 0.06a	0.14*	353.063 ± 7.75a	0.19*	8.469 ± 0.32a	0.61*	0.837 ± 0.006a	0.89*
COR	1.571 ± 0.06b	0.22*	185.654 ± 15.35c	0.38*	7.235 ± 0.14b	0.97	0.732 ± 0.011b	0.65*
NAV	2.069 ± 0.07a	0.15*	294.250 ± 12.09b	0.21*	7.194 ± 0.16b	0.96	0.815 ± 0.013a	0.74*
	WUE	S/C	WUEi	S/C	PRI	S/C	NDVI	S/C
BEN	3.166 ± 0.23c	3.29*	19.166 ± 1.85b	2.23*	0.098 ± 0.002a	0.31*	0.749 ± 0.007c	0.98
LAD	4.644 ± 0.17a	5.16*	43.916 ± 3.10a	1.89*	0.100 ± 0.001a	0.16*	0.762 ± 0.001bc	0.92*
CAL	3.971 ± 0.18b	4.41*	24.107 ± 0.98b	3.22*	0.099 ± 0.002a	0.27*	0.771 ± 0.001ab	0.94*
COR	4.674 ± 0.15a	4.36*	42.313 ± 2.88a	2.40*	0.095 ± 0.001ab	0.32*	0.779 ± 0.002a	0.94*
NAV	3.523 ± 0.14bc	6.17*	25.056 ± 1.17b	4.55*	0.092 ± 0.001b	0.40*	0.752 ± 0.005c	0.96*

This research can also aim at appropriate timing and rate of cold deacclimation and the ability of oilseed rapeseeds to reacclimate, which are important components of winter survival of perennials in temperate zones. In association with the progressive increase in atmospheric CO₂, temperate winters are becoming progressively milder, and temperature patterns are becoming irregular with increasing risk of unseasonable warm spells during the colder periods of plants' annual cycle. Because deacclimation is mainly driven by temperature, these changes pose a risk for untimely/premature deacclimation, thereby rendering plant tissue vulnerable to freeze-injury by a subsequent frost. Research also indicates that elevated CO₂ may directly impact deacclimation [150]. Loss of freezing tolerance is additionally associated

with substantial changes in cell/tissue-water relations and carbohydrate metabolism; the latter also impacted by temperature-driven, altered respiratory metabolism.

4.1.3 Research limitations

Some limitations in this study can be seen in the way of artificial cultivation of rapeseeds in controlled conditions. From the theory (however, this fact we confirmed also in a reality) we know, that DHN accumulation of selected cultivars can be different in comparison of field to “chamber” results. Many factors play significant roles in DHN accumulation, carbohydrate storage and FT progress, e.g. photoperiod length and concomitant changes in temperatures, different temperature of roots + temperature influenced mineral uptake etc. Some of these factors, which many of them are detrimentally linked together, influence the adaptability of individual cultivars and therefore the accumulation or de-novo synthesis of protective compounds. Another limitation is that every cultivar has its threshold of vernalization. After the vernalization requirements are much fulfilled, the DHN accumulation continues to slowly decrease (in weeks) until the suitable conditions allow the plant to shift from vegetative to generative stage. The rapid decrease in DHN is mostly connected to energetic depletion and homeostasis disturbance. The precise vernalization requirements were not studied here, because the rapid (pre)selection technique was looked for. The study confirming the real impact of gasometry-related data upon cold, which should be studied in future on real changes of biomass via growth characteristics and/or allocation of nutrients within the plants.

4.1.4 Implications of finding for next research

The present study combines physiological and biochemical data to give a partial answer on the FT process in selected winter rape cultivars after CA. Future work should focused on detailed observations of DHN temporal dynamics, related to the developmental stages. The time-consuming direct evaluation of FT of the whole plants from field (and not only leaf segments from chamber) should be further involved in verification of DHN/FT correlation. However, in order to generalize such conclusions, as well as to be able to consider DHN accumulation, P_n , and WUE_i as reliable indirect indicators of FT in winter oilseed rape, it will be necessary to verify these relationships in a wider range of genotypes and data should be compared from both, fields and “chambers” cultivations. Qualitative differences in the range of accumulated dehydrin proteins can be explained either by different dehydrins accumulated or by a presence of different allelic variants for the same protein in the two genotypes. Also a detailed mass spectrometry analysis of individual DHN bands

should be done to be sure how many different DHNs is actually in each individual band and if there is a difference between cultivars. Mutants of selected DHN genes should be prepared to finally answered the question about individual DHN protein contribution into final FT and clarified the location of individual DHNs. The cultivar BEN-related mapping population can be very useful study material for dissecting the relationship between FT and metabolism.

We already did some additional research on selected cvs in terms of their response on the chlorophyll fluorescence level, and selected dehydrin genes (ERD 10, ERD 15, Cor 25, Bn 115) transcriptomics on younger and mature leaves were done (will be published later).

4.2 The different geographic origin of winter rapeseed cultivars is only a part of their adaptability to drought – proteomic profiles of water-savers and water-spenders are revealed

Notice: All figure and table numbers refer to original article included in 9.3. chapter. Results showed with “S” (e.g. Fig. S-1) are included in chapter 9.3.1 Supporting Information A. Materials S-8 to S-20 please find with online version of the paper.

4.2.1 Scientific novelty and theoretical significance of the results

The cultivar-dependent differences in *Brassica napus* L. seed yield are significantly affected by drought stress. The seeds filling stage is a less perceptive stage to moderate drought stress due to a sufficient and rapid translocation of assimilates from the stem [93]. However, this stage is feedback connected to sufficient stem assimilates and then to effective stem prolongation period. No surprise, to evaluate the degree of drought stress and its influence on individual cultivar (cv) we have selected this developmental stage for our research. Furthermore, in the warm, humid continental climate of The Czech Republic, different water-use patterns can be useful for drought adaptation of crops, as it is postulated for other similar countries [95-103, 151]. The so-called water-saver plants can maintain satisfactory yield in long-term drought conditions. On the other hand, in a quick drought the second group - water-spenders - showed delayed response to drought (so they seem to be less perceptive to actual water content in the soil profile) [102, 104-106].

Among the proteomics studies and reviews published on *Brassica* spp. to date [107-120], only two differential proteomic studies focused on *B. napus* drought stress response (comparative proteomics on seedling roots [121] and 6 weeks old plant leaves [122]). In this study, we screened – for the first time – for differentially regulated proteins in vernalized winter oilseed rapes under long-term drought stress. In addition, the fact, that four cvs were tested at the same time is not a common article perspective. Proteomics was carried out by

modification of 2DE, the two-dimensional difference gel electrophoresis (2D-DIGE). Since an internal standard composed of both tested variants is loaded, DIGE reduces gel-to-gel variability and thus improves the reproducibility. Unlike gel-free methods, gel-based methods provide a visual representation of the proteome, including intact protein maps in which protein gene products (isoforms) with modifications resulting from Mr and pI changes can be clearly seen [123].

In addition, chosen physiological, biochemical and molecular characteristics during a highly water-demanding stage – the stem prolongation period – are described. The analyses of gasometric, biochemical and water-related characteristics will provide evidence of how proteome modulates and/or is modulated during response to gradual drought stress. This complete information based on vernalized plants was missing so far. Detailed comparison of these changes will enable genotype separation according to their actual behaviour based on soil water content. Using this data, we can distinguish in detail between water-savers and water-spenders strategies. Additionally, proteomics data could evaluate the real impact of drought and the extent of genotype-based adaptability from the longer-time point of view and contribute to understanding the level of phenotype plasticity.

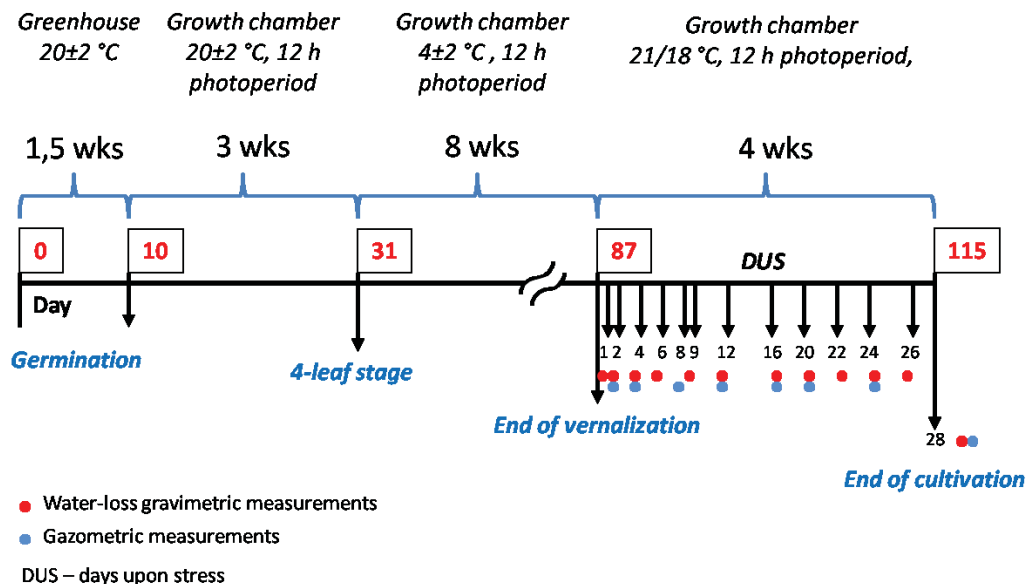
4.2.2 Discussion and synthesis into wider context

Here, the response of leaf proteome to long-term drought (28 days of drought after stress was imposed) was studied in cultivars (cvs): Californium (C), Cadeli (D), Navajo (N), and Viking (V). Analysis of twenty-four 2-D DIGE gels revealed 134 spots quantitatively changed at least 2-fold; from these, 79 proteins were significantly identified by MALDI-TOF/TOF. According to the differences in water use, the cultivars may be assigned to two categories: water-savers or water-spenders. In the water-savers group (cvs C+D), proteins related to nitrogen assimilation, ATP and redox homeostasis were increased under stress, while in the water-spenders category (cvs N+V), carbohydrate/energy, photosynthesis, stress related and rRNA processing proteins were increased upon stress. Taking all data together, we indicated cv C as a drought-adaptable water-saver, cv D as a medium-adaptable water-saver, cv N as a drought-adaptable water-spender, and cv V as a low-adaptable drought sensitive water-spender rapeseed. Proteomic data help to evaluate the impact of drought and the extent of genotype-based adaptability and contribute to the understanding of their plasticity. These results provide new insights into the provenience-based drought acclimation/adaptation strategy of contrasting winter rapeseeds and link data at gasometric, biochemical, and proteome level.

Highlights of the obtained results:

Two water-uptake strategies were found in stem-prolongation phase of rapeseeds: water-savers with high WUE and water-spenders with low WUE. In the water-savers group, accumulation of fewer proteins was changed in contrast to water-spenders. Water-savers showed better nitrogen metabolism, higher ATP conversion proteins, higher accumulation of ROS, signalling, and stress-related proteins. Water-spenders showed high numbers of down-accumulated proteins in carbohydrate/energy metabolism and photosynthesis. This mirrors the intracellular homeostasis disturbance. Two cultivars (Californium and Navajo) showed higher acclimation to drought by saving more available assimilates for growth despite different water-use mechanism.

Fig. 2 The work-flow of experimental design



1. Water-related characteristics revealed two groups of water-saving strategies

Water uptake data showed N+V with rapid and higher water uptake; C+D with moderate to lower water uptake (Fig. 1 in paper 3). The FTWS and NET estimations - based on different calculations - confirm that C+D are water-savers (respond conservatively according to Passioura [102]) and that N+V are water-spenders throughout the whole cultivations. However, both saver or spender strategies can be suitable in winter oilseed rape production in Central Europe. Further, they mirror the geographic origin and ergo, phenotypic traits of cvs (C+D – are French cvs suitable for a dry warmer climate, N – humid medium British climate, V – humid colder German climate).

2. Drought-affected biological processes revealed different strategies of individual genotypes

Physiological and biochemical characteristics (Fig. 2 A-L, Fig. S-4, both in paper 3) showed significant differences between control and stressed plants. Upon drought, the genotypes differed in their response, which implies genotype-based adaptation processes (detailed data in Supporting Information S-8 – S-10).

Slower uptake of water in cvs C+D was connected to slower growth in controls in comparison to faster water use group N+V. Under drought, FW and DW showed different accumulation patterns. DW in treated plants was significantly lower for V and D (Fig. S-4 D). In V, this result is connected to low Pn of this cv (Fig. S-2 E, F). In opposite, higher DW accumulation in treated C+N can be connected to higher accumulation of specific proteins and to better drought adaptability. While C profited from higher gs, E, and Pn under stress (Fig. S-2B, D, F), cv N probably profited from higher assimilates content during earlier phase of stem prolongation. The leaf areas of all cvs were not significantly different between cvs, so the DW accumulation was allocated more into stem. Gasometric and water-related data from the first to the last DAS were plotted in PCA (Fig. 3; correlation table in Supporting Information S-9). The data clustered cvs D+V and C+N together, which mirrored mainly the level of cultivar-based plasticity.

Faster water depletion under stress in N+V resulted in higher (more negative) OP, lower RWC and a rapid decrease of CO₂ assimilation (low WUE and WUE_i, Fig. S-2F, H). Less negative OP in C+D can be then connected to higher leaf area duration, and prolonged growth [100, 101, 103].

The results indicate that under fully saturated conditions, water-spenders (N+V) have high E and gs, and, subsequently, rapid growth. In opposite, in treated conditions, water-spenders rapidly decline all gasometric parameters; however, cv N keeps growing. Interestingly, in water-spenders, WUE and WUE_i are very low in both, control and treated conditions in opposite to water-savers C+D. Water-savers also showed higher values of gasometric parameters when treated/control plants are compared (Fig. S-3). This is in general agreement with Blum [103], that greater WUE is often associated with a slow rate of water use [103]. Blum's effective use of water is likely to be higher for a plant that maximises Pn dependently on the amount of available water.

3. Proteomic analysis of differentially abundant proteins under drought

The table of identified proteins (Supplementary Information S-13) contains all 62

identified protein spots. Among the 62 spots, several proteins with documented relation to abiotic stress were found (e.g., germin-like protein, TIR-NBS type disease resistance protein, HSP 60 and 70, fibrillin and many antioxidants and photosynthesis adaptation/acclimation proteins). The search for protein spots revealing large changes in protein relative abundance (more than ± 3 fold; $p < 0.05$) has revealed 24 protein spots (e.g., elongation factor G, epithiospecifier protein, 2-Cys peroxiredoxin, fibrillin, fructose-1,6-bisphosphatase precursor, ribulose-1,5-bisphosphate carboxylase/oxygenase activase, glyoxalase I, germin-like protein etc.). Desclos-Theveniau et al. [152] found some of these high abundant proteins as “residual” (with long-lasting turnover) to enable quick rapeseed senescence.

4. Functional categories and cellular localization of drought-responsive proteins

To investigate the functional and biological process-based identity of the individual differentially accumulated proteins (*DAP*), the 62 spots (45 *DAP*) were categorized into 7 major groups based on their putative biological functions and are common for all cultivars (Supplementary Information S-13): 1, Amino acid, nitrogen and sulphide metabolism/protein metabolism (4 *DAP*); 2, ATP interconversion (3 *DAP*); 3, Carbohydrate/energy metabolism (9 *DAP*); 4, Photosynthesis (11 *DAP*); 5, Redox homeostasis, ROS and signalling (9 *DAP*); 6, Stress and defence related (6 *DAP*); and 7, Transcription, translation, RNA processing (3 *DAP*). According to these seven functions, we can designate these groups to be most affected by long-term drought after vernalization in chosen winter oilseed rapes (Fig. S-11). These affected functional groups for the *Brassicaceae* family were also confirmed by others [108]. Please, if detailed information about each identified protein is required, see the data in the article.

Amino acid, nitrogen and sulphide metabolism/protein metabolism

In the *Brassicaceae* family, the nitrogen and sulphide compounds are very important for metabolism (thiols, glucosinolates, brassinosteroids) and biotic/abiotic stress adaptation [153, 154]. The drought acclimation process and salinity very often are associated with significant alterations in protein metabolism, because in oilseed rape, the negative effects of drought are quite similar to those for nitrogen limitation [155]. Almost all proteins in this category show a decreasing abundance of proteins in stressed cvs. This can also be a result of a “secondary” effect of drought – the decrease of soil water potential diminishes nutrient availability for plants.

ATP interconversion

Stress factors affect energy metabolism because plant adjustment to an altered

environment generally means an enhanced need for immediately (ATP) available energy. Significant differences were observed in all proteins of this category and showed a higher accumulation of ATP-related proteins in water-savers cvs and the opposite for spenders. This higher demand for ATP can be connected to better homeostasis maintenance under drought.

Carbohydrate/energy metabolism

Maintaining sufficient energy and balanced carbohydrate production is one of the most important pathways in all plants as sessile organisms. This group represents the largest part of identified proteins (17 %). Some carbon/nitrogen metabolism-related proteins identified here showed increased energy demand as well as enhanced cellular activities in the root tissue of rapeseed under drought [121].

When photosynthesis is declined due to drought, the export of photoassimilates from source to sink tissues is inhibited, too. Mueller et al. [88] found extracellular invertase activity the most correlated (low activity enables the export of sucrose from source to sink tissues) with RWC and OP changes in drought-stressed *B. napus*. Except for some proteins (TPI, RPI-A and MDH), all significant protein abundances in this category showed a decreased accumulation in both groups, and thus are probably not well suited for water-behaviour based selection. This general trend is contrary to Koh et al. [122] where non-vernalized seedlings of rapeseed were studied. The water-spenders showed a higher number of down-accumulated proteins and two proteins were up-accumulated (TPI, MDH). This particular result is in congruence with a decrease of ATP and photosynthesis-related protein categories for water-spenders.

Photosynthesis-related proteins

During drought stress, one of the possible ways to achieve development is to maintain the photosynthetic efficiency as high as possible, but avoid the energy and ion imbalances that result from the stress. This can lead to over-excitation of the photosynthetic apparatus, and consequently, to photo-oxidative damage [124]. The ability of plants to adapt and/or acclimate to adverse environments is related to the plasticity and resilience of photosynthesis, which, in combination with other processes, determines plant growth and development [34]. The photosynthetic apparatus should also be considered a major energy sensor because it is modulated by environmental cues and plays a major role in the regulation of phenotypic plasticity [156, 157]. This functional group represents the second largest group of identified proteins. In our study, water stress was slowly imposed by plant transpiration losses (28 days) and by reduction in the biochemical capacity for carbon assimilation and utilization that occurred along with restrictions in gaseous diffusion [124].

Redox homeostasis, ROS and signalling

Cellular redox homeostasis generates signals for the synthesis of defence enzymes and other antioxidant systems coping with stress. Together with photosynthesis and stress-related proteins, the redox homeostasis is likely to integrate all stresses into a cellular response with a stress-adaptive programme [157]. These somehow signalling and/or retrograde feedback signs can help to more deeply understand the behaviour of individual genotypes of winter oilseed rape. Our findings show the anti-oxidant system and ROS production may play a crucial role in dehydration tolerance of rapeseed and should be further examined in detail to help with selection of more stress-adaptable rapeseeds. The general pattern of antioxidant accumulations in our study is similar to other studies [69, 158]. Almost all proteins in this category were increased under drought, which contrasts to Koh et al. [122] study on six-week old rapeseed plants (without vernalization) under 14 days of drought. This could mean that after vernalization (but before flowering), other proteins become important for drought-related adaptation in rapeseed and/or can mirror differences in drought adaptation of the genotypes used.

Stress and defence related proteins

In our study, this group of proteins represents the third most abundant protein group in rapeseeds influenced by drought. Generally, oilseed rapeseed (and other crop plants of the family *Brassicaceae*) contain a unique defence system known as the glucosinolate-myrosinase system or the 'mustard oil bomb' [159] which is one of the best-studied plant defence systems. For its functionality of the glucosinolate-myrosinase defence system, the epithiospecifier protein (SSP 10 and 6410; AAY53488.1; ESP) is necessary. ESP converts glucosinolates at the expense of isothiocyanates [160]. From human health perspective, isothiocyanates are major inducers of carcinogen-detoxifying enzymes [161]. ESP showed a specific function in defence against herbivores and pathogens [160] and acts as a negative regulator of senescence. Both ESP's found in our study differ in pI and Mr and showed quantitative and qualitative changes in accumulation. These ESP gene product changes seem to play an unresolved role in drought-related studies and could be targets of further focus. ESP activity was found to be negatively correlated with the extent of formation of the sulforaphane in broccoli [162]. This also means that upon drought, the general accumulation of health-promoting phytochemicals can decrease. This fact is worthy of future study because the process of secondary metabolites production and drought-related glucosinolate composition and accumulation in response to abiotic stress is still not well known and has demonstrated conflicting trends [154].

Transcription, translation, RNA processing

These differentially accumulated proteins belong to two larger subgroups: rRNA processing and translation, and can be also involved in epigenetic changes under stress influence.

Genotype-based differences and overview on obtained data

For water-spenders, the drought treatment can be connected to higher metabolism disruption, mainly due to rapid water stress onset, supported by other observed changes, discussed above. Rapid water depletion caused more severe disturbance in intracellular water homeostasis in water-spenders (indicated by RWC, OP etc.). These differences profoundly affect cellular, energetic and nitrogen metabolism. Additionally, some measurements of treated plants at the 28 DAS may portray the changes beyond some metabolic threshold, which “edge” is also genotype-specific. Distinct onset of stress, and earliness of genotypes (C+N are intermediate cvs; D is an intermediate/late cv; V is an early cv) can also add some explanation. Additionally, both C+N are generally high yielding, high cold/frost and disease tolerant cvs with lower oil content in seeds (Table S-1). Similar unique responses (each cv used in this study behaves somehow unparalleled to other) for *B. napus* cvs are confirmed in other studies on drought [69, 88, 158].

The differences found in physiological response and in numbers of proteins responsible for the individual biological processes suggest the existence of diverse response strategies to drought between contrasting genotypes. Water-savers showed better nitrogen metabolism, higher ATP conversion proteins accumulation and thus more available energy, as well as higher accumulation of ROS, signalling, and stress-related proteins (Fig. 9). On the other hand, water-spenders showed a unique protein-accumulation response in carbohydrate/energy metabolism, photosynthesis-related, stress-related and rRNA processing proteins and high numbers of down-accumulated proteins, especially in carbohydrate/energy metabolism and photosynthesis, which is in congruence with water-related characteristics, low WUE and net photosynthesis.

The data from the experiments demonstrate that cultivars responded to progressive drought in different ways and at different levels. According to the present stage of knowledge, only the connection between gasometric, physiological and proteomic data seem to be effective in drought-tolerance selection for further targeted and environment-based breeding purposes. However, under a mixed climate profile, both water-use patterns (savers or spenders) can be appropriate for drought adaptation, so there is definitively no clear drought-tolerant “winner.” Interestingly, both groups – savers and spenders – contain drought tolerant genotypes (C+N). Therefore, if we have to decide which cv is more drought tolerant,

there has to be specified the rate of field dry-down, duration of stress and actual plant developmental stage.

4.2.3 Research limitations

The research limitation of this study is obvious for everybody who works with drought stress. That is all the studies have to calculate that drought can be sensed by plants in a different ways even in the case it is same in “technical and physical” point of view (e.g. water content, soil water potential, etc.). The genotype based differences and the phenotypic observations can be only a consequence of a stress and not a cause (plant reaction pattern) of stress. This means that we are sometimes not able to distinguish between what plants did to fight (e.g. active osmotic adjustment) OR what naturally happened because of a drought (passive response due to thickening of cytoplasm). Some cvs also react later than others do so at the particular sampling no or low stress response is observed. The particular limitation is also the lack of knowledge about the seed yield of used treated cultivars. However, for this kind of study, different water-stress regimes will give valuable information about cvs based adaptability.

The different behaviours of four cvs in terms of their gasometric responses to available water, biochemical adaptations, and differentially abundant protein profiles may be a mutual or even an exclusive result of several factors: 1) the complex influence of drought stress on cvs development and 2) the passport, genetic and other “invisible” backgrounds of selected cultivars.

Importantly, some of the biochemical measurements of treated plants at 28 DAS may portray the metabolism beyond the water shortage threshold and thus did not show different values between cvs. It has to be mentioned that this study does not reflect the possibly different genotype-based water uptake rate before stem prolongation or even after flowering. These missing data will actually complete the whole WUE of individual genotype-based scenarios.

4.2.4 Implications of finding for next research

It has to be mentioned that no found proteins were validated in “field” conditions, which seems to be a further implication goal for these data. Using these data in “associative transcriptomics” (or functional transcriptomics) or using genome-wide selection process are possible ways how to evaluate protein data. There are also questions about stress combinations (mentioned in the introduction) as they naturally occur in the fields (heat + drought + nutrient availability, etc.). Also, the mycorrhizal life and presence of “endophytic”

bacteria or fungi in plant tissues or even viruses in the soil or plants definitively plays an enigmatic role in “pot” related research. I am sure, in the near future many of published results can/will be questioned as incorrect, because no data and no inoculation was done for plants as naturally occur in the field experiments.

It can be concluded that no universal strategy can be suggested for breeding for an enhanced drought adaptability in oilseed rape since the individual strategies are often mutually exclusive, i.e., a given genetic material cannot adopt all of them. As the most crucial example, the „trade-off“ principle between water conservation and biomass accumulation as expressed in terms of WUE and EUW can be given [100, 163]. Therefore, knowledge on the environment (a season when drought occurs and the severity of the drought stress) appears to be crucial in the selection of the most suitable breeding strategy. This needs to be further researched by using sophisticated GxExM modelling. The crop model (e.g. APSIM) could provide the desired foresight to test *in silico* the particular plant strategy investigated in this study.

Some additional research is further planned with obtained data using associative transcriptomics (more than 350 accessions of *B. napus*, available at University of York, GB). The goal is to screen for possible donors of drought tolerance and/or sensitivity among cultivars.

4.3 The doubled-haploid regenerants derived from microspores of winter oilseed rapes can be used for selection of adaptable cultivars or new breeds

Notice: All figure and table numbers refer to original article included in 9.4 chapter.

4.3.1 Scientific novelty and theoretical significance of the results

The main aim of this study was to screen for proteins in winter oilseed rape embryos that are differentially regulated under osmotic stress and to explore in detail the proteomic basis of this response to low water availability during the embryo development period. The comparison between embryos proteome and drought-treated leaves proteome will showed, that there is only little similarity between these two systems. To authors best knowledge no similar comparative proteomic and transcriptomic analyses of microspores derived embryos was ever published. Cv Cadeli, however, showed high adaptability in both studies (article 3 and 4). So, if there is a possibility to select for more adaptable genotypes on the base of microspore biomass accumulation in influenced growth media, it is a question which needs to be solved.

4.3.2 Discussion and synthesis into wider context

This proteomic study is a first step for MDE confirmation as a suitable model for follow-up research in characterization of new breeds, new crossings, and can be used for phenotype-based selection tolerant to other worsening effects (other abiotic stresses and their combinations). Of course, the selected microspores have to be subsequently cultivated until seeds and evaluated in field conditions.

Two cultivars of winter oilseed rape were included in this analysis because they differ in their response to drought in mature plants and applied different drought-adaptation strategies. According to our previous study [164], cv. D is a middle drought tolerant water-saver, and V is drought-susceptible water-spender. The cv. V is considered as early and cv. D as intermediate/late cv [165].

Biomass accumulation in treated D was significantly higher (3-fold) than in V. This increase of biomass in PEG-treated cultivation media supports the idea about D with water-saver strategy and lower sensitivity to osmotic stress. Is a known fact, that cvs that are more adaptable also invest more energy into osmolytes and other protective molecules.

Proteins differentially abundant in cv Cadeli

The highest numbers of proteins significantly accumulated in D vs V belong to energy metabolism, redox homeostasis + signalling, transcription and also protein destination, storage and proteolysis. Cv D showed then effective energy-related pathways, higher sensing for ROS related changes in cell compartments, higher protein turnover, and/or synthesis and also increase in cell trafficking system. Below selected proteins with higher abundance in D are described.

Protein differentially abundant in cv Viking

The higher accumulation of proteins in cv V belong to four functional groups: AA, nitrogen and protein metabolism; ATP interconversion; stress and defence-related/detoxification; cell structure. This fact supports the idea about higher need for ATP and nutrient utilization, deeper stress impact, and increased stress-related cell structure changes. This also supports data from slower MDE growth. Below selected proteins with significant changes in V are described.

The comparison between leaf proteome under drought and MDE proteome under osmotic stress

The comparison of this study with our previous study Urban et al. [164], is based on arranging of differential leaf proteome under drought in stem-prolongation stage of both cvs D and V with MDE derived proteome under osmotic stress. The idea of this is to reveal possible association between these two very different studies and developmental stages to confirm possible role of MDE in early selection of more adaptable rapeseed cultivars.

Some proteins between studies are similar or even identical: glutamine synthetase, lactoylglutathione lyase (glyoxalase), atpA gene product, carbonic anhydrase, malate dehydrogenase 1, oxygen-evolving enhancer protein 1-2, L-ascorbate peroxidase, and glutathione S-transferase. Unfortunately, none of these proteins showed even similar patterns in protein accumulation. This result is also visible in comparison of standardized values of protein abundances between the two studies. Interestingly, in MDE only one small chain RuBisCO (CAA30290.1; SSP 2111; RuBisCO ssu precursor) was found in contrast to five rbcL (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit) and nine activases (chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase activase) in leaves. This is probably because of high sugar concentration in media.

Even though, drought is primarily manifested as osmotic stress (low water potential of soil, increasing xylem sap potential, etc.) the simple relationship between substrate dry-down and vapour-saturated low osmotic potential media is definitely not obvious. Also on the proteome level, we cannot easily compare these two cultivation methods and developmental stages. Generally, we can conclude that proteome response on MDE and leaves level are very different from mean abundances in each category or in comparison of Z-scores. In this study, only the MDE biomass accumulation (higher in D) significantly shows adaptability to drought on a non-proteomic level.

Confirmation of selected protein abundances with relative gene expressions

The mRNA expression values have shown their usefulness in a broad range of applications, including the diagnosis and classification of diseases, these results are almost certainly only correlative, rather than causative..

Nine proteins were selected according to their interesting accumulation behaviour across genotypes and treatments. Expression patterns of individual genes are shown in Fig. 9. Some of these gene relative expression profiles (7 DAS) are similar to protein abundance profiles (7 DAS): catalase, peroxiredoxin antioxidant and lactoylglutathione lyase (I call it

"the first group"). The other gene expression profiles are not similar to protein accumulation patterns. However, some genes showed similar expression only after 1 DAS as proteins accumulated after 7 DAS ("the second group"): glutathione S-transferase, ABA modulated tyrosine-phosphorylated protein, and lactoylglutathione lyase.

Alternatively, according to Greenbaum et al. [166], if there is definitively no correlation between mRNA and protein data, both quantities could be used as independent sources of information for use in machine-learning algorithms, for example, to predict protein interactions. On this base, we can postulate genes from first group suitable for gene-targeting at the same time as protein are extracted. The second gene group can probably be used for early selection of embryos in regards to their osmotic stress adaptability. Peroxiredoxin antioxidant and lactoylglutathione lyase can be used for early MDE selection as they are stress-related protein possibly increasing the adaptability of MDE to osmotic stress.

Conclusions

D showed highest number of unique energy-related proteins and then better ability for protein synthesis and adjunctive communication between compartments. On the other hand, V protein profile showed high need for energy (ATP) and increased need for nutrients with significant number of stress-related proteins and cell structure changes. Also, higher number or proteins dealing with non-aerobic metabolism (e.g. alcohol dehydrogenase) were found in V. In V more proteins were generally down-accumulated, which we believe is connected to higher stress and similar trend for V was observed also in our previous study Urban et al. [164]. Taking these findings together, cv D showed quick adaptation to osmotically activated PEG-infused cultivation media, while cv V showed alert-based response with clear signs of damage. Maintenance of the primary metabolism, oxidative stress and signalling seems to be a strategy for D osmotic tolerance. On the other hand, susceptibility might be related to maintenance of the energy consuming homeostatic equilibrium in V.

4.3.3 Research limitations

The upcoming new-generation breeding strategies (epigenetic breeding, stress-memory based breeding, gene editing etc.) are looking for stable but wide genomic variation within established crops. The microspore-derived embryos (MDE) seem to be one of appropriate ways how to manage this goal.

The microspore-derived embryos or regenerants are quite fragile materials. Although protocols for isolated microspore culture vary from laboratory to laboratory, the basic steps of growing donor plants, harvesting floral organs, isolating microspores, culturing and inducing

microspores, regenerating embryos, and doubling the chromosomes, remain the same. Then, the general limitation of this technique it is very time and space consuming, laborious and not cheap. To guard the microspore size is not easy, and to synchronize the microspore development is almost impossible. If some future research will be done, the focus on detailed characterization of protein profiles in control conditions between several cultivars should be evaluated to omit stress treatment and increase the throughput of the system.

4.3.4 Implications of finding for next research

Over the past few years, a large proportion of the research reports on isolated microspore culture have focused on cereal and Brassica species. For some of these species, isolated microspore culture protocols are well established and routinely used in laboratories around the world for developing new varieties, as well as for basic research in areas such as genomics, gene expression, and genetic mapping. Although these species are considered highly responsive to microspore culture, improvements in efficiency are still being made. However, with many species, isolated microspore culture is simply not yet efficient enough at producing DH plants to be cost-effective for breeding programs. There has been a recent resurgence of haploidy research with response being reported in some species once considered recalcitrant. As suggested by Ferrie et Caswell [167], future research programs aimed at elucidating pathways involved in microspore induction and embryogenesis will be of benefit, as will novel approaches to improve the efficiency of microspore culture for DH production. The development of molecular markers for use in determining the gametic origin of regenerated plants, irrespective of their ploidy, would also be beneficial. Finally, the rapid and cheap method for single microspore selection methodology has to be evolved. This allows selecting phenotypically interesting materials. Of course, the selected microspores have to be subsequently cultivated until seeds, crossed with elite parents and evaluated in field conditions.

5 Conclusions

The hypothesis 1) The lower rate of photosynthesis acclimation and lower accumulation of protective molecular components during cold acclimation will cause lower oilseed rapeseed frost tolerance **cannot be rejected**.

The hypothesis 2) The responsiveness of oilseed rape to drought (sensitivity or adaptation) is mainly based on different water-uptake strategies: water-savers will save water in the soil because of slower growth (and lower biomass accumulation). On the other hand, water-spender will have high growth rate and high water uptake. The highly energy-demanding homeostatic equilibrium will be more detrimental in water-spender, as they will deplete soil water more quickly and can undergo severe stress **cannot be rejected. Even though, the results are more complicated** - both strategies, due to e.g. more efficient proteomic profile in some cvs, can unexpectedly show higher dry weight biomass accumulation under drought.

The hypothesis 3) MDE technique is a reliable source of information about distinct cultivars. On the base of MDE-related proteomic results, there is a possibility to distinguish between cultivars if non-lethal osmotic stress is applied **cannot be rejected**.

Thesis main aim and all specific aims were fulfilled.

The responses of selected genotypes of oilseed rape to drought, cold and osmotic stress in different developmental changes were described in 2 papers and 1 manuscript. Experiments in this study lead to deeper understanding of winter oilseed rape behaviour in different environments. The same genotypes were used in almost all studies, allowing wide comparison and postulating further hypotheses. Additional research is planned to complete the knowledge, to test new hypotheses and to prepare more suitable outputs for further use (see chapter 4). All data are based on understanding the physiological and biochemical parameters, accompanied by proteomic-based approach.

The conclusions from physiological and proteomic response to cold:

- The relationship between FT, DHN accumulation, and gasometric acclimation was significant. The presented study indicates that during acclimation of winter rapeseed to cold, the complex interaction of photosynthesis acclimation and DHN accumulation occurs. However, no direct mechanistic link between Pn and FT was found - photosynthesis rather provides the energy necessary for the cellular changes (DHNs)

and other proteins accumulation) required for higher FT.

- The procedures for DHN visualization were optimized for oilseed rapeseeds.
- The absence of some DHNs (especially D47, which were observed as the most in other cultivars) is associated with lower FT in cultivar BEN. This was observed only for BEN cultivar and can be used in further detailed studies of DHN accumulation and DHN relationship with other parameters.
- For the first time, the specific DHN D97 (dehydrin around 97kDa) was shown to be accumulated in rapeseed. Other DHNs were shown to have a “dual” nature not detected in previous studies.
- Interestingly, the rate of Pn in highly FT cultivars (COR and NAV) does not respond to E or GS values after full acclimation (as is common in controls). This decrease in the sensitivity of Pn to the changes in transpiration and stomatal opening can be used as a selective trait as early as after 4 weeks of an acclimation period.
- All genotypes which showed higher FT (COR, NAV) also showed higher net photosynthesis after CA.
- However, in order to generalize such conclusions, as well as to be able to consider DHN accumulation, Pn, and WUEi as reliable indirect indicators of FT in winter oilseed rape, it will be necessary to verify these relationships in a wider range of genotypes. Also, data from both fields and growth-chambers cultivations should be compared.

The effect of progressive drought in stem-prolongation stage on selected oilseed rapeseeds belonging to different geographic origin:

- Two water-uptake strategies were found in the stem-prolongation phase of rapeseeds: water-savers with high WUE and water-spenders with low WUE.
- The 62 differentially identified proteins responding to drought were identified, belonging to 7 functional groups, of them, carbohydrate/energy metabolism, anti-oxidant + ROS and stress/defence related proteins play the most important role in drought-related acclimation of rapeseed.
- In contrast to water-savers, faster water depletion under stress in water-spenders resulted in more negative OP, lower RWC and a rapid decrease of CO₂ assimilation (low WUE and WUEi).
- Under fully saturated conditions, water-spenders (N+V) have high E and gs, and subsequently, rapid growth. In contrast, in treated conditions, water-spenders rapidly decline all gasometric parameters; however, cv N keeps growing.

Interestingly, in water-spenders, WUE and WUE_i are very low in both control and treated conditions, in contrast to water-savers C+D.

- In the water-savers group (cvs C+D), proteins related to nitrogen assimilation, ATP and redox homeostasis were increased under stress.
- In the water-spenders category (cvs N+V), carbohydrate/energy, photosynthesis, stress related and rRNA processing proteins were increased upon stress.
- Taken together, the data indicated:
 - cv C as a drought-adaptable water-saver
 - cv D as a medium-adaptable water-saver
 - cv N as a drought-adaptable water-spender
 - cv V as a low-adaptable drought-sensitive water-spender rapeseed.
- Highest drought acclimation was shown by those genotypes that saved available assimilates for growth in drought, interestingly, despite their different strategy (C + N). This was supported by higher DW accumulation in treated C+N.
- The results mirror the geographic origin of cvs (C+D – are French cvs suitable for a dry warmer climate, N – humid medium British climate, V – humid colder German climate).
- The protein spots analysis revealed candidate proteins which can be further tested as tolerant (or adaptable) cvs markers:
 - Fructose-bisphosphate aldolase 2, Triosephosphate isomerase, Chloroplast beta-carbonic anhydrase, Ferredoxin-NADP reductase, Fibrillin, and Chloroplast elongation factor tub.
- The differences found in physiological response and in numbers of proteins responsible for the individual biological processes suggest the existence of diverse response strategies to drought between contrasting genotypes.
- According to the present stage of knowledge, only the connection between gasometric, physiological and proteomic data seem to be effective in drought-tolerance selection for further targeted and environment-based breeding purposes.

To show the proteome profile of osmotically treated microspore-derived embryos:

- To investigate the functional and biological process-based identity of the individual *differentially accumulated proteins* (DAP), the 63 spots (= 61 DAP) were categorized into 8 major groups based on their putative biological processes:
 - 1, Amino acid, nitrogen and sugars metabolism/protein metabolism (13 DAP)
 - 2, ATP interconversion (1 DAP)

- 3, Energy metabolism (glycolysis, gluconeogenesis, TCA pathway, respiration photosynthesis) (24 DAP)
- 4, Redox homeostasis, ROS and signalling (7 DAP)
- 5, Stress/defence-related/detoxification (8 DAP)
- 6, Transcription (DNA/RNA processing and binding) and protein synthesis (2 DAP)
- 7, Protein destination and storage, proteolysis (5 DAP)
- 8, Cell structure (1 DAP).
- These groups are then the most affected by PEG-related osmotic stress in chosen winter oilseed rape MDEs.
- The highest numbers of proteins significantly accumulated in osmotica-tolerant cultivar D belong to energy metabolism (especially glycolysis), redox homeostasis + signalling (phospholipases, MAPK4), transcription and also protein destination, storage and proteolysis.
 - MDE biomass accumulation in treated D was significantly higher (3-fold) than in V.
 - Cv D showed effective energy-related pathways, higher sensing for ROS related changes in cell compartments, higher protein turnover, and/or synthesis and also an increase in the cell trafficking system.
- The higher accumulation of proteins in cv V belong to four functional groups: AA, nitrogen and protein metabolism; ATP interconversion; stress and defence-related/detoxification; cell structure.
 - This fact supports the idea about a higher need for ATP and nutrient utilization, deep stress impact, and increased stress-related cell structure changes.
 - Also, a higher number of proteins dealing with non-aerobic metabolism (e.g. alcohol dehydrogenase) were found in V.
- Comparison of decreased protein in individual genotypes shows dissimilarities between MDE and drought-influenced leaves proteomes (comparison with data in paper 3) for both cvs especially in redox homeostasis and stress/defence proteins.
 - However, some proteins are similar between studies: glutamine synthetase, lactoylglutathione lyase (glyoxalase I), atpA gene product, carbonic anhydrase, malate dehydrogenase 1, oxygen-evolving enhancer protein 1-2, L-ascorbate peroxidase, and glutathione S-transferase.
 - Unfortunately, none of these proteins showed even similar patterns in protein

accumulation.

- Generally, we can conclude that proteome responses on MDE and leaves level are very different from absolute abundances in each category or in comparison of normalized Z-scores.
- Gene relative expression profiles (7 days after stress) are similar to protein abundance profiles (7 days after stress) for: catalase, peroxiredoxin antioxidant and lactoylglutathione lyase (we call it "the first group").
 - However, the other gene expression profiles are not similar to protein accumulation patterns.
 - Some genes showed similar expression only after 1 DAS as proteins accumulated after 7 DAS ("the second group"): glutathione S-transferase, ABA modulated tyrosine-phosphorylated protein, and lactoylglutathione lyase.
 - We can postulate genes from first group suitable for gene-targeting at the same time as proteins are extracted. The second gene group can be used for early selection of embryos in regards to their osmotic stress adaptability.
- Taking these findings together, cv D showed quick adaptation to osmotically activated PEG-infused cultivation media. Then, maintenance of the primary metabolism, oxidative stress and signalling seems to be a strategy for osmotic tolerance in MDE stage.
- Cv V showed alert-based response with clear signs of damage. Its susceptibility might be related to maintenance of the energy-consuming homeostatic equilibrium in V.
- Because of the differences found in numbers of proteins responsible for separate biological processes, it is reasonable to believe in the existence of diverse response strategies to osmotic stress between chosen contrasting genotypes. This was also proven in drought-related differential leaf proteomics of four cvs in Urban et al. [164].

6 Summary of the results

In Ph.D. thesis, different developmental stages of oilseed rape and three main stresses were addressed by a combination of different approaches.

In paper 1, we review the contribution of proteomic studies to elucidate biological mechanisms underlying stress response in temperate crops, with special attention paid to proteins revealing differential responses between crop genotypes with differential stress tolerance levels. We used this information to design our further research. Paper 2 revealed the importance of energy-related changes (photosynthetic apparatus acclimation, accumulation of protective proteins) in sufficient frost-tolerance of oilseed rapes. The benefit of more complex drought-related studies, based on a comparison of water-related strategies, geographical origin with biochemical and proteomic approach was shown in paper 3. Microspore techniques (paper 4) showed an interesting and valuable approach for further phenotype/proteome based evaluation of materials.

Based on obtained results we propose some traits and techniques for oilseed rape phenotyping against frost and drought stress that can be used in oilseed rape breeding programmes focused on higher adaptable materials:

1) Immunoblotting based DHN accumulation as a technique suitable to select higher FT cvs and new breeds.

2) Use of growth characteristics, together with water-related traits and strategies in an experiment with slow dry-down (weeks). The breeding should also be based on geographic origin of used components.

3) Selection of genotypes with lower decrease of Pn (in comparison to controls) upon cold acclimation temperature (4° C) 3-4 weeks after vernalization begins.

4) The leaf proteome analysis of drought-stressed plants revealed candidate proteins that can be further tested for adaptable cvs: Fructose-bisphosphate aldolase 2 (NP_568049.1), Triosephosphate isomerase (NP_179713.1), Chloroplast beta-carbonic anhydrase (ADI52861.1), Ferredoxin-NADP reductase (BAD07827.1), Fibrillin (NP_192311.1), and Chloroplast elongation factor tub (XP_002869935.1).

4) MDE proteins suitable for early-selection of adaptable embryos are: RuBisCO ssu precursor (CAA30290.1), UDP-glucose 6-dehydrogenase (NP_197053.1), ABA modulated tyrosine-phosphorylated protein (CDY40342.1), PurALPHA-1 (XP_002862885.1), Glutathione S-transferase (XP_013592836.1), and Lactoylglutathione lyase (BAF81517.1).

Unfortunately, some of the proposed methods are neither cheap nor highly throughput. In the near future the laboratory techniques able to select materials with sufficient adaptability to upcoming climate changes needs to be still developed and validated in the field conditions. No universal strategy can be suggested for breeding for enhanced drought adaptability in oilseed rape without knowledge of targeted agro-ecosystem and because the individual strategies are often mutually exclusive, i.e., a given genetic material cannot adopt all of them. The crop models (e.g. APSIM, SSM) could provide the desired foresight to test in silico the particular plant strategy investigated in this study.

7 Summary of the results – in Czech

V doktorské disertační práci jsem se pokusil několika přístupy zodpovědět problematiku stresové odpovědi u různých vývojových stádií brukve řepky olejky.

V prvním přehledovém článku jsme porovnali výsledky proteomických studií stresovaných plodin mírného pásma se zaměřením na diferenční proteomické odpovědi různě odolných genotypů. Tyto informace jsme poté použili i k návrhu vlastního výzkumu. Druhý článek objasnil důležitost změn, souvisejících s energetickými změnami metabolismu (aklimace fotosyntézy, akumulace protektantů) k dosažení dostatečné mrazuvzdornosti řepky olejky. Ve třetím článku byl ukázán přínos studie, založené na porovnání strategie využití vody, biogeografického původu odrůd s biochemickými a proteomickými daty pro získání komplexní představy o chování odrůd během sucha. Kultura mikrosporových embryí (článek č. 4) také přinesla zajímavé a cenné výsledky pro další fenotypové či proteomické hodnocení šlechtitelských materiálů.

Na základě získaných výsledků doporučujeme některé znaky a techniky pro fenotypování odolnosti k mrazu a suchu u řepky olejky, které mohou být použity ve šlechtitelských programech, zaměřených na více adaptabilní materiály:

1) Stanovení akumulace DHN pomocí imunoblottingu je metoda vhodná pro výběr vysoce mrazuvzdorných odrůd či novošlechtěnců řepky

2) Růstové charakteristiky, společně se znaky vodního hospodaření v pokusech s pomalým vysycháním v řádu týdnů. Šlechtitelé by také měli brát v potaz původ používaných komponent.

3) Selektce odrůd a materiálů s nižším poklesem Pn mezi kontrolními a chladově aklimovanými rostlinami po min. 3-4 týdnech působení jarovizační teploty (4°C).

4) Analýza listového proteomu suchem stresovaných rostlin umožnila vybrat některé kandidátní proteiny, které mohou být dále testovány a sledovány u adaptabilních odrůd: Fruktóza-bisfosfát aldoláza 2 (NP_568049.1), triózafosfát izomeráza (NP_179713.1), chloroplastová beta-karboinic anhydráza (ADI52861.1), ferredoxin-NADP reduktáza (BAD07827.1), fibrillíny (NP_192311.1), chloroplast elongation faktor tub (XP_002869935.1).

4) Technika mikrosporových embryí umožnila vybrat proteiny, které jsou vhodné pro ranou selekci adaptabilních embryí: RuBisCO ssu prekurzor (CAA30290.1), UDP-glukóza 6-dehydrogenáza (NP_197053.1), ABA modulovaný tyrozin-forforylovaný protein (CDY40342.1), purALPHA-1 (XP_002862885.1), glutathione S-transferáza (XP_013592836.1), a lactoylglutathione lyáza (BAF81517.1).

Některé z použitých či navrhovaných metod bohužel nejsou ani levné ani objemově propustné. Stále tedy existuje snaha najít vhodnou laboratorní metodu, schopnou selektovat materiály s dostatečnou adaptabilitou k nadcházejícím klimatickým změnám, jejíž výsledky by byly porovnatelné s tolerancí, dosaženou v polních pokusech. Obecná strategie šlechtění k vyšší odolnosti k suchu, která by mohla být šlechtiteli využita – nemůže být aplikována bez znalosti cílového agro-ekosystému a kvůli tomu, že některé fyziologické znaky odolnosti jsou vzájemně neslučitelné. Rostlinné modely (např. APSIM, SSM) ale mohou poskytnout dostatečnou pravděpodobnost úspěchu při testování odolnosti genotypů za použití dat a strategií z této studie.

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9 Attachments

9.1 Paper 1

Title: Biological Networks Underlying Abiotic Stress Tolerance in Temperate Crops—A Proteomic Perspective

Document type: review

Authors: Kosova, K., Vıtamvas, P., Urban, M. O., Klıma, M., Roy, A., Prasıl, I.

Journal specification: *International Journal of Molecular Sciences*, 2015, 16 (9): 20913 – 20942

Supplementary data available at:

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9.2 Paper 2

Title: Significant relationship between frost tolerance and net photosynthetic rate, water use efficiency and dehydrin accumulation in cold-treated winter oilseed rapes.

Document type: original article

Authors: Urban, M O., Klíma M., Vítámvás P., Vašek J., Kučera V.

Journal specification: *Journal of Plant Physiology*, 10.1016/j.jplph.2013.07.012, 2013. 170 (18): 1600 - 1608.

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9.3 Paper 3

Title: Proteomic and physiological approach reveals drought-induced changes in rapeseeds: Water-saver and water-spender strategy

Document type: original article

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9.4 Paper 4 –not published results, manuscript

Title: Proteomic analysis of two drought-tolerance contrasting oilseed rape microspore-derived embryos showed different profile after drought-simulation via infusion of polyethylene-glycol into cultivation media.

Document type: original article, manuscript

Authors: Urban MO, Jelínková I, Klíma M, Renaut J, Planchon S, and Vítámvás P.

Review

Biological Networks Underlying Abiotic Stress Tolerance in Temperate Crops—A Proteomic Perspective

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Abstract: Abiotic stress factors, especially low temperatures, drought, and salinity, represent the major constraints limiting agricultural production in temperate climate. Under the conditions of global climate change, the risk of damaging effects of abiotic stresses on crop production increases. Plant stress response represents an active process aimed at an establishment of novel homeostasis under altered environmental conditions. Proteins play a crucial role in plant stress response since they are directly involved in shaping the final phenotype. In the review, results of proteomic studies focused on stress response of major crops grown in temperate climate including cereals: common wheat (*Triticum aestivum*), durum wheat (*Triticum durum*), barley (*Hordeum vulgare*), maize (*Zea mays*); leguminous plants: alfalfa (*Medicago sativa*), soybean (*Glycine max*), common bean (*Phaseolus vulgaris*), pea (*Pisum sativum*); oilseed rape (*Brassica napus*); potato (*Solanum tuberosum*); tobacco (*Nicotiana tabacum*); tomato (*Lycopersicon esculentum*); and others, to a wide range of abiotic stresses (cold, drought, salinity, heat, imbalances in mineral nutrition and heavy metals) are summarized. The dynamics of changes in various protein functional groups including signaling and regulatory proteins, transcription factors, proteins involved in protein metabolism, amino acid metabolism, metabolism of several stress-related

compounds, proteins with chaperone and protective functions as well as structural proteins (cell wall components, cytoskeleton) are briefly overviewed. Attention is paid to the differences found between differentially tolerant genotypes. In addition, proteomic studies aimed at proteomic investigation of multiple stress factors are discussed. In conclusion, contribution of proteomic studies to understanding the complexity of crop response to abiotic stresses as well as possibilities to identify and utilize protein markers in crop breeding processes are discussed.

Keywords: abiotic stresses; temperate crops; proteomics; protein functions; stress tolerance; multiple stress; protein markers

1. Introduction

Abiotic stress factors, especially cold (low temperatures), drought and salinity, profoundly affect crop growth and development in temperate climate zones which can be defined as regions lying between tropical and polar zones, *i.e.*, between the Tropic of Cancer and the Arctic Circle in the northern hemisphere and between the Tropic of Capricorn and the Antarctic Circle in the southern hemisphere, respectively, with relatively moderate temperatures and significant temperature differences between summer and winter seasons [1]. The major crops grown in temperate climate include cereals such as common wheat (*Triticum aestivum*), durum wheat (*Triticum durum*), barley (*Hordeum vulgare*) and maize (*Zea mays*), potato, leguminous plants such as soybean and alfalfa, sugar beet, oilseed rape, flax, sunflower, tobacco, vegetables such as chicory, pea, tomato and watermelon, and woody crops such as grapevine. Abiotic stress factors represent the major constraints limiting agricultural production and reducing crop yield. Under global climate change, the risk of damaging effects of abiotic stress factors on crop production increases. The risk includes not only an increased aridization and salinization in several regions such as Australia, the Middle East, and southern Europe (Spain), but also an increased frequency of frost damage in temperate areas due to rapid temperature shifts during winter and early spring seasons as a consequence of freeze-thaw cycles [2].

As living organisms, plants tend to maintain homeostasis in their bodies. Plants are poikilothermic organisms, *i.e.*, they cannot actively regulate temperature of their bodies. Basic principles of plant water uptake from soil are also passive as they are governed by differences between soil and plant cell water potential. However, plants actively exchange energy, water, and thousands of chemical compounds between themselves and the environment. Therefore, plants sense changes in their environment and respond to them in order to prevent damage of their bodies (Figure 1). Plant stress response is a dynamic process in which several phases could be distinguished—an alarm phase, an acclimation phase, a resistance phase, an exhaustion phase when stress lasts too long or is too severe, and a recovery phase after a cessation of a stress factor which leads to an establishment of a novel homeostasis [3–5]. Each phase of plant stress responses is aimed at an establishment of novel homeostasis under altered environmental conditions and is therefore accompanied by profound alterations in plant cellular composition. Recently, a boom of high-throughput separation and identification techniques has enabled

researchers to study plant cellular responses in a more complex way using so-called “omics” approaches including structural and functional genomics, transcriptomics, proteomics, and metabolomics. Proteins represent a crucial component of plant stress response since they are directly involved in plant cell structure and metabolism [5]. They are products of genes, but they are much closer to the resulting phenotype since they act as direct effectors of the phenotype, *i.e.*, they constitute plant cell structure and actively participate on metabolism of all cellular components. The total of all proteins in a given tissue at a given time—proteome—is uniquely variable. Unlike the genome, which is only one for a given organism, there are infinite proteomes which depend on an organism’s growth and developmental stage, plant tissue, and cell type as well as on ambient growth conditions. Moreover, one gene can give rise to various protein products due to mechanisms of posttranscriptional (alternative RNA splicing, RNA editing, *etc.*) and posttranslational modifications (PTMs—phosphorylation, acetylation, methylation, ubiquitination, myristoylation, *etc.*). Therefore, the total number of distinct proteins synthesized by a given organism can be several orders higher than the total number of genes encoded by a genome of the given organism. Considering the splendid variety of proteomes, a plant thus possesses an efficient tool to modulate its response to specific environmental conditions.

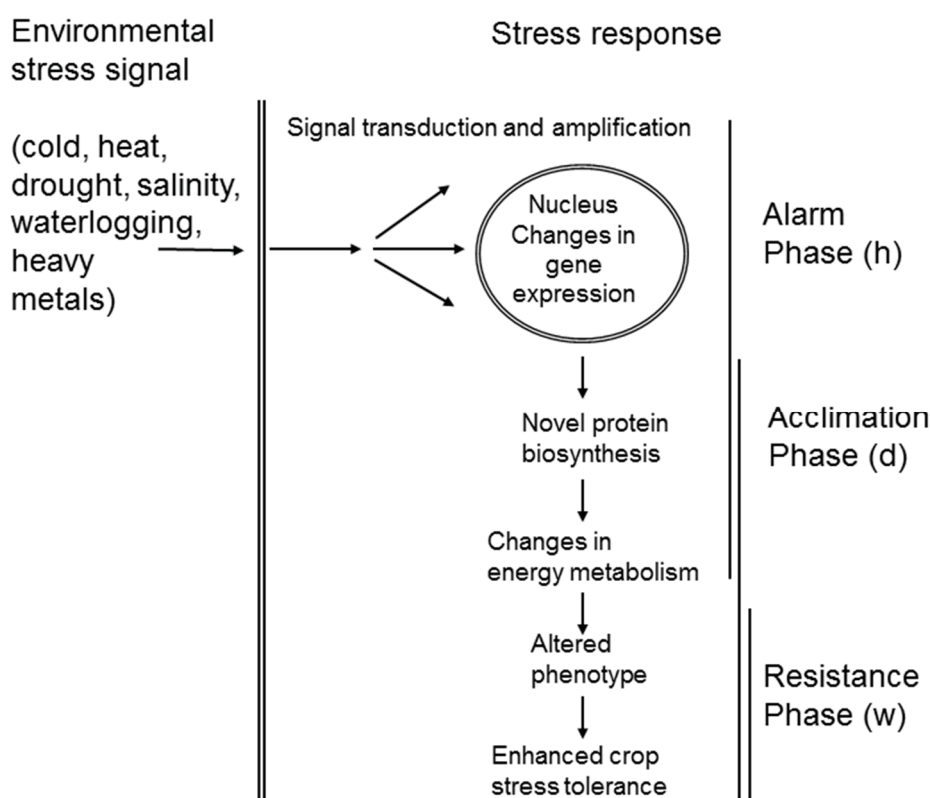


Figure 1. A schematic representation of the dynamics of plant stress perception and stress response at cellular level. The first phase of plant stress response, an alarm phase, is usually very short (hours; h) with respect to the following acclimation phase (days; d) and resistance phase (weeks; w). There are also significant overlaps between the individual processes and phases with respect to their timing.

During the past two decades, high-throughput plant proteomic studies have revealed a boom due to technological advancement in both gel-based and gel-free protein separation and relative quantification

techniques (2D-DIGE, iTRAQ, label-free MS/MS protein quantification) and publication of complete genome sequences of model plants (*Arabidopsis thaliana*) as well as major crops (rice, potato, soybean, maize, barley, common wheat). These factors enabled the researchers to study plant responses to internal and external (environmental) factors including abiotic stresses at proteome level. Study of proteome response of major crop plants to environmental stress is of high interest due to elucidation of biological mechanisms and identification of crucial proteome components underlying an enhanced crop stress tolerance. Results of proteomic studies dealing with proteome response to major abiotic stress factors in temperate crops were already reviewed in several papers including large-scale comprehensive reviews on proteomics of abiotic stresses in crop plants [5,6] as well as more specialized reviews dedicated to specific stress factors, crops or cellular fractions such as reviews on salinity proteomics [7–9], subcellular proteomics of crop plants exposed to stress [10], proteomics of heavy metal stress [11], proteomics of abiotic stresses in wheat and barley [12,13], soybean proteomics [14], proteomics of flooding stress in soybean [15], and others. The aim of the present review paper is to summarize recent results of proteomic studies obtained in major crop plants grown in temperate climate regions and subjected to major abiotic stresses—drought, salinity, cold, frost, waterlogging, and heavy metal stress. The major focus of the review is on contribution of proteomic studies to elucidation of biological mechanisms underlying stress response in temperate crops, with special attention paid to proteins revealing differential responses between crop genotypes with differential stress tolerance levels as well as proteomic studies dealing with the effects of multiple stress factors. In conclusion, future challenges in proteomic studies focused on elucidation of protein roles under stress are discussed and possible applications of proteomic results in crop breeding programs aimed at an improvement of crop stress tolerance are suggested.

2. A Brief Summary of Proteomic Studies on Stress Response in Temperate Crops

Common wheat (*Triticum aestivum*) is the most grown crop worldwide with the third highest total production of ca. 716 million metric tons [16] and its production is still dominant in temperate climate zone although an introduction of photoperiod-insensitive genotypes has enabled wheat production also in tropical zones. Maize, potato, and barley are the second, fourth, and fifth most produced crops worldwide, respectively, with a dominant production also in temperate climate zone.

Unlike model plants such as *Arabidopsis thaliana* and rice, a complete genome annotation is not available for many crops although recently, draft genome annotations were published for major crops grown in temperate climates including maize [17], potato [18], barley [19], common wheat [20], and soybean [21], common bean [22], tobacco [23], tomato [24], oilseed rape [25], sugar beet [26], watermelon [27], and grapevine [28].

Most proteomic studies have dealt with most cultivated cereal crops including common wheat, barley, maize, potato, and soybean whose whole genome sequences are already publicly available. However, several proteomic studies on other temperate crops are being published including field crops such as durum wheat [29,30], Indian mustard (*Brassica juncea*) [31], sunflower [32,33], flax [34,35], leguminous crops such as alfalfa [36–38], and white lupin [39], and vegetables such as chicory [40], and pea [41,42]. The major abiotic stresses studied include temperature stress (cold, frost, heat), water stress (drought, waterlogging), osmotic stress (polyethylene glycol—PEG), salinity, imbalances in

mineral nutrition, and heavy metal stress. An overview of proteomic studies on temperate crops subjected to abiotic stresses listed above is given in Table S1.

3. Dynamics of Crop Stress Response at Proteome Level

3.1. Alarm Phase

Stress Signaling and Gene Expression

An ambient cue is recognized by a plant cell as a signal when it leads to significant changes in physical properties (changes in the fluidity of phospholipid molecules in plasma membrane bilayer under low or high temperatures) or chemical composition of the ambient environment (e.g., dehydration stress or salinity leading to a decrease of water potential or an increase in Na^+ concentration in ambient soil solution). The changes in plasma membrane physico-chemical properties lead to conformational alterations of plasma membrane-associated peripheral or integral signaling protein complexes (e.g., two-component histidine kinases with respect to cold [43,44]; SOS1/SOS2/SOS3 complex in Na^+ signaling; reviewed in [45,46]). The initial signal is then transferred and amplified by several second messengers to the nucleus where the signal induces changes in gene expression leading to alterations in plant transcriptome, proteome and metabolome underlying an active plant stress response. At proteome level, especially when using 2DE based approaches, alterations in protein abundance are scarcely detected due to a relatively low abundance of signaling proteins with respect to other cellular proteins and due to the rapidity of their changes during an alarm phase of stress response. However, at least some signaling proteins such as components of mitogen-activated protein kinase (MAPK) cascade, calcium signaling (calmodulin, calnexin), components of heterotrimeric plasma membrane-located G proteins, phospholipases C and D (PLC, PLD), were detected in stress-treated plants, especially under drought and salinity [47–49]. It was proposed that Ca^{2+} signaling may affect cellular Na^+/K^+ homeostasis via SOS1/SOS2/SOS3 complex [45] and plays also an important role in sensing of osmotic stress [50]. Phospholipases cleave small molecules from plasma membrane phospholipid heads which then act as second messengers. Protein phosphorylation plays an important role in the activity of several signaling proteins—a differential phosphorylation level of not only signaling proteins (MAPK, calcium-dependent protein kinase CDPK, sucrose non-fermenting-related kinase SnRK2, protein phosphatase PP2C), but also transcription factors (ABI5), transport proteins (aquaporins, H^+ -ATPase) and protective proteins (COR/LEA) was found in drought-treated wheat [51] and PEG treated common bean [52]. The 14-3-3 proteins are known as regulatory proteins which can bind several signaling proteins, cell cycle regulating kinases and ion transporters (H^+ -ATPase, K^+ channels) depending on their phosphorylation status; 14-3-3 proteins thus significantly modulate plant stress response [53]. An increase in 14-3-3 proteins was found in copper- and water-stressed wheat [37,54–58], barley [59], in PEG-stressed soybean plasma membrane fraction [50], in salt-stressed maize [49], and others.

In the nucleus, the signal is transformed into the changes in gene expression. Changes in several transcription factors as well as other regulatory proteins such as glycine-rich RNA binding proteins or lectins such as VER2 were found in proteomic studies aimed at wheat response to a long-term cold treatment affecting plant development [60–63]. An increase in bHLH transcription factor was found in

salt-stressed soybean seedlings [64]. The abundance and activity of several transcription factors and regulatory proteins is modulated by several phytohormones upregulated during the alarm phase of stress such as ABA (AREB/ABF; MYB, MYC transcription factors), JA (glycine-rich RNA binding proteins, lectin VER2), SA, and others [62,65].

3.2. Acclimation Phase

3.2.1. Protein Metabolism

Changes in gene expression are coupled with changes in protein metabolism including both protein biosynthesis and degradation. Plant adjustment to an altered environment requires myriads of novel proteins to be synthesized as well as myriads of proteins to be degraded. Therefore, several changes in the abundance of ribosomal proteins include proteins belonging to both eukaryotic and prokaryotic-type (mitochondrial and plastidic) ribosomal subunits. For example, an increase in ribosomal protein L39 involved in accuracy of translation in drought-treated grapevine was found by Vincent *et al.* [66]. A decrease in chloroplast 30S ribosomal protein S10 indicates a down-regulation of chloroplast protein biosynthesis in salt-sensitive canola cultivar Sarigol since protein S10 seems to be crucial for tRNA binding to ribosomal surface and the stability of 30S ribosomal subunit [67]. Moreover, differential abundance of several eukaryotic translation initiation and elongation factors was found. However, the observed change may be related also to processes other than protein biosynthesis. For example, eukaryotic translation initiation factor 5A (eIF5A) reveals multiple regulatory roles in cell cycle regulation—different forms of eIF5A are proposed to affect a switch between cell proliferation and cell death [68]. A lower decrease of eIF5A3 isoform in salt-treated *Triticum aestivum* × *Thinopyrum ponticum* hybrid with respect to its *T. aestivum* parent indicates a higher anti-senescence ability of salt-tolerant hybrid compared to salt-sensitive parent under salinity [54]. Factor eIF5A was also detected in salt-tolerant oilseed rape Hyola 308 while it was absent in salt-sensitive cultivar Sarigol under salt stress [67]. Moreover, alterations in several regulatory proteins involved in mRNA stabilization, processing, and editing, e.g., nuclear-encoded chloroplastic ribonucleoprotein cp29, were found in stressed plants [69,70]. Regulation of cell cycle and programmed cell death (PCD) is also associated with translationally controlled tumor protein homolog (TCTP) which has been characterized as an agent decreasing cytosolic Ca²⁺ levels and thus inhibiting PCD [71]. An increased abundance of TCTP was observed under several stresses including salinity [72], drought [56], and others.

Protein degradation pathways include mainly proteins associated with protein targeting by ubiquitin to proteasome degradation. An increase in proteasome subunits, e.g., 20S proteasome alpha [56,61,73] and alterations in E2 ubiquitin ligase indicating an up-regulation of proteasome-dependent protein degradation were found in cold-treated winter wheat [62].

Alterations in protein metabolism also affect alterations in amino acid metabolism. Several amino acids represent not only protein components, but also precursors of various stress-related compounds and key components of metabolic pathways associated with carbon and nitrogen metabolism. For example, glutamate (glutamic acid) and glutamine represent crucial compounds associated with nitrogen assimilation, glutathione and proline biosynthesis, phenylalanine and tyrosine can be deaminated to yield *trans*-cinnamic acid and *p*-coumaric acid, precursors of lignin components synthesized via the

phenylpropanoid pathway, methionine is a precursor of *S*-adenosylmethionine (SAM) which is not only a universal methyl donor in plant cells, but also a precursor of many stress-related compounds including phytosiderophores (synthesized from SAM in a series of reactions known as Yang cycle), polyamines (spermine, spermidine, putrescine), ethylene and other stress-related compounds. An increase in glutamine synthetase in drought-treated soybean roots and alfalfa leaves, respectively, was consistent with an enhanced content of proline and a decreased osmotic potential [36,74]. An increase in methionine synthase or SAM synthase (SAMS) was found in many proteomic studies dealing with various stress factors including cold [62,75], drought, salinity [76–78], *etc.* Alterations in enzymes involved in phytosiderophore biosynthesis were also found in some studies, e.g., an increase in methylthioribose kinase was found in boron-treated barley [79], while a decrease in IDI2, IDS2, and IDS3 proteins involved in a biosynthesis of mugineic acid (a precursor of phytosiderophores) was found in salt-treated barley [80] indicating a reduction of metal uptake as potential catalyzers of ROS. In contrast, an increase in ferritin levels was found in flax cell culture exposed to elevated cadmium [34] and wheat leaves exposed to salinity [81]. Moreover, several amino acids can be deaminated by aminotransferases to yield oxoacids, which are intermediates of Krebs cycle, a crucial pathway of aerobic respiration.

3.2.2. Energy Metabolism

An active plant response to stress is associated with enhanced demands on energy. Therefore, alterations in energy metabolism were reported in several proteins involved in energy metabolism. Adenosine triphosphate (ATP) represents an immediately available energy source which functions as a cofactor in several energy-demanding reactions. Alterations in enzymes involved in a cleavage of macroergic phosphate bonds such as nucleoside diphosphate kinase (NDPK) were reported in several papers [40,69,75,82,83]. Novel ATP molecules are synthesized in photosynthesis, anaerobic, and aerobic respiration. Thus, alterations in both mitochondrial and chloroplast ATP synthases subunits, especially α , β , γ and ϵ subunits of chloroplast and mitochondrial CF1 complex directly involved in ATP biosynthesis, are reported in relation to stress [36,48,70,84–87]. An active site of ATP synthesis lies in β subunit of CF1 ATP synthase complex which was reported to be declined under drought in wheat [88].

Photosynthesis is highly sensitive to imbalances between primary electron-transport processes and secondary chemical reactions associated with CO₂ assimilation. A discrepancy between the rate of primary and secondary photosynthetic reactions enhances a risk of ROS formation. Changes in photosystem-associated proteins, especially OEE proteins as components of PSII OEC center involved in photolysis of water, were found in several studies [29,56,60,77,83,86,89]. A decrease in RubisCO large and small subunits (RubisCO LSU and SSU) as well as Calvin cycle enzymes phosphoglycerokinase (PGK), phosphoribulokinase (PRK), and transketolase was found in drought- and salt-treated durum wheat [29,30] as well as in cold-treated spring Iranian wheat Kohdasht [60]. In contrast, an increase in proteins with protective functions such as RubisCO activase A [29,30,76,90], a Triticeae-specific thermostable RubisCO activase B [91], and carbonic anhydrase [30,56], were found in stressed plants. Changes in OEE1 and OEE2 proteins were frequently found in salt-treated barley [76,77], durum wheat [29], and tobacco [92], and an increase in

OEE1 protein was observed in drought-treated barley infected by *Piriformospora indica* [56]. Proteomic studies have usually shown an increase in OEE proteins under milder stress and in tolerant plant materials while a decrease under severe stress or in sensitive plant materials [93,94]. Moreover, an increase in RubisCO chaperones CPN60- α , CPN60- β , and 20-kDa co-chaperonin was found under several stresses [29,55,62,69,95–97]. Stress also leads to profound changes in aerobic metabolism. Due to an enhanced risk of ROS formation, a decrease in some components of mitochondrial electron-transport chain such as NADH-dependent ubiquinone oxidoreductase was found in some studies [83,95]. Imbalances in the rates of several aerobic processes lead to an enhanced risk of oxidative stress. Therefore, an increase in alternative electron-transport pathways using alternative oxidase [98,99] as well as in enzymes involved in anaerobic ATP-producing processes such as glycolysis (glyceraldehyde-3-phosphate dehydrogenase GAPDH, triosephosphate isomerase TPI, enolase ENO, pyruvate kinase) and alcoholic fermentation (alcohol dehydrogenase ADH, aldehyde dehydrogenase, formate dehydrogenase) has been found in several studies dealing with waterlogged root cells, but also with seedlings and young plants exposed to severe dehydration stresses such as drought and salinity [29,30,62,73,83,95,100]. In contrast, a decline in the levels of glycolytic enzymes was found during the flax seed development in radioactivity-contaminated environment [35]. However, an increased need for energy can lead to an increase in several proteins related to aerobic metabolism (Krebs cycle, mitochondrial electron-transport chain) as indicated by an enhanced level of thiamine thiazole synthase, dihydrolipoamine acetyltransferase, and other cofactors of dehydrogenase complex catalyzing pyruvate conversion to acetyl-CoA [101]. Differential patterns of changes in several isoforms of glycolysis enzymes were frequently reported in one proteomic experiment as shown for enolase isoforms in drought-treated sunflower roots [33]. Moreover, nuclear isoforms of cytoplasmic glycolytic enzymes can act as important regulators of stress-responsive pathways as reported for a nuclear isoform of ENO encoded by *LOS2* locus and involved in regulation of cold-inducible CBF pathway [102] or for a nuclear GAPDH involved in tRNA transport [103,104].

An enhanced need for energy under stress acclimation also corresponds with a degradation of energy-rich storage compounds such as polysaccharides (starch) and storage proteins. A decrease in enzymes related to carbohydrate anabolism (sucrose synthase 1 yielding UDP-glucose) have been found under cold [62]. A decrease in several storage proteins, e.g., legumin-like, 11S seed storage proteins, *etc.*, were also found [62,75]. In contrast, salinity led to an increase in β -conglycinin, a major storage protein in soybean seeds, in young soybean seedling plants indicating a reduced seedling growth under stress with respect to control [64].

3.3. Resistance Phase

3.3.1. Stress-Protective Proteins

Several stress factors including drought, salinity, but often also cold, frost, and heat, induce cellular dehydration. A decrease in cell water content in plant cells results in a lack of hydration envelopes and an increased risk of an improper protein folding. Protein disulfide isomerase (PDI) catalyzes a reversible cleavage of disulfide bonds and thus affects protein conformation. Alterations in PDI abundance were found in several studies [37,38,75,88,105]. An increased accumulation of several

hydrophilic proteins from COR/LEA family including LEA-II dehydrins [106–109] and LEA-III proteins such as chloroplast-located COR14b protein [110–112] and others [101] was found under cold and drought. Other proteins with chaperone functions include heat shock cognate proteins (HSC) and proteins from HSP superfamily encompassing five families of HSP proteins, HSP110, HSP90, HSP70, HSP60, and small HSP (sHSP) proteins. An increase in several small HSP proteins, but also HSP82 from HSP90 family was found in wheat grain endosperm in developing wheat grains subjected to a heat period [113,114]. An increase in HSP70 and HSC70 was found in watermelon exposed to drought and in tomato exposed to waterlogging, respectively [115,116]. Not only an increase, but also a decrease in some HSP proteins was found in several proteomic studies, e.g., a decrease in HSP70 in drought-treated soybean roots [74] and salt-treated tobacco chloroplast stroma [92], and a decrease in HSP90 in cold-treated winter wheats [75]. An opposite pattern of changes was reported for several sHSP26 protein isoforms in drought-stressed maize leaves [117]. Other proteins with chaperone functions found in proteomic studies include copper chaperone [75,89,118], cystatin [62,119] (cysteine protease inhibitor), serpins [73,84,120,121] (serine protease inhibitor), Zn-dependent metalloproteases [86], DnaK [60], *etc.* Besides their roles as protein chaperones, an interaction of HSP70 with glutathione-related enzymes such as GPX and GR was reported in animal cells thus indicating a role of HSP70 in regulation of cell redox homeostasis [122].

An enhanced risk of protein damage is also reflected by an increase in several ROS scavenging enzymes including catalases, peroxidases, and enzymes associated with ascorbate-glutathione cycle (ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase) alterations of which were found in practically all proteomic studies published. The ROS scavenging enzymes differ not only in their substrate specificity, but also in cellular localization and metal cofactors, e.g., Cu/Zn-SOD (cytosolic), Mn-SOD (mitochondrial) and Fe-SOD (chloroplastic). Glutathione peroxidases (GPX) have cytosolic as well as membrane-associated isoforms and catalyze a reduction of lipid peroxides while lipoxygenases catalyze lipid peroxidation. A decrease in two lipoxygenases was found in soybean roots exposed to flooding [123]. Peroxiredoxins and thioredoxins are small nuclear-encoded, but chloroplast-located proteins regulating protein activity by a reversible reduction of cysteine residues to disulphide bonds. In plants, thioredoxins are known to regulate activity of Calvin cycle enzymes thus affecting efficiency of photosynthesis. Alterations in several thioredoxin forms, e.g., thioredoxin H and thioredoxin M, were observed under stress including drought [89], a brief freezing stress [70], and salinity [76,77,124] while alterations in peroxiredoxins were found in drought-treated sugar beet [82], wheat [89], salt-treated grapevine [66], maize [49], and cold-treated wheat [69]. A precise regulation of ROS production plays an important role in crop responses to several stresses, namely drought [59,82], drought-induced senescence [89], salinity [77], and heavy metal stress [31,34,37]. Whereas most stress factors led to an increase in the levels of ROS-scavenging enzymes due to imbalances in aerobic metabolism and cellular redox status, a decrease in Cu/Zn-SOD levels was found in soybean roots exposed to waterlogging due to hypoxia [123].

Hypoxia stimulates anaerobic metabolism such as glycolysis and fermentation processes, but also biosynthesis of efficient O₂ captures such as hemoglobin. In waterlogged maize root cells, an enhanced abundance of coproporphyrinogen III oxidase which catalyzes oxidative decarboxylation of coproporphyrinogen III to protoporphyrinogen IX in heme, a crucial step in hemoglobin biosynthesis, was found by Yu *et al.* [100]. Hypoxia also results in decreased soil pH. Maintenance of stable cellular

pH necessary for a proper enzyme function is achieved by an enhanced accumulation of cytoplasmic enzymes with buffering capacity such as glutamate decarboxylase (GDC), malate dehydrogenase (MDH), and NADP-malic enzyme (NADP-ME) [100]. An enhanced abundance of GDC, NADP-ME3, and NADP-ME4 was found in waterlogging-tolerant maize line with respect to the sensitive one [100]. Glutathione S-transferases (GST) represent a large family of enzymes catalyzing glutathione (GSH) conjugation to various substrates. They encompass several structural classes marked by Greek letters ϕ , τ , ζ , θ . They are known as enzymes involved in detoxification of heavy metals and xenobiotics including several herbicides; however, their glutathionylating activity could also play an important role in regulation of protein activity (*S*-glutathionylation as a posttranslational modification) and in secondary metabolism [125] (*S*-glutathionylated intermediates in metabolism of terpenes, glucosinolates, thiophenes, allins, *etc.*). Increased GST levels were found under several stresses including wheat exposed to enhanced copper levels [37], cold [69,75], drought [88,94,96,105], salinity [77,81], and cadmium [31].

Pathogenesis-related proteins (PR) encompass a diverse group of 17 protein families (PR2— β -1,3-glucanases; PR3,4,8,11—chitinases; PR5—thaumatin-like; PR6—proteinase inhibitor; PR7—endoproteinase; PR9—peroxidase; PR10—ribonuclease-like; PR12—defensin; PR13—thionin; PR14—lipid-transfer protein; PR15—germins; PR16—germin-like proteins; PR17—unknown function) involved not only in plant protection against pathogen attack, but also in response to abiotic stresses [126]. Several of them reveal glucanase and chitinase activities aimed at cleavage of fungal cell walls while others reveal ROS scavenging activities (peroxidases, some germins and germin-like proteins) or RNase activities (PR10). Alterations in several PR proteins including β -1,3-glucanases [39], thaumatin-like protein [39,62], PR10 [41,66,127,128], TSI-1 protein [93,124], germin and germin-like proteins [62,77,123,129], PR17 [130], and other protective proteins such as lipid transfer proteins [129], and lipocalins [32] were reported under a wide range of abiotic stresses including drought, salinity, cold, and waterlogging. Plant stress acclimation response is associated with a biosynthesis of several specific stress-protective compounds. For example, changes in enzymes involved in flavonoid and isoflavonoid metabolism participating on biosynthesis of several protective compounds such as anthocyanins and phytoalexins were found not only in pathogen-treated plants, but also in pea plants subjected to salinity [41]. An increased level of chalcone synthase (CHS), a crucial enzyme in flavonoid/isoflavonoid biosynthesis pathway, also interacting with methyl jasmonate and salicylic acid (SA) signaling [131], was found in a drought-sensitive sunflower genotype under dehydration [33].

3.3.2. Structural Proteins

Stress also profoundly affects cellular transport and cytoskeleton. An active ion transport in an opposite direction to physicochemical gradients requires energy. An acquisition of stress tolerance in response to salinity or a hyperosmotic stress (PEG treatment) is associated with an enhanced activity of several Na^+ transporters and H^+ transporters involved in Na^+ exclusion or intracellular compartmentation to vacuole coupled with ATP cleavage [50,56,81,124] (V-ATPase, H^+ -PPase) which is often associated with an increased abundance of ATP synthases components. Severe dehydration also affects water transport via aquaporins resulting in an enhanced abundance of aquaporin proteins as well as

aquaporin differential phosphorylation in common wheat exposed to severe osmotic stress [51]. Annexins are cytosolic monomers which can form integral membrane oligomeric complexes enabling transport of several cations including Ca^{2+} and thus involved in cytoplasmic calcium signaling including MAPK kinase cascade and phosphatidylinositol bisphosphate signaling. An increase in annexin level was found in salt-treated potato [93], soybean [95], and tomato plants [124] and a transient elevation was found in drought-treated barley [83] indicating an important role of annexin in abiotic stress signaling process. Voltage-dependent anion channel (VDAC) is a protein complex located in outer mitochondrial membrane which is important for metabolite transport between mitochondria and cytoplasm and which was found increased under several stresses including drought [132], salinity [98], and others. ABC transporters are known to participate on transport of glutathione conjugates into vacuoles and were found elevated under Cd stress [31]. Enhanced actin and β -tubulin levels were found in chicory roots exposed to cold [40], while a decrease in α - and β -tubulin was found in drought-stressed sunflower roots and copper-stressed wheat roots, respectively [33,37].

Stress also reveals profound impacts on plant cell walls. Stress leads to a decreased rate of plant growth and cell division, which also affects cell wall composition. Several proteomic studies dealing with water stress (drought) suggest an increased cell wall lignification, which is reflected by an increased abundance of enzymes involved in lignin biosynthesis such as caffeic acid 3-*O*-methyltransferase (COMT), caffeoyl-coenzyme A *O*-methyltransferase (CCOMT) and phenylalanine ammonia lyase (PAL) [56,74,133,134]. In addition, alterations in enzymes involved in metabolism of cell wall polysaccharides such as cellulose, hemicelluloses, and pectins were found; for example, UDP-glucuronic acid decarboxylase, β -D-glucan exohydrolase, UDP-glucose pyrophosphorylase in salt-treated barley [72], xyloglucan *endo*-transglycosylase and UDP-glucosyl transferase BX9 in drought-, salt- and waterlogging-treated maize roots [49,100,135]. These data indicate a substantial cell wall remodeling in response to stress usually leading to a decreased elasticity due to cell elongation cessation and an increased lignification as a potential barrier against dehydration stress. In contrast, a decreased lignification and increased cell wall loosening were found under waterlogging [78,100]. Decreased levels of β -1,3-glucanases, β -glucosidases, and methionine synthase as a precursor of SAM, a methyl donor for monolignol synthesis, indicate an inhibitory effect of waterlogging on wheat seedling growth [136]. This phenomenon may be associated with decreased ROS and jasmonate levels under flooding [123].

3.4. Comparison of Various Abiotic Stresses, Stress Recovery

Regarding crop stress acclimation responses, plant stress responses include both common as well as specific features. Common features of several abiotic stresses studied (cold, frost, drought, salinity) include cell dehydration, *i.e.*, dehydrative stress, and imbalances in aerobic metabolism, *i.e.*, oxidative stress. Cell dehydration leads to an enhanced biosynthesis of low-molecular osmolytes and hydrophilic proteins as well as chaperones to prevent protein misfolding and aggregation. Imbalances between chloroplast and mitochondrial electron transport chains and enzymatic reactions (Krebs cycle, Calvin cycle) resulting in an enhanced ROS formation lead to an enhanced accumulation of ROS scavenging enzymes. However, several stress factors also reveal specific effects on plant cell structure and metabolism. Heat is associated with an enhanced risk of protein misfolding leading to enhanced levels

of HSPs, especially sHSPs. Salinity can be characterized by a specific ionic effect, *i.e.*, penetration of Na^+ into cell cytoplasm, which leads to an activation of ATP-dependent ion channels resulting in Na^+ exclusion or intracellular compartmentation (vacuolar sequestration). Specific effects of waterlogging include hypoxia, resulting in an activation of anaerobic metabolism (glycolysis, fermentation), and a decreased soil pH resulting in an enhanced abundance of several cytosolic enzymes with buffering capacity such as NADP-ME, MDH, and others. Imbalances in metal nutrients as well as heavy metal stress lead to an enhanced abundance of several proteins with ion chelating functions (ferritin, phytochelatins) as well as ROS scavenging enzymes since free metal ions act as efficient ROS catalyzers (Table 1).

Practically all proteomic studies dealing with crop response to an abiotic stress are focused on plant stress acclimation. However, it should be kept in mind that recovery after a cessation of stress stimulus is equally important since it profoundly affects further plant growth and development. However, recovery after a stress treatment is still being seldom studied in crops. An exception represent a paper on drought-treated wheat cultivars with differential drought tolerance where plant proteome response at one day after rewatering was studied [132] as well as a paper on drought-treated soybean followed by four days of recovery [74]. In wheat, proteome analysis of a rewatering response has revealed an increased abundance of 8 out of 12 glycolysis enzymes in tolerant cultivar Excalibur indicating an enhanced need on energy during a recovery treatment. HCF136 which is a protein involved in repair and assembly of OEC and PSII complexes significantly increased in tolerant Excalibur under rewatering indicating a quick PSII repair after stress cessation. In contrast, dehydration-induced proteins COR410 and SDi-6 revealed a significant decrease at rewatering indicating a cessation of the adverse impacts of a stress treatment. In soybean, rewatering led to an increase in some regulatory proteins potentially involved in the delay of senescence and PCD (eIF5A, MADS-box TF KIP, pentatricopeptide repeat protein) which were downregulated upon drought stress. In common bean, recovery after a long-chilling stress (10 °C per 16 days) as well as short-chilling stress (10 °C per 24 h) was studied by Badowiec and Weidner [137]. Recovery after chilling stress led to a decrease in several stress-related proteins such as LEA1, HSP, GST, SAMS, while an increase in proteins involved in energy metabolism (mtATP synthase $\text{CF1}\alpha,\beta$; pyruvate kinase) indicating an enhanced need for energy to achieve novel homeostasis.

Table 1. Basic characteristics of the major effects of abiotic stresses on plant biological mechanisms including plant stress response. Abbreviations: COR—cold-regulated (protein); GDC—glycine decarboxylase; HSP—heat shock (protein); LEA—late embryogenesis-abundant (protein); MDH—malate dehydrogenase; NADP-ME—NADP malic enzyme; PCD—programmed cell death; ROS—reactive oxygen species; XET—xyloglucan *endo*-transglycosylase.

Stress Factor	Stress Effect	Plant Response
Low temperature (cold, frost)	Imbalance between water uptake and water release—cellular dehydration. Imbalance between non-enzymatic electrontransport reactions and enzymatic reactions (Krebs cycle, Calvin cycle) in chloroplasts and mitochondria—oxidative stress (enhanced ROS formation)	Enhanced biosynthesis of low-molecular osmolytes (proline, sugars, betaines) and hydrophilic proteins (COR/LEA) Enhanced biosynthesis of ROS scavenging enzymes, downregulation of crucial photosynthetic enzymes
Heat	Enhanced risk of protein misfolding	Enhanced accumulation of HSPs, especially sHSPs
Drought	Imbalance between water uptake and water release—cellular dehydration Imbalance between non-enzymatic electron transport reactions and enzymatic reactions (Krebs cycle, Calvin cycle) in chloroplasts and mitochondria—oxidative stress Reduced growth	Enhanced biosynthesis of low-molecular osmolytes (proline, sugars, betaines) and hydrophilic proteins (COR/LEA) Enhanced biosynthesis of ROS scavenging enzymes, downregulation of crucial photosynthetic enzymes Enhanced cell wall lignification
Salinity	Decreased soil water potential—cellular dehydration—osmotic effect Enhanced Na ⁺ penetration—ionic effect	Enhanced biosynthesis of low-molecular osmolytes (proline, sugars, betaines) and hydrophilic proteins (COR/LEA) Enhanced levels of ATP-dependent Na ⁺ /H ⁺ transporters resulting in Na ⁺ exclusion (plasma membrane) or Na ⁺ intracellular compartmentation (tonoplast)
Nutrient deficiencies and heavy metal stress	Enhanced metal ion penetration—oxidative stress	Enhanced levels of metal-chelating proteins (ferritin, phytochelatin, LEA) and pathways involved in their biosynthesis (Yang cycle); enhanced ROS scavenging enzymes

4. Differences in Stress Response between Tolerant and Sensitive Genotypes

Most temperate crop plants represent plant species with a relatively large and diverse genetic pool despite an intensive selection during a breeding process. Although modern cultivars are bred primarily for crop quality and high yield, there are also several landraces and wild relatives adapted to harsh environments. Moreover, modern breeding for high-yielding cultivars in harsh environments such as dry environments in Australia or freezing temperatures in Canada, have also led to a release of tolerant cultivars. In addition, several landraces, wild accessions and relative species such as *Hordeum marinum*, *Solanum commersoni*, *Thinopyrum ponticum*, etc., represent genetic materials adapted to harsh environments, which can be employed as potential genetic resources of alleles underlying an enhanced stress tolerance.

Transcriptomic and proteomic studies focused on comparison of plant genotypes or related species with contrasting stress tolerance, e.g., *Arabidopsis thaliana* (glycophyte) and *Thellungiella salsuginea* (halophyte), have revealed increased levels of stress-inducible proteins in tolerant genotypes even in the absence of stress [138]. Tolerant plants are thus able to efficiently diminish adverse effects of stress when compared to sensitive plants. Tolerant plants reveal constitutively enhanced levels of several stress-responsive proteins including transcriptional regulators [59] (e.g., SWIB/MDM2 protein, Myb protein, B-Peru-like protein involved in anthocyanin biosynthesis) and thus also several stress-protective proteins, ROS scavenging enzymes and proteins involved in metabolism of stress-related phytohormones. For example, constitutively enhanced levels of r40c1 protein which belongs to a class of ABA-induced proteins, and an enhanced level of lipoxygenase (LOX), an enzyme involved in biosynthesis of jasmonic acid, and increased levels of several chaperones (HSP70, HSP90, CPN60- α,β , cyclophilin A), S-adenosylmethionine synthase (SAMS), glutathione-S-transferase (GST), etc., were found in drought-tolerant barley Basrah with respect to drought-sensitive Golden Promise [59] as well as in drought-tolerant wheat Nesser with respect to drought-sensitive Opata [57]. A comparative proteomic study of common wheat (*Triticum aestivum*) and its hybrid *Triticum aestivum* \times *Thinopyrum ponticum* exposed to salinity has revealed an increased abundance of V-ATPase involved in Na⁺ vacuolar compartmentation [54]. Increased levels of ascorbate peroxidase (APX) and catalase (CAT) were found in drought-tolerant maize genotype with respect to the drought-sensitive one [117]. Increased levels of Mn-SOD as well as other mitochondrial redox enzymes and protective proteins were found in salt-tolerant wheat \times *Lophopyrum elongatum* amphiploid with respect to salt-sensitive common wheat cv. Chinese Spring as well as in cold-tolerant winter wheat with respect to the less tolerant one, respectively [97,99]. An increased level of cold-inducible dehydrin proteins WCS120 in common wheat and DHN5 in barley in frost-tolerant winter cultivars with respect to less tolerant winter genotypes as well as spring ones was found not only upon cold, but also at mild cold to optimum growth temperatures [63,139]. Moreover, a differential PTM such as a differential phosphorylation level of YxSKn-type dehydrin DHN5 was reported in differentially drought-tolerant genotypes of durum wheat [107]. Consistent with constitutively increased levels of several stress-protective proteins in tolerant genotypes, a higher stress-inducible increase of some stress-responsive proteins such as HSP70 and thioredoxin H was found in sensitive genotypes with respect to tolerant ones when exposed to a stress treatment [124]. Due to a constitutively increased abundance of several stress-protective proteins in stress-tolerant

genotypes with respect to stress-sensitive ones, tolerant genotypes are able to fulfill the demands on enhanced energy during the stress acclimation process. This fact is reflected by enhanced levels of photosynthesis-related proteins (OEC components; carbonic anhydrase; RubisCO large subunit) and ATP biosynthesis (ATP synthase β subunit) found in tolerant genotypes with respect to sensitive ones [94,96,120,130,140]. Consistent with this hypothesis, a relatively lower decrease in energy-rich storage compounds such as storage proteins (legumin-like protein, 11S seed storage protein) was found in tolerant winter wheats than in less tolerant winter genotypes and sensitive spring genotypes upon cold treatment [62,75].

A lower damage of plant tissues and lower energy costs on stress acclimation in tolerant genotypes in comparison to sensitive ones could also result in a relatively more positive effect on novel protein biosynthesis, plant growth, and development in tolerant genotypes compared to sensitive ones when subjected to stress. An increase in eIF3 and mitochondrial EF-TuM was found in drought-tolerant maize genotype CE704 subjected to six days of dehydration while a decrease in eEF1D was found in drought-sensitive maize genotype 2023 under the same conditions [117]. A relatively lower decrease in eIF5A3 factor regulating not only protein biosynthesis, but also cell cycle (cell division) was found in *Triticum aestivum* \times *Thinopyrum ponticum* hybrid Shanrong 3 with respect to its parental wheat cultivar Jinan 177 under salinity [54]. Consistent with a factor regulating cell division, a relatively higher level of DWARF3, a protein involved in gibberellin biosynthesis, was found in salt-tolerant hybrid with respect to its parent under salt stress indicating a relatively higher rate of cell division and plant development in the tolerant genotype [47,54]. Consistent with a relatively lower growth inhibition in tolerant cultivars vs sensitive ones under stress, a relatively increased level of enzymes involved in cell wall elongation such as xyloglucan *endo*-transglycosylase (XET) was found in drought-tolerant grapevine cultivar with respect to drought-sensitive one [66]. In addition, novel protein biosynthesis may be not impaired to such an extent in tolerant cultivars with respect to sensitive ones as shown by a significant decrease of chloroplast 30S ribosomal protein S10, a protein crucial for binding of tRNA to ribosomal surface and initiation of protein biosynthesis, in salt-sensitive canola cultivar Sarigol compared to no significant change in salt-tolerant cultivar Hyola 308 when exposed to salt stress [67].

5. Combinations of Multiple Stress Factors

In nature, plants are usually exposed to multiple abiotic (and biotic) stresses [141,142]. However, plant stress responses to combined stress treatments are seldom studied. A few proteomic studies dealing with combined stress treatments have shown that plant response to a combined stress treatment is specific when compared to the individual stress factors applied separately. Since no simple assumptions on an additive effect of individual stress treatments can be applied, plant response to combined stress treatments deserves to be studied.

Peng *et al.* [47] compared the effects of drought and salinity as two separate treatments applied on a relatively sensitive bread wheat cv. Jinan 177 and its somatic hybrid with tall wheatgrass *Thinopyrum ponticum* named Shanrong 3. Comparison of proteome response to both treatments has revealed that salinity induced significant alterations in a higher number of proteins than drought as a consequence of an ionic effect of salinity stress.

Rollins *et al.* [91] studied the effects of drought (15% soil water content), heat (36 °C) and a combined drought and heat treatment in two relatively drought-tolerant barley genotypes originating from differential drought environments, Syrian landrace Arta and Australian cultivar Keel, differing in their drought response strategies. Drought led to a reduction in plant growth while maintaining relatively stable proteome composition. In contrast, heat led to enhanced protein damage, especially of PSII components, and thus an enhanced need for energy due to an increased protein turnover. An enhanced abundance of ROS scavenging enzymes and protective proteins (HSPs, a thermostable RubisCO activase B isoform) was found in heat-treated plants indicating an imbalance between the rates of primary (light-dependent) and secondary (light-independent) photosynthetic reactions and an enhanced risk of protein damage under heat stress.

Li *et al.* [143] studied the effect of a spring freezing in combination with either drought or waterlogging (water stresses) on winter wheat cv. Yannong leaves sampled from plants in anther connective tissue formation stage. Differences between the individual treatments and combined treatments were observed. For example, HSP70 decreased in response to a single freeze stress treatment while the same protein increased in response to combined stress treatments. In contrast, decreased levels of chloroplast ATP synthase β subunit and mitochondrial ATP synthase α subunit in both single freezing and combined freezing and waterlogging treatments are consistent with a decrease in Ca^{2+} and Mg^{2+} -ATPase activities and an observed damage of PSII under waterlogging stress.

Yang *et al.* [144] investigated an effect of drought and heat (32 °C) treatment on proteome composition of wheat grain in the stage of terminal spikelet and anthesis. Several proteins revealed a specific response to each stress treatment while only a few common proteins involved in redox metabolism, defense, carbohydrate metabolism, and storage revealed an analogous response to multiple stress treatments. Proteins responding exclusively to heat stress include increased cinnamoyl-CoA reductase, TCTP (translationally-controlled tumor protein), cell division control protein, and heat shock cognate 70 (HSC70) and a decreased 14-3-3 protein.

A comparison of contrasting water stresses—drought and flooding—was carried out by Oh and Komatsu [78] in soybean seedlings. The results have shown differentially regulated stress responses—an increase in enzymes involved in regulation of redox homeostasis was found in drought-stressed plants while an increase in anaerobic metabolism-related (fermentation) enzymes was found in flooded plants. Moreover, an opposite pattern of changes in SAM synthetase (SAMS) was found in drought-treated plants vs flooded ones indicating an increase in SAMS under drought while a decrease in SAMS under flooding. Since SAM is a universal cell methylation agent involved in lignin biosynthesis, the differential pattern of SAMS may be related to changes observed in root cell wall lignification in soybean seedlings under the two treatments corresponding to an increased cell wall lignification under drought while a decreased cell wall lignification under flooding, respectively.

Effects of drought, cold, and herbicide paraquat treatments on pea mitochondrial proteome were compared by Taylor *et al.* [42]. The strongest adverse effects on mitochondrial proteins resulting in an oxidative damage were observed under paraquat treatment, followed by chilling while drought revealed the mildest effects. Mitochondria isolated from stressed pea plants maintained their electron transport chain activity; complexes of mitochondrial electron transport chain were least damaged by oxidative stress, F_1F_0 ATP synthase complex was more damaged while enzymes of carbon metabolism in mitochondrial matrix were significantly modified by oxidation. Moreover, increased lipid peroxidation

and a decrease in inner membrane import proteins were observed. Differential changes in abundance of several HSP proteins with an induction of HSP22 and opposite patterns in several isoforms of HSP70 were also found under both chilling and drought treatments.

6. Conclusions and Future Perspectives

During the past two decades, a boom of high-throughput separation techniques together with whole genome sequencing projects have enabled the development of “Omics” approaches in the study of plant response to environmental cues at transcript, protein, and metabolite levels. Proteome, a whole of proteins present in a given tissue at a given time, represents an important component of plant response to environment since proteins are directly involved in constituting the resulting plant phenotype. Recent publications of complete genome sequence in major crops have enabled the researchers an identification of practically any novel protein detected in a proteomic analysis. However, large genomes of several crops, especially the allohexaploid genome of common wheat (*T. aestivum*), are poorly annotated containing several draft sentences and genes of unknown functions. Moreover, unlike the one genome, an infinite number of proteomes can be described for a given organism depending on tissue type, developmental stage, and environment. Moreover, a given protein can adopt multiple forms differing in their pI and MW values on 2DE gels as a result of several posttranscriptional and posttranslational protein modifications. Therefore, one gene can encode multiple different proteins.

Currently, proteomic studies dealing with stress treatments in crop plants are still dominated by comparative studies focused on total proteome, *i.e.*, comparisons of proteomes in control vs stress-treated plants as well as genotypes with differential responses to a given stress. However, it can be suggested that in the future, proteomic studies will become more specific and focused on a response of a defined tissue or cell line compared to plant organs dominating in current studies due to employment of laser microdissection and other isolation techniques. Moreover, subcellular proteomics will become more common due to improved cell fractionation techniques. Specific protein isoforms and posttranslational modifications associated with characterization of their individual roles in plant response to environmental cues will become increasingly studied since current proteomic studies clearly show that different protein isoforms can reveal a differential response to the same environmental cue. Moreover, study of protein-protein interactions will also become an inevitable part of proteomic research since protein interactions are crucial for a final plant cell response. As an example of an interactomics study applied in crop stress response, a paper of Tardif *et al.* [145] on interaction networks of signaling proteins (Ran-related GTP binding protein, phospholipase C) and transcription factors involved in vernalization regulation (TaVRT1/VRN1, TaVRT2, VRN2, TaFT) in winter wheat studied by classical yeast-two-hybrid approach and validated *in planta* by split-GFP technique can be given.

Abiotic stress factors belong to the main environmental factors affecting crop growth and productivity. The major crops of a temperate climate zone including common and durum wheat, barley, maize, and soybean are grown worldwide in a very diverse environments (including semi-arid and arid areas of Australia and Middle East, temperate climate areas with harsh winter conditions and a high risk of freezing damage, areas endangered by soil salinity, imbalances in mineral nutrition, soil pollution by heavy metals, and other factors). Moreover, in nature, plants usually have to cope with combinations of several diverse stress factors. Proteomic research aimed at understanding crop

responses to abiotic stresses is still in its beginnings despite more than a decade of high-throughput proteomic experiments. One way to use proteomic outputs to be more beneficial e.g. for breeders is to aim studies for detailed characterization of tolerant to sensitive cultivars of different crops and relate these data to particular environment and management systems. The major reasons include a unique response of different plant organs and tissues as well as growth stages, and different stress treatments including stress dynamics. It has also been proven that combined stress treatments induce a unique plant stress response at the proteomic level, which could not be described as a simple additive effect of the individual stress treatments. Therefore, combined stress treatments also need to be studied due to their frequent occurrence in natural conditions. As an example, combined heat and drought stress occurrence, but also freezing in combination with either drought or waterlogging can be given.

Proteomic analyses can lead to an identification of proteins revealing common response to multiple stress treatments as well as proteins responding only to specific stress conditions. Both types of proteins can represent potential candidates for testing new plant materials for their potential stress tolerance during prescreening procedures in crop breeding programs aimed at an improvement of crop stress tolerance. According to Riccardi *et al.* [119], a protein considered a potential stress marker candidate has to fulfill the following two criteria: it has to be induced by a given stress factor and its protein quantitative locus (PQL) has to co-localize with a corresponding quantitative trait locus (QTL) for a given trait associated with stress tolerance. Proteomics of crop response to abiotic stresses has thus a large potential in crop breeding due to its large potential in designing novel breeding materials with specific characteristics (Figure 2). Application of the results of proteomic analyses can lead to selection of key protein markers including specific protein isoforms or PTMs, respectively, for a given crop feature which will be then tested in large sets of breeding materials as a part of routine procedures during the breeding selection. As an example, our results obtained on cold-responsive dehydrin proteins WCS120 in common wheat and DHN5 in barley as potential markers of plant acquired frost tolerance can be given [63,108,109,139]. It can be concluded that in the future, analysis of potential protein markers for some desired trait will probably become a routine part of a modern breeding process.

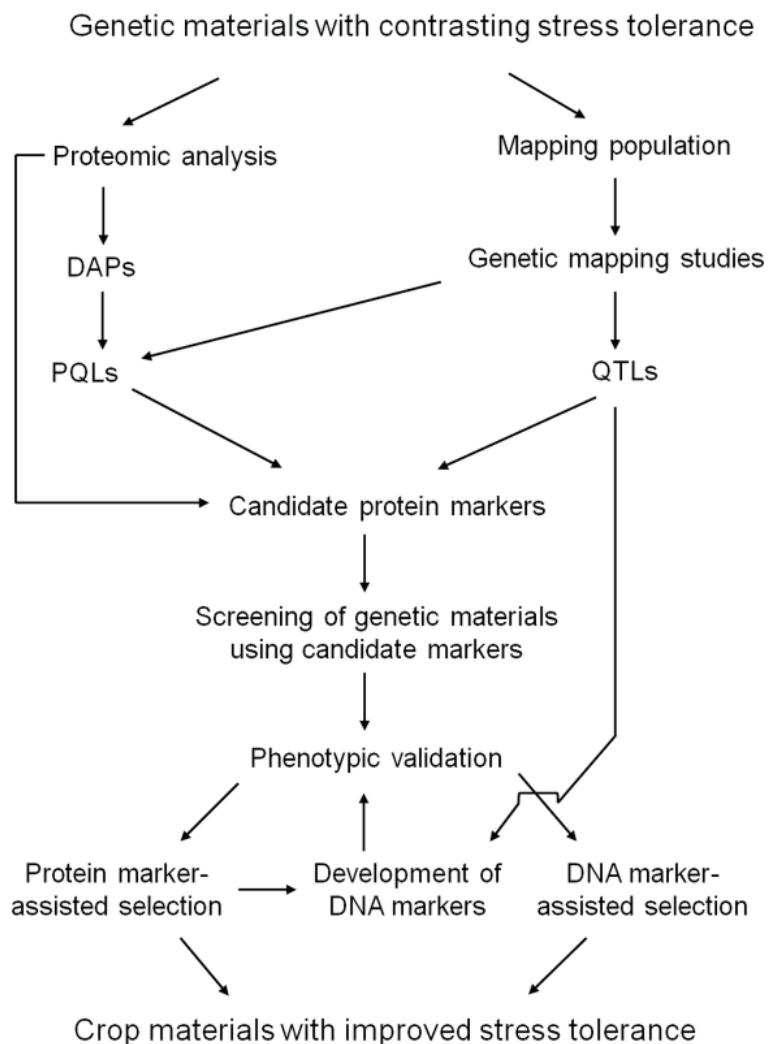


Figure 2. A schematic workflow of the utilization of candidate protein markers in breeding for an improved crop stress tolerance. Abbreviations: DAPs—differentially abundant proteins; PQL—protein quantitative loci; QTL—quantitative trait loci.

Supplementary Materials

Supplementary materials can be found at <http://www.mdpi.com/1422-0067/16/09/20913/s1>.

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Author Contributions

Klára Kosová has outlined the manuscript idea and prepared the manuscript text. Pavel Vítámvás, Milan Oldřich Urban, Miroslav Klíma, Amitava Roy and Ilja Tom Prášil have searched for relevant

literature, prepared literature outline in the form of Supplementary Table and prepared the graphics. Milan Oldřich Urban also prepared the reference list using EndNote programme.

Conflicts of Interest

The authors declare no conflict of interest.

Abbreviations

2Cys-Prx—2-cysteine peroxiredoxin; 2DE—two-dimensional electrophoresis; 2D-DIGE—two-dimensional differential in-gel electrophoresis; β -CAS— β -cyanoalanine synthase; ABA—abscisic acid; ACP—acyl carrier protein; ADH—alcohol dehydrogenase; AGPase—ADP glucose pyrophosphorylase; AOX—alternative oxidase; APX—ascorbate peroxidase; AQP—aquaporin; AsA—ascorbic acid; bHLH—basic helix-loop-helix (protein); BN-PAGE—blue-native polyacrylamide gel electrophoresis; CaM—calmodulin; CCOMT—caffeoyl-coenzyme A *O*-methyltransferase; CHS—chalcone synthase; COMT—caffeic acid *O*-methyltransferase; COR—Cold-regulated (protein); CPN—chaperonin; CS—cysteine synthase; CDPK—calcium-dependent protein kinase; CHS—chalcone synthase; DAP—differentially abundant proteins; DH—double haploid (line); DHAR—dehydroascorbate reductase; DON—deoxynivalenol; ENO—enolase; ESI—electrospray ionization; FBP ALDO—fructose-1,6-bisphosphate aldolase; FRK—fructokinase; FWC—field water capacity; GAPDH—glyceraldehyde-3-phosphate dehydrogenase; GAPDH B—glyceraldehyde-3-phosphate dehydrogenase B form; GDC—glycine decarboxylase; GDH—glutamate dehydrogenase; GLP—germin-like protein; GPX—glutathione peroxidase; GRP—glycine-rich protein; GS—glutamine synthetase; GST—glutathione S-transferase; HPLC—high performance liquid chromatography; Hsc—heat shock cognate protein; IFR—isoflavone reductase; iTRAQ—isobaric tag for relative and absolute quantification; LC—liquid chromatography; LEA—Late embryogenesis-abundant (protein); LOX—lipoxygenase; LTP—lipid transfer protein; LTQ-FTICR—linear quadrupole trap-Fourier transform ion cyclotron resonance; MALDI-TOF/TOF—matrix-assisted laser desorption ionization time-of-flight/time-of-flight (spectrometry); MAPK—mitogen-activated protein kinase; MDAR—monodehydroascorbate reductase; MDH—malate dehydrogenase; MIPS—*myo*-inositol-1-phosphate synthase; MS—mass spectrometry; MSSP2—monosaccharide sensing protein 2; NADP-ME—NADP malic enzyme; NBS-LRR—nucleotide-binding site leucine-rich repeat protein; NEPHGE—non-equilibrium pH gel electrophoresis; NDPK—nucleoside diphosphate kinase; NIL—near-isogenic line; OEE—oxygen evolving enhancer (protein); PBS—phosphate buffer saline; PC—plastocyanin; PDI—protein disulfide isomerase; PDX—pyridoxal biosynthesis protein; PEG—polyethylene glycol; PGK—phosphoglycerokinase; PGM—phosphoglyceromutase; POX—peroxidase; PPase—inorganic pyrophosphatase; PPK—pyruvate phosphate dikinase; PPR—pentatricopeptide repeat (protein); PRK—phosphoribulokinase; Prx—peroxiredoxin; PS—photosystem; PVP—polyvinyl pyrrolidone; qTOF—quadrupole time-of-flight; RubisCO—ribulose-1,5-bisphosphate carboxylase/oxygenase; RubisCO LSU—RubisCO large subunit; RubisCO SSU—RubisCO small subunit; RWC—relative water content; S—sensitive (genotype); SA—salicylic acid; SAMS—S-adenosylmethionine synthase; SBP—sedoheptulose-1,7-bisphosphatase; SHMT—serine hydroxymethyltransferase; SnRK—sucrose non-fermenting-related protein kinase; SOD—superoxide dismutase; SUS1—sucrose synthase 1;

SWC—soil water content; T—tolerant (genotype); t—genotype less tolerant than T; TCA—trichloroacetic acid; TCTP—translationally controlled tumour protein; TF—transcription factor; TLP—thaumatin-like protein; TPI—triose phosphate isomerase; Trx—thioredoxin; TSI-1—tomato salt-induced 1 (protein); V-ATPase—vacuolar ATPase; VDAC—voltage-dependent anion channel; WCS—Wheat Cold-specific (protein); WRAB—Wheat responsive-to-ABA (protein); XET—xyloglucan *endo*-transglycosylase; Y2H—yeast-two-hybrid (screen).

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Physiology

Significant relationships among frost tolerance and net photosynthetic rate, water use efficiency and dehydrin accumulation in cold-treated winter oilseed rapes



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Winter oilseed rape

ABSTRACT

Five winter oilseed rape cultivars (Benefit, Californium, Cortes, Ladoga, Navajo) were subjected to 30 days of cold treatment (4 °C) to examine the effect of cold on acquired frost tolerance (FT), dehydrin (DHN) content, and photosynthesis-related parameters. The main aim of this study was to determine whether there are relationships between FT (expressed as LT₅₀ values) and the other parameters measured in the cultivars. While the cultivar Benefit accumulated two types of DHNs (D45 and D35), the other cultivars accumulated three additional DHNs (D97, D47, and D37). The similar-sized DHNs (D45 and D47) were the most abundant; the others exhibited significantly lower accumulations. The highest correlations were detected between LT₅₀ and DHN accumulation ($r = -0.815$), intrinsic water use efficiency (WUEi; $r = -0.643$), net photosynthetic rate ($r = -0.628$), stomatal conductance ($r = 0.511$), and intracellular/intercellular CO₂ concentration ($r = 0.505$). Those cultivars that exhibited higher Pn rate in cold (and further a significant increase in WUEi) had higher levels of DHNs and also higher FT. No significant correlation was observed between LT₅₀ and E, PRI, or NDVI. Overall, we have shown the selected physiological parameters to be able to distinguish different FT cultivars of winter oilseed rape.

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Introduction

The ability of plants to cope with cold stress is a very complex trait. During cold acclimation (CA), many physiological, biochemical, and molecular changes occur (Fu et al., 2000; Kosová et al., 2012). CA, a period of exposure to low non-freezing temperature, increases plant frost tolerance (FT; Thomashow, 1999). FT seems to

be the main factor influencing the winter survival of winter oilseed rape. Research on FT has recently been focused on the characterization of genes that are up/down-regulated and are important for the capacity of each genotype to develop higher FT (Yamaguchi-Shinozaki and Shinozaki, 2006). However, the acquired FT can be induced by environmental conditions; thus, it is not easily predictable (Thomashow, 1999; Castonguay et al., 2013). FT has several components, e.g., cellular desiccation tolerance, due to the possibility of ice crystal nuclei formation within intercellular spaces (Thomashow, 1999). A rapid method to quantify the FT level lies in an evaluation of the LT₅₀ (the temperature at which 50% of the plants are killed) of plants using the frost test (Janáček and Prášil, 1991). However, a more reliable pre-screening method as well as a better understanding of FT in *Brassica napus* cultivars (cvs) is clearly needed (Snowdon and Luy, 2012).

Decreasing water availability under cold initially leads to an inhibition of photosynthesis, mainly sucrose synthesis. A limited water supply also changes transpiration, stomatal conductance, chlorophyll contents, inhibits photochemical activities, and decreases the activities of enzymes (Savitch et al., 2002; Prasad et al., 2009; Pons, 2012). Photosynthesis provides the energy

Abbreviations: ABA, abscisic acid; AP, alkaline phosphatase; BEN, cultivar Benefit; CA, cold acclimation or cold acclimated; CAL, cultivar Californium; Ci, internal leaf CO₂ concentration; COR, cultivar Cortes; COR/LEA, cold regulated/late embryogenesis abundant; cvs, cultivars; D97, D47, D45, D37 and D35 stand for dehydrin accumulation according to their different molecular masses from protein gel blot analysis (~97, ~47, ~45, ~37 and ~35 kD); DHN, dehydrin; FT, frost tolerance; GS, stomatal conductance; E, transpiration; LAD, cultivar Ladoga; LT₅₀, lethal temperature for 50% of plants; NAV, cultivar Navajo; NBT/BCIP, nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolylphosphate; NDVI, normalized difference vegetation index; PCA, principal component analysis; Pn, net photosynthetic rate; PRI, photochemical reflectance index; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; WUE, water use efficiency (Pn/E); WUEi, intrinsic water use efficiency (Pn/GS).

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necessary for the maintenance of cold acclimation response. Therefore, maintaining a sufficient net photosynthetic rate (Pn) enhances acquired FT in plants. Thus, any negative impact on photosynthesis may influence FT (Huner et al., 1998). The physiological impact of cold on plant physiology can be measured by different methods. Water use efficiency (WUE; some authors use WUE_i for the same ratio) is considered an important determinant of yield under abiotic stress, and even as a component of crop tolerance (Navarrete-Campos et al., 2013). Crop WUE has long been known to increase with limited water supply, even under cold temperatures (Blum, 2009) because it integrates the ratio of net photosynthesis to transpiration or stomatal conductance (Hall et al., 2005). WUE value is affected by genotype, weather conditions, and available soil water.

Spectral indices have been used to study the actual physiological state of plants in a non-destructive way. The most widely used indices, which can be determined by pocket-sized devices, are the Photochemical Reflectance index (PRI); and also the Normalized Difference Vegetation Index (NDVI; Chytký et al., 2011; Porcar-Castell et al., 2012). The PRI is based on the epoxidation state of the xanthophyll cycle pigments, which are related to thermal dissipation during photochemical stress (Gamon et al., 1997; Nylander et al., 2001). NDVI is an indicator of chlorophyll content in plants (Chytký et al., 2011), and it has been proposed as a tool to estimate biomass production and yield in wheat (Prasad et al., 2009). The relationship between the above-mentioned indices and FT has not yet been published.

In several studies (Cellier et al., 1998; Ismail et al., 1999; Kosová et al., 2007; Vítámvás et al., 2007; Vítámvás and Prášil, 2008; Hanin et al., 2011), it has been shown that CA timing, dehydrin (DHN) expression, and/or relative DHN protein accumulation in a plant reveals a significant relationship with plant-acquired FT. DHNs represent a unique group in the family of COR/LEA proteins (Hanin et al., 2011). DHN proteins are present in all higher plants, mostly in young plant organs in the subepidermal tissues, because they are the first tissue influenced by dehydration stress (Hara et al., 2003, 2004, 2005; Xu et al., 2008b; Sun and Lin, 2010; Kosová et al., 2011, 2012). Due to their ability to bind water with a minimum of intracellular hydrogen bonds (thanks to their intrinsically unstructured character), DHNs play many regulatory and defense roles in *Brassica* spp. Relative to cold (Deng et al., 2005; Yao et al., 2005; Xu et al., 2008a,b; Rurek, 2010; Hanin et al., 2011). DHNs protect membranes and other proteins against loss of the water envelope, which could lead to their denaturation (Kosová et al., 2007). In the *Brassicaceae* family, DHNs could be considered possible indicators of FT on the basis of the content of dehydrin proteins in the leaves of cold-treated plants (Klíma et al., 2012). Several other papers have been focused on expression profiles and/or function of DHNs in *Brassica* spp. (Deng et al., 2005; Savitch et al., 2005; Yao et al., 2005; Xu et al., 2008a,b). A few DHNs have been characterized at the molecular and transgenic levels in *B. juncea*. The expression of *BjDHN2* and *BjDHN3* (homologues to *AtDHN* in *A. thaliana* and *RsLEA2* in *Raphanus sativus*) showed an improved FT in transgenic yeast, and the genes were up-regulated under low temperature, drought, salinity, and heavy metals in *B. juncea* (Xu et al., 2008a,b). Expression analysis of *BnDHN1* in *B. napus* seed and siliques indicated that the *Brassica* DHN gene is inducible by water-deficit and low temperature (Yao et al., 2005). Abscisic acid, cold, and salt stresses were the signals for expression of the DHN gene *Bndhn ERD10* (Deng et al., 2005).

The main aim of this study was to determine whether there is a relationship between FT (expressed as LT₅₀ values), accumulation of dehydrins, and other selected physiological characteristics in chosen winter oilseed rape cvs. The results are discussed in relation to the possible role of the above-mentioned parameters in the development of cold acclimation.

Materials and methods

Plant material and growth conditions

Five winter oilseed rape (*Brassica napus* L.) cvs Californium (CAL), Ladoga (LAD), Benefit (BEN), Cortes (COR), and Navajo (NAV) were cultivated in a greenhouse for 10 d in a germination substrate (Agro CS, Česká Skalice, CZ). Young seedlings were transferred to a peat-bark-clay soil substrate RKS II (Agro CS, Česká Skalice, CZ) in plastic pots (120 mm in diameter), and kept in a growth chamber at 20 ± 2 °C (day/night), 70% humidity, 12 h photoperiod, and irradiance of 300 μmol m⁻² s⁻¹. After 4 weeks (controls were measured on the fourth leaf), the plants were exposed to cold treatment (4 ± 2 °C) for another 30 d (stress were measured on the fifth leaf). Plants were fertilized by the liquid fertilizer Vegaflor (Nera Agro, Neratovice, CZ).

Photosynthesis-related characteristics

All parameters were measured in the growth chamber on the youngest fully-developed leaves using the following instruments: TPS-1 (PP Systems, Amesbury, USA) for E, GS, Pn; as well as a PlantPen PRI 200 and PlantPen NDVI 300 (Photon Systems Instruments, Brno, CZ) for the PRI and NDVI, respectively. The measurements started three hours after the beginning of the light period; CO₂ concentration of 380 μL L⁻¹, and a light intensity of 300 μmol m⁻² s⁻¹ at the top of the plants. Ten values were measured at minimum on each of the five plants of the same cultivar, in three biological replications.

Dehydrins accumulation

Proteins soluble upon boiling were extracted from the right halves of the youngest fully-developed leaves of 30 d cold-treated plants in three biological and six technical replications, according to Vítámvás et al. (2010), with some modifications. Briefly, the tissue was ground under liquid nitrogen; 1 g of leaf powder was mixed with 4 mL of Tris-buffer [0.1 M Tris-HCl, pH 8.8, containing “Complete EDTA-free Protease Inhibitor Cocktail Tablets” (Roche)]. The fraction of heat-stable proteins was acquired by boiling the purified supernatant for 15 min. The proteins were precipitated and purified by cold acetone with 1% 2-mercaptoethanol and repeated centrifugation. One protein pellet (extracted from 135 mg of fresh tissue) was dissolved in 40 μL of sample buffer. To divide the proteins by molecular weight, 9 μL of the solution was used per each line of 12.5% SDS-PAGE (Laemmli, 1970). Precision Plus Protein Standards All Blue (Bio-Rad) was used for the estimation of molecular weight. The proteins were electrophoretically transferred to nitrocellulose membrane (0.45 μm, Pharmacia Biotech) and stained by Ponceau S dye (Sigma Life Sciences, St. Louis, USA). For hybridization with the antibody against dehydrin K-segment (Enzo Life Sciences, Inc., Farmingdale, USA) to visualize DHNs, an Immune-Blot Assay kit with Goat Anti-Rabbit IgG Alkaline Phosphatase (AP; Bio-Rad) was used according to the manufacturer's instructions. Membranes with visualized DHNs were scanned by a calibrated densitometer GS-800 (Bio-Rad) at 600 dpi. Determination of relative DHN protein accumulation was carried out using the Quantity One 4.6.7 program (Bio-Rad). To compensate for subtle differences in sample loading, the obtained density was normalized to the density of all visualized Ponceau S-stained protein in each sample. Such normalized spot volumes correspond to the relative abundance of the particular protein in relationship to the constant protein baseline. The volume of each spot (i.e. spot abundance) was expressed as the relative volume.

Table 1
One-way ANOVA. The transpiration rate (E), stomatal conductance (GS), net photosynthetic rate (Pn), intracellular/intercellular CO₂ concentration (Ci/Ca); water use efficiency – calculated as Pn/E (WUE), intrinsic water use efficiency – calculated as Pn/GS (WUEi), photochemical reflectance index (PRI), normalized difference vegetation index (NDVI), dehydrin accumulation (DHN) and lethal temperature for 50% of plants (LT₅₀).

Source of variation	Df	Parameter	Stress values	
			F-value	p-Value
Cultivars	4	E	162.990	0.00E+00
		GS	83.729	0.00E+00
		Pn	48.610	0.00E+00
		WUE	62.237	0.00E+00
		WUEi	76.714	0.00E+00
		Ci/Ca	182.760	0.00E+00
		PRI	11.587	1.24E–07
		NDVI	7.730	2.11E–05
		DHN	306.649	0.00E+00
		LT ₅₀	71.660	0.00E+00

Frost tolerance

FT was assessed on the leaf samples, taken from the left halves of the youngest fully-developed leaves of 30 d cold-treated plants, by means of the electrolyte leakage method (Prášil and Zámečník, 1998) in three biological and two technical replications for each sample. The level of FT was calculated using LT₅₀ (the temperature that results in 50% tissue damage) according to Janáček and Prášil (1991), based on linear regression, fit within the range of temperatures differentiating the resistance.

Statistical analysis of data

To statistically compare the means between individual cvs in cold treatment conditions, one-way and two-way analysis of variance (ANOVA) with interactions and Tukey multiple-comparison tests were used. All statistical analyses were considered significant at $p < 0.05$ in Statistica 10.0 statistical software (StatSoft, Tulsa, OK, USA). The linear relationship between the parameters was verified by graphic analysis of data (data not shown) and the degree of correlation was determined by the Pearson correlation coefficient. To reveal the internal structure of the data, as well as to distinguish cvs groups, principal component analysis (PCA) and factor analysis (supplementary data) were used. For PCA, all values were standardized in order to eliminate the dependency on the different units of the parameters. A scree plot was constructed to show the number of principal components by eigenvalues greater than 1.0. To distinguish relationships and bonds between parameters, the plot component weights were used to assess the relationships among the parameters.

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jplph.2013.07.012>.

Results

According to analysis of variance, all measured parameters differed markedly ($p < 0.01$) between the controls and cold-treated plants (Tables S1 and S2). The effects of genotype on levels of measured parameters under cold were highly significant as well (Table 1; Fig. 1).

Frost tolerance

Analysis of variance confirmed the significant impact of genotype on the degree of FT. The highest FT, expressed as the LT₅₀ value (Fig. 3B), was observed in cvs NAV, COR, and CAL (–13.8, –13.6, and

–13.2 °C, respectively). In contrast to the above cvs, BEN and LAD had significantly lower FT (–11.5 and –12.1 °C, respectively).

Photosynthesis-related characteristics

Mean values of transpiration (E), stomatal conductance (GS), photochemical reflectance index (PRI), WUE and WUEi were significantly lower in stressed plants than in the controls in all tested cvs (Fig. 1A, B, E, F and G; Table S1). With respect to the effect of genotype, the largest differences upon cold were recorded in E values, followed by Ci/Ca, WUE, and PRI (Fig. 1A, D, E and G; Table S1). The other parameters of the cold treatment exhibited smaller differences between cvs, according to homogeneous groups, derived from multiple comparisons among the means. The Pn rate and WUEi were lowest in BEN, medium in LAD and CAL and the highest in COR and NAV. The highest value of GS was observed in BEN, while in all other cvs GS values were not statistically different (see individual graphs in Fig. 1).

Dehydrins accumulation

DHNs of different molecular masses, extracted from the leaves of cold-treated plants, were detected in all cvs (Fig. 2B). Both the qualitative and quantitative changes were observed. While BEN only accumulated two DHNs (D45 and D35), other cvs accumulated three additional DHNs with molecular masses of 97, 47, and 37 kD, respectively. The most abundant DHNs in all of the cvs were the similar-sized D47 and D45 DHNs, while the others exhibited significantly lower accumulations (Fig. 2A). The visualization of individual DHNs and the total accumulation of DHNs in individual cvs are shown in Figs. 2A and 3A, respectively. Based on the overall dehydrin accumulation, the cvs were divided into three homogeneous groups: NAV and COR (30% and 28.8%), CAL and LAD (17.8% and 17.5%), and BEN (4.8%).

Relationship between frost tolerance and other characteristics

Pearson's correlation-based relationships among all characteristics, assessed in cold-treated plants, are shown in Table 2. Among the highly significant correlations, there are some with greater physiological importance. A significant correlation between LT₅₀ and other characteristics was observed for DHN accumulation ($r = -0.815$, see Fig. 3C), followed by WUEi ($r = -0.643$), Pn ($r = -0.628$), GS ($r = 0.511$) and Ci/Ca ($r = 0.505$). On the other hand, no significant correlations were observed between LT₅₀ and E, PRI, or NDVI.

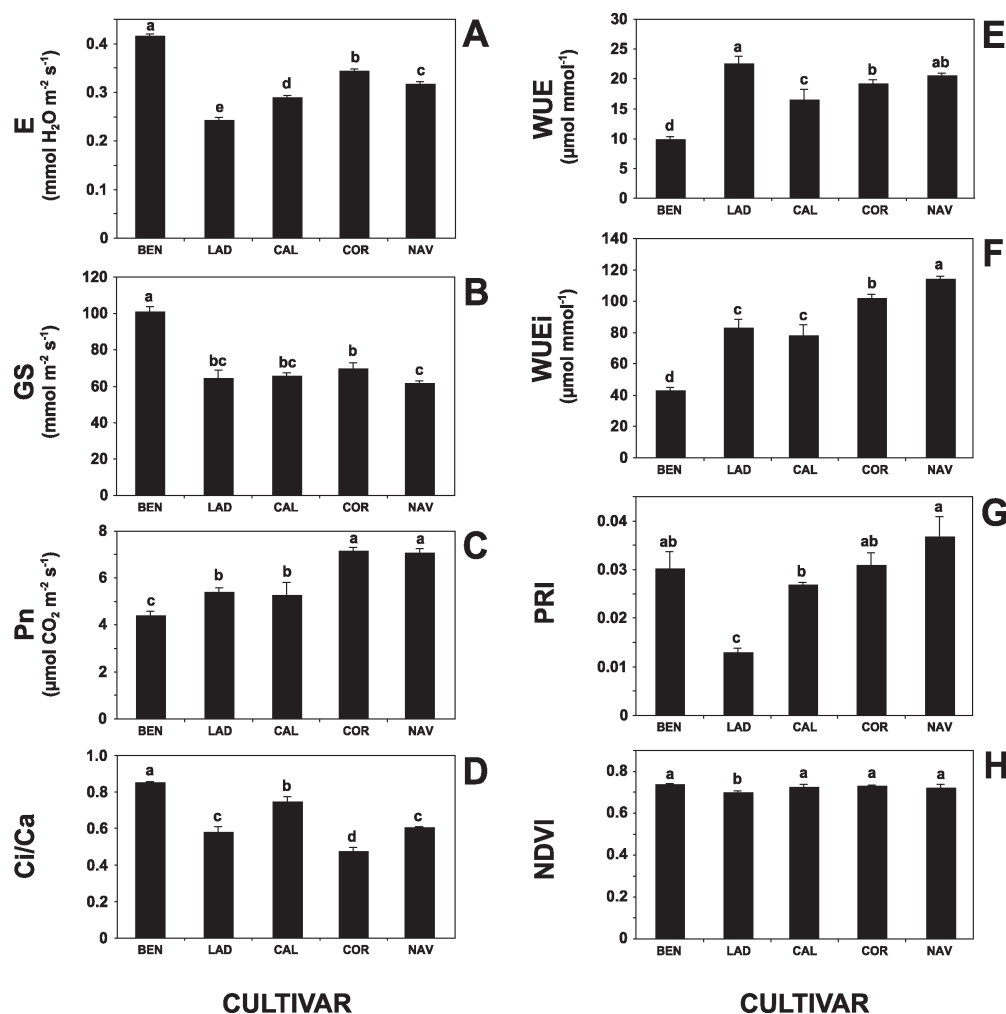


Fig. 1. Photosynthetic parameters, water use characteristics and reflectance indices of cold acclimated of winter oilseed rape leaves. The transpiration rate (E; A), stomatal conductance (GS; B), net photosynthetic rate (Pn; C), intracellular/intercellular CO_2 concentration (Ci/Ca; D), water use efficiency – calculated as Pn/E (WUE; E), intrinsic water use efficiency – calculated as Pn/GS (WUEi; F), photochemical reflectance index (PRI; G), and normalized difference vegetation index (NDVI; H). Five cultivars of winter oilseed rape Benefit (BEN), Ladoga (LAD), Californium (CAL), Cortes (COR), and Navajo (NAV) were subjected to 30 days of cold (4°C). The means \pm SE are shown. The letters denote the statistical significance (as determined by the Tukey HSD test) of mean differences between stress treatments. Only those marked with different letters differ significantly at $p < 0.05$.

Principal component analysis

Based on the plot, only the principal components with an eigenvalue greater than 1.0 were chosen. Sufficient explanation of the variability can be achieved by two factors (principal components; PC). PC1 and PC2 explained 54.03% and 21.15% of the

whole variability, respectively (in total 75.18% of variability was explained; Fig. 4A). The variables with the greatest contribution to PC1 were WUEi (96.3%), DHNs accumulation and WUE (92.7%), Pn (91%), Ci/Ca (83%), and LT_{50} (78%), while the variables with the greatest contribution to PC2 were E (65%), PRI (60%), and NDVI (57%). The plot component weights (Fig. 4A) grouped similar

Table 2

Pearson correlation coefficients (calculated from CA plants) among frost tolerance, dehydrin accumulation and selected photosynthesis-related characteristics. The transpiration rate (E), stomatal conductance (GS), net photosynthetic rate (Pn), water use efficiency as Pn/E (WUE), intrinsic water use efficiency as Pn/GS (WUEi), intracellular/intercellular CO_2 concentration (Ci/Ca); photochemical reflectance index (PRI), normalized vegetation index (NDVI), dehydrin accumulation (DHN) and acquired frost tolerance (LT_{50}).

	E	GS	Pn	WUE	WUEi	Ci/Ca	PRI	NDVI	DHN
GS	0.765*								
Pn	-0.014	-0.411*							
WUE	-0.700*	-0.755*	0.690*						
WUEi	-0.370*	-0.769*	0.878*	0.844*					
Ci/Ca	0.342*	0.616*	-0.767*	-0.765*	-0.809*				
PRI	0.366*	0.047	0.226	-0.150	0.143	0.007			
NDVI	0.417*	0.235	0.046	-0.283*	-0.079	0.124	0.382*		
DHN	-0.246	-0.686*	0.777*	0.626*	0.844*	-0.782*	0.141	-0.046	
LT_{50}	0.043	0.511*	-0.628*	-0.352*	-0.643*	0.505*	-0.183	-0.057	-0.815*

$n = 150$.

* Statistically significant correlations at $p < 0.01$.

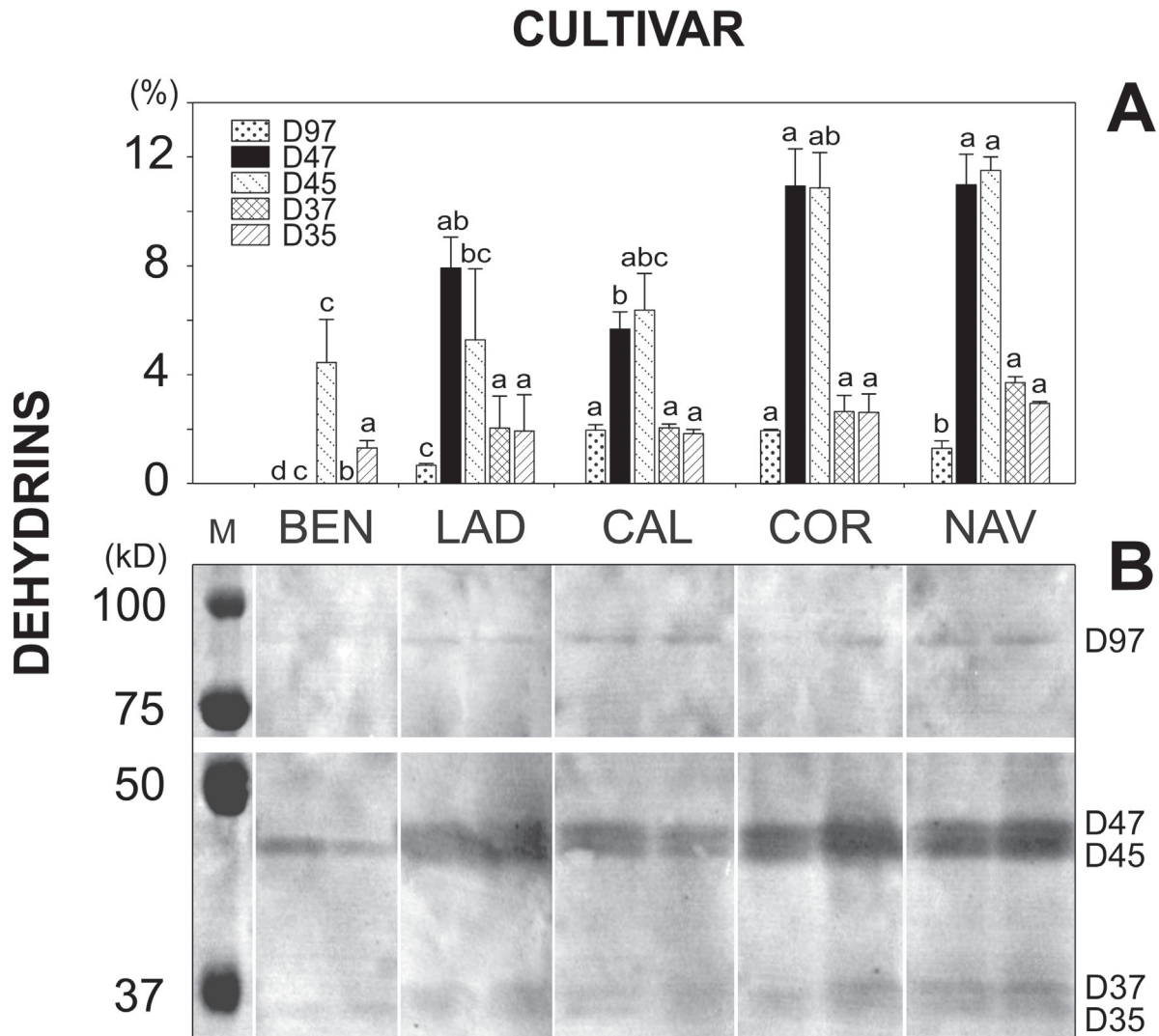


Fig. 2. Individual dehydrin proteins accumulation (A) and representative protein gel blot analysis (B) probed with anti-dehydrin antibody, derived from extracts of cold-stressed leaves of five winter oilseed rape cultivars (two biological replications shown). Benefit (BEN), Ladoga (LAD), Californium (CAL), Cortes (COR), Navajo (NAV), and M, molecular mass standard; equal amounts of each sample volume were loaded onto the gel. D97, D47, D45, D37, and D35 stand for dehydrin accumulation, according to different molecular masses visualized on protein gel blot analysis (~97, ~47, ~45, ~37, and ~35 kD, respectively). The means \pm SE are shown. The letters denote the statistical significance (as determined by the Tukey HSD test) of the same dehydrin molecular mass differences. Only those marked with different letters differ significantly at $p < 0.05$.

variables (WUEi, DHNs, Pn, and LT_{50}) as did factor analysis (Fig. S1). Plot case factor coordinates assembled individual observations in the cvs into three groups (Fig. 4B).

Discussion

The focus of our work was to study whether selected physiological parameters are good indicators of FT for chosen winter oilseed rape cvs when subjected to cold temperatures. In general, winter annuals uniformly exhibited similar or higher rates of Pn in cold-acclimated vs. control plants (Hurry et al., 1995; Savitch et al., 1997; Rapacz, 1998a,b; Rapacz and Janowiak, 1998, 1999; Fu et al., 2000; Savitch et al., 2002, 2005; Dumlao et al., 2012). Cold-acclimated leaves typically show an increase of Pn below the thermal optimum, which results in a lowering of the peak temperature for photosynthesis. This is because cold grown leaves are thicker, with larger cells, higher leaf nitrogen content, and an overall increase of photosynthetic enzymes (Sage and Kubien, 2007). On the basis of the above criteria, some authors suggest that the ability of the photosynthetic apparatus to be acclimatized can be useful as an indirect indicator of the whole cold acclimation

efficiency (Hurry et al., 1995). The energy required to attain the FT is derived from photosynthesis, which plays a crucial role during the first stage of CA (Rapacz, 1998a,b). In the winter oilseed *B. napus* cultivar Tor, Hurry et al. (1995) observed a 21% increased Pn at 5 °C, compared with controls (24 °C). In contrast, in the spring cultivar, the Pn decreased by 92% (from 8.6 to 0.7 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) under the same conditions. Rapacz and Chilmonik (2000) found that the main difference between spring and winter oilseed rape in terms of Pn was the higher efficiency of electron transport in autumn for the latter.

In our experiments, the mean values of E and GS were significantly lower and WUEi significantly higher in stressed plants than in controls in all cvs (Fig. 1A and B; Table S1). The E normally decreases under low temperature conditions, because of a cold-related decrease in the vapor pressure difference between the leaf surface and the atmosphere (Sage and Kubien, 2007; Aroca et al., 2012; Navarrete-Campos et al., 2013). During the reduced availability of water in the plant upon becoming cold, other metabolic processes, including photosynthesis, may be limited until the plants are fully CA. Similar results to ours were obtained in the experiments of Hall et al. (2005) in drought-stressed *Brassica* spp.; by

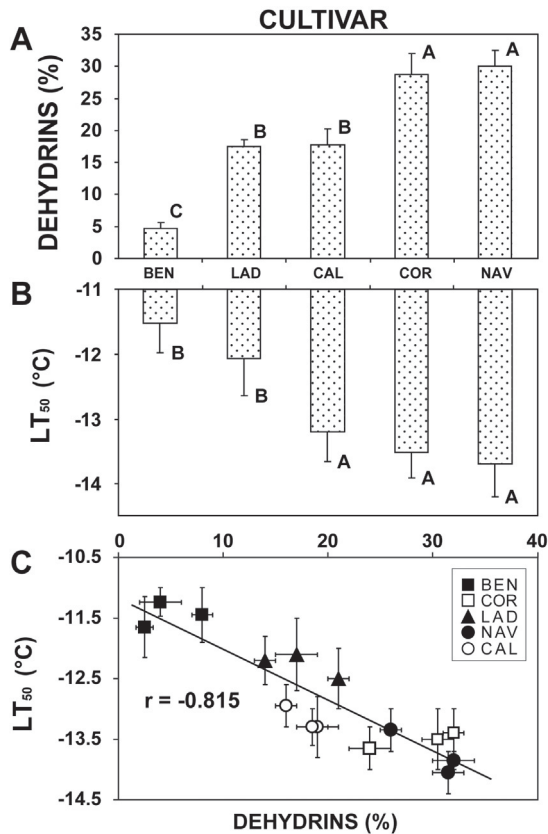


Fig. 3. Dehydrins accumulation and LT₅₀ values of cold-stressed winter oilseed rape leaves. Dehydrin accumulation (A); acquired frost tolerance (B) and Pearson correlation between total DHNs accumulation and acquired frost tolerance, expressed as LT₅₀ values in five rapeseed cultivars after 30 d of cold acclimation in three biological and two technical repetitions each (C), cultivars BEN, LAD, CAL, COR, and NAV. The means \pm SE are shown. Capitals denote (as determined by the Tukey HSD test) cold treatment mean differences between genotypes. Only those marked with different letters differ significantly at $p < 0.05$.

Hurry et al. (1995) in cold-stressed *Brassica* spp.; and in *A. thaliana* (Pons, 2012). However, with respect to the intensity of Pn, Fig. 1C shows that the two cvs (COR and NAV) with the highest FT and DHN (Fig. 3) also had the highest Pn values for these cvs. The Pn rate in COR and NAV do not seem to be reacting on E or GS values after full CA. This can be explained by the fact that the sensitivity of Pn to variations in E and GS generally increase at warmer (not colder) temperatures (Sage and Kubien, 2007). The Ci/Ca ratio also depends on the balance between GS (which increases) and Pn (which decreases) the Ci of the plant. This is because the entry of CO₂ into the leaf interior is influenced by GS, while the Pn rate determines the demand for CO₂ (similarly observed in *A. thaliana*; Pons, 2012). CA is associated with increased O₂ and CO₂ sensitivity at low temperatures, indicating a disproportionate enhancement of the inorganic phosphorus regeneration capacity (Sage and Kubien, 2007). *B. napus* can increase such capacity through the expression of the enzymes via starch and sucrose synthesis (Hurry et al., 1995). Pons (2012) observed higher Pn and carboxylation capacity in frost tolerant *A. thaliana* (the same family as *B. napus*) accession Hel-1 even in lower Ci in contrary to frost susceptible accession CVI-0. Both had similar GS, leaf mass per unit area, Rubisco and chlorophyll content.

Changes in cold conditions are believed to also have primary relationships to receptors of turgor pressure changes in cells (Aroca et al., 2012) and thus also may influence the GS. The significant correlations of LT₅₀ to Ci/Ca and GS (positive correlations), as well as to WUEi (negative correlation) can generally be related to the fact that

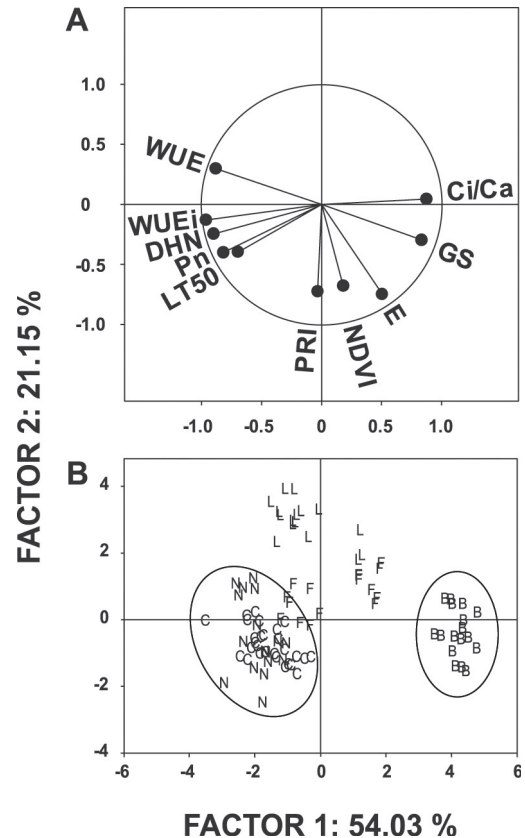


Fig. 4. Principal component analysis. (A) Plot component weights; the circle unit provides a visual indication of how well each variable (i.e. measured parameter) is characterized by PC. Individual variables are represented by a vector and the angle that is formed between them, estimating the level of similarity. Angles $< 90^\circ$ indicate a positive relationship and vice versa; orthogonal angles indicate that the variables are linearly independent (r close to 0). All variables are chosen to be active, the same abbreviations as in Fig. 1; (B) Plot case factor coordinates (the individuals factor plot of two first component scores), axes spanning a total of 75.2% of the explained variation in the data matrix. B and N + C are in an oval to show similar observations. B (Benefit), L (Ladoga), F (Californium), N (Navajo), and C (Cortes). In A and B the percents of explained variability by each principal component are shown.

genotypes more resistant to (cold-related) dehydration prevented such a state by a large portion of the stomata closing (Fig. 1B). This is in agreement with Aroca et al. (2012), who observed that the stomata of the cold-sensitive maize remain open, while those of the tolerant plants close more rapidly. However, the Ci/Ca ratio under colder conditions was lower in cultivars with higher photosynthetic rates and vice versa ($r = -0.767$; see 'Benefit'; Fig. 1D), likely due to higher actual need for CO₂ in higher FT plants and other nonstomatal limitation phase, causing Ci to increase. Stomata can be more closed (and WUEi rises) if the inner CO₂ concentration (Ci) is sufficient enough to saturate carboxylation, contrary to other cultivars. Genotype-related variation in leaf thickness altered the difference in Ci at a Rubisco site (Hall et al., 2005) and answered questions about different Pn rates. Moreover, in some cases, at 10°C, the ceiling imposed on Pn by phosphate regeneration lowers the CO₂ saturation point, causing Pn to become CO₂ insensitive (Sage and Kubien, 2007). Additionally, GS is controlled by the phytohormone ABA and, according to our DHN data, there may be a hypothetical crosslink to ABA-dependent and ABA-independent COR/LEA protein (DHNs) expression as the final products of signaling cascades. It is known that the low temperature brings about an increase in ABA (Thomashow, 1999). Thus, with higher Pn and lower water diffusion from the leaves, our experiments showed that both water use efficiencies increased (WUE and WUEi; Fig. 1E and F). If the plant is able to effectively manage water deficit, high energy-consuming

metabolic processes (e.g., cryo-protective) need not be significantly affected. Therefore, it seems that the observed positive relationship between Pn and WUEi ($r=0.878$) may also be directly related to FT because WUEi also has a connection to DHN ($r=0.844$; see also Fig. 4; Fig. S1). In *Eucalyptus*, increased WUE (caused by the decrease of GS) after drought was used to select for increased FT (Navarrete-Campos et al., 2013). Our results indicated that the higher WUEi values identified cvs that effectively coped with the decrease of water availability during cold conditions (4 °C). Furthermore, the correlation of LT₅₀ to WUEi was $r=0.643$. Cvs-related differences in WUEi may be attributed to the intrinsic ability of each genotype to regulate GS and to the changes in dry matter partition induced by stress. Thus, the higher the WUEi value, the better the ability to face the dehydration of cells. Energy-consuming metabolic processes are significantly influenced by sufficient water availability which implies that higher WUEi is a beneficial indicator of resistant (highly adapted) cultivars even in cold. Cell dehydration can also be decelerated by the increase of soluble carbohydrate pools as products of the higher photosynthetic rate during hardening (Hurry et al., 1995). We hypothesize that COR and NAV are able to sustain large pools of cryo-protective metabolites, and they probably adapt their enzymatic apparatus to work at lower temperatures even under cold-increased amounts of soluble carbohydrates, which normally inhibit photosynthesis, as postulated e.g., by Hurry et al. (1995), Savitch et al. (1997), Rapacz (1998b), Fu et al. (2000), Sage and Kubien (2007) and Dumlao et al. (2012).

Pons (2012) observed that a low growth temperature requires a large investment in the photochemical apparatus to compensate for the reduced enzyme activity. To explain more changes in the physiological response at the individual cvs level, the spectral indices (PRI; NDVI) were used. Our results on PRI showed that, in all cvs, the photoprotective mechanisms increased their activity significantly during CA (Fig. 1G) as PRI value decreased. In the experiments of Gamon et al. (1997), PRI was correlated with Pn under field conditions ($r=0.54$). Hernández-Clemente et al. (2011) showed PRI related to GS and water potential in the conifer forest (*Pinus sylvestris* and *P. nigra*) under water stress. Porcar-Castell et al. (2012) published the correlation of PRI to the quantum efficiency of photochemistry ($r=0.94$) in *P. sylvestris*. In agreement with our results Savitch et al. (2005) confirmed an increase of xanthophyll cycle pigments deepoxidation (which corresponds to PRI decrease) in *B. napus* BNCBF/DREB1 after 2–4 weeks at 4 °C. BNCBF/DREB1 overexpression in *Brassica* not only resulted in increased constitutive freezing tolerance, but also partially regulated chloroplast development to increase photochemical efficiency and even photosynthetic capacity. The lower PRI indicated the increase of the xanthophyll cycle and carotenoid pigments were observed in cvs with lower DHN and LT₅₀ which means lower FT plants (LAD, CAL) suffered under photosynthetic stress more than acclimated cvs. Even so; the reduction of chlorophyll content in the leaves upon cold has been recognized as a general phenomenon (Rapacz and Janowiak, 1998) NDVI values in our experiments showed only small decrease in relation to controls which means there was only small reduction of chlorophyll content in leaves as well. However, the NDVI values may indicate that the deficiency of Rubisco is not the cause. Differences in carboxylation efficiency might be explained by qualitative changes concerning higher activity of ribulose-1,5-bisphosphate carboxylase/oxygenase and sucrose-phosphate synthase (Hurry et al., 1995; Rapacz and Janowiak, 1998; Rapacz and Hura, 2002). Nevertheless, we assume neither the NDVI nor the PRI values are directly suitable to distinguish among cvs in terms of FT. Similar results were obtained in field conditions by Chytky et al. (2011) in 11 spring wheat cvs.

The accumulation of DHNs was another parameter studied in cold-acclimated samples. Interestingly, we were able to visualize other types of DHNs (Fig. 2A and B), which were not visible

in our previous study (Klíma et al., 2012). This is evident from a comparison of the DHN spectrum accumulated in the CAL, used in both experiments. This result was achieved by optimizing visualization procedures; specifically, more concentrated DHN samples, increasing the amount of loaded samples, using a gel with a higher concentration of acrylamide, and longer incubation with the primary antibody. The densities of these “additional” DHNs (D97, D37 and D35) were significantly lower than the others (D47 and D45), which made it difficult to visualize their accumulation. Moreover, in comparison to CA wheat, the DHN accumulation in CA winter oilseed rape is about 100 times lower (Vítámvás et al., 2007, 2010). However, within the *Brassica* species, DHN proteins have previously been visualized in the experiments of Rurek (2010) (mitochondrial fraction of *B. oleracea*), and as the result of cold stress by Klíma et al. (2012) (fraction of proteins soluble upon boiling of *B. napus* and *B. carinata* cvs) also. However, to date, the qualitative differences in DHNs accumulation between *Brassica* species genotypes has not been observed. BEN accumulated only two types of DHNs, while in the other cvs, all five were accumulated (Fig. 1B). In addition, BEN also had the highest (less negative) LT₅₀ values (Fig. 3B). The sum of D45 and D47 DHNs were correlated the same way as the sum total of the DHNs accumulation (data not shown). Consequently, we assume that the absence of some DHNs (especially D47, which was observed as the most abundant in the other cvs; Fig. 2A) is associated with lower FT in BEN. The results indicated that the capability of accumulation of D47 could have a measurable influence on FT of rapeseed genotypes. Therefore, any event (mutation in the most abundant DHNs genes or in their regulation sequences) leading to different levels of accumulation of DHNs could result in different levels of FT of plants also. This observation is in agreement with the correlation between quantitative differences in accumulation of DHNs and FT of genotypes. The strong correlation between the LT₅₀ and the accumulation of DHNs (i.e. the highest value observed in our experiments; $r=-0.815$) has already been confirmed for a number of plants (Cellier et al., 1998; Ismail et al., 1999; Kosová et al., 2007; Vítámvás et al., 2007, 2010; Hanin et al., 2011). Additionally, the accumulation of DHNs in wheat has been considered a reliable indicator of FT, where even reduced contrast genotypes in FT can be distinguished among from each other (Vítámvás et al., 2007, 2010). However, it should be taken into consideration that the overexpression of DHN genes alone generally does not result in an increased plant stress tolerance and supports the idea that FT is a polygenic trait (Lång and Palva, 1992; Yamaguchi-Shinozaki and Shinozaki, 2006; Kosová et al., 2007). Thus, the different accumulation of DHNs in cultivars could indicate that a stress-regulated pathway leading to the accumulation of DHNs was fully functional but the pathway had different levels of regulation in cultivars with different FT. The accumulation of DHNs is only a small part of the very complex CA process (e.g., Kosová et al., 2012). Therefore, other components of the CA process should have a higher probability for the phenotyping of crop FT. Consequently, the close relationships of LT₅₀ with other physiological parameters found are important.

CA is a quantitative trait involving the action of many genes, and current evidence suggests that multiple mechanisms are involved in activating the cold-acclimation response (Thomashow, 1999). This relationship (between measured parameters) is shown by the result of the PCA analysis which grouped the parameters previously correlated with FT (WUEi, Pn, WUE, and DHN; compare Table 2 to Fig. 4A and B and factor analysis in Fig. S1). None of the control measured parameters were able to differentiate between genotypes and/or explain the subsequently observed LT₅₀ values at a significant level (data not shown). By contrast, the results of Pn and WUEi in CA cvs can clearly distinguish between genotypes with a higher FT. The crucial effect of a genotype on all ten measured parameters is shown in Fig. 4B. Cvs were divided, according to the PCA analysis, into the three compact groups with similar FT. BEN was a

representative of a cold susceptible cultivar, NAV and COR as representatives of the most resistant cvs (both distinguished by PC1 where WUEi, DHN, WUE, Pn, Ci/Ca and LT₅₀ have played the major role) and LAD and CAL as an intermediate group, distinguished mostly by PC2. For example BEN is grouped outside not only for its lower Pn, DHN and LT₅₀, but also for its significantly higher value of GS, Ci/Ca and E in cold when coupled with lower WUE a WUEi values.

FT has been recognized as a polygenic trait. The process of CA encompasses biological modification on many levels, e.g., modulation of gene expression, accumulation and degradation of proteins, changes in sugar content and changes in the photosynthetic machinery (Thomashow, 1999). For the full development of FT, it seems to be necessary to have a high rate of Pn (available energy) as well as the synergistic and/or protective effect of the DHNs. The present study indicates that, during acclimation of winter rape to cold, the complex interaction of Pn and DHN production and accumulation occurs. All genotypes which showed higher FT (COR, NAV) also showed higher net photosynthesis after CA. This increase is connected – besides other things – with a higher amount of DHN's present in such tissues contrary to others with lower FT (DHN to FT, $r=0.815$). We do not believe that the observed correlation proves any direct mechanistic link between Pn and FT. As Hurry et al. (1995) and e.g. Savitch et al. (2005) postulate, we rather favor the view that photosynthesis provides the energy necessary for the cellular changes (DHNs and others) required for higher FT. These cellular changes are covered here by DHNs accumulation. Furthermore, in some cases, correlations are linked with other hidden developmental strategies realized among winter cultivars. That implies the lack of photosynthesis acclimation caused by reducing the amount of energy available for plant acclimation and in this way it can cause difficulty in the assessment of FT.

Our hypothesis is that high levels of Pn in the CA winter oilseed rape leaves suggest the success of these cvs in the cold hardening process, and can be used together with WUEi and DHN accumulation as indirect indicators for FT selection. The present study combines physiological and biochemical data to provide a partial answer regarding the FT process in selected winter oilseed rape cvs after CA. Future work should focus on more detailed observations into DHN time dynamics, related to the developmental stages of winter oilseed rape. However, in order to generalize such conclusions, as well as to be able to consider DHN accumulation, Pn, and WUEi as reliable indirect indicators of FT in winter oilseed rape, it will be necessary to verify these relationships in a wider range of genotypes.

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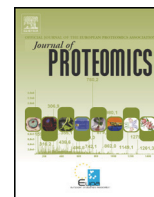
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Proteomic and physiological approach reveals drought-induced changes in rapeseeds: Water-saver and water-spender strategy



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ABSTRACT

The cultivar-dependent differences in *Brassica napus* L. seed yield are significantly affected by drought stress. Here, the response of leaf proteome to long-term drought (28 days) was studied in cultivars (cvs): Californium (C), Cadeli (D), Navajo (N), and Viking (V). Analysis of twenty-four 2-D DIGE gels revealed 134 spots quantitatively changed at least 2-fold; from these, 79 proteins were significantly identified by MALDI-TOF/TOF. According to the differences in water use, the cultivars may be assigned to two categories: water-savers or water-spenders. In the water-savers group (cvs C + D), proteins related to nitrogen assimilation, ATP and redox homeostasis were increased under stress, while in the water-spenders category (cvs N + V), carbohydrate/energy, photosynthesis, stress related and rRNA processing proteins were increased upon stress. Taking all data together, we indicated cv C as a drought-adaptable water-saver, cv D as a medium-adaptable water-saver, cv N as a drought-adaptable water-spender, and cv V as a low-adaptable drought sensitive water-spender rapeseed. Proteomic data help to evaluate the impact of drought and the extent of genotype-based adaptability and contribute to the understanding of their plasticity. These results provide new insights into the provenience-based drought acclimation/adaptation strategy of contrasting winter rapeseeds and link data at gasometric, biochemical, and proteome level. **Significance:** Soil moisture deficit is a real problem for every crop. The data in this study demonstrates for the first time that in stem-prolongation phase cultivars respond to progressive drought in different ways and at different levels. Analysis of physiological and proteomic data showed two different water regime-related strategies: water-savers and spenders. However, not only water uptake rate itself, but also individual protein abundances, gasometric and biochemical parameters together with final biomass accumulation after stress explained genotype-based responses. Interestingly, under a mixed climate profile, both water-use patterns (savers or spenders) can be appropriate for drought adaptation. These data suggest, than complete “acclimation image” of rapeseeds in stem-prolongation phase under drought could be reached only if these characteristics are taken, explained and understood together.

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Abbreviations: C, Californium; Car, carotenoids; Chl, chlorophyll; C_i/C_a , ratio of leaf intercellular $[CO_2]$ to ambient air $[CO_2]$; cv(s), cultivar(s); D, Cadeli; DAS, day(s) after beginning of treatment; DAP, differentially accumulated proteins; DW, dry weight; E, leaf transpiration rate; ET, evapotranspiration change; FTSW, fraction of transpirable soil water; FW, fresh weight; GO, gene ontology; gs, stomatal conductance; mb Diff, differential H_2O concentration in mbar; mb Ref, reference H_2O concentration in mbar; N, Navajo; NMMDS, non-metric multidimensional scaling; NET, normalized evapotranspiration; OP, osmotic potential; PCA, principal component analysis; PEG, polyethylene-glycol; Pn, net photosynthetic rate; PPI, protein-protein interaction network; PRO, proline; RWC, relative water content; SI, supplementary information; SWC, soil water content; TTSW, total transpirable soil water; UPGMA, unweighted pair group method with arithmetic mean; V, Viking; WUE, water use efficiency (Pn/E); WUEi, intrinsic water use efficiency (Pn/g_s).

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1. Introduction

Abiotic stress conditions such as drought cause extensive losses to agricultural production worldwide [1]. Crop production has to double by 2050 to meet the predicted demands of the global population and to achieve crop yields increases at the rate of 2.4% per year (now 1.3%) [2,3]. A combination of different approaches (physiology-based understanding, omics-techniques, QTL mapping, epigenetic breeding and other tools) will likely be needed to significantly improve the abiotic stress tolerance of crops in field conditions, particularly to drought [1]. Environmental water deficiency triggers an osmotic stress-signalling cascade which induces short-term cellular responses to reduce water loss and long-term responses to remodel the transcriptional network, physiological and developmental processes [1]. The dynamic change

in protein abundance and protein phosphorylation is a strategy to mitigate and recover from damaging effects in plants [4,5]. Therefore, in plant abiotic stress studies, it is common to analyze proteomes of stressed plants in contrast to the control ones, attempting to correlate changes in protein accumulation with the plant phenotypic response [6,7]. Additionally, comparisons between genotypes with different sensitivity to drought are crucial to understanding the putative influence of differentially abundant proteins in tolerant genotypes.

Brassica napus (winter form) is the major oilseed crop in temperate regions in Europe and the second most important oil-bearing crop in the world [8]. Winter oilseed rape cultivars have high genetic diversity [9], show variability in frost tolerance and quantitative and qualitative changes in dehydrin accumulation [10]. Some proteomic studies of oilseed rape roots suggest different proteomic profiles between genotypes [11]. However, pathways triggered by plant-environment interactions (such as nitrogen resources), environment-related plant architecture, and, therefore, sink-source relations *in planta*, seem to be globally conserved between the model plant *Arabidopsis* and *B. napus* [12]. These findings encourage the transfer of knowledge from *Arabidopsis* to the crop *B. napus*.

Rapeseed is classified as salinity-tolerant [13], however, it is a drought-sensitive crop. Its sensitivity depends on and varies with the developmental stage [14]: during the seedling stage [15], in the vegetative (stem prolongation) [16] and the reproductive stage (flowering) [17–20]. The seeds filling stage is a less perceptive stage to moderate drought stress due to a sufficient and rapid translocation of assimilates from the stem [21]. However, this stage is connected to sufficient stem prolongation period because prolongation is highly water-demanding stage. To evaluate the degree of drought stress and its influence on individual cultivar (cv), the following physiological traits (e.g., relative water content, proline accumulation, transpiration changes, etc.) are used in screenings for drought tolerance within *Brassica* species in the stem prolongation stage [16]. In the warm, humid continental climate of The Czech Republic (Cfb; Koeppen-Geiger classification [22]), different water-use patterns can be useful for drought adaptation of crops, as it is postulated for other similar countries [23–31]. The so-called water-saver plants can maintain satisfactory yield in long-term drought conditions. On the other hand, in a quick drought, the second group - water-spenders - showed delayed response to drought (so they seem to be less perceptive to actual water content in the soil profile) [30, 32–34]. Water-spenders keep stomata open and thus can sustain higher net assimilation and growth. Furthermore, genotypes that keep growing throughout the decreasing water supply (water-spenders or spenders) perform well in the field whenever there is adequate water in the subsoil and a prospect of rain [30], and thus can possess future productive advantage [28].

High-yielding cultivars are believed to respond to the drought stress by various acclimation strategies including phenotypic plasticity [35], because adaptation to stress has metabolic and energy costs. Highly plastic cultivars should possess very regulated stress-response ability, without influencing crop performance when stress is absent [7,36].

Among the proteomics studies and reviews published on *Brassica* spp. to date [37–50], only few differential proteomic studies are focused on *B. napus* drought stress response (comparative proteomics on seedling roots [11] and 6 weeks old plant leaves [51]). In this study, we screened for differentially regulated proteins in four vernalized winter oilseed rapes under long-term drought stress. Proteomics was carried out by modification of 2DE, the two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) [52]. This characterization will enable preparation of a “data platform” used to explain proteomic results and to make some “genotype” based conclusions.

In addition, selected physiological, biochemical and molecular characteristics during a highly water-demanding stage – the stem prolongation period – are described. The analyses of gasometric, biochemical and water-related characteristics will provide evidence of how proteome modulates and/or is modulated during response to gradual drought

stress. This information based on vernalized plants was missing so far. Detailed comparison of these changes will enable genotype separation according to their actual behaviour based on soil water content. Using this data, we can distinguish between water-savers and water-spenders strategies. Additionally, proteomics data could provide an insight into the real impact of drought on plant cellular homeostasis and the extent of genotype-based adaptability from a longer-time point of view and contribute to understanding the level of phenotypic plasticity.

2. Material and methods

2.1. Plant samples and physiological measurements

The seeds of four winter rapeseed (*Brassica napus* L.) cultivars (cvs) Californium (C), Navajo (N), Viking (V) and Cadeli (D) were germinated and cultivated under semi-controlled conditions (at 20 ± 2 °C day/night) in a greenhouse for 10 d in the substrate RKS II (Agro CS, CZ). For passport and other information on the four accessions, please see [9,53] and Supplementary Information Table S-1. Cvs were kept in a growth chamber to the four-leaf stage at 20 ± 2 °C (day/night), 70% humidity, 12 h photoperiod and irradiance of $300 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. After three weeks, plants were exposed to vernalization temperature (4 ± 2 °C) for another 8 weeks in the same conditions. Plants were transferred into bigger pots filled with the same substrate to the weight 2.0 kg, water soaked and transferred into a growth chamber at $21/18$ °C day/night, 12 h photoperiod (7:00–19:00 CET) with irradiance intensity $\sim 300 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ for another 4 weeks. For the protocol of water imposition (dry down) and detailed workflow of experimental design, please see information in Supporting Information S-1. The watering in well-watered conditions was adjusted to mimicking conditions in the field (similar to Ford et al. [54]). After 28 days of progressive drought, both controls and stressed samples were collected. At the end of stress, both groups were in the middle of stem prolongation stage 33–35 BASF, Bayer, Ciba-Geigy, Hoechst (BBCH) [55]. All biochemical and molecular samples were taken at the 28th day after the beginning of treatment (DAS). For details, please see Supporting Information S-1 and S-3. Five technical from each biological and three independent biological replicates were done. The middle part of the first fully developed leaf (from the top) was harvested from each of the five plants from each technical repetition, frozen in liquid nitrogen as one biological replicate and stored at -80 °C for subsequent protein extraction. The other parts of that leaf were used for all other physiological measurements (e.g. RWC) and for proline extraction. Osmotic potential was measured on the youngest (not fully developed) leaf. All photosynthetic data during cultivation were measured on the same leaf used for protein extraction using open system unit TPS-2 (manual available at: <http://ppsystems.com/tps2-portable-photosynthesis-system/>; PPSystems, Amesbury, MA, USA). Data were taken according to manual instructions and measurements were taken from 10:00–12:00 CET.

The water content changes in pot volume are expressed using three calculations: evapotranspiration (ET; see formula 1 in Supporting Information S-1), soil water content (SWC; see formula 2 in Supporting Information S-1), and fraction of transpirable soil water (FTSW; see formula 3 a 4 in Supporting Information S-1). ET is an evapotranspiration rate change ($\Delta\text{H}_2\text{O}$ [g.day⁻¹]). ET values are based on plant H₂O transpiration and pot H₂O evaporation changes. Dry-weight gravimetric SWC means weight of water (kg) in the potted soil related to the weight of oven-dried soil from each particular pot (kg) to the constant weight. After harvest, FTSW for every day was calculated according to references in [56,57]. The FTSW values represented the portion of remaining transpirable soil water (until a precisely defined and observable threshold) and were used as the indicator of stress, so that experiments could be rigorously compared and evaluated. Formula 3 is FTSW evaluated according to plant Pn or E, while formula 4 is based strictly on ET changes without using physiological data. According to Kholová et al. [57], the double data standardization of transpiration (NET) within individual

genotype and control was carried out in order to reduce plant-to-plant variation.

2.2. Protein extraction, 2D-DIGE analysis, protein identification and database search

For total protein extraction, the trichloroacetic acid (TCA)/acetone method followed by protein extraction into phenol and subsequent protein precipitation using ammonium acetate was used as described in [58–60]. For details about protein extraction, please see Supporting Information S-3. Dry protein pellets were resolved in lysis buffer according to GE Healthcare manual for 2D-DIGE analysis, pH of the solution was adjusted to 8.5 by 50 mM NaOH and protein concentration was determined by 2D Quant kit (GE Healthcare). The protein samples (30 µg) were labelled with CyDye® minimal dyes (GE Healthcare) according to manufacturer's instructions. For detailed information about 2D-differential gel electrophoresis and image analysis, please see Supporting Information S-4 and S-5. Protein identification was carried out using MALDI-TOF/TOF. For protein identification, the excised proteins were processed as described in [61] with details in Supporting Information S-6. All spots with two or more identified proteins were discarded from further analysis. The data obtained from MALDI-TOF/TOF analysis, grouping the MS and maximum 10 MS/MS spectra with highest values for each spot, were used for protein identification using MASCOT v 2.2 (Matrix Science Inc., Boston) on a ProteinPilot-platform (ABSciex) by searching against NCBI database (www.ncbi.nlm.nih.gov), downloaded on 7 April 2016 and limited to the taxonomy of Tracheophyta. All parameters used in the protein identification are described in Supporting Information S-14.

2.3. Bioinformatic analysis of proteins, biological functions of identified proteins

Molecular functions of proteins were searched in AgBase GORetriever [62] (http://agbase.msstate.edu/cgi-bin/tools/goretriever_select.pl). For Gene Ontology annotation (GO), GOSlim Viewer (http://www.agbase.msstate.edu/cgi-bin/tools/goslimviewer_select.pl) was used to characterize general cellular components, biological functions and biological processes (Ag Base version 2.00; Select GOSlim set: Plant). The protein accession versions were created according to NCBI database (<http://www.ncbi.nlm.nih.gov>; NCBI nr 20151110, 76068736 sequences; 27658295194 residues). Cellular localisation of identified proteins was determined using TargetP 1.1 [63] server (<http://www.cbs.dtu.dk/services/TargetP/>). To characterize protein responses for each individual cultivar, GOModeler [64] was used to create “net effect” of protein density tested on 16 hypotheses (<http://agbase.msstate.edu/cgi-bin/tools/GOModeler.cgi>). Proteins were Z-score standardized and sorted into clusters according to their mode of accumulation using PermutMatrix [65] (version 1.9.4.; Supporting Information S-2). To understand interactions of identified proteins, a protein-protein interaction network (PPI) was created on identified proteins blasted against the *Arabidopsis thaliana* TAIR10 protein database (<http://www.arabidopsis.org>) with the intention of obtaining annotated protein entries for PPI tools. Only results with the highest score and lowest E value were considered as relevant for each protein. For PPI map, the on-line analysis tool STRING 9.1 [66] was used. Biological processes, molecular functions and cellular components were predicted by BiNGO 3.0.2 [67] as a plugin in Cytoscape 3.1.1 [68] (<http://cytoscape.org>). For detailed information about used bioinformatic analyses, please see Supporting Information S-7.

2.4. Statistical analysis of physiological, biochemical and protein data

Exploratory Data Analysis (EDA) was used to determine statistically important characteristics of measured (Pn, gs, E, mb, etc.) and derived physiological parameters (WUE, WUEi, etc.) of the data set.

Combination of statistical tests together with diagnostics graphs were used for descriptive statistics (mean, variance, etc.), verification of normality and homogeneity of the data and for detection of the outliers. Linear dependence of the parameters of interest was determined by correlation and regression analysis. The differences among group of means were estimated either by one-way and two-way ANOVA or by Kruskal-Wallis ANOVA (nonparametric one-way ANOVA for non-homogeneous variance). Comparisons were performed for all physiological or derived parameters. For deeper understanding of the relationship between measured characteristics, principal components analysis (PCA) was used. The same method was applied to a protein dataset. Diagnostic indicators, such as Scree plot, loading plot and total amount of explained variability were used to find an optimal model. To verify PCA results, different mathematical methods were used, namely a hierarchical clustering method (CLU) and non-metric multidimensional scaling (NMMDS). The most appropriate clustering method was chosen according to a canonical correlation coefficient and delta parameter values using NCSS 2000 software (Hintze) [69]. To estimate the ideal number of dimensions and quality of the resulting NMMDS model, stress and alienation parameters as well as diagnostic graphs were used. All statistical tests, with the exception of canonical correlation coefficient and delta parameters, were computed in STATISTICA v. 12 (StatSoft, Inc.). Cluster analysis of the protein spots relative abundance and selected physiological and biochemical measurements (Supporting Information S-2) were carried out using PermutMatrix software (version 1.9.4) [65]. PDQuest-based statistics of protein abundance calculation is described in Supporting Information S-5.

3. Results and discussion

Four cultivars of winter oilseed rape (for passport data, see Table S-1) were vernalized and then exposed to long-term and progressive drought stress. The 28th day after the start of water withdrawal (DAS), the plants were analysed at gasometric, biochemical and leaf proteome levels. The different behaviours of four cvs in terms of their gasometric responses to available water, biochemical adaptations and differentially abundant protein profiles may be a mutual or even an exclusive result of several factors: 1) the complex influence of drought stress on cvs development and 2) the passport, genetic and other backgrounds of selected cultivars.

3.1. Water-related characteristics revealed two groups of water-saving strategies

Fig. 1 showed higher transpiration rates for N + V, and slower water depletion for C + D, plotted to different FTSW. This water-related behaviour is in accordance with photosynthetic measurements (Fig. 2 A–C) that show the same pattern of gasometric values for these two groups of controls. Mogensen et al. [21] showed a similar FTSW inflection point (around 0.3–0.45) for spring rapeseed form. The soil water content changes during cultivation are shown in Fig. S-1 and showed a gradual decrease of water content in treated plants. Also gasometric changes throughout the whole cultivation (Fig. S-2) and its S/C ratio (stressed/control values; Fig. S-3) showed distinct behaviour of cvs. ET and SWC upon stress significantly correlated to all gasometric values (S-10). The groups C + D and N + V differed in their ability to evapotranspire water for each FTSW_{Pn, E, and ET} (Fig. 1 and Fig. S-2). C + D transpire less water than N + V and have highest WUE and WUEi (Fig. S-2). It is worth to mention here, that the leaf area of treated cvs were not statistically different between cultivars (data not shown).

Water uptake data showed N + V with rapid and higher water uptake; C + D with moderate to lower water uptake (Fig. 1). The FTWS and NET estimations - based on different calculations - confirm that C + D are water-savers (respond conservatively according to Passioura [30]) and that N + V are water-spenders throughout the whole cultivations. However, both saver or spender strategies can be suitable in

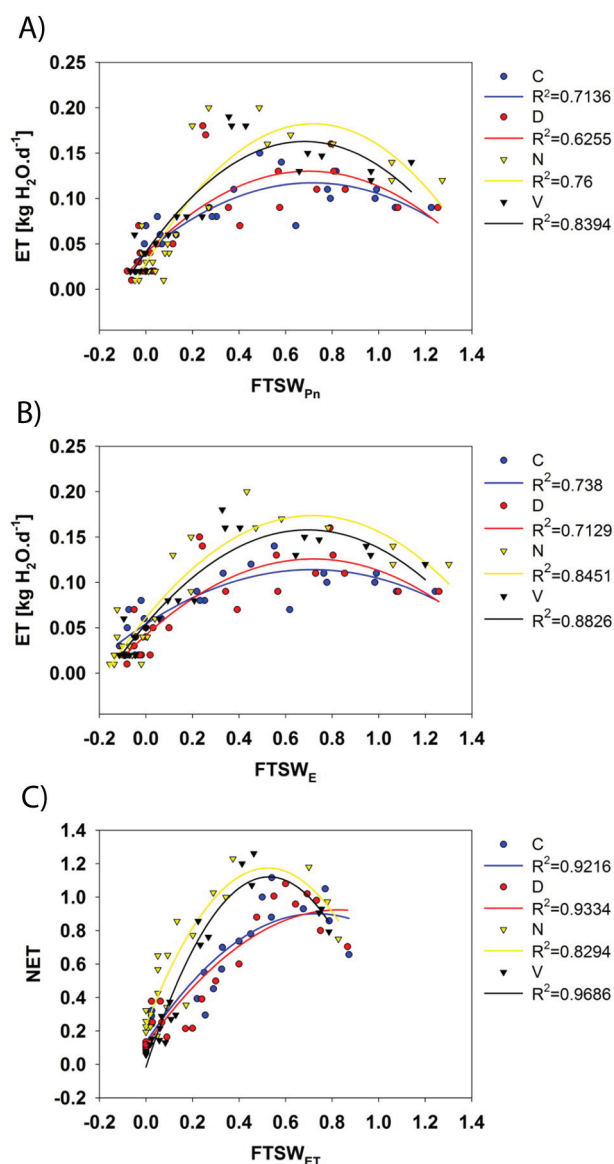


Fig. 1. Transpiration behaviour (ET; $\text{kg H}_2\text{O}\cdot\text{d}^{-1}$) of two groups C + D and N + V based on different fractions of transpirable water contents. Fraction of transpirable soil water (FTSW) according to values: A, net assimilation values (FTSW_{Pn}), where Pn of treated plants is ~0; B, leaf transpiration (FTSW_E), where E of treated plants is about 10% of controls; C, evapotranspiration changes (FTSW_{ET}). In case of C, FTSW is related to normalized transpiration (NET). Data are plotted from treated plants only. Genotype-related data are visualized by quadratic polynomial curves. Genotypes: C-Californium, D-Cadeli, N-Navajo, and V-Viking.

winter oilseed rape production in Central Europe. Further, they mirror the geographic origin and ergo, phenotypic traits of cvs (C + D – are French cvs suitable for a dry warmer climate, N – humid medium British climate, V – humid colder German climate).

3.2. Drought-affected biological processes revealed different strategies of individual genotypes

Physiological and biochemical characteristics (Fig. 2 A–L, Fig. S-4) showed significant differences between control and stressed plants. Upon drought, the genotypes differed in their response, which implies genotype-based adaptation processes (detailed data in Supporting Information S-8–S-10).

Physiological data showed higher fresh weight (FW) and dry weight (DW) biomass accumulation in controls of N + V (Fig. S-4 A, B). This growth is connected to higher water uptake, higher g_s , E and C_i/C_a

(Fig. 1, Fig. 2, Fig. S-2 A, C). Slower uptake of water in cvs C + D was connected to slower growth in controls in comparison to faster water use group N + V. This result is fully supported by data in Fig. 2, Fig. S-2, Fig. S-5, where lower gasometric values for controls of C + D are reflected in lower FW or DW biomass accumulation. Under drought, FW and DW showed different accumulation patterns. DW in treated plants was significantly lower for V and D (Fig. S-4 D). In V, this result is connected to low Pn of this cv (Fig. S-2 E, F). In opposite, higher DW accumulation in treated C + N can be connected to higher accumulation of specific proteins and to better drought adaptability. While C profited from higher g_s , E, and Pn under stress (Fig. S-2B, D, F), cv N probably profited from higher assimilates content during earlier phase of stem prolongation. The unexpected shift in higher DW biomass accumulation for CS is also reflected in Fig. S-5 and Fig. S-9 (C + N clustered together). The leaf areas of all cvs were not significantly different between cvs, so the DW accumulation was allocated more into stem. Gasometric and water-related data from the first to the last DAS were plotted in PCA (Fig. 3; correlation table in Supporting Information S-9). PCA clustered controls into C + D and N + V; however, in treated plants, N + V + D and separated C were clustered. These findings are further supported by the results of NMMDS (Supporting Information S-12 and Fig. S-6). All measured parameters taken together resulted in different cvs order (Fig. S-5), where hierarchical clustering of 19 gasometric, biochemical and water-related parameters for controls and treated plants is shown. The data clustered cvs D + V and C + N together, which mirrored mainly the level of cultivar-based plasticity. The similar result to Fig. S-5 is shown in ratio of protein abundances (S/C) in Fig. S-9, where PCA of all proteins abundances (based on ratio S/C) is plotted.

In controls, D has middle Pn even in very low g_s and E which concord to low C_i/C_a (Fig. 2D) and thus higher utilization of CO_2 inside the leaf. We speculate that this is linked to the different gasometric scenario of cv D (high mesophyll conductance and better carboxylation efficiency as suggested by Chaves et al. [70]). In stressed plants, the lowest Pn, g_s and E were observed in N, which also corresponds to the high C_i/C_a ratio under stress and subsequent low CO_2 fixation. At the end of treatment, only C had positive Pn in stressed plants. Other cvs had negative Pn already at 20 DAS. There is no clear explanation for this observation in cv C, however, the positive Pn at the late stage of drought can be connected to higher DW, PRO, higher RWC with help of high ROS and also to better carboxylation efficiency. The differences in cold tolerance of rapeseeds are partially explained by differences in Pn and WUE in cold-treated rapeseed plants [10]. Under drought, RWC values (Fig. 2 E) were significantly lower for each genotype ($p < 0.05$), but similar for all controls. This reflects sufficient saturation of control plants, and at the same time, different strategies of the genotypes in drought. In stressed plants, the lowest RWC was found in N and V, which is related to their higher rate of water extraction from the pot. The osmotic potential values (OP) for all controls (Fig. 2 F) were significantly ($p < 0.05$) higher (closer to 0) than for treated plants but did not differ between genotypes. Within the stressed plants, OP was the lowest (most negative) for N and V, which can be related to RWC and different rate of water use. The ETs ($\Delta\text{H}_2\text{O} \cdot \text{day}^{-1}$; Fig. 2 G) represent the changes in daily evapotranspiration within a pot. The ET values in our study are similar to those in Mueller et al. [16]. Faster water depletion under stress in N + V resulted in higher (more negative) OP, lower RWC and a rapid decrease of CO_2 assimilation (low WUE and WUE_i, Fig. S-2 F, H). Less negative OP in C + D can be then connected to higher leaf area duration, and prolonged growth [28,29,31].

PRO may be a suitable ‘marker’ for osmotic adjustment in juvenile *Brassica* plants [18,71]. Proline (PRO; Fig. 2I) accumulation was significantly ($>10\times$) lower in all controls ($p < 0.05$) compared to stressed plants. Unfortunately, PRO showed only genotype-based accumulation with no clear connections to other data probably because of spatial (leaf age) and metabolic (source-sink status) peculiarities of each cv. PRO accumulation correlates with abiotic stress and plays a multifunctional role: as a mediator of osmotic adjustment in rapeseed in the

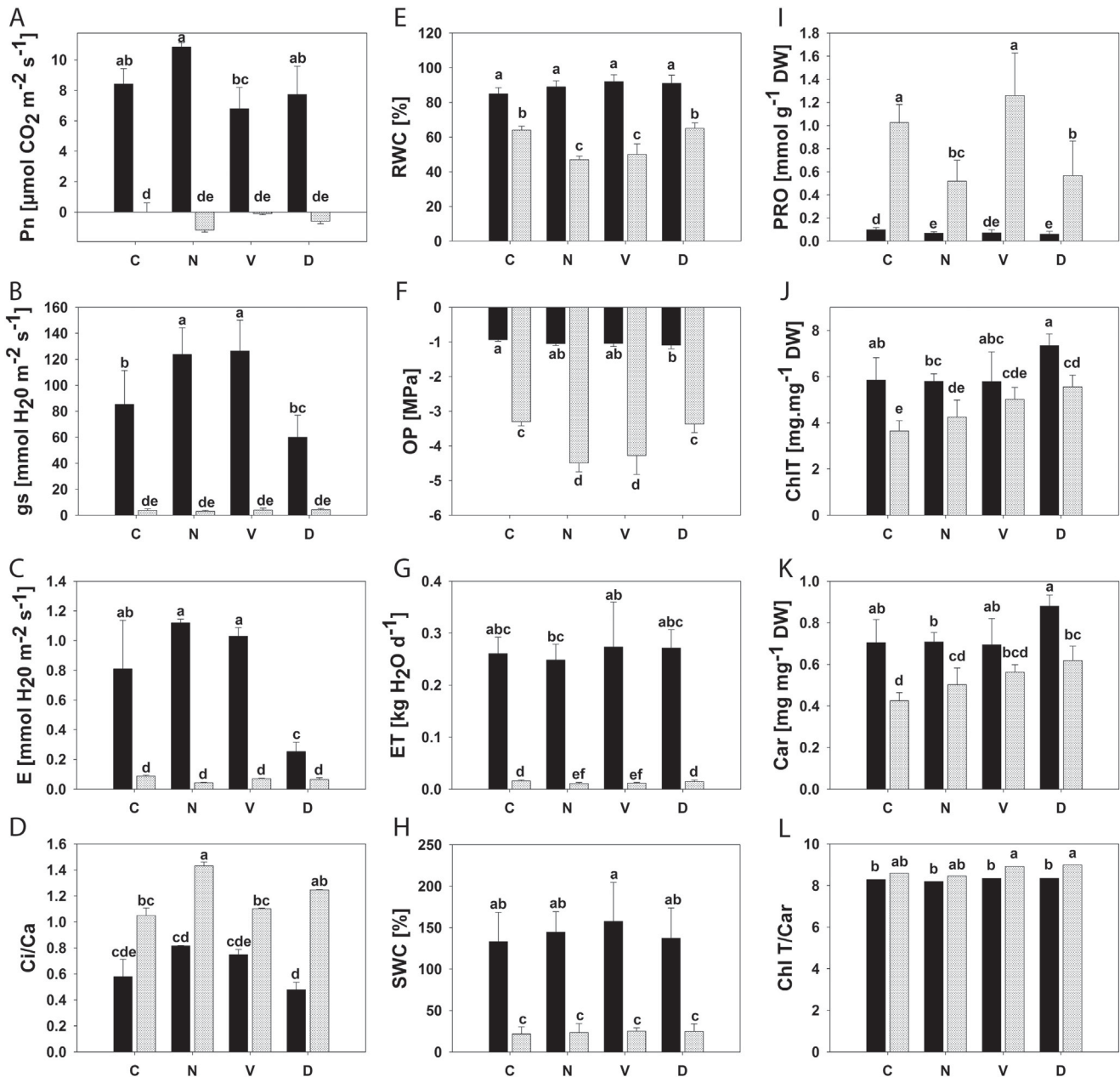


Fig. 2. Effect of long-term drought stress on the biochemical characteristics of four winter oilseed rape genotypes showing different behaviour on individual cvs. Five rapeseed plants were used for each experiment and three independent experiments were performed. Values are medians \pm SD, compared by Student *t*-test ($p < 0.05$). Black columns are controls, dotted gray columns are treated plants. Different letters indicate significant differences at 0.05 level using Duncan's multiple range test. (A) Pn; (B) gs; (C) E; (D) Ci/Ca; (E) RWC; (F) OP; (G) ET; (H) SWC; (I) PRO; (J) Chl total; (K) Car; (L) Chl total/Car. Genotypes: C-Californium, N-Navajo, V-Viking, and D-Cadeli.

defence mechanisms [20,72], as a scavenger or free radicals [73], as an inhibitor of programmed cell death, protein stabiliser [74], and as a stress-related signal [75]. Because PRO is an easily remobilized nitrogen reserve substrate for growth [76], the higher the accumulation in the leaves of particular cvs, the lower the nitrogen need for growth. Chl *a*, Chl *b*, Car and total Chl (ChIT) concentrations were significantly ($p < 0.05$; Fig. 2 J–L) higher for all controls except for V. The ratios (Chl *a* + *b* and ChIT/Car) of photosynthetic pigments are similar within genotypes and treatments with an increase in stressed plants. This finding implicates Chl *a* content increase for all genotypes under drought. ChIT and Car were reduced in treated plants mainly in C and D, while ChIT/Car was significantly different only for V and D between treatments.

The results indicate that under fully saturated conditions, water-spenders (N + V) have high E and gs, and, subsequently, rapid growth. In opposite, in treated conditions, water-spenders rapidly decline all

gasometric parameters; however, cv N keeps growing. Interestingly, in water-spenders, WUE and WUE_i are very low in both, control and treated conditions in opposite to water-savers C + D. Water-savers also showed higher values of gasometric parameters when treated/control plants are compared (Fig. S-3). This is in general agreement with Blum [31], that greater WUE is often associated with a slow rate of water use [31]. Blum's effective use of water is likely to be higher for a plant that maximises Pn dependently on the amount of available water. Ryan et al. [34] showed that high WUE maize cvs respond to increasing vapour pressure deficit earlier with increased stomatal sensitivity than low WUE.

Importantly, some of the biochemical measurements of treated plants at 28 DAS may portray the metabolism beyond the water shortage threshold and thus did not show different values between cvs. It has to be mentioned that this study does not reflect the possibly

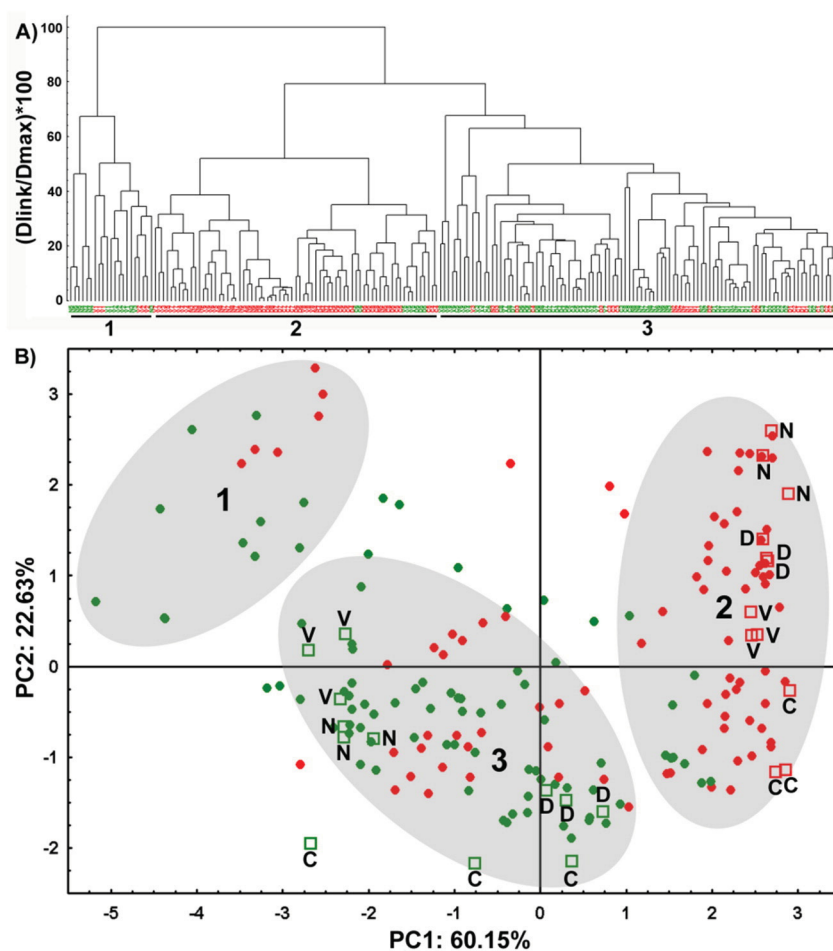


Fig. 3. The composite graph of PCA and cluster analysis based on photosynthetic and water-related data from the whole cultivation. Result of the cluster analysis in the form of dendrogram (A), which was created based on the Euclidean distances using UPGMA method, revealed three main clusters (1–3) of plants. First cluster contains mostly control plants at the beginning of measurement, the second cluster almost all control plants regardless of DAS together with stressed plants at the beginning of measurement. In the third cluster stressed plants in later phases of measurements is shown. CLU analysis is in concordance with the result of PCA method (B) as can be seen on 2D projection using two PC axes that explain almost 85% of data variability. To highlight the concordance of CLU and PCA results, the clusters 1–3 were picked out by gray ovals. The green colour indicates control plants (the very last measurements–28th DAS are in green boxes); red colour indicates the stressed plants (the very last measurements–28th DAS are in red boxes). Genotypes: C-Californium, N-Navajo, V-Viking, and D-Cadeli.

different genotype-based water uptake rate before stem prolongation or even after flowering. These missing data will actually complete the whole water-use efficiency of individual genotype-based scenarios.

3.3. Proteomic analysis of differentially abundant proteins under drought

The representative 2D-DIGE gel with highlighted 134 extracted spots is shown in Fig. 4 with an example of good matching (Fig. S-7). PCA distinguished control, well-watered plants from drought stressed plants in all cultivars (Fig. S-8A) and showed a similar pattern of genotype distribution as presented in Figs. 1, 2 and Fig. S-2. Cvs C + D and N + V are clustered together in both controls and stressed variants. The protein spots belonging to different clusters according to cluster analysis (present in Fig. 5 and in Supplementary Information S-17) are placed in the centre of the PCA scatter plot revealing the fact that protein spots exhibit opposite accumulation patterns within genotypes and thus, the mean is centred close to zero (Fig. S-8 and S-9).

Meanwhile – opposite to S8 – when stress/control protein abundances were plotted in PCA in S9, three groups were visible (1-C + N; 2-D; 3-V). This result mirrors clustering in Fig. S-5. This means that in stress/control ratios of protein abundances, there is other hidden information, putting D and V away from C + N. One explanation is, that S/C ratio contains the information about drought-related growth. Furthermore, this result (C + N clustered together) is supported by GOModeler

output (GOM; Fig. 6; Supplementary Information S-18). On the base of GOM results, better overview can be distinguished and cultivars differences are more apparent (for GOM calculation details, please see Supplementary Information S-7). Cvs C + N showed highest net effects (many proteins up-accumulated in stress) of selected hypothesis on its proteome, cv D is in the middle and V showed the lowest net effect (many proteins down-accumulated in stress). Additionally, according to column clustering in Fig. 5, V control (VC) is clustered together with other stressed cvs. This steep decrease of protein accumulations in treated tissues of V can be partially explained by disrupted cellular metabolism - rapid water depletion metabolism changes caused more severe disturbance in intracellular water homeostasis in water-spenders indicated by RWC, OP etc. Cv V has the lowest net effect sum shown in Fig. 6 = – 18.96. In accordance with the above-mentioned data, it can be hypothesized that stressed cv V does not have enough energy for maintenance and stress protection (supported in Figs. S-2 and S-3).

Only proteins that changed in their abundance at least twice in consequence of treatment and/or genotype-based changes according to Student's *t*-test ($p < 0.05$) were intended for analysis. According to these parameters, 109 from the 134 selected spots were identified. After manual discarding of 9 spots with multiple and 21 spots with unsatisfactory identification (below significant protein or ion score), respectively, the set of 79 spots was successfully identified (62 spots in

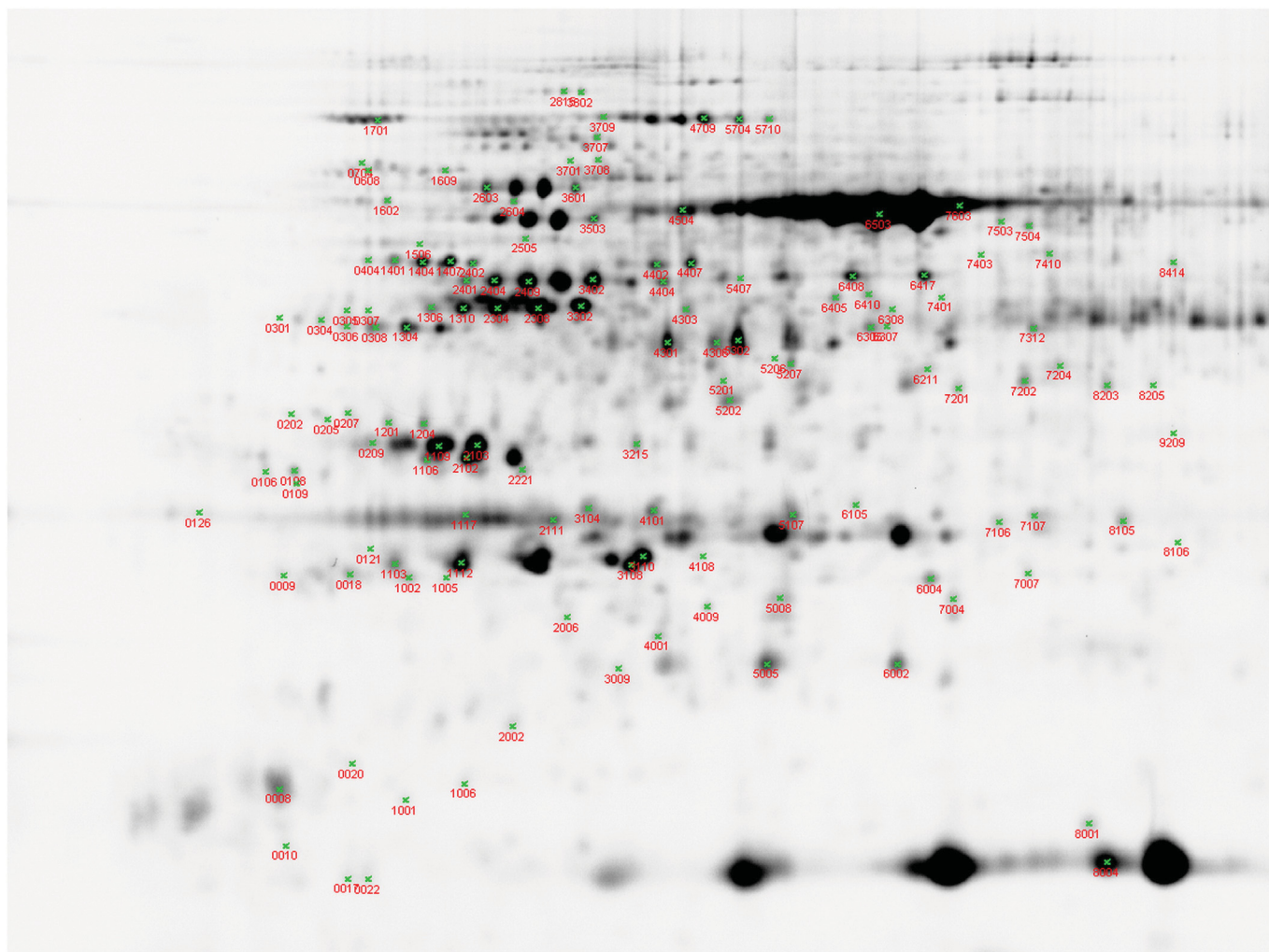


Fig. 4. Representative proteome map of total proteins from leaves of winter oilseed rape, separated by 2D-DIGE. At least two times up- or down-accumulated proteins ($p < 0.05$, 24 cm IPG strip, pl 4–7, 1.5 mm thick gel) are marked with the corresponding spot numbers. In total, 134 spots chosen for cutting and identification are shown. In total, twenty-four images were used to analyze the DAPs.

the set of biologically relevant comparisons, and 17 proteins from “uncommon” – biologically non-relevant ratios; details in Supplementary Information S-5) and was chosen for detailed analysis. The final lists of identified proteins are available in Supplementary Information S-13 and S-19.

All significantly identified spots reveal clustering into four main clusters according to their accumulation pattern in controls and treated genotypes (Fig. 5). In Fig. 5B, the average profile of selected clusters helps to understand the general pattern of each cluster. Generally, cluster 1 and 3 showed treatment-based changes and in cluster 2 and 4, cultivars-based differences in protein accumulation are visible. In Fig. 5, cluster 1 (CLU1, red colour, 20 spots) showed DC, CC and NC have higher accumulation of proteins in this cluster. The highest number of proteins in this cluster belongs to carbohydrate/energy metabolism and photosynthesis, which indicate that they are important for metabolism of D, C and N. Cluster 2 (CLU2, yellow colour, 21 spots) showed water-savers C + D, both control and treated plants, have higher accumulation of proteins in this cluster in which carbohydrate/energy and photosynthesis play the major role. Cluster 3 (CLU3, green colour, 38 spots) showed all treated genotypes (C, N, V and D) have higher accumulation of proteins. In CLU3, proteins involved in photosynthesis, redox/ROS and stress response show the highest accumulation. This cluster also showed some connection between V control (VC) proteins with all other treated ones (as visible in column clustering). Cluster 4 (CLU4,

blue colour, 21 spots) showed average abundance clustering that NC and NS, and VC have higher accumulation of proteins in this cluster. Protein metabolism (amino acid, nitrogen, and sulphide), carbohydrate/energy, photosynthesis, redox/ROS, and stress/defence proteins are mostly accumulated in this cluster. This cluster revealed proteins important for water-spenders N + V. In Fig. 5, the high protein abundance variability in both controls and treated cultivars could be related to the severe water condition of plants where some damage of plants could influence physiological and quantitative proteomics data. In some part of plants of one cultivar, the higher ratio of senescence process or cell death could emerge and the same is true of different responses to drought between cultivars. We observed this phenomena in drought-treated barley, too [59]. Therefore, the variability in the data set could be an additional explanation of plant status (stress response and plant damage).

According to Venn diagrams (Fig. 7), the N + V showed the highest number of unique spots differently accumulated in both (genotype-based - Fig. 7 upper and treatment-based - Fig. 7 lower) variants. Both water-spender cvs can be characterized by faster water shortage and then longer period of drought stress for N + V (see no unique proteins in water-saving cv C). This is in accordance with a hypothesis that stress-sensitive genotypes (more and/or rapidly stressed) synthesize and accumulate more proteins upon stress than the tolerant ones. Unfortunately, for some of differentially abundant proteins we cannot easily recognize if their accumulation is a consequence or cause of drought.

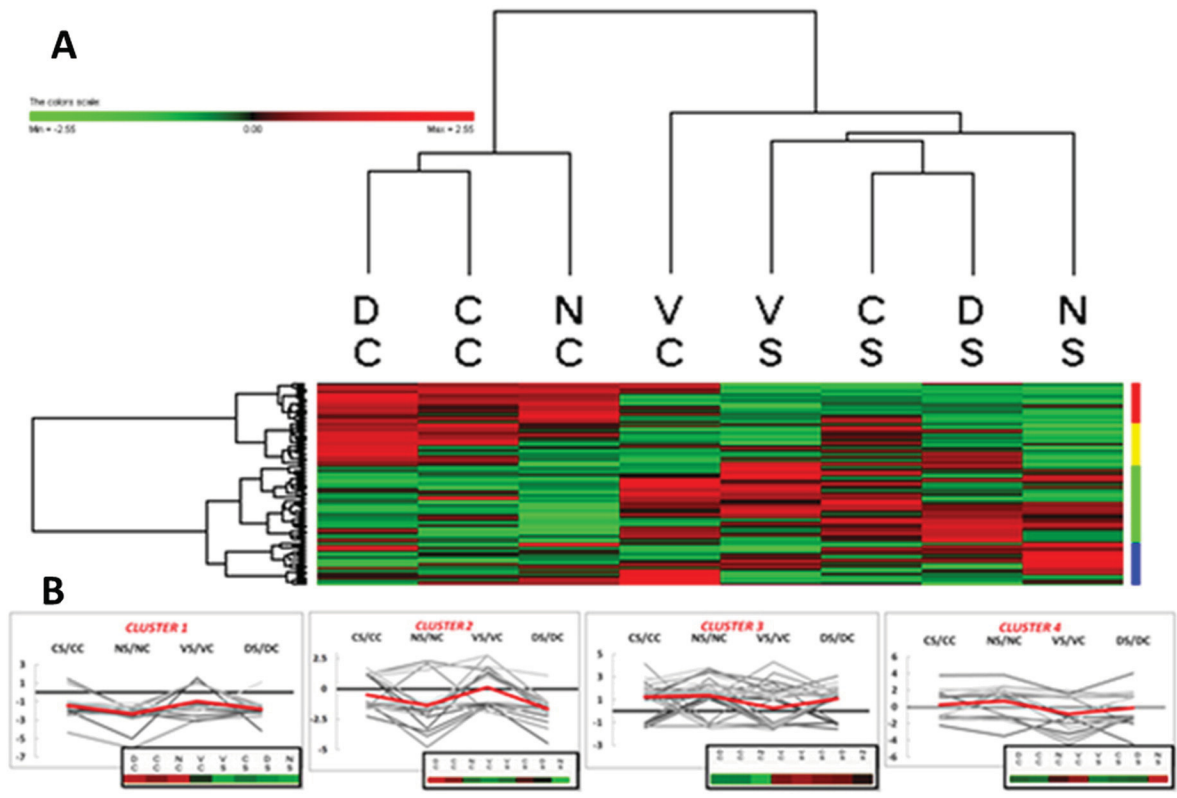


Fig. 5. Hierarchical clustering of differentially accumulated spots. (A) Heat map of hierarchical clustering consisting of 100 spots differentially accumulated in leaves of winter oilseed rape (*Brassica napus* L.) during the course of drought. Controls (C) and treated plants (S) of four genotypes C, N, V, and D. Clustering was done simultaneously for spot expression profiles (rows) and every treatment (columns) to obtain the best clustering result. The four clusters are highlighted from top to down (cluster 1-red; cluster 2-yellow; cluster 3-green; cluster 4-blue). Dendrogram was constructed in PermutMatrix using Ward's method. According to Z-score standardization, the red colour in heat map indicates higher accumulation of protein and vice versa for green colour. (B) The relative protein densities in individual clusters are shown (clusters 1–4; from left to right). The relative protein densities expressed as stress/control values (e.g. CS/CC = genotype Californium, density of protein x in stress/density of protein x in control) in the order CS/CC, NS/NC, VS/VC, and DS/DC are shown in the graphs. See details in Supplementary Information S-17. The red line is a mean of protein densities; the black line is zero. The small heat maps in the right corners of these graphs show the average value of protein accumulation for each cluster (based on Z-score) showing which variant is affected [visualized in the same order as the main heat map (A) on the top DC, CC, NC, VC, VS, CS, DS, and NS].

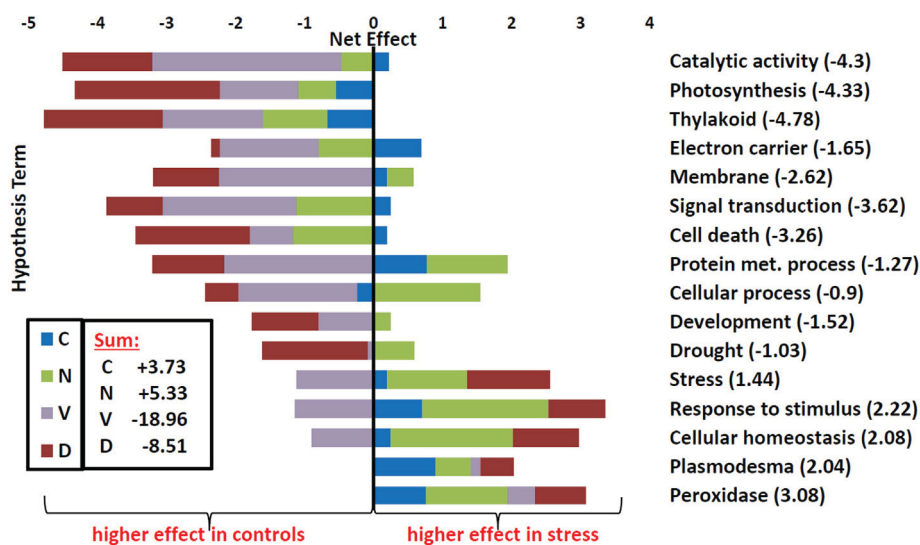


Fig. 6. GOModeler output. GOModeler based graphical summary of proteome net effects for each of 16 chosen hypothesis terms (right side) showing grouping C + N, D and V. Net effect is a product of quantitative value (logarithm of protein abundance expressed as stress density/control density) with qualitative value (+1, 0, or -1; assessed by GOModeler according to GO annotation of each protein). In brackets behind each hypothesis term, the sum of net effects for each particular hypothesis is shown. In the right upper corner, the sum of all net effects for individual genotype is calculated. Positive value (right side of the graph) means higher protein net effect in stressed plants; negative values means higher net effect in controls. Zero net effect is indicated by value of zero (e.g. cv C in Development). The length of individual colour parts (not the position in the line) testifies to the net effect value for individual genotypes. Genotypes: C-Californium, N-Navajo, V-Viking, and D-Cadeli.

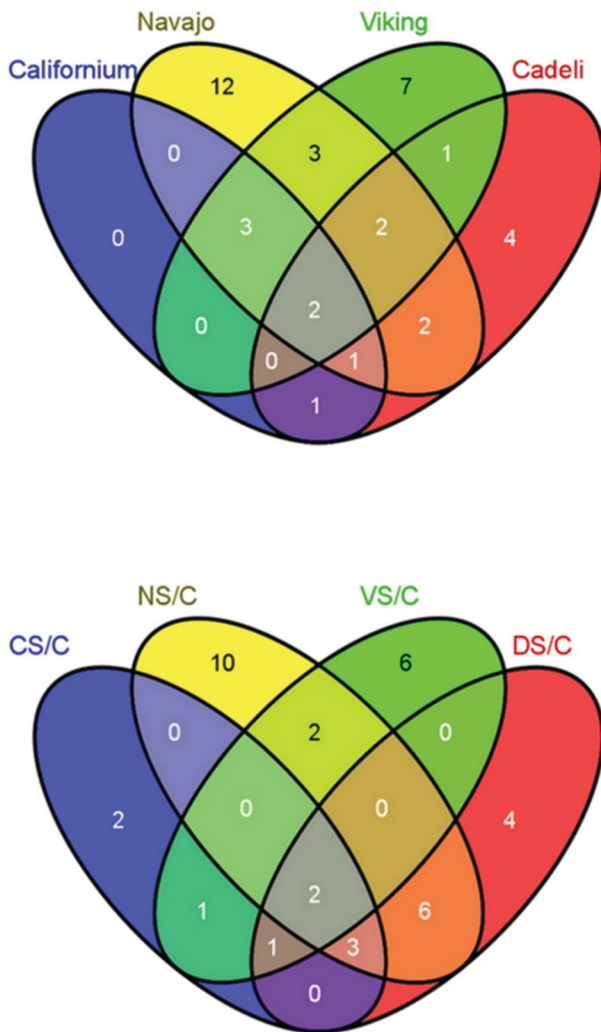


Fig. 7. Venn diagrams. Venn diagrams showing the numbers of unique protein spots ($n = 38$) exhibiting statistically significant differences (at least ± 2 -fold change between stress and control sample; $p < 0.05$) between genotypes (upper), between stress/control (S/C) samples (lower). Genotypes: C – Californium, N – Navajo, V – Viking, D – Cadeli.

Among the 62 biologically relevant spots, 10 spots were more abundant in treated samples of every genotype (e.g. SSP 1002, 2002, and 7204); 16 have lower abundance (e.g. SSP 1506, 5710, and 7504) and 36 spots showed a mixed pattern of genotype-based abundance. In Supplementary Information S-10, distribution of the identified proteins based on abundance of stress/control values is visible.

Twenty-one spots were not sufficiently identified, probably due to a lack of sequence similarity or low abundance; nevertheless, these spots were included in Permut Matrix clusters and PCA analysis so we can describe their behaviour based on their clustering. The table of identified proteins (Supplementary Information S-13) contains all 62 identified protein spots. Among the 62 spots, several proteins with documented relation to abiotic stress were found (e.g., germin-like protein, TIR-NBS type disease resistance protein, HSP 60 and 70, fibrillin and many antioxidants and photosynthesis adaptation/acclimation proteins). The search for protein spots revealing large changes in protein relative abundance (more than ± 3 fold; $p < 0.05$) has revealed 24 protein spots (e.g., elongation factor G, epithiospecifier protein, 2-Cys peroxiredoxin, fibrillin, fructose-1,6-bisphosphatase precursor, ribulose-1,5-bisphosphate carboxylase/oxygenase activase, glyoxalase I, germin-like protein etc.). Desclos-Theveniau et al. [77] found some of these high abundant proteins as “residual” (with long-lasting turnover) to enable rapeseed senescence.

3.4. Functional categories and cellular localization of drought-responsive proteins

The number of identified proteins suggests rapeseed drought acclimation to be an active and energy-demanding process, supported by changes in photosynthesis, redox and ROS signalling, also with RNA processing.

To investigate the functional and biological process-based identity of the individual differentially accumulated proteins (DAP), the 62 spots (45 DAP) were categorized into 7 major groups based on their putative biological functions and are common for all cultivars (Supplementary Information S-13): 1, Amino acid, nitrogen and sulphide metabolism/protein metabolism (4 DAP); 2, ATP interconversion (3 DAP); 3, Carbohydrate/energy metabolism (9 DAP); 4, Photosynthesis (11 DAP); 5, Redox homeostasis, ROS and signalling (9 DAP); 6, Stress and defence related (6 DAP); and 7, Transcription, translation, RNA processing (3 DAP).

According to these seven functions, we can designate these groups to be most affected by long-term drought after vernalization in chosen winter oilseed rapes (Fig. S-11). These affected functional groups for the *Brassicaceae* family were also confirmed by others [38].

The relative protein abundance scheme – showed as \log_2 S/C accumulation – simplifies the overview over protein distribution (Fig. 8). The other schemes of protein classifications are presented by protein-protein interaction network STRING (Fig. S-12), by over-represented molecular functions and biological processes in BiNGO (Fig. S-13 and S-14), and by GOSlim schemes (Fig. S-15). The STRING scheme showed associations between accumulated proteins even at the high confidence level and add 23 predicted association protein partners (available in below Fig. S-12). The output from all BiNGO structures supports the results from protein identification and from functional categorization of DAP.

The detailed graphical scheme, summarizing selected proteins different accumulation patterns between water-savers and water-spenders, is shown in Fig. 9. Below, only proteins with contrasting accumulation between water-savers and water-spenders or with an important influence of drought adaptation mechanisms are discussed in detail.

3.5. Amino acid, nitrogen and sulphide metabolism/protein metabolism

In the *Brassicaceae* family, the nitrogen and sulphide compounds are very important for metabolism (thiols, glucosinolates, brassinosteroids) and biotic/abiotic stress adaptation [78,79]. The four proteins included in the chemical reactions and pathways: nitrogen fixation, nitrification, denitrification, assimilatory/dissimilatory nitrate reduction and sulphide interconversion significantly changed their accumulation under drought in rapeseed leaves. The drought acclimation process and salinity very often are associated with significant alterations in protein metabolism, because in oilseed rape, the negative effects of drought are quite similar to those for nitrogen limitation [76].

Glutamine synthetase precursor (SSP 2401; CAA73062.1; EC 6.3.1.2; GS2) accumulation decreased in all treated genotypes; however, significant decrease was found only in N. GS2 is an ATP-dependent plastidic enzyme that plays an essential role in the metabolism of nitrogen (according to STRING visualization, there is a connection to NIT1; Fig. S-12). According to some authors [80,81], plants with low GS2 have a diminished capacity for photorespiration and decreased tolerance to high-intensity light (see also STRING GS2 connection to photosynthetically active proteins and carb/energy metabolism), so they are photoinhibited more severely by high-intensity light compared to control plants. If GS2 accumulation could directly protect a plant from drought-related photoinhibition, then C and V could better photo-acclimate because of a lower decrease of GS2 in controls and a higher accumulation of ATP (atpA gene products, ATPase vacuolar and VHA-A) in stressed plants at the same time.

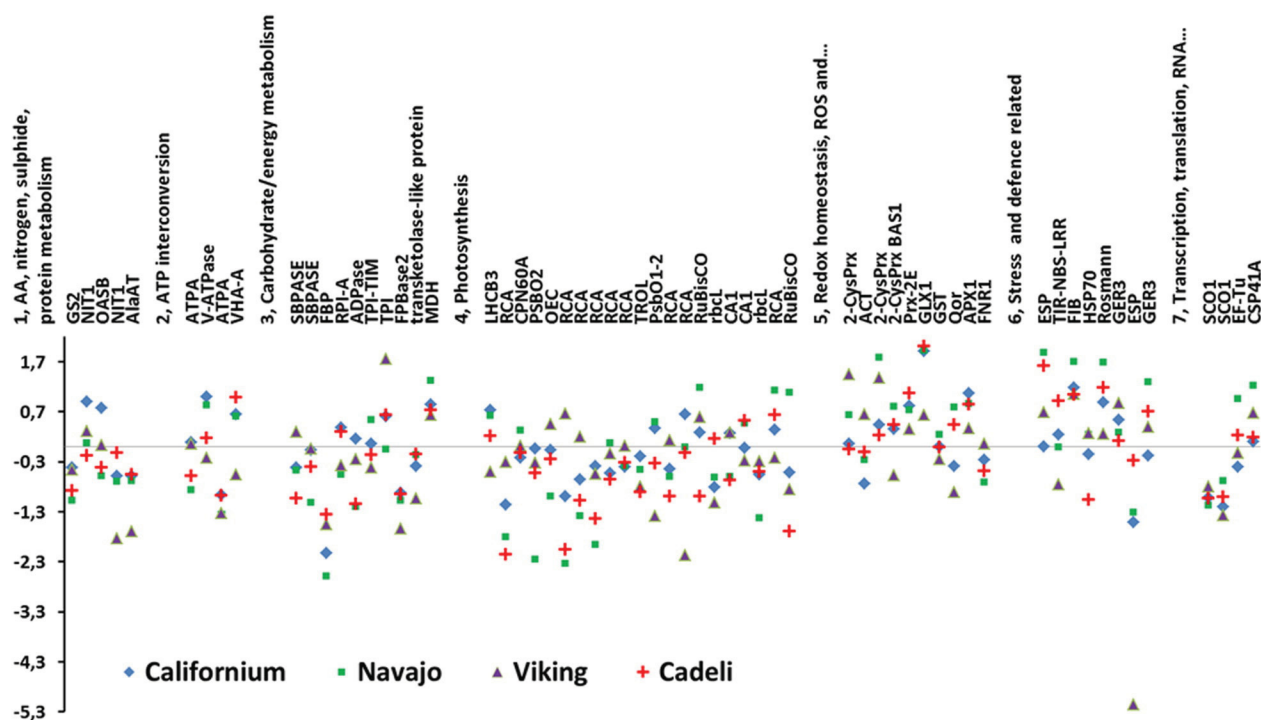


Fig. 8. Relative protein abundance (\log_2 stress/control) scheme of 62 identified proteins, divided according to functional groups. Proteins above zero line showed higher accumulation in treated plants and vice-versa for proteins below zero line.

In *Brassicaceae*, a new family of nitrilases has evolved - the nitrilase 1 homologs - that are able to hydrolyze nitriles that result from the catabolism of glucosinolates, the typical secondary metabolites of the *Brassicaceae* [82]. Nitrilase 1 (SSP 4303 and 6405; ABM55733.1; EC 3.5.5.1; NIT1) has been identified in two spots differing in pI and accumulation patterns. The latter NIT1 was significantly lower in V. Between controls, NIT1 accumulation in C was $3\times$ higher than in other cvs. Furthermore, the enzyme nitrilase is suggested to play an important role in auxin biosynthesis [83] and in phytohormone crosstalk [84].

Alanine-2-oxoglutarate aminotransferase 1 (SSP 7504; XP_002893277.1; AlaAT) catalyzes transamination reaction between L-alanine and 2-oxoglutarate and the reverse reaction between L-glutamate and pyruvate. AlaAT plays a crucial role in nitrogen metabolism, and in the regulation of serine, citrulline and glycine contents in leaves [85]. In drought-related lack of ATP, the concerted modulation of alanine and glutamate pathways allows for the substitution of ATP-dependent enzymes GS by AlaAT [86]. This adaptation saves ATP, regenerates NAD(+), and saves carbon in the form of alanine (a carbon/nitrogen storage readily remobilized upon recovery). A significant decrease in this protein was observed in V (> -3.2) which is likely related to overall lower abundance of almost all proteins connected to ATP interconversion in V (ATPA, VHA-A; see below).

It is well known that water deficit can significantly decrease the amount of nitrogen assimilated into amino acids and proteins [87]. Furthermore, oilseed rape has a low nitrogen use efficiency (NUE), mainly due to its low nitrogen remobilization efficiency (NRE) observed during the vegetative phase when sequential leaf senescence occurs [76,88]. On the other hand, in the field, nitrogen-deprived *B. napus* plants exhibited less pronounced symptoms of wilting, probably because of smaller leaves and closed stomata [76].

Brassica species, in general, have high sulphur demand during vegetative growth for protein synthesis [86]. In this study, we identified O-acetylserine (thiol) lyase oasB (SSP 5201; CBL74423.1; OASB) which is connected to glutathione metabolism. Sulphides are necessary elements for *Brassicaceae* development and secondary metabolism, and they influence nitrogen-use efficiency. Not surprisingly, cvs of *B. napus* with

high sulphur-use efficiency were more tolerant to PEG-induced drought stress [86].

Almost all proteins in this category show a decreasing abundance of proteins in stressed cvs. This can also be a result of a “secondary” effect of drought – the decrease of soil water potential diminishes nutrient availability for plants.

3.6. ATP interconversion

This group includes proteins in chemical reactions and pathways involving ATP, a universally important coenzyme and enzyme regulator with increased relevance under any kind of stress-related changes. Changes in several enzymes involved in ATP metabolism, especially coupling factors, were found in our study. The major sources of novel ATP molecules represent processes of both anaerobic and aerobic respiration and photosynthesis.

The chloroplastic ATP synthase coupling factor is a key element in the drought stress responses [6]. Chloroplastic atpA gene product (SSP 2603 and 3601; YP_005089937.1; ATPA) produces ATP from ADP in the presence of a proton gradient across the membrane. This protein was present in two spots. However, ATPA accumulation patterns showed a decrease in water-spenders cvs.

Two DAP were identified as vacuolar ATPases in rapeseed response to drought. V-type proton ATPase catalytic subunit A (SSP 3707; NP_178011.1; VHA-A) and nucleotide-binding subunit of vacuolar ATPase (SSP 2604; AAC36485.1; V-ATPase) was accumulated in all genotypes except V. A decrease in both of these important proteins in V shows lower energy-related acclimation and/or need. These two ATPases couple ATP hydrolysis to the build-up of an H^+ gradient, but V-ATPases do not catalyze the reverse reaction. The V-ATPase is found in the membranes of vacuoles, the Golgi apparatus and in other coated vesicles in eukaryotes. To replenish water deficit within the systems, roots also developed mechanisms such as enhanced pumping of protons into vacuoles. In Mohammadi et al. [11], authors showed on drought-stressed rapeseed roots that V-ATPase, HSP 90, and elongation

Drought during prolongation stage – *Brassica napus* L. winter form

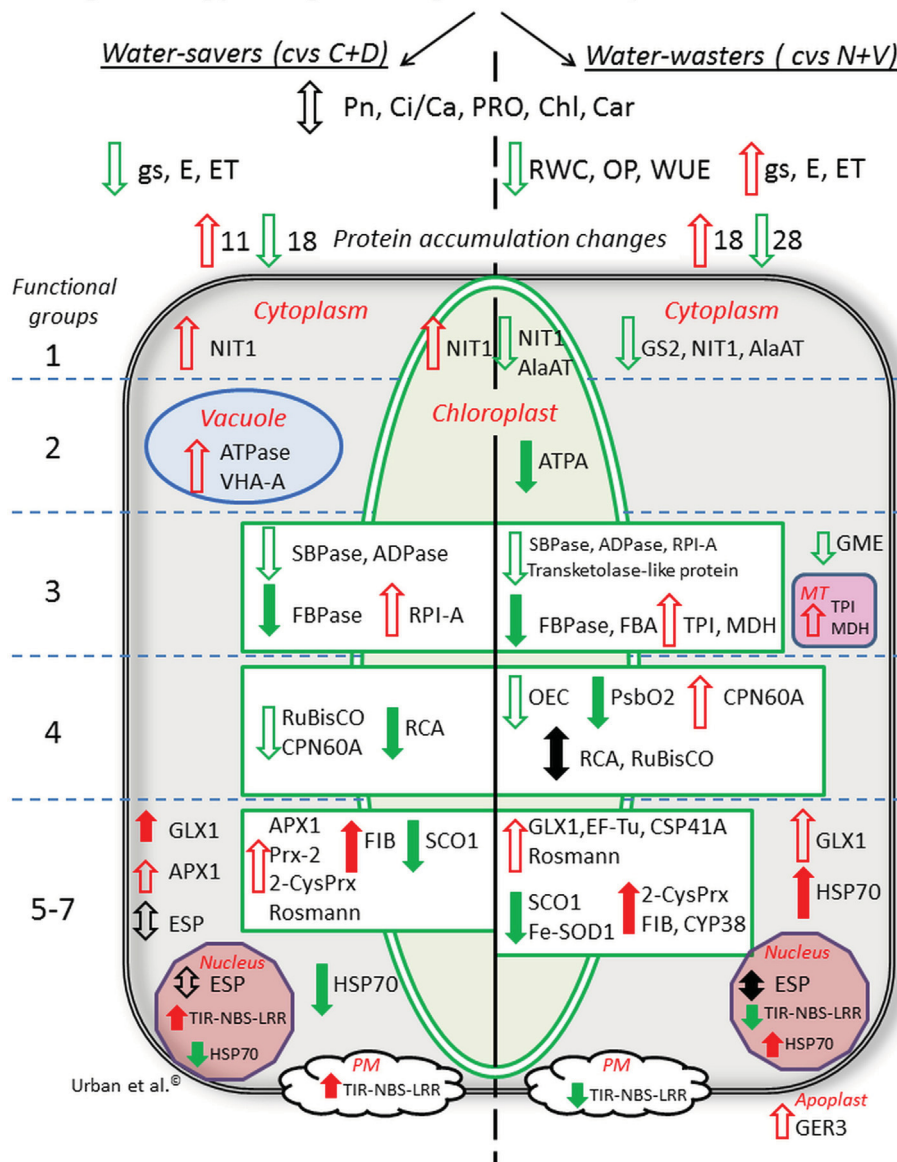


Fig. 9. The schematic representation of water-spenders and water-savers characteristics. The schematic representation of proteins differentially accumulated between water-savers (left side) and water-spenders (right side) with regard to protein functional groups (1–7). Legend: Pn (assimilation rate; $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), gs (stomatal conductance to water vapour; $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), E (transpiration rate; $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), Ci/Ca (ratio of leaf intercellular $[\text{CO}_2]$ to ambient air $[\text{CO}_2]$), RWC (relative water content; %), OP (osmotic potential; MPa), ET (evapotranspiration rate; $\text{kg H}_2\text{O d}^{-1}$), PRO (proline; $\text{mmol g}^{-1} \text{ DW}$), ChlT (chlorophylls in total; $\text{mg mg}^{-1} \text{ DW}$), Car (carotenoids; $\text{mg mg}^{-1} \text{ DW}$). Protein names: NIT1 - nitrilase; GS2 - plastidic glutamine synthetase; AlaAT - alanine-2-oxoglutarate aminotransferase 1; ATPase - nucleotide-binding subunit of vacuolar ATPase; VHA-A - V-type proton ATPase catalytic subunit A; ATPA - atpA gene product; SBPase - sedoheptulose-bisphosphatase; ADPase - glucose-1-phosphate adenyllyltransferase; FBPase - fructose-1,6-bisphosphatase precursor; RPI-A - ribose 5-phosphate isomerase A; FBA - fructose-bisphosphate aldolase 2; TPI - triose phosphate isomerase; MDH - malate dehydrogenase 1; GME - GPD-D mannose epimerase; CPN60A - RuBisCO large subunit-binding protein subunit alpha; RCA - RuBisCO activase; OEC - photosystem II subunit O-2; PsbO2 - oxygen-evolving enhancer protein 2, chloroplastic; GLX1 - glyoxalase 1; APX1 - L-ascorbate peroxidase; ESP - epithiospecifier protein; TIR-NBS-LRR - TIR-NBS-LRR class disease resistance protein; HSP70 - heat shock protein 70; Prx-2 - peroxiredoxin-2E; 2-CysPrx - 2-Cys Peroxiredoxin; Rosmann - NAD(P)-binding Rossmann-fold-containing protein; FIB - fibrillin; SCO1 - elongation factor G; EF-Tu - chloroplast elongation factor tub; CSP41A - chloroplast stem-loop binding protein-41; Fe-SOD1 - Fe superoxid dismutase 1; GER3 - germin-like protein. Arrows: up - up-accumulation; down - down-accumulation of proteins; both directions - accumulation is mixed; single arrow - one protein accumulation; bold arrow - two or more protein accumulation (accumulation of different gene products).

factor EF-2 have a role in drought tolerance of rapeseed. Also RuBisCO is regulated by ATP and inhibited by ADP.

Stress factors affect energy metabolism because plant adjustment to an altered environment generally means an enhanced need for immediately (ATP) available energy. Significant differences were observed in all proteins of this category and showed a higher accumulation of ATP-related proteins in water-savers cvs and the opposite for spenders. This higher demand for ATP can be connected to better homeostasis maintenance under drought.

3.7. Carbohydrate/energy metabolism

Maintaining sufficient energy and balanced carbohydrate production is one of the most important pathways in all plants as sessile organisms. Sugars play a central regulatory role in many vital processes besides serving the energetic function, and are considered to be important signals that regulate plant metabolism and development [73]. Starch also serves as a transient reserve of carbohydrate, which is used to support respiration, metabolism, and growth at night.

This group represents the largest part of identified proteins (17%). Some carbon/nitrogen metabolism-related proteins identified here (TPI, MDH, ADPase etc.) showed increased energy demand as well as enhanced cellular activities in the root tissue of rapeseed under drought [11]. It is reasonable that in STRING (Fig. S-12), this group showed important intra- and inter-protein interactions with all other groups because gluconeogenesis and glycolysis share a series of six reversible reactions.

Sedoheptulose-1,7-bisphosphatase (SSP 306 and 1304; XP_002876336.1; SBPase) is a critical protein in the gluconeogenesis pathway. SBPase has a key role in regulating the photosynthetic Calvin cycle and is normally down-regulated under abiotic stress [6] which was confirmed in this study, too. These two spots are both decreased in accumulation (except for V in SSP 306). The deeper decrease was observed for N. These results somehow mimic the photosynthetic parameters of N, where N showed the highest fall in Pn, gs, E and highest Ci in stressed plants compared to controls (Fig. 2).

Fructose-1,6-bisphosphatase precursor (SSP 1506; AAD12243.1; FBPase) catalyzes a hydrolysis of fructose-1,6-bisphosphate into fructose-6-phosphate, and is also a rate-limiting step in the gluconeogenesis pathway. A large decrease in free energy makes this reaction irreversible. FBPase is also important in contributing to starch and/or fatty acid synthesis in the developing embryos of oilseed rape [89] and in sugar partitioning [90]. This protein is down-accumulated >2 times in all genotypes, especially in C (−4) and N (−6).

Aldolase has been implicated in many non-catalytic functions, based upon its binding affinity for multiple other proteins including F-actin, α -tubulin, phospholipase D, glucose transporter GLUT4 and V-ATPase. Fructose-bisphosphate aldolase 2 (SSP 5302; NP_568049.1; EC 4.1.2.13; FBA) catalyzes reversible cleavage of fructose-1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Glycolysis uses this forward reaction while gluconeogenesis and Calvin cycle (anabolic pathways) use the reverse reaction. However, these reactions are not possible under energy deficit as for FBPase. Lower accumulation of FBA under drought was observed only in water-spender cvs (in accord with Abreu et al. [6]), which is connected (the same for FBPase) with lower accumulation ATP-related proteins. Down-accumulation of FBA was observed during severe drought in barley [59]. On the contrary, Yin et al. [91] found FBA important in maintaining plant physiological functions during biotic stress (wound-response) in leaves of *B. napus*. Based on the important role of FBPase and FBA, we hypothesize that their significant decrease can act somehow as a “signal” that will slow down stem prolongation and delay flowering.

Ribose 5-phosphate isomerase A (SSP 2111; XP_002882374.1; RPI-A) in plants is a part of the Calvin cycle as ribulose 5-phosphate, which is a CO₂ receptor in the first dark reaction of photosynthesis. This reaction leads to the conversion of phospho-sugars into glycolysis intermediates, which are precursors for the synthesis of amino acids, vitamins, nucleotides, and cell wall components. This protein showed an interesting accumulation pattern – an increase in savers C + D and a decrease in spenders N + V, which makes RPI-A a possible selection target (interestingly, *B. napus* rpi2 mutants accumulated less starch in the leaves and flower significantly later than wild-type [92]). The treated plants of N and V showed a reduction of photosynthetic efficiency, which with higher growth in controls can be some clue in understanding RPI-A decrease upon stress (similar to SBPase; S4).

Chloroplastic glucose-1-phosphate adenyltransferase small subunit (SSP 2505; Q9M462.1; EC 2.7.7.27; ADPase) also called ADP-glucose pyrophosphorylase – plays a role in starch biosynthesis and sucrose metabolism. ADPase showed lower accumulation in N, V, and D except for C where ADPase is increased. The intensity of starch biosynthesis depends on the activity of ADPase.

Triosephosphate isomerase (SSP 3104; NP_179713.1; EC 5.3.1.1; TPI) is a glycolytic enzyme which plays an important role in several metabolic pathways and is essential for efficient energy production. TPI is drought-accumulated only in C and N and thus can play some

role in the observed adaptability of these cvs. We observed also other triosephosphate isomerase (SPP 5107; AAA03449.1; EC 5.3.1.1; TPI). This cytosolic TPI increased in all genotypes except in N. Researchers demonstrate that a large reduction of cytosolic TPI alters the distribution of carbon in plant primary metabolism [93]. TPIs, both plastidic and cytosolic, play an important role in adaptation and should be further studied in stress-related experiments.

Transketolase-like proteins (SSP 5710; CAB82679.1, and 3709; AAO29950.1; TKL) catalyze the reversible transfer of a two-carbon ketol group from fructose-6-phosphate or sedoheptulose-7-phosphate to glyceraldehyde-3-phosphate. According to Rocha et al. [94], transketolase could act as a stress sensor involved in the adaptation process and regulation of carbon allocation. These proteins showed significantly lower accumulation in V.

Malate dehydrogenase (SSP 7204; NP_564625.1; EC 1.1.1.37; MDH) reversibly catalyzes the oxidation of malate to oxaloacetate using the reduction of NAD⁺ to NADH. This reaction is a part of many metabolic pathways, including the citric acid cycle and gluconeogenesis. A drought and heat stress combination was found to involve the conversion of malate to pyruvate generating NADPH and CO₂ and thereby alleviates the effects of stress on photosynthesis [1]. The source for conversion of malate to pyruvate is starch breakdown coupled with energy production in the mitochondria [1]. MDH was significantly increased only in cv N (however, close to 2-fold up-accumulation in all cvs), which can support the idea about its important role in water transport and need of NADPH reduction power upon drought. MDH accumulation in barley crowns upon drought was clearly shown in Vítámvás et al. [59].

When photosynthesis is declined due to drought, the export of photoassimilates from source to sink tissues is inhibited, too. Mueller et al. [16] found extracellular invertase activity the most correlated (low activity enables the export of sucrose from source to sink tissues) with RWC and OP changes in drought-stressed *B. napus*. Except for TPI, RPI-A and MDH, all significant protein abundances in this category showed a decreased accumulation in both groups, and thus are not well suited for water-behaviour based selection. This general trend is contrary to Koh et al. [51] where non-vernalized seedlings of rapeseed were studied. The water-spenders showed a higher number of down-accumulated proteins and two proteins were up-accumulated (TPI, MDH). This particular result is in congruence with a decrease of ATP and photosynthesis-related protein categories for water-spenders.

3.8. Photosynthesis-related proteins

During drought stress, one of the possible ways to achieve development is to maintain the photosynthetic efficiency as high as possible, but avoid the energy and ion imbalances that result from the stress. This can lead to over-excitation of the photosynthetic apparatus, and consequently, to photo-oxidative damage [70]. The ability of plants to adapt and/or acclimate to adverse environments is related to the plasticity and resilience of photosynthesis, which, in combination with other processes, determines plant growth and development [6]. The photosynthetic apparatus should also be considered a major energy sensor because it is modulated by environmental cues and plays a major role in the regulation of phenotypic plasticity [95,96].

LHCII type III chlorophyll *a/b* binding protein (SSP 121; CAA43804.1; LHCB3) transfers light energy to one chlorophyll *a* molecule at the reaction centre of a photosystem. LHCB3 abundance is increased in all genotypes except V, which can be connected to patterns in photosynthetic (very low Pn), and biochemical measurements (chlorophyll content, etc.). Down-regulation or disruption of any member of the LHCB family (six major families) reduced responsiveness of stomatal movement to ABA, and therefore, it resulted in a decrease in plant tolerance to drought stress in *A. thaliana* [97]. This also means that LHCBs play a role in guard cell signalling in response to ABA.

Chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase activase (SSP 404, 1306, 1310, 1401, 1404, 1407, 2304, 2402, 8004;

AFH35543.1; RCA) is one of the most important and limiting regulating factors of photosynthesis. Because of general impairment of ATP production and an increase of leaves temperature due to decreased transpiration during drought stress, RCA may be an important factor determining the response of plants to climate change [98]. Some results provide clear evidence that RCA also has a potential to improve crop plant productivity under drought or salinity stresses [99]. Abundances of RCA protein gene products (isoforms) decreased in almost all genotypes; however, between controls, cv V showed the highest accumulation of RCA. Interestingly, Bandehagh et al. [41] found an increased abundance of RCA in salinity-tolerant cvs. In most plants, the sensitivity of RCA to the ratio of ATP/ADP is modified by the stromal reduction/oxidation state through another small regulatory protein, thioredoxin [100]. Thioredoxin detoxification system through 2-Cys peroxiredoxin BAS1 (SSP 1005) was decreased in treated V. We hypothesize that, this fact can generally decrease the cv V sensitivity to some RCA gene products, concluding that further genotype-based research needs to be done.

Chloroplastic RuBisCO large subunit-binding protein subunit alpha (SSP 608; P21239.2; CPN60A) belongs to chaperonin (HSP60) family and showed an increased abundance in water-spenders and a decreased abundance in water-savers cvs. The higher accumulation of RCA's and CPN60A's in controls of N and V can be connected to the different rate of growth and/or an increased need for photoassimilates. CPN60 is usually up-accumulated in high light-resistant mutants exposed to excess light and in heat and cold treated plants [101].

The extrinsic photosystem II protein of 33 kDa, which stabilizes the water-oxidizing complex, is here represented by two forms (PsbO1–2 and PsbO2). Oxygen-evolving enhancer protein 2 (SSP 1103; P11594.2; PsbO2) accumulation decreased in all genotypes, mainly in N (>–5). Bandehagh et al. [41] found PsbO2 decrease in both, tolerant and sensitive rapeseed leaves upon salt stress. A similar protein is oxygen-evolving enhancer protein 1–2 (SSP 2221; NP_190651.1; PsbO1–2), which accumulation increased in C and N, decreased in V and D, and thus can be connected with higher ATP needs (electron chain in thylakoids) for better acclimation of C + N upon stress. The function of PsbO1 in *Arabidopsis* is mostly in support of PS I activity because it regulates the turnover of the D1 protein [102]. Cvs V and D showed a decrease of both PsbO proteins. Photosystem II subunit O-2 (SSP 1106; XP_002877774.1; OEC) is known as the manganese-stabilising protein as it is associated with the manganese complex of the OEC. OEC decreased abundance in all genotypes except V where accumulation of OEC is increased under drought. OEC was found to be accumulated in salt-sensitive rapeseed cv Sarigol under salt stress [41].

Thylakoid rhodanese-like protein (SSP 1602; NP_567209.1; TROL) is a nuclear-encoded component required for anchoring ferredoxin-NADP reductase (see SSP 7201) to the thylakoid membranes and for sustaining efficient linear electron flow. TROL abundance decreased in all genotypes, mainly in V and D. NADPH synthesis, mediated by FNR-TROL interaction, may be the source element in metabolic retrograde signal-transduction pathway linking light reactions with nuclear gene expression [103].

Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RuBisCO) has two protein accession numbers presented in the data which differ in pI and Mr. but belong to the same protein cluster 2 (SSP 4001; AFC79531.1 and SSP 8414; AEK33920.1). Both SSPs showed mixed accumulation pattern within cvs. According to Desclos-Theveniau et al. [77], this protein can become an indicator for identifying new *B. napus* genotypes with improved NUE and also could be related to high-photosynthesizing genotypes (in this case cv N) under drought. However, the mixed pattern of accumulation within cultivars shows its limited usage under long-term drought. RuBisCO together with LHCII contain up to 40–50% of the nitrogen in the mesophyll cell, which makes their degradation crucial for nitrogen remobilization. To a similar protein group belong two other down-accumulated proteins: chloroplastic rbcL gene products (SSP 6503 and 7503; YP_005089960.1; rbcL) which are related to RuBisCO.

Chloroplast beta-carbonic anhydrases (SSP 7106 and 7107; ADI52861.1; EC 4.2.1.1; CA1) have opposite accumulation pattern for both SSP's, which could make this protein an interesting target of further drought-related research. CAs proteins are involved in the CO₂ signalling pathway, which controls gas-exchange between plants and the atmosphere by modulating stomatal movements [104] and promotes water use efficiency by influencing the internal conductance [105].

This functional group represents the second largest group of identified proteins. In our study, water stress was slowly imposed by plant transpiration losses (28 days) and by reduction in the biochemical capacity for carbon assimilation and utilization that occurred along with restrictions in gaseous diffusion [70].

3.9. Redox homeostasis, ROS and signalling

Cellular redox homeostasis generates signals for the synthesis of defence enzymes and other antioxidant systems coping with stress. Together with photosynthesis and stress-related proteins, the redox homeostasis is likely to integrate all stresses into a cellular response with a stress-adaptive programme [96]. These somehow signalling and/or retrograde feedback signs can help to more deeply understand the behaviour of individual genotypes of winter oilseed rape. Oxidative stress is caused by the presence of low levels of antioxidant or by the increased ROS production due to environmental stresses. Extreme generation of ROS elevates the sensitivity of proteins to proteolysis [74].

In our study, we found three unique proteins (four gene products SSP 9, 1002, 1005, and 2002) with general peroxiredoxin activity. Peroxiredoxins (Prx) are known to play an important role in combating the reactive oxygen species generated at the level of electron transport activities in the plant exposed to different types of biotic and abiotic stresses. Prx also modulate redox signalling during development and adaptation and were shown to protect DNA from damage in vitro and in vivo [106]. In our study, all Prx were upregulated in almost all cvs. The results of Kim et al. [107], suggest that in *Brassicaceae*, Prx isotypes play specific roles in the cells in timely and spatially different manners, but they also cooperate with each other to protect the plant.

2-Cys peroxiredoxins catalyze the transfer of electrons from sulfhydryl residues to peroxides and are ubiquitous among all organisms. 2-Cys peroxiredoxin (SSP 9 and 1002; AAG30570.1; 2-Cys Prx) has been reported to localize to chloroplasts and perform antioxidative and chaperone roles [108] during plant development and photosynthesis. 2-Cys Prx showed an increased accumulation in all stressed plants in both SSP's (but especially for water-spenders group).

Peroxiredoxin-2E (SSP 2002; NP_190864.1; Prx-2E) was found to be increased in all genotypes after drought stress. Prx-2E with BAS1 (SSP 1005) are involved in detoxification provided through the thioredoxin system and may be involved in chloroplast redox homeostasis [109].

Glyoxalases are known to be differentially regulated under stress conditions and their overexpression in plants confers tolerance to multiple abiotic stresses. The glyoxalase system is a set of at least two enzymes (glyoxalase 1 and 2) that carry out the glutathione-dependent detoxification reactive aldehydes that are produced as a normal part of metabolism. Glyoxalase 1 (SSP 3225; Q39366.1; EC 4.4.1.5; synonym: lactoyl glutathione lyase; GLX1) was significantly accumulated (>3.5) in treated plants in all genotypes (except for V). *B. juncea* glyoxalase I is surprisingly modulated by calcium/calmodulin complex [110]. Glyoxalase I activity decreased in rapeseed seedlings upon exposure to salt stress [111].

L-Ascorbate peroxidase (SSP 6105; CAA55209.1; APX1) showed a significant increase in all genotypes under drought stress conditions. Ascorbate is a multifunctional metabolite that plays a key role in hydrogen peroxide (H₂O₂) removal in the chloroplasts and cytosol of higher plants [74]. H₂O₂ is also playing a signalling function modulation in plant phenotype [95] so APX is a powerful part of the complex response to any biotic and/or abiotic stress. In *Arabidopsis*, APX1 was found to be

specifically required for the tolerance of plants to drought and heat stress combination [1].

Ferredoxin-NADP reductase (SSP 7201; BAD07827.1; EC 1.18.1.2; FNR1) plays a key role in regulating cyclic and non-cyclic electron flow to meet the demands of the plant for ATP and reducing power. It is involved in the final step in the linear photosynthetic electron transport chain with TROL (see SSP 1602 in functional group 4). Bandehagh et al. [41] found lower FNR1 accumulation in salinity-susceptible salt-treated leaves of rapeseed. STRING scheme showed the cooperation of FNR1 to GS2 and AlaLaT and other photosynthetic and energetic metabolism. Overexpression of the LHCBs and chloroplastic ferredoxin-NADP(H) reductase in crop plants followed by abiotic stress tolerance assessment can be a priority for future crop breeding [6].

Our findings show the anti-oxidant system and ROS production may play a crucial role in dehydration tolerance of rapeseed and should be further examined in detail to help with selection of more stress-adaptable rapeseeds. The general pattern of antioxidant accumulations in our study is similar to other studies [100,112]. Almost all proteins in this category were increased under drought, which contrasts to Koh et al. [51] study on six-week old rapeseed plants (without vernalization) under 14 days of drought. This could mean that after vernalization (but before flowering), other proteins become important for drought-related adaptation in rapeseed and/or can mirror differences in drought adaptation of the genotypes used.

3.10. Stress and defence related proteins

In our study, this group of proteins represents the third most abundant protein group in rapeseeds influenced by drought. Generally, oilseed rapeseed (and other crop plants of the family *Brassicaceae*) contain a unique defence system known as the glucosinolate-myrosinase system or the 'mustard oil bomb' [113] which is one of the best-studied plant defence systems.

For functionality of the glucosinolate-myrosinase defence system, the epithiospecifier protein (SSP 10 and 6410; AAY53488.1; ESP) is necessary. ESP converts glucosinolates at the expense of isothiocyanates [114]. From human health perspective, isothiocyanates are major inducers of carcinogen-detoxifying enzymes [115]. ESP showed a specific function in defence against herbivores and pathogens [114] and acts as a negative regulator of senescence. Both ESP's found in our study differ in pl and Mr. and showed quantitative and qualitative changes in accumulation. These ESP gene product changes seem to play an unresolved role in drought-related studies and could be targets of further focus. ESP activity was found to be negatively correlated with the extent of formation of the sulforaphane in broccoli [116]. This also means that upon drought, the general accumulation of health-promoting phytochemicals can decrease. This fact is worthy of future study because the process of secondary metabolites production and drought-related glucosinolate composition and accumulation in response to abiotic stress is still not well known and has demonstrated conflicting trends [79].

The fibrillins are a large family of chloroplastic structural proteins that have been linked with stress tolerance and disease resistance [117]. Fibrillin (SSP 207; NP_192311.1; FIB) abundance profile is significantly increased for all genotypes upon drought. Fibrillins are involved in plastoglobule structural development [118], chromoplast pigment accumulation, hormonal responses, protection of the photosynthetic apparatus from photo-damage, and plant resistance to a range of biotic and abiotic stresses [117]. FIB is probably involved in light/cold stress-related jasmonate (JA) biosynthesis and contributes to the protection of photosystem II (PS II) against light stress [117]. Because FIB is involved in carotenoids accumulation (in chromoplasts), it helps remove several types of ROS and acts as precursors to signalling molecules that control plant development and abiotic/biotic stress response [74,119]. The area of fibrillin research reveals substantial growth potential and will contribute to better understanding of the mechanisms of plant stress tolerance and plastid structure and functions. Because FIB is

potential drought-adaptation indicator, its cultivar-based accumulation pattern upon different drought stages needs to be further confirmed.

Heat shock protein 70 (SSP 1701; AAF27639.1; HSP70) is the most important HSP known to be involved in various plant abiotic stress responses [101]. HSPs assist other nascent proteins to achieve the spatial structure and ultimately help the cell protect against stress factors, such as heat. HSP70 proteins accumulated higher in water-spenders and down-accumulated in water-savers cvs. A decrease of typical stress-related protein HSP70 in treated D might be explained by its slowest growth under drought (as supported by Mohammadi et al. [11] and Vítámvás et al. [59]). Results of Hu et al. [120] suggest that HSP70 may play a crucial role in ABA-induced antioxidant defence of maize to drought and heat combination. According to several studies (reviewed by Wang et al. [7]; Timperio et al. [101]), HSP70 are mostly down-regulated in susceptible genotypes.

NAD(P)-binding Rossmann-fold-containing protein (SSP 3215; NP_565868.1) showed higher accumulation mainly in N (>3) and D (>2). The Rossmann fold is a super-secondary protein structural motif found in proteins that bind nucleotides, such as enzyme cofactors FAD, NAD, and NADP [121]. Numerous putative proteins belonging to the family have been annotated as malate dehydrogenase (MDH) or lactate dehydrogenase (LDH). According to Bottoms et al. [121], this protein is able to keep enzyme hydrated and thus showed a dehydrin-like strategy.

Peptidyl prolyl *cis-trans* isomerase CYP38 (SSP 304; XP_002884278.1; CYP38) belongs to the cyclophilin family. CYP38 is responding to biotic stress and when under drought. We found CYP38 also accumulated in barley under drought [59]. In our study here, CYP38 was up-accumulated in water-spenders cvs (see "uncommon" ratios).

Germin-like protein (SSP 5008 and 7004; AAB51566.1; GER3) showed higher accumulation in water-spenders cvs. Generally, GER or cupins are defined by their sequence homology to barley germins and are present ubiquitously in plants; they play diverse roles in plant development, stress and defence responses [122–124]. By participation in glyoxylate cycle, GER is involved in carbohydrate biosynthesis from fatty acids. Rietz et al. [123] identified a family of 14 germin-like genes from *Brassica napus* (BnGLP), although their role in overall metabolism is not fully understood. This involvement in the protection of plants from environmental stress of various types has led to numerous plant breeding studies that have found links between GLPs and QTLs for disease and stress resistance [125].

3.11. Transcription, translation, RNA processing

These differentially accumulated proteins belong to two larger subgroups: rRNA processing and translation, and can be also involved in epigenetic changes under stress influence.

Two gene products of elongation factor G (SSP 2815 and 3802; NP_564801.1; SCO1) are chloroplast-localized elongation factors EF-G involved in protein synthesis in plastids and in proplastids re-differentiation into chloroplasts [126]. EF-G catalyzes the GTP-dependent ribosomal translocation steps during translation elongation and ribosome recycling phases of protein synthesis [127]. SCO1 protein abundances were significantly decreased in all stressed plants.

Chloroplast elongation factor tub (SSP 4402; XP_002869935.1; EF-Tu) has an essential function in the elongation phase of mRNA translation. Recent studies have shown that EF-Tu plays an important role in heat tolerance in maize and non-heading Chinese cabbage [128]. EF-Tu showed mixed a pattern of abundance. In Mohammadi et al. [11], authors showed that elongation factor EF-2 has a role in the drought tolerance of rapeseed roots.

Chloroplast stem-loop binding protein-41 (SSP 6004; NP_191873.1; CSP41A) participates in chloroplast ribosomal RNA metabolism, is required for chloroplast integrity [129] and is also involved in the regulation of the circadian system. CSP41A showed higher accumulation in N. It is proposed that CSP41 complexes may serve to stabilize non-

translated target mRNAs and precursor rRNAs during the night when the translational machinery is less active in a manner responsive to the redox state of the chloroplast [129].

3.12. Genotype-based differences and overview on obtained data

Analysis of physiological and proteomic data showed two different water regime-related strategies. The first group (C + D) is saving water in all conditions (higher WUE in both, control and treated conditions) and the opposite is true for spenders N + V.

In the water-savers group, fewer proteins were up-accumulated (11 proteins; Fig. 9) and less down-accumulated (18) in contrast to a higher accumulation in the water-spenders group (18 and 28, respectively). For water-spenders, the drought treatment can be connected to higher metabolism disruption, mainly due to rapid water stress onset, supported by other observed changes, discussed above. Rapid water depletion caused more severe disturbance in intracellular water homeostasis in water-spenders (indicated by RWC, OP etc.). These differences profoundly affect cellular, energetic and nitrogen metabolism. Differential clustering based on protein accumulation (Fig. S-8) and clustering of physiological parameters (Fig. 5) is clearly visible for controls as well as for treated plants because cultivars differed – besides other factors – in respect to their geographic origin indicating also a range of different backgrounds. Additionally, some measurements of treated plants at the 28 DAS may portray the changes beyond some metabolic threshold, which “edge” is also genotype-specific. Distinct onset of stress, and earliness of genotypes (C + N are intermediate cvs; D is an intermediate/late cv; V is an early cv) can also add some explanation. Additionally, both C + N are generally high yielding, high cold/frost and disease tolerant cvs with lower oil content in seeds (Table S-1). Similar unique responses (each cv used in this study behaves somehow unparalleled to other) for *B. napus* cvs are confirmed in other studies on drought [16,100,112].

Taken together, the strategy of water-savers (C + D) is based on regulation of gasometric characteristics to further profit from available water. However, cv C acclimated most rapidly from all other cvs on some levels (e.g. proline, GOM based results) and also grew more under stress than D, despite both cvs having showed water-saver adaptations. In contrast, cvs N + V (which drastically decreased the gasometric parameters and metabolism and accumulated high amounts of osmoticas) are sensu stricto less perceptive to water amount in the pot.

However, cv N acclimates to drought at the proteomic level and grows better under stress, similarly to cv C. The data showed that cvs C + N are able to “combat” drought in similar proteome-based way (Fig. 5; Fig. S-9) despite their different physiological and biochemical responses. The C + N “respond conservatively” [30] in terms of biomass accumulation upon stress, by harmonizing their metabolic and proteomic profile. The information and results obtained in our study implies both cvs C + N can adapt/acclimate to drought more easily and save more available assimilates in stem for further seed production. This was also supported by higher accumulation of above ground dry weight biomass upon stress for these two cvs.

4. Conclusions

The differences found in physiological response and in numbers of proteins responsible for the individual biological processes suggest the existence of diverse response strategies to drought between contrasting genotypes. Water-savers showed better nitrogen metabolism, higher ATP conversion proteins accumulation and thus more available energy, as well as higher accumulation of ROS, signalling, and stress-related proteins (Fig. 9). On the other hand, water-spenders showed a unique protein-accumulation response in carbohydrate/energy metabolism, photosynthesis-related, stress-related and rRNA processing proteins and high numbers of down-accumulated proteins, especially in

carbohydrate/energy metabolism and photosynthesis, which is in congruence with water-related characteristics, low WUE and net photosynthesis.

Under a mixed climate profile, both water-use patterns (savers or spenders) can be appropriate for drought adaptation, so there is definitely no clear drought-tolerant “winner.” Interestingly, both groups – savers and spenders – contain drought tolerant genotypes (C + N). Therefore, if we have to decide which cv is more drought tolerant, there has to be specified the rate of field dry-down, duration of stress and actual plant developmental stage.

The data from the experiments demonstrate that cultivars responded to progressive drought in different ways and at different levels. According to the present stage of knowledge, only the connection between gasometric, physiological and proteomic data seem to be effective in drought-tolerance selection for further targeted and environment-based breeding purposes.

Conflict of interest

The authors declare no conflict of interests.

Transparency document

The Transparency documents associated with this article can be found in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2016.11.004>.

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Proteomic analysis of oilseed rape microspore-derived embryos showed different profile after drought-simulation via media infusion with polyethylene-glycol

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Abstract

The changes in microspore-derived embryos (MDE) proteome of rapeseeds coping with osmotic stress are still unknown. The PEG-induced osmotic stress was studied in cotyledonary MDE of two genotypes: Cadeli (D), and Viking (V). We previously described the D and V to be contrasting in their water-related characteristics under drought in stem prolongation period. Out of the all differentially abundant protein spots, 156 representative protein spots have been selected for MALDI-TOF/TOF identification and 63 proteins have been successfully identified and divided into 8 functional groups. Several protein abundances were evaluated by using qRT-PCR. Biomass accumulation in treated D was significantly higher (3-fold) than in V, so we can claim D as a tolerant to osmotic stress. We propose different mechanisms to cope drought in the genotypes studied. Cv D showed tolerance strategy thanks to accumulation of proteins in energy metabolism, redox homeostasis, protein destination and signalling functional groups. While V protein profile shows high need for energy (ATP) and nutrients with significant number of stress-related proteins and cell

structure changes. MDE proteome profile is also discussed together with leaf proteome results from our previous study (Urban et al. 2017).

Keywords: Microspore, Proteomics, Osmotic stress, Brassica napus, Selection

Abbreviations:

cv(s), cultivar(s); D, Cadeli; DAS, day(s) after beginning of treatment; DAP, differentially accumulated proteins; DW, dry weight; FW, fresh weight; GO, Gene Ontology; MDE, microspore-derived embryos; PCA, principal component analysis; PEG, polyethylene-glycol; SI, supplementary information; V, Viking

Introduction

Plant breeding is focused on continuously increasing crop production to meet the needs of an ever-growing world population, improving food quality to ensure a long and healthy life and address the problems of global warming and environment pollution, together with the challenges of developing novel sources of biofuel. A combination of different approaches (mechanistic understanding, -omics, QTL mapping and other tools) will likely be needed to significantly improve the abiotic stress tolerance of crops in the field [1]. Major crops growing in our future fields are likely to be exposed to a greater range and number of abiotic and biotic conditions, as well as their combination [2]. To mitigate and recover from such damaging effects plants have evolved various adaptive strategies like dynamic changes in protein abundance.

Biotechnologies provide powerful tools for plant breeding, and among these ones, tissue culture, particularly haploid and doubled haploid technology, can effectively help to select superior plants [3]. Haploids (Hs), which are plants with gametophytic chromosome number, and doubled haploids (DHs), which are haploids that have undergone chromosome duplication, represent an attractive biotechnological method to accelerate plant breeding because latter can produce entirely homozygous lines. Isolated plant microspores can be diverted from their normal gametophytic pathway towards sporophytic development. In this transformation, besides other stresses such as heat or cold [4] shock, starvation [5], or endogenous auxin biosynthesis [6], media osmotics play a very significant role, too [7].

Brassica napus (winter oilseed rape) is the major oilseed crop in temperate regions of Europe and China and the second output oil crop in the world [8]. The Czech Republic (CZ) thanks to

much elaborated agronomy belongs to countries with biggest and most quality-based winter oilseed rape production in EU. The first draft of genome sequence of *B. napus* has been created Chalhoub et al. [9], but a lot of follow-up work will be necessary due to its complex genome and homologous regions of A and C genomes. The complete nucleotide sequence of the rapeseed chloroplast and mitochondrial genome was determined also by Hu et al. [8]. The recent proteomics articles and reviews of *Brassicaceae*. family have been published [10-22] Despite the importance of oilseed rapes worldwide, to date only three drought-focused comparative proteomic study was aimed on *B. napus* (seedling roots [23], 20 days old seedlings [24], and on mature plants [25]).

In *B. napus*, androgenesis or microspore embryogenesis is widely used to generate homozygous lines for breeding purposes. Protocols for the induction of microspore embryogenesis and the subsequent regeneration of DH plants have been successfully developed for more than 200 species [26]. For some species, isolated microspore culture protocols are well established and are routinely used in laboratories around the world for developing new varieties, as well as for basic research in areas such as genomics, gene expression, and genetic mapping [27]. In *B. napus* DHs production was firstly described in 1977 by Thomas et Wenzel [28]. The induction of microspore embryogenesis produces dramatic changes in different aspects of the cell physiology and structure [29, 30]. Not surprisingly, there are still some challenges to be solved, therefore, the DHs are significant part of research interest worldwide. Between 2010 - 2016, 296 and 44 article topics and titles, respectively, focused on “microspore” and “*B. napus* + microspore” were published. From the most recent, Kitashiba et al. [31] identified loci associated with embryo yield in microspore culture of Brassica rapa. Zhang et al. [32] modified the microspore protocol by adding histone deacetylase inhibitors to improve the rate of microspore embryogenesis and the frequency of direct plant regeneration in Pakchoi (*B. rapa* ssp. *chinensis* L.). By comparing intervarietal substitution lines, eight genomic regions containing genetic factors controlling the of direct embryo to plant conversion rate in rapeseed were identified by Kampouridis et al. [33]. The use of microspore culture and marker-assisted selection greatly shortened the time required to obtain elite DH resistant lines against Cabbage Fusarium wilt [34].

Plant stress response represents a dynamical process where several phases with a unique proteome composition can be distinguished [35]. In plant abiotic stress studies, it is common to analyze proteomes by contrasting stressed plants against control ones, attempting to correlate changes in protein accumulation with the plant phenotypic response [36].

Additionally, comparisons between genotypes with different sensitivity towards drought are crucial to understand the putative influence of differentially abundant proteins in tolerant genotypes. It is worthy to study different genotypes and look for proteins possibly correlated to drought adaptability and acclimation, because the responsiveness to changes of multiple environmental parameters in existing genotypes is vital [37].

The main aim of this study is to screen for proteins in oilseed rape embryos that are differentially regulated under osmotic stress and to explore in detail the basis of this response to a lack of water during the MDE development. To authors best knowledge, no similar comparative proteomic analysis (both, MDE together with leaf proteome) was published.

Experimental Procedures

Plant samples and measurements

The seeds of two winter oilseed rapes (*Brassica napus*. L.) cultivars (cvs), Viking (V) and Cadeli (D) were germinated and plants were cultivated under controlled conditions according to Urban et al. [25]. After vernalization, plants were cultivated as described in below section. For detailed information about cvs, please see Table S- 1.

Microspore culture treatment with antimitotic agents

Microspore cultures were carried out according to the basic protocol in Klíma et al. [38]. In short: young flower buds with microspores at mid-uninucleate and late-uninucleate developmental stages were collected from donor plants grown under controlled conditions in a culture chamber (light intensity 84 $\mu\text{mol}/\text{m}^2/\text{s}$, 22/20°C day/night and photoperiod 16/8 h). Microspores were isolated from flower buds after the microspore developmental stage observation. Freshly isolated and purified microspores were resuspended in NLN liquid medium supplemented with corresponding amounts of particular doubling agent stock solutions to get the final concentrations of trifluralin 10 $\mu\text{mol}/\text{l}$. Microspores in 60-mm plastic Petri dishes containing 6 ml of suspension were incubated for 18 h at 30°C in the dark. The microspores were purified after incubation by centrifugation, resuspended in a fresh NLN medium and cultivated in the dark at 30°C with anti-mitotic agent. After three weeks, embryos at torpedo and early cotyledonary stage (at least 2 mm in length) on the Petri dishes

were placed on a shaker (70 rpm) under continuous light at 22°C until embryos grown and turned green. Cotyledonary embryos at least 4 mm in length were transferred to a solid differentiation medium (DM) with benzylaminopurine (0.2 mg/l), indolyl acetic acid (0.2 mg/l) and 2% sucrose, solidified by 0.8% agar and maintained at 22/20°C, with photoperiod 10/14 h and a light intensity of 300 $\mu\text{mol}/\text{m}^2/\text{s}$. To lower the media water potential, sterile PEG 4000 30% w/v solution was poured on the top of solid matrix, and then poured out after 24 h, according to [39]. The control media was not treated. The pH of both medias and PEG solution, was adjusted at pH 5.8. After one day (for transcriptomic study) and 7 days (for transcriptomic and proteomic studies) of cultivation, one third of the embryos was weighed and frozen immediately in liquid nitrogen for protein extraction, second third was weighed and frozen for transcriptomic study and the last part was used for biomass accumulation and other physiological characteristics. Only embryos similar in shape and size were used for further study. Three biological replicates with five technical repetitions each were done. Details about experimental procedures are presented in SI.

Protein extraction, 2D-DIGE analysis, MS-based spot identification and database search

Total soluble proteins were extracted from embryos as described in [40] with some modifications as described in details in [25]. Dry protein pellets were resolved in lysis buffer according to GE Healthcare manual for 2D-DIGE analysis, pH of the solution was adjusted to 8.5 by 50 mM NaOH and protein concentration was determined by 2D Quant kit (GE Healthcare). The protein samples (15 μg) were labelled with CyDye® minimal dyes (GE Healthcare) according to manufacturer's instructions. Samples were run on 11 cm IPG strips with pI range 5-8. Image capture of gels was done using the PharosFX Plus (Bio-Rad) at a resolution of 100 μm . Densitometric analysis of scanned images was carried out using PDQuest Advanced 8.0.1 (Bio-Rad). Protein spot normalization was carried out using local regression model, and spot manual editing was carried out using group consensus tool. The differentially abundant protein spots (at least a 2 fold change; $p < 0.05$) were chosen for spot excision (ExQuest Spot Cutter; Bio-Rd) and identification from preparative gels (2-DE of 200 μg of internal standard sample) stained by Bio-Save Coomassie G-250 stain (Bio-Rad). Each biological replicate of protein samples was created as a bulk from five technical repetitions. Samples of VC and DS were dyed four times, samples DC and VS were dyed six times. Cy3- and Cy5-labelled samples were randomly combined and Cy2-labelled internal standard was added to form mixed sample for loading onto IPG strip.

For protein identification, the excised proteins were processed as described in [41]. Briefly, each sample was washed initially in a 50 mM ammonium bicarbonate solution containing 50% (v/v) methanol and dehydrated using a 75% (v/v) acetonitrile (ACN) solution. Proteins were then digested in 8 μ L of trypsin Gold (Promega), 5 ng/ μ L trypsin in 20 mM ammonium bicarbonate. After extraction with 50% (v/v) ACN containing 0.1% (v/v) trifluoroacetic acid (TFA), the peptides were dried at 50°C and spotted on MALDI-TOF target plates. A volume of 0.7 μ L of 7 mg/mL α -cyano-4-hydroxycinnamic acid in 50% (v/v) ACN containing 0.1% (v/v) TFA was added. A MALDI peptide mass spectrum was acquired using the AB Sciex 5800 TOF/TOF (AB Sciex, Foster City, CA, USA), and the 10 most abundant peaks, excluding known contaminants, were selected and fragmented. The ProteinPilot™ software 4.0.8085 was used for database searches with an in-house MASCOT platform (version 2.3, Matrix Science, www.matrixscience.com, London, UK). All proteins were identified by search against NCBI nr database 20151110 (76068736 sequences; 27658295194 residues) with the taxonomy Viridiplantae (<http://www.ncbi.nlm.nih.gov>) containing 3269297 sequences and downloaded on October 15, 2016. All searches (combined MS and 10 MS/MS spectra) were carried out using a mass window of 100 ppm for the precursor and 0.5 Da for the fragments. During the different searches, the following parameters were defined: two missed cleavages, fixed carbamidomethylation of cysteine, variable oxidation of methionine or tryptophan, and tryptophan to kynurenine or double oxidation to N-formylkynurenine. All identifications were manually validated and extra precursors were selected for fragmentation if the obtained data were judged as insufficient. When high quality spectra were not matched to sequences, a sequence was determined manually and in the current data set could be linked to the identified protein by allowing for more missed cleavages, semitryptic peptides, or specific modifications. Only spots considered for discussion were the ones that have unique and significant protein identification. The spots, which contained more than one protein, were not considered in the study. Out of the all differentially abundant protein spots (894 normalized spots – Boolean of all normalized spots) just 212 spots were assessed by PDQuest as quantitatively changed at least ± 2 -fold. From these, the present in at least 80% of gels and revealing less than 50% variability in their standard deviation (SD) density values relative to the mean sample values have been selected for protein spot excision (156 spots).

RNA isolation and qRT-PCR

Samples for studying expression of genes identified to encode osmotically induced proteins were collected as a bulk of 8 – 10 biological replicates (embryos) and were stored at -80 °C. Total RNA was extracted using the RNeasy plant mini kit (Qiagen) according to manufacturer's instruction. Contaminating DNA was removed using the DNA-free™ Kit (Ambion). RNA was quantified using spectrophotometric measurements (OD260) and sufficient quality was assessed (OD260/280 ratio and OD260/230 ratio) by BioSpec Nano (Shimadzu). Total RNA were stored at -80 °C. Complementary DNA templates were prepared using Standard Reverse Transcription Protocol (Promega) and stored at -20 °C. The qRT-PCR was performed on the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems) using Power SYBR® Green PCR Master Mix (Applied Biosystems) in a 96-well reaction plate using parameters recommended by the manufacturer (2 min in at 50 °C, 10 min at 95 °C and 40 cycles of 15 s 95 °C, 1 min of 60 °C, 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C). The three technical replicates and no-template controls were included. The specificity of amplification was determined by dissociation curve analyses. Description of primers for real-time PCR is given in Table S- 3. All the gene expression levels were normalized to Actin gene expression (BnAct), chosen as endogenous control. Relative quantity of the target gene expression levels was performed using the comparative $\Delta\Delta CT$ method according to [42].

Bioinformatic analysis of proteins, biological functions of identified proteins

Molecular functions of proteins were searched in AgBase GORetriever [43] (http://agbase.msstate.edu/cgi-bin/tools/goretriever_select.pl). For Gene Ontology annotation (GO) GOSlimViewer (http://www.agbase.msstate.edu/cgi-bin/tools/goslimviewer_select.pl) was used to characterize general cellular components, biological functions and biological processes (Ag Base version 2.00; Select GOSlim set: Plant). Proteins were sorted into clusters accordingly to their mode of accumulation using Permut Matrix [44] (version 1.9.4.) on the base of Z-score standardization of protein density data. For more detailed information about bioinformatic analyses, please see SI. In order to draw venn diagrams we used “Venn Diagram Plotter”, available on <http://omics.pnl.gov/software/venn-diagram-plotter>.

For data used in associative transcriptomics, the protein accession versions were blasted to *Arabidopsis* (*taxid:3701*) in NCBI blastp (<https://blast.ncbi.nlm.nih.gov>), and manually searched for a gene locus in TAIR (www.arabidopsis.org). Blastp search set: Database: Non-redundant UniProtKB/SwissProt sequences; Molecule Type: Protein; Update date:

2016/12/11; Number of sequences: 465342; *Arabidopsis* (taxid:3701); Matrix: BLOSUM62. For associative transcriptomics, only proteins with increased accumulation in treated samples (DS/DC; DS/VS or with significant increase in DC/VC) were used as “proteins accumulated in D” and similarly for V. Proteins with similar accumulation across genotypes and/or treatments were not used in associative transcriptomics.

Statistical analysis of differentially accumulated of physiological, biochemical and protein data

Exploratory Data Analysis (EDA) was used to determine statistically important features of measured of the data set. Combination of statistical tests together with diagnostics graphs were used for descriptive statistics (mean, variance etc.), verification of normality and homogeneity of the data and detection of the outliers. Linear dependence of the parameters of interest was determined by correlation and regression analysis. For a deeper understanding of relationship between measured characteristics principal components analysis (PCA) was used. The same method was also applied to a protein dataset. Diagnostic indicators, such as Scree plot, loading plot and total amount of explained variability were used to find an optimal model. All statistical tests were computed in STATISTICA ver.12 (StatSoft, Inc.). Cluster analysis of the final protein spots relative abundance has been carried out using Permut Matrix software [44] (version 1.9.4). For all cluster analyses, Z-score transformation of data was carried out. Euclidean distances (dissimilarity) and Ward’s criteria (rows linkage rule) were used for the analysis. For every Permut Matrix analysis, the highest rows and columns objective functions (R) and sum of all pairwise distances of neighbouring rows or columns (S; shortest path length) were chosen to describe patterns within genotypes and treatments.

Results and Discussions

Two cultivars of winter oilseed rape (detailed description in Table S-1) were included in this analysis because they differ in their response to drought in mature plants and applied different drought-adaptation strategies. According to our previous study [25], cv. D is a middle drought tolerant water-saver, and V is drought-susceptible water-spender. The cv. V is considered as early and cv. D as intermediate/late cv [45]. Embryos were placed in the control and PEG activated media and harvested after 24 h (1 day after stress began – 1 DAS; for transcriptomic use only) and after 7 days (7 DAS; for both, transcriptomic and proteomic use).

Biomass accumulation and other physiological characteristics shows better adaptation of Cadeli to PEG-infused media

The MDE fresh weight (FW) changes after 1 DAS (C1 and S1) and 7 DAS (C2 and S2) is shown in Fig. 1. The osmotic potential of liquid PEG solution (30 g w/v) was measured by WESCOR Psypro as -1.05 MPa. The osmotic potential of solid media in control conditions was -1.11 MPa, and -1.55 MPa for treated solid media. There is no statistical difference between MDE biomass accumulation until treated embryos 7 DAS. Biomass accumulation in treated D was significantly higher (3-fold) than in V. This increase of biomass in PEG-treated cultivation media supports the idea about D with water-saver behaviour. Cv D MDE metabolism can be possibly better adapted and then able to growth under higher osmotical pressure too. This increase in biomass is similar to growth under control conditions for D. In Fig. 1 we can see cv V is losing its MDE weight (however, not significantly) in the after 7 days in controls. The other hypothesis can be leaded by idea about high and rapid accumulation of osmolytes in D vs V, which could decrease (to more negative values) MDE osmotic pressure to stabilize cytosol against PEG-driven dehydration.

Proteomic analysis of embryos

Two drought-tolerance contrasting genotypes of winter oilseed rape cultivars (D and V), and two different treatments (control and PEG-induced drought simulation in cultivation media) after 7 DAS have been compared in the proteomic experiment. Despite the embryos were the same age, treated MDE of both cvs were slightly dwarfish (Fig. 2). The representative 2D-DIGE gel of MDE with highlighted 156 spots showing protein resolution is shown in Fig. 6. All normalized (894 spots; data not shown) and chosen for identification (156) protein spots across the gels were included in the PCA testing to identify sample outliers and to group samples from different stages of treatment for each cultivar. Two PCAs were prepared: 1) analysis is based on all protein abundances of all individual gels (Fig. 3) and 2) analysis is based on averaged values of protein abundances of individual samples (DC, DS, VC, and VS; Fig. 4). PCA based on all values (Fig. 3) distinguishes both genotypes in all factors 1-3 and explain 33 % of data variability. PCA based on averages of abundances (Fig. 4) distinguishes between controls samples from treated samples between cultivars, despite the fact DC+VS

and DS+VC were projected closer to each other. This result is based on the fact, that these groups share high numbers of similarly oriented (down- or up-accumulated) proteins (see Fig. 5) and is supported also by PermutMarix clustering analysis (data not shown). Factors 1-2 based projection of Fig. 4 data explains almost 66 % of data variability. The protein spots belonging to 156 chosen spots are placed in center of the PCA's plots, which reveals that spots exhibit contradictory accumulation patterns within genotypes (and ergo the mean is centred close zero).

The heat map was created on the base of standardized protein abundances of all 156 chosen protein spots (standardization by Z-score values) in Permut Matrix. In Fig. 7, individual clusters are visible and visualized by colours (on the right side in the picture). From the heat map, clusters belonging to individual samples are visible. All differently accumulated spots (156 spots) reveal clustering into 9 main clusters according to their accumulation pattern in controls and treated genotypes (see Tab. 1).

These 9 clusters were divided according to genotypes and treatments as follows: Cluster 1 – proteins accumulated mainly in DC; cluster 2 – proteins accumulated mainly in DS; cluster 3 – proteins with generally higher abundance in cv D; cluster 4 – proteins accumulated in VC, cluster 5 – proteins mainly accumulated in VS; cluster 6 – proteins with generally higher abundance in cv V; cluster 7 – proteins accumulated mainly in controls; cluster 8 – proteins accumulated mainly in treated samples, and cluster 9 – proteins with mixed patterns of accumulation.

Some proteins (e.g., cobalamin-independent methionine synthase, glyceraldehyde-3-phosphate dehydrogenase) have been found in more protein spots, however belong to different clusters. It refers to the one of the main advantage of gel/based method – that is possibility to visualize and quantify different gene products and/or posttranslational modification of the same proteins that could have different or opposite accumulation under same growth condition.

Generally, cv D showed high number of proteins changed in AA, protein, and energy metabolism (protein groups 1, and 3). Cv V showed high number or proteins changed in AA, protein and energy metabolism and in stress/defence-related processes (groups 1, 3, and 5). Interestingly, if combined both treated cvs together (cluster 8), they showed accumulation in energy metabolism and redox homeostasis, ROS and signalling (groups 3 and 4).

According to Venn diagrams (Fig. 5 A), Cadeli showed the highest numbers of unique spots differently up- or down-accumulated in controls and treated variant. Cv V showed high numbers of genotype-based variant. This result can be partially explained by different growth

rates in control and treated conditions (Fig. 1). On the contrary to data shown in Fig. 5 A, when S/C protein ratio was used in treatment-based variant (Fig. 5 B) significantly more protein were down-accumulated in VS/VC ratio. In DS/C, only 5 and 13 were down- and up-accumulated, respectively. For VS/C, 25 and 16 were down- and up-accumulated, respectively. This result showed cv V as more influenced by changes in cultivation and its homeostasis was disturbed.

Eleven spots were not sufficiently identified, probably due to a lack of sequence similarity or low abundance; nevertheless, these spots were included in Permut Matrix clusters and PCA analysis so we can describe its behaviour on the base of its clustering. The table of identified proteins (Tab. 3) contains all 63 successfully identified spots. The search for identified protein spots with large changes in protein abundances (more than ± 3 fold; $p < 0.05$) has revealed 7 protein spots (aspartate aminotransferase, AT2G47510-fumarate hydratase 1, rubisco, peroxiredoxin antioxidant, peroxidase 12, jasmonate inducible protein, elongation factor EF-2-like protein). Highly accumulated proteins belong mostly to protein and energy metabolism, and to stress/defence-related proteins.

Functional categories and cellular localization of drought-responsive proteins

Detailed biological functions of individual differently accumulated proteins were determined by GO Retriever output in domain of biological processes according to their NCBI Accession version. To investigate the functional and biological process-based identity of the individual *differentially accumulated proteins* (DAP), the 63 spots (61 DAP) were categorized into 8 major groups (Fig. 8 A) based on their putative biological processes: 1, Amino acid, nitrogen and sugars metabolism/protein metabolism (13 DAP); 2, ATP interconversion (1 DAP); 3, Energy metabolism (glycolysis, gluconeogenesis, TCA pathway, respiration photosynthesis) (24 DAP); 4, Redox homeostasis, ROS and signalling (7 DAP); 5, Stress/defence-related/detoxification (8 DAP); 6, Transcription (DNA/RNA processing and binding)/Protein synthesis (2 DAP); 7, Protein destination and storage, proteolysis (5 DAP); 8, Cell structure (1 DAP). On the base of previous published results, we compared MDE functional groups with leaf proteome groups (Fig. 8B).

According to these eight protein function groups, we can designate these groups to be most affected by PEG-related osmotic stress in chosen winter oilseed rape MDEs. Because of the differences found in numbers of proteins responsible for separate biological processes influenced by osmotic stress, it is reasonable to believe in existence of diverse response

strategies to drought between chosen contrasting genotypes. This was also proven in drought-related differential leaf proteomics of four cvs in Urban et al. [25].

Generally, significant decrease in accumulation of proteins could be explained by reduction of growth rate and/or different development strategy under osmotic drought conditions (like the compensation for an extension of the growing period).

The brief introduction into functional groups regarding *Brassica napus* MDE specific response to osmotic stress

Amino acid, nitrogen and sugars metabolism/protein metabolism

The proteins in this group are included in the chemical reactions and pathways involving organic or inorganic compounds that contain nitrogen and sulphide interconversion with sugars and protein metabolism. Sugars play a central regulatory role in many vital processes besides serving the energetic function and are considered as important signals which regulate plant metabolism and development [46]. This group represents the second biggest part of protein identification. In *Brassicaceae* family, the nitrogen and sulphide compounds are important from metabolism (thiols, glucosinolates, brassinosteroids) and biotic and/or abiotic stress adaptation point of view [47]. Not surprisingly, cvs of *B. napus* with high sulphur- an nitrogen-use efficiency are more tolerant to PEG-induced drought stress [48]. To this functional category belong proteins which are significantly down-accumulated in DC vs VC (e.g. isocitrate dehydrogenase, glutamine synthase, sucrose synthase and glyoxalase). Some proteins are accumulated in treated D vs V (aspartate aminotransferase, cobalamin-independent methionine synthase, and nodulin).

ATP interconversion

This group includes proteins in chemical reactions and pathways involving ATP, a universally important coenzyme and enzyme regulator which relevance increase under any kind of stress-related changes. Genotype and treatment changes in one enzyme involved in ATP metabolism were found in our study. The major sources of novel ATP molecules represent processes of both an anaerobic and aerobic respiration and photosynthesis [49, 50].

Energy metabolism (glycolysis, gluconeogenesis, TCA pathway, respiration, photosynthesis)

To maintain sufficient energy and balanced carbohydrate production is one of the most important pathways in all plants as sessile organisms. In contrast to Urban et al. [25], here we joint energetic metabolism related proteins with photosynthesis-related ones. The ability of plants to adapt and/or acclimate to adverse environments is related to the plasticity and resilience of photosynthesis, which, in combination with other processes, determines plant growth and development [36]. This group represents the biggest part of protein identification (Fig. 8). Some carbon/nitrogen metabolism related proteins identified here (e. g. malate dehydrogenase) showed also increased energy demand as well as enhanced cellular activities in the root tissue or rapeseed upon drought [23].

Most protein spots were up-accumulated in cv D in comparison to the same spots from cv V. Also between treatments (S/C of individual genotypes) most protein were significantly changed in cv V (e.g. glyoxysomal beta-ketoacyl-thiolase, fumarate hydratase 1, FBA, phosphoenolpyruvate carboxykinase etc.). These changes support connection between the growth (Fig. 1) and energy metabolism proteins. Cv D grew significantly more in treated conditions, probably because its better energy metabolism accumulated proteins in stress.

Unfortunately, there is still no clear output revealing some genotype more or less tolerant to stress according to its changes in energy metabolism-related proteins. More proteins were accumulated in cv D (both in controls and treated samples). Nevertheless, this is only quantitative point of view, several proteins play important qualitative role, too.

Redox homeostasis, ROS and signalling

Together with stress/defence-related proteins, this category contains the third most abundant protein accumulations. Then, cellular redox homeostasis is significantly affected by stress-induced production of ROS, however, generate signals for the synthesis of defence enzymes and other antioxidant systems against stress. With the photosynthesis and stress proteins the redox homeostasis and signalling are likely to integrate all stresses into a cellular response with a stress-adaptive programme [51]. Spots found in this study show the anti-oxidant system and ROS production play a crucial role in MDE tolerance and should be further examined to help in selection for adaptable rapeseeds. An increase in several ROS scavenging enzymes was reported practically in all proteomic studies dealing with plant stress response since imbalances in energy metabolism during stress treatments are associated with an enhanced risk of oxidative stress [52]. Proteins in this category showed up-accumulation in both treated cvs. Interestingly, several proteins showed higher accumulation in controls of V in comparison to low accumulation in D (e.g. alcohol dehydrogenase class III, S-

nitrosogluthathione reductase, peroxiredoxin antioxidant etc.). Such a similar trend was clearly visible in the leaf proteome study of this cv V in comparison to other cvs too [25].

Stress and defence related

Half of proteins in this group showed down-accumulation in treated vs control samples. Only MLP-like protein 329 (SSP 4114) was accumulated in treated samples for both cvs. Cv D generally showed lower accumulation of stress and defence related proteins than cv V. In DS/VS ratio, only catalase and ABA modulated tyrosine-phosphorylated proteins were more accumulated.

Transcription, protein synthesis/protein storage/cell structure

This is an artificial category, jointing resting three small categories together (categories 6-8). Proteins in DNA/RNA processing and binding/protein synthesis showed higher accumulation in treated D versus treated V. Cruciferin cru2/3 subunit significantly increased in both treated cvs, while tubulin decreased.

Proteins differentially abundant in cv Cadeli (cluster 1-3 and selected spots from cluster 9)

The highest numbers of proteins significantly accumulated in D vs V belong to energy metabolism, redox homeostasis + signalling, transcription and also protein destination, storage and proteolysis. Cv D showed then effective energy-related pathways, higher sensing for ROS related changes in cell compartments, higher protein turnover, and/or synthesis and also increase in cell trafficking system. Below selected proteins with higher abundance in D are listed.

Aspartate biosynthesis is mediated by the enzyme aspartate aminotransferase [53] (SSP 2404) and both cytosolic and plastidic form play central role in nitrogen metabolism and its storage [54] and increase after infection of necrotrophic pathogen [55]. One gene product of aspartate aminotrasferase is accumulated in cv D, the other in cv V. In the chloroplasts and in non-green plastids of plants, aspartate is the precursor for the biosynthesis of different amino acids and derived metabolites that play distinct and important roles in plant growth, reproduction,

development or defence. This protein probably plays a dual role, in both, somatic embryogenesis and stress, as proposed by Almeida et al. [56].

Proteins fluG-like (SSP 5705, 5724, and 5726) were searched by Blastp and proved similar to nodulin/glutamate-ammonia ligase-like proteins (NodGS). NodGS belongs to the glutamine synthetase family. Recent studies highlight the importance of nodulin-like proteins for the transport of nutrients, solutes, amino acids or hormones and for major aspects of plant development [57]. Some of nodulins showed aquaporin activity [58], facilitating all water, hydrogen peroxide and even arsenite transports out of cytosol. Doskočilová et al. [59], pointed the role for NodGS in root morphogenesis and microbial elicitation. However, the role of NodGS in abiotic stress is still unknown.

AT4g37510/F6G17_160 (SSP 5601; EC 1.6.5.5; NDH-1) is a subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I) and functions in the transfer of electrons from NADH to the respiratory chain [60]. NDH-1 belongs to quinone reductases (QRs) which are flavoproteins that protect organisms from oxidative stress. The function of plant QRs has not as yet been addressed *in vivo* despite biochemical evidence for their involvement in redox reactions [61].

The AT2G47510 (SSP 416, FUM1) is recognized as a fumarase 1, which is a mitochondrial-localized protein and plays important role in the tricarboxylic acid cycle (TCA). FUM1 was down-accumulated in both treated samples, however, highly accumulated generally in cv D in contrast to V.

Among the other energy metabolism-related proteins with significantly higher accumulation in treated samples of cv. D (DS/DC) is UDP-glucose 6-dehydrogenase (SSP 3506). UDP-glucose dehydrogenase (UGD) plays a key role in the nucleotide sugar biosynthetic pathway, as its product UDP-glucuronic acid is the common precursor for many sugar residues found in the cell wall [62]. Importance of UDP GlcA for plant primary cell wall formation was also shown by Reboul et al. [63].

Two proteins from redox homeostasis, ROS and signalling group were accumulated only in DS: S-nitrosogluthathione reductase (SSP 2412, GSNOR) and mitogen-activated protein kinase 4 (SSP 5403, MAPK4). NO may react with glutathione (GSH) to form GSNO, which is considered the main reservoir of NO in cells [64]. The redox-active molecule nitric oxide (NO) is known to modulate plant responses to stressful conditions, plant immunity [65], crosstalk in salt tolerance [66], photosynthetic apparatus protection and improved shoot and root growth upon drought in sugarcane [64] etc.

The MAPK generally are important factors in the regulation of signal transduction in response to biotic and abiotic stresses [67]. Gawronski et al. [68] and others concluded that MAPK4 is a complex regulator of chloroplastic retrograde signalling for photosynthesis, growth, and immune defence in Arabidopsis. MAPK4 is also recognized as salicylic acid-independent regulator of growth [68], expression of brassinosteroid-related genes in rice [69], and general abiotic/biotic stress response in barley [70].

The peroxiredoxin antioxidant (SSP 5214) and hydroxyacylglutathione hydrolase 3 (SSP 5220) were accumulated in both treated cvs significantly. This result is similar to Urban et al. [25]. Peroxiredoxins (Prx) are known to play an important role in combating the reactive oxygen species generated at the level of electron transport activities in the plant exposed to different types of biotic and abiotic stresses. Kim et al. [71] suggest that in Brassicaceae Prx isotypes play specific roles in the cells in timely and spatially different manners, but they also cooperate with each other to protect the plant. Hydroxyacylglutathione hydrolase 3 (SSP 5220; ETHE) is also called persulfide dioxygenase and is located in mitochondria. ETHE catalyzes the oxidation of persulfides in the mitochondrial matrix and is essential for early embryo development in Arabidopsis [72, 73].

Surprisingly, no protein from stress-related group was significantly up-accumulated in DS. Ascorbate peroxidase (SSP 5204; APX) was accumulated in DC/VC ratio, and catalase (SSP 1502) with ABA modulated tyrosine-phosphorylated protein (SSP 4216) were accumulated in DS/VS ratio. APX was found to be specifically required for the tolerance of Arabidopsis plants to drought and heat stress combination [1]. In the thylakoid lumen, APX is essential for photoprotection as a cofactor for violaxanthin de-epoxidase, a key enzyme in the formation of nonphotochemical quenching. It has to be mentioned that H₂O₂ is playing a signalling function modulation plant phenotype [74], therefore, the APX is a powerful part of complex response to any biotic and/or abiotic stress.

Also transcriptional factor Pur ALPHA-1 (SSP 5314; PurA) from RNA processing showed accumulation in DS. PurA is a single-stranded DNA-binding protein that plays a role in cell growth and differentiation by modulating both transcriptional and translational controls of gene expression.

SSP 4711 was blasted to elongation factor EF-2-like protein LOS1 (SSP 4711; EF2). This spot was significantly reduced in VS and accumulated in DS. The DS/VS ratio is more than 11 x higher in DS. LOS1 encodes a translation elongation factor 2-like protein that is involved in cold-induced translation, however, LOS means “low expression of osmotically responsive genes.”

To protein destination and storage group belong three protein spots: 26S proteasome ATPase subunit, cruciferin cru2/3 subunit and Clp ATPase. 26S proteasome ATPase subunit (SSP 2407) belongs to AAA+ (ATPases Associated with a wide variety of cellular Activities). This superfamily represents an ancient group of ATPases. Members of the AAA+ ATPases function as molecular chaperons, ATPase subunits of proteases, helicases, or nucleic-acid stimulated ATPases. This protein showed significant ratio in DC/VC.

Clp ATPase (SSP 6710) belongs also to AAA+. This protein is sometime called HSP93-III and is involved in protein import into chloroplast stroma, chloroplast. This protein showed higher value in DS/VS ratio.

Protein differentially abundant in cv Viking (cluster 4-6 and selected spots from cluster 9)

The higher accumulation of proteins in cv V belong to four functional groups: AA, nitrogen and protein metabolism; ATP interconversion; stress and defence-related/detoxification; cell structure. This fact supports the idea about higher need for ATP and nutrient utilization, deeper stress impact, and increased stress-related cell structure changes. This also supports data from slower MDE growth (Fig. 1). Below selected proteins with significant changes in V are described.

Glutamine synthetase precursor (SSP 5408; EC 6.3.1.2; GS) accumulation decreased in VS and also in relation DC/VC. GS is an ATP-dependent plastidic enzyme that plays an essential role in the metabolism of nitrogen and in photorespiration where is a key enzyme. According to studies [59, 75] plants with low GS2 have diminished capacity for photorespiration and decreased tolerance to high-intensity light, so they are photoinhibited more severely by high-intensity light compared with control plants. GS accumulation can directly protect plant from drought-related photo-inhibition, so D could probably better photo-acclimate because of lower decrease of GS in treated conditions.

Glyoxalases are known to be differentially regulated under stress conditions and their overexpression in plants confers tolerance to multiple abiotic stresses [76]. The glyoxalase system is a set of at least two enzymes (glyoxalase 1 and 2) that carry out the glutathione-dependent detoxification of methylglyoxal and the other reactive aldehydes that are produced as a normal part of metabolism. Putative lactoylglutathione lyase (synonym: glyoxalase 1;

SSP 7201) in both treated genotypes was significantly accumulated, as also shown in Urban et al. [25].

Chloroplastic *atpA* gene product, also called NADH dehydrogenase (SSP 8504; ATPA) produces ATP from ADP in the presence of a proton gradient across the membrane. ATP production is significantly higher in VS/VC ratio and no change in DS/DC at the same time. This protein was accumulated in treated V samples and highly down-accumulated in DS/VS ratio (-6.6 fold), which show higher energy need of V. This accumulation pattern is contradictory to Urban et al. [25].

In energy-related protein category, the glyceraldehyde-3-phosphate dehydrogenase (SSP 418) and chloroplast beta-carbonic anhydrase like 1 (SSP 1208; CA1), together with malate dehydrogenase 2 (SSP 2312), F21D18.28 (blasted pyridine nucleotide-disulphide oxidoreductase or dihydrolipoamide dehydrogenase; SSP 3502; PYROXD), and fructose-bisphosphate aldolase (SSP 3319) were uniquely accumulated in VS. CAs are ubiquitous enzymes involved in fundamental processes like photosynthesis, respiration, pH homeostasis and ion transport. CAs proteins are involved in the CO₂ signalling pathway, which controls gas-exchange between plants and the atmosphere by modulating stomatal movements [77, 78]. CA promotes water use efficiency by influencing the internal conductance [79, 80].

Phosphoglucomutase 1 (SSP 5602, PGM1) catalyzes the bidirectional interconversion of glucose-1-phosphate (G-1-P) and glucose-6-phosphate (G-6-P) via a glucose 1,6-diphosphate intermediate. PGM1 shows higher accumulation in both, VC and VS.

MLP-like protein 329 (SSP 4114, NP_565265.1; MLP329) is a pathogenesis-related protein with still no clear function within plants and predicted location in nucleus and/or chloroplast [81]. In both cvs, MPL329 was increased in treated conditions. Interestingly, in V this protein is more than 3x higher accumulated than in D. Possible role in cytokinin signalling is mentioned in Černý et al. [81].

Glutathione S-transferase (SSP 4203; gi|87294807; GST) is cytosolic dimeric protein involved in cellular detoxification by catalyzing the conjugation of glutathione (GSH) with a wide range of endogenous and xenobiotic alkylating agents. GST showed increased abundance only in VS. GST is a part of the plant protection mechanisms against toxic O₂ intermediates, together with superoxide dismutases, catalases, ascorbate peroxidases and glutathione peroxidases [36]. Other results suggest that a lowering of the glutathione redox status during embryo development may represent a metabolic switch needed for increasing the endogenous levels of ABA, which is required for successful completion of the developmental program [82].

Jasmonate inducible protein (SSP 6717) containing jacalin-like lectin domain is a lectin. Its accumulation is significantly lower in VS/C, however, in comparison to DC is 4.7 x higher. Jacalin-like lectins are sugar-binding protein domains mostly found in plants. Proteins containing this domain may bind mono- or oligosaccharides with high specificity. The domain is also found in the salt-stress induced protein from rice.

Cruciferin cru2/3 (SSP 5203; CRU) is a storage protein (also called 11S globulin) localized on rough endoplasmic reticulum. CRU principal function appears to be the major nitrogen source for the developing plant, can be classified, on the basis of their structure, into different families. This family is a member of the 'cupin' superfamily on the basis of their conserved barrel domain. Gábrišová et al. [83] showed increased abundance of cupin fragments in radio contaminated flax contributing to growth and reproduction. CRU showed low relative accumulation in DC and high in VS. However, in S/C ratios, both cvs showed significant CRU up-accumulation upon stress.

Selected proteins with similar accumulation pattern according to treatments, genotypes or with mixed pattern of accumulation (cluster 7-9)

Proteins in this part did not show differences between treatments. Some of them show mixed pattern of their gene products (isoforms). Therefore, they were similarly up- or down-accumulated despite treatment in both cvs.

Cobalamin-independent methionine synthase (SSP 3619 and 3713; MetE) was found to have mixed accumulation. The predicted function of the cobalamin-independent methionine synthase isozyme is closely related to ethylene biosynthesis [84] and then probably also in stress-related signalling.

Rubisco ssu precursor (SSP 2111) showed very high increase in DS/DC (7 x) and significant decrease in VS/VC ratio. However, VC showed 9 x high accumulation than in DC, but, interestingly, higher accumulation in DS vs VS.

Fructose-bisphosphate aldolase 3 (SSP 2322 and 3319; EC 4.1.2.13; FBA) catalyzes reversible cleavage of fructose-1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Glycolysis uses this forward reaction while gluconeogenesis and the Calvin cycle, which are anabolic pathways, use the reverse reaction. However, these reactions are not possible under energy deficit. The level of FBA was found to decline under

salt stress in most of the plants studied (reviewed by Abreu et al. [36]). Yin et al. [85] found FBA important in maintaining plant physiological functions during wound-response in leaves of *B. napus*. Aldolase has also been implicated in many non-catalytic functions, based upon its binding affinity for multiple other proteins including tubulin, and phospholipase D also found (decreased in both treated samples) in this study.

Malate dehydrogenase (SSP 2312 and 2319; EC 1.1.1.37; MDH) reversibly catalyzes the oxidation of malate to oxaloacetate using the reduction of NAD⁺ to NADH. This reaction is part of many metabolic pathways, including the citric acid cycle and gluconeogenesis. MDH showed mixed abundance in stressed, which cannot support idea about its important role in water transport and need of NADPH reduction power, as was postulated in our previous study [25].

In short, phospholipases D alpha 1 (SSP 6705, and 6712), phospholipase D alpha 2 (SSP 6716), catalase (SSP 1502), ABA modulated tyrosine-phosphorylated protein (SSP 4216), both transcription-related proteins (SSP 4711, and 5314), and Clp ATPase (SSP 6710) showed increased abundance in DS vs VS. Phospholipase D alpha 1 (SSP 6705, 6712; PLD 1) and phospholipase D alpha 2 (SSP 6716; PLD 2) are important enzymes of the phospholipid metabolism. Phospholipases D (PLD) and their products phosphatidic acid are now considered to be one of the key elements of numerous physiological processes in plants including the salicylic acid signalling pathway [86]. Distefano et al. [87] showed that *pld* Arabidopsis mutants were more tolerant to severe drought than wild-type plants. This finding suggest that, in wild-type plants PLD disrupt membranes in severe drought stress and, in the absence of the protein (PLD knock-out) might drought-prime the plants, making them more tolerant to severe drought stress. Interestingly, all PLD's decreased in treated samples, however, D vs V comparison showed always higher abundance of PLD's in D.

Generally, results of protein abundances related to energy metabolism (glycolysis, TCA pathway, respiration etc.) showed the higher protein accumulation in cv D which again supports results obtained from MDE weight changes (Fig. 1), and makes a platform to understand similar changes in other protein functional groups.

The comparison between leaf proteome under drought and MDE proteome under osmotic stress

The comparison of this study with our previous study Urban et al. [25], is based on arranging of differential leaf proteome under drought in stem-prolongation stage of both cvs D and V with MDE derived proteome under osmotic stress. The idea of this is to reveal possible association between these two very different studies and developmental stages to confirm possible role of MDE in early selection of more adaptable rapeseed cultivars.

In a simplified way, if we compare number of proteins in each functional category between MDE and leaves, there is a significant increase of protein metabolism in MDE. The protein destination and cell structures are missing in leaf proteomes, so we can't compare them. On the other side, ATP interconversion, redox homeostasis, and protein synthesis are higher in leaf proteome of these two cvs. If we compare individual numbers of increased protein in each genotype, we can see similarities between MDE and leaves proteome in ATP interconversion and redox homeostasis in V. Comparison of decreased protein in individual genotypes for each functional category shows similarities between MDE and leaves proteomes for both cvs especially in redox homeostasis and stress/defence proteins.

Some proteins between studies are similar or even identical: glutamine synthetase, lactoylglutathione lyase (glyoxalase), atpA gene product, carbonic anhydrase, malate dehydrogenase 1, oxygen-evolving enhancer protein 1-2, L-ascorbate peroxidase, and glutathione S-transferase. Unfortunately, none of these proteins showed even similar patterns in protein accumulation. Interestingly, in MDE only one small chain RuBisCO (CAA30290.1; SSP 2111; rubisco ssu precursor) was found in contrast to five rbcL (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit) and nine activases (chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase activase) in leaves. This can be attributed to the high sugars content present in the media.

Even though, drought can be primarily manifested also as an osmotic stress (low water potential of soil, increasing xylem sap potential, etc.) the simple relationship between substrate dry-down and vapour-saturated low osmotic potential media is definitely not obvious. Also on the proteome level, we cannot easily compare these two cultivation methods and developmental stages. Generally, we can conclude that proteome response on MDE and leaves level are very different from mean abundances in each category or in comparison of Z-scores. The information about individual proteins behaviour comparison are already described in the above text. In this study, only the MDE biomass accumulation (higher in D) significantly shows adaptability to drought on a non-proteomic level. The D revealed middle-

drought tolerant water-saver strategy, in contrast to drought-susceptible water-spender strategy in V.

Confirmation of selected protein abundances with relative gene expressions

The mRNA expression values have shown their usefulness in a broad range of applications, including the diagnosis and classification of diseases, these results are almost certainly only correlative, rather than causative. In the end it is most probably the concentration of proteins and their interactions that are the true causative forces in the cell, and it is the corresponding protein quantities that we ought to be studying [88].

Nine proteins were selected according to their interesting accumulation behaviour across genotypes and treatments. The chosen proteins were as follows (SSP, name): 1502, catalase; 7201, putative lactoylglutathione lyase; 6705/6712, phospholipase D alpha 1; 5214, peroxiredoxin antioxidant; 6717, jasmonate inducible protein; 4216, ABA modulated tyrosine-phosphorylated protein; 1608, sulfite reductase; 4805, 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase; and 4203, glutathione S-transferase. Expression patterns of individual genes are shown in Fig. 9.

Some of these gene relative expression profiles (7DAS) are similar to protein abundance profiles (7DAS): catalase, peroxiredoxin antioxidant and lactoylglutathione lyase (we call it "the first group"). The other gene expression profiles are not similar to protein accumulation patterns. However, some genes showed similar expression only after 1 DAS as proteins accumulated after 7 DAS ("the second group"): glutathione S-transferase, ABA modulated tyrosine-phosphorylated protein, and lactoylglutathione lyase.

Alternatively, according to Greenbaum et al. [88], if there is definitively no correlation between mRNA and protein data, both quantities could be used as independent sources of information for use in machine-learning algorithms, for example, to predict protein interactions. On this base, we can postulate genes from first group suitable for gene-targeting at the same time as protein are extracted. The second gene group can probably be used for early selection of embryos in regards to their osmotic stress adaptability. Peroxiredoxin antioxidant and lactoylglutathione lyase can be used for early MDE selection as they are stress-related protein possibly increasing the adaptability of MDE to osmotic stress.

Conclusions

Developing embryos derived from microspores are challenging and interesting targets of abiotic or biotic treatments. The upcoming new-generation breeding strategies (epigenetic breeding, stress-memory based breeding, gene editing etc.) are looking for stable but wide genomic variation within established crops. The microspore-derived embryos (MDE) seem to be one of appropriate ways how to manage this goal. The technique itself is not easy and far away from extensive usage, however, is already established for several crops.

This proteomic study is a first step for MDE confirmation as a suitable model for follow-up research in characterization of new-breeds, new crossings, and can be used for phenotype-based selection tolerant to other worsening effects (other abiotic stresses and their combinations). Of course, the selected microspores have to be subsequently cultivated until seeds and evaluated in field conditions.

Cultivars D showed higher biomass accumulation under osmotic stress because of high number of proteins belong to energy metabolism (especially glycolysis), redox homeostasis + signalling (phospholipases, MAPK4), transcription and also protein destination, storage and proteolysis. On the contrary, the higher accumulation of proteins in cv V belong to four groups: AA, nitrogen and protein metabolism; ATP interconversion, stress and defence-related/detoxification; cell structure. D showed highest number of unique energy-related proteins and then better ability for protein synthesis and adjunctive communication between compartments. On the other hand, V protein profile showed high need for energy (ATP) and increased need for nutrients with significant number of stress-related proteins and cell structure changes. Also, higher number of proteins dealing with non-aerobic metabolism (e.g. alcohol dehydrogenase) were found in V. In V more proteins were generally down-accumulated, which we believe is connected to higher stress and similar trend for V was observed also in our previous study Urban et al. [25].

Taking these findings together, cv D showed quick adaptation to osmotically activated PEG-infused cultivation media, while cv V showed alert-based response with clear signs of damage. Maintenance of the primary metabolism, oxidative stress and signalling seems to be a strategy for D osmotic tolerance. On the other hand, susceptibility might be related to maintenance of the energy consuming homeostatic equilibrium in V.

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Conflict of interests Disclosure

The authors declare no conflict of interests.

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Figures

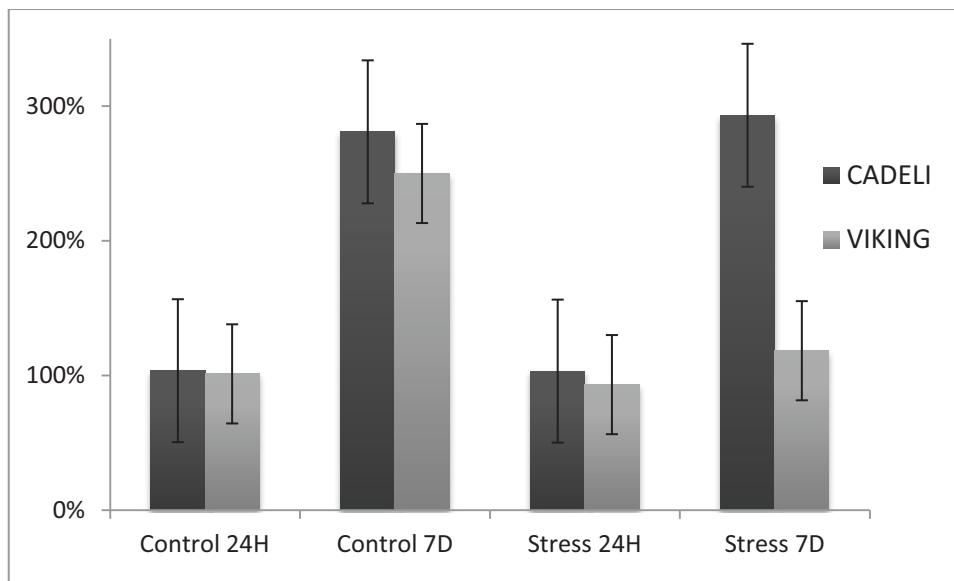


Fig. 1 Embryos relative weight changes are lower in Viking MDE in comparison to Cadeli genotype after one (24 H) or seven days of cultivation. Asterisks above the error bars indicate significant changes between cvs by the Student's t-test (* $p < 0.05$).

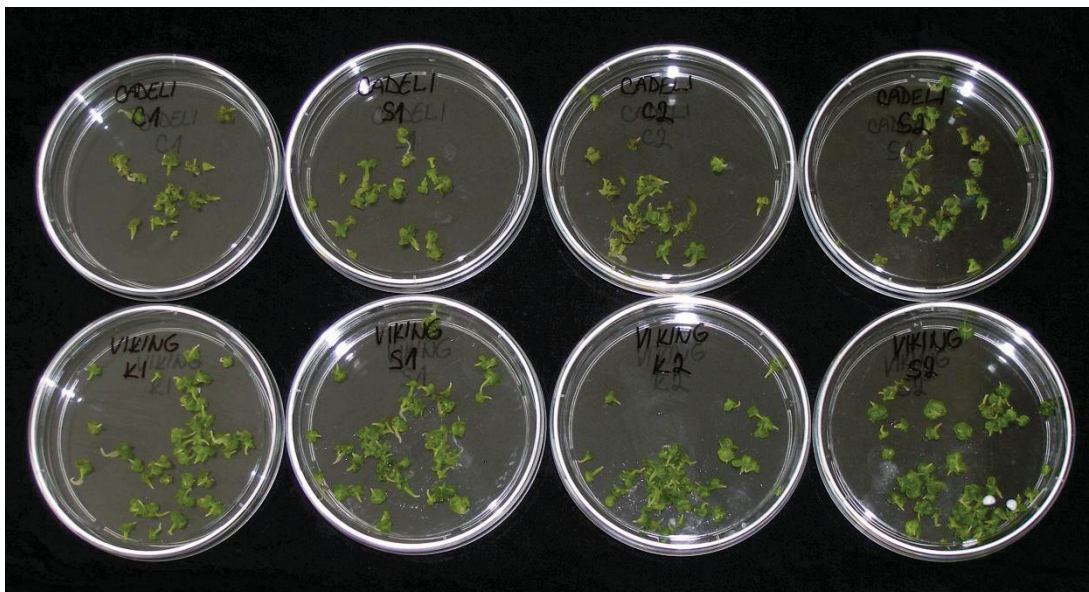


Fig. 2 Microspore-derived embryos. Upper row, cv Cadeli; lower row, cv Viking. From left to right: C1, S1, C2, and S2. C1, S1 – controls and treated samples for the first sampling (24 hours after stress); C2, S2 – controls and treated samples for the second sampling (7 days DAS).

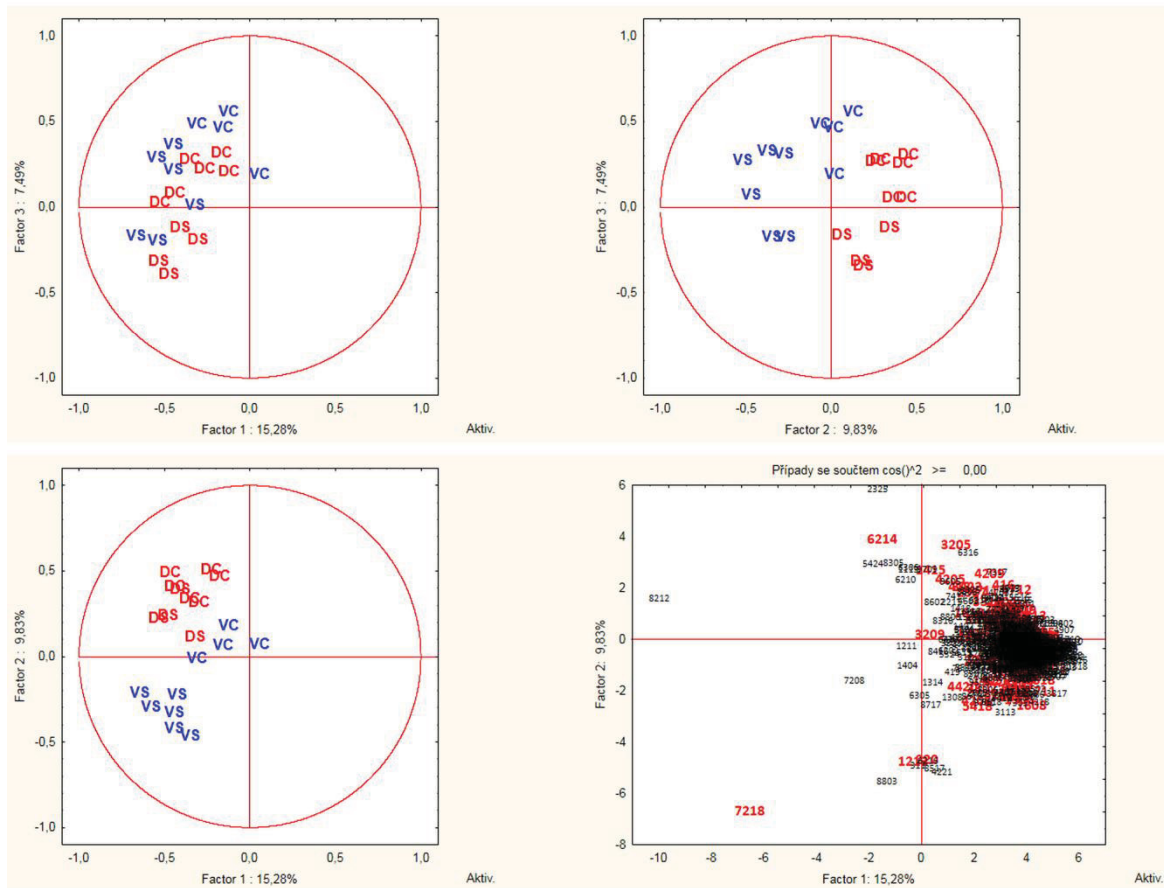


Fig. 3. Principal component analysis (PCA) analysis of all matched proteins spots (894) chosen for further analysis by PDQuest regarding the individual experimental variants. Analysis was prepared using protein abundances between two cvs Cadeli (D) and Viking (V). Differentially abundant protein spots revealing high relative abundance reproducibility (less than 50% variation in protein spot relative abundance within the individual replicates in the whole sample set) are indicated in red. DC – Cadeli control, DS – Cadeli treated samples, VC – Viking control, VS – Viking treated samples. To show individual cases the PC1, PC2 and PC3 factors based-projections of data (d) were created and explain almost 33% of variability.

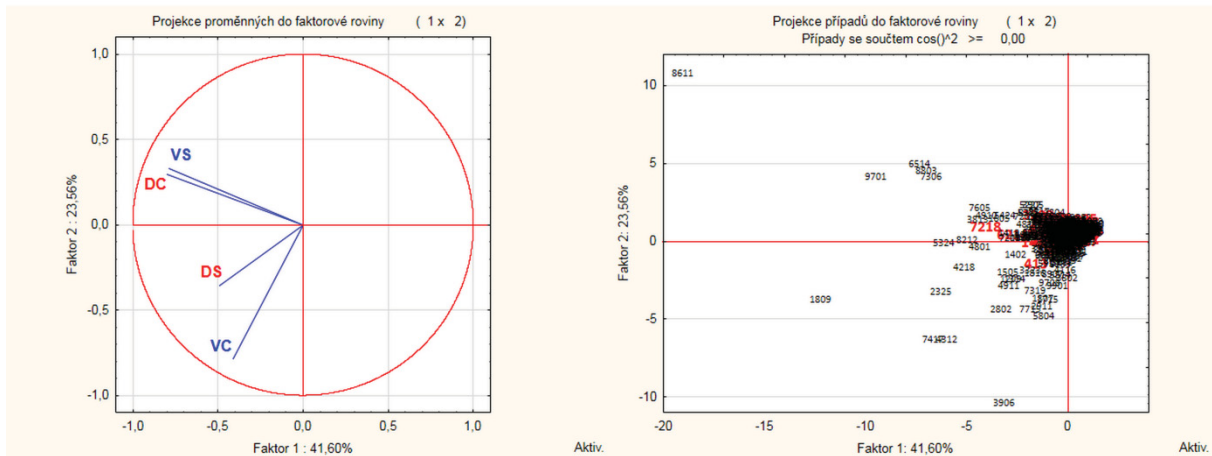


Fig. 4. Principal component analysis (PCA) of all normalized proteins (894 spots) between two cvs Cadeli (D) and Viking (V). Analysis is based in average protein abundances (see difference to Fig. 3). Differentially abundant protein spots chosen spots for further analysis by PDQuest (156 spots) revealing high relative abundance reproducibility (less than 50% variation in protein spot relative abundance within the individual replicates in the whole sample set) are indicated in red according to the individual clusters. DC – Cadeli control, DS – Cadeli treated samples, VC – Viking control, VS – Viking treated samples. To show individual cases the PC1 and PC2 factor based-projections of data were created and explain almost 66% of variability.

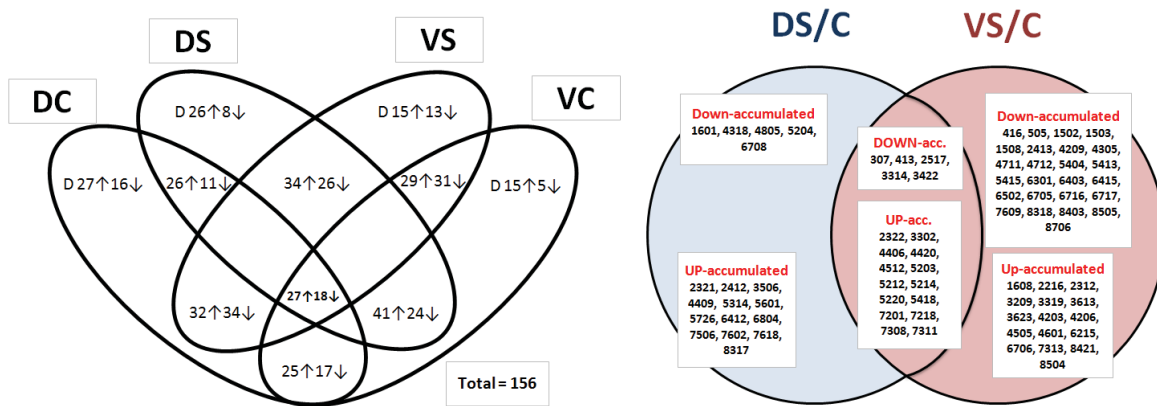


Fig. 5 Venn diagram of identified and non identified spots (A) showing all differentially abundant protein spots (at least 2 fold change at $p < 0.05$ and $SD \leq 0.5$; $n = 156$) revealing differential abundance between PEG-treated and control samples (DC-Cadeli control, DS-Cadeli stress, VC- Viking control, VS-Viking stress). Venn diagram showing SSPs of unique identified protein spots (B; at least ± 2 fold change at $p < 0.05$ and $SD \leq 0.5$; $n = 63$) revealing differential abundances between PEG-treated and control samples among genotypes. DS/C -

Rows : - Objective function : R=0.590
 - Sum of all pairwise distances of neighboring rows (path length): S=69.828
 - Linkage rule: Ward's criteria
 - Tree Seriation rule: Multiple-fragment heuristic (MF)
 Columns : - Objective function : R=0.769
 - Sum of all pairwise distances of neighboring columns (path length): S=58.717
 Dissimilarity : - Euclidean distance

The colors scale:

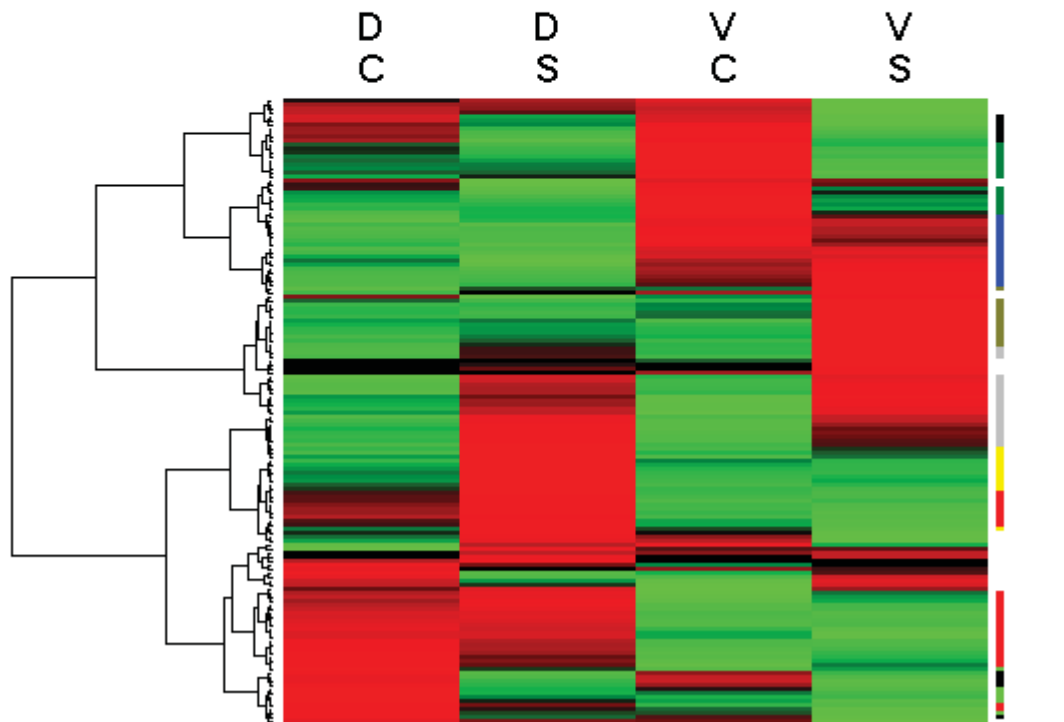


Fig. 7 Short heat map (Permut Matrix) of all 156 proteins, clustered according to the row Z-score values, calculated from protein abundances. The red values mean higher protein abundance in the samples, the green values mean lower protein abundance in the sample according to the variant (DC – Cadeli control, DS – Cadeli stress, VC – Viking control, VS – Viking stress). On the right side, the colour marks show the 9 different clusters, revealing the information about the proteins accumulation patterns. Clusters: 1 light green - higher in DC; 2 light yellow – higher in DS; 3 red – higher in both DC and DS; 4 green – higher in VC; 5 dark yellow – higher in VS; 6 blue – higher in both VC and VS; 7 black – higher in both controls, 8 grey – higher in both treated samples; 9 white – miscellaneous patterns.

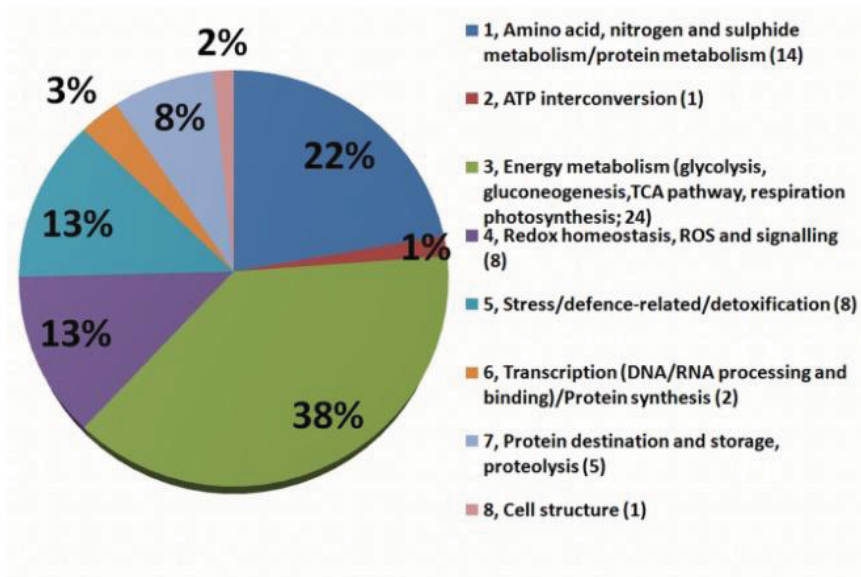


Fig. 8 The functional classification of proteins differentially accumulated after 7 DAS in MDE (A; n = 63) The numbers in brackets are number of proteins involved within each process.

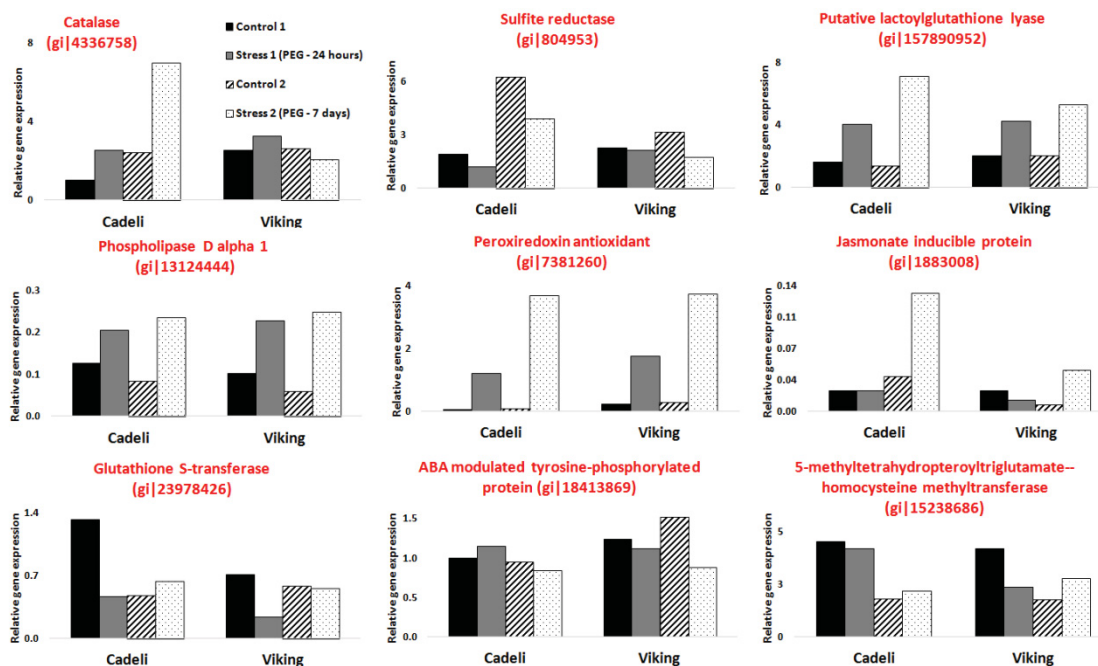


Fig. 9 Relative gene expressions of nine selected genes according to protein identification. Control 1 – control after 24 h after transfer to solid medium; Stress 1 – treated samples after 24 h after transfer; Control 2 – control after seven days after transfer; Stress 2 – treated samples after seven days after transfer.

Tables

Table S- 1. Agronomical characteristics and personal observations of used cultivars

Cultivar	Country of origin/ Company	Common characteristics (from official producer websites and GRIN Czech 1.9.1)	Our personal observations upon cultivars
Viking (line)	Germany NPZ (Norddeutsche Pflanzenschutz) (year of registration 2002)	https://grincech.vurv.cz/gringlobal/accesiondetail.aspx?id=60847 <ul style="list-style-type: none"> - early cultivar (flowering time) - small/intermediate size (low plant, smaller leaves) - very rapid autumn development and rosette formation - quick spring regeneration - very high tolerance to lodging before harvest - low/lower cold and frost tolerance - strong root system and rapid root development - tolerance to fungal disease and pests - middle seed yield and low TSW - very low GLS (lowest within known rapeseed cultivars) and very low erucic acid content - high oil content in seeds - more suitable for warm agriculture areas 	<p>Higher germination rate in high temperature (42°C day, 20°C night). Highest osmotic adjustment of leaves in drought stress. LOW root:shoot ratio under drought stress. Relative expression of COR25, ERD10 and ERD15 higher in controls than in drought stressed plants but significantly lower than Californium and Navajo.</p>
Cadeli (line)	France Monsanto SAS Monsanto Technology LLC (year of registration 2007)	https://grincech.vurv.cz/gringlobal/accesiondetail.aspx?id=60875 <ul style="list-style-type: none"> - intermediate/late cultivar (flowering time) - middle size (middle plant, middle leaves) - rapid autumn development and rosette formation - long flowering period - middle tolerance to lodging before harvest - low/lower cold and frost tolerance - strong root system and rapid root development - good/very good tolerance to fungal disease and pests - high oil content (very high oleic acid = oil stability) - higher adaptability to drought in warmer conditions - low seed yield and high TSW - very low GSL content - more suitable for warm and drier agriculture areas 	<p>Higher germination rate in high temperature (42°C day, 20°C night). High root:shoot ratio under drought stress. Middle dehydrins content at low (4°C) temp. Relative expression of COR25, and ERD10 higher in controls than in drought stressed plants but significantly lower than for Californium and Navajo (comparable to Viking).</p>

Table S- 2. Clustering of proteins according to their abundance between variants

Cluster number	Colour of cluster	Uniquely accumulated proteins in variants	Number of spots	Most abundant protein processes – protein functional categories (DIFFP)
1	light green	DC	3	3 (2x),5
2	light yellow	DS	6	1,2,3(2x),4,6
3	red	DC+DS	12	1(2x),3(7x),5,6,7
		cv Cadeli	21	1(3x),2,3(11x),4,5(2x),6,7
4	dark green	VC	7	1(2x),3(2x),4,5,8
5	dark yellow	VS	5	1,2,3(2x),7
6	blue	VC+VS	7	1(3x),3(3x),5
		cv Viking	19	1(6x), 2, 3(7x), 4,5(2x), 7,8
7	black	DC+VC	3	1,3,5
8	grey	DS+VS	5	3(2x),4(3x)
9	white	miscellaneous	10	1(2x),3,4(3x),5(3x),7

Table S- 3. The list of primer sequences used for qRT-PCR

Target gene	Primers forward / reverse	Amplicon size	NCBI accession no.
Brassica juncea catalase	CGCTCTCAAACCAAACCCAA / TTAAGCTCTCAGGGTGGTGG	83 bp	AF104454.1
A.thaliana sulfite reductase	TGAGCTTGGTCTAGTGGGTG / ATCTGTGCTGGTTCGGTGT	75 bp	Z49217.1
Brassica rapa putative lactoylglutathione lyase	ACAAAGGGCAACGCATATG C / TTCAGCGCTTTTGTACACAT CA	62 bp	AB300312.1
Brassica napus phospholipase D alpha 1	CATGTTACGCACCATCAGA AG / GAGTTTTGTTGGTGGGATCG A	102 bp	XM_013841229.1
Brassica napus Peroxiredoxin antioxidant	CGTCCTCTTCTCTCACCTG / AGCGTATTTCCCATCGCAC	73 bp	AF139817.1
Brassica napus jasmonate inducible protein	CTGGAGCTGTATGGGACGAT / CCATCTGTGCCTTGCCAAC	70 bp	Y11482.1

Brassica rapa glutathione S-transferase U5-like	CCGAGCAGACAAGAGAACTG / ATCACACTTCCGGCGACTAA	109 bp	AY567976.1
Brassica rapa uncharacterized protein At5g02240	AAAGAAGGAGGTGCACGAGA / CTCGAACAGCAATGCCTGAA	120 bp	XM_009124548.1
Brassica rapa 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase 1-like	CGCCCAGAAGATCGTTGAAG / TGGTGACTCTTGGGGAAGAC	122 bp	XM_009122789.1

Tab. 3 – Differentially abundant proteins, divided into 8 functional groups

XP_002862885.1	5314	pur ALPHA-1 [Arabidopsis lyrata subsp. lyrata]	2	37.8	6.6	0.84 ± 0.4	2.25 ± 0.4	0.86 ± 0.1	0.73 ± 0.3	2.673	-1.178	-1.030	3.059	UP	DNA-binding protein
7. Protein destination and storage, proteolysis, secretion															
CDX84088.1	1616	BnaC08g07650D [Brassica napus]	6	63.9	5.6	0.8 ± 0.3	0.6 ± 0.4	1.42 ± 0.3	1.27 ± 0.3	-1.141	-1.117	-1.780	-2.010	DOWN	non characterized protein with possible role in intracellular trafficking, secretion, and vesicular transport
AAT52191.1	2407	26S proteasome ATPase subunit [Pisum sativum]	3	51.0	5.8	2.21 ± 0.2	1.5 ± 0.1	0.56 ± 0.1	0.99 ± 0.3	-1.468	1.760	3.923	1.518	UP	protein catabolic process
CAA40979.1	5203	cruciferin cru2/3 subunit [Brassica napus]	5	18.8	6.6	0.35 ± 0.1	0.78 ± 0.4	0.55 ± 0.2	2.19 ± 0.6	2.213	3.947	-1.571	-2.803	DOWN	nutrient reservoir activity
XP_013585177.1	5212	PREDICTED: uncharacterized protein LOC106294119 [Brassica oleracea var. oleracea]	5			0.4 ± 0.3	0.89 ± 0.1	0.35 ± 0.2	2.48 ± 0.4	2.217	6.955	1.136	-2.761	DOWN	Protein of unknown function with possible role as a putative lipoprotein
NP_001190035.1	6710	Clp ATPase [Arabidopsis thaliana]	9	77.2	6.9	1 ± 0.3	1.36 ± 0.4	1.01 ± 0.3	0.66 ± 0.3	1.357	-1.514	-1.009	2.036	UP	protein metabolic process
8. Cell structure															
AAQ81585.1	8505	putative tubulin alpha-2/alpha-4 chain [Brassica napus]	4	52.6	7.4	1.78 ± 0.3	0.99 ± 0.4	3.53 ± 0.4	0.95 ± 0.6	-1.794	-3.702	-1.979	1.043		microtubule-based process

Curriculum vitae

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1996 – 1999 **High Industrial School** in Prostějov, field of study: Machine-building and technical administration

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Bachelor Thesis, 2006: The agriculture in the semi-arid regions in Sudan in consideration of Sorghum (*Sorghum sp.*). Grade: A (excellent)

Master Thesis, 2009: The improvement of in vitro microspore technique of oilseed rape (*Brassica napus L.*): role of polyethylene glycol (PEG) and amiprofos methyl (APM) on embryogenesis and doubled haploid production. Grade: A (excellent)

Hobbies:

Nature, science, music, singing, travelling, squash, cycling, cross-country skiing, etc. Since 2003 I've been also a teacher of English, firstly at the private language school, since 2005 as a private teacher. I am also the steward of municipal archival materials in my home village Smržice.

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Topic (written in Czech language): The influence of abiotic stress on the germination of selected oilseed rapes (*Brassica napus* L.). The BSc. thesis supervisor: RNDr. Olga Vránová, Ph.D. Thesis author: BSc. Eva Jurníčková. Palacký University Olomouc. Thesis defended: 27. May 2016.

In Summary:

8 already published peer-reviewed articles:

- 2 articles as first author (2013, 2016)
- 6 articles as a co-author

1 first-author original article in preparation (2016)

6 chapters in books (in 2 chapters as a first-author, one chapter in English)

9 articles in *Úroda* (Jrec) and 6 full articles in other proceeding materials and books

Publications (or data) partially or fully used ONLY for Ph.D. thesis and defence

Scientific articles (peer-reviewed with IF > 1):

Kosová, K., Vítámvás, P., Urban, M. O., Klíma, M., Roy, A., Prášil, I. Biological Networks Underlying Abiotic Stress Tolerance in Temperate Crops—A Proteomic Perspective. *International Journal of Molecular Sciences*, 2015, 16 (9): 20913 – 20942

Urban, M. O., Klíma, M., Vítámvás, P., Vašek, J., Kučera, V. Significant relationship between frost tolerance and net photosynthetic rate, water use efficiency and dehydrin accumulation in cold-treated winter oilseed rapes. *Journal of Plant Physiology*, 10.1016/j.jplph.2013.07.012, 2013, 170 (18): 1600 - 1608.

Urban, M. O., Vašek, J., Klíma, M., Krtková, J., Kosová, K., Prášil, I. T. and Vítámvás, P. Proteomic and physiological approach reveals drought-induced changes in rapeseeds: Water-saver and water-spender strategy. *Journal of Proteomics*, 2017, 152, pp. 188-205, DOI: 10.1016/j.jpro.2016.11.004.

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Chapters in Books (in Czech language):

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