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Drought stress response in winter wheat varieties – changes in leaf proteins and proteolytic activities

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Abstract – Radiation mutagenesis has been used in sustainable agriculture as a tool for increasing plant variability and providing new lines for selection. This necessitates a comparison, by using suitable stress markers, of the newly created lines with some well-established varieties, which are stress tolerant or susceptible. Drought is one of the most frequently encountered stresses with deleterious effects on plant performance and crop yield. Winter wheat seedlings (soil cultures at 3–4th leaf stage) from one mutant line (M181/1338K), one drought-tolerant (Guinness) and one sensitive variety (Farmer) were subjected to severe drought stress by water withholding, followed by recovery. Changes in leaf protein profiles, the amount of Rubisco large subunit (RLS), some specific chloroplast proteins such as Rubisco binding protein (RPB), Rubisco activase (RA), the chaperone subunit clpA/C of clp protease, as well as the activities of exo- and endo-peptidases were analyzed. At the protein level, some differences were found in the drought response of genotypes – stability of RLS and RBP in M181/1338K and Guinness, diminution of RLS and increase in RBP in Farmer. RA presented strong up-regulation at recovery in Guinness but decreased in content under drought in M181/1338K and Farmer. Increase in ClpA/C level was found in all compared varieties under stress. Strong increase in total proteolytic activity was detected under drought only in Farmer. Inhibitory analysis revealed a predominance of cysteine and serine protease types. Aminopeptidase activities remained higher at recovery in M181/1338K and Farmer. Results are discussed in terms of genotype-linked different stress coping strategies.

Keywords: chaperones, drought, proteolysis, recovery, Rubisco, wheat

Introduction

Cereals (wheat, rice, maize) are crops providing staple food for over 4.5 billion people and drought is a major abiotic stress that affects 1/3 to 2/3 of wheat production worldwide, decreasing wheat grain yield by between 17% and 70% (Ahmad et al. 2018). Selection for wheat varieties that maintain relatively stable yield under adverse environmental conditions is of prime importance for sustainable agriculture. Studies on genotypes with contrasting drought tolerance are useful to reveal the important features needed for survival and yield maintenance under water constraint (Bhargava and Sawant 2013). Another major consideration is the restricted variability in crops used in current farming practices. Induced mutagenesis has been used for long time as a tool for increasing plant variability (Ahloowalia and Maluszynski 2001). The most important criterion for drought tolerance is the crop yield, which generally depends on plant performance, biomass production and nutrient reallocation in developing grains (Zhang et al. 2018). Changes at the protein level are at the basis of phenotypic plasticity and adaptation to various stresses including drought (Zang and Komatsu 2007, Demirevska et al. 2008, Kidrič et al. 2014a, Hasanuzzaman et al. 2018). Of particular interest is the detection of molecular markers able to distinguish between drought-tolerant and susceptible genotypes, if possible at an early developmental stage.

Growth and photosynthesis are among the primary processes affected by water constraint. Under moderate water stress, stomatal closure is the main factor limiting photosynthetic activity, while under severe drought metabolic impairment also takes place (Deng et al. 2018). Inactiva-

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tion of the key photosynthetic enzyme in C3 plants – ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (EC 4.1.1.39) strongly contributes to the non-stomatal limitation of photosynthesis under drought (Medrano et al. 2008, Perdomo et al. 2017). Rubisco accounts for about 30–60% of the total soluble protein in plants. This abundant protein constitutes a large pool of stored leaf amino nitrogen, which can be readily remobilized under stress and in senescence (Makino et al. 1984, Feller et al. 2008). Under conditions of limited photosynthesis at prolonged/severe water stress, the reuse of carbon and nitrogen stored in Rubisco could serve for maintaining metabolism, providing resources for stress defense and for successful grain filling. Rubisco degradation could be both intra- and extra-plastidial, mediated mainly by proteases located in vacuoles. Transportation of Rubisco-containing particles from chloroplasts to vacuoles has been documented (Buet et al. 2019).

Molecular chaperones are helper proteins that assist proper protein folding and assembly into complexes, keeping or restoring the native conformation of proteins after misfolding, damage or aggregation, or targeting misfolded/damaged proteins for degradation (Vaseva et al. 2012). Chloroplasts contain important proteins with a chaperone nature, which help in maintaining the amount and activity of Rubisco, such as Rubisco-binding protein (RBP, or chaperonin 60 – Cpn60) and Rubisco activase (RA). Rubisco, RBP and RA could associate to each other by protein–protein interactions (Demirevska-Kepova et al. 1999). RBP guides the ATP dependent process of the Rubisco holoenzyme assembly by encapsulating Rubisco large subunits (RLS). Besides, it could keep and protect a sequestered pool of RLS under unfavorable conditions. It has been established that under heat shock the folding capacity of chaperonins is suppressed while their binding affinity towards unfolded proteins is increased (Llorca et al. 1998). It has been recently revealed that chloroplast eukaryote genomes encode multiple Cpn60 genes, which can be divided into α and β subtypes, and chaperonins exist as hetero-oligomers containing both subtypes, which could accommodate different specific substrates (Zhao and Liu 2018). Besides RLS, which remains the most important substrate for RBP, other chloroplast proteins such as NDH subunit NdhH and ATPase synthase γ subunits are also among the client proteins of RBP (Zhao and Liu 2018). The Cpn60β subtype of *Arabidopsis* can associate with RA in a high molecular mass complex during heat stress, and thus RBP can have a role in preventing RA from thermal denaturation (Zhao and Liu 2018). It seems that RBP is particularly important for the stability of several chloroplast proteins. The activity of Rubisco is regulated by RA, the function of which is to remove the tightly bound inhibitory sugar phosphates from inactive Rubisco (Salvucci et al. 1985, Bhat et al. 2017). RA belongs to the ATPase family associated with various cellular activities (AAA+ proteins), a class of chaperone-like proteins catalyzing the assembly, operation and disassembly of protein complexes of other macromolecules (Sánchez de Jiménez et al. 1995, Neuwald et al. 1999).

Generally, the amount of Rubisco is correlated to that of total leaf protein (Nagy et al. 2013) and depends on the species, plant age and the type of stress, for example the amount of Rubisco in wheat seedlings was not affected by drought or high temperature, but it decreased in rice and maize under water deficit (Perdomo et al. 2017). RBP content is correlated positively with the amount of Rubisco under normal conditions (Hemmingsen 1990). Rubisco and RBP are among drought responsive proteins in wheat flag leaves (Deng et al. 2018). Comparing wheat varieties with contrasting drought tolerance using a proteomic approach, Cheng et al. (2015) reported an increase in the content of RBP in the tolerant and a decrease in RA content in the sensitive variety. Experiments with Bulgarian wheat varieties at seedling stage (Demirevska et al. 2008) and grain-filling stage (Vassileva et al. 2012) revealed higher content of RBP in the tolerant cultivars under water deficit than in the sensitive ones. Under severe drought at seedling stage, the amount of RBP drastically increased and was positively correlated to the amount of Rubisco (Demirevska et al. 2008). These observations particularly indicate RBP as a potential marker for the selection of stress tolerant varieties, especially at an early developmental stage.

Alterations in the steady state level of individual proteins result from the fine balance between synthesis and degradation. Proteolysis is essential for cells in non-stress conditions as well as under stress (Vaseva et al. 2012). In the stroma of chloroplasts, one of the proteases regulating protein levels by breakdown and recycling is the ATP-dependent caseinolytic (Clp) protease – a complex multi-subunit enzyme analogous to the proteasome. The chaperone subunits of this protease (ClpA-like chaperones) belong to the HSP 100/Clp proteins of the AAA+ chaperone group (Neuwald et al. 1999) with ATP dependent unfoldase activity. Clp protease has mainly essential and constitutive roles in chloroplasts by exerting protein quality control (Zheng et al. 2002). At least 19 potential substrates for clp have been revealed by a proteomic approach, among them enzymes involved in photosynthetic carbon fixation, nitrogen metabolism and chlorophyll/heme biosynthesis, RNA maturation, protein synthesis and maturation, which underlines the vital importance of clp protease for chloroplast function (Stanne et al. 2009). Despite this essential constitutive role of clp protease, some evidence points at stress engagement of the clp system. Nakashima et al. (1997) reported induction by water stress and senescence of a nuclear gene (erdl), encoding the chloroplast-targeted homolog of Clp protease regulatory subunit in *Arabidopsis thaliana* (L.) Heynh. Muthusamy et al. (2016) have found a potential role for Clp in heat, cold, salt and biotic stress responses in wheat.

In view of the multiple pathways for degradation of chloroplastic proteins, the role of extra-plastidial proteolysis seems to be particularly important under unfavorable conditions, especially that of the vacuolar proteases (Roy-Macauley et al. 1992, Martínez et al. 2007). The bulk degradation of unnecessary proteins could fuel the central and secondary metabolism by amino acids and provide building

blocks for new protein synthesis (Demirevska et al. 2008, Kidrič et al. 2014a). Exopeptidases, especially aminopeptidases, which release amino acid residues from the N-terminus of proteins, also contribute to recycling and balance in the amino acid pool. Besides, elimination of a residue from the N-terminus and exposure of different residues can influence protein stability and regulate protein half-life via the N-end rule (Walling 2006). Aminopeptidases (APs) are very active in seeds, growing tissues, and senescing plant parts (Matsui et al. 2006). Evidence was found of up-regulation of Leu-AP in the tomato under osmotic stress, wound stress and hormonal treatment (Chao et al. 1999). A loss of activity phenotype of Arabidopsis Leu-AP2 is reported to be early senescent and stress-sensitive (Waditee-Sirisattha et al. 2011). Up-regulation of APs and general mobilization of the proteolysis under drought has been well documented at activity level (Zagdańska and Wisniewski 1996, 1998) and as protein abundance (Cheng et al. 2015).

This study aimed at analyzing leaf protein profiles, the amount of some chloroplast chaperone proteins and the general proteolysis in a winter wheat mutant line and two wheat varieties differing in drought resistance, in order to better characterize the new mutant line and estimate the usefulness of certain specific protein markers for selection purposes.

Materials and methods

Winter wheat (*Triticum aestivum* L.) seeds were obtained from the selection of the Konstantin Malkov Institute of Plant Genetic Resources, Sadovo, south Bulgaria. Two well established varieties (Guinness and Farmer) and one new and relatively less characterized line (M181/1338K) were used. Plants were grown in pots (9.5 cm diameter, 12 cm deep, 18 plants per pot – 6 plants of each genotype distributed in three sectors) in a mixture of leached meadow cinnamonic soil (400 g, pH 6.2, optimally fertilized with NPK) and sand in a ratio of 3:1. Relative soil humidity 70% of the maximal field capacity was maintained by daily watering. Growth chamber conditions were: day/night temperatures of 25/21 °C, 250 μmol. m^{-2} s^{-1} photosynthetically active radiation and 14/10 h light/dark photoperiod. Drought stress was applied to 20 day-old seedlings with developed second and emerging third leaf by withholding irrigation for 6 days. Recovery from stress was effectuated by restoring optimal irrigation for 4 days after the drought treatment. In parallel, control plants were maintained at optimal irrigation by daily watering to attain up to 70% of the maximal field capacity. Plant material for analyses (2nd leaves) was collected at a fixed hour in the afternoon at the end of the drought treatment period (treated – D, and age controls of drought – CD, from 26 day-old seedlings) and at the end of recovery period (recovered – R, and age controls of recovery – CR, from 30 day-old seedlings). In some cases, a separate control at the beginning of the treatment (C0, 26 day-old seedlings) was also collected. For biochemical analyses, appropriate quantities of leaves were quick frozen in liquid nitrogen and stored at –70 °C.

The aboveground biomass (shoot fresh weight – FW) was determined gravimetrically on seven individual plants. Leaf water deficit (WD) was estimated in triplicate on leaf segments from second leaves, using the formula WD% = (TW – FW) / TW × 100, where FW – fresh weight, TW – weight of the same leaf material at full turgidity (after floating one night at 4 °C in 20 ml distilled water). Relative electrolyte leakage (EL%) from the same leaves was determined by conductivity measuring of the electrolytes leaked in the water at full turgidity of leaves (initial conductivity). Conductivity of the same fluid was measured after boiling leaves in it for 10 minutes and cooling down (total conductivity, all electrolytes have been released from leaves). Measurements for EL% were in triplicate, calculating the ratio of initial to total conductivity. Leaf dry weight (DW) was measured after drying plant material for 8 h at 105 °C to constant weight.

For SDS-PAG electrophoresis and immunoblotting, leaf material (250–500 mg, 2nd leaf) was ground to a fine powder in liquid nitrogen. Proteins were extracted in 2 mL ice-cold 100 mM Tris–HCl buffer (pH 7.6) containing 10 mM $MgCl_2$, 2 mM EDTA, 2 mM phenylmethane-sulfonyl fluoride, 0.005% Triton-X-100 (v/v), 20 mM β-mercapthoethanol, and 50 mg Polyclar AT, and centrifuged at 15000 g for 30 min at 4 °C (Sigma 2-16K). The content of total soluble protein was determined by the method of Bradford (1976) at 595 nm using bovine serum albumin as a standard. Proteins were separated by SDS-PAGE in 12% resolving and 5% stacking gel, according to Laemmli (1970), using a Mini Protean II Dual Slab Cell (Bio-Rad), with equal protein quantity loading on the starts (30 μg per sample). Broad range SDS protein MW standards (6.5–200 kDa, Bio-rad) were used to estimate protein relative mobility. Separated proteins were stained with Coomassie colloidal or were transferred into nitrocellulose membrane (Bio-Rad) as described by Mitsuhashi and Feller (1992) using mini Trans Blot system (Bio-Rad). Transfer conditions were: 1 h at 100 V / 0.19–0.20 A. Transfer effectiveness was estimated using pre-stained MW markers (26.6–180 kDa, Sigma). Nitrocellulose membranes were blocked in TBS buffer (0.1 M Tris, pH 7.6, 0.15 M NaCl) containing 3% nonfat dry milk for 60 min at room temperature. RLS, RA, RBP and ClpA/C were identified with antibodies against the corresponding proteins as previously described (Demirevska et al. 2008) in TTBS buffer (TBS with 0.05% Tween 20) supplemented with 1% nonfat dry milk. Goat–anti-rabbit-IgG (for bridging) and peroxidase–anti-peroxidase soluble complex were used to enhance the sensitivity of the antigen–antibody reaction as previously described (Mitsuhashi and Feller 1992). The peroxidase reaction was developed with 4-chloro-alpha-naphtol (Sigma). Stained bands from electrophoresis and immunoblotting analyses were scanned and processed using ImageJ 1.30v software (National Institutes of Health, NIH, Maryland, USA). After background subtraction, the total area of each peak was taken in arbitrary units. Peak intensities of three separate SDS gels were calculated as percentages of the total area of measured peaks in a line. Then, peak intensity ratios of protein bands in drought-treated or recovered samples to

the area of corresponding bands in the respective controls was calculated. Immunoblotting results (three separate blots for each specific protein) were similarly processed. As one to three bands were revealed per specific protein, in this case peak areas were used instead of percentages.

For proteolytic activities, leaf material (250–500 mg, 2nd leaf) was ground to a fine powder in liquid nitrogen. Proteins were extracted in 2 mL 0.1 M sodium acetate buffer, pH 5.4, containing 0.1% v/v β-mercaptoethanol and 50 mg Polyclar AT. Endopeptidase and aminopeptidase activities were measured as previously described (Simova-Stoilova et al. 2010). Aminopeptidase activity was estimated spectrophotometrically with Leu-p-nitroanilide substrate at pH 7.0. Endopeptidase activity was assayed using azocasein as a substrate at pH 5.0. Inhibitory analysis was performed by pre-incubation for 30 min on ice of mixed samples of all variants (as controls, drought, and recovery) with distinct protease inhibitors (dichloro isocoumarin, benzamidin and soybean trypsin inhibitor for serine proteases, E – 64, N – ethylmaleimide and Na iodacetate for cysteine proteases; pepstatin for aspartate proteases and 1,10 phenantroline – for metalloproteases). Inhibitors were added from stocks in the recommended working concentrations. Results are given in percentage of inhibition compared to respective controls without inhibitors (only solvent added).

Data were statistically analyzed by pairwise comparison of mean values (pair-sample *t*-test embedded in the graphical program Origin Pro 8) within each variety; the basal levels between varieties were also compared pairwise. Significant differences between D and CD, R and CR, CR and CD within each variety are indicated on tables and figures by one, two or three asterisks at the significance levels $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Results

The previously established experimental scheme for soil drought stress at seedling stage was applied, putting all varieties in sectors in the same pot in order to obtain the same stress level (Fig. 1 – drought stressed and control 26 day old plants, just before plant material collection). The increase in water deficit of the second leaves from 4–5% in control to 65–68% in drought-treated plants, and in electrolyte leakage from 3–5% in controls to 30–48% in drought-treated plants (indicative of membrane damage) defined the applied stress as severe (Tab. 1). Diminution in leaf protein content was observed in stressed plants, with a tendency to restoration at recovery; as well as in control plants between 26 and 30 days (Tab. 2). Water withholding was imposed during a period of active shoot growth (increase in the aboveground biomass of control plants by 59% on average between the beginning of stress treatment and the harvest of drought-treated plants, data not shown), while a significant biomass increment was not seen in control plants during the subsequent recovery period (Tab. 2, shoot FW of controls). Drought treated plants resumed growth at recovery, as can be seen in shoot biomass increase (Tab. 2). Along with the restoration in protein content, this observation indicates that the imposed severe stress was recoverable.

Fig. 1. Wheat plants exposed to 6-day-long drought stress (D) and control, appropriately irrigated plants (CD). The compared varieties Guinness, Farmer and line M181/1338K were grown in sectors in the same pot in order to obtain uniform stress conditions.

Tab. 1. Water stress severity in wheat varieties Guinness, Farmer and M181/1338K. WD – relative leaf water deficit, EL – relative electrolyte leakage from leaf membranes. Values are given as means ± standard deviation from 3 replicates. Statistically significant differences between controls and drought treatment within each variety are indicated by two or three asterisks at the significance levels $^{**}P < 0.01$ and $^{***}P < 0.001$, respectively.

Variety/Treatment	WD (%)	EL (%)
Guinness		
Control	4.25± 0.66	3.635±0.2498
Drought	65.497±1.669***	29.973±10.696**
M181/1338K		
Control	3.853±1.189	3.739±0.392
Drought	67.397±4.398***	29.765±10.787**
Farmer		
Control	5.25±1.14	4.84±1.667
Drought	68.693±3.731***	48.28±19.413**

Leaf soluble protein SDS-electrophoretic patterns (Fig. 2, Tab. 3) revealed 17 well separated protein bands (Fig. 2A). A diminution in the dominant Rubisco LS band was clearly seen in the Farmer variety under drought stress, along with its partial cleavage. Prominent stress-related changes were found in some bands in the lower MW range, namely a significant decrease under stress of the band with appar-

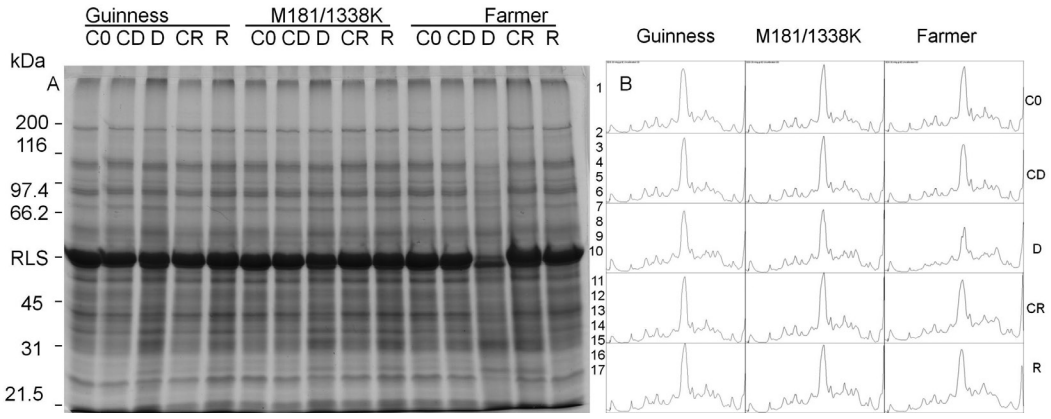

Fig. 2. Leaf protein pattern from Guinness, M181/1338K and Farmer wheat varieties after 12% SDS-PAGE and Coomassie staining. A – Representative SDS-PAGE, B – peak intensity plots of the same separation (ImageJ profiles). Numbers correspond to protein bands quantified in Tab. 3. Proteins were extracted at the beginning of treatment (C0), control of drought (CD), drought (D), recovery (R) and control of recovery (CR). Samples with equal protein quantity of 30 μg were loaded per lane.

Tab. 2. Growth parameters and leaf protein content in controls, drought-stressed and recovered winter wheat seedlings, varieties Guinness, Farmer and line M181/1338K. CD – control for drought, D – drought, CR – control for recovery, R – recovery, FW – fresh weight, DW – dry weight. Values are given as means ± standard deviation from seven replicates for aboveground plant biomass and three replicates for protein content. Statistically significant differences between values of D to CD, R to CR and CR to CD within each variety are indicated by one, two or three asterisks at the significance levels *P < 0.05, **P < 0.01 and ***P < 0.001, respectively.

Variety/Treatment	Shoot FW (g per plant, n=7)	Leaf protein content (mg g^{-1} DW, n=3)
Guinness		
CD	0.343 ± 0.041	212.5 ± 19.7
D	0.090 ± 0.015 ***	125.7 ± 7.1 **
CR	0.364 ± 0.083	146.6 ± 18.1 *
R	0.265 ± 0.082 *	145.5 ± 19.8
M181/1338K		
CD	0.301 ± 0.067	211.7 ± 21.6
D	0.084 ± 0.031 ***	121.0 ± 10.2 **
CR	0.269 ± 0.046	138.4 ± 6.5 *
R	0.216 ± 0.055	144.8 ± 17.8
Farmer		
CD	0.403 ± 0.136	169.4 ± 4.4
D	0.037 ± 0.006 ***	151.4 ± 7.6 *
CR	0.416 ± 0.084	129.6 ± 15.7 **
R	0.192 ± 0.048 ***	119.7 ± 24.8

ent MW 45 kDa in all varieties, as well as an increase in relative content of two bands in the region 36 – 30 kDa and of about 70 kDa. In Farmer, a diminution under drought stress was detected in some band intensities in the range of 158, 88 and 26 kDa. These dynamic protein changes prompted us to look for detection of some individual proteins using available antibodies.

Immunoblotting results on specific detection of Rubisco LS, Rubisco binding protein, Rubisco activase and ClpA/C protease (Fig. 3, Tab. 3) confirmed the stability of Rubisco LS in Guinness and M181/1338K and its diminution by

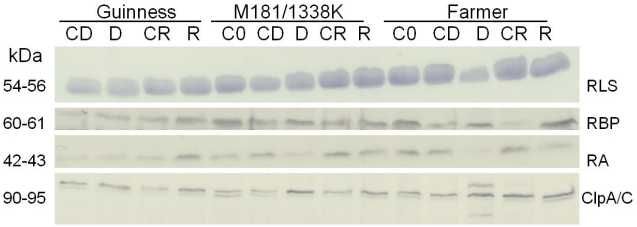

Fig. 3. Immunoblot analysis of wheat leaf extracts from Guinness, M181/1338K and Farmer – at the beginning of treatment (C0), control of drought (CD), drought (D), recovery (R) and control of recovery (CR). Polyclonal antibodies against Rubisco large subunit (RLS), Rubisco binding protein (RBP), Rubisco activase (RA) and the regulatory subunits of caseinolytic protease (ClpA/C) were used for revealing specific proteins. Samples with equal protein quantity are compared. For RLS, RBP and RA the load was 30 μg per lane, for ClpA/C – 40 μg per lane.

54% in Farmer, which was a recoverable change. The chaperonin 60 – RBP, which in controls had the highest basal level in M181/1338K and lowest in Farmer, increased under drought twofold in Farmer and even more on recovery. Thus, in treated plants the highest relative level of RBP was detected in Farmer, intermediate in M181/1338K and lowest in Guinness. A raise in RBP content was detected in Guinness only on recovery. RA basal content was highest in Farmer and lowest in Guinness, with maintenance of the same level under drought and an increase by 35% in Guinness at recovery. In M181/1338K and Farmer, RA diminished under drought, most in the variety Farmer; the level of RA was not completely restored on recovery in Farmer. ClpA/C protein was revealed as two bands, with appearance of a third in Farmer under drought stress. Strongly responsive to stress was the upper band, which remained at an elevated level on recovery.

Strong up-regulation of acid protease activity (Fig. 4A) and Leu-AP activity (Fig. 4B) was observed under drought

in the variety Farmer. In the mutant line M181/1338K and in the sensitive variety, aminopeptidases seem to play some role in recovery from drought. The inhibitory analysis using protease inhibitors specific to distinct protease classes (Tab. 4) pointed at a relative increase in the activity of serine and cysteine proteases under stress, whereas that of aspartate and metalloproteases presented a relative decrease in % of total activity.

Tab. 3. Relative peak volume ratios of SDS-electrophoretic protein bands or immunoblot specific protein bands in wheat varieties Guinness, Farmer and line M181/1338K. D/CD – ratio drought treatment to control of drought; R/RC – ratio recovery to control of recovery. RLS – Rubisco large subunit, RBP – Rubisco binding protein, RA – Rubisco activase, ClpA/C – regulatory subunit of caseinolytic protease. Asterisks denote significant changes in peak volumes comparing treatment to control at *P < 0.05. Significant increase in ratios is emphasized by **bold**, diminution – by *italics*. Band No. corresponds to the indicated ones in Fig. 2A.

Band No/ protein	kDa	Guinness		M181/1338K		Farmer	
		D/CD	R/CR	D/CD	R/CR	D/CD	R/CR
1	236	**1.403***	1.179	**1.411***	1.013	**1.597***	*0.624**
2	158	0.792	1.015	0.895	1.011	*0.665**	1.203
3	142	1.288	1.245	0.998	**1.357***	**1.363***	**2.146***
4	108	0.799	1.181	0.845	0.929	0.722	1.260
5	92	1.023	1.135	1.110	**1.452***	0.974	**1.769***
6	88	0.941	1.131	0.898	1.118	*0.596**	1.149
7	80	1.169	1.261	1.220	**1.340***	1.139	0.775
8	70	**1.335***	**1.386***	**1.401***	1.320	**1.448***	1.082
9	62	1.271	1.054	0.898	1.305	1.023	1.060
10	56	0.871	0.868	0.927	0.882	*0.628**	1.006
11	50	0.922	0.805	1.080	0.876	1.062	0.737
12	45	*0.424**	0.901	*0.491**	0.957	*0.417**	**1.908***
13	40	0.974	0.913	0.865	0.916	1.075	1.212
14	36	1.286	1.117	1.184	0.991	1.181	1.329
15	34	**1.797***	1.175	**1.544***	1.231	**1.721***	*0.628**
16	30	**2.395***	**1.729***	**1.403***	**2.130***	**2.488***	0.889
17	26	0.940	**1.339***	0.669	1.141	*0.369**	**1.806***
RLS	54–56	0.969	1.159	1.305	1.083	*0.465**	1.016
RBP	60–61	1.106	**1.349***	0.965	1.172	**2.002***	**3.626***
RA	42–43	1.287	**1.848***	*0.575**	0.788	*0.253**	*0.507**
ClpA/C	90–95	**1.370***	**1.866***	**2.033***	**1.544***	**2.458***	0.784

Discussion

The previously established experimental scheme for soil drought stress at seedling stage was applied, putting all varieties in sectors in the same pot (Simova-Stoilova et al. 2016) in order to obtain similar stress levels for the three genotypes compared. Our results on diminution in the aboveground plant biomass and leaf protein content support previous large screening studies (Vassileva et al. 2019), where the variety Guinness was revealed as drought tolerant and the variety Farmer as drought sensitive. The variety Guinness is a mutant created from parental variety Katya by gamma irradiation (50 Gy) of the seeds; with high productivity potential and ecological plasticity, but relatively small grain size. The variety Farmer is a mutant created from parental variety Pobeda by gamma irradiation (50 Gy) of the seeds; and also presents high productivity potential, along with a short stem in comparison to the parental variety (Vitanova and Rachovska 2009, Rachovska and Uhr 2010). Parental varieties Katya and Pobeda have been recognized in the last 20 years in Bulgaria as standards for high tolerance to drought and to cold, respectively: these traits seem to be conserved and even enhanced in the new varieties. Guinness has been considered to be relatively more drought- than cold-tolerant while Farmer is more cold- than drought- tolerant, and both of them are resistant to economically important plant pathogens (Vassileva et al. 2019). Very little is known about line M181/1338K, which was obtained from the variety Katya by physical mutagenesis (treatment of seeds with 100 Gy) and has improved yield potential as well as resistance to economically important plant pathogens.

The equal water status changes under applied stress allowed us to compare genotypes in terms of differences in molecular adaptation mechanisms to drought at the protein level, by estimating the content of important chloroplast proteins. The variety Guinness and its sibling, the mutant line M181/1338K had remarkable stability of RLS and RBP under drought in contrast to the variety Farmer, in which the RLS band was strongly diminished in conjunction with sharp up-regulation of RBP. These results are a bit different from the previously observed correlation among RLS and RBP in other varieties (Demirevska et al. 2008) probably due to differences in experimental conditions (younger plants, first leaf instead of second leaf). On the other hand,

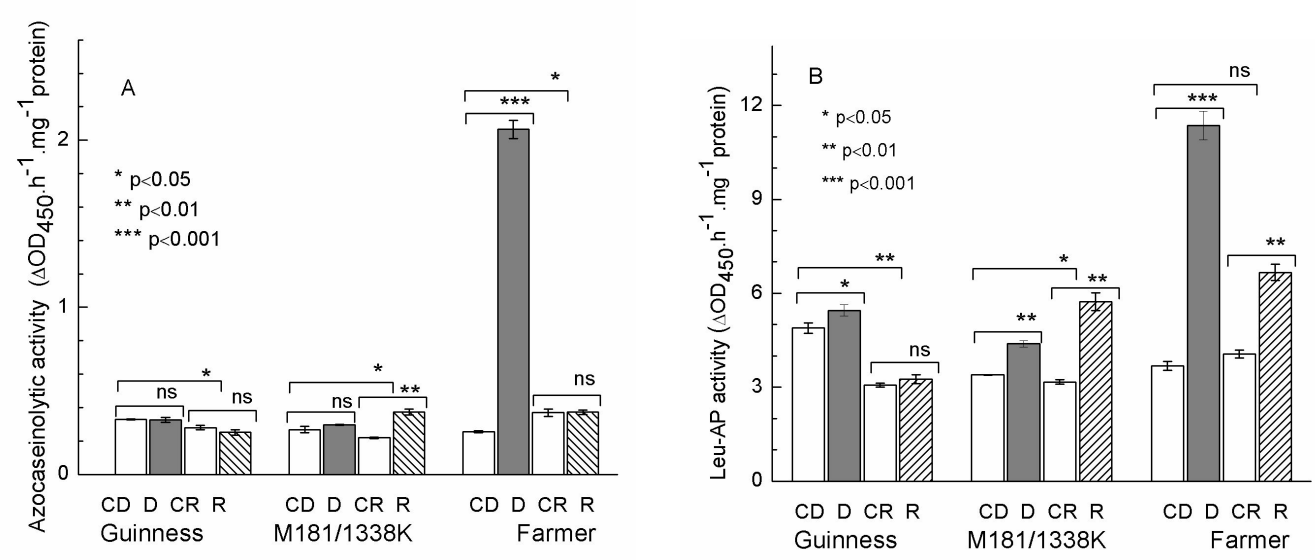

Fig. 4. Total proteolytic activity (A) and aminopeptidase activity (B) in leaf extracts from Guinness, M181/1338K and Farmer. CD – e control of drought, D – drought, CR – control of recovery, R – recovery after drought treatment. Statistically significant differences between values of D to CD, R to CR and CR to CD within each variety are indicated by one, two or three asterisks at the significance levels *P < 0.05, **P < 0.01 and ***P < 0.001, respectively; ns – non-significant differences. Azocasein and leucine-p-nitroanilide were used as substrates for total protease and aminopeptidase activities, respectively.

the increase in RBP could indicate the enhanced misfolding, degradation and loss of function of Rubisco under drought stress (Cheng et al. 2015). The diminution in the dominant Rubisco LS band in Farmer variety under drought stress, along with its partial cleavage, could be due to degradation by proteases as this variety also presented the highest rise in proteolytic activity under the applied drought stress. Thus, a plausible explanation of RLS diminution and RBP increase in Farmer could be the strong induction of general proteolysis, together with the possible function of RBP to rescue RLS and other client proteins under stress conditions (change in the role of RBP from helper to keeper as described by Llorca et al. 1998). The complete restoration of RLS content in recovered Farmer along with further increase in RBP level are indicative of the high plasticity of this variety. RA was unchanged under drought in Guinness, but was diminished in M181/1338K and even more in Farmer. In recovery, RA content increased in Guinness; the same tendency was registered in M181/1338K and in Farmer but without complete restoration. These changes possibly reflect different stress-coping strategies – Guinness maintains active photosynthesis under drought due to the high Rubisco stability/activity whereas Farmer exploits more the storage function of Rubisco as a source of readily reusable amino acids while photosynthesis is inhibited. In this respect line M181/1338K is close to the variety Guinness. Strong increase in proteolytic activity under drought was previously observed in the cold-tolerant parental variety Pobeda while the drought-resistant parental variety Katya presented a very small increase in proteolysis under drought (Simova Stoilova et al. 2010). Interestingly, response of plants to cold stress was linked to increased proteolytic activity, especially of cysteine proteases, and a role of proteases in cold adaptation of wheat has been suggested (Pinedo et al. 2000, Frolova et al. 2011). Probably the observed different stress adaptation strategies reflect the fact that wheat varieties Pobeda/progeny Farmer are more cold- than drought-tolerant whereas varieties Katya/progenies Guinness, M181/1338K are more drought- than cold-tolerant. The role of the tradeoff between the double function of Rubisco as key photosynthetic enzyme and nitrogen store in tolerance to different kinds of stress needs further elucidation.

Tab. 4. Analysis of proteolytic activity in mixed extracts from wheat varieties Guinness, Farmer and line M181/1338K. Results are expressed as percentage of inhibition of azocaseinolytic activity at pH 5 (samples in duplicate) after pre-incubation of mixed samples (from all 3 varieties) with the respective protease inhibitors for 1 h at 4 °C. CD – control of drought, D – drought, R – recovery, RC – control of recovery.

Type of protease	Protease inhibitor	Working concentration	CD	D	CR	R
Serine-	benzamidine	1 mM	8.04	11.17	14.41	19.49
	Soybean Trypsin inhibitor	50 μM	17.86	31.38	43.22	37.29
	E 64	10 μM	45.34	71.87	48.95	47.01
Cysteine-	N-ethylmaleimide	10 mM	44.64	53.99	60.17	46.66
	Na iodacetate	5 mM	43.75	76.60	59.39	55.08
Aspartate-	Pepstatin A	20 μM	24.62	12.75	21.15	30.6
Metallo-	1,10phenantroline	10 mM	27.69	13.41	30.13	27.60

A response common to all studied varieties was the increase in Clp A/C chaperone (HSP100 family) subunit of the chloroplast clp system under severe drought. Greatest increase was detected in Farmer, along with the appearance of an extra band with lower molecular mass. The clp system has an essential role in protein quality control in chloroplasts (Zheng et al. 2002, Stanne et al. 2009) under both normal and stressful conditions. Abiotic stresses with a dehydration component (drought, salt, and freezing) could be damaging to proteins, as diminution in cellular volume, macromolecular crowding and oxidative injury can increase the number of denatured, aggregated or oxidatively-damaged, inactive proteins (Hoekstra et al. 2001, Muthusamy et al. 2016). It could be speculated that at greater stress-induced damage of proteins, the greater will be the involvement of the protein quality control system. In this respect the highest increase in Clp A/C chaperone was found in Farmer, lowest – in Guinness; M181/1338K clp response was again close to the variety Guinness.

Recovery from drought stress is an active process of restoration of cellular function, repair of damaged structures, and return to normal metabolism and as such it is very important for stress adaptation. Recovery processes in Guinness were linked to elevated content of chaperones such as RBP, RA and clpA/C, whereas in M181/1338K only clpA/C was maintained at a significantly higher level than in the recovery control. At recovery, the RA content in Farmer still remained half of the controls, and RBP level was highly elevated (most probably linked to restoration of Rubisco quantity). Unlike Guinness, M181/1338K presented elevated total protease and aminopeptidase activities; increased aminopeptidase activity was also registered at recovery in Farmer. Our previous studies on drought stress response in wheat varieties at seedling stage revealed a general increase in Leu-AP activity under stress but without significant differences among varieties (Simova-Stoilova et al. 2010). Another study of AP activities with various substrates pointed towards higher AP activity in the tolerant wheat variety Yantar on recovery, which highlights the importance of amino acid metabolism in the recovery from stress (Simova-Stoilova et al. 2016). It seems that aminopeptidases play some role in recovery from drought in certain wheat varieties, without a clear link with drought tolerance. Drought responsiveness of various aminopeptidases was found in leaves of *Phaseolus vulgaris* L. (Budič et al. 2016) and in a desiccated resurrection plant *Ramonda serbica* Pančić (Kidrič et al. 2014b).

This study reveals the important role of certain chloroplast chaperone proteins in drought stress response and different strategies of stress adaptation depending on the wheat genotype. It proves the usefulness of induced mutagenesis in developing new genotypes, although the use of specific chloroplast chaperone proteins as markers for selection purposes needs further elucidation. Controversial results have been reported so far about changes in Rubisco, RBP and RA content under drought stress. Rubisco steady state level is mediated by the action of the Rubisco interacting proteins and proteases. Flag leaves of drought-tolerant wheat varieties are reported to contain higher amounts of Rubisco, whereas lower Rubisco content and accelerated senescence are registered in sensitive varieties (Nagy et al. 2013) as a tradeoff between the double function of Rubisco as key photosynthetic enzyme and nitrogen store. In alfalfa leaves, drought induced decreases in RBP and RA content along with up-regulation of proteases that could degrade Rubisco (Aranjuelo et al. 2011). RA was significantly up-regulated in both tolerant and sensitive wheat genotypes upon exposure to drought stress at seedling stage (Faghani et al. 2015). This controversy could probably be solved if results are interpreted in terms of different stress adaptation strategies instead of changes in individual proteins.

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