Plant Growth Regul (2008) 56:97–106
DOI 10.1007/s10725-008-9288-1

ORIGINAL PAPER

Rubisco and some chaperone protein responses to water stress and rewatering at early seedling growth of drought sensitive and tolerant wheat varieties

Klimentina Demirevska · Lyudmila Simova-Stoilova · Valya Vassileva · Urs Feller

Received: 20 November 2007 / Accepted: 9 May 2008 / Published online: 31 May 2008

Abstract Four wheat varieties differing in their drought tolerance were subjected to severe but recoverable water stress at seedling stage. Growth parameters, leaf water deficit (WD) and electrolyte leakage (EL) were used to evaluate the stress intensity and the extent of recovery. The physiological response of the varieties was quite similar under severe drought. Leaf protein patterns and levels of some individual proteins relevant to ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) maintenance were studied in control, stressed and recovering plants by electrophoresis and immunoblotting. The bands representing Rubisco large subunit (RLS), N- and C-terminus of RLS, Rubisco activase (RA) and Rubisco binding protein (RBP, cpn 60), as well as the chaperone and proteolytic subunits of the Clp protease complex were identified using polyclonal antibodies. Under drought conditions RLS, Clp proteases and especially RBP were enhanced, whereas the RA band was only slightly affected. The drought tolerant varieties had higher RBP content in the controls and drought treated plants. Its concentration could be a potential marker for drought tolerance.

K. Demirevska (✉) · L. Simova-Stoilova · V. Vassileva
Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bdg. 21, Sofia 1113, Bulgaria
e-mail: klimdemi@yahoo.com

U. Feller
Institute of Plant Sciences, University of Bern, 3013 Bern, Switzerland

Keywords Chaperones · Early seedling growth · Drought stress and recovery · *Triticum aestivum* L. · Rubisco

Abbreviations

Clp	ATP dependent calpain protease
Cpn	Chaperone
EL	Electrolyte leakage
FW	Fresh weight
MW	Molecular weight
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RA	Rubisco activase
RBP	Rubisco binding protein
RLS	Rubisco large subunit
RLS-C	C-terminus of RLS
RLS-N	N-terminus of RLS
RSS	Rubisco small subunit
RT	Room temperature
RuBP	Ribulose-1,5-bisphosphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
WD	Water deficit

Introduction

Many regions of the Earth are often or permanently exposed to drought, which is one of the most widespread environmental stresses (Bray 1997).

Drought causes physiological and metabolic changes in wheat plants resulting in growth inhibition, stomata closure with consecutive reduction of transpiration, decrease in chlorophyll content, inhibition of photosynthesis, changes in protein biosynthesis, their maintenance or degradation (Lawlor and Cornic 2002). According to Riccardi et al. (2004) plant response to water deficit (WD) shows some genetic variations. Drought tolerance is achieved by modulation of gene expression and accumulation of specific protective proteins and metabolites (Reddy et al. 2004; Zang and Komatsu 2007).

The key photosynthetic enzyme in C_3 plants is ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (EC 4.1.1.39), localized in the soluble fraction of chloroplasts. Rubisco inactivation contributes to the non-stomatal limitation of photosynthesis under drought stress (Lawlor 2002; Lawlor and Cornic 2002; Reddy et al. 2004). However, the amount of Rubisco protein is little affected by moderate and even prolonged severe drought (Medrano et al. 1997). Data exist also about a reduction in Rubisco amount in stressed plants (Majumdar et al. 1991; Parry et al. 2002) which may be related to a general stimulation of senescence and/or to oxidatively damaged Rubisco. In plants Rubisco accounts for about 30–60% of the total soluble protein. Thus, Rubisco constitutes a large pool of stored leaf nitrogen (20–30%) that can be quickly remobilized under stress and senescence (Makino et al. 1984; Feller et al. 2008).

The Rubisco holoenzyme assembly in chloroplast stroma is an ATP-dependent process that requires the presence of another protein with chaperone function—Rubisco binding protein (RBP) or cpn 60 (Musgrove et al. 1987). According to Hemmingsen (1990) the cpn 60 content is coordinated positively with that of Rubisco under normal conditions. Very limited data are available to date concerning the response of cpn 60 to stress conditions. In a susceptible variety of *Sorghum bicolor* L. markedly higher concentration of the chloroplastic cpn 60 were observed under high light and high temperature (Jagtap et al. 1998). Accumulation of cpn 60 opposite to RLS was noticed in *Nicotiana tabacum* seedlings under NaCl and cold stress but not under heat and UV-B stress (Holland et al. 1998). To our knowledge, there are no data on the response of RBP to drought.

The activity of Rubisco is regulated by the chloroplast protein Rubisco activase (RA) that possesses ATPase activity (Salvucci et al. 1985). The function of RA is to remove tightly bound sugar phosphates from the active centers of Rubisco. It is considered that RA protein is not a conventional enzyme and belongs to the ATPase family associated with various cellular activities (AAA+ proteins), a class of chaperone-like proteins acting on other macromolecules and catalyzing mechanical processes, such as assembly, operation and disassembly of protein complexes (Sanchez de Jimenes et al. 1995; Portis 2003; Neuwald et al. 2006). Parry et al. (2002) reported decreased RA and lower activation state of Rubisco at low relative water content. The increase in abundance of RA was observed by Salekdeh et al. (2002) in rice under drought. Haupt-Herting et al. (2001) explained the increase abundance of RA that could function to reactivate Rubisco in carboxylase reaction or for protective energy dissipation by photorespiratory oxygenase reaction. Another possibility is to protect chloroplast protein synthesis from drought stress as a chaperone (Rokka et al. 2001). Chaperons including RBP and RA are supposed to interact transiently with other proteins, promoting different processes. These proteins might associate to each other by protein–protein interactions facilitating directly Rubisco assembly and activation (Demirevska-Kepova et al. 1999).

The level of proteins, their breakdown and recycling depend on another important protein group—proteases. Different classes of proteases are involved in the response to drought, especially those located in plant vacuoles (Roy-Macauley et al. 1992; Martinez et al. 2007). A special protease in the chloroplast stroma is the ATP-dependent Clp protease—a complex serine type multi-subunit enzyme with catalytic subunits (Clp P) and ATPase chaperone subunits belonging to ATP-dependent HSP 100/Clp proteins of the AAA+ chaperone group (Neuwald et al. 2006; Sakamoto 2006). ClpA-like chaperones possess ATP-dependant unfoldase activity and assist the processive degradation of proteins by Clp protease complex. It is generally accepted that the role of this protease is essential and constitutive (Zheng et al. 2002).

Rubisco still remains an enzyme with a very complex and poorly elucidated regulation of its activity and quantity (Houtz and Portis 2003). During the past few years the investigations concerning Rubisco and its changes under different stress conditions were reconsidered with a special emphasis on the important

role of RA and RBP (Portis 2003; Haupt-Herting et al. 2001; Rokka et al. 2001; Demirevska-Kepova et al. 2005). Data about the response of RBP as chaperone and the common response of Rubisco, RA and RBP under stress conditions are quite limited. Very little is known about the functioning of Clp proteolytic system and its physiological role in higher plants under stress conditions.

This work is focused on the possible integrative response of Rubisco as a key photosynthetic enzyme, RA—as a Rubisco actvity regulator, RBP and Clp proteolytic system—as possible factors determining Rubisco content in leaves under drought stress and during recovery. Wheat (*Triticum aestivum* L.) was chosen because it is a widely cultivated crop plant exhibiting high sensitivity to water deprivation. Additional point in this study was to compare wheat varieties with different drought tolerance. The comparison was made at the seedling stage under severe but recoverable drought stress in order to evaluate the possibility of using these proteins as potential early markers for evaluation of variety differences in drought tolerance.

Materials and methods

Plant material and growth conditions

The experiments were carried out in a growth chamber with four winter wheat varieties. Katya and Sadovo are well established and cultivated varieties from South Bulgaria. Zlatitza and Miziya are new varieties from North Bulgaria selection. All varieties are high yielding under optimal water supply. Variety Katya was considered as drought tolerant (Kalapos et al. 1996). Variety Sadovo was less tolerant to drought than Katya (Simova-Stoilova et al. 2006). Under field conditions the varieties Zlatitza and Miziya showed a drought sensitivity index according to Fisher and Maurer (1978) of 0.657 and 1.282, respectively (unpublished data). Thus, Zlatitza is a drought tolerant and Miziya is a drought sensitive variety.

Plants were grown in pots (9.5 cm diameter, 12 cm deep) containing 400 g leached meadow cinnamonic soil (pH 6.2) at day/night temperatures of 25/21°C, 150 μmol m^{-2} s^{-1} photosynthetically active radiation and 14-h photoperiod. In order to avoid nutrient limitation, before planting the soil was fertilized to reach content of mobile nitrogen—3 mg/100 g, P_2O_5—18 mg/100 g and K_2O—25 mg/100 g dry soil (optimum for cultivation of most crops in this soil type). Soil moisture was controlled daily by gravimetric measurements of the pots. The missing water was added to maintain relative soil humidity 70% of the maximal field capacity (FC). The maximum FC was 47% for this type of soil (saturation point). Thus irrigation maintained 70% FC (32.9% soil moisture content). Until 7th day of growth all plants received optimal water supply. Drought treatment was imposed on 8-day-old plants (fully developed first leaf and expanding second one) for a period of 7 days followed by 3 days period of recovery by optimal watering. At day 7 of drought the soil moisture content was 18.9% (57% FC). The control plants were watered daily during the whole period. Leaf samples were taken from the control and stressed plants after 7-day-drought (14-day-old plants) and from age control and recovery plants (17-day-old plants). All analyses were performed on the fully developed first leaf. The leaf material (0.5 g fresh weight per variety and treatment harvested from 2 to 3 pots for controls and recovered and from 7 to 9 pots for stressed plants) was frozen in liquid nitrogen.

Growth parameters, water deficit and electrolyte leakage

As a standard growth parameter the shoot fresh weight (FW) was determined. WD of the first leaf was calculated in percentages according the formula (TW − FW)/TW, where TW is the leaf weight at full turgidity and FW is the leaf FW. Membrane integrity was evaluated by the relative electrolyte leakage (EL) from 2 cm leaf segments floating on distilled water for 24 h at 4°C according to Nunes and Smith (2003). Briefly the initial conductivity of the effusate and the conductivity after boiling the segments for 10 min in the same solution and cooling were measured using a conductivity meter (final conductivity). The relative EL was calculated as a percent of the initial to the final conductivity.

SDS-PAGE, immunoblot analysis and protein quantification

The soluble leaf proteins were extracted from 0.5 g leaf material in ice-cold 100 mM Tris–HCl buffer (pH 8.0),

containing 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM EDTA (disodium salt), 2 mM phenylmethanesulfonyl fluoride, 12.5% glycerol (v/v), 20 mM β-mercaptho-ethanol and 2% (w/v) Polyclar. After centrifugation (15 min, 15,000*g*, 4°C), the supernatant was boiled in sample buffer for SDS-PAGE. The proteins were separated by 12% SDS-PAGE with a *Mini Protean II Dual Slab Cell* (Bio-Rad) according to Laemmli. Samples with protein quantity equivalent to the same FW were loaded for all variants. Protein markers (14–66 kDa, Sigma) were used. The gels were stained with Coomassie brilliant blue R-250 or transferred into nitrocellulose membrane (Bio-Rad) as described by Mitsuhashi and Feller (1992) using Trans Blot system (Bio-Rad). Prestained SDS MW Standards (31.2–174.6 kDa, Sigma) were used to control the effectiveness of the transfer. The membrane was blocked in TTBS buffer (0.1 M Tris, pH 7.9, 0.15 M NaCl, 0.1% Tween 20) containing 1% Ovalbumin for 60 min. RLS, RA and RBP were identified using antibodies against the corresponding proteins (Demirevska-Kepova and Simova 1989; Demirevska-Kepova and Juperlieva-Mateeva 1990). Polyclonal antibodies against synthetic oligopeptides representing amino acid sequences near the N-terminus or near the C-terminus of RLS were prepared as reported previously (Parrott et al. 2007). Antibodies against ClpP and ClpA were raised in rabbits against a polypeptide representing an amino acid sequence near the C-terminus of ClpP from wheat (RTGKPFWVVSEDMERDVFMS) and against a polypeptide representing an amino acid sequence starting at position 535 in the ClpA homologue from *Brassica napus* (EKVSTDESDLLLKMEETLHK). Goat–anti-rabbit-IgG (for bridging) and peroxidase–anti-peroxidase soluble complex were used to enhance the sensitivity of the antigen–antibody reaction as described earlier (Mitsuhashi and Feller 1992). The peroxidase reaction was developed with 4-chloro-alpha-naphtol (Sigma).

The content of total soluble proteins was measured by the method of Bradford (1976) at 595 nm with bovine serum albumin as a standard.

Data processing and statistical analysis

The results presented in Fig. 2 are based on nine replicates of FW and three replicates for WD, EL and protein concentration. Data from representative of three independent experiments are given. They are analyzed using a multifactor ANOVA analysis (StatGraphicsPlus Version 2.1) at least significant difference $P < 0.05$. Different letters above columns indicate significant differences whereas the same letters show no significant difference.

Electrophoresis and immunoblotting analyses were repeated three times and the obtained results were similar. A representative picture of the results is given. The stained bands were scanned and processed using ImageJ 1.30v software (National Institutes of Health, NIH, Maryland, USA). Data are expressed as the total area occupied by each pick. The Induction Factor as percent area of protein bands in stressed/recovered gels to the percent area of corresponding bands in controls was calculated. The staining methods after electrophoretic and immunoblot analysis are not strictly quantitative because of background and other effects.

Results

Physiological responses to water stress and recovery

The extent of the water stress was determined by analysing changes in growth parameters of the plants, the WD and EL (Fig. 1). The growth of the second leaf was inhibited in drought treated plants in all 14-day-old varieties and they did not develop third leaf. In recovered 17-day-old plants the growth was resumed, the 3 days leaf appeared. The age controls of 17-day-old recovered plants were more developed than the controls of 14-day-old plants. As a result of the drought treatment, shoot FW significantly diminished (Fig. 1a). Growth response to the drought treatment was the same comparing varieties. A tendency of higher shoot FW was seen for Sadovo 17-day-old controls compared to other three varieties. The leaf WD remained unchanged during the first 4 days of water deprivation, then sharply increased to reach 55–60% at day 7 (severe water stress). Drought tolerant varieties did not present significant differences in WD at day 7 drought compared to sensitive ones (Fig. 1b). After rewatering the plants restored completely their water status. At the 7th day of drought treatment, the EL sharply increased (2–3 times compared to the controls) (Fig. 1c). The membrane injury seems to be reparable because after

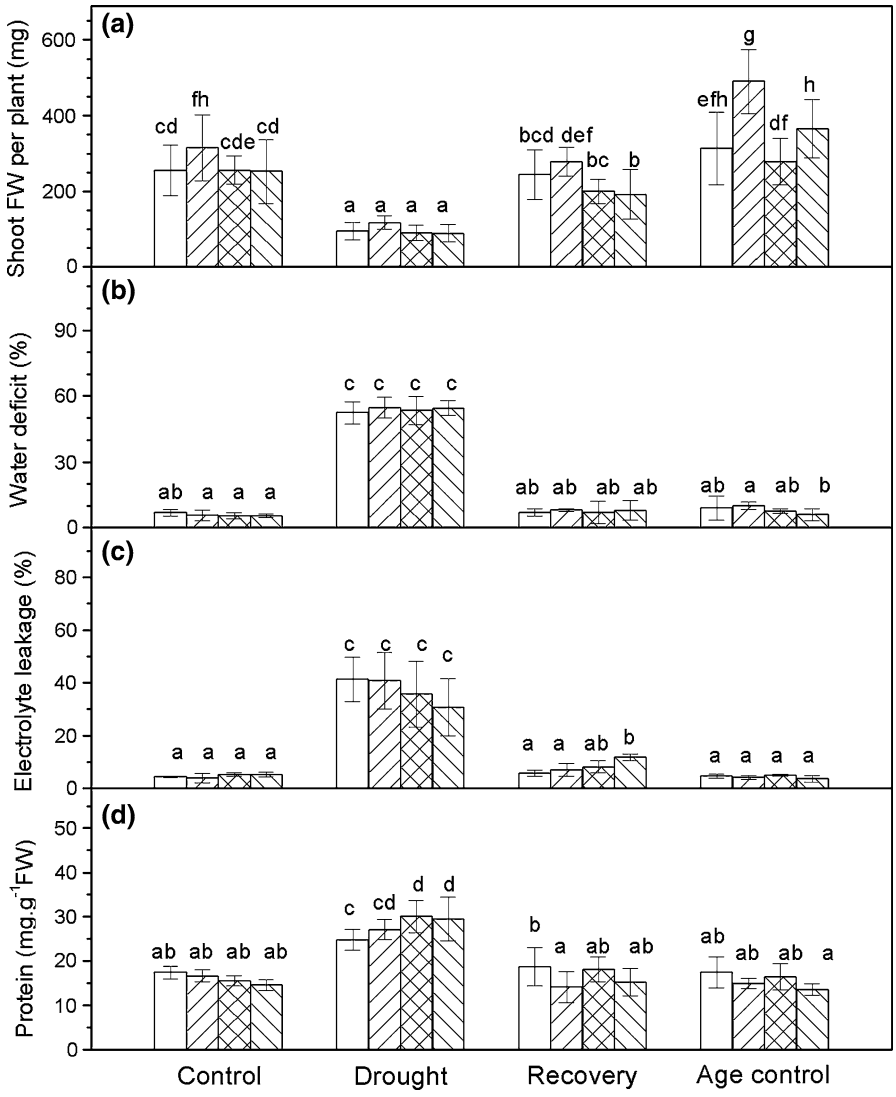

Fig. 1 Shoot fresh weight (**a**), leaf water deficit (**b**), electrolyte leakage (**c**) and leaf soluble protein (**d**) in of 14-day-old control plants and drought-treated plants for 7 days, in 17-day-old recovered plants and age control for recovery. Varieties: Katya—white columns; Sadovo—columns striped to the right; Zlatitza—cross columns; Miziya—columns striped to the left. Means and standard deviations were given. Significant differences ($P < 0.05$) between variants are indicated by different letters

recovery the EL was restored to the values of the control plants. Comparing the varieties studied, the WD and EL showed quite similar rates under dehydration. The 17-day-old controls had similar WD and EL as 14-day-old controls. In drought treated leaves the quantity of soluble proteins in the leaf extracts on a FW basis approximately doubled compared to the control and recovered plants due to leaf dehydration (Fig. 1d). Comparing the protein concentration in both controls no senescence effect concerning the diminution of protein content was observed. From the all results presented in Fig. 1 we could not separate the physiological response of drought tolerant from drought sensitive varieties at early seedling stage.

Protein responses to water stress and recovery

SDS-PAGE analyses on a 12.5% gel of the four wheat varieties studied were performed after dehydration and recovery and compared to the respective controls (Fig. 2). The protein pattern was rather conserved for all variants however some differences were detected in the area of high MW proteins as well as of low MW proteins in drought stressed plants. The RLS was revealed as a predominant band of 55–56 kDa. Under drought conditions the RLS showed an increase (on a FW basis) of the band intensity compared to the controls for all varieties.

Immunoblot analysis showed more changes in the abundance of some individual proteins in wheat leaves

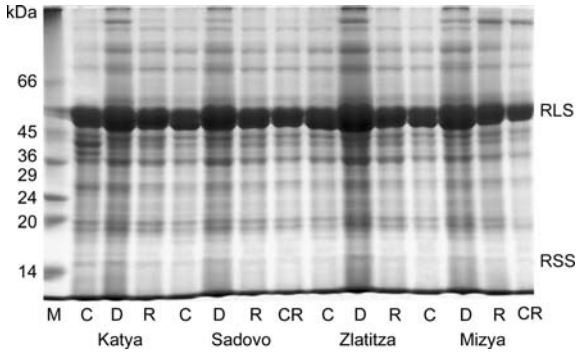

Fig. 2 Leaf protein pattern after 12% SDS-PAGE of extracts from wheat varieties—Katya, Sadovo, Zlatitza and Mizya in control conditions (C), drought (D), recovery after drought (R) and age control for recovery (CR). M—Dalton Mark Standard Mix (Sigma). Samples with protein quantity equivalent to 5 mg FW were loaded per lane. The position of Rubisco subunits are indicated in the right—RLS and RSS

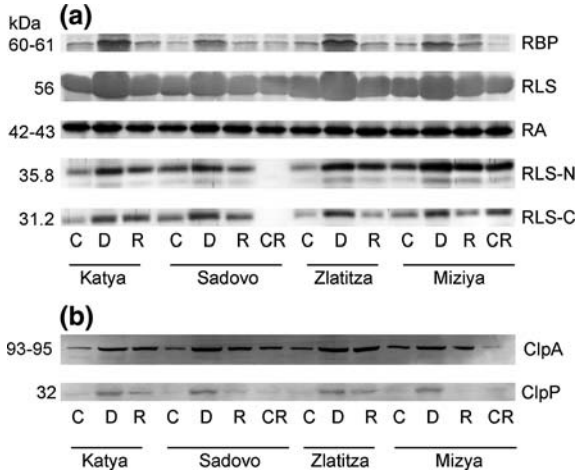

Fig. 3 Immunoblot analysis of extracts from wheat varieties—Katya, Sadovo, Zlatitza and Mizya in control conditions (C), drought (D), recovery after drought (R) and age control for recovery (CR) using polyclonal antibodies against RLS, RBP, RA, RLS-N and RLS-C (**a**) and against ClpA and ClpP (**b**). Samples with protein quantity equivalent to 5 mg FW were loaded per lane

under treatment and control conditions (Fig. 3). The intact RLS was recognized around 56 kDa, RA—as a single 42–43 kDa polypeptide and RBP—as overlapping 60–61 kDa bands. Severe drought induced a significant (about three times) increase in RBP quantity for all varieties. The intensity of RBP bands were about 50% higher in control and drought treated variants of drought tolerant varieties Katya and Zlatitza compared with the controls and drought treatments of sensitive varieties Sadovo and Mizyia (Table 1). Changes in RLS were similar as those observed in Fig. 2. The content of RA was only slightly affected (Fig. 3a). Some degradation products of RLS were detected with antibodies against RLS-N and RLS-C termini between 30 and 40 kDa (Fig. 4). Among these fragments, the N-terminus (35.8 kDa) and C-terminus (31.2 kDa) of RLS were revealed, which were weaker in the controls of the drought tolerant varieties. Their intensities were increased under stress conditions and diminished in recovery. An enhanced response of Clp proteases under severe drought stress and after recovery was observed (Fig. 3b). The antibody against ClpA detected an intense band (92–95 kDa), probably corresponding to plant ClpC or ClpD subunits whereas the antibody against ClpP detected one band (32 kDa). The drought tolerant variety Katya possessed the higher induction of Clp proteases under drought and recovery period. The quantitative changes were more clearly seen in the plots of the immunoblots (Fig. 5) and Table 1.

Discussion

Water stress is one of the major stress factors on plant metabolism affecting plant growth parameters, membrane integrity, total protein quantity and quality. In order to obtain more obvious protein changes among the varieties, severe but recoverable drought stress was applied. Under these conditions the physiological response of the varieties was quite similar at seedling stage.

In the present study, immunoblotting analyses showed that RBP content substantially increased after 7 days of severe but reversible drought stress, whereas the RA level was only slightly affected. Obviously, the wheat seedlings under these treatment conditions maintained Rubisco protein quantity. In our drought conditions the amount of Rubisco is conserved and even enhanced. A similar tendency of increase in Rubisco content was observed in sunflower leaves subjected to a long-term WD, especially in the drought-tolerant sunflower hybrid (Pancović et al. 1999). Pelloux et al. (2001) also noticed an increased quantitiy of RLS in *Pinus halepensis* M. subjected to drought stress. Pääkkönen et al. (1998) detected an increase in Rubisco content under drought stress in

Table 1 The average area occupied by each band of immunoblots (Fig. 5) of extracts from wheat varieties—Katya, Sadovo, Zlatitza and Mizya in control conditions (C), drought (D), recovery after drought (R) and age control for recovery (CR) using polyclonal antibodies against RLS, RBP, RA, RLS-N and RLS-C (a) and against ClpA and ClpP (b)

Varieties	Variants	RBP		RLS		RA		RLS-N		RLS-C		ClpA		ClpP	
		Area	%	Area	%	Area	%	Area	%	Area	%	Area	%	Area	%
Miziya	CR	3829	58	16860	83	16723	108	18690	127	15834	101	4025	25	6492	77
	R	13092	198	21382	106	17473	113	20926	143	9389	60	12404	78	3056	36
	D	**20750**	**314**	**33270**	**165**	**18556**	**120**	**29256**	**199**	**20997**	**134**	**20126**	**127**	**13120**	**155**
	C	6599	100	20197	100	15437	100	14669	100	15626	100	15818	100	8459	100
Zlatitza	R	9244	94	23856	107	18533	114	17473	191	9278	111	20318	124	11508	199
	D	**31181**	**319**	**35837**	**160**	**17736**	**109**	**25164**	**276**	**25348**	**303**	**23109**	**141**	**15471**	**268**
	C	9787	100	22379	100	16324	100	9127	100	8364	100	16427	100	5775	100
Sadovo	CR	7513	138	18761	93	15801	118	149	10	4370	3	16865	121	3778	70
	R	7675	140	19851	98	16795	125	11278	81	17755	102	19091	138	6452	120
	D	**19410**	**355**	**30191**	**149**	**17074**	**128**	**20940**	**151**	**30488**	**175**	**24236**	**175**	**15261**	**284**
	C	5455	100	20270	100	13386	100	13875	100	17399	100	13882	100	5381	100
Katya	R	9772	106	22692	100	13975	95	11107	128	20893	203	16180	378	9558	350
	D	**31195**	**337**	**39519**	**175**	**17602**	**120**	**18524**	**214**	**11386**	**207**	**14753**	**344**	**14205**	**520**
	C	9258	100	22635	100	14716	100	8651	100	10313	100	4283	100	1730	100

Values are given in arbitrary units and in percentage from the control of the respective variety. The data are processed with ImageJ software after minimizing background effect

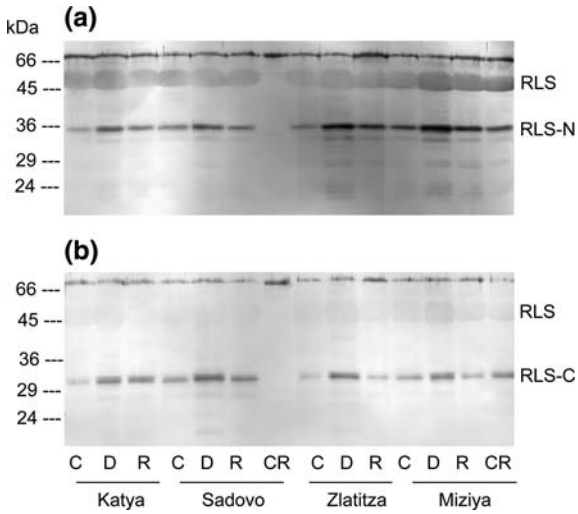

Fig. 4 Immunoblots of extracts from wheat varieties—Katya, Sadovo, Zlatitza and Mizya in control conditions (C), drought (D), recovery after drought (R) and age control for recovery (CR) using antibodies against RLS-N (**a**) and RLS-C (**b**). Samples with protein quantity equivalent to 5 mg FW were loaded per lane

birch (*Betula pendula* Roth.). Probably the higher Rubisco content could be related to quickly restoring this protein function during the recovery process.

Water deficit, as a result of osmotic stress, is expected to lead to increased protein aggregation and denaturation, making production of molecular chaperons more necessary (Zang and Komatsu 2007). According to Lawlor (2002) the decreased production of ATP in the chloroplasts of drought stressed plants serves as a signal triggering synthesis of chaperone molecules. Thus, RBP and RA could play an enhanced role as chaperones in plant cells under drought stress, maintaining Rubisco at the appropriate content, necessary for quick recovery. In our study the amount of RBP drastically increased under drought and positively correlated to the amount of Rubisco.

After immunoblotting the revealed RA increased slightly under severe drought conditions (on a FW basis). On the other hand, heat stress in wheat leaves increases substantially the expression of RA protein (Law and Crafts-Brandner 2001; Salvucci et al. 2001), identified as a thermolabile protein. Haupt-Herting et al. (2001) have explained the increased abundance of RA with its function to reactivate Rubisco.

Besides the intact RLS, some small fragments were additionally detected in control plants. Their intensities increased under drought stress conditions. They could be a consequence of non-enzymatic Rubisco

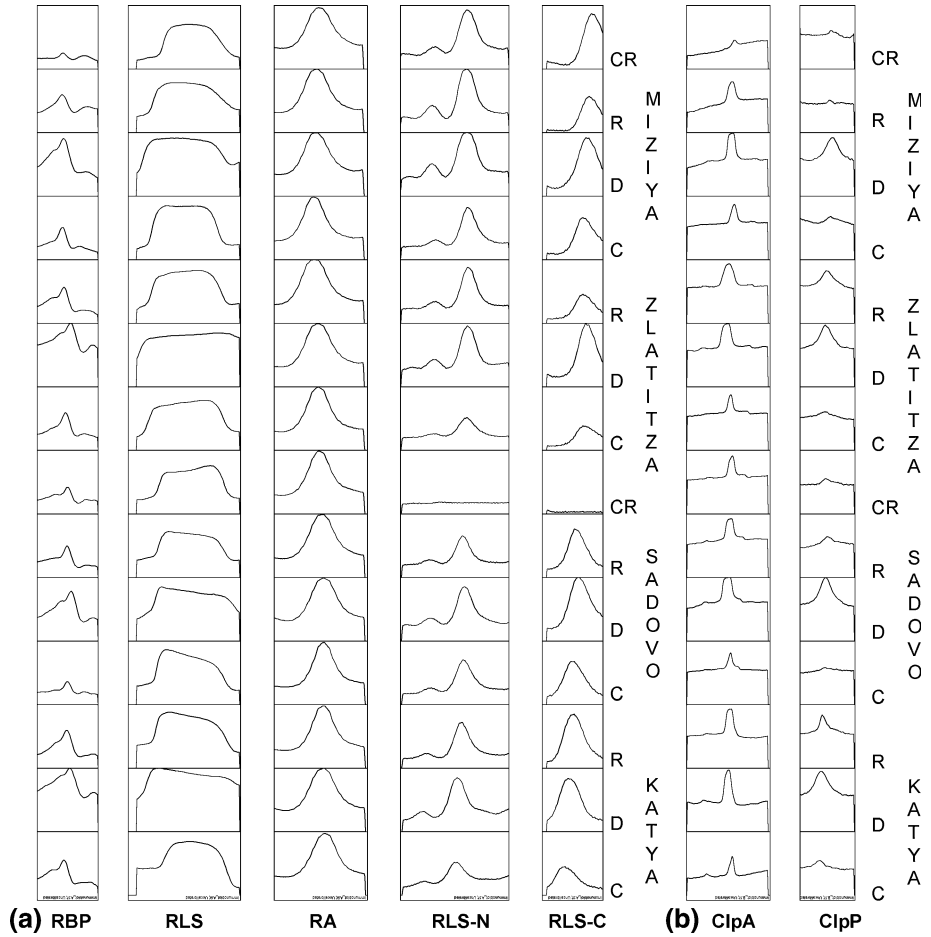

Fig. 5 Plots of the immunoblots of extracts from wheat varieties—Katya, Sadovo, Zlatitza and Mizya in control conditions (C), drought (D), recovery after drought (R) and age control for recovery (CR) using polyclonal antibodies against RLS, RBP, RA, RLS-N and RLS-C (**a**) and against ClpA and ClpP (**b**). Horizontaly—the respective proteins; vertically—varieties and treatments

degradation. Ishida et al. (1997) demonstrated RLS fragmentation products. The same authors suggested a proteinase-independent mechanism in which RLS could be directly degraded by active oxygen probably via the hydroxyl radicals generated in the wheat chloroplasts. Severe drought is connected with oxidative stress (Reddy et al. 2004). It could be considered that under certain physiological conditions some fragmentation of the RLS into RLS-N and RLS-C might occur. The investigated Clp proteases could not participate in the Rubisco fragmentation under stress conditions because they degrade their substrates to short peptides that are not detectable on electrophoresis (Sakamoto 2006).

The function of the Clp proteases in plant stress response and their physiological substrates are far from being elucidated. In our experiments Clp proteases were more abundant under severe drought. It is generally believed that in plants these proteins are primarily constitutive proteins, on the contrary to the bacterial cells, where they are strongly induced by different stress treatments (Zheng et al. 2002). Our studies are in agreement with the findings of Nakashima et al. (1997), who showed up-regulation of Clp protease subunit homologue in *Arabidopsis* under water stress and senescence. The increased quantity of Clp proteases detected immunologically supports the findings of Zagdanska and Wishnewski (1998), who have found an enhanced activity of ATP-dependent proteases in the leaves of wheat plants subjected to drought. However, Rubisco seems to be quite stable in drought stress conditions. The

physiological substrates of Clp proteases under dehydration still remain to be elucidated.

From the results presented it can be concluded that Rubisco content is maintained and the ATP-dependent chaperone proteins studied are enhanced under severe drought stress after which wheat plants could be recovered. Some differences between sensitive and tolerant varieties exist concerning RBP. The drought tolerant varieties Katya and Zlatitsa have higher level of RBP in the control and drought stressed plants compared to the sensitive varieties. The high content of this protein could be useful as a marker for drought tolerance. Clp proteins are also responsive to drought stress and recovery but their link to drought tolerance is not so clear.

In conclusion, the application of a severe but recoverable drought stress at seedling stage on four wheat varieties led to a coordinated response of Rubisco, RA, RBP and Clp proteases, which could contribute to the plant tolerance to dehydration. The observed increase of Rubisco and other chaperone proteins that are of great importance for Rubisco stability and activity, could be related to the protection of plant metabolism and functioning in adverse environmental conditions.

Acknowledgments This study was supported by grants from the Swiss National Science Foundation, SCOPES (project DILPA) and from the Ministry of Education and Science of Republic Bulgaria (projects CC 1503 and PISA). The authors are grateful to Dr. I. Stancheva for her advices on growing wheat plants in soil conditions and to B. Juperlieva-Mateeva and A. Kostadinova for their excellent technical assistance.

References

Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254. doi:10.1016/0003-2697(76)90527-3

Bray E (1997) Plant responses to water deficit. Trends Plant Sci 2:48–54. doi:10.1016/S1360-1385(97)82562-9

Demirevska-Kepova K, Juperlieva-Mateeva B (1990) Purification of Rubisco large subunit binding protein from barley and preparation of polyclonal antisera against it. Compt Rend Acad Sci Bulg 43:101–104

Demirevska-Kepova K, Simova L (1989) Isolation and purification of ribulose-1, 5-bisphosphate carboxylase/oxygenase from barley leaves. Bulg J Plant Physiol 15: 3–10

Demirevska-Kepova K, Simova L, Kjurkchiev S (1999) Barley leaf Rubisco, Rubisco binding protein and Rubisco activase and their protein/protein interactions. Bulg J Plant Physiol 25:31–44

Demirevska-Kepova K, Hölzer R, Simova-Stoilova L, Feller U (2005) Heat stress effects on Rubisco, Rubisco binding protein and Rubisco activase in wheat leaves. Biol Plant 49(4):521–525. doi:10.1007/s10535-005-0045-2

Feller U, Anders I, Mae T (2008) Rubiscolytics: fate of Rubisco after its enzymatic function in a cell is terminated. J Exp Bot Advance Access Nov 1:1–10

Fisher RA, Maurer R (1978) Drought tolerance in spring wheat cultivars I. Grain yield response. Aust J Agric Res 29:897–912. doi:10.1071/AR9780897

Haupt-Herting S, Klug K, Fock HP (2001) A new approach to measure gross CO_2 fluxes in leaves. Gross CO_2 assimilation, photorespiration, and mitochondrial respiration in the light in tomato under drought stress. Plant Physiol 126:388–396. doi:10.1104/pp.126.1.388

Hemmingsen SM (1990) The plastid chaperonin. Semin Cell Biol 1:47–54

Holland N, Belking A, Holland D, Pick U, Edelman M (1998) Stress-responsive accumulation of plastid chaperonin 60 during seedling development. Plant J 13:311–316. doi:10.1046/j.1365-313X.1998.00028.x

Houtz RL, Portis AR Jr (2003) The life of ribulose 1,5-bisphosphate carboxylase/oxygenase—Posttranslational facts and mysteries. Arch Biochem Biophys 414:150–158

Ishida H, Nishimori Y, Sugisawa M, Makino A, Mae T (1997) The large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase is fragmented into 37-kDa and 16-kDa polypeptides by active oxygen in the lisates of chloroplasts from primary leaves of wheat. Plant Cell Physiol 38(4):471–479

Jagtap V, Bhargava S, Streb P, Feieraben J (1998) Comparative effect of water, heat and light stresses on photosynthetic reactions in *Sorghum bicolor* (L.) Moench. J Exp Bot 49:1715–1721. doi:10.1093/jexbot/49.327.1715

Kalapos T, van den Boogaard R, Lambers H (1996) Effect of soil drying on growth, biomass allocation and leaf gas exchange of two annual grass species. Plant Soil 185:137–149. doi:10.1007/BF02257570

Law RD, Crafts-Brandner ST (2001) High temperature stress increases the expression of wheat leaf ribulose-1,5-bisphosphate carboxylase/oxygenase activase protein. Arch Biochem Biophys 386:261–267. doi:10.1006/abbi.2000.2225

Lawlor DW (2002) Limitation to photosynthesis in water stressed leaves: stomata vs. metabolism and role of ATP. Ann Bot (Lond) 89:871–885. doi:10.1093/aob/mcf110

Lawlor DW, Cornic G (2002) Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant Cell Environ 25:275–294. doi:10.1046/j.0016-8025.2001.00814.x

Majumdar S, Ghosh S, Glick BR, Dumbroff EB (1991) Activities of chlorophyllase, phosphoenolpiruvate carboxylase and ribulose 1,5-bisphosphate carboxylase in the primary leaves of soybean during senescence and drought. Physiol Plant 81:473–480. doi:10.1111/j.1399-3054.1991.tb05087.x

Makino A, Mae T, Ohira K (1984) Relation between nitrogen and ribulose-1,5-bisphosphate carboxylase in rice leaves

from emergence through senescence. Plant Cell Physiol 25:429–437

Martinez DE, Bartoli CG, Grbic V, Guiamet JJ (2007) Vacuolar cysteine proteases of wheat (*Triticum aestivum* L.) are common to leaf senescence induced by different factors. J Exp Bot 58:1099–1107. doi:10.1093/jxb/erl270

Medrano H, Parry MAJ, Socias X, Lawlor DW (1997) Long term water deficit inactivates Rubisco in subtertanean clover. Ann Appl Biol 131:491–501. doi:10.1111/j.1744-7348.1997.tb05176.x

Mitsuhashi W, Feller U (1992) Effects of light and external solutes on the catabolism of nuclear-encoded stromal proteins in intact chloroplasts isolated from pea leaves. Plant Physiol 100:2100–2105

Musgrove JE, Jonson RA, Ellis RJ (1987) Dissociation of the ribulose bisphosphate carboxylase large subunit binding protein into dissimilar subunits. Eur J Biochem 163:529–534. doi:10.1111/j.1432-1033.1987.tb10900.x

Nakashima K, Kiyosue T, Yamaguchi-Shinozaki K, Shinozaki K (1997) A nuclear gene, erdl, encoding a chloroplast-targeted Clp protease regulatory subunit homolog is not only induced by water stress but also developmentally up-regulated during senescence in *Arabidopsis thaliana*. Plant J 12:851–861. doi:10.1046/j.1365-313X.1997.12040851.x

Neuwald AF, Aravind L, Spouge JL, Koonin EV (2006) AAA+: a class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. Genome Res 9:27–43

Nunes MES, Smith GR (2003) Electrolyte leakage assay capable of quantifying freezing resistance in rose clover. Crop Sci 43:1349–1357

Pääkkönen E, Vahala J, Pohjolai M, Holopainen T, Kärenlampi L (1998) Physiological, stomatatal and ultrastructural ozone responses in birch (*Betula pendula* Roth.) are modified by water stress. Plant Cell Environ 21:671–684. doi:10.1046/j.1365-3040.1998.00303.x

Pancović D, Sakač Z, Kevrešan S, Plesničar M (1999) Acclimation to long-term water deficit in the leaves of two sunflower hybrids: photosynthesis, electron transport and carbon metabolism. J Exp Bot 50:127–138. doi:10.1093/jexbot/50.330.127

Parrott DL, McInnerney K, Feller U, Fischer AM (2007) Steam-girdling of barley (*Hordeum vulgare*) leaves leads to carbohydrate accumulation and accelerated leaf senescence, facilitating transcriptomic analysis of senescence-associated genes. New Phytol 176:56–69. doi:10.1111/j.1469-8137.2007.02158.x

Parry MAJ, Andralojc PJ, Khan V, Lea PJ, Keys AJ (2002) Rubisco activity: effect of drought stress. Ann Bot (Lond) 89:833–839. doi:10.1093/aob/mcf103

Pelloux J, Jolivet Y, Fontaine V, Banvoy J, Dizengremel P (2001) Changes in Rubisco and Rubisco activase gene expression and polypeptide content in *Pinus halepensis* M subjected to ozone and drought. Plant Cell Environ 24:123–131. doi:10.1046/j.1365-3040.2001.00665.x

Portis AR Jr (2003) Rubisco activase—Rubisco's catalytic chaperone. Photosynth Res 75:11–27. doi:10.1023/A:1022458108678

Reddy AR, Chaitanya KV, Vivekanandan M (2004) Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. J Plant Physiol 161:1189–1202. doi:10.1016/j.jplph.2004.01.013

Riccardi F, Gazeau P, Jacquemot M-P, Vincent D, Zivy M (2004) Deciphering genetic variation of proteome responses to water deficit in maize leaves. Plant Physiol Biochem 42:1003–1011. doi:10.1016/j.plaphy.2004.09.009

Rokka A, Zhang L, Aro EM (2001) Rubisco activase: an enzyme with a temperature-dependent dual function? Plant J 25:463–471. doi:10.1046/j.1365-313x.2001.00981.x

Roy-Macauley H, Zuily-Fodil Y, Kidric M, Pham Thi AT, Viera da Silva J (1992) Effect of drought stress on proteolytic activities in *Phaseolus* and *Vigna* leaves from sensitive and resistant plants. Physiol Plant 85:90–96. doi:10.1111/j.1399-3054.1992.tb05268.x

Sakamoto W (2006) Protein degradation machineries in plastids. Annu Rev Plant Biol 57:599–621. doi:10.1146/annurev.arplant.57.032905.105401

Salekdeh GH, Siopongco J, Wade LJ, Chareyazie B, Bennett J (2002) A proteomic approach to analyzing drought- and salt-responsiveness in rice. Field Crops Res 76:199–219. doi:10.1016/S0378-4290(02)00040-0

Salvucci ME, Osteryoung KW, Crafts-Brander SJ, Vierling E (2001) Exceptional sensitivity of rubisco activase to thermal denaturation in vitro and in vivo. Plant Physiol 127:1053–1064. doi:10.1104/pp.127.3.1053

Salvucci ME, Portis AR, Ogren WL (1985) A soluble chloroplast protein catalyses ribulosebisphosphate carboxylase/oxygenase activation in vivo. Photosynth Res 7:193–201. doi:10.1007/BF00037012

Sanchez de Jimenes E, Medrano L, Martines-Barajas E (1995) Rubisco activase, a possible new number of the molecular chaperone family. Biochemistry 34:2826–2831. doi:10.1021/bi00009a012

Simova-Stoilova L, Vassileva V, Petrova T, Tsenov N, Demirevska K, Feller U (2006) Proteolytic activity in wheat leaves during drought stress and recovery. Gen Appl Plant Physiol Spec Issue 32(1–2):91–100

Zagdanska B, Wishnewski K (1998) ATP-dependent proteolysis contributes to the acclimation-induced drought resistance in spring wheat. Acta Physiol Plant 20:41–48. doi:10.1007/s11738-998-0041-1

Zang X, Komatsu S (2007) A proteomic approach for identifying osmotic-stress-related proteins in rice. Phytochemistry 68:426–437. doi:10.1016/j.phytochem.2006.11.005

Zheng B, Halperin T, Hruskova-Heidingsfeldova O, Adam Z, Clarke AK (2002) Characterization of chloroplast Clp proteins in *Arabidopsis*: localization, tissue specificity and stress responses. Physiol Plant 114:92–101. doi:10.1034/j.1399-3054.2002.1140113.x