AMINOPEPTIDASE ACTIVITIES IN ROOTS AND LEAVES OF DROUGHT STRESSED WINTER WHEAT SEEDLINGS

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Researce Article

Summary: In order to evaluate the role of aminopeptidases (APs) in drought response and their potential as protein markers to distinguish between stress tolerant and sensitive varieties, various AP activities were studied in roots and leaves of winter wheat seedlings, subjected to severe but recoverable soil drought stress. Two varieties with contrasting drought tolerance - Yantar (drought tolerant) and Miziya (sensitive) were compared. Activity changes under severe water stress and subsequent recovery were related to changes in the pools of the major redox buffers ascorbate and glutathione, changes in protein profiles and total proteolysis in roots and leaves. Glutathione was responsive to drought both in roots and leaves, with increased total pool and transient rise in the oxidized form; stronger response in the roots of Yantar was observed. The sensitive variety had higher ascorbate content in leaves under stress. Severe drought led to reversible changes in protein profiles and increase in major protease bands in leaves but not in roots. AP activities were partly independent from the predominant endoprotease activities. Highest activities in roots were detected with substrates releasing terminal leucine, lysine and metionine. In stressed leaves AP activities toward most of the substrates increased under drought, without clear differences comparing varieties. Activities tested with Gly-pNA were raised in leaves only in recovery from stress. In roots, the tolerant variety Yantar presented increased AP activities under stress with most of the substrates used except Leu-pNA and Phe-pNA, whereas the sensitive variety Miziya had almost unchanged AP activities. Based on activity profile changes, at least two different AP enzymes should exist in wheat. It remains to be established which activities towards different substrates reflect distinct aminopeptidases.

Keywords: Aminopeptidase; drought; recovery; ascorbate; glutathione; *Triticum aestivum L*.

Abbreviations: AP – aminopeptidase; DMSO – dimethylsulfoxide; DTNB – dithionitrobenzoic acid; DTT – dithiotreitol; EDTA – ethylendiaminetetracetic acid; FW – fresh weight; MDA – malondialdehyde; PAR – photosynthetic active radiation; pNA – para-nitroanilide; PMSF – phenylmethylsulfonylfluoride; ROS – reactive oxygen species; SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA – trichloroacetic acid; TW – weight at full turgidity; WD – water deficit.

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INTRODUCTION

among Drought is the most deleterious stresses with respect to plant productivity, especially in the temperate regions of the world; yield losses due to field drought may account up to 50% of the production under optimal conditions (Ludlow and Muchow 1990). Wheat (Triticum aestivum L.) is among the basic crops providing staple food for human population. Elucidating the mechanisms for adaptation of wheat plants to drought is of uppermost importance in order to reveal suitable markers assisting selection for stress tolerant cultivars, especially applicable at early developmental stage. In this respect, several groups of drought inducible proteins have been studied in wheat, both in primary leaves at early developmental stage and in flag leaves of field grown wheat plants (Demirevska et al 2008). Lately, studies on the root system came into focus, since this is the first organ which faces soil drought and transmits stress signals over the whole plant (Davies and Zhang 1991, Kohli et al 2012, Paez-Garcia et al 2015). Differences in root and leaf response to drought are found with respect to growth, antioxidative protection and metabolism, amino acids being the most affected metabolic class (Gargallo-Garriga et al 2014, Maraghni et al 2014, Chmielewska et al 2016).

Changes at protein level are the basis for phenotypic plasticity and adaptation to various stresses including drought (Demirevska et al 2008, Kidrič et al 2014a). Alterations in the steady state level of individual proteins result from the fine balance between synthesis and degradation. Proteolysis, both in its processing and complete degradation modes, is essential for cells in nonstress conditions as well as under stress (Vaseva et al 2012). The main functions of the proteolytic system are to exert protein quality control by removal of damaged/unnecessary proteins, to fuel the central and secondary metabolism by amino acids, to provide building blocks for new protein synthesis, to fit the hormonal regulation via degradation of short living signal proteins. Modification at the N terminus could also determine the fate of a given protein (Walling 2006, Kidrič et al 2014a). The numerous functions and the big physiological significance of proteolytic enzymes is reflected in the considerable amount of protease encoding genes. For example, there are 1131 known putative peptidases and peptidase homologues in Triticum aestivum L. according to the MEROPS (http://merops.sanger.ac.uk, database Rawlings et al 2016). Among them, aminopeptidases (APs, EC 3.4.11) have a special place in the protease network.

exopeptidases which are APs release amino acid residues from the N-terminus of proteins. They belong mostly to the metallo-protease (M1, M17, M18 and M24 families) and serine protease (family S33) catalytic classes; thiol dependency of the activity is also reported for some of them (Tishinov et al 2009, Kidrič et al 2014a). Presence of several APs is documented in different plant organs and in various subcellular locations - cytosol, plasma membrane, plastids, mitochondria, associated with meiotic chromosomes (summarized by Walling 2006). Some APs have broad substrate specificity with preference for bulky hydrophobic amino acids at the N-terminus, while others are characterized with narrow substrate specificity (Blätter and Feller 1988, Miazek and Zagdanska 2008, Waditee-Sirisattha et al 2011, Budič et al 2016). APs are partners of the major cell proteolytic systems like the proteasome and proteases involved in autophagy, dealing with the terminal degradation of small peptides (Walling 2006). Besides the general recycling function, cleavage of the ultimate and exposure of different penultimate amino acid residue at the N-terminus of a protein could regulate it's halflife, as this is a site for co-translational and post-translational modifications, which can influence protein stability, localization or activity (Walling 2006). Moreover, a role in glutathione turnover is reported for Leu-AP, thus linking APs with the cell redox status (Kumar et al 2015). An additional function is found for some Leu-APs as molecular chaperones (Scranton et al 2012, DuPrez et al 2016). For these reasons APs are regarded as multifunctional proteins. Like the other peptidases, APs are also subjected to post-translational regulation and sometimes changes at the transcript level do not necessarily coincide with changes in protein abundance/activity. Activity changes are considered more closely related to AP function in vivo than changes at the transcript level (Budič et al 2016).

APs are relatively abundant in seeds, young fast-growing tissues and sites of damage and injury, but they also remain active in senescing plant parts (Matsui et al 2006). In development, APs take part in resource mobilization, in germination, meiosis, mitosis, protein trafficking and signal transduction processes (Peer

2011). There are only few studies on the involvement of APs in stress response. Upregulation of Leu-AP at the transcript, protein and activity levels was found in tomato plants under osmotic stress, wound stress and hormonal treatment (Chao et al 1999). Transcripts of Prolyl-AP gene were upregulated by NaCl, drought, and heavy metal stresses (Szawłowska et al 2011, Sun et al 2013, Wang et al 2015). Studies in transgenic plants, focused on Prolyl-AP overexpression or silencing in Arabidopsis, are in favor of positive regulation of tolerance to salt and drought stress, which coincide with higher free proline content and higher Prolyl-AP in overexpressing plants both in control and stress conditions (Sun et al 2013). Loss-of activity phenotype of Arabidopsis Leu-AP2 is reported as early senescent and stress-sensitive (Waditee-Sirisattha et al 2011). In barley, screening for low temperature-induced genes identified Met-AP, which expression was induced also by abscisic acid treatment, and overexpression of this gene conferred stronger freezing tolerance to Arabidopsis transgenic plants (Jeong et al 2011). Five partially purified APs – three metallopeptidases with broad substrate specificity (active against substrates with N terminal Ala and Lys), and two serine APs with narrow specificity toward N- terminal Phe, were found to be drought responsive in various extent in Phaseolus vulgaris leaves (Budič et al 2016). Leaf APs activities in the resurrection plant Ramonda serbica were significantly higher in desiccated state than in rehydrated plants and in regularly watered plants, implying involvement of APs in the recovery of vegetative tissues from desiccation (Kidrič et

al. 2014b). Upregulation of APs and general mobilization of the proteolytic system for enhanced protein turnover under drought has been documented in proteomic studies as well (Ghosh and Xu 2014; Cheng et al 2015). In wheat seedlings general increase in AP activity was observed under dehydration stress (Miazek and Zagdanska 2008, Simova-Stoilova et al 2010). Water stress during grain filling in wheat enhanced the rate of monocarpic senescence and increased both endopeptidase and exopeptidase activities in flag leaves (Srivalli and Khanna-Chopra 1998). Along with other enzymes, the AP profiling is used in plant breeding to differentiate among varieties (Baes and Van Cutsem 1992, Walling and Gu 1996). Comparing common bean drought-tolerant and sensitive varieties, differences in aminopeptidase activities are reported in drought-stressed leaves (Hieng et al 2004). However, Leu-AP activity in the leaves of three different wheat genotypes at seedling stage presented general increase under severe water deficit, without distinction among varieties (Simova-Stoilova et al 2010).

In order to evaluate the role of exopeptidases in drought response and their potential as protein markers to distinguish between stress tolerant and sensitive varieties, APs profiling with a larger set of substrates is needed. In this study we report changes in AP activities in two winter wheat varieties with different sensitivity to drought (yield based), testing a panel of AP substrates. Plants were subjected to severe but recoverable soil drought stress at the seedling stage. AP activity changes under stress and subsequent recovery were compared in leaves and roots and were related to general stress parameters, redox buffer changes, protein profiles and total proteolysis.

MATERIALS AND METHODS

Plant material, growth conditions and stress treatment

Two winter wheat varieties differing in drought tolerance, based on drain yield under field terminal drought, were used the drought tolerant variety Yantar and the drought sensitive one Miziva (Simova-Stoilova et al 2006, 2008). Experimental design and physiological response of wheat plants to drought treatment has been described in details elsewhere (Simova-Stoilova et al 2006). Briefly, plants were grown in plastic pots filled with 500 g of leached meadow cinnamonic soil under optimal NPK fertilization, relative soil humidity at 70% of field capacity, 180 µE.m⁻².s⁻¹ PAR, 25°/21°C and 16 h photoperiod. In order to obtain uniform development of the stress, seeds of both varieties under comparison were sown in sectors in the same pots. Drought was imposed on 8-day old seedlings by withholding watering for 7 days, followed by 3 days recovery under optimal water supply. Age controls of the stressed and recovered plants were included in the experimental scheme, which were watered daily. Biochemical analyses were performed on samples from the first leaf, which was fully expanded at the beginning of the treatment, and on total root biomass. Roots were cleaned out of soil, washed quickly but thoroughly with tap water followed by distilled water, and blotted dry. Samples were quickly frozen in liquid nitrogen and stored at -70°C until biochemical analyses.

Stress intensity parameters

Leaf water deficit (WD) was calculated in percentages according the formula (TW-FW)/TW, where TW is leaf weight at full turgidity (leaf segments floating in distilled water for 24h at 8°C), FW – the actual leaf fresh weight at sampling. For proline and malondialdehyde (MDA) determination, 0.5 g FW leaf material was homogenized in 5 ml of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 10000 g for 30 min. Proline content was analysed according to Bates et al (1973). Lipid fragmentation resulting from peroxidation was estimated using the thiobarbituric acid reactive substances assay. Optical density at 440, 532 and 600 nm was read and MDA content was calculated as described by Hodges et al (1999).

Antioxidant compounds

The state of redox buffers ascorbate and glutathione was analysed as described by Zaharieva and Abadía (2003) starting from the same extract of 0.5g FW sample in 3 ml 2% w/v metaphosphoric acid and using micro methods. The ascorbate content (total and reduced) was assayed by reduction of Fe³⁺ to Fe²⁺ by ascorbate in acidic solution and complexation of Fe^{2+} with α, α' -dipyridyl, giving a pinkcolored product. The absorbance at 525 nm was read and ascorbate content was quantified using a standard curve. Oxidised ascorbate was estimated from the difference between total and reduced ascorbate. Glutathione content was assayed with dithiobisdinitrobensoic acid (DTNB) and glutathione reductase. Absorbance at 412 nm was read and total glutathione the content was

estimated using a standard curve. Oxidised glutathione was estimated by derivatization with divinylpiridine.

Protein profiles and protease zymograms

Leaf material (0.5 g FW) or root material (1g FW) was homogenized in 3+1 ml ice-cold 100 mM Tris-HCl buffer pH 7.5 containing 5 mM DTT, 20 mM MgCl,, 2 mM EDTA, 0,05% TX-100, 2mM PMSF, 50 mg Polyclar AT (polyvinlylpolypyrrolidone). After centrifugation at 14000 g for 40 min at 4°C, total soluble protein content was measured (Bradford, 1976). Samples for SDS electrophoresis were prepared after equalizing protein content to 1.5 mg/ml for leaves and 0.3 mg/ml for roots and concentrating samples with 50% w/v TCA (final 10%). The protein pellet was washed twice with 80% v/v acetone and dissolved with suitable volume of Laemmli sample buffer. The 12.5 % SDS-PAGE of leaf and root soluble proteins was performed according to Laemmli (1970) loading equal protein quantity per lane.

For protease activities in gel staining, leaf and root proteins were extracted with 100 mM sodium phosphate buffer pH 6.5 containing 0.1% TX-100, 1mM EDTA, 5 mM cysteine, 5 mM DTT and 5 mM CaCl₂. After separation of samples (mixed 1:1 with Laemmli buffer, without boiling) in 10% SDS-PAGE with immobilized 0.5 % BSA, protease activities were developed as previously described (Simova-Stoilova et al 2010).

Aminopeptidase activities

Aminopeptidase activities were assayed with micro methods according to

Salgó and Feller (1987). Briefly, enzyme extracts were prepared from 0.5g FW leaf or 1 g FW root material in 2+1 ml 100 mM sodium acetate buffer pH 5.5 containing 0.1% v/v β -mercaptoethanol, 50 mg Polyclar AT, and centrifugation at 14000 g for 40 min at 4°C. Protein content was measured according to Bradford (1976). A set of p-nitroanilide (pNA) substrates was used to measure aminopeptidase activities: Ala-pNA, LeupNA, Gly-pNA, Lys-pNA, Arg-pNA, Phe-pNA, Pro-pNA, Met-pNA. Freshly prepared substrate solutions contained 2mM pNA substrate (first dissolved in dimethylsulfoxide - DMSO) in 50 mM phosphate buffer pH 7.0, final 1% DMSO. The reaction mixtures consisted of (per well of microtitration plate): 200 µl substrate solution, 20 µl leaf or 60 µl root extract (with protein content in the extract brought to 1 mg/ml for leaves and of 0.2-0.4 mg/ml for roots). The reaction was started by adding the extracts and proceeded at room temperature. The release of pNA was followed at 405 nm every 10 minutes for one hour.

Statistical analysis

Results were based on three technical replicates from two independent experiments presenting similar trends of changes. Data are given as means and standard deviation from a representative experiment. Multifactor ANOVA (Statgraphics plus version 2.1) at significance level P < 0.05 was used for comparison among all variants from a tissue. Electrophoretic analyses for proteins and activity staining (different extractions as described) were repeated twice and similar results were obtained; representative pictures are shown.

RESULTS

Stress intensity and the response of the main redox buffers

According to our previously established experimental conditions. recoverable stress severe drought imposed on 8-days old winter wheat seedlings resulted in strong but reversible leaf water deficit (56-63% WD), proline accumulation (about 17fold increase in drought stressed leaves of both varieties) and increased MDA levels (about 2.5-fold rise under drought) (Fig. 1). No significant differences in these parameters between the two tested varieties under stress were registered. In addition, the redox buffers ascorbate and especially glutathione were highly responsive to the applied stress (Fig. 2). Along with the total rise in glutathione pool under severe drought, an increase in the oxidized forms was registered in leaves (Fig. 2C) - for glutathione in both varieties (40-46% of GSSG in the total pool under drought compared to 16-26% in control leaves), for ascorbate (Fig. 2A) - more in the drought sensitive variety (8% and 21% of oxidized leaf ascorbate under drought in Yantar and Miziya, respectively). Compared to leaves, roots presented less antioxidant compounds in absolute values and higher proportion of oxidized redox buffers (about 86-89% of GSSG in the total pool under drought), with stronger rise in glutathione pool in the tolerant variety Yantar - almost doubled when compared to Miziya (Fig. 2D). No significant changes in root ascorbate content were detected under drought in Yantar (Fig. 2B). An increase in oxidised ascorbate content in the total pool was registered in Miziya at recovery



Figure 1. General stress parameters in the leaves of age control of drought (CD), drought stressed for 7 days (D), and recovered (R) plants and age control of recovery (CR) plants. A – leaf water deficit; B – oxidative damage to membranes; C – proline content. Different letters above columns denote statistically significant differences.



Figure 2. Main redox buffers in leaves and roots of age control of drought (CD), drought stressed for 7 days (D), and recovered (R) plants and age control of recovery (CR) plants. A – Ascorbate content in leaves, B – ascorbate content in roots, C – glutathione content in leaves, D – glutathione content in roots. White parts of the columns – oxidized forms. Different letters above columns denote statistically significant differences.

(Fig. 2B). Taken together, data point at higher secondary oxidative stress in roots under drought, which has been counteracted mainly by glutathione in both variaties. An increased ascorbate content was documented in the droughtsensitive variety Miziya.

Protein profile changes and protease zymograms

Protein yield from leaves was about ten times higher than from roots; in both varieties under severe drought total soluble protein content remained unchanged in leaves but increased in roots (on a FW basis). Changes in protein profiles of leaves and roots under severe drought and subsequent recovery were followed by SDS-PAGE (Fig. 3) by loading equal protein amount derived from the tested treatment groups. The applied drought stress leaded to reversible changes in proteins both in leaves and in roots. Prominent but reversible changes in Rubisco LS under drought could be clearly seen in both varieties (40-50% loss in intensity). The tolerant variety Yantar restored RLS content after stress release, whereas Rubisco LS band was not fully recovered in the sensitive variety Miziya.

The protease activity profiling in leaves and roots under severe drought and subsequent recovery is presented in Fig. 4. A reversible increase in the intensity of



Figure 3. 12% SDS-PAGE profiles of leaf (1-6) and root (7-12) soluble proteins. Variants as follows: 1-3, 7-9 – Yantar; 4-6, 10-12 – Miziya; 1,4,7,10 – drought controls; 2,5,8,11 – drought treatment; 3,6,9,12 – recovery from drought. Equal protein quantity of 50 and 10 μ g per band was loaded for leaves and roots, respectively. The place of Rubisco large subunit (RLS) is indicated.

the upper bands was detected after stress in the leaves of both tested varieties. In drought stressed root samples the intensity of the revealed signals were less affected with the tolerant variety showing weaker basal intensity staining.

Aminopeptidase activities

The results for drought response of aminopeptidases in wheat seedlings tested with eight different substrates - two for release of glycogenic amino acids (AlapNA and Gly-pNA), two for ketogenic amino acids (Leu-pNA and Lys-pNA), four for amino acids fueling directly Krebs cycle through 2-oxoglutarate (Arg-pNA and Pro-pNA), succinyl-CoA (Met-pNA) and fumarate (Phe-pNA) - are presented in Fig. 5 (leaf activities) and Fig. 6 (activities in roots). In leaves, severe drought caused an increase in AP activities towards most of the substrates used with the exception of Phe-pNA (Fig.

(Fig. 5A, strong increase in both varieties in recovery). Generally, in recovery AP activities were diminished compared to drought but remained above the ones detected in the age-controls. Recovered Yantar leaves had similar or slightly higher AP activities toward Leu-pNA (Fig. 5B) and Pro-pNA (Fig. 5C) than the ones detected in drought-stressed samples. An interesting observation has been made for AP profiles releasing leucine, proline and alanine (Fig. 5B,C,E) - they all exhibited similar activity responses to the treatments, probably reflecting one AP with broad substrate specificity or a group of APs regulated similarly. In general, the tolerant variety presented higher AP activities in recovery toward the substrates Leu-pNA, Pro-pNA, Ala-pNA, Gly-pNA (Fig. 5A,B,C,E,) than the susceptible one. A relatively bigger difference between the tolerant and the sensitive variety in the

5H, no significant changes) and Gly-pNA



Figure 4. In gel activity staining for major proteolytic activities in leaves (1-8) and in roots (9-16). Variants: 1,9 - Miziya drought control; 2,10 - Yantar drought control; 3,11 - Miziya drought; 4,12 - Yantar drought; 5,13 - Miziya recovery control; 6,14 - Yantar recovery control; 7,15 - Miziya recovery; 8,16 - Yantar recovery. Protein load 35 µg for leaves and 15µg for roots. The place of the upper bands is indicated.

response of AP activities to the applied stress was documented in roots (Fig. 6). The tolerant variety Yantar presented increased AP activities at severe drought with most of the substrates used (except Leu-pNA and Phe-pNA Fig. 6B and H), whereas the sensitive variety Miziya had almost unchanged AP activities in roots under stress. Based on activity profiles, two groups of substrates with similarity in the response could be distinguished - the first group includes Gly-pNA, Pro-pNA, Ala-pNA, Lys-pNA and Arg-pNA (Fig. 6 A,C,E,F,G), and the other one comprises Leu-pNA, Met-pNA and Phe-pNA (Fig. 6 B,D,H). Probably these groups reflect the existence of at least two different APs in roots. Compared to leaves, roots presented higher AP activities toward Leu-pNA and Lvs-pNA (releasing ketogenic aminoacids, Fig.6 B and F), as well as toward MetpNA (releasing met which is important for the one-carbon metabolism, Fig. 6D). This observation could reflect specific metabolic demands in the root tissue.

DISCUSSION

Data presented here follow previous work on the antioxidative protection and proteolytic response in wheat leaves under drought, using the same experimental scheme (Simova-Stoilova et al 2006, 2008, 2010). The tested drought sensitive (Miziya) and drought tolerant variety (Yantar) were subjected to severe but recoverable drought stress. Genotype-dependent differences in proline accumulation have been observed in wheat seedlings subjected to drought, in relation to the stress intensity (Yadav et al 2004). MDA accumulation is regarded as a sign for oxidative damage to membranes and development of secondary oxidative stress. As membrane oxidative injury and proline accumulation were not significantly different in the leaves of the two varieties under comparison, it can be concluded that both experienced equal stress intensity. Proline has multiple functions in stress adaptation, recovery,



Figure 5. Aminopeptidase activities in leaf extracts from age control of drought (CD), drought stressed for 7 days (D), recovered (R) and age control of recovery (CR) plants. Activities are expressed in arbitrary units $-\Delta OD_{405}$.h⁻¹.mg⁻¹protein. White columns – CD, dark grey columns – D, light grey columns – R, stripped columns – CR. Different letters above columns denote statistically significant differences.



Figure 6. Aminopeptidase activities in root extracts from age control of drought (CD), drought stressed for 7 days (D), recovered (R) and age control of recovery (CR) plants. Activities are expressed in arbitrary units $-\Delta OD_{405}$. h⁻¹.mg⁻¹protein. White columns – CD, dark grey columns – D, light grey columns – R, stripped columns – CR. Different letters above columns denote statistically significant differences.

and signaling (Szabados and Savoure 2009). Proline accumulation under osmotic stresses, besides its effect of osmotic adjustment and protection of membranes and proteins from denaturation, could also help for ROS scavenging and buffering cellular redox potential (Ashraf and Foolad 2007). In this respect, apart from up-regulation of proline synthesis and/or translocation from storage compartments (Szabados and Savoure 2009), some role of prolyl-specific APs in maintaining the free proline pool in cells has also been claimed (Szawłowska et al 2011, Sun et al 2013, Wang et al 2015). In this study an enhancement of pro-AP activity was found under drought in the frame of overall increase of AP activities using several substrates. This activity could also contribute to the total proline pool.

The focus of the present study was on the involvement of aminopeptidases in the stress response, having in view their multiple roles in plants as part of the total proteolytic system which upregulation is a relatively late response to severe drought, as well as a possible link between AP activities and the major redox buffers, especially glutathione. Besides the reported role of Leu-AP in glutathione turnover by degrading Cys-Gly (Cappiello et al 2004, Kumar et al 2015), the release of amino acids by APs can directly provide glutamate, cysteine, glycine or can fuel Krebs cycle, supplying precursors of the necessary amino acids for glutathione synthesis. Ascorbate pool cannot be directly linked to AP activities but these major redox buffers are closely interconnected via the ascorbate-glutathione cycle, the main ROS detoxification system in cytosol, mitochondria plastids and (Noctor

secondary oxidative stress is commonly observed under many stresses including drought. The ROS scavengers ascorbate and glutathione, which intracellular concentration is very high, typically in the range of 1-10 mM, are essential for direct protection of cell constituents from indiscriminate damage (Chaudiere and Ferrari-Iliou 1999). Like proline, both redox buffers have multiple additional functions. Ascorbate is the major primary antioxidant reacting directly with ROS $(OH^{\bullet}, O_{2}^{\bullet})$ and $^{1}O_{2}$). Besides, as a secondary antioxidant ascorbate acts as a cofactor for violaxanthin de-epoxidase for the formation of zeaxanthin; it is also involved in the regeneration of α -tocopherol by reducing its oxidized form, thus helping in membrane damage prevention (Noctor and Foyer 1998). Additionally, ascorbate serves as a precursor for synthesis of oxalate and tartrate, acts as cofactor for monooxygenase and dioxygenase type enzymes, keeps metal ions in the active site of enzymes in a reduced state, has a role in the control of growth and development processes, cell division and elongation, in cell wall metabolism, acts as a co-factor for the biosynthesis of ethylene, gibberellins and abscisic acid (Zhang 2013). Glutathione is the predominant non-protein thiol, redoxbuffer and substrate for keeping ascorbate in reduced form in the ascorbateglutathione pathway, but also has multiple additional roles in detoxification of xenobiotics, pathogen response, in heavy metal tolerance as phytochelatin precursor, in signaling of Sulphur status (Tausz et al 2004). Dynamic changes in the pools and oxidation/reduction state of the antioxidant metabolites are described

and Foyer 1998). Development of

under drought with increased pools at the beginning of the water stress and diminution when the stress becomes more severe (Dalmia and Savhney 2004). Some impairment in the dynamics of ascorbate and glutathione in drought response is reported in Arabidopsis - glutathione being involved in the early response, to signal drought stress from roots to leaves, whereas ascorbate remaining unchanged in most cell compartments until late stages of drought (Koffler et al 2014). In our study redox buffers presented total rise in their pools under severe drought with some increase in their oxidized forms. Compared to leaves, roots contained less ascorbate and glutathione in absolute values and higher proportion of their oxidized state, in concert with the higher oxidative strain in roots under soil drought stress. Glutathione pool was more mobilized in the tolerant variety Yantar (compared to the sensitive one), without significant changes being observed in root ascorbate pool. As for variety Miziya, the ascorbate pool was more involved especially at recovery. It is tempting to speculate that enhancement of root glutathione pool in the tolerant variety could be partly due to stronger raise in AP activities, which could supply more substrates for glutathione synthesis.

The applied stress led to reversible changes in protein profiles in both varieties. Protein loss was inversely related to increase in some bands of proteolytic activity in leaves. Similar relation was registered for other wheat varieties under the same experimental conditions (Simova-Stoilova et al 2010). As a part of the total proteolytic system, the response of AP activities under severe drought in leaves could be related

to their function in terminal peptide degradation and amino acid recycling a function in partnership with the major proteolytic systems in cells (Walling 2006). Besides, the released amino acids could fuel anaplerotically the tricarbonic acid cycle, two - providing the glycogenic amino acids alanine and glycine, two the ketogenic amino acids leucine and lysine, the rest four - amino acids going to the Krebs cycle through 2-oxoglutarate (arginine and proline), through succinvl-CoA (metionine) and through fumarate (phenylalanine). Additionally, the released amino acids could be used as building blocks for de novo synthesis of proteins (leaves, recovery) or as intermediates for other syntheses (roots, drought, highest activities releasing ketogenic amino acids which could provide acetyl CoA for various syntheses). In both these cases. AP activities seem to be rather independent from the major proteolytic activities revealed by the zymograms. The reported broad substrate specificity for some of the APs (Kidrič et al 2014a) obstructs detection of distinct APs with the different substrates used in the study. Assays implementing protease inhibitors would differentiate between metallo, serine of cysteine type APs. In a previously published work with young spring wheat seedlings (4-6 days) two aminopeptidases were detected (Miazek and Zagdanska 2008): one metalloenzyme with broad substrate specificity (Phe-, Leu-, Arg-, Ala- and Gly-β-NAs) and second cysteine type enzyme with narrow substrate specificity (Phe- and Leu-β-NAs). For better distinction of different APs, at least an initial purification step should be run (Blätter and Feller 1988, Budič et al 2016). Nevertheless, in our study severe

drought resulted in increased AP activities in leaves towards most of the substrates used, with the exception of Phe-pNA and Gly-pNA. The latter two activities raised during the recovery phase. Based on similarities in activity profiles, at least two distinct APs might be involved in drought response – one with a broad and the other with a narrower substrate specificity. The tolerant variety Yantar presented higher AP activities in recovery toward five of the used substrates, which highlights the importance of amino acid metabolism in the recovery from stress. Interestingly, the roots of the two varieties presented more differences in the response of AP activities to the applied stress. Increased AP activities were found in the tolerant variety Yantar at severe drought with most of the substrates used, whereas the sensitive variety Miziya had almost unchanged AP activities in roots under stress. It is worth mentioning the strong increase in gly-AP activity role in recovery from stress, which probably reflects a distinct AP with a special function. It remains to be established which activities towards different substrates reflect distinct aminopeptidases.

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