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SEMI-QUANTITATIVE RT-PCR ANALYSIS OF SELECTED PROTEASE INHIBITORS IN DROUGHT-STRESSED *TRITICUM AESTIVUM*

Vaseva I.[1*], G. Zehirov[1], V. Stoychev[1], E. Kirova[1], L. Simova-Stoilova[1], J. Sabotič[2], J. Šuštar-Vozlič[3], V. Meglič[3], M. Kidrič[2]

[1]Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Sofia, Bulgaria, address: Acad. G. Bonchev Str., Bld. 21, 1113, Sofia, Bulgaria

[2]Jožef Stefan Institute, Ljubljana, Slovenia, address: Jamova cesta 39, SI-1000 Ljubljana, Slovenia

[3]Agricultural Institute of Slovenia, Ljubljana, Slovenia, address: Hacquetova 17, SI-1000, Ljubljana, Slovenia

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Summary: Proteases and their specific inhibitors are ubiquitously distributed and play a key regulatory role in many biological processes. Gene expression and activity of certain proteases has been shown to increase in *Triticum aestivum* L. leaves under drought, with a major contribution of cysteine proteases, especially in sensitive wheat varieties. However, little is known about the stress response of protease inhibitors (PIs) and their role in the regulation of intracellular proteolysis. In this study the changes in transcript abundance of some protease inhibitors (belonging to cystatin and serpin classes) were evaluated by semi-quantitative RT-PCR in leaves and roots of winter wheat seedlings from two varieties with differing tolerance. The expression of two cysteine proteases in the same samples was also assessed. The expression of the studied genes was compared in the tolerant variety "Katya" and the more susceptible to water deprivation variety "Sadovo", applying severe but recoverable soil drought. Growth inhibition and stress related parameters confirmed the relatively higher drought sensitivity of variety "Sadovo". Serpin transcript abundance in control roots was higher than in the leaves. An opposite trend was documented for cystatins – the level of their expression was stronger in the non-treated leaves compared to roots. Drought stress inhibited PI expression in roots, while varying effects on the transcript levels were detected in the leaves of water deprived plants. The levels of the two cysteine protease transcripts under drought exhibited organ-specific response – they declined in roots, and increased in leaves. Further detailed studies using more sensitive methods are necessary to evaluate the potential of protease inhibitors as biochemical markers for drought tolerance.

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Keywords : Cystatin; drought; leaves; roots; serpin; wheat.

Abbreviations: EL – electrolyte leakage; FW – fresh weight; MDA – malondialdehyde; PI - protease inhibitors; WD – water deficit; Ta – annealing temperature.

*Corresponding author: irina.vaseva@abv.bg

INTRODUCTION

Drought is one of the most deleterious stresses affecting growth, productivity and survival of plants (Chaves and Oliveira, 2004). With water supply challenges in terms of global climate change the introduction of plant science achievements into agricultural practice can assist in optimizing crop yield and survival under unfavourable environment. Such benefit is the so-called "marker-assisted breeding" which is based on different biochemical and genetic markers that are linked to important traits such as disease resistance, stress tolerance, high yield, etc.

Proteases and their specific inhibitors are ubiquitously distributed and play a key regulatory role in many biological processes (Grudkowska and Zagdanska, 2004; Kidrič et al., 2014). The importance of proteolytic activity under drought comprises the following major points: rearrangement of metabolism through selective degradation of key enzymes; degradation of short-lived proteins involved in cell signaling; removal of oxidatively damaged, improperly folded or irreversibly denatured proteins; recycling of carbon-starvation-related amino acids and hastening of senescence under source-sink regulation; protection against potential biotic stress (Vaseva et al., 2011). Besides their obvious role in plant protection against pests and pathogens, increasing evidence supports the essential role of naturally occurring protease inhibitors (PI) for regulating the activity of endogenous proteases as well (Roberts and Hejgaard, 2008; Benchabane et al., 2010). Gene expression and activity of certain proteases has been shown to increase in wheat under drought, with a major contribution of cysteine proteases especially in sensitive wheat varieties (Grudkowska and Zagdanska, 2004; Simova-Stoilova et al., 2010). The changes of plant PI expression profiles are still scarcely addressed.

The cysteine protease inhibitors (cystatins) belong to MEROPS inhibitor family I25, clan IH (Rawlings et al., 2014). They inhibit mainly peptidases of families C1 (papain family) and C13 (legumain family) which are largely involved in intracellular protein degradation. Cystatins not only have the capacity to regulate normal physiological processes, but they also participate in defence mechanisms against biotic and abiotic stress (Pernas et al., 2000; Diop et al., 2004; Massonneau et al., 2005). Recently an improved osmotic tolerance has been reported for transgenic yeasts overexpressing plant cystatin gene (Jangpromma et al., 2014).

Serine PIs block the activity of serine proteinases. Plants contain a variety of serine protease inhibitors, which can be divided into at least 12 families (Rawlings et al., 2014). In the present study, the expression of two serpin genes under severe drought was monitored. Serpins are the so-called "suicide" type inhibitors. They undergo a unique and dramatic conformational change when bind their target proteases (Roberts and Hejgaard, 2008). It has been suggested that plant serpins differ in their physiological function compared to the PIs of the same class from the animal kingdom. Plant serpins, a large part of which belong to clade P of the serpin superfamily, were shown to participate in defence against insect predators (Roberts and Hejgaard, 2008). In Arabidopsis another serpin

type which belongs to Clade B was identified – AtSerpin1. It has been shown that the major *in vivo* target of AtSerpin1 is protease RD21 (Lampl et al., 2010; Fluhr et al., 2012). Previous studies demonstrated that RD21 was up regulated under drought and senescence (Koizumi et al., 1993; Yamada et al., 2001).

The semi-quantitative RT-PCR profiling of two cystatins (WC-1 and WC-4) and two serpins (serpin 1 and serpin 2) in plants from two winter wheat varieties differing in drought tolerance was performed in order to outline the effect of water deprivation on the transcript abundances of the two PI classes and to distinguish probable candidates for molecular markers.

MATERIALS AND METHODS

Plant material, growth and treatment

Two winter wheat varieties differing in drought tolerance – „Katya", considered as highly drought-tolerant, and „Sadovo" – a standard and widely distributed variety which is less drought-tolerant (Simova-Stoilova et al., 2006; 2010) were used. Plants were grown as soil cultures in leached meadow cinnamon soil (pH 6.2, optimally fertilised with N, P and K) under 190 $\mu E.m^{-2}.s^{-1}$ PAR at 21– 25°C, 16-h photoperiod and relative soil humidity of 70%. Drought stress was imposed on 8-day-old plants with a fully developed first leaf and expanding second one, by withholding irrigation for 7 days, followed by 3 days of recovery in which watering was resumed. Control plants (C, 15-days-old and RC, 18-days-old) were watered daily. Analysis was performed with samples derived from the first leaf and from roots.

Analysis of stress intensity

Samples were taken at a fixed hour in the morning. To estimate growth inhibition, shoot length and shoot fresh weight were registered in control, drought stressed and recovered plants. Leaf water deficit (WD) expressed in percentage was determined by measurement of the actual leaf fresh weight (FW), the weight of the same leaves at full turgidity (TW) and calculated using the formula (TW-FW)/ TW. Membrane integrity of the first leaf was evaluated by the measurement of the ratio between electrolyte leakage from 2 cm leaf segments floating on distilled water for 16 h at 8°C, and the total leaf electrolyte content released after boiling segments for 10 min in the same effusate (expressed in percentage). Leaves used for proline and malondialdehyde (MDA) assays were frozen and stored in liquid nitrogen until analyses. Proline was determined by the method of Bates et al. (1973). Lipid peroxidation was estimated using the thiobarbituric acid reactive substances assay according to Hodges et al. (1999).

Semi-quantitative two step RT-PCR

The semi-quantitative two step RT-PCR analysis was performed with first fully developed leaves and roots. After harvesting the samples were quickly frozen in liquid nitrogen and preserved at -80°C until analysis. The expression of the genes was evaluated at the beginning of the stress treatment (Day 8), after 7 days of water deprivation (Day 15) and after a 3-day recovery period (Day 18) (Figs. 1, 2 and 3). Total RNA was extracted from 100 mg plant material (first true leaf or roots) with RNeasy Plant mini Kit (QIAGEN). RNA samples (400 ng) were reversely transcribed at 37°C

for 1 h with 2 mM anchored oligo(dT_{23}) primer (Sigma-Aldrich) using Omniscript Reverse Transcription Kit (QIAGEN). PCR reactions (50 μL) containing 2 μL RT assay were performed with HotStart Taq polymerase (QIAGEN) according to the manufacturer's protocol. The cycling conditions were 15 min at 95°C, 30 cycles of 94°C for 1 min, amplification for 40 sec and 72°C for 1 min. The amplification temperatures were as follows: Ta=63°C for Ta.61026 thiol protease (AY253445), WCP2 peptidase (AB109216), and cystatin WC-4 (DQ279930); Ta=58°C for cystatin WC-1 (DQ279929), serpin-2 (FJ705437) and α-tubulin (U76558); Ta=60°C for serpin-1 (FJ705436). The final extension step was for 10 min at 72°C. The expression of α-tubulin was used as internal control for normalization of the expression levels of the studied genes (Fig. 1C). The used primer sequences are listed in Table 1. RT-PCR reactions were loaded on at least two different ethidium bromide-stained 1% agarose gels, and quantification of bands revealed during the different runs was performed with ImageJ 1.30v software (National Institutes of Health, Bethesda, MD, USA). Figures 1, 2 and 3 visualize gel images from one representative experiment. Processed data represent percentage of the average area of the ethidium bromide-stained agarose gel occupied by each band. The graphs (Figs. 1, 2 and 3) depict target PI genes and the α-tubulin expression ratio.

RESULTS

The parameters characterizing the intensity of the stress experienced by the plants are presented in Table 2. The applied drought stress significantly affected shoot biomass in both varieties. The growth of the aboveground parts was resumed in the recovered plants. The drought resistant variety "Katya" developed smaller leaves and shorter shoots compared to variety "Sadovo". The relative water content measured in the stressed leaves characterized the applied drought as strong but reversible. This was further supported by the increased content of stress markers detected in the water-deprived plants. The tolerant variety "Katya" developed water deficit of about 59%, while in the sensitive variety "Sadovo" it reached 71%.

Proline accumulation, increased membrane permeability (determined by electrolyte leakage) and membrane lipid peroxidation (MDA content) are some of the most commonly used stress-markers for characterization of the stress intensity experienced by plants. The higher electrolyte leakage in the age controls of recovery could indicate early signs of natural senescence of the tested first leaf. The susceptible variety "Sadovo" experienced more severe leaf membrane damages as a result of the applied stress. The MDA content measured in the leaves of the drought-sensitive variety was also distinctly higher than the one detected in the samples of the tolerant variety "Katya". After the recovery MDA levels reached the controls, which indicated that the applied stress, despite its strong physiological effect remained reversible.

The accumulation of serpin 1 and 2 transcripts in the two wheat varieties is presented in Fig. 1. The control roots of both varieties "Katya" and "Sadovo" exhibited higher serpin expression compared to leaves (Fig. 1A, B). In the recovered var. "Sadovo" organs the accumulation of serpin transcripts was

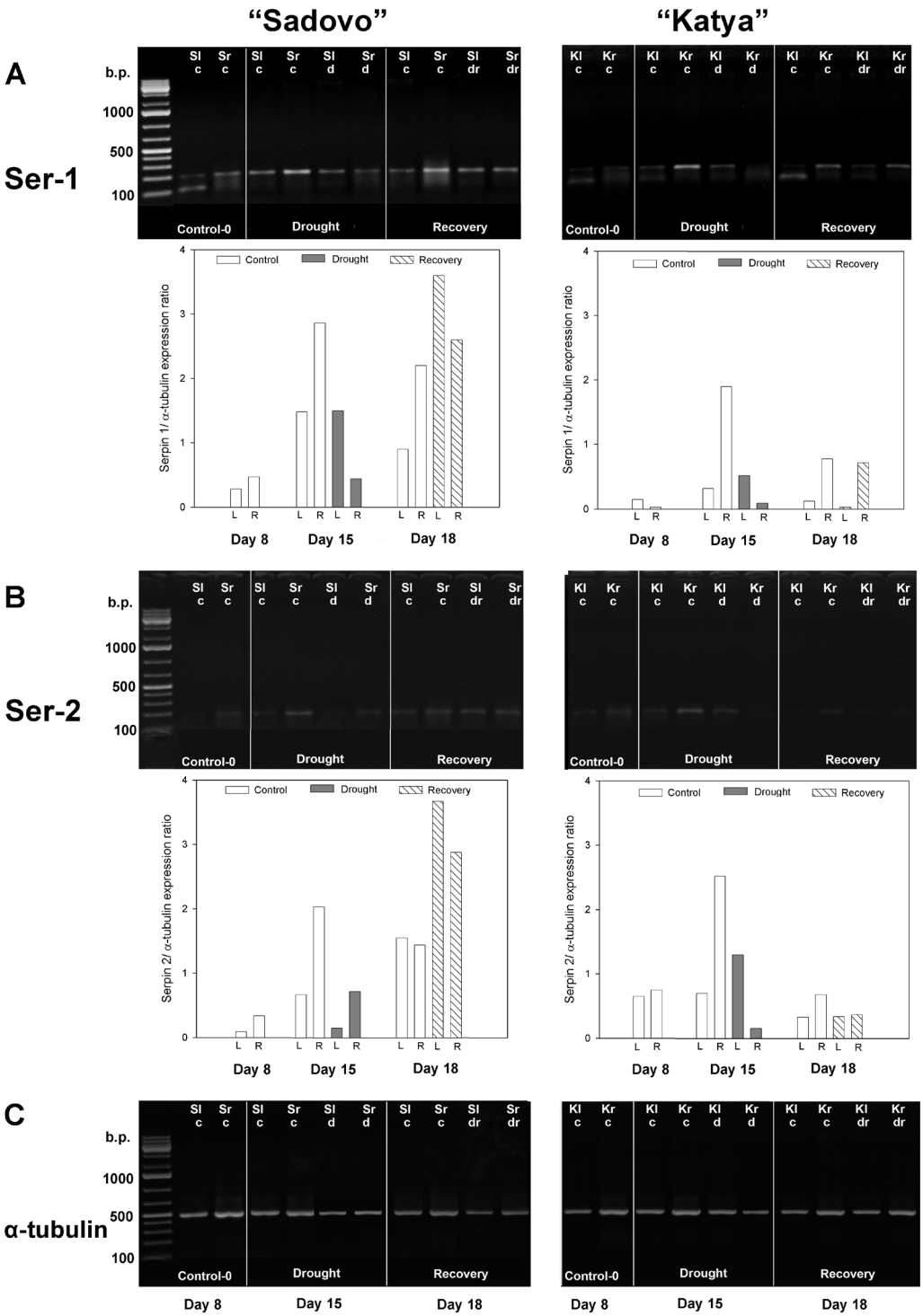

Figure 1. Expression of serpin genes in leaves (l) and roots (r) of two winter wheat varieties, "Sadovo" (S) and "Katya" (K). **A**. Ser-1 (FJ705436.1); **B.** Ser-2 (FJ705436.1) normalized to α-tubulin (U76558) (**C**). Expression profiles were monitored at day 8 (beginning of the stress period), day 15 (7 days of water deprivation), and day 18 (3 days of recovery by resuming watering).

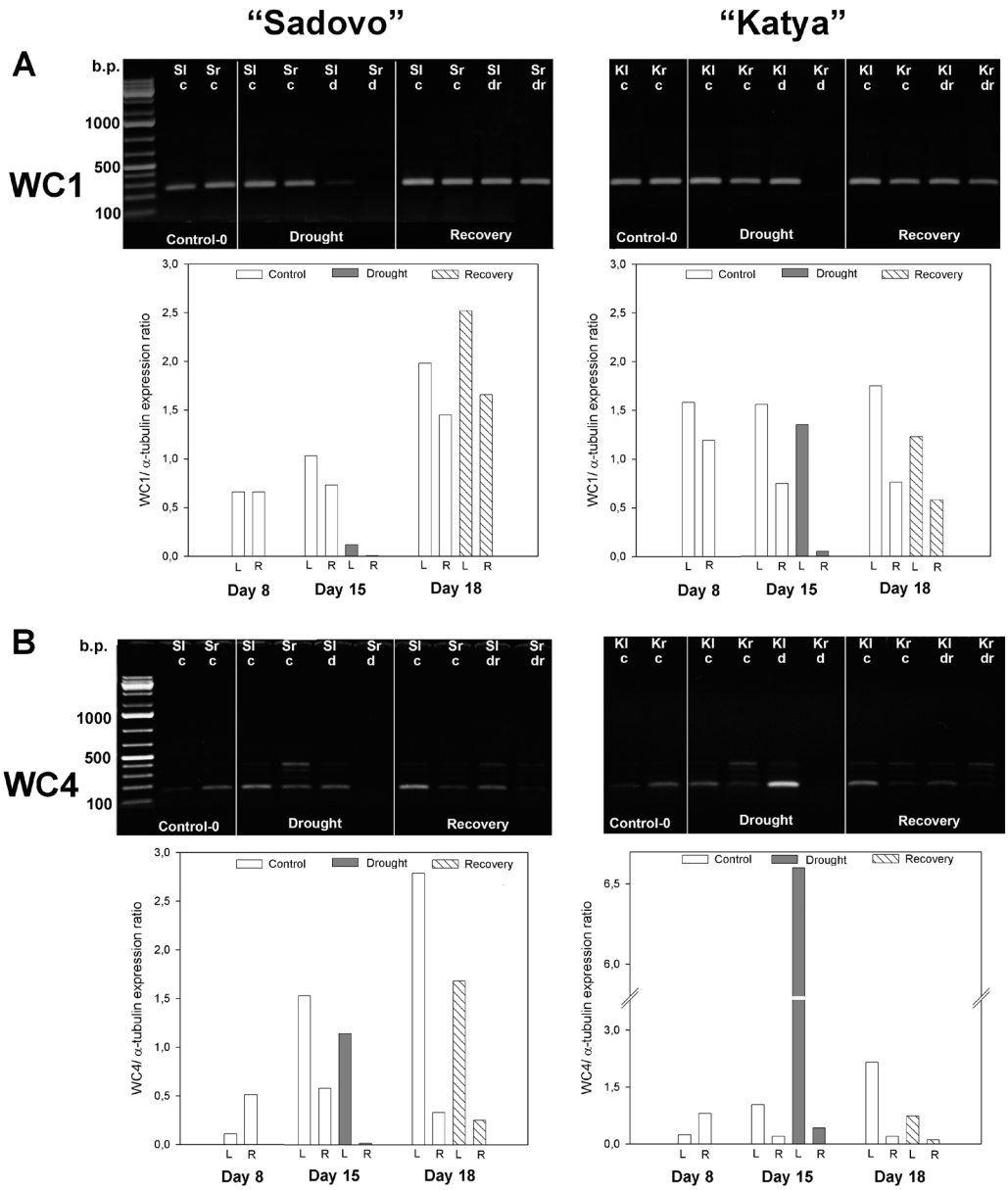

Figure 2. Expression of cystatin genes in leaves (l) and roots (r) of two winter wheat varieties, "Sadovo" (S) and "Katya" (K). **A.** WC1 single domain cystatin (DQ279928.1); B WC4 single domain cystatin (DQ279930) normalized to α-tubulin (U76558). Expression profiles were monitored at day 8 (beginning of the stress period), day 15 (7 days of water deprivation), and day 18 (3 days of recovery by resuming watering).

relatively higher than the one observed in rehydrated var. "Katya" leaves and roots (Fig. 1A, B).

The control plants of both varieties exhibited higher cystatin expression in leaves than in roots (Fig. 2). The expression profiles of WC1 and WC4 clearly indicated that the divergent PI transcript accumulation provoked by drought was more distinct in leaves (Fig. 2A, B). The tolerant variety "Katya" contained higher cystatin transcripts in the drought-stressed

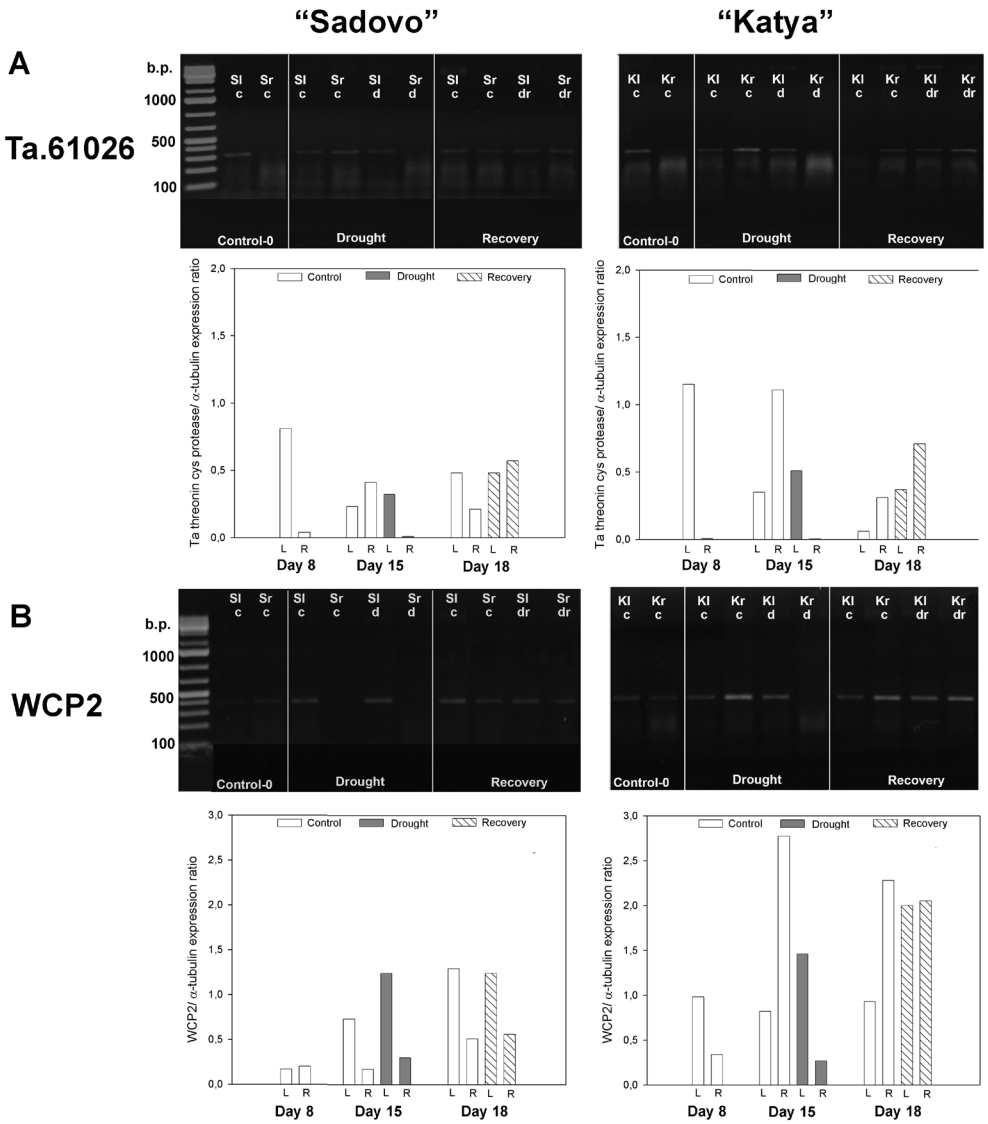

Figure 3. Expression of two cysteine proteases in leaves (l) and roots (r) of two winter wheat varieties, "Sadovo" (S) and "Katya" (K). **A.** Ta.61026 putative thiol protease (AY253445); **B**. WCP2 peptidase (AB109216) normalized to α-tubulin (U76558). Expression profiles were monitored at day 8 (beginning of the stress period), day 15 (7 days of water deprivation), and day 18 (3 days of recovery by resuming watering).

leaves than var. "Sadovo". Under drought, WC1 expression was suppressed in both "Sadovo" samples (leaves and roots), and in "Katya" roots, while it remained relatively stable in drought-stressed "Katya" leaves (Fig. 1A). WC1 transcript content in the recovered plants was similar to the controls after the stress relief (Fig. 1A).

The semi-quantitative RT-PCR analyses of two cysteine proteases: Ta.61026 – a putative thiol protease, and WCP2 – peptidase of papain type (Fig. 3) showed that the leaves accumulated higher transcript amounts compared to the roots – almost no signal of Ta.61026 thiol protease was documented in the root

Table 1. Primers used in semi quantitative RT-PCR.

Primer	Description	Sequence (5'-3')
Cys-protease inhibitors		
WC	Forward	CGCCCGCTTCGCCGTCTC
WC1	Reverse	AGCTGGGACGCGCCTTATGAGTTA
WC4	Reverse	TACAGCTTCTTTGCCCCGCCTTCA
Ser-protease inhibitors		
Serpin	Forward	CCACCGAYGTCYGCCTCTC
Serpin 1	Reverse	TCCCGAGTGTGGCGACGAGTTG
Serpin 2	Reverse	CCAGCGCCGGCAGTAATGAGG
Cys proteases		
TA.61026	Forward	CTGCACGAGCGGCGAGATGT
TA.61026	Reverse	CGAGCACGGGCCAAAGTATTC
WCP2	Forward	TTCCGCTCGTTGGCTCTCCTC
WCP2	Reverse	CCGCCCCCTCGACAACATCTC
Tubulin primers		
tubulin	Forward	AGCGCCTTTGAGCCTTCGTCC
tubulin	Reverse	TCATCGCCCTCATCACCGTCC

Table 2. Stress intensity parameters measured in leaves.

Parameter	variety Sadovo				variety Katya			
	C	D	R	RC	C	D	R	RC
Shoot FW [g]	1.49	0.274	0.789	1.76	1.335	0.220	0.70	1.356
Shoot Length [cm]	28.8	20.3	25.4	32.7	28.5	16.57	23.5	29.7
Water deficit [%]	5.66	71.10	13.79	14.38	5.3	59.15	11.9	4.72
Electrolyte leakage [%]	2.84	65.44	6.49	13.24	5.32	46.74	6.4	11.0
Proline [$\mu g.g^{-1}FW$]	8	6960	7	8	11	7600	9	10
MDA [$nmol.g^{-1}FW$]	9.05	56.46	10.55	12.99	12.48	24.95	11.5	12.2

For shoot fresh weight (FW) and shoot length, n=8, for water deficit and electrolyte leakage, n=2, for proline and MDA n=3. Mean values are given. C – controls (same age as drought treated, 15 days old plants), D – drought, R – recovery, RC – controls of recovery (18 days old plants).

samples. The transcript abundances of these cysteine proteases increased in the leaves and diminished in the roots under drought in both varieties. After recovery cys-protease transcripts did not show a clear trend.

DISCUSSION

Marker-assisted selection is an important and more widely accepted field in agricultural biotechnology. Recent studies have reported on the promising potential of certain protease inhibitors as biochemical markers for selection of pathogen resistant varieties (Feldman et al., 2014; Yarullina et al., 2014). It has been shown that potato protease inhibitor PLPKI could be used as a suitable biochemical marker to help breeders in the selection of cultivars with high degree of horizontal resistance (Feldman et al., 2014). Another study reported an infection-induced up-regulation of a gene, which codes for a protease inhibitor in wheat leaves with reduced disease symptoms (Yarullina et al., 2014).

In the present study, we performed evaluation of expression of some protease inhibitor genes as an initial step towards more detailed characterization and analysis of their potential as markers for drought tolerance. An interesting trend regarding serpin and cystatin transcript abundance in the control plants was observed – serpins were predominantly expressed in roots while cystatins were presented at higher levels in leaves. It could be linked to different protease composition in the respective organs and the presumed role of protease inhibitors in regulating distinct endogenous proteases.

Several widely used stress markers are monitored for the assessment of severity of drought – free proline and MDA contents, and electrolyte leakage, the latter being an informative parameter for cellular membrane integrity. Proline plays an important role in the adaptation of organisms to drought, regulating the osmotic pressure in the cell and contributing to water balance improvement. Besides, it protects membranes and proteins from denaturation and has the ability to scavenge free radicals (Ashraf and Foolad, 2007). The amount of free proline in cells is highly responsive to dehydration of the cytoplasm and therefore, it is an indicator for the strength of the stress. The other biochemical marker MDA is related to the oxidation of lipids, resulting in membrane damage. The physiological outcome of lipid peroxidation comprises change in membrane potential, increased permeability to H + and other ions, and membrane rupture followed by release of cellular/organellar content (as vacuolar hydrolytic enzymes for example). Some of the products of lipid peroxidation are cytotoxic (Mittler, 2002). The values of MDA content and electrolyte leakage, as well as the accumulation of proline in the water-deprived wheat plants characterized the applied drought as severe but recoverable. One of the most consistent observations regarding the expression profiles of the studied PIs under severe water deprivation was that cystatins responded to dehydration to a higher extent compared to serpins, and that the most distinct changes were observed in leaves. These data are in agreement with previous studies which report a considerable increase in the activity of intracellular proteases under drought in leaves with a major contribution of cysteine proteases

(Grudkowska and Zagdanska, 2004). Levels of proteases are increasingly seen to be associated with stress tolerance (Kidrič et al., 2014). Some experimental evidence suggests that drought-sensitive species and cultivars have higher proteolytic activity compared to resistant ones (Simova-Stoilova et al., 2006; 2010). In order to relate these results to the changes in PI expression profiles we plan to apply more sensitive techniques such as Real Time qRT-PCR to monitor protease inhibitor gene expression in increased number of winter wheat varieties.

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