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Transcript Profiling of Serine- and Cysteine Protease Inhibitors in *Triticum aestivum* Varieties with Different Drought Tolerance

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A high number of protease inhibitors (PI) have been identified in diverse plant species but information about their role in plant stress responses is still fragmentary. Transcript profiling of six published serine and cysteine protease inhibitor sequences in water-deprived plants from four winter wheat *(Triticum aestivum)* varieties with varying tolerance was performed in order to outline PIs predominantly accumulating under drought. Expression was analyzed by real time RT-qPCR. Considerable transcript accumulation of Bowman–Birk type PI WALI3 (BBPI) was detected in drought stressed leaves suggesting an important regulatory role of BBPI in adjustment of protein metabolism in leaves under dehydration. Serpin transcripts were less represented in water-deprived plants. Transient accumulation of cystatin transcripts revealed organ-specificity. Under drought cystatin and serpin expression in the leaves of the most drought tolerant variety "Katya" tended to preserve relatively stable levels close to the controls. This preliminary data will serve for future detailed study of regulation of proteolysis in winter wheat subjected to unfavorable environmental factors for development of molecular-based strategies for selection of tolerant varieties.

Keywords: Bowman–Birk protease inhibitors, cystatin, drought, serpin, wheat

Introduction

Changes in protein composition, expression and post-translational modifications are substantial part of plant development and response to various abiotic and biotic stresses. Proteases are the principal enzymes controlling cellular protein complement and the steady state level of individual proteins through selective protein breakdown (López-Otín and Bond 2008). Endogenous protease inhibitors (PIs) play role in regulation of the proteolytic activity and are ubiquitously distributed in animals, plants and microorganisms (Mosolov and Valueva 2005; Kidrič et al. 2014). Plant PIs have a major role in the defense against insect and pathogen attack. Accumulating evidence point out their active involvement in abiotic stresses (Kidrič et al. 2014) and recently stress responsive elements were identified in the promotor region of some PIs (de Almeida et al. 2012).

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PIs comprise of large and structurally diverse group, classified into 48 families on the basis of similarities in amino acid sequences, and on the basis of three dimensional structure – to 26 clans. Continuously updated database of PIs is available at http://merops.sanger.ac.uk (Rawlings et al. 2014).

Cystatins belong to MEROPS PI family I25, clan IH (Rawlings et al. 2014). They mainly inhibit peptidases of families C1 (papain family) and C13 (legumain family). Some studies suggest that cystatins participate in the defence against biotic and abiotic stress (Pernas et al. 2000; Diop et al. 2004; Massonneau et al. 2005; Zhang et al. 2008). Overexpression of some plant cystatins has conferred osmotic stress tolerance (Jangpromma et al. 2014; Quain et al. 2014).

Plants also contain variety of serine protease inhibitors, which can be divided into at least 12 families (Rawlings et al. 2014). Serpins are unique by their suicide-type irreversible inhibitory mechanism and may be involved in stress accelerated senescence and plant cell death (PCD). Serpin genes are up-regulated by salt stress in *Arabidopsis* (Ma et al. 2006).

Bowman-Birk PIs (BBPIs) are canonical inhibitors of serine protease of the trypsin and chymotrypsin type (de Almeida et al. 2012). A BBPI was shown to be involved in the tolerance to salt stress in wheat (Shan et al. 2008) and *Arabidopsis* (Wang et al. 2014). BBPI was up regulated by water deficit in peanut leaves (Dramé et al. 2013).

The aim of the present study was to monitor PI transcript accumulation in four winter wheat varieties with differing drought tolerance under dehydration and after recovery.

Materials and Methods

Plant material, growth and treatment

Plants from four Bulgarian winter wheat varieties (Simova-Stoilova et al. 2006) with differing drought tolerance (cv. "Katya – drought-tolerant, cv. "Yantar", cv. "Miziya" and cv. "Sadovo" – less drought tolerant) were grown in leached meadow cinnamon soil (pH 6.2, optimally fertilised with N, P and K) under 180 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR at 16-h photoperiod, 21°/25 °C day/night temperature and 70% relative soil humidity maintained by daily watering. Each pot, containing 20 plants (five plants per variety), was considered as an independent biological repeat. Plants from the four tested wheat varieties were grown in one and the same pot to exclude variations in water supply and water deprivation during the different experimental stages. Drought stress was imposed on 8-day old seedlings with fully expanded first leaf and developing second one, by withholding irrigation for 7 days, followed by 3 days of recovery. Controls were watered daily. Leaf (material collected from the first leaf only) and root samples from three independent biological experiments were frozen in liquid nitrogen and stored at –80 °C until analyzed by RT-qPCR.

Relative water content

Relative water content (RWC) was measured in the first fully expanded leaf after 7 days of water deprivation (which equals to 15 days after germination – 15 DAG), and after 3 days of recovery (18 days after germination – 18 DAG). Leaf material for determination of RWC was taken from at least five individual plants of each variety obtained from 3 to 5 biological repeats (different pots). Fresh weight (FW) was immediately recorded after their excision. Turgid weight (TW) was measured 24 h after soaking leaves in distilled water at 4 °C. Dry weight (DW) was measured after drying for 24 h at 104 °C. Leaf relative water content (RWC) was calculated according to Barrs and Weatherley (1968): RWC (%) = [(FW-DW)/(TW-DW)] × 100.

RNA extraction, synthesis of cDNA and RT-qPCR

Total RNA was extracted from 100 mg plant material with RNeasy Plant mini Kit (QIAGEN). DNase-treated RNA samples (0.2 μg) were reverse transcribed with Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific) according to the manufacturer's instructions. RT-qPCR was performed with 'PikoReal' Real-Time PCR System with Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific) according to the protocols provided by the manufacturer. Primers used to amplify the studied PIs (GenBank IDs: serpin-1 – FJ705436.1, serpin-2 – FJ705437.1, WALI3 – L11881.1, TaMDC1 – AB223039.1, WC1 – DQ279928.1 and WC4 – DQ279930) and the reference gene tubulin (GenBank ID: U76558, for normalization of relative quantification) are listed in Table 1. The qPCR was performed on three independent biological repeats and the analy-

Table 1. Primers used in RT-qPCR experiments

Primer	Description	Sequence (5'-3')
Cys-protease inhibitors		
TaMDC1 TaMDC1	Forward Reverse	CGCCGTCGACGAGCACAACA GCCACGGCTTGACCCAGACCTT
WC WC1 WC4	Forward Reverse Reverse	CGCCCGCTTCGCCGTCTC AGCTGGGACGCGCCTTATGAGTTA TACAGCTTCTTTGCCCCGCCTTCA
Ser-protease inhibitors		
Serpin	Forward	CCACCGAYGTCYGCCTCTC
Serpin1	Reverse	TCCCGAGTGTGGCGACGAGTTG
Serpin 2	Reverse	CCAGCGCCGGCAGTAATGAGG
WALI	Forward	GGTGACCAGGCAATTTTTCTTT
WALI 3	Reverse	CTTGAACTGGAGGAGCGACGAT
Tubulin primers		
Tubulin	Forward	TTCTCCCGCATCGACCACAAGTTT
Tubulin	Reverse	TCATCGCCCTCATCACCGTCC

Table 2. An overview of relative serine (Serpin-1, Serpin-2 and Bowman–Birk WALI3) and cysteine inhibitors (WC1, WC4 and TaMDC1) expression in drought stressed and recovered plants from four winter wheat varieties: "Yantar" (Y), "Miziya" (M), "Katya" (K) and "Sadovo" (S)

PIs		Organ															
		Leaves								Roots							
		Relative Expression in Treatment															
		Drought				Recovery				Drought				Recovery			
		Y	M	K	S	Y	M	K	S	Y	M	K	S	Y	M	K	S
Ser-PIs	*Serpin-1*	<0.1	<0.1	* 0.7 ±0.5	<0.1	0.1	0.2 ±0.1	1.1 ±0.7	1.5 ±1.0	2.8 ±1.0	10.3 ±4.5	*** 17.2 ±1.9	1.0 ±0.3	** 0.8 ±0.1	0.1	** 0.8 ±0.3	1.8 ±0.5
	Serpin-2	<0.1	<0.1	*** 0.5 ±0.1	<0.1	3.9 ±1.7	0.6 ±0.1	** 0.1	0.8 ±0.1	1.1 ±0.3	1.5 ±1.1	1.7 ±0.3	1.5 ±0.9	** 8.1 ±1.3	0.1	** 7.9 ±5.2	0.1
	WALI3	*** 99.3 ±13.3	133.0 ±22.9	167.8 ±31.0	220.7 ±37.1	24.7 ±0.5	13.3 ±3.3	*** 3.8 ±0.8	62.0 ±26.8	5.2 ±0.6	4.9 ±0.1	* 3.9 ±0.8	5.7 ±0.9	8.1 ±0.1	*** 3.8 ±0.1	10.5 ±0.6	10.9 ±1.2
Cys-PIs	*WC1*	14.5 ±6.6	2.4 ±1.0	* 0.4 ±0.3	15.9 ±10.6	n.d.	2.3 ±1.6	* 0.4 ±0.3	2.6 ±0.9	1.9 ±1.0	3.8 ±1.0	*** 0.4 ±0.3	1.0 ±0.5	0.4 ±0.1	0.8 ±0.1	0.8 ±0.4	1.3 ±0.8
	WC4	1.1 ±0.1	*** 2.6 ±0.1	1.7 ±0.4	0.9 ±0.1	1.0 ±1.0	0.7 ±0.2	** 2.2 ±0.7	** 1.4 ±0.6	** 8.3 ±1.9	23.4 ±4.1	22.2 ±1.1	18.4 ±2.7	1.5 ±0.1	1.4 ±0.1	*** 2.2 ±0.2	1.2 ±0.1
	TaMDC1	0.9 ±0.1	1.8 ±0.3	1.4 ±0.1	1.7 ±0.1	0.4 ±0.0	0.3 ±0.1	1.1 ±0.8	0.7 ±0.3	4.7 ±1.2	6.9 ±0.1	10.7 ±0.8	8.9 ±1.7	1.2 ±0.4	0.9 ±0.5	* 2.0 ±0.4	1.0 ±0.2

Notes: The average expression rates above 0.1 are represented ± standard deviation from three independent biological repeats. The values above 5-fold are marked with a grey background. ANOVA single factor analysis was applied to evaluate statistically different PI expression among the tested varieties. Significant differences are indicated for $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***) n.d. – not detected.

ses were carried out in three technical replicates of 10.0 μL reaction volumes using 1 μL RT reaction derived from 0.2 μg total RNA, with the following cycling parameters: DNA polymerase activation at 95 °C for 15 min – 1 cycle; denaturation at 95 °C for 15 sec, annealing/extension at 60–63 °C for 1 min – 40 cycles, melting curve analysis, with an increase in temperature of 0.5 °C/s from 60 to 95 °C. Triplicate of no template control (NTC) and reverse transcription negative (RT–) reactions were included in all experiments. Performances of qPCR assays were evaluated by amplification of five 10-fold dilutions of cDNA template with the following acceptable high performance: r^2 value ≥ 0.995 and amplification efficiency = 100% ± 10% [efficiency (%) = $10^{-1/\text{slope}} - 1$].

Calculation of relative gene expression using the $2^{-\Delta\Delta Cq}$ method

Relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen 2001). Cq values of three technical replicates from three independent biological experiments were averaged. Then, ΔCq was calculated by normalizing Cq of the target PI gene to Cq of the reference tubulin gene. The average ΔCq values were calculated for the relevant controls. ΔΔCq was calculated by normalizing ΔCq of the samples derived from drought treated plants to average ΔCq of the respective controls.

Statistical analysis

The RT-qPCR data obtained from three independent biological repeats was subjected to ANOVA single factor analyses to evaluate differential expression caused by the treatment. Significant differences are indicated in Table 2 for $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***).

Results

Relative water content

The imposed 7-days water deprivation resulted in significant decrease in RWC of the first fully developed true leaf (Fig. 1) where the accumulation of PI transcripts was analyzed. Drought stressed plants of the sensitive variety "Miziya" exhibited the lowest RWC of 21% after 7 days of water deprivation and it was able to recover it only to 64% (Fig. 1). The performance of the other two varieties ("Sadovo" and "Yantar") was comparable with "Katya" which exhibited the highest recovery of RWC – 96%. These data confirmed the severity and reversibility of the applied stress.

RT-qPCR

The relative expression of the studied PI genes upon drought and recovery is represented in Table 2.

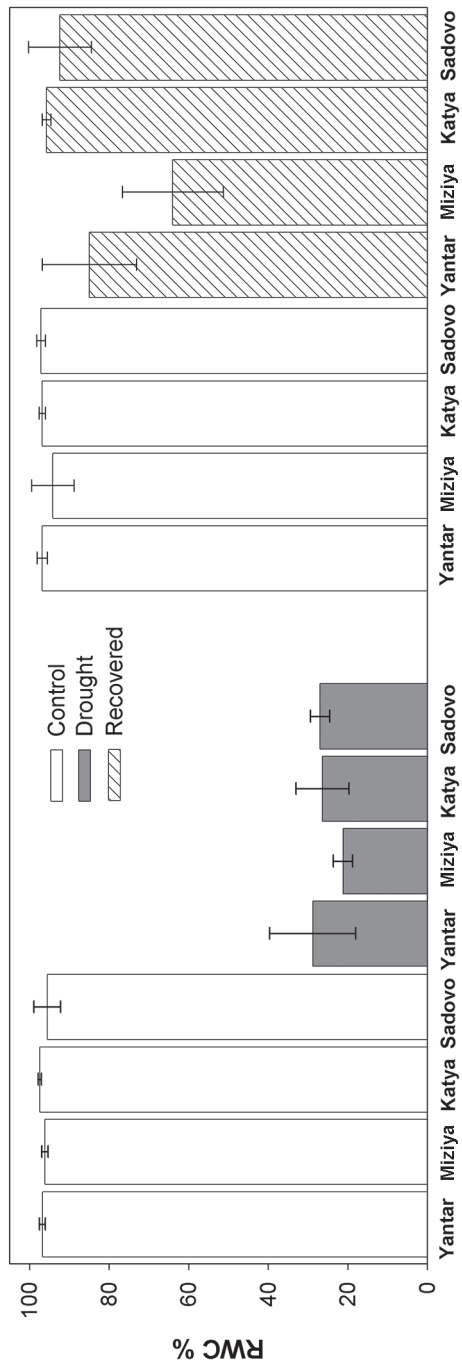

Figure 1. Relative water content (RWC) measured in the first fully expanded leaf after 7 days of water deprivation (15 days after germination – 15 DAG), and after 3 days of recovery (18 days after germination – 18 DAG). Bars represent the standard deviation ($n \geq 5$) among samples derived from at least three biological repeats

Serine protease inhibitors

Considerable transcript accumulation of Bowman–Birk serine protease inhibitor WALI3 (100 to 200 fold compared to non-stressed plants) was detected in dehydrated leaves (Table 2). WALI3 transcript content remained still above the controls upon recovery with drought tolerant "Katya" showing the lowest levels – only 4 times higher than the relevant control (Table 2). The recovered plants from "Sadovo", "Yantar" and "Miziya" showed respectively 62-, 25- and 13-fold levels than their watered controls. WALI3 expression in roots was less influenced by dehydration compared to leaves. The measured WALI3 levels were up to 6 times higher than the controls in the dehydrated roots and remained a bit higher or similar in the recovered plants. Water-deprived leaves of "Katya" preserved relatively stable content of serpin transcripts. The expression of serpins 1 and 2 in the first true leaf was inhibited by drought (Table 2). Serpin-1 expression in roots under drought displayed varying expression and the highest levels were detected in the tolerant variety "Katya". After re-watering the tolerant variety "Katya" and the less drought tolerant "Yantar" exhibited similar serpin contents in roots – serpin-1 transcripts dropped to the control levels while serpin-2 transcripts remained significantly high. Serpin-2 expression in the recovered roots of the other two wheat varieties "Miziya" and "Sadovo" remained below the control.

Cysteine protease inhibitors

The accumulation of cystatin transcripts in stressed plants revealed organ-specificity (Table 2). Cystatin WC1 transcript content increased under drought in the leaves of all the varieties but "Katya" (Table 2). The same trend was observed in drought stressed roots – cv. "Katya" accumulated less WC1 transcripts compared to the rest of the tested winter wheat varieties (Table 2). WC1 transcript levels in recovered roots reached control levels in all varieties except "Yantar" where they remained lower than the ones in the non-stressed age controls. Recovered leaves from the tested varieties exhibited different WC1 transcript profiles with "Miziya" and "Katya" showing similar contents of the transcript as the ones measured in dehydrated leaves. The content of WC1 in "Yantar" was below detection level while in "Sadovo" WC1 transcripts decreased from 16- to 3-fold compared to the control. Water shortage provoked transient accumulation of WC4 and TaMDC1 transcripts in roots which dropped to control level upon stress relief (Table 2). Slightly increased WC4 levels were detected in leaves of the drought sensitive cv. "Miziya" while TaMDC1 expression in leaves was not affected by drought (Table 2). The statistically significant differences among WC4 and TaMDC1 expression profiles under dehydration did not show clear relation to the differences in drought tolerance of the four wheat varieties.

Discussion

Studies on protein stress-responsiveness provide important information regarding discovery of genes conferring stress tolerance. Elucidating the differential expression of such genes in resistant and susceptible varieties could contribute to novel strategies in marker-assisted breeding of crops with improved tolerance to biotic and abiotic stresses (Feldman et al. 2014; Yarullina et al. 2014). In the present study we monitored the expression of a published *T. aestivum* Bowman–Birk type proteinase inhibitor WALI3 under drought and recovery. Earlier study has demonstrated that WALI3 expression is induced by aluminium treatment (Snowden and Gardner 1993). The observed high transcript accumulation of BBPI WALI3 transcripts in drought stressed wheat leaves suggests that this inhibitor has an important regulatory role in adjustment of protein metabolism under dehydration. Another Bowman–Birk type PI WRSI5 was found to be involved in the tolerance to salt stress in wheat (Shan et al. 2008). The same authors observed increased expression of WRS15 in roots exposed to drought and oxidative stress. Induced BBPI expression under drought was registered also in peanut leaves by Dramé et al. (2013) who documented an earlier strong accumulation of the BBPI transcripts in the tolerant peanut variety. Such differential accumulation of WALI3 transcripts in regard to varying drought susceptibility of the tested wheat varieties failed to show a clear trend in the present study. However, the dehydrated leaves of the most drought tolerant "Katya" had very high WALI3 transcript content (167-fold of the control) which dropped quickly to only 4-fold after recovery (Table 2). WALI3 expression profile distinguished "Katya" from the other three tested varieties, which preserved relatively higher transcript levels after rehydration.

Serpins are involved in processes regulating senescence and programmed cell death (PCD) in vegetative tissues (Fluhr et al. 2012; Lampl et al. 2013). In this study they showed differential expression in drought-stressed and recovered leaves while maintained relatively stable transcript content in roots. The observed reverse trends of serpin accumulation in leaves and roots under drought may be attributed to processes related to the stress-induced premature leaf senescence. A clear trend of stable leaf serpin expression, with levels close to the controls in the drought-tolerant variety was documented. This observation could be linked to the higher potential of "Katya" to tolerate suboptimal water availability as shown earlier (Simova-Stoilova et al. 2006, 2010).

The expression of cysteine protease genes under various stresses is related to reorganization of metabolism, remodeling of cell protein components, degradation of damaged or unnecessary proteins and nutrient remobilization (Martínez et al. 2012). Transcript cystatin abundance under drought showed certain organ specificity (Table 2). WC1 transcripts accumulated mainly in drought-stressed leaves while WC4 and TaMDC1 were predominantly expressed in roots. Previously published data showed that the prevailing drought responsive proteases in wheat leaves were of cysteine type and that the drought-sensitive winter wheat varieties had higher proteolytic activity compared to the resistant ones (Simova-Stoilova et al. 2006, 2010). Although PI transcript profiling did not exhibit clear trend in this regard, the most consistent observation was that the drought tolerant "Katya" preserved relatively stable levels of cys-PI transcripts in leaves without evidence

for drastic changes provoked by dehydration (Table 2). This could be linked to lower endogenous proteolysis under water deprivation documented for this variety (Simova-Stoilova et al. 2010). Transcripts of the multidomain cystatin TaMDC1 have been shown to accumulate in wheat seedlings under cold, drought, salt and ABA treatment (Christova et al. 2006). We observed up-regulated TaMDC1 expression only in drought stressed roots (Table 2) with highest levels in the most drought-tolerant variety.

These preliminary data, although failed to outline a reliable discriminant PI gene, demonstrated that under drought both serpins and cystatin WC1 transcript contents remained significantly stable in the tolerant winter wheat "Katya". The considerable BBPI transcript accumulation with subsequent fast drop in the level of the transcript upon recovery in the same variety is an observation which deserves to be addressed in future studies. A broader list of varieties with different genetic background is necessary to validate the potential of the PIs as molecular markers for drought tolerance in winter wheat.

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