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Involvement of proline and non-protein thiols in response to low temperature and cadmium stresses in wheat

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Abstract

The aim of this study was to investigate the effects of low temperature (4 °C), cadmium sulphate (100 µM), or their combination on content of free proline (Pro), glutathione (GSH), and phytochelatin (PCs) in wheat (*Triticum aestivum* L.) leaves. Results revealed an increase in proline and phytochelatin accumulation in leaves of wheat seedlings along with enhanced cold tolerance at the low temperature, CdSO₄, and their combination. Moreover, there were increases in mRNA content of *TaP5CS* and *TaPCS1* genes, encoding Δ^1 -pyrroline-5-carboxylate synthase (P5CS) and phytochelatin synthases (PCS), respectively. A rapid increase in glutathione content was found within 1 h of exposure to the low temperature and its combination with CdSO₄, followed by a drop. However, upregulation of the *TaGS1* gene, encoding glutathione synthetase (GS), was maintained during 7 d. A significant decrease in glutathione content on the seventh day of exposure to the low temperature, CdSO₄, and their combination was most probably due to its involvement in cadmium detoxification and/or in phytochelatin synthesis. Our data suggest that proline, glutathione, and phytochelatin may be important for plant tolerance to low temperature and cadmium stress in wheat.

Additional key words: glutathione, glutathione synthetase, phytochelatin, phytochelatin synthases, Δ^1 -pyrroline-5-carboxylate synthase.

Introduction

Plants in their natural environment are often subjected to various stresses, such as extreme temperatures, heavy metals, drought, salinity, *etc.*, and thus have evolved a multitude of defense mechanisms to increase their tolerance (Nakabayashi and Saito 2015). Adaptation of plants to stresses is linked to mobilization of antioxidants including enzymes and non-enzymatic compounds such as proline, glutathione, tocopherols, flavonoids, and others (Waśkiewicz *et al.* 2014). Proline (Pro), as a multifunctional amino acid, has diverse roles in stress protection such as stabilization of proteins, subcellular structures and membranes, scavenging reactive oxygen species (ROS), and mediating shuttling chemical energy required for recovery from stress (Rejeb *et al.* 2014, Kaur and Asthir 2015). It has been demonstrated that Pro is synthesized from either glutamate or ornithine, whereas under stress conditions, the glutamate pathway usually dominates (Stein *et al.* 2011). Accumulation of Pro in

response to various environmental stresses in plants has been well established (Kishor and Sreenivasulu 2014).

Glutathione (GSH) is a thiol-containing low-molecular-mass tripeptide (γ -Glu-Cys-Gly) with multiple functions in plants (Noctor *et al.* 2012, Gill *et al.* 2013, Delorme-Hinoux *et al.* 2016). Besides the control of ROS accumulation, GSH takes part in the regulation of growth, development, cell cycle, gene expression, and protein activity due to its effect on redox state of the cells (Pivato *et al.* 2014, Diaz-Vivancos *et al.* 2015). This compound is also involved in storage and transport of sulphur, signal transduction, and enzymatic regulation (Anjum *et al.* 2012). Moreover, GSH plays an important role in plant adaptation to many environmental stresses such as drought, salinity, temperature extremes, heavy metal, *etc.* There are several possible ways for GSH to be involved in heavy metal tolerance: it protects cells from oxidative stress, and it is also a ligand for metal ions and so enables

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Abbreviations: f.m. - fresh mass; GS - glutathione synthetase; GSH - reduced glutathione; LT₅₀ - temperature which induced death of 50 % of palisade cells of leaf mesophyll; MDA - malondialdehyde; PCs - phytochelatin; PCS - phytochelatin synthases; Pro - free proline; ROS - reactive oxygen species.

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their transport into the vacuole (Gallego *et al.* 2012). Furthermore, GSH is a substrate for synthesis of phytochelatins (PCs), which are directly involved in heavy metal detoxification (Pal and Rai 2010, Anjum *et al.* 2012). The PCs are small thiol peptides with the general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ ($n = 2\text{-}11$) (Pivato *et al.* 2014). Due to their ability to bind metals, PCs are generally considered to be essential cellular chelating agents, and so they contribute to metal tolerance by metal detoxification. In addition, PCs also play a role in the long-distance transport of metals including Cd (Chen *et al.* 2006). Moreover, an increase in PCs content has also been observed in response to salt, heat, and UV-B radiation (Zagorchev *et al.* 2013). Probably, the role of PCs may not be restricted to the chelation of metal ions, and their involvement in response to other abiotic stresses

as antioxidants or proteins protectors can be proposed for further studies (Zagorchev *et al.* 2013).

Despite the fact that proline and non-protein thiols participate in cold and metal tolerance, their specific roles in plant adaptation to stresses are still debated. Moreover, the molecular and metabolic responses of plants to a combination of low temperature and cadmium cannot be directly extrapolated from plant responses to each of these individual stresses. However, to date there are no studies available on the role of low-molecular-mass protectors to the combined effect of different stress factors. For this reason, we analyzed the effect of low temperature, cadmium, and their combination on Pro, GSH, and PCs content and biosynthesis in wheat plants. The findings can improve our understanding of the mechanisms underlying plants tolerance to combined stresses.

Materials and methods

Seeds of winter wheat (*Triticum aestivum* L. cv. Moskovskaya 39) were purchased from the Tula Research Institute of Agriculture, Tula, Russia. Seedlings were cultivated on a Hoagland nutrient solution (pH 6.2 to 6.4) in a growth chamber with a 14-h photoperiod, a photo-synthetic photon flux density of $180\ \mu\text{mol m}^{-2}\text{s}^{-1}$, a temperature of 22 °C and a relative humidity of 60 - 70 %. Seven-day-old seedlings were exposed to a low temperature (4 °C), cadmium sulphate (100 μM), or their combination for 7 d. Then, they were rinsed with distilled water and blotted with tissue paper for fresh mass (f.m.) measurements.

Experiments were carried out using the equipments of the Core Facility of the Karelian Research Center of the Russian Academy of Sciences. Cold tolerance of plants was evaluated by a temperature which induced death of 50 % of palisade cells of leaf mesophyll after 5 min exposure to testing temperature (LT_{50}) in a TZhR-02-20 thermoelectric microcooler (Interm, Moscow, Russia). Temperature was lowered at an interval of about 0.4 °C (Talanova *et al.* 2009). Viability of palisade cells was determined in a LOMO Micmed-2 light microscope (LOMO, Moscow, Russia) according to coagulation of cytoplasm and chloroplast disruption.

Lipid peroxidation was estimated spectrophotometrically by determining malondialdehyde (MDA) formed in leaves using the thiobarbituric acid method according to Stewart and Bewley (1980). Leaves (0.1 g) were homogenized with 2 cm³ of 5 % (m/v) thiobarbituric acid in 20 % (m/v) trichloroacetic acid and centrifuged at 10 000 g and 4 °C for 15 min. The mixture was heated at 95 °C in a water bath for 30 min, cooled quickly in an ice-bath, and centrifuged at 10 000 g for 5 min. Absorbance of the supernatant was measured at 532 nm and corrected for a non-specific turbidity by subtracting the absorbance at 600 nm. Malondialdehyde content was

calculated using a coefficient of absorbance of $155\ \text{mM}^{-1}\text{cm}^{-1}$.

Proline analysis was performed according to Bates *et al.* (1973). Wheat leaves (0.5 g) were homogenized in 3 % (m/v) sulfosalicylic acid, and the homogenate was centrifuged at 5 100 g for 5 min. A reaction mixture, which consisted of 2 cm³ of the supernatant, 2 cm³ of 0.2 % (m/v) ninhydrin solution, and 2 cm³ of glacial acetic acid, was incubated in a boiling water bath for 1 h followed by storage in an ice bath. Proline content was monitored spectrophotometrically at 520 nm using L-proline as a standard.

Glutathione and three major PCs (PC2, PC3, and PC4) were extracted and analyzed after derivatization by reverse-phase HPLC following the protocol described by Sneller *et al.* (2000). Leaves were ground in liquid nitrogen. Non-protein thiols were extracted by homogenization of 20 mg leaf material in 2 cm³ of ice-cold 6.3 mM diethylenetriaminepentaacetic acid (Sigma, St. Louis, USA) and 0.1 % (m/v) trifluoroacetic acid (Merck, town?, USA). The homogenate was centrifuged at 10 000 g and 4 °C for 10 min. The supernatant was filtered. After derivatization with monobromobimane, GSH and PCs were separated on a Phenomenex Luna 5u C18 column at 37 °C. The sample was injected and eluted with a slightly concave gradient of 12 - 25 % (v/v) methanol for 15 min, and then a linear gradient of 25 to 50 % methanol from 15 to 50 min. Phytochelatins content was calculated as nanomols of the sulphydryl equivalent per gram of fresh mass using GSH as a standard (Sigma).

Frozen leaf tissues were homogenized with liquid nitrogen. Total RNA was extracted using a TRizol reagent (Evrogen, Moscow, Russia) as instructed by the manufacturer. The total RNA was treated with RNase-free DNase (Syntol, Moscow, Russia) to remove genomic DNA. The purity of RNA samples and their

concentrations were determined spectrophotometrically (*SmartSpecPlus*, *Bio-Rad*, Hercules, USA): samples with A_{260}/A_{280} ratios within 1.8 - 2.0 were used for further analysis. The total RNA (1 µg) was reverse-transcribed using a *MMLV RT* kit (*Evrogen*) following the supplier's recommendations. Real-time quantitative PCR was performed using the *iCycler iQ* detection system (*Bio-Rad*). Analyzes were performed using a *SYBR Green* PCR kit (*Evrogen*). The PCR conditions consisted of denaturation at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 56 °C for 40 s, and extension at 72 °C for 45 s. A dissociation curve was generated at the end of each PCR cycle to verify that a single product was amplified using *iCycler iQ*. To

minimize sample variations, mRNA expression of a target gene was normalized relative to the expression of a housekeeping gene *actin*. The mRNA content of target genes (*TaP5CS*, *TaGSI*, and *TaPCS1*) were quantified in comparison to the *actin* by the $\Delta\Delta C_t$ method (Livak and Schmittgen 2001). Primers were designed (using the *Primer Design* program), and they are listed in Table 1 Suppl.

All experiments were performed at least three times. Data were subjected to analysis of variance and to principal component analysis. Data are presented as means $\pm$ standard errors. The Fisher least significant difference test was used to compare means. Differences at $P \leq 0.05$ were considered as statistically significant.

Results

The results of our study demonstrate that both the individual and combined effects of the low temperature (4 °C) and CdSO₄ (100 µM) reduced fresh mass of wheat seedlings (Table 1). On the seventh day, the fresh mass decreased by 40, 15, and 42 % at the low temperature, CdSO₄, or their combination, respectively, compared with the control. However, the magnitude of the negative effect due to the “combined stresses” was not additive.

Cold tolerance of the leaf cells of wheat seedlings significantly increased after 1 - 5 h at the low temperature and continued to rise reaching a maximum on the sixth to seventh day (Fig. 1A). Interestingly, cadmium treatment also led to an increase in cold tolerance but to a lower extent and reached a maximum after 1 d of exposure. The combination of the low temperature and CdSO₄ induced an increase in cold tolerance after 5 h of exposure and remained unchanged until the end of the experiment (Fig. 1A).

Additionally, MDA content of leaves increased after 24 h of 4 °C exposure and remained increased throughout the experiment (Fig. 1B). In the presence of CdSO₄, MDA content increased after 3 d without any further change. The combined stress induced an increase in MDA content already after 1 h, followed by a further substantial rise (Fig. 1B).

The low temperature, CdSO₄, and their combination caused an increase in Pro content of leaves. In particular, Pro accumulation slightly increased within 5 h of exposure to 4 °C and dramatically rose after 7 d up to 67 µmol g⁻¹ (f.m.) (Fig. 2A). Cadmium treatment for 5 h also induced an apparent increase in proline content, however, a significant increase was observed after 7 d but much less than at the low temperature. In contrast, the combined stress resulted in an increase in leaf Pro content up to 40 µmol g⁻¹ (f.m.) on the seventh day (Fig. 2A).

The increase in GSH content of leaves was noted within 1 h of 4 °C exposure alone or in combination with CdSO₄, whereas GSH content decreased during 1 - 7 d (Fig. 2B). No significant difference in GSH content was observed at a short-term (1 - 24 h) CdSO₄ treatment, however, after 2 d GSH content dropped, and at the end of the experiment, it was 2-fold smaller as compared with the initial level.

Analysis of PCs content showed their accumulation within 1 h at 4 °C, but after 48 h PCs content did not change during further exposure (Fig. 2C). A rapid increase in PCs content was observed within 1 h of CdSO₄ treatment followed by a further increase up to the seventh day. The combined stress also led to an increase in PCs content, but lower than by CdSO₄ alone (Fig. 2C).

Table 1. The effect of a low temperature of 4 °C, cadmium sulphate (100 µM), and their combination on wheat seedling fresh mass [mg seedling⁻¹]. Means $\pm$ SEs, $n = 27$; different letters indicate significant differences between treatments at $P < 0.05$ (ANOVA).

Time [h]	Control	4 °C	CdSO ₄	4 °C + CdSO ₄
0	159 $\pm$ 3 ^g	159 $\pm$ 3 ^g	159 $\pm$ 3 ^g	159 $\pm$ 3 ^g
24	186 $\pm$ 8 ^c	161 $\pm$ 2 ^{fg}	180 $\pm$ 5 ^e	163 $\pm$ 4 ^{fg}
48	223 $\pm$ 5 ^c	162 $\pm$ 4 ^{fg}	209 $\pm$ 3 ^d	163 $\pm$ 3 ^{fg}
96	277 $\pm$ 8 ^a	164 $\pm$ 2 ^{fg}	248 $\pm$ 8 ^b	167 $\pm$ 5 ^{efg}
144	289 $\pm$ 9 ^a	168 $\pm$ 2 ^{fe}	248 $\pm$ 6 ^b	169 $\pm$ 3 ^{efg}
168	293 $\pm$ 11 ^a	175 $\pm$ 3 ^e	248 $\pm$ 6 ^b	169 $\pm$ 5 ^{efg}

The increase in Pro content in wheat leaves correlated with the accumulation of transcripts of the *TaP5CS* gene encoding Δ^1 -pyrroline-5-carboxylate synthase (Fig. 3). An increase in the mRNA content of the *TaP5CS* gene was observed within 24 h of exposure to 4 °C and

reached a maximum on the seventh day. The influence of CdSO₄ and its combination with the low temperature also led to an enhancement of *TaP5CS* transcript accumulation but less than under the low temperature alone.

Fig. 1. The effect of a low temperature of 4 °C, 100 μ M CdSO₄, and their combination on lethal temperature for 50 % of palisade cells (LT₅₀) (A) and malondialdehyde (MDA) content (B) in wheat leaves. Means $\pm$ SEs, $n = 18$ for LT₅₀ and $n = 12$ for MDA. Different letters indicate significant differences at $P < 0.05$ (ANOVA).

The individual and combined effects of the low temperature and CdSO₄ led to a higher content of *TaGSI* gene transcripts. In particular, such upregulation was apparent within 1 h. The upregulated state of this gene was maintained during 1 - 7 d. Transcription of *TaGSI* did not change after 1 d of CdSO₄ exposure, but it was higher than control on the seventh day. Upregulation of *TaGSI* in response to the combination of the low temperature and CdSO₄ was not additive (Fig. 3).

Both individual exposure and combined exposure to the low temperature and CdSO₄ resulted in accumulation of *TaPCS1* gene transcripts. Leaves exposed to CdSO₄ or to the low temperature showed *TaPCS1* upregulation within 1 h, which rose up to the seventh day (Fig. 3). The combined stress also resulted in the accumulation of *TaPCS1* mRNA throughout the experiment.

Principal component analysis was performed to display the maximum amount of variation in a data

profile within a few principal components and to understand relations between variables (Fig. 1 Suppl.). As could be expected, physiologically related parameters and processes tended to display correlated output values. The analysis of some parameters considering three types of treatment (4 °C, cadmium, combination of them) showed two components, PC1 accounting for 72.4 % and PC2 representing 23.2 % of total variance. The PC1 was loaded on parameters related to fresh mass, Pro, GSH, and PCs, whereas PC2 was loaded on LT₅₀ and MDA. For instance, GSH, PCs, and MDA accumulations were negatively correlated with LT₅₀, Pro, and fresh mass, whereas a positive correlation was found between PCs content and fresh mass. Moreover, LT₅₀ and Pro content seem to depend on low temperature exposure, PCs content and fresh mass were mainly impacted by cadmium, whereas MDA and GSH were linked to the combined stress (Fig. 1 Suppl.).

Discussion

It is generally accepted that environmental stresses are detrimental to plants by reducing their growth and biomass production. However, the inhibition of growth in response to low positive temperatures is one of the prerequisites for the adaptation of winter cereals to cold (Klimov 2009). In the present study, fresh mass of wheat seedlings treated with the low temperature reduced, while their cold tolerance increased. Moreover, accumulation of Pro was observed within 5 h of low temperature exposure and further increased substantially after 7 d. The results are consistent with the data of Pro involvement in a

chilling-induced osmotic stress as a key osmolyte and antioxidant promoting cold tolerance of rape (Gaveliené *et al.* 2014) and chickpea (Kaur *et al.* 2011). Moreover, the low temperature induced an increase in *TaP5CS* mRNA. The highest amount of *TaP5CS* transcripts correlated with Pro content of leaves. Content of MDA increased at 24 h under the low temperature but then did not change. These results suggest that proline as well as other non-enzymatic antioxidants played an important role in ROS scavenging and cell protection from oxidative stress.

Fig. 2. The effect of a low temperature of 4 °C, 100 µM CdSO₄, and their combination on free proline, reduced glutathione (GSH), and phytochelatin (PCs) content in wheat leaves. Means ± SEs, *n* = 12. Different letters indicate significant differences at *P* < 0.05 (ANOVA).

Along with Pro, GSH is a vital component with multiple roles for eliminating ROS and improving stress adaptation (Noctor *et al.* 2012, Zagorchev *et al.* 2013). Our results show that a short-term influence of 4 °C also led to an increase in PCs. However, PCs content remained enhanced, while GSH content dropped. The decrease in GSH content was most probably due to an increased use of GSH for PCs synthesis. Alternatively, the drop in GSH level could be caused by a presumed competition for glutamate use between GSH and Pro (Fig. 2).

Fig. 3. The effect of a low temperature of 4 °C, 100 μ M CdSO₄, and their combination on *TaP5CS* (A), *TaGS1* (B), *TaPCS1* (C) gene transcriptions in wheat leaves. An initial gene transcription at 22 °C is taken as 1. Means $\pm$ SEs, $n = 9$, different letters indicate significant differences between treatments $P < 0.05$ (ANOVA).

In plants treated with the low temperature for 7 d, cold tolerance was highest as well as PCs content. Moreover, *TaPCS1* transcription in leaves increased at the low temperature and reached the highest level on the seventh day. Numerous studies on PCs have been

published but despite an undoubted role in heavy metal stress response, their response to other stresses is unclear. Although, some reports suggested that PCs activity may be affected by stress generally, for example, by a high temperature, salinity (Zagorchev *et al.* 2013), and UV radiation (Bhargava *et al.* 2005). Further studies are needed to fully understand their putative role in plant stress response.

Previously, we have noted Cd accumulation in roots of plants treated with 100 μ M CdSO₄ for 1 h and an increase in Cd accumulation in leaves during the next 7 d (Repkina *et al.* 2015). This is typical for wheat as well as for some other plants species which mainly accumulate heavy metals in roots for preventing their entry into the aboveground parts (Kovacs and Szemmelweis 2017). Nevertheless, cadmium exposure led to a decrease in wheat fresh mass accumulation but less compared with the low temperature (Table 1). The negative effect of Cd on growth have also been reported by Talanova *et al.* (2000), Farooq *et al.* (2013), Li *et al.* (2013), and Han *et al.* (2014). Interestingly, an induced cold tolerance in wheat was also detected after Cd exposure but less than that induced by low temperature. Cadmium as well as other stresses lead to ROS generation that in turn trigger plant ROS-scavenging mechanisms, which include non-enzymatic compounds (Foyer and Noctor 2012). The present research shows that MDA content only slightly changed under CdSO₄ treatment, which can be a result of effective ROS scavenging and so prevention of lipid peroxidation. However, CdSO₄ caused less Pro accumulation and *TaP5CS* mRNA content compared to the cold treatment. Apparently, this effect can link to the fact that unlike a low temperature, CdSO₄ does not lead to direct osmotic effects and, therefore, not require any accumulation of an osmolyte such as Pro. Perhaps in plants treated with Cd, Pro plays a relatively minor role in stress tolerance.

Our data indicate that Cd caused a greater decrease in GSH content compared to 4 °C but resulted in a greater increase in PCs accumulation. Several reports have noted the increase in PCs under heavy metal stress as key chelators (Pal and Rai 2010, Gallego *et al.* 2012). Also, *TaPCS1* gene expression was correlated with PCs accumulation under stress. On the contrary, the dramatic drop in GSH amount could be caused by a direct GSH involvement in cadmium detoxification and simultaneously for PCs synthesis. Though, *TaGS1* gene transcripts were elevated during 7 days in cadmium treated plants. These results suggest that under cadmium stress, in contrast to the chilling effect, glutamate is more used for GSH synthesis than for Pro synthesis.

We noted that wheat response to a combination of the low temperature and CdSO₄ was similar to that to the low temperature alone. This stress combination significantly reduced Cd accumulation in wheat in comparison with CdSO₄ alone (data not shown). This can be caused by an effect of low temperature on nutrient transport in plants

although a decrease in fresh mass and an elevated MDA content in wheat were documented.

A significant increase in Pro and PCs content was also induced by the combination of the low temperature and Cd, but it was less than at the low temperature or CdSO₄ alone (Fig. 2). Also the combined effect of these factors led to *TaP5CS*, *TaPCSI*, and *TaGSI* transcript accumulations. A drop in GSH content was also observed but lower than that at cadmium stress alone. Thus, it is suggested that there was no acute substrate competition, and glutamate was used for synthesis of both Pro and GSH because Pro is needed as an osmolyte, and non-protein thiols as key chelators for heavy metal detoxification.

In the present study, an increase in wheat cold tolerance was observed under a combined effect of low temperature and cadmium, but it did not reach the level

obtained by cold exposure alone, which can be a consequence of cadmium toxicity to plant cells and tissues. According to our results, the combined stresses impact did not lead to an additive effect. Therefore, their combination should be regarded as a new state of abiotic stress in plants. However, an interaction between low temperature and cadmium seems to be predominantly controlled by low temperature, which contributes more to the occurrence of stress. The increase of some metabolites with multiple functions (Pro, GSH, and PCs) associated with plant defense mechanisms should contribute to the plant protection from both an individual stress as well as combined stresses. Assessing their physiological responses and adaptive mechanisms under combined rather than individual stresses may prove to be more useful since plants are often exposed to multiple stresses in their natural environment.

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