Proline metabolism-related gene expression in four potato genotypes in response to drought stress

Y. LIU[1,2], L. WANG[1,3], Y. LI[4], X. LI[5], and J. ZHANG[1,4]*

*Gansu Provincial Key Laboratory of Aridland Crop Science, Gansu Agricultural University,
Lanzhou 730070, P.R. China[1]*
College of Agronomy, Gansu Agricultural University, Lanzhou 730070, P.R. China[2]
College of Life Science and Technology, Gansu Agricultural University, Lanzhou 730070, P.R. China[3]
College of Horticulture, Gansu Agricultural University, Lanzhou 730070, P R. China[4]
Qingyang Academy of Agricultural Sciences, Qingyang 745000, P.R. China[5]

Abstract

Drought severely limits potato yield. The aim of this work was to study a response of plantlets of four potato genotypes to polyethylene glycol (PEG 6000)-induced drought stress in both physiological and molecular levels. The drought-tolerant and drought-sensitive genotypes were identified based on plantlet growth, chlorophyll content, lipid peroxidation, free proline content, and proline metabolism-related gene expressions. We found that PEG-induced stress increased malondialdehyde (MDA) and proline content, and drought-tolerant plantlets exhibited lower MDA and proline content than sensitive genotypes. In addition, PEG up-regulated Δ-1-pyrroline-5-carboxylate synthase (*P5CS*) and pyrroline-5-carboxylate reductase (*P5CR*) gene expressions and down-regulated pyrroline dehydrogenase (*PDH*) and Δ-1-pyrroline-5-carboxylate dehydrogenase (*P5CDH*) gene expressions. Genotype B plantlets exhibited lower *P5CS* and *P5CR* expressions and higher *PDH* and *P5CDH* expressions compared with the other plantlets. The results suggest that significant cultivar differences among potato plantlets in response to PEG-induced drought stress are exhibited in root length, MDA content, proline accumulation, and proline metabolism-related gene expressions.

Additional key words: chlorophyll, *in vitro* cultivation, malondialdehyde, PEG-induced drought stress, *Solanum tuberosum*.

Introduction

Productivity of potatoes in some regions is low because of drought and salt stress (Wang *et al.* 2005). Until now, only a small amount of drought-tolerant cultivars are cultivated. Nevertheless, the International Potato Center (CIP) in Peru maintains 4 354 landraces of potato and 2 414 accessions of wild relatives to meet an international requirement for potato genetic resources (Ellis *et al.* 2015). To locate drought-resistant potato plants for growing in northwestern China, we obtained 119 plantlets from the CIP for assessment. We aimed to analyze *in vitro* plantlets and to provide a fast, effective approach to study physiological responses to drought stress according to Tewary *et al.* (2000).

We chose polyethylene glycol (PEG) to induce drought stress, similar to drought found in nature because PEG does not penetrate into plants, and it is able to reduce soil water availability (Larher *et al.* 1993, Gopal *et al.* 2008, Rai *et al.* 2011). We examined physiological and molecular changes in four *in vitro* grown potato genotypes obtained from the CIP in response to PEG-induced drought stress. The four plantlets were chosen out of 119 due to their growth in northern China (B > A > C > D). The aim was to provide insights into the physiological and molecular differences among drought-tolerant and drought-sensitive potato genotypes. The physiological characteristics chosen were chlorophyll content, malondialdehyde (MDA) content (an important indicator of lipid peroxidation), and proline accumulation, which has been reported to

correlate closely with drought and salinity stress in plants (Delauney and Verma 1993, Gupta and Huang 2014). The free proline content is regulated by the reciprocal action of its biosynthesis and degradation. Glutamate and ornithine are two different proline precursors that are converted to glutamic-γ-semialdehyde, catalyzed by the rate-limiting enzyme Δ1-pyrroline-5-carboxylate synthetase (P5CS), and ornithine δ-aminotransferase (OAT), respectively (Spoljarevic *et al.* 2011). Glutamic-γ-semialdehyde is spontaneously converted to pyrroline-5-carboxylate (P5C), which is further converted to proline catalyzed by P5C reductase (P5CR). Two enzymes proline dehydrogenase (PDH) and P5C dehydrogenase (P5CDH) are responsible for proline degradation (Xue *et al.* 2009). Therefore, the molecular measurements chosen were expression of proline biosynthesis-related genes.

Materials and methods

Plants and cultivation: The CIP391047.34, CIP385499.11, CIP394611.112, and CIP391919.3 are the four tetraploid potato (*Solanum tuberosum* L.) genotypes, from the CIP, that are referenced here as plant genotypes A, B, C, and D, respectively. They were propagated *in vitro* on a solidified Murashige and Skoog (1962; MS) medium containing 3 % (m/v) sucrose and 0.5 % (m/v) agar (pH 5.8 ±1). Plantlets (1 cm long) containing axillary buds were cultured in 150 cm^3 triangular flasks and grown at a 16-h photoperiod, an irradiance of 200 μmol m^{-2} s^{-1} (white fluorescent lamps), and a temperature of 23 ± 2 °C. Plantlets with at least one leaf and one axillary bud were sub-cultured in the MS medium supplemented with 0 (control), 2.5, 5.0, 7.5, 10.0 % (m/v) PEG 6000 to induce water stress. There were six plantlets in each triangular flask and each replication contained seven triangular flasks (three replications). Leaf samples were collected after a 20-d treatment for analyses.

Chlorophyll content was measured using a method of Wang *et al.* (2011). The chlorophyll was extracted from the plant tissue using 80 % (v/v) acetone, then the solution was vigorously shaken for 10 s, and lastly it was stored at 4 °C overnight. Afterwards, the solution was centrifuged at 10 000 *g* to remove the debris. Absorbance was determined at 645 and 663 nm using a spectrophotometer (*UV-2100*, *Shimadzu*, Kyoto, Japan), and chlorophyll content was calculated according to Arnon *et al.* (1949).

Lipid peroxidation: Malondialdehyde content, an important indicator of lipid peroxidation, was determined according to Hodges *et al.* (1999) with a slight modification. Approximately 0.5 g of fresh leaves was homogenized with a mortar and pestle using 5 cm^3 of 20.0 % (m/v) trichloroacetic acid. The homogenate was centrifuged at 10 000 *g* and 4 °C for 15 min, and 1 cm^3 of supernatant was added to 2 cm^3 of 0.5 % (m/v) thiobarbituric acid containing 20 % (m/v) trichloroacetic acid. The samples were heated at 95 °C in a water bath for 30 min after vortexing, and the reaction was terminated

by quick cooling on ice. Samples were centrifuged at 10 000 *g* and 4 °C for 10 min. Absorbance was measured (an *UV-2100* spectrophotometer) at 450, 532, and 600 nm, respectively. Content of MDA was calculated according to Turan and Tripathy (2013).

Proline content: The total proline content was measured by the ninhydrin reaction method (Bates *et al.* 1973). Approximately 0.1 g of fresh leaves was homogenized using 3 % (m/v) aqueous sulfosalicylic acid, and then the solution was centrifuged at 10 000 *g* (4 °C, 10 min) to remove the debris. Then, 2 cm^3 of the supernatant was mixed with 2 cm^3 of acid ninhydrin and 2 cm^3 of glacial acetic acid in a test tube and incubated at 100 °C for 1 h. After that, the reaction was terminated in an ice bath. The product was extracted with 4 cm^3 of toluene, and absorbance was measured at 520 nm on a UV–vis spectrophotometer (*UV-1601PC*, *Shimadzu*). Proline content was determined from a standard curve.

Extraction of RNA and cDNA synthesis: The total RNA was extracted from the four potato genotypes using a *PureLink* plant RNA reagent kit (*Invitrogen*, Carlsbad, CA, USA) as described in the manufacturer's instructions. Then, it was adjusted to 500 ng mm^{-3} using a *Nanodrop ND-1000* spectrophotometer (*NanoDropTechnologies*, Rockland, DE, USA), Genomic DNA was removed and cDNA was synthesized using the *SuperScript*[III] first-strand synthesis system (*Invitrogen*).

Real-time quantitative PCR was conducted using the *Mx3000P QPCR* system (*Stratagene*, La Jolla, USA) with the *MxPro v. 3.00* software. The quantitative PCR (qPCR) was performed three times for each sample in a total reaction volume of 25 mm^3 consisting of 100 ng of template cDNA, 0.2 μM forward and reserve primers, and 12.5 mm^3 of 2× *SYBR Premix Ex Taq* (*TaKaRa*, Dalian, China). The thermal cycling process was as follows: an initial denaturation cycle at 95 °C for 5 min followed by 40 cycles at 95 °C for 5 s, annealing at 60 °C for 10 s, elongation at 72 °C for 10 s, and melting curve detection at 65 - 95 °C . The potato *elongation factor-1* (AB061263) was selected as a constitutive control for template normalization. Relative gene expression was calculated using the 2$^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Primers are listed in Table 1 Suppl.

Statistical analysis: Data were expressed as means ± SEs. All data were statistically analyzed through linear regression analysis to determine significant differences among treatments at $P \leq 0.05$ by using the *SPSS* statistical software (*v.17.0, SPSS Inc.*, Chicago, IL, USA).

Results

Polyethylene glycol-induced stress hindered the growth of all four plant genotypes regardless of concentration, as seen on day 20 (Table 1 and Fig. 1 Suppl). Linear regression was performed using *GraphPad*, and both coefficients and

Table 1. Effect of polyethylene glycol (PEG)-induced stress on the growth of potato plantlets *in vitro* for 20 d. Means ± SEs, *n* = 3. Values in a column with the same letters are not statistically different at $P \geq 0.05$ by the Duncan test.

Genotypes	PEG concentration [%]	Number of new leaves [plant^{-1}]	Number of roots [plant^{-1}]	Root length [cm plant^{-1}]
A	control	5.33 ± 0.45a	5.00 ± 0.38a	9.83 ± 0.35a
	2.5	3.00 ± 0.33b	2.33 ± 0.21b	5.70 ± 0.33b
	5.0	1.33 ± 0.16c	1.67 ± 0.33bc	2.37 ± 0.14c
	7.5	1.00 ± 0.23c	1.33 ± 0.19bc	1.67 ± 0.20cd
	10.0	0.67 ± 0.13c	0.67 ± 0.12c	0.37 ± 0.11d
B	control	6.00 ± 0.37a	4.67 ± 0.51a	9.27 ± 0.42a
	2.5	5.00 ± 0.56a	2.33 ± 0.36b	6.10 ± 0.49b
	5.0	3.33 ± 0.24b	1.67 ± 0.15bc	5.80 ± 0.33b
	7.5	3.00 ± 0.34b	1.67 ± 0.22bc	6.60 ± 0.28b
	10.0	2.00 ± 0.21c	0.67 ± 0.13c	0.37 ± 0.09c
C	control	5.00 ± 0.23a	4.67 ± 0.44a	6.67 ± 0.39a
	2.5	2.67 ± 0.36b	2.00 ± 0.28b	3.68 ± 0.26b
	5.0	1.67 ± 0.27c	1.33 ± 0.25c	1.40 ± 0.30c
	7.5	0.67 ± 0.15d	1.00 ± 0.11c	0.77 ± 0.21d
	10.0	0.67 ± 0.09d	0.67 ± 0.12c	0.27 ± 0.13d
D	control	4.67 ± 0.48a	4.00 ± 0.58a	6.23 ± 0.55a
	2.5	2.00 ± 0.32b	1.67 ± 0.29b	3.30 ± 0.36b
	5.0	1.33 ± 0.17bc	1.33 ± 0.22c	1.07 ± 0.29c
	7.5	0.67 ± 0.21cd	0.67 ± 0.15cd	0.47 ± 0.25d
	10.0	0.33 ± 0.08d	0.33 ± 0.11d	0.10 ± 0.06d

intercepts were calculated. Genotypes A, C, and D were severely impacted by PEG-induced stress in comparison to genotype B when examining the number of new leaves (intercepts $P = 0.000623$), roots (intercepts $P = 0.03$), and root length (intercepts $P = 0.0152$). Overall, it was shown that already 2.5 % PEG-induced stress significantly decreased the number of new leaves, roots, and root lengths compared to the control in all genotypes (Table 1 and Fig. 1 Suppl). Leaf and root growth were greatly affected in all four potato genotypes under 5 and 7.5 % PEG. The maximum root length of plant genotypes A, B, C, and D were in ranges 1.67 - 2.37 cm, 5.80 - 6.60 cm, 0.77 - 1.40 cm, and 0.47 - 1.07 cm, respectively. All four plant genotypes were seriously injured under 10 % PEG-induced stress. Furthermore, there was no visible rooting of plantlets of C and D genotypes. However, in genotype B, the roots and leaves remained present (Fig.1 Suppl). The results suggest that B was the most tolerant genotype, whereas D was the most sensitive genotype to PEG-induced stress.

After 20 d of growth in MS medium, genotype B displayed the highest above-ground fresh mass (95.37 mg per plant), which was 1.56-times higher than of A, 1.87-times higher than C, and 2.35-times higher than of D genotypes.The ratios of above-ground and root fresh masses to dry masses were 22.87 and 28.25 in genotype B, respectively, which were roughly 1.2- and 1.5-times higher than in the other genotypes (Table 2 Suppl.). The results suggest that genotype B retained a larger water content than the other plant genotypes during PEG-induced stress (Table 2 Suppl.).

Chlorophyll content in genotype B [0.46 mg g^{-1}(f.m.)] was 1.41-times higher than in genotype A, 1.38-times higher than in genotype C, and 1.51-times higher than in genotype D when grown on the control MS medium. The PEG-induced stress significantly decreased chlorophyll content in all four genotypes ($P < 0.05$). Genotype D showed the most dramatic chlorophyll decrease with reduction ranging from 67.74 to 80.65 %, whereas genotype B chlorophyll content decreased only by 36.96 to 52.17 % in comparison to the control (Fig. 1). Chlorophyll content of genotype B was 2.39 - 2.79 (2.5 % PEG), 2.09 - 2.51 (5 % PEG), 3.56 - 4.12 (7.5 % PEG), and 2.56 - 3.66 (10 % PEG) times higher than that of other genotypes. These results suggest that genotype B had the best photosynthetic capability among the four plant genotypes examined. Genotype D showed the lowest chlorophyll content [0.06 mg g^{-1}(f.m.)] at 10 % PEG-induced stress. There were no significant differences in chlorophyll content among different PEG concentrations, which suggests that all plantlets were sensitive to PEG-induced stress, and chlorophyll synthesis could have beeen greatly reduced even when they were exposed to a low PEG concentration.

Content ofMDA was measured as an indicator of membrane damage. In the control plantlets, MDA content was 9.2, 4.4, 10.2, and 11.3 μmol g^{-1}(f.m.) for genotypes A, B, C, and D, respectively. As PEG-induced stress increased, MDA significantly increased ($P < 0.05$) in genotypes A, C, and D with ranges 58 - 76 %, 43 - 89 %, and 34 - 79 %. Genotype D showed the highest MDA content of 20.2 μmol g^{-1}(f.m.) under 10 % PEG stress. There was no significant difference in MDA content in
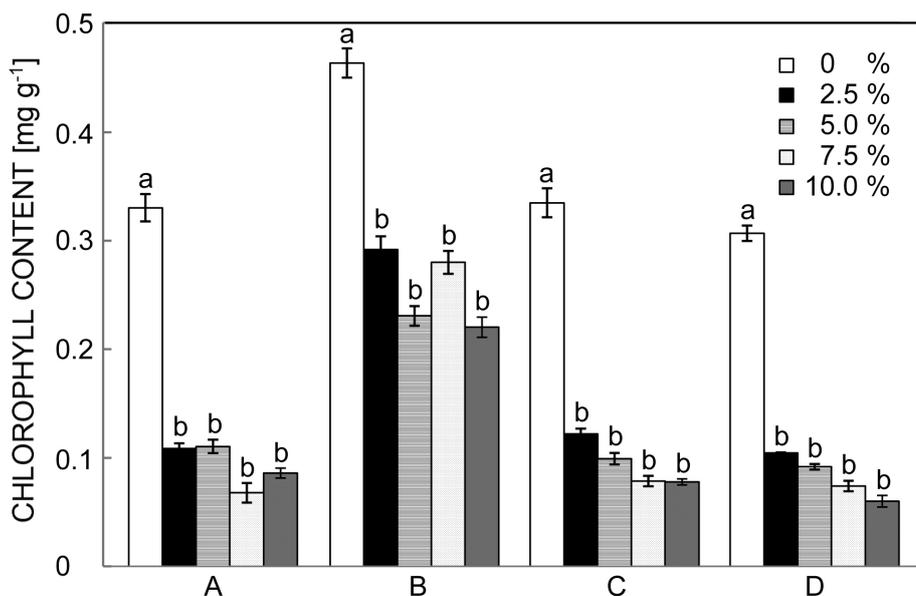
Fig. 1. Chlorophyll content of *in vitro* grown plantlets of four potato genotypes (A - D) treated with different polyethylene glycol PEG concentrations for 20 d. Means ± SEs, *n* = 3, different letters indicate significant differences between treatments at *P* < 0.05.
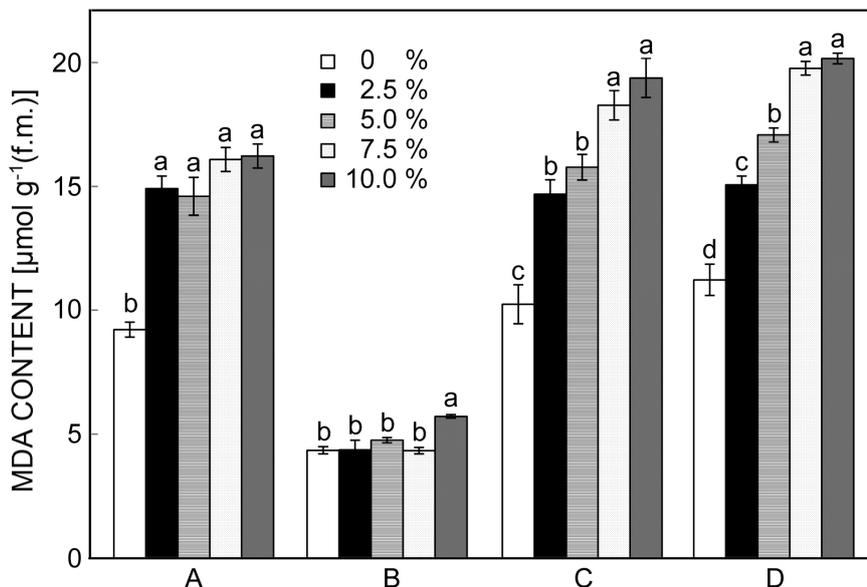

Fig. 2. Effect of different polyethylene glycol concentrations on malondialdehyde (MDA) content in *in vitro* grown potato plantlets of four genotypes (A - D). Means ± SEs, *n* = 3, different letters indicate significant differences between treatments at *P* < 0.05.

genotype B between the control and 2.5 - 7.5 % PEG treatment; it increased only by 31 % under 10 % PEG, which was 4-times lower than of genotype D. The results show that the extent of membrane damage in genotype D was much higher than in the other genotypes, and genotype B showed the lowest degree of membrane damage (Fig. 2).

Distinct differences in free proline content were observed between the four plant genotypes under PEG-induced stress. Proline content of plantlets increased significantly (*P* < 0.05) with the increase in PEG concentration in genotypes A, C, and D. For genotype D, proline content increased 16-, 38-, 52-, and 57-times

compared with the control plantlets under 2.5 to 10 % PEG and reached its highest content of 1.15 mg g⁻¹(f.m.) under 10 % PEG, which was about 2.5-times higher than in genotype A and 1.9-times higher than in genotype C. Proline content in genotype B significantly increased (*P* < 0.05) under 2.5 % PEG compared to the control, but it was almost the same when the PEG concentration increased to 5 or 7.5 %. However, 10 % PEG dramatically increased proline content, but it was still two times lower than that of genotype D (Fig. 3).

The PEG-induced water stress caused a significant increase in *P5CS* gene expression in the four genotypes,
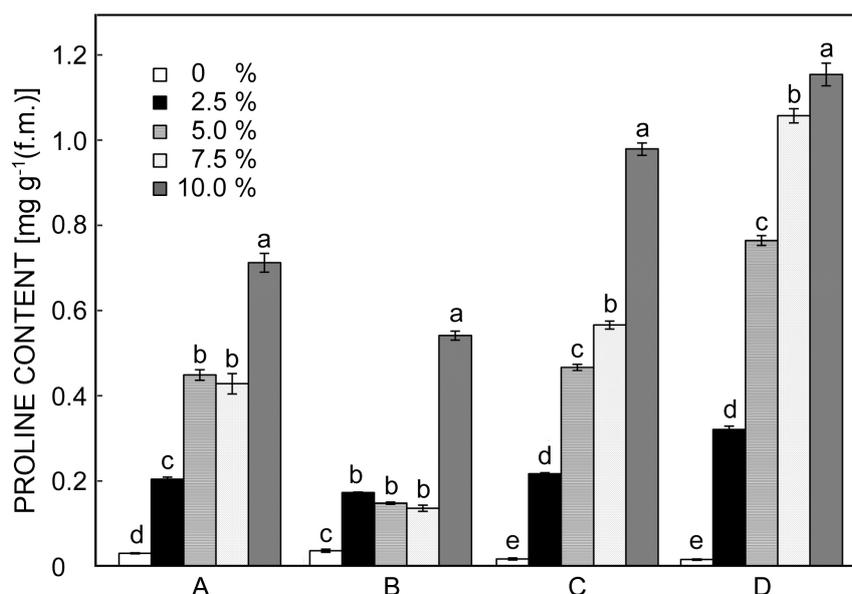
Fig. 3. Effect of different polyethylene glycol concentrations on free proline content of *in vitro* grown potato plantlets of four genotypes (A - D). Means ± SEs, *n* = 3, different letters indicate significant differences between treatments at $P < 0.05$.

and the expression was highest at 10 % PEG. Genotype B showed the lowest expression and genotype D showed the highest expression (Fig. 4). For A and B, there was no significant difference in *P5CS* gene expression between the control and 2.5 and 5 % PEG-induced stresses, but when PEG concentration increased up to 7.5 and 10 %, *P5CS* gene expression incrased. For genotypes C and D, *P5CS* was highly expressed already when treated with 2.5 % PEG. The *P5CS* expression profile correlated with proline accumulation, which suggests that *P5CS* expression limitated glutamate pathway of proline biosynthesis under PEG-induced stress.

The *OAT* gene expression significantly increased in genotypes A, C, and D already at 2.5 % PEG-induced stress and in genotype B at 5 % PEG-induced stress (Fig. 4). The greatest *OAT* gene expression increase was observed in genotype D under all PEG treatments, which was 1.36 and 2.19 times higher than in genotype A, 2.19 - 2.94 times higher than in genotype B and 1.26 - 1.82 times higher than in genotype C. The lowest *OAT* gene expression was found in genotype B under all PEG concentrations.

The *P5CR* expression profile largely overlapped that of *P5CS*. Among the four plant genotypes under water stress, genotype D showed a dramatic increase of *P5CR* gene expression under every PEG-induced stress, followed by genotypes C, A, and B (Fig. 4). For the drought-tolerant genotype B, *P5CR* gene expression increased only 2- to 3.92-times compared to the control, which was much lower than in drought-sensitive genotype D, which had 3.04- to 9.13-times higher P5CR expression than the control.

In contrast, *PDH* and *P5CDH* gene expressions decreased with the increase in PEG concentration (Fig. 4). Expressions of *PDH* and *P5CDH* genes significantly decreased in the four plant genotypes treated with 2.5 % PEG, and genotype D showed the greatest decreases in their expressions of 34 and 33 %, respectively. *PDH* and

Expressions of *P5CDH* genes dramatically decreased by 78 and 80 % in genotype D when treated with 5 % PEG whereas there was no significant differences for the two gene expressions in genotypes A, B, and C between 2.5 and 5 % PEG treatments. Under 7.5 and 10 % PEG, *PDH* and *P5CDH* gene expressions were relatively lower in genotype D, but higher in genotype B in comparison to the other genotypes. The results show that *PDH* and *P5CDH* gene expressions in genotype D were significantly inhibited under all PEG treatments. However, *PDH* and *P5CDH* expressions in genotype B were not significantly altered when exposed to 2.5 - 7.5 % PEG.

For plant genotypes A, B, C, and D, proline accumulation displayed a positive correlation to MDA content and to *P5CS* and *P5CR* gene expressions under PEG-induced drought stress ($P < 0.01$ or $P < 0.05$) whereas *OAT* gene expression significantly correlated only with proline accumulation ($P < 0.01$ or $P < 0.05$) in genotype D (Table 3 Suppl.). Proline accumulation shows a strong, negative correlation with maximum root length and *PDH* and *P5CDH* gene expressions in the four genotypes under PEG-induced drought stress ($P < 0.01$ or $P < 0.05$).

Discussion

Crops, including potato, can be seriously affected by various biotic and abiotic stresses, and drought belongs to the most important environmental stresses that limits crop productivity (an average crop yield reduction by 50 % or more; Boyer 1982). Therefore, it is essential to promote the development of more drought-tolerant potato genotypes by effectively screening plants, particularly under simulated drought stress. To achieve the goal of attaining food security worldwide, further genetic research needs to be explored, and complex mechanisms related to
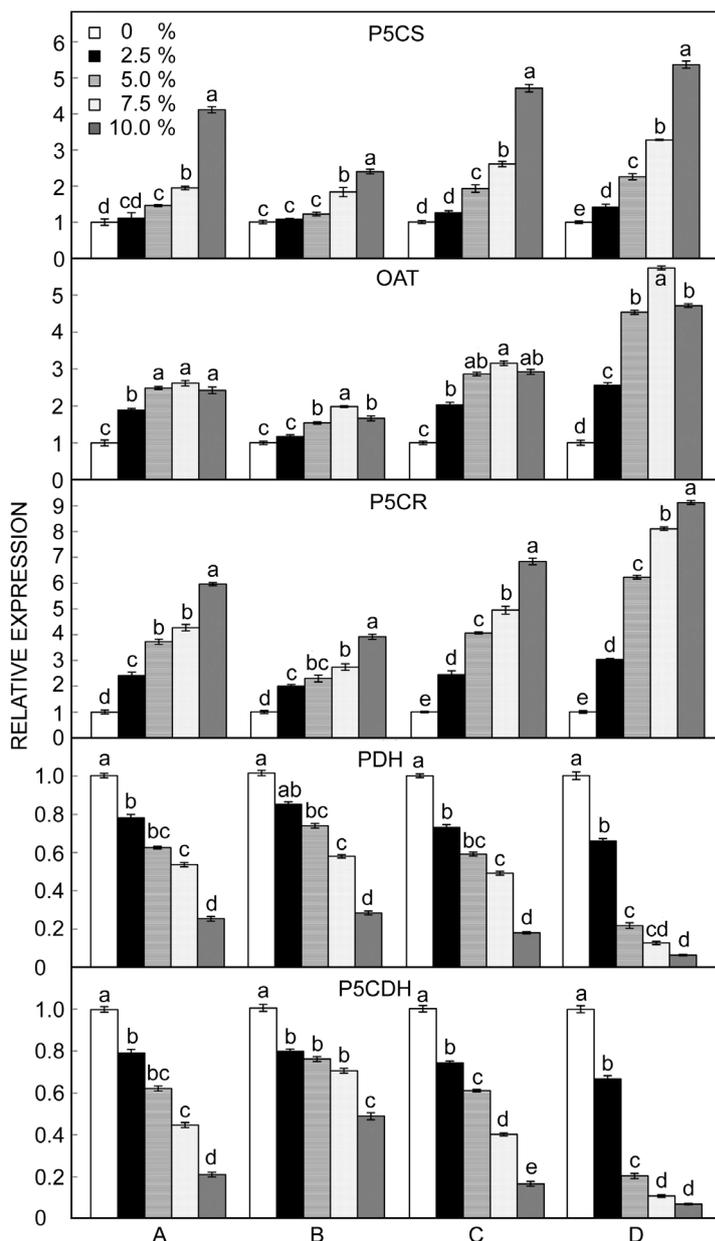
Fig. 4. Effects of different polyethylene glycol concentrations on expressions of proline synthesis-related genes in *in vitro* grown potato plantlets of four genotypes. *P5CS - Δ-1-pyrroline-5-carboxylate synthase*, *OAT - ornithineaminotransferase*, *P5CR - pyrroline-5-carboxylate reductase*, *PDH - pyrroline dehydrogenase*, *P5CDH - Δ -1-pyrroline-5-carboxylate dehydrogenase*. Means ± SEs, $n = 3$, different letters indicate significant differences between treatments at $P < 0.05$. Meanswere derived from three replicates.

drought-induced stress need to be fully understood.

In this study, plant growth, plant biomass, content of chlorophyll, MDA, and proline, as well as expressions of genes involved in proline biosynthesis were analyzed in four potato genotypes. We applied increasing concentration of PEG to induced stress to the plantlets.

Proline accumulation during stress has been suggested as an indicator of drought-tolerance. To date, the role of proline accumulation in response to drought stress is controversial, and it is unclear whether the proline increase is correlated with drought-tolerance. One theory indicates that proline accumulation under stress enhances

salt- or drought-tolerance of the plant, whereas contrasting studies suggest that proline accumulation is a result of osmotic stress, which is a symptom of stress injury rather than an indicator of stress tolerance in plants (Liu and Zhu 1997, Nayyar and Walia 2003, Demiral and Türkan 2005, Rampino *et al.* 2006, Wang and Han 2009). In our current research, we found that proline content was related to drought-tolerant or drought-sensitive characteristics. There were significant differences in the amount of proline accumulation in the four plant genotypes under 5.0 - 10 % PEG. The earliest proline accumulation and highest proline amount were observed in drought-sensitive genotype D

whereas only a slight increase of proline accumulation was observed in drought-tolerant genotype B in response to 5.0 and 7.5 % PEG-induced stress. Under 10 % PEG, proline content of genotype B significantly increased compared to the control, but it was still much lower than that of genotype D (Fig. 3). The data strongly supports the theory that proline accumulation is a sign of stress-induced plant injures.

To further understand proline biosynthesis mechanisms, the expressions of proline metabolism-related genes were also analyzed. The *P5CS* and *P5CR* genes were up-regulated with the increase of PEG concentration, but the drought-tolerant genotype B showed only a slight increase under the severe stress. There was a positive correlation between proline content and *P5CS* and *P5CR* expressions (Fig. 4 and Table 3 Suppl.). Expression of *OAT* gene increased under 2.5 - 7.5 % PEG, but a decrease was observed when the plants were subjected to 10 % PEG. These results suggest that the ornithine pathway not only contributes to proline accumulation, but it may also be involved in glutamate synthesis (Kishor *et al.* 2014, Kubala *et al.* 2015).

Proline degradation is the reverse process of proline biosynthesis, which also regulates proline content. Therefore, *PDH* and *P5CDH*, genes related to proline degradation, were inhibited by PEG-induced stress resulting in proline accumulation. There was a negative correlation between proline content and *PDH* and *P5CDH* gene expressions (Fig. 4 and Table 3 Suppl.). In drought-tolerant genotype B, *PDH* and *P5CDH* gene expressions were much higher than those found in drought-sensitive genotype D under severe PEG-induced stress. These results suggest that drought-tolerant potato plant genotypes accumulated less proline than the drought-sensitive genotype under PEG-induced stress, which is attributed to the relatively low *P5CS* and *P5CR* expressions and high *PDH* and *P5CDH* expressions. Therefore, downregulating *P5CS* and *P5CR* genes or upregulating *PDH* and *P5CDH* genes in potato may be beneficial for future development of drought-tolerant genotypes on a large scale.

In our study, proline accumulation positively correlated with MDA content and *P5CS* and *P5CR* gene expressions and negatively correlated with maximum root length and *PDH* and *P5CDH* gene expressions under PEG-induced drought stress. In conclusion, we demonstrated that drought-induced stress alters maximum root length, MDA content, proline accumulation and proline metabolism-related gene expressions in different genotypes of potato. Our results provide molecular mechanisms and strategies to increase potato yield under drought stress.

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