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The positive roles of exogenous putrescine on chlorophyll metabolism and xanthophyll cycle in salt-stressed cucumber seedlings

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Abstract

The effects of foliar spray of putrescine (Put; 8 mM) on chlorophyll (Chl) metabolism and xanthophyll cycle in cucumber seedlings were investigated under saline conditions of 75 mM NaCl. Exogenous Put promoted the conversion of uroporhyrinogen III to protoporphyrin IX and alleviated decreases in Chl contents and in a size of the xanthophyll cycle pool under salt stress. Moreover, the Put treatment reduced the activities of uroporphyrinogen III synthase, chlorophyllase, and Mg-dechelatase and downregulated the transcriptional levels of glutamyl-tRNA reductase, 5-aminolevulinate dehydratase, uroporphyrinogen III synthase, uroporphyrinogen III decarboxylase, and chlorophyllide *a* oxygenase, but significantly increased the expression levels of non-yellow coloring 1-like, pheide *a* oxygenase, red chlorophyll catabolite reductase, and violaxanthin de-epoxidase. Taken together, these results suggest that Put might improve Chl metabolism and xanthophyll cycle by regulating enzyme activities and mRNA transcription levels in a way that improved the salt tolerance of cucumber plants.

Additional key words: chlorophyll biosynthesis; chlorophyll degradation; de-epoxidation; energy dissipation; light-harvesting complex; pigments.

Introduction

More than 800 million hectares of land (6% of the world total land area) is affected by salt (Wang *et al*. 2015). As one of the most adverse environmental stresses on plants, salt stress has a negative effect on important plant physiological processes, including growth, photosynthesis, water absorption, nutrient imbalance, and yield (Parihar *et al*. 2015).

The most common polyamines (PAs) are diamine Put, triamine spermidine (Spd), and tetraamine spermine (Spm). These low molecular-mass aliphatic amines exist in a wide range of plants (Bagni and Tassoni 2001). Many studies have focused on the relationships between PAs and abiotic stresses in recent years. Several studies have shown that PAs can alleviate the inhibition of plant physiological processes induced by stresses, such as the inhibition of photosynthetic apparatus activities and polyamines biosynthesis at physiological and molecular levels (Ioannidis and Kotzabasis 2007, Hu *et al*. 2012). The photosystem comprises Chl and carotenoid (Car) pigments, which harvest light energy and transform it into biochemical energy in higher plants. Li *et al*. (2015) showed that exogenous Spd protects tomato seedlings against the damage induced by saline-alkaline stress by regulating Chl metabolism. In addition, Chl metabolism

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Abbreviations: A – antheraxanthin; ALA – δ-aminolevulinic acid; ALAD – 5-aminolevulinate dehydratase; Car – carotenoids; CAO – chlorophyllide *a* oxygenase; Chl – chlorophyll; CBR – chlorophyll *b* reductase; Chlase – chlorophyllase; CHLH – Mg chelatase H subunit; DEPS – de-epoxidation of the xanthophyll cycle; DOE – days of experiment; FM – fresh mass; HEMA1 – glutamyl-tRNA reductase; MDCase – Mg dechelatase; Mg-Proto IX – Mg proporphyrin IX; NOL – NYC1-like; NPQ – nonphotochemical quenching; NYC1 – non-yellow coloring 1; PAO – pheide *a* oxygenase; PAs – polyamines; PBG – porphobilinogen; PBGD – porphobilinogen deaminase; PChl – protochlorophyllide; PPH – pheophytinase; PPO – protoporphyrinogen IX oxidase; ProtoIX – protoporphyrin IX; Put – putrescine; RCCR – red chlorophyll catabolite reductase; Spd – spermidine; Spm – spermine; UroIII – uroporhyrinogen III; UROD – uroporphyrinogen III decarboxylase; UROS – uroporphyrinogen III synthase; V – violaxanthin; VAZ – xanthophyll cycle pool; VDE – violaxanthin de-epoxidase; Z – zeaxanthin.
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not only influences the formation of Chl-protein complexes but also regulates other physiological processes, including cell death and chloroplast–nucleus communication (Tanaka and Tanaka 2006).

Car comprise a class of pigments that are widespread in living organisms. Moreover, Car transfer light energy to Chls and play a crucial role in photoprotection against oxidative damage (Horton and Ruban 2005). The xanthophyll cycle exists in higher plants and involves three pigments: violaxanthin (V), antheraxanthin (A), and zeaxanthin (Z) (Hieber *et al*. 2000). Z has been convincingly shown to establish tolerance of photo-oxidative stress through an independent process of nonphotochemical quenching (NPQ), which protects the thylakoid membrane from oxidative damage (Johnson *et al*. 2007). Li *et al*. (2013) cloned the violaxanthin de-epoxidase (*VDE*) gene of cucumber, and their results showed that the gene expression of *CsVDE* and the de-epoxidation of the xanthophyll cycle (DEPS) rapidly increased under high light stress. However, it remains unclear whether a close relationship exists between Put and salt tolerance through changes in the Chl metabolism pathway and the xanthophyll cycle.

In our previous study, Put was found to regulate cucumber growth, glycolysis, the Krebs cycle metabolism, carbohydrate metabolism, and photosynthetic efficiency under salt stress (Shu *et al*. 2012, Yuan *et al*. 2015, Zhong *et al*. 2015). In this study, we examined the effects of exogenous Put on pigment contents, enzyme activities, the expressions of genes involved in Chl-metabolism pathways and the xanthophyll cycle in leaves of cucumber seedlings in order to further evaluate the roles of Put in regulating Chl metabolism and the xanthophyll cycle. The purpose of this study was to elucidate the protective mechanism of Put by altering the contents of photosynthetic pigments in salt-stressed cucumber seedlings.

Materials and methods

Plant material and growth conditions: The cucumber seeds (*Cucumis sativus* L., cv. Jingyou No. 4) were germinated in a thermostat at 28°C and sown in quartz sand. The experiments were performed in a plant growth chamber, which was maintained at 28 ± 1°C (day) and 18 ± 1°C (night) with a maximum PPFD of approximately 1,200 μmol m^{-2} s^{-1} and a relative humidity of 60–70%. The seedlings were transplanted to plastic containers (40 × 30 × 15 cm) containing half-strength Hoagland solution (pH 6.5 ± 0.1, EC of 2.0–2.2 dS m^{-1}) when two true leaves had expanded. The nutrient solution was aerated using an air pump at intervals of 30 min to maintain dissolved oxygen concentration at 8.0 ± 0.2 mg L^{-1} throughout the experiment.

Experimental approach: After precultivation for 3 d in the nutrient solution, the seedlings were treated as follows:

Treatment	Seedling growth	Leaf spraying
Control (C)	half-strength Hoagland solution	distilled water
Put	half-strength Hoagland solution	8 mM Put
NaCl	half-strength Hoagland solution + 75 mM NaCl	distilled water
NaCl + Put	half-strength Hoagland solution + 75 mM NaCl	8 mM Put

The Hoagland solution was renewed every two days. The containers were arranged in a completely randomized block design with three replicates per treatment (36 plants per treatment). The cucumber leaves were sprayed with 100 mL of 8 mM Put every day until the end of the experiment. An equal volume of distilled water was also sprayed on the leaves in the control and salt stress treatment. After 1, 3, 5, and 7 d of the experiment (DOE), the pigment content of the third fully expanded leaf from the top of each plant was analyzed. The enzyme activities and expression of genes involved in Chl metabolism were analyzed after treating the cucumber seedlings for 7 DOE.

Pigment determination: We analyzed the effects of Put on the pigment content of cucumber seedlings under salt stress using HPLC, using a modified version of the method of García-Plazaola and Becerril (1999). Leaf discs were collected and immediately ground to a fine powder, which was extracted in 1 mL of 100% (v/v) acetone. The homogenate was centrifuged at 15,000 × *g* for 10 min at 4°C, and the supernatant was collected. Fifteen microliters of the filtered (0.2 μm nylon filter) supernatant was subjected to HPLC (*Agilent Technologies 1200 series*, *Agilent Technologies*, *California,* USA) and separated on a *Spherisorb C18* column (4.6 by 250 mm, 5 μm Kromasil) at 30°C. Pigments were eluted using a linear gradient from 100% (v/v) solvent A [acetonitrile:methanol:Tris-HCl buffer (0.1 M, pH 8.0), 84:2:14] to 100% (v/v) solvent B (methanol:ethyl acetate, 68:32) for 12 min, followed by 6 min of solvent B, followed by a gradient from 100% B to 100% A for the next 7 min. The solvent flow rate was 1.2 mL min^{-1}, and pigment absorption was detected at 445 nm. The injection volume was 20 μL. De-epoxidation of the xanthophyll cycle (DEPS) was calculated as

(A + Z)/(V + A + Z) (%). Chl *a* and Chl *b* were extracted in 80% acetone until the leaf turned white, and the contents were calculated according to Moran (1982).

Determination of Chl precursors: The concentration of 5-aminolevulinic acid (ALA) was calculated based on the absorbance at 553 nm according to Dei (1985). Leaf samples were homogenized in 100 mM Tris buffer (pH 8.0) containing 50 mM mercaptoethanol and then centrifuged at 8,000 × *g* for 15 min at 4°C. The supernatant was then added to Ehrlich's reagent, and the content of porphobilinogen (PBG) was calculated based on the absorbance at 555 nm (Bogorad 1962). The cucumber leaves were ground with Tris buffer (pH 7.2) on ice, the homogenate was then centrifuged at 5,000 × *g* for 10 min at 4°C, and the content of uroporphyrinogen III (UroIII) was calculated based on the absorbance at 405.5 nm according to the method of Bogorad (1962). Protoporphyrin IX (ProtoIX), Mg proporphyrin IX (Mg-ProtoIX), and protochlorophyllide (PChl) were extracted from the leaves using 80% alkaline acetone (acetone:1% ammonia, 4:1), and their concentrations were determined based on the absorbance of the solution at 575, 590, and 628 nm (*JH-752, Jinghua Inc.*, Shanghai, China), respectively (Hodgins and Huystee 1986).

PBGD, UROS, Chlase and MDCase activity assays: Fresh leaves were ground on ice with 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM mercaptoethanol, 2 mM phenylmethyl sulfonyl fluoride and 2 mM EDTA-Na_2. The homogenate was centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant was then heated at 65°C for 20 min, cooled in an ice bath, and centrifuged again. Porphobilinogen deaminase (PBGD, EC 2.5.1.61) activity was calculated based on the absorbance at 405.5 nm (*JH-752, Jinghua Inc.*, Shanghai, China) according to the method of Riminton (1960) and expressed as mmol(hydroxymethylpyridine) g^{-1}(protein) h^{-1}. Uroporphyrinogen III synthase (UROS, EC 4.2.1.75) activity was determined as follows. Fresh leaves were homogenized with 0.1 M phosphate buffer (pH 8.0) containing 5 mM mercaptoethanol, and the homogenate was centrifuged at 10,000 × *g* for 10 min at 4°C; 0.9 mL of the supernatant and 0.1 mL solution of 4 mM porphobilinogen were then mixed and incubated for 5 min at 37°C. I_2/KI solution was added, and the solution was mixed with 0.05 mL of 1% $Na_2S_2O_3$ and 0.1 mL of acetocaustin. The resulting mixture was stirred and then centrifuged at 8,000 × *g* for 10 min at 4°C. The UROS activity in the supernatant was measured based on the absorbance at 405 nm (*JH-752, Jinghua Inc.*, Shanghai, China) and was calculated as mmol(uroporhyrinogen III) g^{-1}(protein) h^{-1}. Chlorophyllase (Chlase, EC 3.1.1.14) and Mg dechelatase (MDCase) were extracted based on the method of Costa *et al*. (2005). Leaf samples were ground with 50 mL of 80% acetone on ice; the resulting homogenate was centrifuged at 8,000 × *g* for 10 min at 4°C, and the pellet was collected. The resulting acetone powder was dried under nitrogen gas and then homogenized in 5 mL of 50 mM potassium phosphate (pH 7.0) containing 50 mM KCl and 0.24% *Triton X-100* for 1 h at 30°C. After centrifugation at 12,000 × *g* for 10 min at 4°C, the supernatant was assayed for Chlase and MDCase based on the absorbance at 665 nm and 686 nm (*JH-752, Jinghua Inc.*, Shanghai, China), respectively, according to the method of Costa *et al.* (2005). The enzyme activity was expressed as the increment of optical density at 665 nm and 686 nm per gram of fresh mass (FM) per hour, respectively.

Total RNA extraction and qRT-PCR analysis: Total RNA was extracted from cucumber leaves as described in the TRI reagent protocol (*Takara Bio Inc*.). The RNA was then reverse-transcribed using the *PrimeScriptTM RT* reagent kit and *gDNA Eraser* (*Takara*, Shiga, Japan) in a 20 μL reaction mixture containing 1 μL of total RNA from each sample. qRT-PCR was performed using a *StepOnePlus*™ Real-Time PCR system (*Applied Biosystems*, New York, USA). The PCR reactions were carried out in a 20 μL reaction mixture containing 2 μL of cDNA, 10 μL of *SYBR Premix Ex Taq™ II*, 0.8 μL of each specific primer, 0.4 μL of ROX reference dye, and 6 μL of ddH_2O. The reactions were performed in triplicate using the following thermocycler conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and 60°C for 1 min. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Primers were designed based on sequences in the *NCBI* and *Cucumber Genome Database* and are listed in Table 1.

Statistical analysis: All biochemical analyses were performed using six biological replicates. The data were tested for significance using *SAS* software (*SAS Institute*, Cary, NC, USA) using *Duncan*'s multiple range test ($P<0.05$).

Results

Photosynthetic pigments: Compared with the control, the contents of Chl *a*, Chl *b*, and total Chl were slightly lower after salt treatment for 3 DOE, and the lowest value was reached after 7 DOE (Fig. 1). The ratio of Chl *a*/*b* decreased with duration of the exposure to NaCl and reached a minimum value after 5 DOE (Fig. 1*D*). However, the application of exogenous Put stabilized the ratio of Chl *a*/*b* at a certain level and increased the contents of Chl *a*, Chl *b*, and total Chl by 26.0, 18.9, and 24.1% after 7 DOE, respectively, compared with the plants, which were exposed to salt stress alone.

Xanthophyll cycle: Put alone did not significantly affect the contents of V, A, and Z or the size of xanthophyll cycle

Table 1. Primer sequences used in real-time quantitative RT-PCR assays. *ALAD* – 5-aminolevulinate dehydratase; *CAO* – chlorophyllide a oxygenase; *CHLH* – Mg-magnesium chelatase H subunit; *HEMA1* – glutamyl-tRNA reductase; *NOL* – NYC1-like; *NYC1* – non-yellow coloring 1; *PAO* – pheide a oxygenase; *PBGD* – porphobilinogen deaminase; *PPH* – pheophytinase; *PPO* – protoporphyrinogen IX oxidase; *RCCR* – red chlorophyll catabolite reductase; *UROD* – uroporphyrinogen III decarboxylase; *UROS* – uroporphyrinogen III synthase; *VDE* – violaxanthine deepoxidase.

Gene	Primer sequence (5'→3')
HEMA1	Forward 5'- ATCCACCTAAATCTTCTAATCTCT-3′
	Reverse 5'- GACAACAATGCTGCTTCTT-3′
ALAD	Forward 5'- ATAGACTTGGATGGAGAC-3′
	Reverse 5'- TTAGGGAATAACACAACAG-3′
PBGD	Forward 5'- AGCAGCAGGTAGAGAAGA-3′
	Reverse 5'- TAACAGCAGCAGATTGGAT-3′
UROS	Forward 5'-CGGCACCTGTTGATTACG-3′
	Reverse 5'-GCGACTTATCCCACTCCTT -3′
UROD	Forward 5'-ACTTTGGTCTGCTGGTAG-3′
	Reverse 5'-TGCTCGCCTTTATGGATAA-3′
PPO	Forward 5'-CATTCCACAGTTCTTGATT-3′
	Reverse 5'-CACCGCATACATAGTTTC-3′
CHLH	Forward 5'-AAGTTCGTCGTGTAGTTC-3′
	Reverse 5'- CTGTGAGTGATGATTGGTA-3′
CAO	Forward 5'- CCTTGTATGGTGCTATCG-3′
	Reverse 5'- AGTCTTGTCTTCTGTCTTG-3′
NYC1	Forward 5'- GGCAGACAGAATCCGAAAC-3′
	Reverse 5'- ATGATGAAGGCACAAACG-3′
NOL	Forward 5'- CAGGAGTTTCAGTCTTTC-3′
	Reverse 5'- TGTAAGGAGGAACCATAG-3′
PPH	Forward 5'- GCAATGTGACGCCCTTAACT-3′
	Reverse 5'- CATCGAACAGGTCATTGGTG-3′
PAO	Forward 5'- GGGCATTGAAAACTGGAAGA-3′
	Reverse 5'- TTACTTGGCGATCAAAAATGG-3′
RCCR	Forward 5'- ATCTCATCAACGCTATCG-3′
	Reverse 5'- TTCTGGTAATACTCAACATCA-3′
VDE	Forward 5'- TCCTGGCATACTCTACAACCATAAC-3′
	Reverse 5'- CATACCCATCCCAAGCATCGT-3′

pool (VAZ) and DEPS throughout the treatment period compared with the control (Fig. 2). Salt stress markedly declined the contents of V and the size of VAZ but increased the content of A during the treatment time (Fig. 2*A–C*). In addition, the contents of Z and DEPS were significantly elevated and reached a maximum after 3 DOE under salt stress. Thereafter, the content of Z rapidly decreased and was lower than that of the control at 7 DOE; however, the value of DEPS remained significantly higher than that of the control (Fig. 2*C,H*). However, the contents of V and Z were significantly upregulated after spraying with the Put under salt treatment and increased to 12.8 and 107.3%, respectively, compared to salt treatment alone at 7 DOE. The content of A did not significantly differ between the salt-treated plants and those sprayed with Put (Fig. 2*B*). Moreover, Put increased the size of VAZ and the ratio of DEPS by 19.5 and 22.6%, respectively, compared with salt stress after 7 DOE (Fig. 2*G,H*).

Under the nonstress conditions, Put exerted no significant influence on β-carotene content; however, β-carotene content decreased by exposure to salt stress for 3 DOE. In contrast, Put greatly enhanced the content of β-carotene, compared with the plants exposed to salt stress (Fig. 2*F*). In addition, contents of lutein and neoxanthin did not change throughout the stress period in all treatments (Fig. 2*D,E*).

Chl precursors: Put exerted no significant influence on the contents of ALA, PBG, and UroIII under the nonstressed conditions, but increased ProtoIX, Mg-ProtoIX, and PChl. Salt stress induced marked increases in ALA, PBG, and UroIII and significant decreases in the contents of ProtoIX, Mg-ProtoIX, and Pchl compared with the control. However, under salt stress, exogenous Put decreased the contents of ALA, PBG, and UroIII by 7.5, 9.7, and 39.5%, respectively, but increased the contents of ProtoIX, Mg-ProtoIX, and PChl by 55.1, 61.6, and 60.5%, respectively (Fig. 3).

PBGD, UROS, Chlase and MDCase activities: Under nonstressed conditions, exogenous Put showed no significant effects on the activities of UROS, MDCase, and Chlase (Fig. 4*B,C,D*), but increased PBGD activity by 37.0% (Fig. 4*A*). Salt stress significantly increased the activities of UROS, Chlase, and MDCase compared with the control. However, Put effectively decreased the activities of UROS, Chlase, and MDCase by 30.9, 14.0, and 22.7% in the salt-stressed cucumber leaves, respectively. However, no significant differences in the activities of PBGD and Chlase were found between the plants subjected to the salt treatment and those subjected to the salt treatment with Put.

Expression of genes related to Chl metabolism: As shown in Fig. 5, compared with the control, salt stress increased the gene expressions of glutamyl-tRNA reductase (*HEMA1*), 5-aminolevulinate dehydratase (*ALAD*), uroporphyrinogen III synthase (*UROS*), and uroporphyrinogen III decarboxylase (*UROD*), but decreased the transcriptional levels of protoporphyrinogen IX oxidase (*PPO*) and Mg chelatase H subunit (*CHLH*) and showed no significant effects on the gene expressions of porphobilinogen deaminase (*PBGD)* and chlorophyllide a oxygenase (*CAO*). However, the application of exogenous Put significantly increased *PPO* and *CHLH* expression by 32.4 and 37.7%, respectively, and decreased the gene expressions of *HEMA1, ALAD, PBGD, UROS, UROD*, and *CAO* by 31.6, 20.8, 22.9, 55.6, 66.4, and 37.7% , respectively, under salt stress.

Five candidate genes related to Chl degradation were analyzed. We found that Put decreased the gene expression of non-yellow coloring 1 (*NYC1),* NYC1-like (*NOL*),

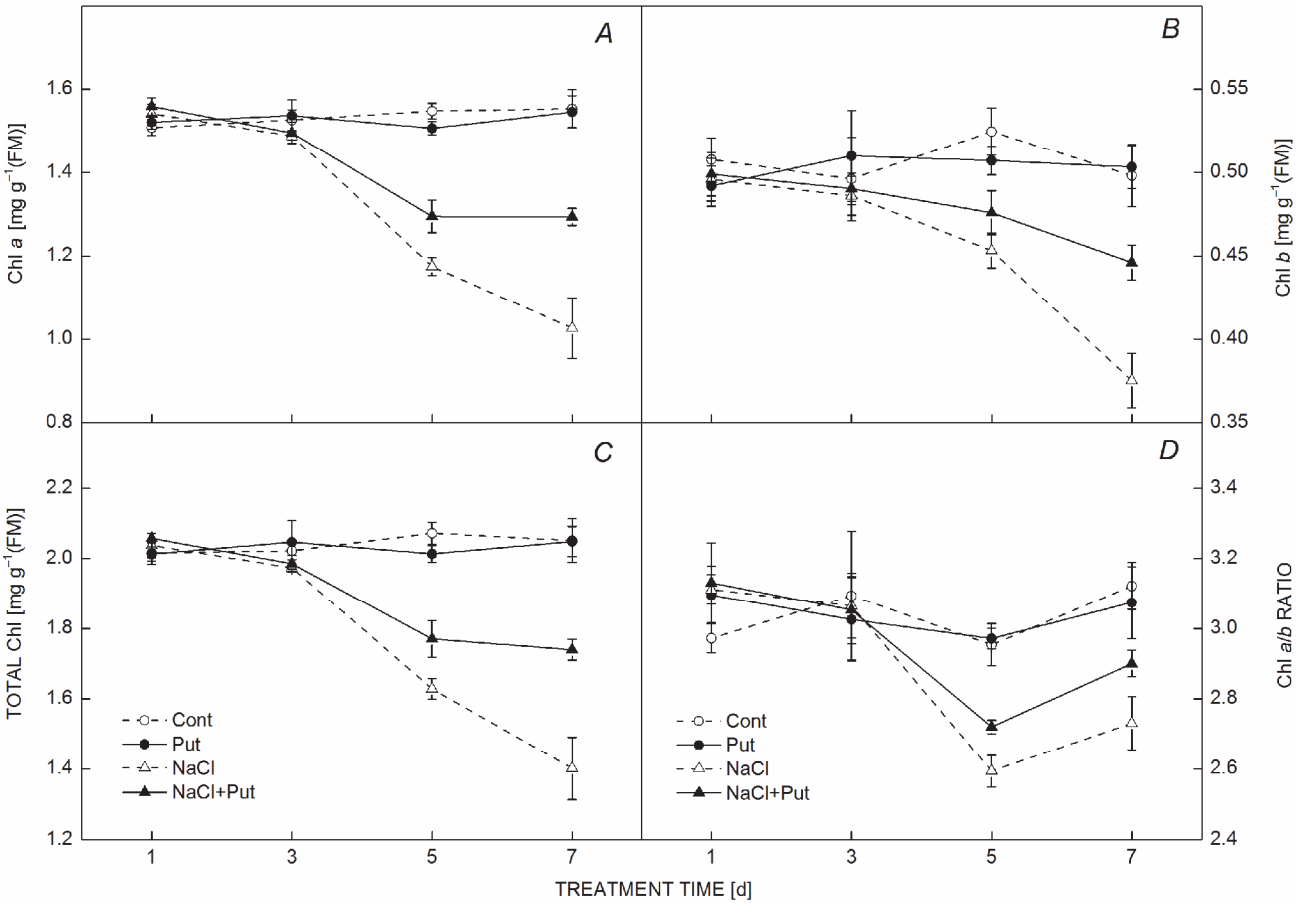

Fig. 1. Effect of exogenous putrescine on chlorophyll contents in the leaves of cucumber seedlings under salt stress. The data were presented as mean ± SE. Chl *a*(*b*) – chlorophyll *a*(*b*); FM – fresh mass.

pheophytinase (*PPH*), pheide *a* oxygenase (*PAO*), and red Chl catabolite reductase (*RCCR*) under nonstressed conditions. When the cucumber seedlings were exposed to 75 mM NaCl for 7 d, the salt stress exerted no significant effects on the expression of *NYC1, NOL, PAO*, and *RCCR* and *PPH* expression markedly increased compared with the control plants. Application of exogenous Put decreased the expression of *NYC1* and *PPH* under normal and salt-stress conditions. Interestingly, compared with salt stress, Put further up-regulated the transcriptional levels of *NOL*, *PAO*, and *RCCR* by 49.7, 71.3, and 26.0%, respectively (Fig. 6).

***VDE* gene expression**: Compared with the nonstressed conditions, the application of exogenous Put increased the expression of *VDE* in cucumber leaves. Salt stress significantly decreased the gene expression of *VDE* throughout the experimental period. However, Put markedly increased the expression of *VDE* under salt stress by 284.3, 49.0, 39.2, and 70.9% after 1, 3, 5, and 7 DOE, respectively, compared with the salt stress treatment (Fig. 7).

Discussion

Light energy, which is used for plant photosynthesis, is harvested by pigments including Chl *a*, Chl *b*, and Car, which are bound to pigment-binding proteins in the thylakoid membrane. It has been demonstrated that LHCII binds approximately 50% of total Chl, which, in addition to Chl *a*, also bind Chl *b* and xanthophylls; in contrast, the core antenna complexes contain only Chl *a* (Caffarri *et al*. 2001, Jennings *et al*. 2015). Thus, an imbalance of Chl metabolism affects the absorption and transmission of light energy and the stability of the conformation of complexes in higher plants (Horn *et al*. 2007). In the present study, exogenous Put alleviated the reduction of Chl *a* and Chl *b* and stabilized the ratio of Chl *a*/*b* under salt stress (3–7 DOE) (Fig. 1). The former decrease and the latter increase of Chl *a*/*b* indicated that the degradation of Chl *b* was faster than the degradation of Chl *a* under salt treatment for 5 DOE (Fig. 1*D*). These results suggested that the extent of degradation of Chl *a* and Chl *b* was not consistent in different periods of stress. Therefore, we speculated that the effects observed in the Put-treated plants under salt stress were related to the regulation of Chl metabolism.

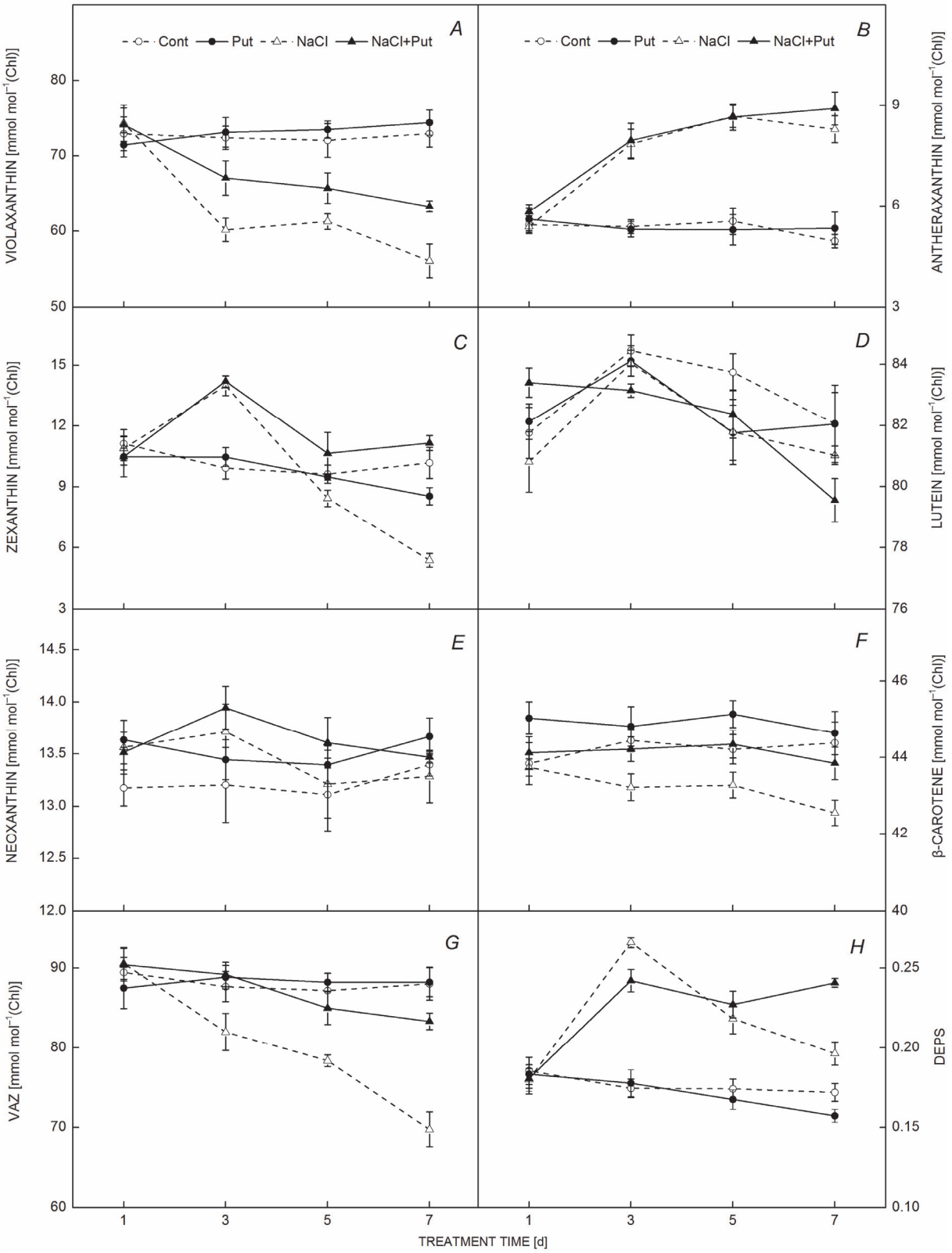

Fig. 2. Effect of exogenous putrescine on xanthophyll cycle components and carotenoids in the leaves of cucumber seedlings under salt stress. The data were presented as mean ± SE. DEPS – de-epoxidation of the xanthophyll cycle.

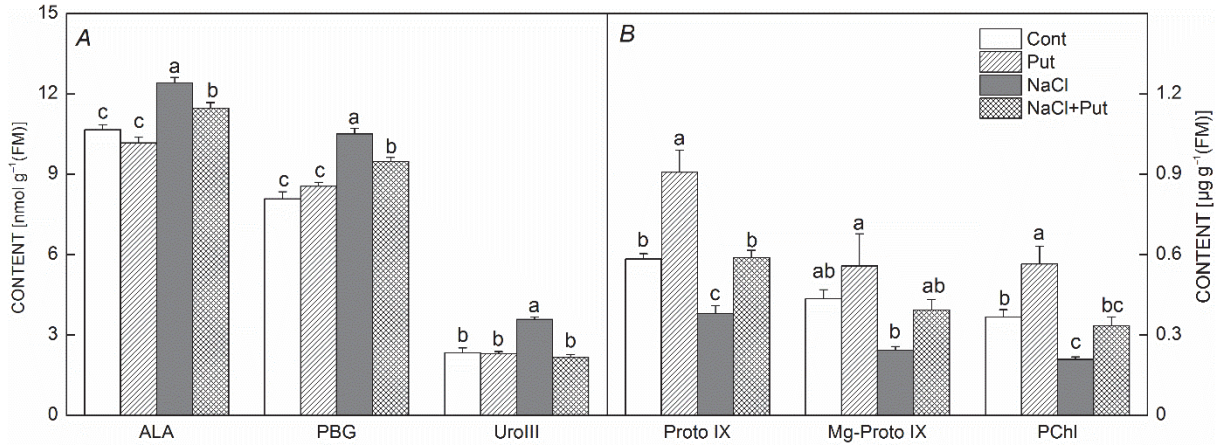

Fig. 3. Effect of exogenous putrescine on the contents of chlorophyll biosynthesis precursors in the leaves of cucumber seedlings exposed to 75mM NaCl for 7 days. The data were presented as means ± SE. ALA – δ-aminolevulinic acid; Mg-Proto IX – Mg proporphyrin IX; PBG – porphobilinogen; PChl – protochlorophyllide; Proto IX – protoporphyrin IX; UroIII – uroporhyrinogen III.

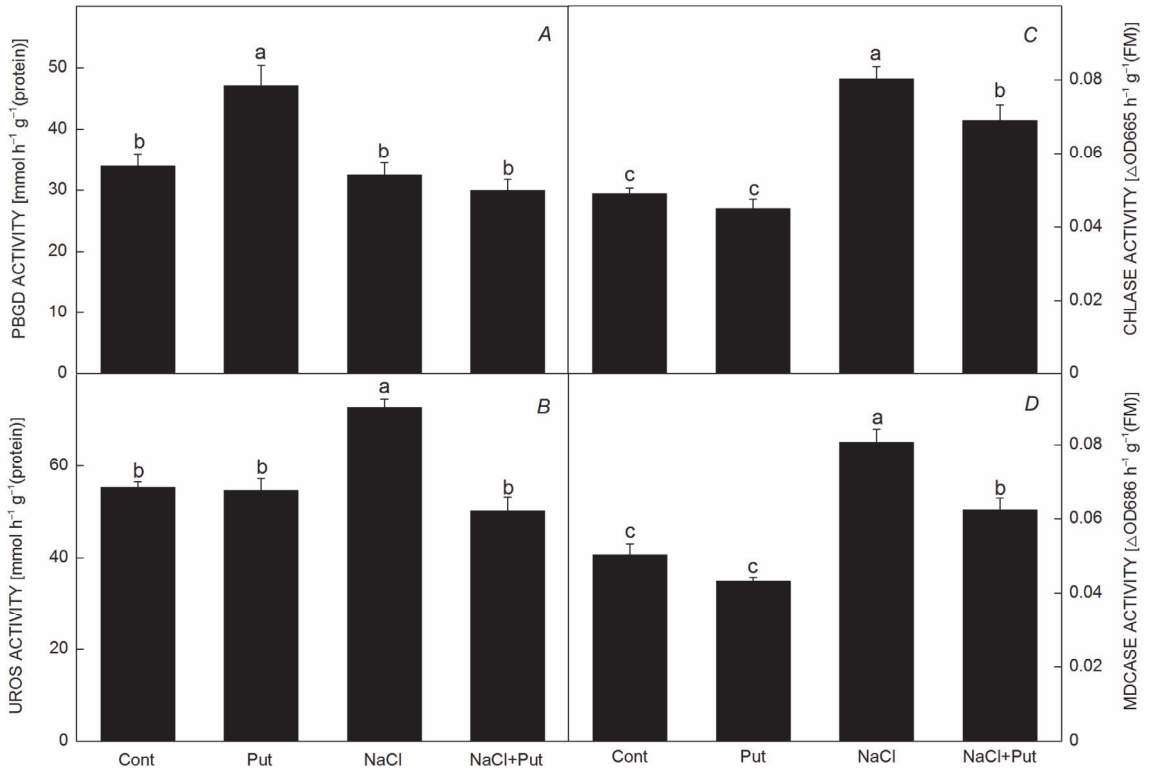

Fig. 4. Effect of exogenous putrescine on the activities of key enzymes of the chlorophyll metabolism pathway in the leaves of cucumber seedlings exposed to 75mM NaCl for 7 days. The data were presented as means ± SE. Chlase – chlorophyllase; MDCase – Mg dechelatase; PBGD – porphobilinogen deaminase; UROS – uroporphyrinogen III synthase.

In Chl anabolism, the transformation of glutamic acid into Mg-protoIX occurs in the chloroplast stroma. Mg-protoIX is then converted to Chl *b* through a series of reactions that occur in the thylakoid membrane (Porra 2008). The disruption of any reaction steps in the Chl biosynthesis pathway might cause a significant accumulation of intermediates prior to the site of obstruction. In addition, as potential chloroplast signals, Chl intermediates might regulate growth rates, photosynthetic gene expression, photosynthetic efficiency, and cell-death processes (Alawady and Grimm 2005). In the present study, we found that Put effectively alleviated the accumulation of ALA, PBG, UroIII, and the reduction of ProtoIX, Mg-ProtoIX, and PChl in salt-stressed cucumber seedlings (Fig. 3). Hence, our results proved that Put relieved the degree of obstruction of the transformation from UroIII to

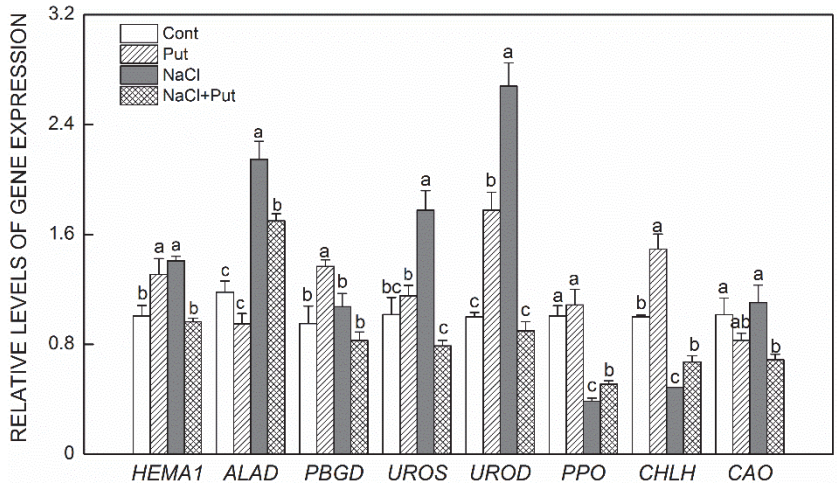

Fig. 5. Effect of exogenous putrescine on the expression of key genes of the chlorophyll biosynthesis pathway in the leaves of cucumber seedlings exposed to 75 mM NaCl for 7 days. The data were presented as mean ± SE. *ALAD* – 5-aminolevulinate dehydratase; *CAO* – chlorophyllide a oxygenase; *CHLH* – Mg-agnesium chelatase H subunit; *HEMA1* – glutamyl-tRNA reductase; *PBGD* – porphobilinogen deaminase; *PPO* – protoporphyrinogen IX oxidase; *UROD* – uroporphyrinogen III decarboxylase; *UROS* – uroporphyrinogen III synthase.

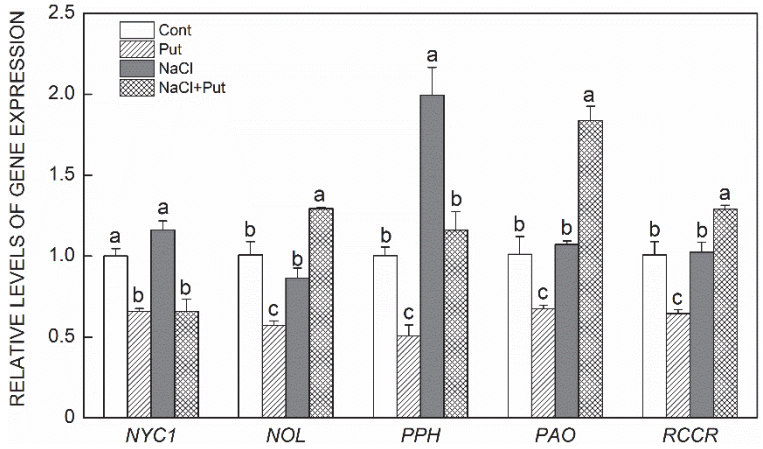

Fig. 6. Effect of exogenous putrescine on the expression of key genes of the chlorophyll degradation pathway in the leaves of cucumber seedlings exposed to 75mM NaCl for 7 days. The data were presented as mean ± SE. *NOL* – NYC1-like; *NYC1* – non-yellow coloring 1; *PAO* – pheide a oxygenase; *PPH* – pheophytinase; *RCCR* – red chlorophyll catabolite reductase.

ProtoIX, probably by decreasing UROS activity in the salt-stressed cucumber leaves (Fig. 4*B*). Furthermore, we speculate that the role of Put in improving the salt tolerance of cucumber seedlings might involve chloroplast signal regulation.

Our results showed that exogenous Put regulated the changes in gene expression that were induced by salt stress to ensure the normal operation of Chl anabolism. The degradation of Chl *b* is the initial step of Chl breakdown, and the transformation of Chl *b* to Chl *a* is catalyzed by Chl *b* reductase (CBR), which is encoded by *NYC1* and *NOL*. The expression levels of *CAO*, *NYC1*, and *NOL* might involve the regulation of Chl *a*/*b* and Chl antenna size (Masuda *et al.* 2003, Jia *et al*. 2015, Sato *et al*. 2015). In this study, exogenous Put decreased the expression level of *CAO* in cucumber seedlings under salt stress (Fig. 5); therefore, we supposed that the mechanism partially involved an increase in the Chl *a*/*b* ratio. The disparity in the expression of *NYC1* and *NOL* under salt stress in presence of exogenous Put suggested that *NYC1* and *NOL* might play different important roles in regulating the conversion of Chl *b* to Chl *a*. The retention of Chl *b* is a very important factor for LHCII stability. When *NYC1* is expressed, Chl *b* is degraded, possibly causing a structural change of LHCII, and allows for the breakdown of Chl *a* and some Car (Sato *et al*. 2009, Hörtensteiner 2013). Moreover, the observed increase in the Chl *a*/*b* ratio was concomitant with high expression levels of the *NOL* gene under salt stress with exogenous Put. In addition, we supposed that the low expression level of the *NYC1* gene might inhibit the degradation of Chl *a* and LHCII.

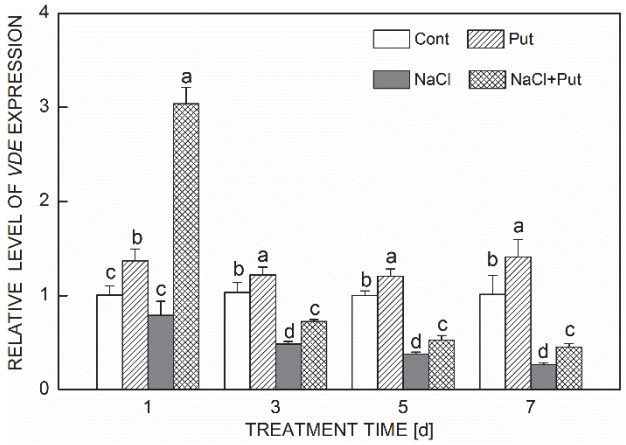

Fig. 7. Effect of exogenous putrescine on violaxanthin deepoxidase (*VDE)* gene expression in the leaves of cucumber seedlings under salt stress. The data were presented as mean ± SE.

After the conversion of Chl *b* to Chl *a*, Chl *a* is degraded by chlorophyllase and is then converted to primary fluorescent Chl catabolite (FCC) by PAO and RCCR. Thereafter, FCC is translocated to the vacuole and transformed to NCC. When Chl and its derivatives are present in excessive amounts, they cause cell death in plants. Therefore, an orderly and effective process for Chl degradation can maintain cell activity and healthy growth in plants (Hörtensteiner 2013). In this study, we found that exogenous Put further increased the transcriptional levels of *PAO* and *RCCR* compared with those observed under salt-stress conditions (Fig. 6). It has been suggested that Put might alleviate the accumulation of pheophorbide *a* and RCC and further promote the degradation of RCC, thereby decreasing the cell toxicity of plants under salt treatment (Nan and Greenberg 2006, Adriana *et al*. 2007). We also found that Put decreased the activity of MDCase in salt-stressed cucumber leaves (Fig. 4*D*). Together, these results indicated that exogenous Put could stabilize the metabolic balance of Chl and protect the cell from suffering photodamage through the regulation of MDCase activity and the expression of genes involved in the Chl metabolism pathway in cucumber seedlings under salt stress.

Photodamage occurs mainly in PSII under stress conditions due to chloroplast oxidative damage (Li *et al*. 2003). In the present study, salt stress resulted in significant decreases in V and Z, which were accompanied by the decrease in the VAZ size after treatment with salinity for 3 d. However, the presence of exogenous Put increased the level of DEPS in the cucumber seedlings leaves under salt treatment (Fig. 2). In addition, Put application greatly enhanced the content of β-carotene, compared to plants exposed to salt stress, a finding that is consistent with that reported by Sáez *et al*. (2013). It has been reported that the *VDE* gene plays a vital role in enhancing the ability of plants to dissipate excess energy and protect thylakoid membranes from photo-oxidation under heat stress (Michel *et al*. 2004). In this work, we found that stressed plants, whichwere treated with Put, exhibited an increased transcriptional level of *VDE* throughout the whole experimental period (Fig. 7). This finding suggests that exogenous Put improved the conversion of V to the final product Z and played a crucial role in dissipating excess excitation energy by enhancing the transcriptional level of *VDE*.

In conclusion, the results of the present study demonstrated that exogenous Put plays an important role in the salt stress tolerance of cucumber seedlings by alleviating the degree of blocked conversion, regulating the expression of genes involved in chlorophyll metabolism and enhancing the photosynthetic pigment contents and de-epoxidation of the xanthophyll cycle, which can increase the photosynthetic capacity and thermal energy dissipation of cucumber seedlings under salt stress (Fig. 1S, *supplement available online*).

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