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BRIEF COMMUNICATION

Ethanolamine induced modification in glycine betaine and proline metabolism in *Nicotiana rustica* under salt stress

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Abstract

The present study aimed to investigate the effects of ethanolamine on glycine betaine and proline metabolism in *Nicotiana rustica* under salt stress. The *in vitro* grown tobacco (*Nicotiana rustica*) plants were pretreated with ethanolamine (at concentrations 70, 130, 270, and 530 µM for biochemical analysis and only at the concentration of 530 µM for molecular analysis) and then transferred to Murashige and Skoog medium containing 200 mM NaCl for 3 weeks. Our results showed that ethanolamine promoted glycine betaine biosynthesis by an increase in betaine aldehyde dehydrogenase (*BADH*) gene expression and BADH enzymatic activity. Moreover, ethanolamine pretreatment possibly reduced proline content in salt stressed plants *via* its negative effect on Δ-pyrroline-5-carboxylate synthase (*P5CS*) gene expression and P5CS enzymatic activity and its positive effect on proline dehydrogenase (*PDH*) gene expression and PDH activity.

Additional key words: betaine aldehyde dehydrogenase, Δ-pyrroline-5-carboxylate synthase, proline dehydrogenase.

According to Zhu (2007), no toxic substance restricts plant growth more often than salt. One of the most common responses of plants to alleviate NaCl-induced stress is production of compatible organic solutes such as proline and glycine betaine (GB) (Serraj and Sinclair 2002). In osmotically stressed tissues, proline mainly arises from the glutamine pathway by the activity of Δ-pyrroline-5-carboxylate-synthetase (P5CS; a rate limiting enzyme in proline biosynthesis pathway) and P5C reductase (P5CR) (Delauney and Verma 1993). After rehydration, proline is degraded by proline dehydrogenase (PDH) and P5C dehydrogenase (P5CDH) (Yoshiba *et al.* 1999). PDH catalyzes the rate-limiting step in-proline degradation. Another osmolyte is GB and it is synthesized from serine *via* ethanol-amine, choline, and betaine aldehyde (Rhodes and Hanson 1993). Betaine aldehyde dehydrogenase (BADH) is a key enzyme in this pathway (Rhodes *et al.* 2002). In many plants such as tobacco, the natural accumulation of GB is lower than sufficient to ameliorate the adverse effects of dehydration caused by various environmental stresses (Yancey 1994, Subbarao *et al.* 2001). Therefore, exogenous application of components of its biosynthetic pathway may lead to an increase in the content of GB and thus can be a good strategy to overcome detrimental effects of environmental stresses. Although much effort has been devoted to genetically engineered plants for overproduction of various osmoprotectants, there has been little success in achieving the desired protection of plants. Alternatively, in some plants, increased resistance to abiotic stresses has been achieved by exogenous application of various organic solutes (Ashraf and Foolad 2007). In the present study, an ethanolamine (EA) was applied on tobacco plants to investigate its effect on improvement of resistance to salt stress by increasing glycine betaine biosynthesis. EA is a naturally occurring compound in plant cells. It has also been suggested that external EA might function as a signal for initiating stress tolerance and may serve as a membrane stabilizer, too (Mascher *et al.* 2005b). The aim of present study was to reveal if EA

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Abbreviations: BADH - betaine aldehyde dehydrogenase; EA - ethanolamine; GB - glycine betaine; P5CR - pyrroline-5-carboxylate reductase; P5CS - Δ-pyrroline-5-carboxylate synthase; PDH - proline dehydrogenase.

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application promotes GB biosynthesis and whether induction of GB biosynthesis by EA interacts with the metabolism of proline.

In vitro grown tobacco (*Nicotiana rustica* L.) plants (4-week old) were transferred to Murashige and Skoog (1962; MS) medium supplemented with 0, 70, 130, 270, and 530 µM EA and kept in a growth chamber (temperature of 25 °C, 16-h photoperiod, and irradiance of 44 µmol m^{-2} s^{-1}) After 2 d, plants were transferred to MS medium with or without 200 mM NaCl for 3 weeks. For real-time quantitative PCR analysis, only 530 µM of EA was used. The effect of EA on gene expression in salt treated plants was determined in 10-d intervals.

Proline content was measured according to the method of Bates *et al.* (1973). P5CS activity was measured according to the malachite green assay (An et al. 2013). PDH enzyme activity was assayed by following the $NADP^+$ reduction at 340 nm based on the method of Ruiz *et al.* (2005). Dried finely-ground plant material (0.5 g), was used to measure GB content as described by Grieve and Grattan (1983). BADH activity assay was carried out in a reaction mixture containing 50 mM Hepes-KOH (pH 8), 5 mM dithiothreitol, 1 mM EDTA, 1 mM betaine aldehyde, 1 mM NAD^+, and enzyme extract according to the method of Weretilnyk *et al.* (1989). Protein content was measured following the method of Bradford (1976), with BSA as a standard.

Total mRNA was extracted by using the *RNesasy* plant mini kit (*Qiagen*, Hilden, Germany), according to the instructions of manufacturer. Then, cDNA was synthesized with a *Revert Aid First Strand* cDNA synthesis kit (*Fermentas*, USA) according to the manufacturer's instructions. Real-time qPCR reactions were carried out on a real-time PCR *Chromo4* (*Bio-Rad*, Hercules, USA) using primers described in Table 1 Suppl. The reaction cycles were as follows: 95 °C for 30 s, 40 cycles of 95° C for 5 s, 60 °C for 20 s, followed by 72 °C for 30 s according to the protocol *SYBR Premix EX Taq* (*Takara*, Japan). Gene expression was calculated in relation to the expression of *Elongation Factor 1a* (*EF1α*) gene using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Statistical analysis was performed with the statistical software package for social science (*SPSS v. 21*). Differences between control and treated plants were analyzed by two-way *ANOVA*, taking $P < 0.05$ as significant, according to Duncan's test.

Our experiment showed that EA pretreatment enhanced GB content of salt treated plants when compared with salt treated plants without EA pretreatment (Table 1). Hitz *et al.* (1982) showed that application of ^{14}C labelled EA in barley plants results in increased conversion of [^{14}C]EA to phosphatidyl choline and betaine over time. These findings confirmed the elevation of GB synthesis induced by exogenous EA. Our BADH activity assay showed a remarkable increase in enzyme activity of EA pretreated plants under salt stress (Table 1). Increase in BADH activity could be the probable cause of increase in GB content. *BADH* gene expression was also up regulated in EA pretreated plants under salt stress (Fig. 1*A*). It is possible to assume that EA application resulted in phosphocholine increase (Rontein *et al.* 2001) and this compound led to an increase in betaine aldehyde amount in turn (Rontein *et al.* 2002). Betaine aldehyde content enhancement could subsequently result in an increase of *BADH* mRNA level and its enzyme activity and finally led to enhancement of GB content in salt treated plants which received EA pretreatment compared to salt treated plants without EA. From previous data and present results, it is obvious that change in proline content is the common response of tobacco plants to salt stress. Interestingly, our results revealed that EA pretreatment dramatically reduced proline content of salt treated plants (Table 1). This observation was consistent with results of Kogan *et al.* (2000). According to available data, it can be speculated that it is possible that plant injuries were lower in salt

Table 1. Effect of pretreatment with ethanolamine at different concentrations (0, 70, 130, 270 and 540 µM) on GB content [µmol g^{-1}(d.m.)], BADH activity [nmol mg^{-1}(prot.) min^{-1}], proline content [µmol g^{-1}(f.m.)], P5CS activity [µmol Pi mg^{-1} (prot.) min^{-1}], and PDH activity [nmol(NAD) mg^{-1} (prot.) min^{-1}] in salt stressed plants (+salt) and control plants (-salt). Means ± SDs, *n* = 3. Means within a row followed by different letters indicate significant differences at $P < 0.05$ based on Duncan's test.

Parameters		0 µM EA	70 µM EA	130 µM EA	270 µM EA	530 µM EA
GB content	+ salt	0.360±0.105c	2.010±0.57a	2.025±0.457a	2.014±0.175a	1.032±0.39b
	– salt	0.076±0.066d	0.120±0.015d	0.230±0.0808cd	0.120±0.014d	0.140±0.014d
BADH activity	+ salt	0.674±0.014d	2.709±0.086a	2.173±0.142b	1.407±0.088c	2.168±0.15b
	– salt	0.030±0.0e	0.682±0.0068d	0.675±0.013d	0.672±0.007d	0.682±0.014d
Proline content	+ salt	29.800±2.00a	4.110±0.90b	6.935±1.50b	4.800±0.50b	5.900±0.5b
	– salt	0.665±0.04c	1.110±0.90c	1.800±0.10c	1.800±0.50c	2.000±0.5c
P5CS activity	+ salt	0.697±0.093a	0.550±0.026b	0.510±0.054b	0.559±0.037b	0.599±0.051b
	– salt	0.354±0.013c	0.335±0.0156c	0.342±0.066c	0.367±0.035c	0.312±0.071c
PDH activity	+ salt	0.860±0.59d	3.440±0.85b	2.380±0.238c	2.440±0.325bc	5.100±1.4a
	– salt	0.380±0.165d	0.359±0.156d	0.345±0.149d	0.352±0.152d	0.320±0.138d

treated plants pretreated with EA than in those non-pretreated with EA (Mascher *et al.* 2005a,b, Rajaeian *et al.* 2011) and so proline content was lower in EA pretreated plants. On the other hand, previous studies have shown that GB has a negative effect on proline response under stress conditions (Larher *et al.* 1993). From present results and according to Larher *et al.* (1993), it is possible to assume that EA increased GB content of plants under salt stress but declined proline content. We found that EA reduced P5CS activity while stimulated PDH activity in salt treated plants (Table 1). Further, EA application resulted in down-regulation of *P5CS* gene expression in plants under salt stress on day 1 and 21 after salt treatment (Fig. 1*B*). A decrease in proline content induced by EA seemed to be due to down-regulation of *P5CS* gene expression. The subsequent decline in P5CS activity decreased *de novo* synthesis of proline in salt treated plants. It is possible that after induction of GB biosynthetic pathway by EA, from the stand point of saving metabolic energy, *P5CS* gene expression was blocked. It is also possible to assume that proline content decline in salt treated plants pretreated with EA could be due to an increase in its degradation. Real-time qPCR analysis of *PDH* gene on day 1 of salt stress showed that *PDH* transcription declined in salt treated plants after EA pretreatment when compared with salt treated plants without EA pretreatment (Fig. 1*C*). Considering the improvement of plant condition under salt stress *via* EA protective effect, it was postulated that proline catabolism was not necessary in order to provide energy and redox adjustment under this condition. Under EA treatment, adopted strategies to create resistance to salt stress were different from the ones in plants without EA pretreatment. EA pretreatment elevated *PDH* mRNA content in the plants under salt stress on day 11 (Fig. 1*C*). Considering the marked increase in *BADH* transcription on this day by EA pretreatment, we assumed that after activation of GB synthesis by EA, proline catabolism was enhanced to provide structural requirement and ATP to GB synthesis.

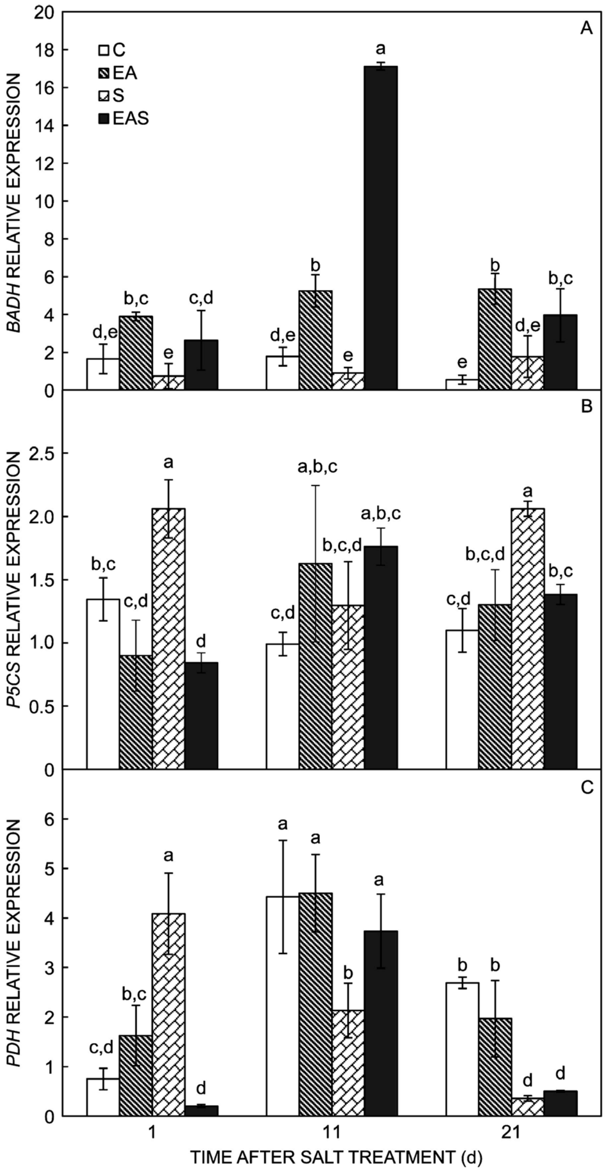

Fig. 1. Effect of 540 µM ethanolamine pretreatment on *BADH* (*A*), *P5CS* (*B*), and *PDH* (*C*) relative gene expressions at 1, 11, and 21 d after treatments. Measurements were done in control plants (C), EA pretreated plants (EA), salt stressed plants (S) and salt stressed plants pretreated with EA (EAS). Means ± SDs, $n = 3$. Different letters indicate significantly different data at $P < 0.05$ based on Duncan's test.

In conclusion, our present observations suggested that EA application induced GB biosynthesis probably *via* an increase in *BADH* mRNA content and enhanced its enzyme activity. Thus, enhanced GB content as a strong osmolyte improved plant resistance against salt stress. Changes in GB metabolism by EA led to a decline in P5CS activity and its gene transcription level and also up-regulation of *PDH* gene expression, an increase in PDH enzyme activity and, subsequently, a significant decrease in proline content in salt treated plants.

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