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Full Length Research Paper

Nitric oxide enhances osmoregulation of tobacco (*Nicotiana tobacam* L.) cultured cells under phenylethanoid glycosides (PEG) 6000 stress by regulating proline metabolism

Xue Ke[1,2,3], Zaiquan Cheng[1], Wenguang Ma[4] and Ming Gong[2]*

[1]Biotechnology and Genetic Germplasm Institute, Yunnan Academy of Agricultural Sciences, Kunming 650223, P. R. China.
[2]School of Life Sciences, Yunnan Normal University; Engineering Research Center of Sustainable Development and Utilization of Biomass Energy, Ministry of Education; Key Laboratory of Biomass Energy and Environmental Biotechnology of Yunnan Province; Kunming 650500, P. R. China.
[3]College of Water Conservancy and Civil Engineering, China Agricultural University, Beijing 100083, P. R. China.
[4]Yunnan Academy of Tobacco Agricultural Sciences, Yuxi 653100, Yunnan, P. R. China.

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This study was carried out to investigate the effect of the intracellular signaling molecule nitric oxide (NO) on osmoregulation of tobacco cells under osmotic stress caused by phenylethanoid glycosides 6000 (PEG 6000). The results show that the PEG stress induced a specific pattern of endogenous NO production with two peaks in tobacco cells *in vivo*. Treatments with the NO donor sodium nitroprusside (SNP) significantly improved vitality and re-growth capacity, lowered cell death rate and alleviated the damage of tobacco cells caused by PEG 6000 stress. Further study indicated that SNP treatments led to relatively lower cell solute potential and higher water potential, which was beneficial for maintaining cell pressure potential under PEG stress. These results indicate that NO could improve the tolerance of tobacco cells to osmotic stress by enhancing their osmoregulation capacity. In addition, SNP treatments increased the accumulation of proline, one of the important organic osmoregulators in the tobacco cells under normal culture condition as well as PEG stress. The investigation on proline metabolism pathways demonstrated that the SNP-induced proline accumulation might be a combined result of sequential activation of several key enzymes of proline biosynthesis, including glutamate dehydrogenase and Δ1-pyrroline-5-carboxylate synthetase of glutamate pathway, and arginase and ornithine aminotransferase of ornithine pathway, and the inhibition of proline dehydrogenase of proline degradation pathway. All of these results suggest that NO takes part in the response and adaptation of tobacco cells to osmotic stress by enhancing their osmoregulation capacity and proline accumulation.

Key words: Nitric oxide, osmoregulation, proline, tobacco suspension cells, osmotic tolerance.

INTRODUCTION

Osmotic stress commonly caused by drought and salinity is one of the major environmental factors limiting plant productivity and distribution (Verslues et al., 2006; Wakeel et al., 2011). To counteract osmotic stress, plants have evolved diverse response and adaptation mechanisms, and they are of four distinct types: (1) to synthesize kinds of compatible solutes that contribute to osmotic adjustment, such as the most common solutes proline and glycinebetaine (Liu et al., 2010; Hoque et al., 2008; Abbas et al., 2010), (2) by increasing the activity of enzymes involved in the antioxidant defense system, such as superoxide dismutase (SOD) (Wang et al., 2010), (3) by signal function of hormone, for instances,

*Corresponding author. E-mail: gongming63@163.com or biochem312@163.com. Tel: +86 871 5941370. Fax: +86 871 5516759.

abscisic acid (ABA)-induced proline accumulation was contributed to osmotic tolerance (Stewart, 1980) and (4) to express some specific proteins, such as aquaporin and late embryogenesis abundant (Lea) proteins (Siefritz et al., 2001; Uehlein et al., 2003). These response and adaptation of plants to osmotic stress are often dependent on the function of intracelluar second messenger molecules, and one of these molecules is nitric oxide (NO).

NO is a lipophilic molecule and is known for its universal signaling properties. It involves not only in plant growth and development, but also in plant response to abiotic and biotic stress (Wendehenne et al., 2004; Arasimowicz and Floryszak-Wieczorek, 2007). Previous works on biological function of NO provided an indirect suggestion that NO acts as a regulator during osmotic stress, although in plants its synthesis by nitric oxide synthase (NOS) is still unknown (Besson-Bard et al., 2008; Guo, 2006; Crawford, 2006). For example, exogenous NO was able to induce stomatal closure, increase relative water content and Lea proteins accumulation in wheat (Mata and Lamattina, 2001). NO donor sodium nitroprusside (SNP) could promote the activity of antioxidant enzymes and plasma membrane H+-ATPase under drought and salt stress (Zhao et al., 2004; Lei et al., 2007). Exogenous NO also relieved oxidative damage of leaves and seedlings in wheat and cucumber (Chen et al., 2004; Fan et al., 2006). During osmotic stress caused by salinity and drought, although endogenous NO production or nitric oxide synthase (NOS)-like activity could be detected in tobacco and Arabidopsis (Guo et al., 2003; Gould et al., 2003; Zhao et al., 2007), but it is unclear how NO is involved in osmoregulation of plant cells.

Until now, only a little experimental data indicated that NO takes part in proline synthesis. The NO donor SNP treatment induced proline accumulation in under drought stress (Lei et al., 2007), and NO-induced cold acclimation and freezing tolerance of Arabidopsis was associated with activity changes of proline synthesis enzyme Δ1-pyrroline-5-carboxylate synthetase (P5CS) and proline degradation enzyme proline dehydrogenase (PDH) (Zhao et al., 2009). However, the mechanism of NO-induced proline accumulation in plant cells under osmotic stress condition is still unknown, and the effect of NO on proline metabolism is worth studying carefully. It is well known that proline metabolisms in higher plants includes two biosynthetic pathways, one is glutamate pathway regulated by two key enzymes glutamate dehydrogenase (GDH) and P5CS, and the other ornithine pathway regulated by two key enzymes arginase and ornithine aminotransferase (OAT), as well as one catabolic pathway regulated by a key enzyme PDH (Szabados and Savouré, 2009).

Thus, in this study, using tobacco suspension cells as experimental material, we investigated the involvement of NO in intracellular osmoregulation and proline metabolism under osmotic stress induced by phenylethanoid glycosides (PEG) 6000.

MATERIALS AND METHODS

Plant materials

Tobacco suspension cells were cultivated at our lab as previously described (Li and Gong, 2008). To induce callus, sterilized stem marrow was placed on Murashige and Skoog (MS) culture medium and cultured for 4 weeks in the dark at 25°C. The medium (pH 5.8) contains 0.3 mg•mL^{-1} KH_2PO_4,10 mg•L^{-1} V_{B1},10 mg•L^{-1} V_{B6}, 5 mg•L^{-1} nicotinic acid, 2 mg•L^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D), 3% (W/V) sucrose, 1 g•L^{-1} casein hydroly-sate acid, 0.5 mg•L^{-1} 6-benzylamino purine (6-BA) and 0.8% agar. White loose callus was taken into 50 mL flask to culture suspension cells on an incubator in the dark at 25°C, 110 r•min-1. Liquid medium for suspension cells was cultured on MS medium plus 0.1 mg•L^{-1} kinetin (Kt) without agar. The subculture period was seven days. After subculture, suspension cells grew for 4 days and were used for the experiment.

Osmotic stress treatments

Osmotic stress on the suspension cells was applied by the addition of phenylethanoid glycosides 6000 (PEG 6000). SNP and 2-(4-carboxyphenyl)-4,4,5,5-tetra-methylimidazoline-1-oxyl-3-oxide cPTIO) were added 15 min before the stress treatment, and equal volume (or weight) of the liquid medium was added into the control sample. Filtrated and washed cells were collected for experiments at each time point.

NO measurement

NO measurements were performed as described by Planchet and Kaiser (2006), using NO sensitive fluorophore 4,5-diaminofluorescein-2 diacetate (DAF-2DA) and by Vitecek et al. (2008), using the method of modified Griess.

Method of DAF-2DA

Stress-treated cells were collected and 0.1 g of cells was resuspended in a liquid medium including PEG 6000. After incubation with 10 μmol•L^{-1} DAF-2 DA for 15 min, fluorescence intensity was measured (EX: 495 nm, EM: 515 nm, slit: 2.5 nm) by a fluorescence spectrophotometer (RF-5300PC; Shimadzu, Kyoto, Japan). The fluorescence intensity of dye mixed liquid medium without cells was used as the blank value.

Method of modified Griess: Self-made instruments according to Vitecek et al. (2008) were used for NO content detection. Briefly, a stream of humidified air was pumped at 40 mL•min-1 flow rate through an enclosed flask into an incubator which has suspension cells for culturing. This stream was passed through the first trap containing Griess reagent to remove traces of HNO_2 and then passed through a column containing the strong oxidizing agent CrO_3. The air stream got through the second Griess reagent trap that collects any NO that was oxidized to NO_2. Optical density (OD) value of the second trap at 540 nm was detected as NO content once 1 h. This method could be utilized for real-time measurement of NO synthesis by replacing new Griess reagent trap.

Measurements of physiological indices

Cell vitality was estimated as described by Gong et al. (1998) and by Steponkus and Lanphear (1967). 0.1 g cells were incubated in 1

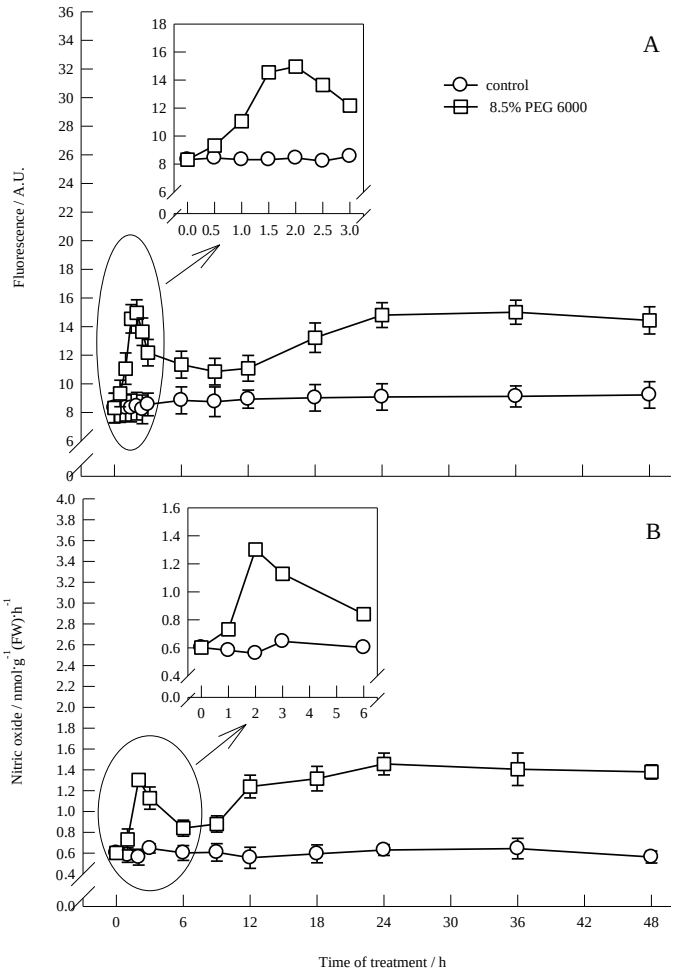

Figure 1. Changes of nitric oxide content in tobacco cultured cells under PEG 6000 stress. A, the change of NO measured by DAF-2DA, the arrow shows the change within 0 to 3 h. B, The change of NO detected by Griess method, the arrow shows the change within 0 to 6 h. Each bar represents means ± SE of at least six measurements in two independent experiments.

mL of 2,3,5-triphenyltetrazolium chloride (TTC) solution at 25°C for 12 h. Cells were collected in 1 mL of 95% ethanol after centrifugation and incubated in boiling water bath for 5 min. OD_{530} nm value of supernatants were measured. Cell death was estimated by Evan's blue staining as described by Yamamoto et al. (2004). Cell regrowth capacity was measured according to Ishikawa et al. (1995). Stress-treated cells was washed five times in a liquid medium and transferred to fresh MS medium to grow and the weight of the cells was measured at 7th day after transferring.

Detection of water potential, solute potential and pressure potential

Water potential (Ψw) of cells was detected as described by Gong et al. (1989). Filtrated cells were immediately put into the sample chamber (C-52, Wescor, USA) of the instrument (Psypro Dew Point Microvolt-meter, Wescor, USA) for Ψw measurement. Equilibrium time was 30 min. Solute potential (Ψs) of cells was detected by a permeameter (FM-9J, SHMU, China). Pressure potential (Ψp) of cells was calculated by the formula Ψw = Ψs + Ψp.

Proline determination

Proline content was measured as reported by Bates et al. (1973). 0.5 g cells were ground in 1 mL 3% sulfosalicylic acid. The homogenate was boiled for 15 min and centrifuged. Then 200 µL supernatants were mixed with 200 µL dH_2O, 200 µL acetic acid and 400 µL acidic-ninhydrin, and absorbance at 520 nm was recorded after incubating at 100°C for 1 h.

Enzyme activity assay

GDH activity was detected by the method of Robinson et al. (1991). P5CS activity was assayed according to Garcia-Rios et al. (1997). Arginase activity was determined according to Alabadí et al. (1996). OAT was extracted according to Lutts et al. (1999). Cells were ground in 100 mmol•L^{-1} K-Pi extraction buffer (pH 7.9) containing 1 mmol•L^{-1} ethylenediaminetetraacetic acid (EDTA), 15% glycerol and 10 mmol•L^{-1} mercaptoethanol. After centrifugation, supernatants were used for activity assay according to Charest and Phan (1990). 0.2 mol•L^{-1} Tris-KOH reaction buffer (pH 8.0) contains 5 mmol•L^{-1} ornithine, 10 mmol•L^{-1} α-ketoglutarate and 0.25 mmol•L^{-1} NADH. The reaction was initiated by adding of 0.2 mL enzyme extracting solution and the absorbance reduction of NADH was monitored at 340 nm. OAT activity was expressed as oxidative NADPH per milligram proteins within 1 min. PDH activity was assayed according to Sánchez et al. (2001).

RNA extraction and RT-PCR

Total RNA of cells used for reverse transcription-polymerase chain reaction (RT-PCR) was isolated using the TRIZOL reagents (Invitrogen) according to its instruction. RT-PCR was carried out using RNA PCR Kit (AMV) Ver.3.0 (TaKaRa) according to the manufacturer's protocol. Based on NCBI information, specific primers were synthesized by Invitrogen and used to amplify the CDS of proline metabolism relative enzyme genes. Primers were as follows: 5'-TGAA TGCCTTAGCACAGC-3' and 5'-GCAACAATCTTCCCACCT-3' for GDH (theoretical amplified fragment length 463 bp); 5'-AGGCTCAAACGCTCACTC-3' and 5'-CAAGGGCAACAGCATCTC-3' for OAT (336 bp); 5'-TTTTACGGCGTTCCTACC-3' and 5'-TTCCCATCTCAGCAAATCAC-3' for PDH (432 bp); 5'-CGATTGAACACGGCATAG-3' and 5'-ACCTCAGGACAGCGGAA C-3' for ACTIN (524 bp). The PCR thermal cycles were performed as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, then 50°C(GDH), or 54°C(OAT), **or** 52°C(PDH), or 55°C(ACTIN) 30 s, 72°C 1 min, at the end of cycles, 72°C 5 min and preserved at 4°C. The light intensity of each gel electrophoresis band of transcripts was estimated by the software Quantity One (Bio-Rad), and the ratio of band intensity (RBI) was calculated as the band light intensity of GDH, OAT and PDH respectively divided by that of ACTIN.

RESULTS

Pattern of PEG 6000-induced NO production

NO production in tobacco cells induced by PEG 6000 stress showed a specific pattern. As shown in Figure 1A and B, the PEG stress induced a rapid increase of NO content within 3 h, peaked at about 2 h (see embedded

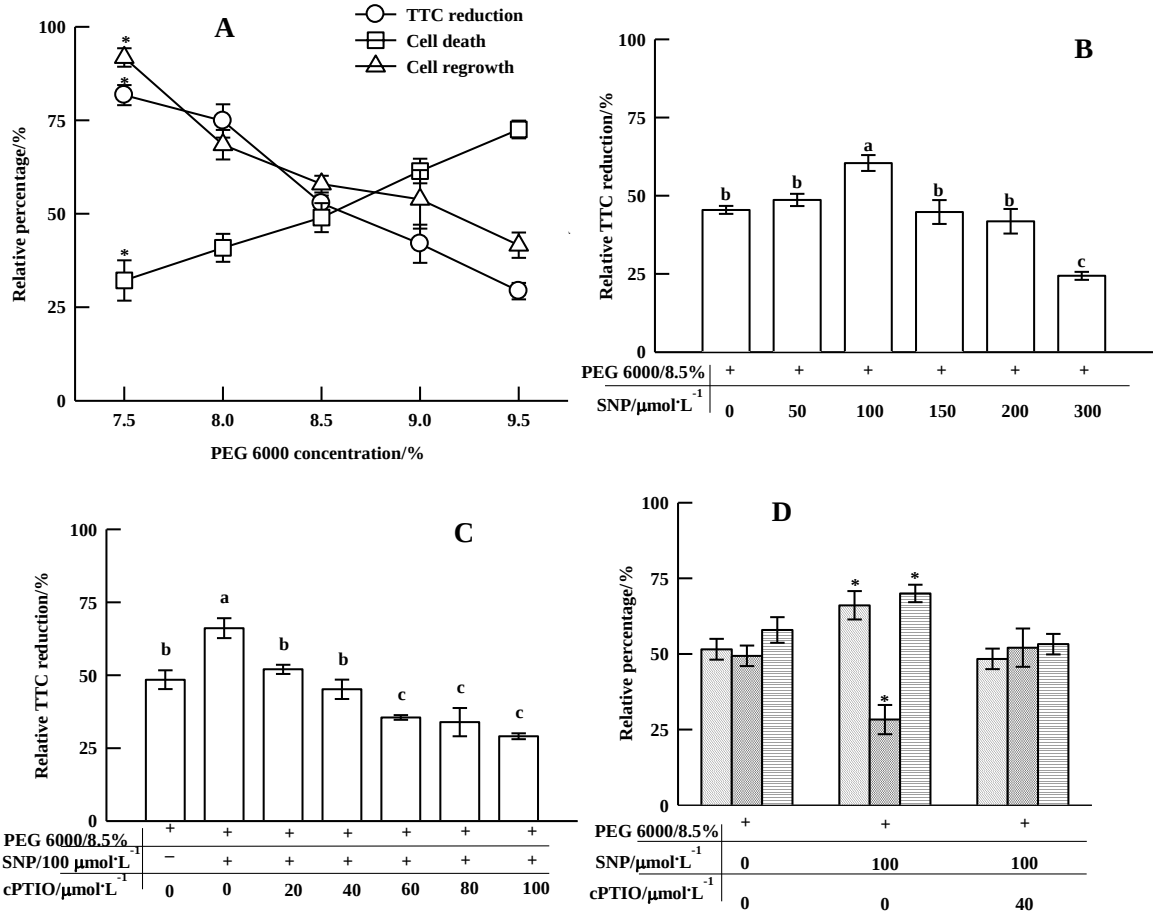

Figure 2. Effects of SNP pretreatment on TTC reduction, cell death and regrowth in tobacco cultured cells under PEG 6000 stress. Relative percentage is the ratio of values of PEG 6000 treatment sample to control sample (the value as 100%). (**A**) effects of PEG 6000; (**B**) effects of SNP; (**C**) effects of cPTIO; (**D**) effects of 100 μmol·L^{-1} SNP and 40 μmol·L^{-1} cPTIO. Each bar represents means ± SE of at least six measurements in two independent experiments. * or different letters indicate significant difference compared to the control (P<0.05).

small figures), and the values of NO fluorescence (Figure 1 A) and the content (Figure 1 B) of the cells at 2 h under PEG stress were respectively 1.8 and 2.3-folds higher than the control. With the prolonged PEG stress, a sustained NO accumulation peak (or plateau) was observed after 12 h PEG stress. The fluorescence and content of NO in the stressed-cells at 48 h were respectively 56.5 and 145.1% higher than that of the control.

Effect of SNP on osmotic tolerance of the cells

Applied concentration of PEG 6000 was selected according to semi-lethal dose for tobacco cells at 48 h. As shown in Figure 2A, under 7.5 to 9.5% (W/V) PEG 6000 treatment, cell vitality, indicated by 2,3,5-triphenyltetrazolium chloride (TTC) reduction, and cell regrowth capacity decreased while cell death raised with the increase of PEG 6000 concentration. Since the values of these three physiological indices of the cells treated with 8.5% PEG 6000 was about 50% of the control under 48 h stress, 8.5% PEG 6000 was used for the following stress treatment.

Under this stress condition, 150 μmol•L^{-1} NO donor sodium nitroprusside (SNP) treatment could significantly increase cell vitality by 32.7% higher than that of non-SNP pretreatment cells at 48 h (Figure 2B). This effect of SNP treatment was inversed or eliminated by NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl- 3-oxide (cPTIO) pretreatment with a proper dose of 40 μmol•L^{-1} (Figure 2C). Further experiment showed that cell regrowth capacity was raised and cell death rate decreased significantly by the SNP pretreatment as compared with the controls without SNP treatment (Figure 2D). Similarly, the above-mentioned effects of SNP on cell regrowth and death were reversed by cPTIO pretreatments (Figure

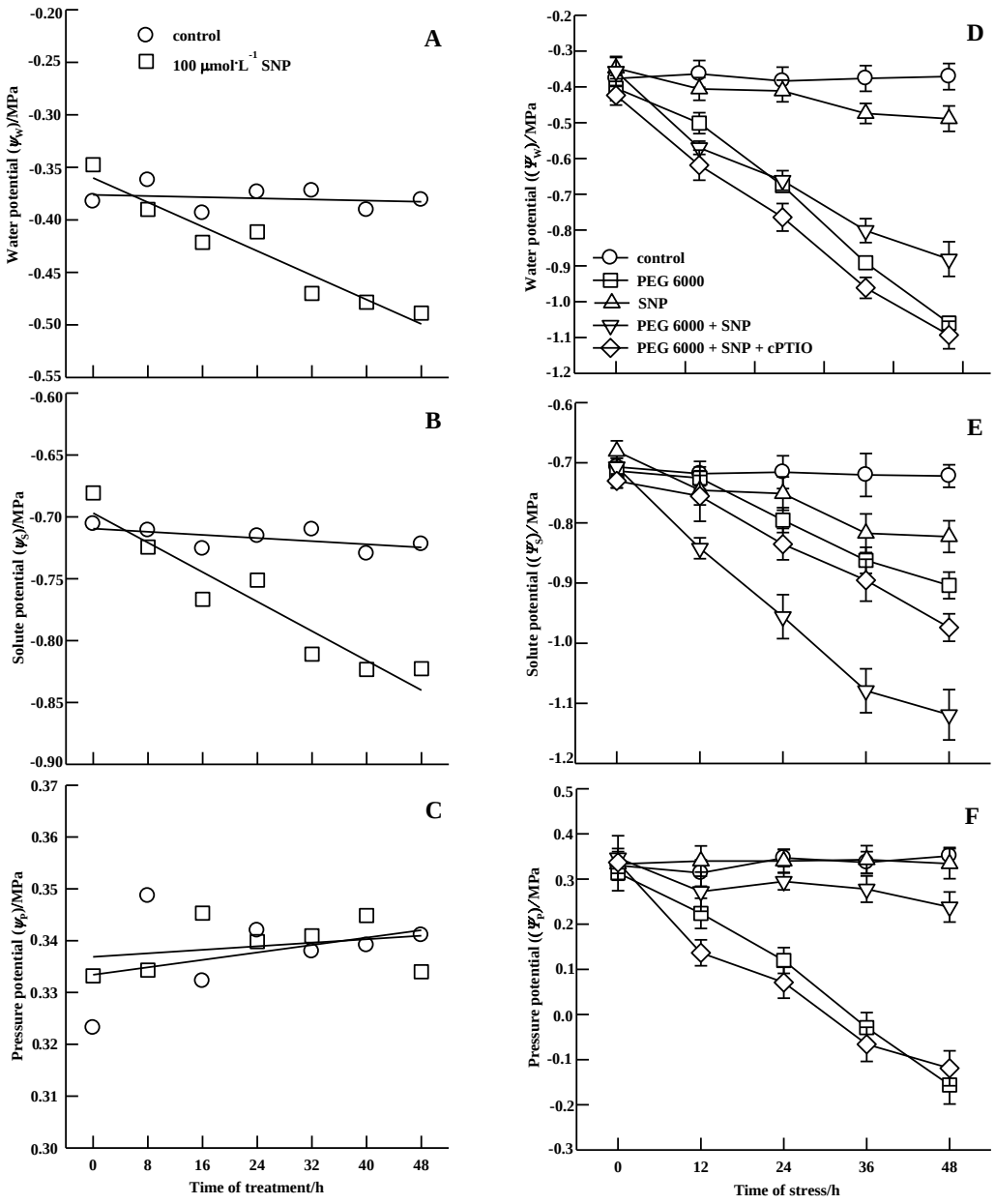

Figure 3. Effects of SNP pretreatments on water potential (ψ_w, **A** and **D**), solute potential (ψ_s, **B** and **E**) and pressure potential (ψ_p, **C** and **F**) of tobacco cultured cells under normal culture condition (left part of the figure) and PEG 6000 stress (right part of the figure). Each value represents means ± SE of at least six measurements in two independent experiments.

2D). These data implied that the NO donor SNP could improve tolerance of the tobacco cells to osmotic stress.

Effect of SNP treatment on osmoreguation of tobacco cells

To investigate the involvement of NO in intracellular osmoregulation process, effect of SNP treatment on the water status of tobacco cells were detected under normal culture condition and PEG stress. In our experiment, there were correlation between SNP treatment and cell water potential (R2 = 0.9277), and between SNP treatment and cell solute potential (R2 = 0.9007), but there was little correlation between SNP treatment and cell pressure potential (R^2 = 0.0817). In general, SNP treatment led to a slight decline of cell water and solute potential but had little effect on pressure potential under normal culture condition (Figure 3A, 3B, 3C).

Furthermore, SNP treatment led to more reduction of

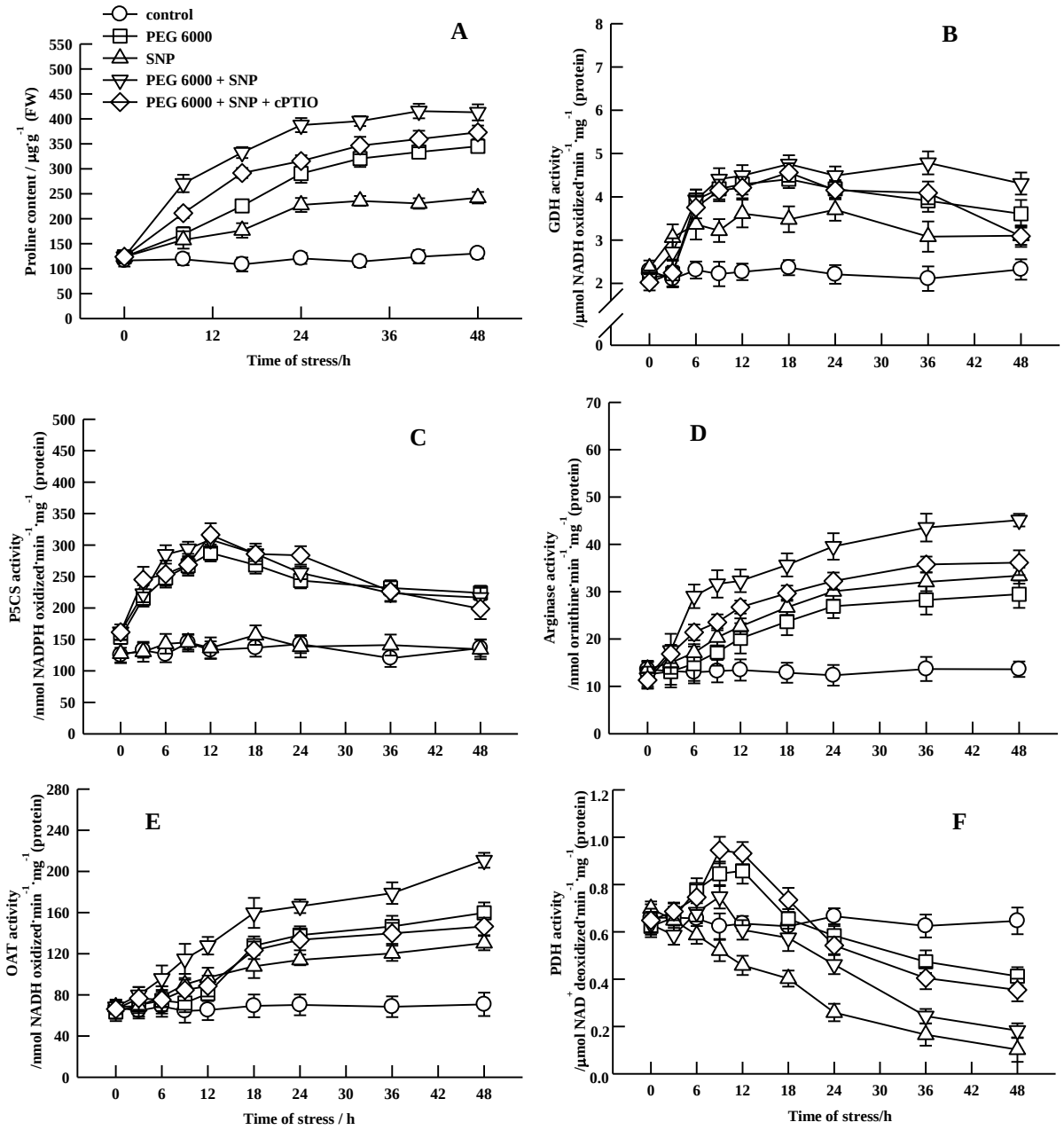

Figure 4. Effects of SNP and cPTIO pretreatments on proline accumulation and the activity of proline metabolic enzymes in tobacco cultured cells under PEG 6000 stress. (**A**) the effects on proline accumulation. (**B**) the effects on GDH activity. (**C**) the effects on P5CS activity. (**D**) the effects on arginase activity. (**E**) the effects on OAT activity. (**F**) the effects on PDH activity. Each value represents means ± SE of at least six measurements in two independent experiments.

cell solute potential, and less reduction of water potential as compared with the controls without SNP treatment under the PEG stress. As a result, the SNP-treated cells could maintain a relatively higher pressure potential than the controls under the PEG stress. The effect of SNP could be reversed by cPTIO pretreatment (Figure 3D, 3E, 3F). These results indicate that NO took part in osmoregulation of tobacco cells.

Effect of SNP on proline accumulation of tobacco cells

Based on the above-mentioned effect of SNP on osmoregulation of cells, proline content was determined as a main osmotic solute. As shown in Figure 4A, SNPtreatment led to a continuous accumulation of proline in the cells both grown under normal condition and PEG

6000 stress for 48 h. Under the PEG stress, proline content of tobacco cells with SNP treatment significantly increased ($P<0.05$), being 19.7% higher than that without SNP treatment at 48 h. Similarly, cPTIO pretreatment could greatly eliminate SNP-induced proline accumulation under the PEG stress.

Effect of SNP on activities key enzymes of proline metabolism

In order to investigate the mechanism of SNP-induced proline accumulation, activities of key enzymes of proline metabolism was determined in tobacco cells. These enzymes include GDH and P5CS of glutamate biosynthetic pathway, arginase and OAT of ornithine biosynthetic pathway and PDH of catabolic pathway (Szabados and Savouré, 2009). As shown in Figure 4B, both SNP-pretreatment and PEG 6000 stress led to a significant increase of GDH activity in tobacco cells. Under PEG 6000 stress, SNP pretreatment was further enhanced by GDH activity by 19.4% at 48 h ($P<0.05$). This SNP-enhanced activity, however, could be eliminated by cPTIO pretreatment.

P5CS activity increased rapidly within 12 h under PEG 6000 stress, but it is noteworthy that SNP or cPTIO pretreatment did not lead to significant change of the enzyme activity under the stress condition in our experiment ($P>0.05$) (Figure 4C). PEG stress greatly increased arginase activity, and SNP treatment could further enhance the activity by 52.8% at 48 h in tobacco cells than the control without SNP treatment. cPTIO pretreatment almost reversed the SNP effect on arginase activity (Figure 4D).

The assay of OAT activity showed that the activity was firstly little change during PEG stress from 0 to12 h and then increased continuously after 12 h PEG stress. SNP treatment significantly increased OAT activity, being 32.0% higher than the control with only PEG treatment at 48 h, meanwhile, this SNP-induced OAT activity was inhibited by cPTIO pretreatment (Figure 4E). The change of PDH activity demonstrated a different pattern. As shown in Figure 4F, PEG stress led to an early increase of PDH activity up to 12 h, being 35.1% higher than the control cells grown in normal condition, then PDH activity decreased continuously with the prolonged PEG stress from 12 to 48 h. SNP treatment inhibited the PEG-induced early increase of PDH activity and accelerate the drop of PDH activity during the PEG stress from 12 to 48 h. In addition, the SNP effect on PDH activity was inhibited by cPTIO pretreatment.

Effect of SNP on gene expression of related enzymes of proline metabolism

The gene expression of related enzymes for proline metabolism was performed using RT-PCR analysis with gene-specific primers. As shown in Figure 5, the results indicate that SNP influenced the gene expression of three enzymes of proline metabolism. PEG 6000 stress enhanced the transcript accumulation of GDH and OAT genes and lowered the transcript accumulation of PDH gene. Furthermore, the transcript accumulation of GDH gene mainly took place at earlier stage of PEG 6000 stress, while that of OAT gene was mainly at the later stage of the stress. These results are in accordance with the changes of GDH and OAT activity (Figures 4B, 4E). SNP treatment enhanced the expression intensity of GDH and OAT genes, and decreased the expression intensity of PDH gene under PEG 6000 stress at 48 h. In addition, these effects of SNP on the gene expression were inhibited by cPTIO pretreatment. By the way, we also tried to detect expression level of the genes encoding P5CS and arginase, but unfortunately failed to detect any PCR product in tobacco cells although we successfully amplified the fragments of P5CS from maize seedlings (Yang et al., 2009).

DISCUSSION

Pattern of endogenous NO production during osmotic stress in tobacco sells

Our present results, by two ways of NO detection show that at earlier stage of PEG 6000 stress, a small peak of NO production occurred, the value being about two-folds higher than the control cells grown in normal condition. As the time of PEG stress prolonged, the second peak of NO production appeared, the maximum value being about at 24 h and maintained at a higher level up to 48 h (Figure 1). According to available literatures, time of NO production under different stresses in various plants was from a few minutes (Sakihama et al., 2002) to several hours (Lamotte et al., 2004; Garces et al., 2001; París et al., 2007; Wang and Wu, 2005), even to dozens of hours (Prats et al., 2005; Tada et al., 2004; Zhao et al., 2008). In our present experiment, the PEG stress-induced NO production in tobacco cells was of specific pattern with two peaks. This phenomenon of NO production has not been reported in plants yet, and it seems need to further conform. Although, the reason could be due to the time of NO production which focused on either short time span or long time span in previous reports, which were different from our experiment. We suggest that the first peak could contribute to signaling function of NO itself, and the second broad peak represented NO accumulation caused by intracellular oxidative stress under prolonged PEG stress. This situation is similar to another signaling molecule H_2O_2 (Baptista et al., 2007; Chen et al., 2009).

Exogenous NO alleviated the damage of osmotic stress to tobacco cells by osmoregulation

As a favorable release reagent of exogenous NO, SNP

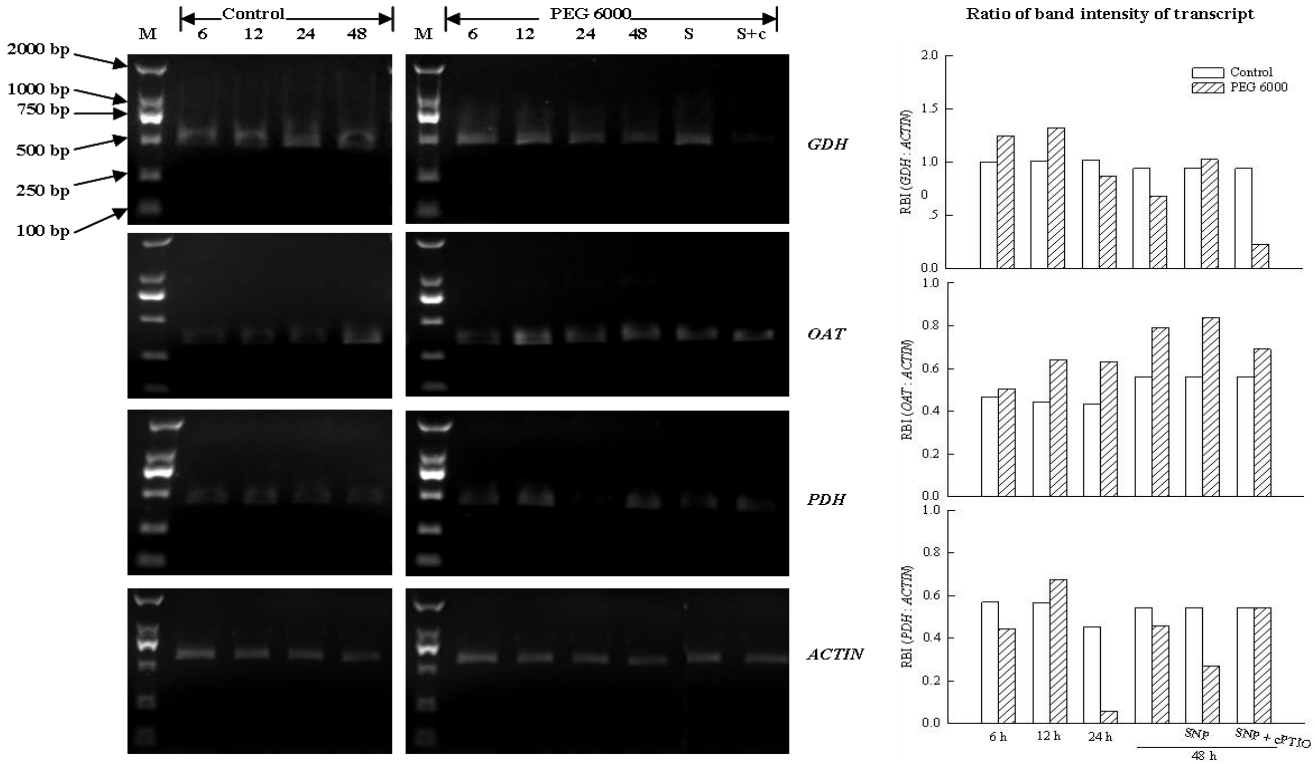

Figure 14. Effects of SNP and cPTIO pretreatment on expression of *GDH*, *OAT* and *PDH* genes in tobacco cultured cells under PEG 6000 stress. The left part is the image of gel electrophoresis of three genes transcripts. Lane M, maker; lanes 6, 12, 24, 48, treatment time (h); S, SNP; S + c, SNP + cPTIO. The right part is the ratio of band intensity of transcripts. The ratio of band intensity (RBI) was calculated as light intensity of *GDH*, *OAT* and *PDH* bands respectively divided by that of *ACTIN* (*GDH*:*ACTIN*, *OAT*:*ACTIN*, *PDH*:*ACTIN*).

treatments significantly improved vitality and regrowth capacity, lowered cell death rate and alleviated the damage of tobacco cells caused by PEG 6000 stress, and this SNP effect could be reversed or inhibited by NO scavenger cPTIO (Figure 2). These results indicate that exogenous NO could enhance the tolerance of tobacco cells to osmotic stress, being consistent with previous reports that NO could alleviate oxidative damage caused by salinity in plants (Chen et al., 2004; Fan et al., 2006).

The SNP-induced osmotic tolerance of cells could be due to its effect on intracellular osmoregulation of tobacco cells. SNP treatments led to relatively lower cell solute potential and higher water potential, which was beneficial for maintaining cell pressure potential to some extent and avoiding occurrence of plasmolysis under the PEG stress (Figure 3). These results indicate that NO could improve the tolerance of the tobacco cells to osmotic stress by enhancing their osmoregulation capacity.

Effect of NO on regulation of proline accumulation and metabolism under osmotic stress

Proline is one of the main osmoregulators in plant cells. A lot of previous studies indicated that

proline respond to osmotic stress in plants. For examples, PEG-induced proline accumulation was detected in barley leaves, tomato cells and leaves (Pérez-Alfocea and Larher, 1995; Handa et al., 1986; Tully et al., 1979). Under NaCl stress, proline content increased largely in rice and cabbage (Hien et al., 2003; López-carrión et al., 2008). Both PEG and NaCl could induce proline accumulation in Ipomoeapes-caprae leaves (Sucre and Suárez, 2011). Several studies showed that NO could improve proline accumulation in plants except for the study of López-carrión et al. (2008), who reported that SNP and NaCl treatment led to reduction of proline content in tomato leaves. Our present experiment shows that proline accumulation in tobacco cells was enhanced by SNP treatment, being about 20% higher than control without SNP under PEG stress, and cPTIO pretreatment inhibited the SNP effect (Figure 4A). This inconsistency between their result and ours may be ascribed to different concentration of SNP treatment. The SNP concentration they used (250 to 1000 μmol•L^{-1}) was much higher than that in our experiments. In fact, the effect of 200 to 300 μmol•L^{-1} SNP on cell vitality (Figure 2B) and proline accumulation (data not shown) was inferior to that of 100 μmol•L^{-1} SNP treatment in tobacco cells in our present experiment.

Several environmental factors, such as salinity, drought and osmotic stress, frequently affect enzyme activity and gene expression of proline metabolism. Hien et al. (2003) reported that proline accumulation in rice under NaCl and mannitol stress was regulated by expression of P5CS gene. López-carrión et al. (2008) reported that under NaCl stress, proline accumulation in cabbage was the result of inhibition of PDH but enhancement of P5CS and OAT activities. There is limited evidence showing the involvement of NO in proline metabolism in plants. Zhao et al. (2009) reported that NO improved P5CS activity and decreased PDH activity to regulate proline accumulation. Our present results show that SNP treatment had different effects on the activities of key enzymes of proline metabolism in tobacco cells. Generally, SNP could improve GDH activity by about 20%, arginase by about 50% and OAT by about 30%, decrease PDH activity by about 50%, and had little effect on P5CS activity (Figures 4B, 4C, 4D, 4E, 4F), and results about gene expression of GDH, OAT and PDH was according to the corresponding enzyme activities (Figure 5). The SNP-induced proline accumulation might be a combined result of the sequential activation of several key enzymes of proline biosynthesis, including GDH and P5CS of glutamate pathway, and arginase and OAT of ornithine pathway, and the inhibition of PDH activity of proline degradation pathway.

This work suggests that NO was involved in response of tobacco cells to osmotic stress and enhanced their stress tolerance by osmoregulation. The regulation of NO on proline metabolism could be an important mechanism for tobacco cells to respond and adapt to osmoticstress. Further research work is needed to illustrate NO signaling transduction in regulating proline metabolism, including interaction between NO and receptors of osmotic stress signals.

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Abbreviations

NO, Nitric oxide; **PEG 6000,** phenylethanoid glycosides 6000; **SNP,** sodium nitroprusside; **DAF-2DA,** diaminofluorescein-2 diacetate; **cPTIO,** 2-(4-carboxyphenyl)-4,4,5,5-tetra-methylimidazoline-1-oxyl-3-oxide; **GDH,** glutamate dehydrogenase; **P5CS,** Δ^1-pyrroline-5-carboxylate synthetase; **OAT,** ornithine aminotransferase; **PDH,** proline dehydrogenase.

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