DOI: 10.1007/s10535-016-0674-7 BIOLOGIA PLANTARUM 61 (3): 529-539, 2017

Glucose-6-phosphate dehydrogenase plays critical role in artemisinin production of *Artemisia annua* under salt stress

J.W. WANG[1]*, H. TIAN[1,2], X. YU[1], and L.P. ZHENG[3]

College of Pharmaceutical Sciences, Soochow University, Suzhou 215123, P.R. China[1]
Institute of Medicinal Plants, Yunnan Academy of Agricultural Sciences, Kunming 650205, P.R. China[2]
Department of Horticulture, School of Architecture, Soochow University, Suzhou 215123, P.R. China[3]

Abstract

Artemisinin, a natural sesquiterpenoid isolated from *Artemisia annua* L., is regarded as the most efficient drug against malaria in the world. Artemsinin production in NaCl-treated *A. annua* seedlings and its relationships with the glucose-6-phosphate dehydrogenase (G6PDH) activity and generation of H_2O_2 and nitric oxide (NO) were investigated. Results revealed that artemisinin content in the seedlings was increased by 79.3 % over the control after 1-month treatment with 68 mM NaCl. The G6PDH activity was enhanced in the presence of NaCl together with stimulated generation of H_2O_2 and NO. Application of 1.0 mM glucosamine (GlcN), an inhibitor of G6PDH, blocked the increase of NADPH oxidase and nitrate reductase (NR) activities, as well as H_2O_2 and NO production in *A. annua* seedlings under the salt stress. The induced H_2O_2 was found to be involved in the upgrading gene expression of two key enzymes in the later stage of artemisinin biosynthetic pathway: amorphadiene synthase (*ADS*) and amorpha-4,11-diene monooxygenase (*CYP71AV1*). The released NO being attributed mainly to the increase of NR activity, negatively interacted with H_2O_2 production and enhanced gene expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGR*). Inhibition of NO generation partly blocked NaCl-induced artemisinin accumulation, and NO donor strongly rescued the decreased content of artemisinin caused by GlcN. These results suggest that G6PDH could play a critical role in NaCl-induced responses and artemisinin biosynthesis in *A. annua*.

Additional key words: hydrogen peroxide, NADPH oxidase, nitrate reductase, nitric oxide, reactive oxygen species.

Introduction

Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) catalyzes the first committed step of the oxidative pentose phosphate pathway (OPPP) controlling metabolic intermediates for biosynthetic processes and the major reducing equivalents for cellular redox state (Kletzien *et al*. 1994). In higher plants, G6PDH responds to environmental and biological stresses such as metal toxicity (Esposito *et al*. 1998), pathogenesis (Šindelář *et al*. 2002), drought, heat stress (Rizhsky *et al*. 2004), and salt stress (Wang *et al*. 2008). As biosynthesis of secondary metabolites often occurs in plants subjected to stresses, G6PDH activity might be involved in the

Submitted 28 December 2015, *last revision* 12 July 2016, *accepted* 14 July 2016.
Abbreviations: ADS - amorphadiene synthase; BSA - bovine serum albumin; cPTIO - 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; CYP71AV1 - amorpha-4,11-diene monooxygenase; DPI - diphenylene iodonium; DXR - 1-deoxy-D-xylulose-5-phosphate reductoisomerase; EDTA - ethylenediaminetetraacetic acid; FPP - farnesyl diphosphate; G6PDH - glucose-6-phosphate dehydrogenase; GlcN - glucosamine; HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HMGR - 3-hydroxy-3-methylglutaryl coenzyme A reductase; HPLC - high-performance liquid chromatography; IPP - isopentenyl diphosphate; L-NAME - *Nω*-nitro-L-arginine methyl ester; MEP - 2-*C*-methyl-D-erythritol-4-phosphate; MTT - 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; MVA - mevalonate; NO - nitric oxide; NOS - NO synthase; NR - nitrate reductase; OPPP - oxidative pentose phosphate pathway; PES - phenazine ethosulfate; PVDF - polyvinylidene difluoride; qPCR - quantitative PCR; ROS - reactive oxygen species; SA - salicylic acid; SOD - superoxide dismutase; TCA - trichloroacetic acid.
Acknowledgements: The authors are grateful to the Graduate Program of Higher Education in Jiangsu Province (No. CXLX13-841), the Suzhou Scholar Program (No. 14317363), and the projects sponsored by the NSFC (No. 81273487) for financial support of this work.
∗ Corresponding author; fax: (+86) 512 65882089, e-mail: jwwang@suda.edu.cn, bcjwwang@gmail.com

accumulation of these metabolites under stresses. It has been reported that taxol biosynthesis was in accordance with G6PDH activity induced by a fungal elicitor (Yu *et al*. 2004, 2005). UV-induced accumulation of phenolic secondary metabolites occurred with the increased G6PDH activity in dark germinated *Vicia faba* (Shetty *et al*. 2002).

Artemisia annua L. is a traditional Chinese medicinal herb well recognized for its synthesis of antimalarial artemisinin. It was found to be a widespread species that frequently exposes to the stressed environments such as drought, mineral deficiency, and salinity (Marchese *et al*. 2010, Aftab *et al*. 2011, Kjær *et al*. 2013). Previous studies on the response of *A. annua* to soil salinity have been mainly concerned on photosynthetic performance, oxidation damage, and artemisinin production (Prasad *et al*. 1998, Qureshi *et al*. 2005, Qian *et al*. 2010). Although salt stress negatively affected plant growth, a comparatively low salinity (50 and 100 mM NaCl) stimulated artemisinin content in *A. annua* (Aftab *et al*. 2011). Therefore, a simple and efficient procedure has been established to enhance artemisinin yield by exposing *A. annua* seedlings to salinity stress at 4 - 6 g dm^{-3} NaCl, enhancing the content about 2-fold (Qian *et al*. 2010). Such studies are also helpful for growing *A. annua* on saline soil to use such land rationally.

In plants, it has been demonstrated that G6PDH is involved in responses to salt stress (Wang *et al*. 2008). G6PDH acts as a regulator of cell redox balance by coordinating the generation of reduced coenzyme NADPH, reactive oxygen species (ROS), and nitric oxide (NO) for signalling as well as defensive responses to salt stress (Liu *et al*. 2007, Zhang *et al*. 2013b). On the other hand, it has been demonstrated that ROS was not involved only in the biological transformation of some precursors of artemisinin (Brown and Sy 2004) but actually played important signalling roles in artemisinin biosynthesis (Nguyen *et al*. 2011). We showed earlier, that NO induced or potentiated the related responses which were mediated by ROS signal during the elicitation of fungal oligosaccharides (Zheng *et al*. 2010) or cerebrosides (Wang *et al*. 2009). However, there has been so far neither report concerning G6PDH activity in *A. annua*, nor regarding its regulation on artemisinin biosynthesis. Therefore, as a follow-up to our previous characterization of artemisinin biosynthesis under abiotic stress (Zhang *et al*. 2013a, Pan *et al*. 2014), we wish to evaluate the G6PDH activity in *A. annua* during salt stress. In this work, the physiological function of G6PDH in maintaining cell redox by the generation of ROS and NO was studied. To understand the mediation of G6PDH on artemisinin production under salt stress, the effect of NaCl treatment on the metabolic pathways for artemisinin biosynthesis was investigated by using inhibitors specific to G6PDH, ROS, and NO generation, respectively.

Materials and methods

Plant culture and NaCl treatments: The seeds of *Artemisia annua* L. (cv. CQF39) were obtained from Yunnan Academy of Agricultural Sciences (Yunnan, China). Seeds were surface-sterilized with 20 % (m/v) sodium hypochlorite for 15 min and then washed three times with sterile water. Seeds were germinated in a tray between moist filter papers in the dark at 25 ± 2 °C for 15 - 20 d. Then seedlings were transplanted to plastic pots (12.5 cm high and 12.5 cm in diameter, 1 plant per pot) containing peat-moss and *Perlite* at the ratio of 1:1 (v/v) and irrigated every other day with 20 cm^3 of normal nutrient (NN) solution containing 33 mg dm^{-3} NH_4NO_3, 38 mg dm^{-3} KNO_3, 8.8 mg dm^{-3} $CaCl_2 \cdot 2H_2O$, 7.4 mg dm^{-3} $MgSO_4 \cdot 7H_2O$ and 3.4 mg dm^{-3} KH_2PO_4. Cultures were grown at a temperature of 25 ± 2 °C, about a 70 % relative humidity, a 16-h photoperiod, and a photosynthetically active radiation of 150 μmol m^{-2} s^{-1} provided by cool-white fluorescent lamps (*Philips* 250 W, Einhoven, the Netherlands). Forty-five days after germination, *A. annua* seedlings at the 6-leaf stage were used for the salinity treatment. NaCl was added to NN solution at the designed salt concentrations (34, 68, 102 and 136 mM). The seedlings were irrigated every other day with 20 cm^3 of these NN + NaCl solutions and NN solution without NaCl served as the control. The experiment lasted 4 weeks. Experiments were conducted in triplicates (five plants per replicate). The fully expanded leaves from the same position (about 2 - 3 cm from the plant top) were sampled after the treatment.

Treatments with inhibitors: Glucosamine (GlcN) was used as inhibitor of G6PDH activity (Ju *et al.* 2009, Liu *et al*. 2013). 1.0 mM GlcN alone or with 68 mM NaCl was added into the NN solution to inhibit the activity of G6PDH. The control was set as only NN solution without NaCl and GlcN. For tests on the effect of NO on NaCl-induced responses and artemisinin synthesis, 50 μM sodium nitroprusside (SNP), 500 μM *Nω*-nitro-L-arginine methyl ester (L-NAME), 20 mM NaN_3, and 500 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) were added as NO donor, NO synthase (NOS) inhibitor, nitrate reductase (NR) inhibitor, and NO scavenger, respectively. Their dosages used in the experiments were chosen based on our previous study (Wang and Wu 2004). For tests on the effect of ROS, 50 μM NADPH oxidase inhibitor diphenylene iodonium (DPI) was applied alone or with NaCl to the above-mentioned NN solution. Its dosage used in the experiments was chosen based on the previous report (Zhao *et al*. 2007).

Determination of artemisinin content: The plants from each treatment were carefully collected and washed with distilled water to remove adhering foreign particles. Leaf samples were dried at 50 °C in an oven until constant mass and dry masses of different groups were recorded. Dry leaf material (100 mg) was used for the extraction of artemisinin and quantification using a high-performance liquid chromatography (HPLC) method (Zhao and Zeng 1985). *Agilent 1260* (Wilmington, USA) system was equipped with 250 × 4.6 mm, Ø 5 μm *Agilent HC-C18* column. Samples were eluted with methanol/0.01 M Na_2HPO_4 + NaH_2PO_4 buffer (pH 7.2) (1:1, v/v) at 1 cm^3 min^{-1} and monitored at 260 nm. Concentration of artemisinin was measured using a calibration curve of an artemisinin standard (*Sigma*, St. Louis, USA).

Determination of endogenous hydrogen peroxide and nitric oxide content: H_2O_2 content from the control and salt-treated seedlings was determined by the method of Velikova *et al*. 2000. About 500 mg of fresh leaf tissue was homogenized in an ice bath with 5 cm^3 of 0.1 % (m/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 10 000 *g* and 4 °C for 15 min, 0.5 cm^3 of the supernatant was added to 0.5 cm^3 of 10 mM potassium phosphate buffer (pH 7.0) and 1 cm^3 of freshly prepared 1 M KI, and the reaction mixture was kept in the dark for 30 min. The absorbance of the mixture was measured at 390 nm. The content of H_2O_2 was calculated from a standard curve.

NO content was determined by haemoglobin assay as described by Murphy and Noack (1994) with some modifications. Fresh leaves (500 mg) were incubated with 5 cm^3 of 100 U catalase and 100 U superoxide dismutase (SOD) for 5 min to remove endogenous ROS and homogenized in 2 cm^3 of homogenization buffer (50 mM triethanolamine hydrochloride, pH 7.5, containing 0.5 mM EDTA, 1 mM leupeptin, 1 mM pepstatin, 7 mM glutathione, and 0.2 mM phenylmethylsulfonyl fluoride). After 5 min incubation with 5 mM oxyhaemoglobin, the homogenate was pelleted (10 000 *g* for 5 min) and the supernatant was collected for spectroscopic analysis at 421 nm using a *Shimadzu UV-2600* (Kzoto, Japan) spectrophotometer.

Determination of nitrate reductase, NADPH oxidase, and G6PDH activities: The activity of NR was assayed following the method of Lu *et al*. (2011). The extraction and assay of NADPH oxidase followed the method reported by Martinez *et al*. (1998). The enzyme activity [nmol(NADPH oxidized) g^{-1}(f.m.) min^{-1}] was calculated using a coefficient of absorbance of 6.22 mM^{-1} cm^{-1} for NADPH at 340 nm. Protein content of the extract was determined by the method of Bradford (1976) with bovine serum albumin as standard.

The G6PDH was extracted according to the method described by Esposito *et al*. (1998) with some modifications. Fresh leaves (0.5 g) were homogenized in ice-cold 0.1 M Tris-HCl buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiothreitol, and 4 % (m/v) polyvinylpyrrolidone. The homogenate was centrifuged at 10 000 *g* and 4 °C for 20 min. The supernatant was used for the enzymatic assays. G6PDH activity was determined spectrophoto-metrically by monitoring the reduction of NADP at 340 nm. Assays were performed at 25 °C in a reaction mixture (3 cm^3) containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.6, 2 mM $MgCl_2$ and 0.8 mM $NADP^+$. The reaction was initiated by adding 5 mM glucose-6-phosphate (*Sigma*).

NADPH measurement: NADPH was determined according to Gibon and Larher (1997) with some modifications. NADPH was extracted by the addition of 200 mm^3 of 0.5 M NaOH per 25 mg of ground fresh tissue, vortexed and boiled for 5 min. This was followed by centrifugation at 10 000 *g* and 4 °C for 10 min. The supernatant (50 mm^3) was neutralized with the same volume of 0.5 M HCl and 100 mm^3 of mix [200 mM Tricine-KOH, pH 9.0, 2 mM 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromid (MTT), 0.4 mM phenazine ethosulfate (PES), 16 mM EDTA] for determination of NADPH. Two units of G6PDH (*Sigma*) and 20 mM glucose-6-phosphate were added and samples were incubated in the dark at 37 °C for 1 h. Reactions were stopped by adding 2.65 M NaCl, and then centrifuged at 12 000 *g* and 4 °C for 15 min. The resulting formazan pellets were resuspended in 500 mm^3 of ethanol, and the absorbance at 570 nm was measured in the microplate reader (*ELx800*, *Bio-TEK*, Winooski, USA). Concentration of NADPH was determined after background subtraction using ranging from 1 to 10 μM.

Western-blot analysis: A 50 μg aliquot of proteins was solubilized and separated in 10 % (m/v) acrylamide gel. For Western blot analysis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes with a wet *Trans-Blot* cell (*Bio-Rad*, Hercules, USA). After transfer, the membrane was blocked for 1 h with 5 % (m/v) non-fat milk. The membrane was used in cross-reactivity assays with the primary antibody against G6PDH (produced in goat, antigen source from baker's yeast *Saccharomyces cerevisiae*) and incubated at 4 °C overnight. After washing, an affinity-purified rabbit anti-(goat IgG)-horseradish peroxidase conjugate (*CWBIO*, Beijing, China) secondary antibody was added and incubated at room temperature for 1 h. The blots were washed and then developed by use of a super enhanced chemiluminescence detection kit (*Applygen Technologies*, Beijing, China). The protein bands were visualized after exposure of the membranes to *Kodak* X-ray film.

RNA extraction and real-time quantitative PCR: Leaves of *A. annua* were sampled randomly after

different treatments, immediately frozen in liquid nitrogen and store at -80 °C. Total RNA was isolated from different treated and control leaves by using the *RNAprep* pure plant kits (*Tian Gen Biotech*, Beijing, China) according to the manufacturer's instructions. The concentration and purity of RNA were determined by measuring A_{260} and A_{280}. Reverse transcription and fluorescent quantitative PCR were performed with purchased first strand cDNA synthesis kit (*Fermentas*, Burlington, Canada) and *FastStart* universal *SYBR Green Master* kit (*Roche Diagnostics*, Switzerland). Amplifications were performed in *CFX96 Touch* real-time PCR detection System (*Bio-Rad*), with initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 15 s, and a final extension at 72 °C for 10 min. The housekeeping gene *β-actin* was chosen as the internal reference. The primers designed for the validation of the target genes from *A. annua* are listed in Table 1.

Statistical analysis: Duncan's multiple range test ($P < 0.05$) was selected where appropriate. The data were expressed as mean ± standard deviation (SD) of at least three independent experiments with at least three replicates for each.

Table 1. Gene primer sequences used in real time qPCR analysis.

Gene	5'-forward-3'	5'-reverse-3'
HMGR	TTGTGTGCGAGGCAGTAAT	CCTGACCAGTGGCTATAAAGA
FPS	GTATGATTGCTGCGAACGATGGA	CGGCGGTGAATAGACAATGAATAC
DXR	GGTGATGAAGGTGTTGTTGAGGTT	AGGGACCGCCAGCAATTAAGGT
ADS	AGAAGAACGCACCTTGTTTAAGAG	ACCATACGCATCATAAGTGTCATC
CYP71AV1	CACCCTCCACTACCCTTG	GACACATCCTTCTCCCAGC
DBR2	CTCAAGGGGATGCTGATTTG	GGTAATCCGTGTACCCAACG
Actin	CCAGGCTGTTCAGTCTCTGTAT	CGCTCGGTAAGGATCTTCATCA

Results

In this investigation, *A. annua* seedlings were exposed to 34 - 136 mM NaCl for one month (Table 2). Significant reductions in shoot length, root length, and shoot and root dry masses were observed with increasing salt concentration. However, artemisinin content was enhanced under all salt treatments. Artemisinin content was increased by 79.3 % over the control after 68 mM NaCl treatment and this NaCl concentration was used in subsequent experiments.

We measured the G6PDH activity in *A. annua* leaf tissues during NaCl treatment (Fig. 1*A*). In the early stage of salt treatment (0 - 5 d), the activity of G6PDH increased rapidly and was 1.68-fold higher compared to control on the fifth day. Afterwards, the enzyme activity was slightly reduced, but it was steadily higher than in control. In order to verify the function of G6PDH under salt stress, we added the inhibitor (1.0 mM GlcN) to the medium (Fig. 2). The inhibitory effect of 1.0 mM GlcN on G6PDH activity was 56.71 % under control conditions and 67.21 % in the presence of 68 mM NaCl (Fig. 2*A*). Moreover, Western-blot analysis further demonstrated that application of GlcN resulted in a decrease in the amount of G6PDH protein in leaves (Fig. 2*B*).

The content of H_2O_2 was measured in order to characterize the internal ROS status under salt stress (Fig. 1*B*). H_2O_2 content was induced rapidly and reached

Table 2. Effect of different concentrations of NaCl for one month on growth and artemisinin content of *Artemisia annua.* Means ± SDs of three replicates and each replicate consisted of five plants. In columns, means followed by different letters are significantly different at $P < 0.05$.

NaCl [mM]	Height [cm]	Root length [cm]	Shoot dry mass [mg plant^{-1}]	Root dry mass [mg plant^{-1}]	Artemisinin content [mg g^{-1}(d.m.)]
0	10.6 ± 0.5 a	7.1 ± 0.9 a	29.0 ± 2.0 a	3.9 ± 0.6 a	0.92 ± 0.01 a
34	9.0 ± 0.3 b	6.0 ± 0.4 ab	24.3 ± 2.1 b	2.8 ± 0.2 b	1.18 ± 0.03 b
68	7.5 ± 0.3 c	5.2 ± 0.6 b	22.0 ± 1.0 bc	2.3 ± 0.2 c	1.65 ± 0.12 c
102	6.5 ± 0.3 d	3.5 ± 0.3 c	13.7 ± 0.6 d	1.5 ± 0.3 d	1.36 ± 0.02 d
136	5.1 ± 0.4 e	2.6 ± 0.2 d	10.3 ± 0.6 e	0.9 ± 0.1 e	1.10 ± 0.07 b

a peak after 4 d. Simultaneously, NaCl treatment induced a gradual increase in the intracellular NO content in leaves, reaching a peak on day 8, which was 8.1-fold higher than that of the control (Fig. 1*C*). The increase of both H_2O_2 and NO production was inhibited by 1.0 mM GlcN (Fig. 3). The inhibitory effects of 1.0 mM GlcN on H_2O_2 and NO content under 68 mM NaCl were 28.6 and 22.4 %, respectively.

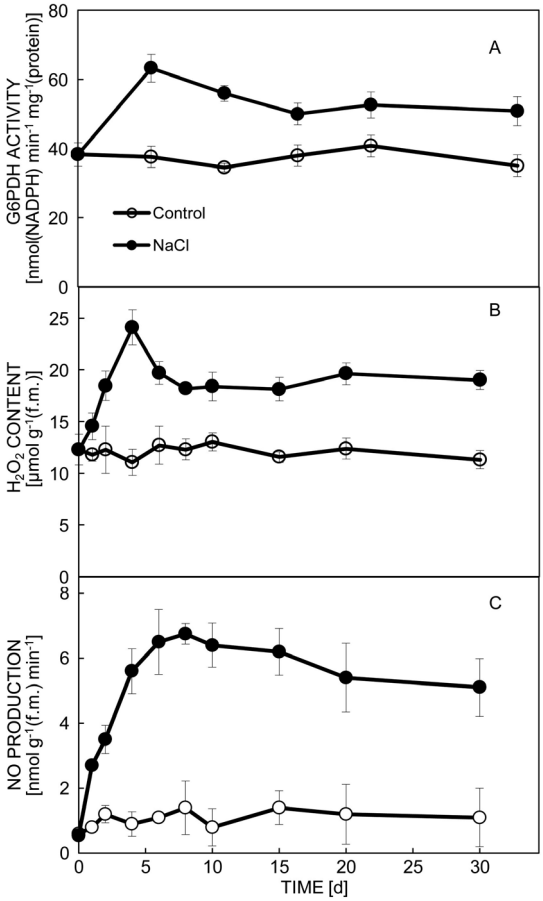

Fig. 1. Time courses of G6PDH activity (*A*), H_2O_2 content (*B*), and NO production (*C*) in leaves of *Artemisia annua* under 68 mM NaCl. Means ± SDs of three replicates, and each replicate consisted of five plants.

NaCl-treated seedlings had higher NADPH oxidase activity than the controls (Fig. 4). The accumulation of H_2O_2 under salt stress may result from the NADPH oxidation by the NADPH oxidase, and DPI, a NADPH oxidase inhibitor, reduced the amount of salt-induced H_2O_2 (Fig. 3*A*). The salt induced NO increase was effectively blocked by either L-NAME, an inhibitor of NOS activity, or NaN_3, an inhibitor of NR activity, suggesting the occurrence of a NOS-like enzyme and NR in *A. annua* seedlings after NaCl treatment (Fig. 3*B*). The inhibitory effect of 20 mM NaN_3 and 500 µM L-NAME on NO production was 44.8 and 17.2 %, respectively. Stimulation of NADPH content and NR activity under salt stress was also markedly blocked by 1 mM GlcN (Fig. 4), indicating a possible relationship between G6PDH and NO production *via* the NADPH-dependent reduction catalyzed by NR.

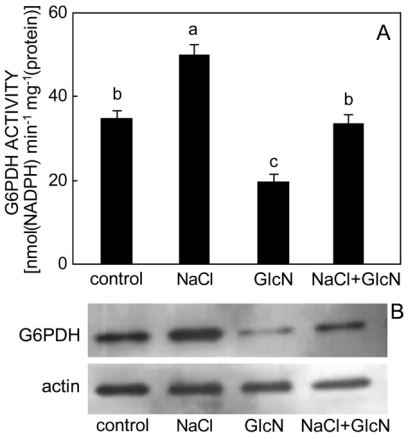

Fig. 2. Modulation of G6PDH activity in leaves of *Artemisia annua* by the inhibitor GlcN under control or NaCl treatments for one month (*A*). Means ± SDs of three replicates, and each replicate consisted of five plants. *Bars* with different letters are significantly different at $P < 0.05$ according to Duncan's test. *B* - Western-blot analysis of G6PDH expression in leaves of *A. annua*. In this experiment, 68 mM NaCl, and 1.0 mM GlcN were used for a month.

In order to understand better the interaction of NO and H_2O_2 production under salt stress, different inhibitors such as L-NAME, NaN_3, and DPI for NOS, NR, and NADPH oxidase, were used to determine the NO and H_2O_2 production, respectively (Fig. 3). The induced H_2O_2 production was stimulated effectively by L-NAME and NaN_3 (Fig. 3*A*). When treated with DPI, an inhibitor of NADPH oxidase, NaCl-induced NO production increased correspondingly (Fig. 3*B*), suggesting a relationship between NO and the NaCl-induced oxidative burst.

To understand the possible correlation between G6PDH activity and artemisinin content under salt stress, we measured artemisinin content under GlcN + NaCl treatment (Fig. 5). Although GlcN in the absence of NaCl could slightly but nonsignificantly reduce artemisinin content, the increase of artemisinin content induced by salt stress was blocked considerably in the presence of 1 mM GlcN (Fig. 5). Although SNP alone did not induce artemisinin biosynthesis, the SNP significantly enhanced the NaCl-induced artemisinin content (NaCl + SNP *vs*. SNP). Moreover, SNP was able to rescue the inhibition of artemisnin content by GlcN in seedlings under salt treatments (NaCl + GlcN *vs*. NaCl + GlcN + SNP). On the other hand, the NaCl-induced artemisinin synthesis was suppressed by both cPTIO and DPI (NaCl *vs*. NaCl + cPTIO, NaCl + DPI). All these results show positive effects of G6PDH, NO, and H_2O_2 on the biosynthesis of artemisinin in seedlings under salt treatment.

To observe the transcription changes of artemisinin

biosynthetic genes under salt treatment, the relative expression of genes encoding vital enzymes were measured (Fig. 6). The mRNA content of 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGR*), amorphadiene synthase (*ADS*) and amorpha-4,11-diene monooxygenase (*CYP71AV1*) was stimulated. However, the enhanced expression of these genes under salt treatment was inhibited significantly in the presence of 1.0 mM GlcN (NaCl + GlcN *vs*. NaCl; Fig. 6*B-D*). The NaCl-induced expressions of *HMGR*, *ADS*, and *CYP71AV1* were suppressed by DPI, while *HMGR* expression was also decreased by cPTIO (NaCl *vs*. NaCl + cPTIO; Fig. 6*B*). All these results reveal a complicated relationship among G6PDH, NO, and ROS in the activation of artemisinin biosynthetic genes under salt stress.

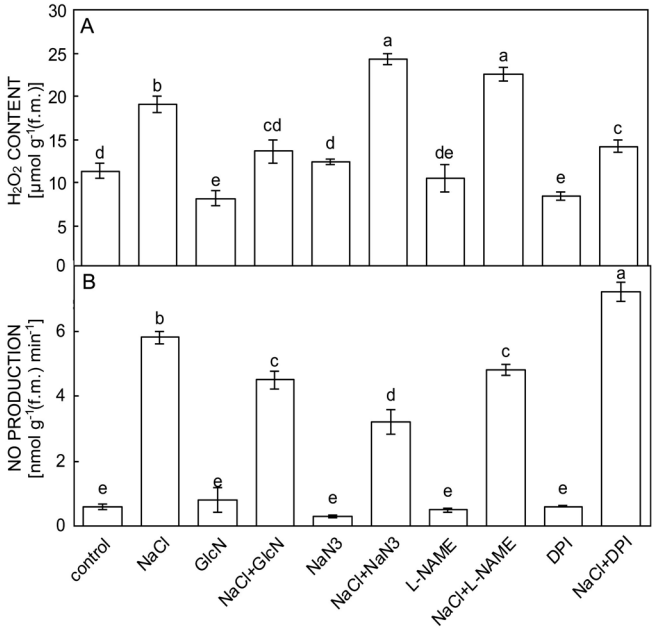

Fig. 3. Responses of H_2O_2 content (*A*) and NO production (*B*) to G6PDH inhibitor GlcN (1.0 mM), NO inhibitors (20 mM NaN_3, 500 μM L-NAME), and NADPH oxidase inhibitor DPI (50 μM), under control or NaCl treatment (68 mM for 1 month). Means ± SDs of three replicates and each replicate consisted of five plants. *Bars* with different letters are significantly different at $P < 0.05$ according to Duncan's test.

Discussion

It has been reported that G6PDH played a central role to maintain cell redox balance for mediating plant responses to NaCl stress (Nemoto and Sasakuma 2000, Wang *et al*. 2008). Our experimental results showed that G6PDH activity in *A. annua* could be stimulated under 68 mM NaCl (Fig. 1). Western blot analysis revealed that the increase of G6PDH activity was mainly due to the increased accumulation of G6PDH protein, which could be partially prevented by 1.0 mM GlcN (Fig. 2). Previous reports demonstrated that G6PDH activity might be involved in the biosynthesis of secondary metabolites such as taxol or phenolics under stresses (Shetty *et al*. 2002, Yu *et al*. 2005). In our study, the NaCl-induced increase of artemisinin content was blocked in presence of 1.0 mM GlcN (Fig. 5), suggesting that G6PDH was implicated in mediating the NaCl-induced artemisinin biosynthesis. To our knowledge, the present study is the first report on G6PDH activity and its physiological roles in *A. annua*.

As a key enzyme of OPPP, G6PDH controls the carbon flow and produces NADPH to maintain the cellular redox state and other precursors or cofactors to meet cellular needs for some biosynthetic routes (Kletzien *et al*. 1994). The present results showed NADPH content increased 2.3-fold under salt stress and the stimulation of NADPH content was almost completely abolished by 1.0 mM GlcN (Fig.4), indicating the possible role of G6PDH for providing NADPH. Although there have been reports regarding NADPH as a substrate of NADPH oxidase in generating ROS for stress signal transduction (Liu *et al*. 2007, Wang *et al*. 2008), we observed the rapid enhancement of H_2O_2 content with NADPH accumulation during salt treatment (Fig. 1*B*). Moreover, our results showed that if G6PDH activity was inhibited, NADPH oxidase activity decreased (Fig. 4*B*), leading to a repression in induced H_2O_2 accumulation (Fig. 3*A*), in accordance with the previous studies (Yu *et al*. 2004, Wang *et al*., 2008). The DPI, the inhibitor of

the NADPH oxidase, also decreased H_2O_2 generation in the seedlings under salt treatment (Fig. 3*A*), further indicating that H_2O_2 production probably results from the activation of NADPH oxidase.

Like ROS, NO is also an important defence signalling molecule in response to salinity (Siddiqui *et al*. 2011). It has been reported that NO could mediate ROS production, enhance the antioxidant defence system, induce the photosynthetic pigments, and upregulate the expression of stress-related genes in plants subjected to salinity (Fan *et al*. 2007, Liu *et al*. 2007). In present study, NaCl-induced NO reached a maximum around day 8 and then slowly decreased as the time of treatment progressed (Fig. 1*C*). A close temporal relationship between the increase of G6PDH activity and NO and H_2O_2 production in *A. annua* seedlings was observed (Fig. 1). NADPH and NO production were blocked by 1.0 mM GlcN (a G6PDH inhibitor) in the presence of 68 mM NaCl (Figs. 3*B*, 4*C*), suggesting that the increased G6PDH activity probably played a key role in NO production induced by salt stress. Moreover, the NaCl-induced NO production was inhibited by the NOS inhibitor (L-NAME) and NR inhibitor (NaN_3) (Fig. 3*B*), which suggests that both NR and NOS were probably involved in the induced NO production. Plants have several candidate pathways for NO synthesis, including non-enzymatic conversion of nitrite to NO, NR-dependent NO formation, and arginine-dependent NO formation catalyzed by NOS-like enzyme, the main source of NO in animals (Besson-Bard *et al*. 2008). Although many studies using NOS inhibitors and mutants demonstrated that NOS-like activity was associated with NO under salt stress (Zhao *et al*. 2004, 2007, Valderrama *et al*. 2007), some researchers also found that NR activity could be an important source of NO under salt stress (Siddiqui *et al*. 2011). Liu *et al*. (2007) showed that G6PDH played an important role in NO production, which was fully dependent on NR activity in *Phaseolus vulgaris* roots under salt stress. Our present study demonstrated NR was a main source of NO in *A. annua* under NaCl treatment, and its activity could be stimulated in the presence of induced G6PDH (Fig. 4*A*). On the

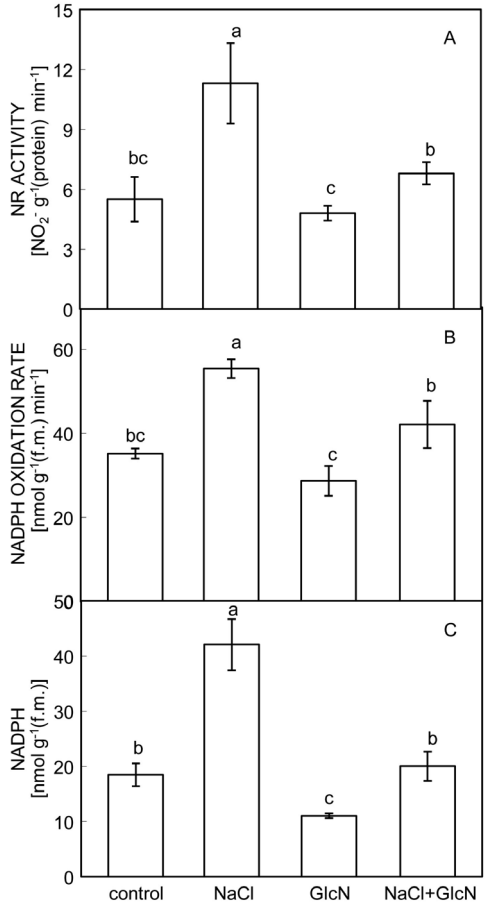

Fig. 4. Responses of activities of NR (*A*) and NADPH oxidase (*B*) and NADPH content (*C*) to G6PDH inhibitor GlcN (1.0 mM) under control or NaCl treatment (68 mM for 1 month). Means ± SDs of three replicates, and each replicate consisted of five plants. *Bars* with different letters are significantly different at $P < 0.05$ according to Duncan's test.

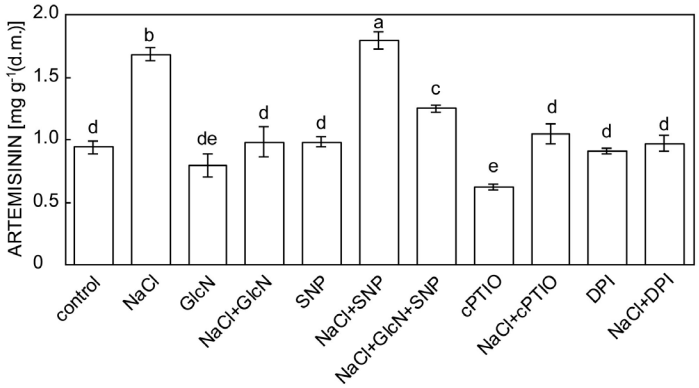

Fig. 5. Responses of artemisinin content to G6PDH inhibitor GlcN (1.0 mM), NO donor SNP (50 μM), NO scavenger cPTIO (500 μM) and NADPH oxidase inhibitor DPI (50 μM), respectively, under control or NaCl treatment (68 mM for 1 month). Means ± SDs of three replicates and each replicate consisted of five plants. *Bars* with different letters are significantly different at $P < 0.05$ according to Duncan's test.

other hand, our present study indicated that the NaCl-induced NO production increased further after the inhibition of NADPH oxidase by DPI, while the inhibition of NO would stimulate H_2O_2 accumulation (Fig. 3*A*). Such relationship between NO and H_2O_2 (Fig. 3*A*,*B*) during the salt stress in this study is consistent with other reports in plants responding to wound (Orozco-Cárdenas and Ryan 2002) or yeast elicitor (Zhao *et al*. 2007). The inhibition of H_2O_2-producing enzyme guaiacol peroxidase and the stimulation of H_2O_2-scavenging enzymes, such as catalase and ascorbate peroxidase, by NO could be attributed to the decrease of H_2O_2 production (Małolepsza and Różalska 2005).

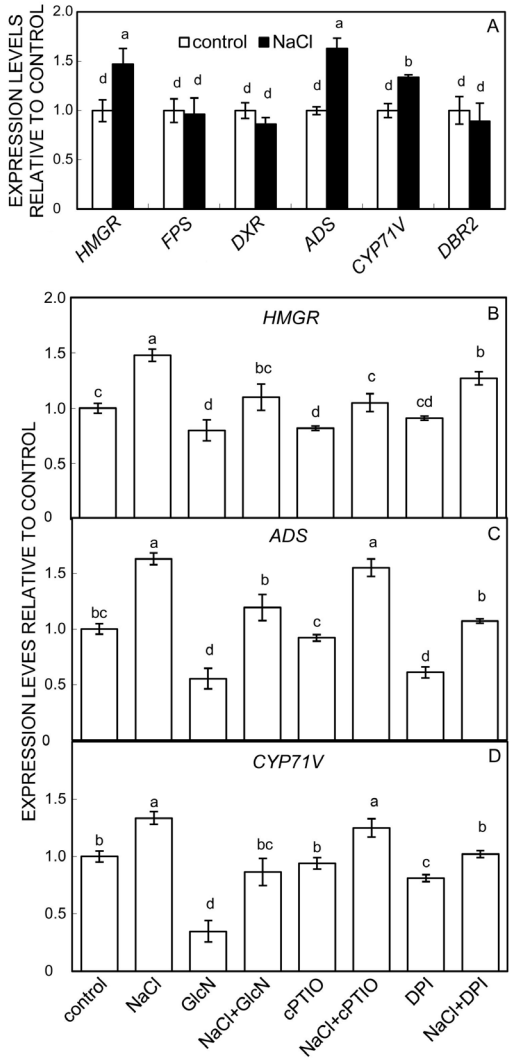

Fig. 6. The q-PCR analysis of artemisinin biosynthetic gene transcription (*A*) in leaves after 1-month NaCl treatment at 68 mM, and responses of the expression of *HMGR* (*B*), *ADS* (*C*) and *CYP71AV1* (*D*) genes to G6PDH inhibitor GlcN (1.0 mM), NO scavenger cPTIO (500 μM), and NADPH oxidase inhibitor DPI (50 μM), respectively, under control or NaCl treatment. Means ± SDs of three replicates, and each replicate consisted of five plants. *Bars* with different letters are significantly different at $P < 0.05$ according to Duncan's test.

Also, the contrasting NO and H_2O_2 accumulation may result from the possible co-consumption between NO and $O_2^{\bullet-}$ to form peroxynitrite for cell apoptosis mediation as a defence response (Zago *et al*. 2006, Zhao *et al.* 2007).

The activation of the primary metabolism pathway OPPP by G6PDH is a defence reaction in plants under stress (Kletzien *et al*. 1994). In our present study, NaCl-induced artemisinin production was related to G6PDH activity of *A. annua* seedlings under salt treatment (NaCl *vs*. NaCl + GlcN in Fig. 5). Artemisinin is derived from a common precursor isopentenyl diphosphate (IPP) from both the cytosolic mevalonate (MVA) pathway and the plastidic 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (Fig. 7) (Towler and Weathers 2007, Sangwan *et al*. 2010). NADPH generated by the G6PDH is a factor limiting incorporation of the reaction catalyzed by HMGR in the MVA pathway and by 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) in the MEP pathway, which is required for isopentenyl diphosphate (IPP) synthesis (Olofsson *et al*. 2011). The first committed step toward artemisinin biosynthesis is the cyclization of farnesyl diphosphate (FPP) to amorpha-4,11-diene by ADS, which can be efficiently converted into artemisinic alcohol by a cytochrome P_{450} monooxygenase CYP71AV1 in the presence of NADPH (Bertea *et al*. 2005). The subsequent reactions still need NADPH as cofactor to participate in bioconversion of artemisinin derivates (Fig. 7). Moreover, glyceraldehyde-3-phosphate (G3P), an intermediate of the pentose-phosphate pathway in which G6PDH participates, is also an initial substrate for the MEP pathway for artemisinin biosynthesis.

In the end of biosynthetic pathway of artemisinin, the oxidation reaction of the $\Delta^{4,5}$ double bond in both artemisinic acid and dihydroartemisinic acid *in vivo* was involved in the biological transformations to the 1,2,4-trioxane system of artemisinin (Brown and Sy 2004). NaCl-induced G6PDH stimulated H_2O_2 production in *A. annua* seedlings and the oxidative stress in turn enhanced artemisinin content (Fig. 5). ROS catalyzed by induced NADPH oxidase was generated through exposure to NaCl treatment, and might be responsible for conversion of the immediate precursors into artemisinin production. There also was a significant increase in *HMGR*, *ADS*, and *CYP71AV1* transcripts in NaCl-treated seedlings (Fig. 6*A*), indicating a high capacity to produce artemisinin precursors. The qPCR data presented here clearly demonstrated that DPI treatment resulted in significant decrease in induced transcription of *HMGR*, *ADS*, and *CYP71AV1* genes (NaCl *vs.* NaCl + DPI; Fig. 6). It can be suggested that the expression of these key genes could be modulated by ROS under salt treatment. In our previous report, a cerebroside elicitor induced NO burst and some defence responses leading to the artemisinin accumulation in *A. annua* hairy roots (Wang *et al*. 2009). On the other hand, NO produced in elicited plant cells also induces the

biosynthesis of many other secondary metabolites (Zhang *et al*. 2012). Our present study demonstrated the NaCl-induced NO production and its interaction with oxidative burst as well as their regulation of artemisinin content (Fig. 5). Here, NO inhibitors L-NAME and cPTIO suppressed NaCl-induced artemisinin accumulation, whereas NO donor SNP promoted it. However, only *HMGR* expression was sensitive to NO scavenger cPTIO under salt treatment (NaCl *vs*. NaCl + cPTIO; Fig. 6). Although the occurrence of NO contributes to strengthening the transcription of genes encoding key enzymes involved in the biosynthesis of those target secondary metabolites, such as shikonin (Wu *et al*. 2009), little effort has been made to reveal the signal transduction steps. Since NO may cause other nonspecific effects in plants (Siddiqui *et al*. 2011), NO may be also indirectly involved in NaCl-induced artemisinin biosynthesis.

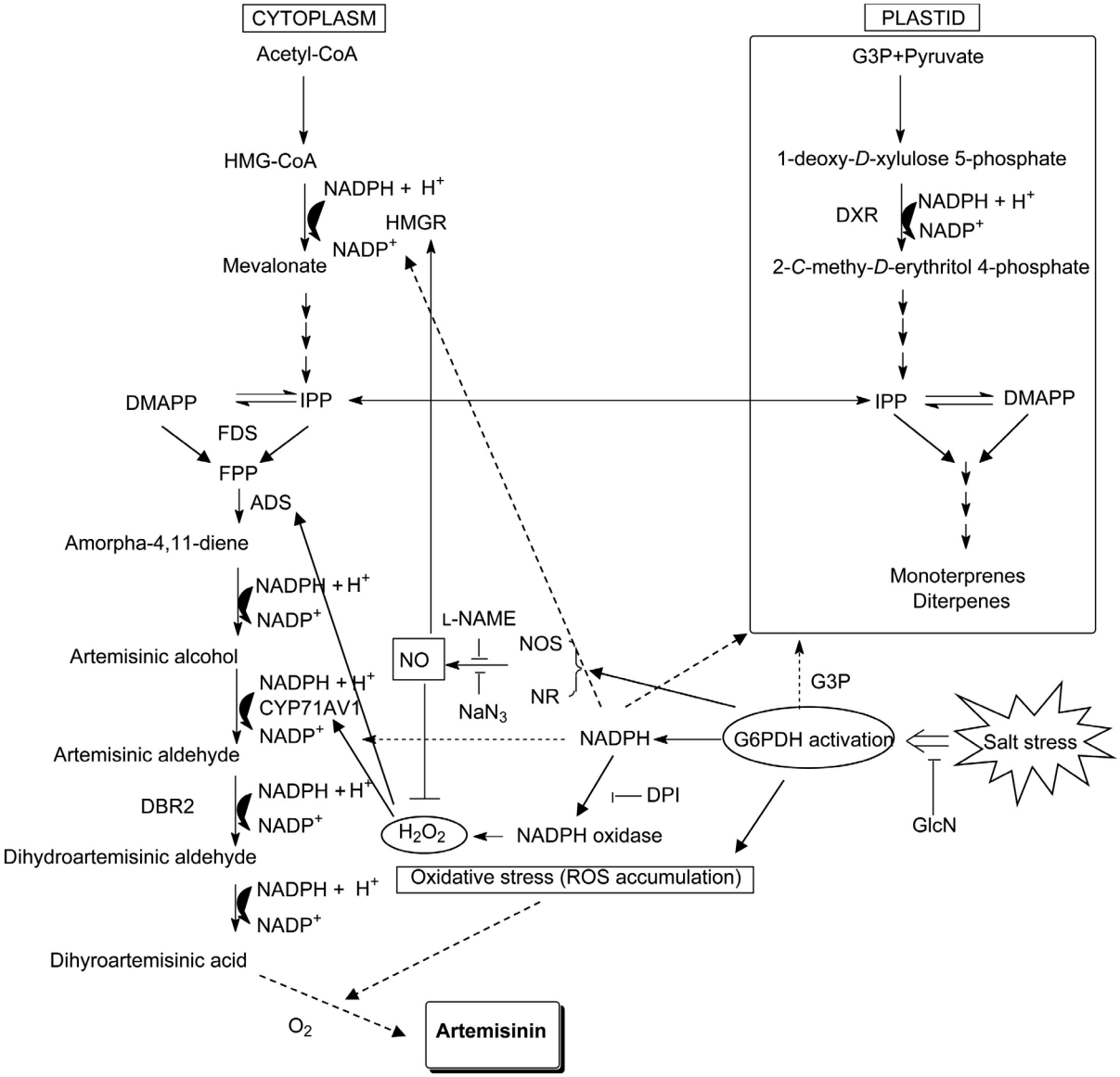

Fig. 7. A proposed model of G6PDH involvement in artemisinin biosynthetic pathway under salt stress (HMG-CoA: hydroxymethylglutaryl CoA, DMAPP: dimethylallyl diphosphate, IPP: isopentenyl diphosphate, FDS: farnesyl diphosphate synthase, FPP: farnesyl diphosphate, G3P: glyceraldehyde-3-phosphate, DXR: deoxylulose-5-phosphate reductoisomerase, ADS: amorpha-4,11-diene synthase, CYP71AV1: amorphadiene-12-hydroxylase, DBR2: artemisinic aldehyde Δ11(13) reductase, NOS: NO synthase, NR: nitrate reductase, L-NAME: *Nω*-nitro-L-arginine methyl ester, DPI: diphenylene iodonium, GlcN: Glucosamine, G6PDH: glucose-6-phosphate dehydrogenase. *Dashed lines* indicate the more uncertain steps; *arrrows* point to inhibitor application).

Conclusion

In our present studies, NaCl-induced G6PDH could contribute to the salt stress response by the activation of NADPH oxidase and NR, which in turn resulted in H_2O_2 and NO accumulation, respectively. The precursors and cofactor NADPH induced under the salt stress could be required in MVA and MEP pathway for artemisinin synthesis. ROS not only could facilitate bioconversion of the immediate precursors into artemisinin but also upregulated the expression of key genes including *HMGR*, *ADS*, and *CYP71AV1* in artemisinin biosynthetic pathway. Furthermore, we demonstrated that NO was involved in the interaction with H_2O_2 production and enhanced *HMGR* expression. Inhibition of NO generation partly blocked NaCl-induced artemisinin accumulation, and NO donors strongly rescued the decreased content of artemisinin caused by GlcN. Thus, the enhanced G6PDH could facilitate to maintain a steady-state content of H_2O_2 and NO, and modulate gene expression for artemisinin by changing redox status under salt stress. During this process, G6PDH could play a central role in modulation of artemisinin biosynthesis.

References

Aftab, T., Khan, M.M.A., Da Silva, J.A.T., Idrees, M., Naeem, M.: Role of salicylic acid in promoting salt stress tolerance and enhanced artemisinin production in *Artemisia annua* L. - J. Plant Growth Regul. **30**: 425-435, 2011.

Bertea, C.M., Freije, J.R., Van der Woude, H., Verstappen, F.W., Perk, L., Marquez, V., De Kraker, J.W., Posthumus, M.A., Jansen, B.J., de Groot, A., Franssen, M.C., Bouwmeester, H.J.: Identification of intermediates and enzymes involved in the early steps of artemisinin biosynthesis in *Artemisia annua*. - Planta med. **71**: 40-47, 2005.

Besson-Bard, A., Pugin, A., Wendehenne, D.: New insights into nitric oxide signaling in plants. - Annu. Rev. Plant Biol. **59**: 21-39, 2008.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Brown, G.D., Sy, L.K.: *In vivo* transformations of dihydroartemisinic acid in *Artemisia annua* plants. - Tetrahedron **60**: 1139-1159, 2004.

Esposito, S., Carillo, P., Carfagna, S.: Ammonium metabolism stimulation of glucose-6P dehydrogenase and phosphoenolpyruvate carboxylase in young barley roots. - J. Plant Physiol. **153**: 61-66, 1998.

Fan, H., Guo, S., Jiao, Y., Zhang, R., Li, J.: Effects of exogenous nitric oxide on growth, active oxygen species metabolism, and photosynthetic characteristics in cucumber seedlings under NaCl stress. - Front. Agr. China **1**: 308-314, 2007.

Gibon, Y., Larher, F.: Cycling assay for nicotinamide adenine dinucleotides: NaCl precipitation and ethanol solubilization of the reduced tetrazolium. - Anal. Biochem. **25**: 153-157, 1997.

Ju, H.W., Koh, E.J., Kim, S.H., Kim, K.I., Lee, H., Hong, S.W.: Glucosamine causes overproduction of reactive oxygen species, leading to repression of hypocotyl elongation through a hexokinase-mediated mechanism in *Arabidopsis*. - J. Plant Physiol. **166**: 203-212, 2009.

Kjær, A., Verstappen, F., Bouwmeester H., Ivarsen, E., Fretté, X., Christensen, L.P., Grevsen, K., Jensen, M.: Artemisinin production and precursor ratio in full grown *Artemisia annua* L. plants subjected to external stress. - Planta **237**: 955-966, 2013.

Kletzien, R.F., Harris, P.K., Foellmi, L.A.: Glucose-6-phosphate dehydrogenase: a "housekeeping" enzyme subject to tissue-specific regulation by hormones, nutrients, and oxidant stress. - FASEB J. **8**: 174-181, 1994.

Liu, J., Wang, X.M., Hu, Y.F., Hu, W., Bi, Y.R.: Glucose-6-phosphate dehydrogenase plays a pivotal role in tolerance to drought stress in soybean roots. - Plant Cell Rep. **32**: 415-429, 2013.

Liu, Y.G., Wu, R.R., Wan, Q., Xie, G.Q., Bi, Y.R.: Glucose-6-phosphate dehydrogenase plays a pivotal role in nitric oxide-involved defense against oxidative stress under salt stress in red kidney bean roots. - Plant Cell Physiol. **48**: 511-522, 2007.

Lu, D., Dong, J.F., Jin, H.H., Sun, L.N., Xu, X.B., Zhou, T., Zhu, Y., Xu, M.J.: Nitrate reductase-mediated nitric oxide generation is essential for fungal elicitor-induced camptothecin accumulation of *Camptotheca acuminata* suspension cell cultures. - Appl. Microbiol. Biotechnol. **90**: 1073-1081, 2011.

Małolepsza, U., Różalska, S.: Nitric oxide and hydrogen peroxide in tomato resistance: Nitric oxide modulates hydrogen peroxide level in *o*-hydroxyethylorutin-induced resistance to *Botrytis cinerea* in tomato. - Plant Physiol. Biochem. **43**, 623-635, 2005.

Marchese, J.A., Ferreira, J.F., Rehder, V.L., Rodrigues, O.: Water deficit effect on the accumulation of biomass and artemisinin in annual wormwood (*Artemisia annua* L., Asteraceae). - Braz. J. Plant Physiol. **22**: 1-9, 2010.

Martinez, C., Montillet, J.L., Bresson, E., Agnel, J.P., Dai, G.H., Daniel, J.F., Geiger, J.P., Nicole, M.: Apoplastic peroxidase generates superoxide anions in cells of cotton cotyledons undergoing the hypersensitive reaction to *Xanthomonas campestris* pv. *malvacearum* race 18. - Mol. Plant Microbe interact. **11**: 1038-1047, 1998.

Murphy, M.E., Noack, E.: Nitric oxide assay using hemoglobin method. - Methods Enzymol. **233**: 240-250, 1994.

Nemoto,Y., Sasakuma, T.: Specific expression of glucose-6-phosphate dehydrogenase (G6PDH) gene by salt stress in wheat (*Triticum aestivum* L.). - Plant Sci. **158**: 53-60, 2000.

Nguyen, K.T., Arsenault, P.R., Weathers, P.J.: Trichomes + roots + ROS = artemisinin: regulating artemisinin biosynthesis in *Artemisia annua* L. - *In Vitro* cell. dev. Biol. Plant **47**: 329-338, 2011.

Olofsson, L., Engström, A., Lundgren, A., Brodelius, P.E.: Relative expression of genes of terpene metabolism in different tissues of *Artemisia annua* L. - BMC Plant Biol. **11**: 45, 2011.

Orozco-Cárdenas, M., Ryan, C.A.: Nitric oxide negatively modulates wound signaling in tomato plants. - Plant Physiol. **130**: 487-493, 2002.

Pan, W.S., Zheng, L.P., Tian, H., Li, W.Y., Wang, J.W.: Transcriptome responses involved in artemisinin production in *Artemisia annua* L. under UV-B radiation. - J. Photochem. Photobiol. B: Biol. **140**: 292-300, 2014.

Prasad, A., Kumar, D., Anwar, M., Singh, D.V., Jain, D.C.: Response of *Artemisia annua* L. to soil salinity. - J. Herbs Spices med. Plants. **5**: 49-55, 1998.

Qian, Z.H., Gong, K., Zhang, L., Lv, J.B., Jing, F.Y., Wang, Y.Y., Guan, S.B., Wang, G.F., Tang, K.X.: A simple and efficient procedure to enhance artemisinin content in *Artemisia annua* L. by seeding to salinity stress. - Afr. J. Biotechnol. **6**: 1410-1413, 2010.

Qureshi, M.I., Israr, M., Abdin, M.Z., Iqbal, M.: Responses of *Artemisia annua* L. to lead and salt-induced oxidative stress. Environ. exp. Bot. **53**: 185-193, 2005.

Rizhsky, L., Liang, H., Shuman, J., Shulaev, V., Davletova, S., Mittler, R.: When defense pathways collide. The response of *Arabidopsis* to a combination of drought and heat stress. - Plant Physiol. **134**: 1683-1696, 2004.

Sangwan, N.S., Kumar, R., Srivastava, S., Kumar, A., Gupta, A., Sangwan, R.S.: Recent developments on secondary metabolite biosynthesis in *Artemisia annua* L. - J. Plant Biol. **37**: 1-24, 2010.

Shetty, P., Atallah, M.T., Shetty, K.: Effects of UV treatment on the proline-linked pentose phosphate pathway for phenolics and L-DOPA synthesis in dark germinated *Vicia faba*. - Process Biochem. **37**:1285-1295, 2002.

Siddiqui, M.H., Al-Whaibi, M.H., Basalah, M.O.: Role of nitric oxide in tolerance of plants to abiotic stress. - Protoplasma **248**, 447-455, 2011.

Šindelář, L., Šindelářová, M.: Correlation of viral RNA biosynthesis with glucose-6-phosphate dehydrogenase activity and host resistance. - Planta **215**: 862-869, 2002.

Towler, M.J., Weathers, P.J.: Evidence of artemisinin production from IPP stemming from both the mevalonate and the nonmevalonate pathways. - Plant Cell Rep. **26**: 2129-2136, 2007.

Valderrama, R., Corpas, F.J., Carreras, A., Fernández-Ocaña, A., Chaki, M., Luque, F., Gómez-Rodríguez, M.V., Colmenero-Varea, P., del Río, L.A., Barroso, J.B.: Nitrosative stress in plants. - FEBS Lett. **581**: 453-461, 2007.

Velikova, V., Yordanov, I., Edreva, A.: Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. - Plant Sci. **151**: 59-66, 2000.

Wang, J.W., Wu, J.Y.: Involvement of nitric oxide in elicitor-induced defense responses and secondary metabolism of Taxus chinensis cells. - Nitric Oxide **11**: 298-306, 2004.

Wang, J.W., Zheng, L.P., Zhang, B., Zou, T. Stimulation of artemisinin synthesis by combined cerebroside and nitric oxide elicitation in *Artemisia annua* hairy roots. - Appl. Microbiol. Biotechnol. **85**: 285-292, 2009.

Wang, X.M., Ma, Y.Y., Huang, C.H., Wan, Q., Li, N., Bi, Y.R.: Glucose-6-phosphate dehydrogenase plays a central role in modulating reduced glutathione levels in reed callus under salt stress. - Planta **227**: 611-623, 2008.

Wu, S., Qi, J., Zhang, W., Liu, S., Xiao, F., Zhang, M., Xu, G., Zhao, W., Shi, M., Pang, Y., Shen, H., Yang, Y.: Nitric oxide regulates shikonin formation in suspension-cultured *Onosma paniculatum* cells. - Plant Cell Physiol. **50**, 118-128, 2009.

Yu, L.J., Lan, W.Z., Chen, C., Yang, Y.: Glutathione levels control glucose-6-phosphate dehydrogenase activity during elicitor-induced oxidative stress in cell suspension cultures of *Taxus chinensis*. - Plant Sci. **167**: 329-335, 2004.

Yu, L.J., Lan, W.Z., Chen, C., Yang, Y., Sun, Y.P.: Importance of glucose-6-phosphate dehydrogenase in taxol biosynthesis in *Taxus chinensis* cultures. - Biol. Plant. **49**: 265-268, 2005.

Zago, E., Morsa, S., Dat, J.F., Alard, P., Ferrarini, A., Inzé, D., Delledonne, M., Breusegem, F.V.: Nitric oxide- and hydrogen peroxide-responsive gene regulation during cell death induction in tobacco. - Plant Physiol. **141**: 404-411, 2006.

Zhang, B., Zheng, L.P., Li, W.Y., Wang, J.W. Stimulation of artemisinin production in *Artemisia annua* hairy roots by Ag-SiO_2 core-shell nanoparticles. - Curr. Nanosci. **9**: 363-370, 2013a.

Zhang, B., Zheng, L.P., Wang, J.W.: Nitric oxide elicitation for secondary metabolite production in cultured plant cells. - Appl. Microbiol. Biotechnol. **93**: 455-466, 2012.

Zhang, L., Liu, J., Wang, X.M, Bi, Y.R.: Glucose-6-phosphate dehydrogenase acts as a regulator of cell redox balance in rice suspension cells under salt stress. - Plant Growth Regul. **69**: 139-148, 2013b.

Zhao, J., Fujita, K., Sakai, K.: Reactive oxygen species, nitric oxide, and their interactions play different roles in *Cupressus lusitanica* cell death and phytoalexin biosynthesis. - New Phytol. **175**, 215-229, 2007.

Zhao, L.Q., Zhang, F., Guo, J.K., Yang,Y.L., Li, B.B., Zhang, L.X.: Nitric oxide functions as a signal in salt resistance in the calluses from two ecotypes of reed. - Plant Physiol. **134**: 849-857, 2004.

Zhao, M.G., Tian, Q.Y., Zhang, W.H.: Nitric oxide synthase-dependent nitric oxide production is associated with salt tolerance in *Arabidopsis*. - Plant Physiol. **144**: 206-217, 2007.

Zhao, S.S., Zeng, M.Y.: [title in English] Spektrometrische hochdruck-flüssigkeits-chromatographische (HPLC) Untersuchungen zur Analytik von Qinghaosu. - Planta med. **51**: 233-237, 1985.[In German]

Zheng, L.P., Zhang, B., Zou,T., Chen, Z.H., Wang, J.W.: Nitric oxide interacts with reactive oxygen species to regulate oligosaccharide-induced artemisinin biosynthesis in *Artemisia annua* hairy roots. - J. med. Plants Res. **4**: 758-765, 2010.