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RESEARCH PAPERS

Influence of Potassium Nitrate on Antioxidant Level and Secondary Metabolite Genes under Cold Stress in *Panax ginseng*

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Abstract—The overall survival mechanism and secondary metabolite synthesis under cold stress conditions (4°C) at exogenous supply with KNO_3 were studied in mountain ginseng adventitious root cultures. Expressed sequence tags encoding antioxidant enzymes, such as catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and also ginsenoside-related secondary metabolites were obtained, and expression studies were carried out using quantitative real-time PCR. Chilled adventitious root cultures grown in vitro with or without an exogenous supply with K^+ were differentially regulated. Expressions of *PgCAT, PgAPX*, and *PgGPX-1* were increased, whereas *PgGPX-2* and all ginsenoside-related secondary metabolite genes showed slight down-regulation upon chilling stress. Interestingly, the most genes were up-regulated at the increased potassium supply except the cytochrome P450 gene, which shows clearly the specific effect of potassium on the antioxidant level and secondary metabolite gene expression involved in the survival mechanism. In addition, we studied the activities of catalase, ascorbate peroxidase, guaiacol peroxidase, and phenylalanine ammonia-lyase, which showed similar pattern of changes. Adventitious root dry weight and relative water content were found to increase at 0.05 mM KNO_3. The high level of potassium is needed for *Panax ginseng* under chilling conditions to increase its cold tolerance through activating the antioxidant system as well as to increase ginsenoside-related secondary metabolite transcripts.

Keywords: Panax ginseng, potassium nitrate, chilling, gene expression, antioxidants, ginsenoside-related genes.

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INTRODUCTION

ROS generation in plants has been described under both biotic and abiotic stresses. The low-temperature stress affects membrane fluidity and alters membrane structure and stability [1]. Like drought stress, chilling and frost conditions are responsible for photooxidative damage in the chloroplasts due to the impairment of photosynthetic C metabolism. Under these circumstances, absorbed light energy exceeds the capacity of chloroplasts to use it for CO_2 fixation; it reacts with O_2 and converts it to ROS [2]. The most important ROS forms include superoxide anion-radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($\cdot O_2 \cdot$), and hydrogen peroxide (H_2O_2). Whether ROS will act as damaging, protecting, or signaling molecules depends on the maintenance of the equilibrium between ROS production and scavenging at the proper site and time.

In order to scavenge the production of free radicals, plants have evolved complex mechanisms, which include superoxide dismutates (SOD), the water–water cycle (WWC), the ascorbate–glutathione cycle (AGC), the glutathione peroxidase cycle (GPXC), and catalase (CAT). Despite of the antioxidant system functioning, oxidative damage still occurs in plants either due to uncontrolled ROS production or to inefficient ROS scavenging [3]. Potassium (K^+), being an abundant cation in plants, constitutes about 3–5% of plant total dry weight. It plays a specific role in plant

Abbreviations: AGC—ascorbate–glutathione cycle; APX—ascorbate peroxidase; CAT—catalase; EST—expressed sequence tag; GPX—glutathione peroxidase/guaiacol peroxidase; PAL—phenylalanine ammonia-lyase; RWC—relative water content; qRT-PCR—quantitative real-time PCR; SOD—superoxide dismutase; WWC—water–water cycle.

survival under various environmental stress conditions by protecting cells from oxidative damage through balancing the nutritional status [4] and by increasing ginseng saponin production [5]. Under chilling conditions, plants can resist cold stress when the K^+ concentration is in the luxury range [6], and the accumulation of K^+ by plants before initiation of stress acts like an insurance strategy to allow plants to survive sudden environmental abiotic stress conditions.

A number of studies related to potassium role at environmental stress conditions have been previously reported for wheat and maize [7, 8].

Panax ginseng (Korean ginseng) is a perennial herb belonging to the family Araliaceae, and its roots are known to have various medicinal properties, including antioxidant, antistress, antidiabetic, and anticancer ones. The major active component of this plant is ginsenoside, a triterpenoid saponin, which accumulates under various biotic and abiotic stresses. Moreover, some researchers reported that this plant responds to various environmental stresses by enhancing the differential expressions of defense-related genes [9]. To the best of our knowledge, no reports comparing the antioxidant level and expressions of ginsenoside genes in *P. ginseng* related to exogenous KNO_3 supply under cold stress (4°C) conditions were published. Hence, our study was directed to investigate the protective role of exogenous supply with K^+ under cold stress, in the in vitro grown mountain ginseng adventitious roots.

MATERIALS AND METHODS

Plant materials and KNO_3 treatments. Adventitious root cultures of mountain *Panax ginseng* C.A Meyer were obtained from Korean Ginseng Center and Ginseng Resource Bank, Kyung Hee University. The roots were maintained at a regular subculture in every four weeks. To investigate whether exogenous KNO_3 under cold stress enhances root cold tolerance, one-month-old adventitious roots were used for the treatments as will be described below. For KNO_3 treatments, the roots were placed in MS medium [10] with different KNO_3 concentrations (0.05, 0.1, and 0.2 mM) for one month. The KNO_3-treated roots were kept at 4°C to induce chilling stress. In all cases, treated samples were collected at 1, 3, 5, and 7 days. Controls (MS only) and treated roots were grown at 25°C and an irradiance from cool-light fluorescent lamps of 30–50 mmol/(m^2 s) with a 16-h photoperiod. In all cases, treated and untreated cultured roots were immediately frozen in liquid nitrogen and stored at –70°C until required. The treated samples were further used for enzyme assays and mRNA transcript analysis of various antioxidant and ginsenoside-related genes.

RNA synthesis, construction of cDNA library, and qRT-PCR. RNA was extracted from mountain ginseng adventitious root cultures using RNeasy kit (Qiagen, USA) according to the manufacturer's instruction. The concentration and quality of isolated RNA was measured using a nano-value spectrophotometer (GE, United States). To obtain the full strand of cDNA, 1 μg of total RNA was subjected to reverse transcription using Power cDNA synthesis kit (Invitrogen, United States) following manufacturer's instructions. Quantitative real-time RT-PCR was performed in RT rotary analyzer (Rotor-Gene 6000, Corbett Life Science, Australia) using 100 ng of cDNA in a 10 μL reaction volume used as a template for reverse transcription using SYBR® Green Sensimix Plus Master Mix (Quantace, England) [9]. The housekeeping gene encoding actin protein was used as a standard, and to normalize the Ct value, untreated mountain ginseng adventitious root culture was used as a control in this experiment. Fluorescence was detected and measured in the qRT-PCR thermocycler, and the increase of the fluorescence corresponding to the exponential increase of the product was used to determine the threshold cycle (Ct) in each reaction, using the formula $2^{-\Delta\Delta Ct}$. All qRT-PCR reactions were performed in triplicates.

Selection of antioxidant and ginsenoside-related genes from the EST database. The selection of antioxidant ESTs and secondary metabolite genes was performed using Ginseng EST database [11] and Blastx program at the Plant Genome Database server (http://www.plantdb.org/plantGDB.cgi/blast/plant GDB blast). Blastn and Tblastx programs were used with the exception value (E-value) less than 10^{-4}. Using ginseng EST searches, we identified and selected various candidates of antioxidant genes, such as *PgCAT*, *PgAPX*, and *PgGPX*, ginsenoside-related genes like those for squalene epoxidase, β-amyrin synthase, cytochrome P450, and sterol-related gene, namely this for cycloartenol synthase, based on their open reading frames (ORF). From the gene sequences obtained, the specific gene primers (Tables 1, 2) were designed for each antioxidant gene and secondary metabolite gene.

Relative water content (RWC). After 30 days of subculture in MS and modified MS with increasing KNO_3 under cold stress (4°C), the roots were collected and their fresh weight and dry weight were recorded. In order to calculate fresh weight, roots were washed with distilled water and blotted with tissues before fresh weights were recorded. The fresh weight samples were oven-dried at 60°C to a constant dry weight. Relative water contents (RWCs) of treated and untreated roots were calculated using the following formula [7]:

$$\text{RWC}\ (\%) = [(\text{fresh weight} - \text{dry weight})/\text{fresh weight}] \times 100.$$

Enzyme extraction. Treated and untreated adventitious roots were homogenized with a mortar and pestle in liquid nitrogen. To the obtained fine root powder, the extraction buffer (25 mM sodium phosphate, pH 7.8) was added; the homogenate was centrifuged at 18000 *g* for 30 min at 4°C, and the supernatant was fil-

Table 1. Primers for antioxidant gene transcription used in the present study

No.	Gene	EST No.	Primers (5'-3')
1	*PgCAT*	EU327037	**F** : CAAGGATGGGAAAGCACA **R**: TGGTTACATCGAGTGGGTCA
2	*PgAPX*	DC03contig333	**F** : CTGGACCAACAACCCTCTCA **R**: CCTCAGCAAATCCCAATCCCAGTTCA
3	*PgGPX-1*	DC04010_C01	**F**: GTCAAGGATGCTAGAGGGAATG **R**: GTCCCTGGCTCCTGTGCT
4	*PgGPX-2*	DC06001-1-H05-044	**F**: GCCAGGATCAAATCCAGAAA **R**: AGGTGATGTTGTCGGTGGAT
5	*Actin*	–	**F**: GTGATCTTACAGATAGCTTGATGA **R**: AGAGAAGCTAAGATTGATCCTCC

Table 2. Primers for ginsenoside-related gene transcription used in the present study

No.	Protein encoded	EST No.	Primers (5'-3')
1	cycloartenol synthase	DC03034B08.ab1	**F**: AAGGTTTAGTAACTGCTGGGAGGA **R**: TGCAATGGTGTTGCGTCTCT
2	β-amyrin synthase	DC0_contig_81	**F**: GAAGGAACTGATTCGCTTCGTT **R:**CCGCTAGATAAGTCTTTCCCCA
3	squalene epoxidase	DC0_4_Contig280	**F**: GTAGCTCCTCAGGTTCCAAAACA **R**: TGGAGATCGCGTAAAGGTCTAAG
4	cytochrome P450	BAD15331.1	**F**: TCTGCAATATTAAACCTTCCAGCC **R**: AATTCCTTTGTGGGGATGAGAAGG

tered through Whatman No 1 filter paper. The supernatant of each treated sample was used as a crude extract to assay enzyme activities. All operations were carried out at 0–4°C.

Total catalase, ascorbate peroxidase, and guaiacol peroxidase activities. CAT (EC. 1.11.1.6) activity was determined using the method described previously [12] by monitoring the disappearance of H_2O_2 at 240 nm. The reaction mixture (3 mL) contained 0.059 M H_2O_2 and 0.05 M potassium phosphate buffer, pH 7.0. The solution was incubated for 4–5 min to achieve temperature equilibration. Later, the reaction was initiated by the addition of 100 μL of crude enzyme extract and a decrease in absorbance was recorded spectrophotometrically. One unit of CAT is defined as the amount of enzyme required to decompose one micromole of H_2O_2 per minute at 25°C and pH 7.0.

APX (EC.1.11.1.11) activity was estimated according to the method described previously with little modification [13]. Oxidation of ascorbate to dehydroascorbate (DHA) was measured spectrophotometrically at 265 nm in 3 mL of the reaction mixture containing 50 mM KOH (pH 7.6), 0.1 mM EDTA, 0.05 mM ascorbate, 100 μL of the crude extract, and 0.1 mM H_2O_2.

GPX (EC.1.11.1.12) activity was measured based on the rate of decomposition of hydrogen peroxide by peroxidase, with guaiacol as a hydrogen donor, by measuring the color development at 470 nm and at 25°C [14].

Phenylalanine ammonia-lyase activity. PAL (EC.4.3.1.5) activity was measured by the method described previously [15]. Fresh tissue (0.2 g) was homogenized in chilled Tris–HCl (50 mM, pH 8.0) supplemented with 0.5 mM EDTA and 1% polyvinylpyrrolidone. PAL activity was measured by monitoring the reaction product trans-cinnamate at 290 nm. The reaction mixture contained 50 mM Tris–HCl (pH 8.0), 20 mM L-Phe, and 100 μL of the enzyme extract. PAL activity was calculated as a difference in the optical density for 30 min, and one enzyme unit was defined as the amount causing an increase of 0.01 in A_{290}/min.

Experimental design and statistical analysis. Twenty different treatments were used in experiment. Twelve flasks contained three levels of KNO_3 and the roots subjected to cold stress (4°C) stress for 1, 3, 5, and 7 days; four flasks contained roots subjected to only chilling (no exogenous KNO_3) for 1, 3, 5, and 7 days; and four flasks were kept at 25°C for 1, 3, 5,

Table 3. Effect of cold stress (4°C) and KNO_3 supplementation on dry weight content of mountain ginseng adventitious roots

Concentration of KNO_3, mM	Dry weight, g/flask			
	1 day	3 days	5 days	7 days
Control	0.41 ± 0.01	0.44 ± 0.12	0.45 ± 0.18	0.47 ± 0.03
0	0.37 ± 0.02	0.34 ± 0.03	0.32 ± 0.02	0.41 ± 0.02
0.05	0.43 ± 0.01	0.45 ± 0.02	0.48 ± 0.01	0.52 ± 0.01
0.1	0.39 ± 0.03	0.39 ± 0.01	0.45 ± 0.03	0.48 ± 0.16
0.2	0.39 ± 0.03	0.41 ± 0.03	0.46 ± 0.03	0.44 ± 0.05

Notes: Controls were grown at 25°C in MS medium. Values represent the three independent replicates.

Table 4. Effect of cold stress (4°C) and increasing KNO_3 concentration on the relative water content (RWC) of mountain ginseng adventitious roots

Concentration of KNO_3, mM	RWC, %			
	1 day	3 days	5 days	7 days
Control	87.62 ± 0.29	88.21 ± 0.41	88.93 ± 0.25	88.02 ± 0.12
0	82.71 ± 0.24	81.92 ± 0.28	83.41 ± 0.51	84.52 ± 1.29
0.05	90.29 ± 0.84	90.85 ± 0.12	92.22 ± 0.43	93.26 ± 0.51
0.1	87.95 ± 0.36	87.75 ± 0.36	86.91 ± 0.20	88.62 ± 0.57
0.2	87.75 ± 0.34	88.17 ± 0.47	88.60 ± 0.29	88.36 ± 1.28

Notes: Controls were grown at optimum temperature of 25°C in MS medium. Values represent the three independent replicates.

and 7 days and used as control. The obtained data were subjected to statistical analysis using the Student *t*-test.

RESULTS AND DISCUSSION

Plants are commonly exposed to chilling in the range from 5 to 10°C even in warm climates. A decline in temperature below these values results in membrane instability [16] and development of oxidative stress in plants. Previously, it has been observed that there is an increase in antioxidant enzyme activities and secondary metabolites when plants are subjected to biotic and abiotic stresses [17]. Down-regulation of some terpenoid-related genes under cold stress was reported [18]. To date, no reports specifically demonstrated the effect of K^+ under cold stress conditions on the antioxidant level or on the level of ginsenoside-related genes in *P. ginseng*.

Potassium, an important macronutrient, maintains membrane stability by increasing frost resistance even under unexpected low-temperature conditions, in particular by activation of some enzymes [19]. Previously, it was reported that ginseng saponin content was enhanced at 60 mM potassium concentration in the suspension cultures [5]. In the present study, we attempted to investigate whether potassium improves the survival policy under cold stress by increasing the antioxidant levels reducing ROS production and by maintaining the stable or increased level of secondary metabolite gene expression. We also analyzed growth parameters, water content, enzyme activities, and transcript levels of various antioxidant enzyme and ginsenoside pathway-related genes in one-month-old subcultured mountain ginseng roots (with increasing KNO_3) under chilling (4°C) conditions. In different treatments, root dry weight, water content, and the transcript levels of antioxidant enzyme and secondary metabolite genes seem to be significantly affected ($P < 0.05$).

Upon chilling, the root dry weight was reduced when compared to that of roots grown under optimum conditions. However, exogenous potassium presence (0.05, 0.1, and 0.2 mM) under chilling stress increased the root dry weight in all K^+-treated samples as compared with chilled roots without K^+ treatment. From the above concentrations tested, particularly strong effect was observed for treatment with 0.05 mM K^+ for 7 day (Table 3). Similar pattern of changes was observed for RMC in root samples subjected to chill-

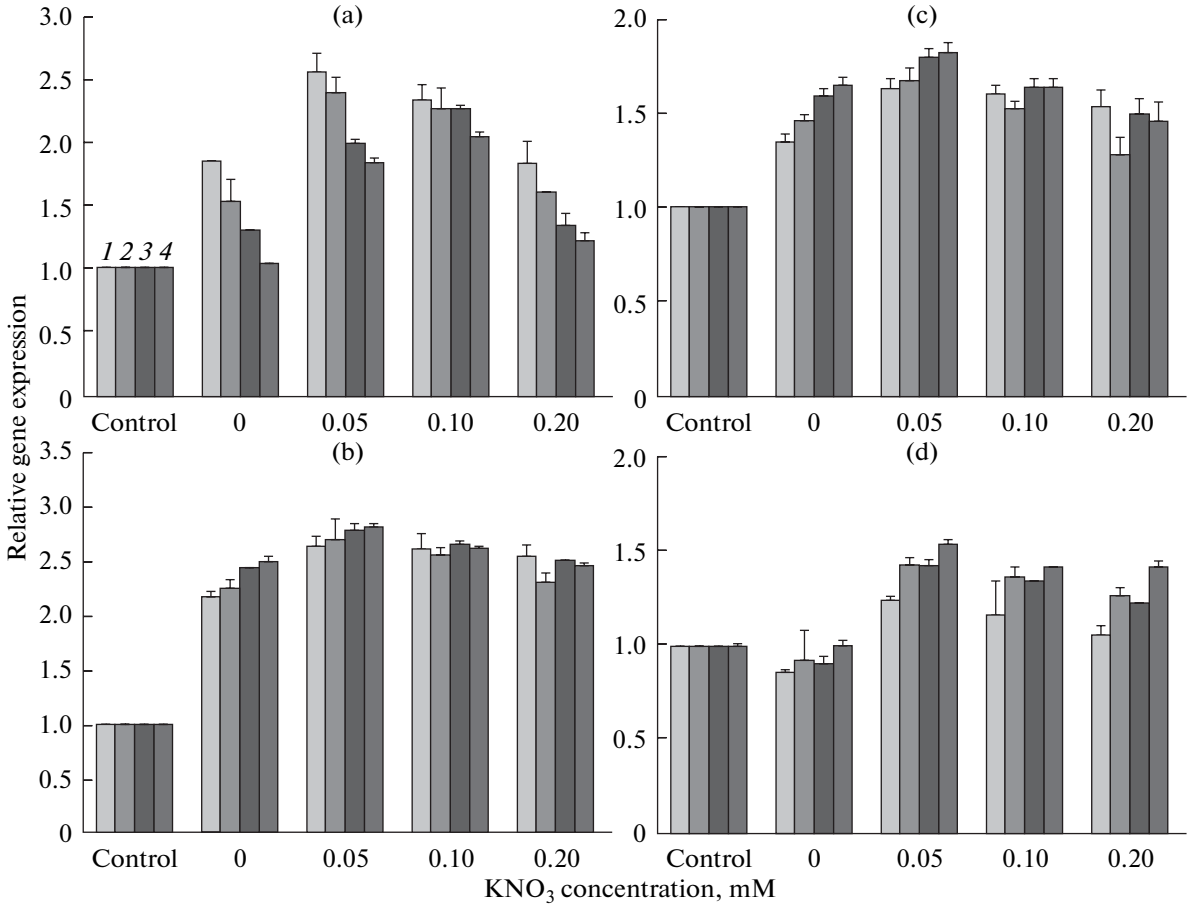

Fig. 1. Differential expression of various genes encoding antioxidant enzymes at different K^+ concentrations under cold stress. (a) *PgCAT*; (b) *PgAPX*; (c) *PgGPX-1*; (d) *PgGPX-2*.

Adventitious root cultures of mountain ginseng were grown in MS medium under increasing KNO_3 concentration for 1 month. These roots were kept at 4°C for cold stress conditions for 1 (*1*), 3 (*2*), 5 (*3*), and 7 (*4*) days. Control roots were kept at 25°C. The increase in the transcript mRNA levels was determined by qRT-PCR. Data represents the means ± SD for three independent replicates.

ing and treated with exogenous KNO_3 (Table 4). This shows that potassium can increase the root weight and water content even under chilling conditions. Similar results were reported for hybrid maize [8].

In further experiments we studied the effects of chilling and K^+ supply on expression of genes encoding antioxidant enzymes and activities of corresponding enzymes.

As evident from Fig. 1, chilling enhanced expression of *PgCAT*, *PgAPX*, and *PgGPX-1* genes and did not affect expression of *PgGPX-2* gene. Expression of *PgCAT* gene decreased with increasing time of chilling, whereas expression of other tested genes was not changed or slightly increased. Increased KNO_3 supply during root growing enhanced the transcript levels of *PgCAT* and *PgGPX-2* genes and essentially did not affect transcription of *PgAPX* and *PgGPX-1* genes.

Table 5 present effects of chilling on the background of increased K^+ supply on activities of antioxidant enzymes and PAL. Chilling of roots grown on MS medium only activated all enzymes tested, except GPX after 7 days of chilling. The effects of K^+ presence in medium were much weaker than those of chilling. However, in some cases K^+ supply resulted in further enzyme activation; its strongest effects were observed for 0.05 M KNO_3 in the case of CAT, APX and PAL activities.

Increased CAT activity was associated with photorespiratory detoxification of H_2O_2 via mitochondrial electron transport systems [20]. APX and CAT are involved in the detoxification of H_2O_2 and thereby reduce the oxidative stress in plants. The role of potassium (to minimize the oxidative stress by maintaining the level of NADPH-oxidase activity and photosynthetic electron transport system [21]) justifies the activation of antioxidants by supplying KNO_3. An increase in PAL activity under chilling stress and K^+ supply results in the accumulation of phenolic compounds [22], indicating K^+ involvement in the phenylpropanoid metabolism.

Table 5. Effect of cold stress (4°C) and increasing KNO_3 concentration on activities of antioxidant enzymes and PAL

Concentration of KNO_3, mM		Activity of enzyme, U/g protein			
		CAT	APX	GPX	PAL
1 day					
Control	–	56.43 ± 0.23	45.23 ± 0.16	9.54 ± 0.05	62.70 ± 0.21
	0	130.40 ± 0.52	115.70 ± 0.18	16.86 ± 0.07	94.30 ± 0.28
	0.05	151.30 ± 0.32	163.70 ± 0.02	22.30 ± 0.09	98.30 ± 0.16
	0.1	138.50 ± 0.24	158.30 ± 0.03	21.14 ± 0.18	86.40 ± 0.22
	0.2	117.20 ± 0.19	147.32 ± 0.17	19.37 ± 0.28	79.20 ± 0.17
3 days					
Control	–	53.34 ± 0.17	56.45 ± 0.23	11.46 ± 0.21	65.20 ± 0.23
	0	125.70 ± 0.31	122.03 ± 0.19	22.78 ± 0.12	100.20 ± 0.18
	0.05	158.90 ± 0.28	157.70 ± 0.08	25.27 ± 0.28	120.60 ± 0.16
	0.1	143.20 ± 0.21	150.90 ± 0.26	24.53 ± 0.04	112.40 ± 0.11
	0.2	134.40 ± 0.07	148.10 ± 0.37	19.17 ± 0.06	107.30 ± 0.17
5 days					
Control	–	59.73 ± 0.16	59.56 ± 0.39	16.92 ± 0.24	58.41 ± 0.07
	0	121.20 ± 0.18	125.10 ± 0.13	20.14 ± 0.17	110.30 ± 0.23
	0.05	167.70 ± 0.28	175.30 ± 0.15	24.53 ± 0.14	114.60 ± 0.09
	0.1	148.20 ± 0.21	169.70 ± 0.17	26.10 ± 0.18	117.90 ± 0.05
	0.2	150.30 ± 0.29	159.30 ± 0.23	21.80 ± 0.28	114.10 ± 0.08
7 days					
Control	–	62.70 ± 0.29	65.20 ± 0.04	58.41 ± 0.31	61.35 ± 0.21
	0	110.60 ± 0.21	130.23 ± 0.07	25.09 ± 0.28	117.20 ± 0.26
	0.05	171.10 ± 0.05	170.30 ± 0.21	26.50 ± 0.21	119.40 ± 0.37
	0.1	156.30 ± 0.10	165.30 ± 0.28	23.70 ± 0.18	122.50 ± 0.13
	0.2	152.60 ± 0.06	160.10 ± 0.24	20.83 ± 0.09	112.20 ± 0.18

Notes: Adventitious root cultures were grown on MS nutrient solution and with modified MS medium (increased level of KNO_3). Treated rootlets and untreated root cultures were grown at 25°C and untreated root cultures were used as control.

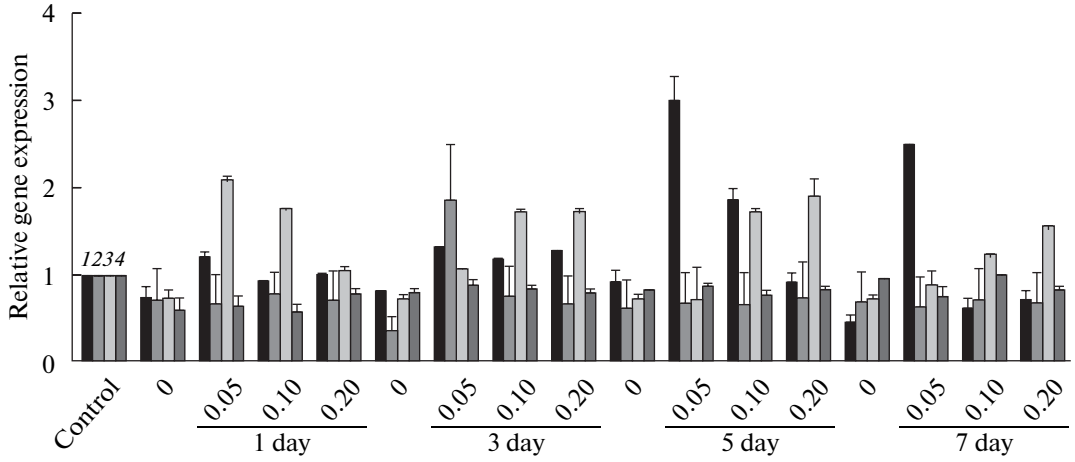

Fig. 2. Differential expressions of selected secondary metabolite genes encoding squalene epoxidase, cycloartenol synthase, β-amyrin synthase, and cytochrome P450, denoted as *1*, *2*, *3*, and *4*.

Adventitious root cultures of mountain ginseng were grown in MS medium under increasing KNO_3 concentration for 1 month. These roots were kept at 4°C for cold stress conditions for 1, 3, 5, and 7 days. Control roots were kept at 25°C. The relative gene expression was obtained using qRT-PCR. Data represents means ± SD for three independent replicates.

The first committed step in the triterpenoid ginsenoside biosynthesis involves the conversion of squalene into oxidosqualene by squalene epoxidase. Oxidosqualene cyclization leads to protopanaxadiol type ginsenosides, with the help of oxidosqualene synthase genes like those encoding cycloartenol synthase, β-amyrin synthase, cytochrome P450, and so on [23].

In this study, expression of few important genes related to ginsenoside biosynthesis was studied under chilling and K^+ supply. Overall secondary metabolite transcripts, i.e., ginsenoside pathway-related genes were not found to be significantly down-regulated upon cold stress. However, with increased K^+ supply the genes were up-regulated compared to those in the absence of K^+ supply. Expression of the gene encoding squalene epoxidase attained maximum (up to 3.02-fold) under 0at 5 days of cold treatment, and the transcript found to be decreased under higher K^+ concentration. This justifies the involvement of potassium in increasing transcription of ginsenoside synthesis-related genes. Expression of the gene encoding cycloartenol synthase was gradually increased (1.3-fold) in 3 days of cold stress under .05 mM KNO_3. Transcript of β-amyrin synthase gene showed increased expression patterns (2.1-fold) at 0.05 mM at 1 day of cold treatments and decreased thereafter. Finally, cytochrome P450 expression seemed to be slightly down-regulated under potassium supply during the entire experiment (Fig. 2). These results indicate that potassium is required under cold stress condition to activate transcription of various ginsenoside synthesis-related genes.. The initial increase in potassium concentration increased the saponin production in *P. ginseng* [5].

In conclusion, exogenous KNO_3 can improve the low-temperature tolerance in ginseng mainly by activating antioxidant enzyme activities and also by improving the expressions of secondary metabolite genes. Antioxidant gene expression and enzyme activity at 0.05 mM KNO_3 concentration was most effective under cold stress conditions. However, the secondary metabolite genes found to be differentially up-regulated with increased supply of KNO_3. Overall, present study showed that 0.05 mM KNO_3 was considered as the best concentration under cold stress conditions.

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SPELL: 1. Zheng, 2. Chemma, 3. Pulla, 4. Khorolragchaa