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ORIGINAL ARTICLE

Tracking dynamics of enzyme activities and their gene expression in *Picrorhiza kurroa* with respect to picroside accumulation

Varun Kumar[1] · **Kirti Shitiz**[1] · **Rajinder Singh Chauhan**[1] · **Hemant Sood**[1] · **Chanderdeep Tandon**[2]

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Abstract Picrosides, the terpenoids synthesized by *Picrorhiza kurroa*, have ample usage in medicine. Identification of the regulatory enzymes involved in picroside biosynthesis needs to be explored for improving the level of these secondary metabolites. Current efforts are based on the analysis of secondary metabolism in picroside biosynthesis but its interpretation is limited by the lack of information on the involvement of primary metabolic pathways. The present study investigated the connection of primary metabolic enzymes with the picrosides levels in *P. kurroa*. The results showed changes in the catalytic activities as well as in the gene expression profiles of hexokinase, pyruvate kinase, isocitrate dehydrogenase, malate dehydrogenase, and $NADP^+$-malic enzyme in congruence with picroside-I content under different conditions of *P. kurroa* growth, which indicates the role of these enzymes in the accumulation of picrosides. The significant correlation coefficients ($p<0.05$) observed between gene expression and enzyme activity underline the role of integrative studies for a better understanding of connecting links between metabolic pathways leading to picroside biosynthesis. This is apparently the first report on the involvement of glycolytic and TCA cycle enzymes in the accumulation of picrosides in *P. kurroa*.

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✉ Chanderdeep Tandon
tandonchanderdeep@yahoo.com

[1] Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, 173234 Solan, HP, India

[2] Department of Biotechnology, Amity Institute of Biotechnology, Noida 201313, India

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Introduction

Picrorhiza kurroa is a medicinally important endangered plant species of family Plantaginaceae, formerly known as Scrophulariaceae. This species is distributed between 3000 and 5000 m above mean sea level in the Himalayan region (Chettri et al. 2005). Indiscriminate and extensive harvesting and lack of organized cultivation of plant has threatened its status in wild and listed as 'endangered' species by International Union for Conservation of Nature and Natural Resources (Nayar and Sastri 1990). Picrosides, the terpenoids with an iridane skeleton of monoterpene origin, are synthesized by *Picrorhiza* and are widely used in many drug formulations *viz.* picroliv, katuki, arogya, livplus and livcare, due to their various pharmacological properties like hepatoprotective, antioxidant (particularly in liver), antiallergic, antiasthmatic, anticancerous and immunomodulatory activity (Kumar et al. 2014). In view of the medicinal applications of picrosides and merging of the plant species to extinction, there is a need to pinpoint the key sites for metabolic engineering so as to modulate the accumulation of secondary metabolites.

Picrosides are synthesized from iridoid moieties (Kawoosa et al. 2010; Gahlan et al. 2012), derived from geranyl pyrophosphate (GPP), which is synthesized by the sequential head-to-tail addition of isopentenyl pyrophosphate and its allelic isomer dimethylallyl pyrophosphate (Wise and Croteau 1998). Synthesis of isopentenyl pyrophosphate and dimethylallyl pyrophosphate may proceed *via* cytosolic mevalonate (MVA) and plastidic methylerythritol phosphate

(MEP) pathways (Mahmoud and Croteau 2002; Hampel et al. 2006) as shown in Fig. 1. This indicates that the supply of GPP is critical in realizing the yield of picrosides. Therefore, studies on regulation of genes and enzymes in GPP biosynthesis assume central importance. Previous studies have shown that regulatory genes in terpenoids biosynthesis are *hmgr* and *dxs*, which encode 3-hydroxy-3-methylglutaryl coenzyme A reductase and 1-deoxy-D-xylulose-5-phosphate synthase, respectively (Kawoosa et al. 2010). Recently, the involvement of phenylpropanoid pathway in the biosynthesis of picrosides has been reported (Gahlan et al. 2012; Kumar et al. 2013) which provides broader class of targets which could be triggered for the enhancement of picrosides biosynthesis. Secondary metabolic pathways are dependant on primary metabolic pathways for the supply of precursors. Though there are several reports on the involvement of secondary metabolism in picroside biosynthesis, the effect of primary carbohydrate metabolism on the accumulation of picrosides is yet to decipher.

Thus, in the light of above facts, the present study was envisaged to explore the connection of primary metabolic enzymes (glycolytic and TCA cycle enzymes) with the levels of picrosides in *P. kurroa* by examining the changes in the activities of selected enzymes and their gene expression profiles under differential conditions of plant growth *viz.* Sairopa (field grown), and tissue culture conditions at 15 °C (metabolite accumulating) and 25 °C (metabolite non-accumulating). To our knowledge, this is the first report on the involvement of glycolytic and TCA cycle enzymes in the accumulation of picrosides in *P. kurroa*.

Materials and methods

Plant sample

Picrorhiza kurroa plants were collected from nursery (Sairopa, Himachal Pradesh, 4500 m altitude, 31°38′–31°54′ N and 77°20′–77°45′ E) and maintained in pots under the glass house at Jaypee University of Information Technology (31.0163° N; 77.0702° E), Waknaghat, India. The shoot samples of *P. kurroa* were collected from plants grown in glass house as well as those grown in tissue culture under different temperature conditions *viz.* 15 and 25 °C. It has been previously documented that the accumulation of picroside-I in tissue culture grown *P. kurroa* shoots was achieved at 15 °C but not at 25 °C (Sood and Chauhan 2010). All plant samples were immediately stored at −80 °C for further analysis of picroside-I content and isolation of total RNA. Freshly grown shoots were used for the enzymatic assay studies.

Selection of enzymes of the primary metabolism

Enzymes of the primary metabolism were selected by looking into the interconnection between primary and secondary metabolic pathways (Fig. 1). The enzymes selected for studying the role of primary metabolism in the accumulation of secondary metabolites included those of glycolysis (hexokinase, HK; pyruvate kinase, PK), TCA cycle (isocitrate dehydrogenase, ICDH; malate dehydrogenase, MDH) and $NADP^+$- malic enzyme ($NADP^+$- ME). The details of the primers with annealing temperatures are provided in the Table 1.

Extraction and quantification of picroside-I

For extraction of picroside, field grown (Sairopa) as well as tissue culture grown (15 and 25 °C) shoots of *P. kurroa* were homogenized in a prechilled mortar and pestle using liquid nitrogen. Each powdered sample (100 mg) was suspended in 10 mL of 80 % methanol and vortexed followed by sonication at room temperature (25 °C) for 30 min. The samples were then centrifuged at 10,000 rpm for 15 min. The supernatants thus obtained were filtered through 0.22 μm filter (Millipore), diluted 10-fold with 80 % methanol and used for quantification of P-I by HPLC as per the method of Pandit et al. (2013). The experiment was performed in triplicates.

Extraction of enzymes

One gram shoots of *P. kurroa* growing under different conditions *viz.* Sairopa (field grown), and in tissue culture at 15 and 25 °C, were homogenized in a prechilled mortar and pestle using liquid nitrogen with 5 mL of extraction buffer, which varied in composition for different enzymes: HK and MDH [0.1 M Tris-Cl pH 7.5 containing 50 mM β-mercaptoethanol, 5 mM $MgCl_2$, 50 mM sucrose, 1 % Triton X-100 and 1 % insoluble PVP (*w/v*)]; PK [50 mM Tris-Cl pH 7.5 containing 10 mM $MgSO_4$, 5 mM β-mercaptoethanol, 20 % ethylene glycol (*v/v*) and 1 % insoluble polyvinylpyrrolidone (*w/v*)]; ICDH [50 mM Na-phosphate pH 7.5 containing 5 mM $MgCl_2$ and 1 % insoluble polyvinylpyrrolidone (*w/v*)], and $NADP^+$-ME [0.1 M Tris-Cl pH 7.5 containing 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 10 % glycerol (*v/v*), 1 mM PMSF and 1 % insoluble polyvinylpyrrolidone (*w/v*)]. The resulting homogenate was centrifuged at 10,000×*g* for 30 min. The supernatant thus obtained was dialyzed overnight against ten times diluted extraction buffer lacking polyvinylpyrrolidone. The overnight dialyzed extract was centrifuged at 10,000×*g* for 20 min and the supernatant so obtained (referred to as crude extract) was used for enzyme assays. The experiment was performed three times in replicates.

Enzyme assays

The activities of enzymes in crude extracts prepared from shoots were determined using coupled assays. The absorbance was recorded using a UV–VIS spectrophotometer (SPECTRA SCAN UV 2700, Thermo Scientific). The standard assay

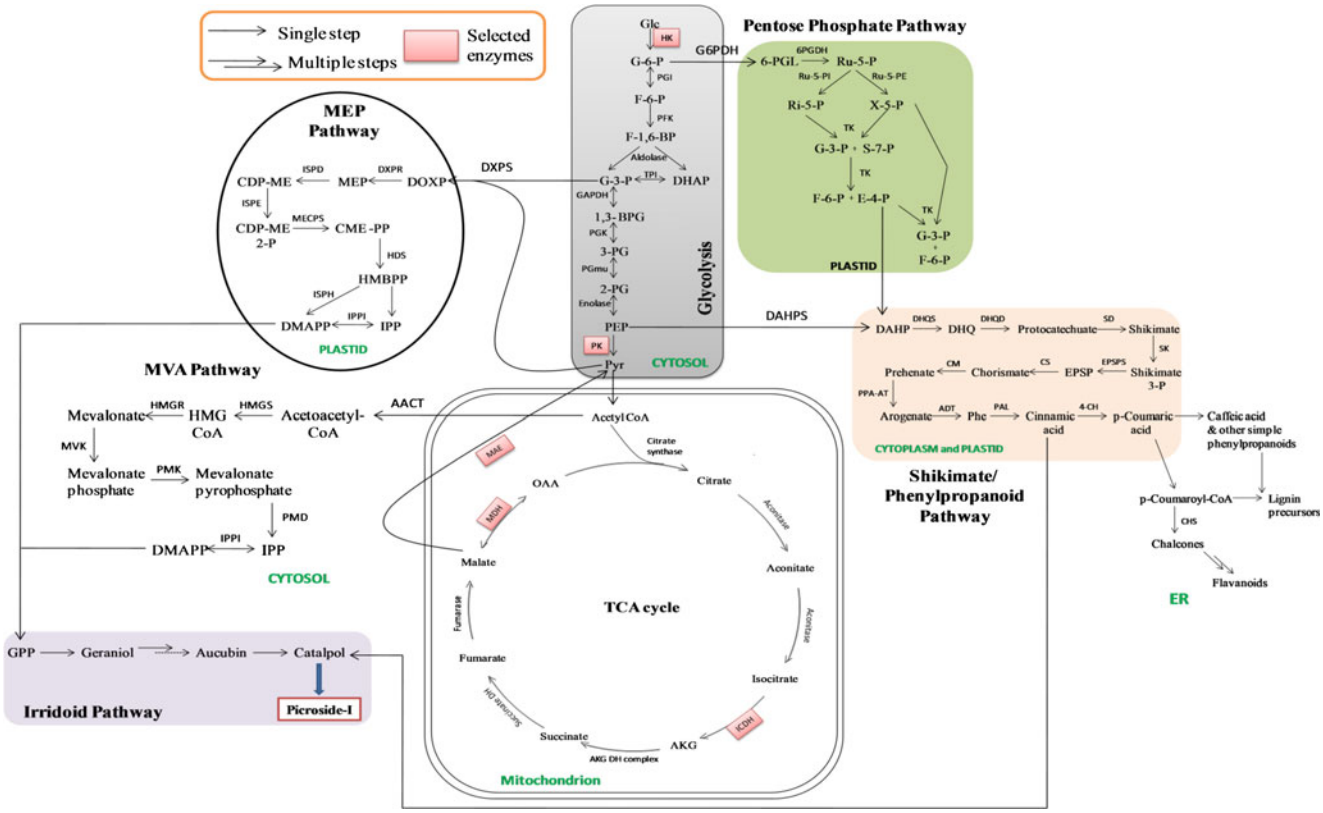

Fig. 1 Interconnection between primary and secondary metabolism. The metabolic network was reconstructed from the KEGG pathway included glycolysis (*grey*), the pentose phosphate pathway (*pale green*), the TCA cycle, mevalonate pathway, non mevalonate pathway, shikimate/ phenylpropanoid pathway (*pink*) and iridoid biosynthetic pathway (*light purple*). Abbreviations for the components are included in Supplementary table 1

mixture for HK contained 50 mM Tris-Cl buffer pH 7.5, 5 mM $MgCl_2$, 1 mM $NADP^+$, 5 mM glucose, 2.5 mM ATP, G-6-P dehydrogenase (2.25 U) and 200 µL crude extract in a total volume of 3.0 mL. The reaction carried out at 30 °C was initiated by the addition of ATP. The enzyme activity has been expressed as µmol $NADP^+$ reduced min^{-1} at 340 nm under the specified conditions. The standard assay mixture for PK contained 50 mM Tris-Cl buffer pH 7.2, 10 mM $MgSO_4$, 50 mM KCl, 0.1 mM NADH, 1.8 mM ADP, 1.2 mM PEP, LDH (2 U) and 50 µL crude extract in a total volume of 1.0 mL. The reaction carried out at 30 °C was initiated by the addition of PEP. The enzyme activity has been expressed

Table 1 Primer sequences used in qPCR based expression analysis

Genes	Primer sequence (Forward primer-FP and Reverse primer-RP)	Fragment size (bp)	Annealing temperatures (°C)
26S	FP 5'-CACAATGATAGGAAGAGCCGAC-3'	500	58
	RP 5'-CAAGGGAACGGGCTTGGCAGAATC-3'		
Hexokinase	FP 5'-ATGCTCCTTACCTACGTTCA-3'	108	52
	RP 5'-TCCTAACTGAACCCTCAAGA-3'		
Pyruvate kinase	FP 5'-AGCTTGTGGCTAAGTACAGG-3'	128	53
	RP 5'-TCCCCTGAATATGAGACTGT-3'		
Isocitrate dehydrogenase	FP 5'-TCGACATGATAACGTGGATA-3'	112	52
	RP 5'-TGTTATGACCTTGAGGCTCT-3'		
Malate dehydrogenase	FP 5'-CTGATTCTCAAGGAATTTGC-3'	114	51
	RP 5'-TACCTGCACTTTCAACCTCT-3'		
Malic enzyme	FP 5'-CAGCAGATCCTCACTTCTTC-3'	117	52
	RP 5'-CACATCCTTCAATCTCACCT-3'		

as μmol NADH oxidized min^{-1} at 340 nm under the specified conditions. The assay mixture for ICDH contained 50 mM Tris-Cl buffer pH 7.5, 5 mM $MgCl_2$, 1 mM $NADP^+$, 10 mM DL-isocitrate and 100 μL crude extract in a total volume of 3.0 mL. The reaction carried out at 30 °C was initiated by the addition of DL-isocitrate. The enzyme activity has been expressed as μmol $NADP^+$ reduced min^{-1} at 340 nm under the specified conditions. The activity of MDH was assayed in a reaction mixture containing 50 mM potassium phosphate buffer pH 7.0, 0.15 mM NADH, 1 mM oxaloacetate and 100 μL crude extract in a total volume of 3.0 mL. The reaction carried out at 30 °C was initiated by the addition of oxaloacetate. The enzyme activity has been expressed as μmol NADH oxidized min^{-1} at 340 nm under the specified conditions. The assay mixture for $NADP^+$-ME contained 50 mM Tris-Cl buffer pH 7.5, 10 mM $MgCl_2$, 0.5 mM NADP, 10 mM L-malate and 100 μL crude extract in a total volume of 3.0 mL. The reaction carried out at 30 °C was initiated by the addition of L-malate. The enzyme activity has been expressed as μmol $NADP^+$ reduced min^{-1} at 340 nm under the specified conditions. The enzyme assays were performed three times in replicates.

Protein estimation

Protein content in all the extracts was estimated by Lowry's method (Lowry et al. 1951). The experiment was performed three times in replicates.

RNA isolation and cDNA preparation

Total RNA was isolated from shoot samples of *P. kurroa* growing under different conditions using RNA isolation kit (Qiagen) including DNase digestion as per the manufacturer's instructions. The quality of RNA was checked by agarose gel electrophoresis on 1 % (*w/v*) gel stained with ethidium bromide and visualized by observing UV fluorescence using gel documentation system (Alpha Innotech, USA). For cDNA preparation, 5 μg of RNA was reverse transcribed using M-MuLV reverse transcriptase (GeNei TM) with oligo-dT primer. The resulting cDNA was quantified with the help of a ND-2000 UV spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The experiment was performed in triplicates.

Gene expression analysis of primary metabolic pathway enzymes using qPCR

Gene expression studies of different enzymes (HK, PK, ICDH, MDH and $NADP^+$- ME) were carried out by taking equal quantity of cDNA (100 ng), measured using a ND-2000 UV spectrophotometer. The qPCR was performed using gene specific primers (Table 1) three times in replicates on a CFX96 system (Bio-Rad Laboratories; Hercules CA). The qPCR reaction mixture (12.5 μL) contained 1 μL cDNA (100 ng), 0.5 μL of each primer (10 pmol each), 6.25 μL iQ™ SYBR Green Supermix (BIO-RAD) and 4.25 μL nuclease free H_2O. The conditions for qPCR were: initial denaturation at 95 °C for 3 min, 39 cycles each of denaturation at 95 °C for 10 s, annealing at 51–58 °C for 20 s and extension at 72 °C for 20 s followed by a final melt curve analysis. 26S rRNA was taken as an internal control for normalization.

Statistical analysis

The quantitative gene expression analysis was obtained by calculating the mean ± SD from ≤3 individual experiments done in replicates. The statistical analysis was done by using two-way ANOVA and followed by a Bonferroni test using GraphPad prism software version 6.0. The correlation analysis was performed by calculating Pearson coefficient using Excel (Microsoft, USA) and significant levels were tested at $P<0.05$ using the Student's *t*-test.

Results

In this study, enzyme activity and gene expression profiles of five selected enzymes *viz.* HK, PK, ICDH, MDH and $NADP^+$-ME of primary metabolic pathways were monitored and correlated with picroside-I content under differential growth conditions of plant *viz.* Sairopa (field grown), and grown in tissue culture at 15 °C and 25 °C.

Levels of picroside-I under different conditions of plant growth

Picroside-I content exhibited a lot of variation in shoots of plants under different growth conditions. The level of picroside-I in *P. kurroa* shoots of Sairopa (field grown) was 1.4 %, which was 2.8-fold ($p<0.001$) greater as compared to that in shoots of plants grown in tissue cultures at 15 °C (0.5 %) ($p<0.01$). In contrast, picroside-I was not detected in the shoots of plants growing in tissue culture at 25 °C (Supplementary Fig. 1). The HPLC chromatograms and UV spectra of the P-I standard and the samples are provided in Supplementary Fig. 2.

Activity profiles of the selected enzymes under different conditions of plant growth

The activity profiles of glycolytic (HK and PK) and TCA cycle (ICDH and MDH) enzymes were similar exhibiting the highest activity in Sairopa (field grown) followed by tissue culture plants growing at 15 °C and at 25 °C. However, the magnitude of variation in the activity of various enzymes in

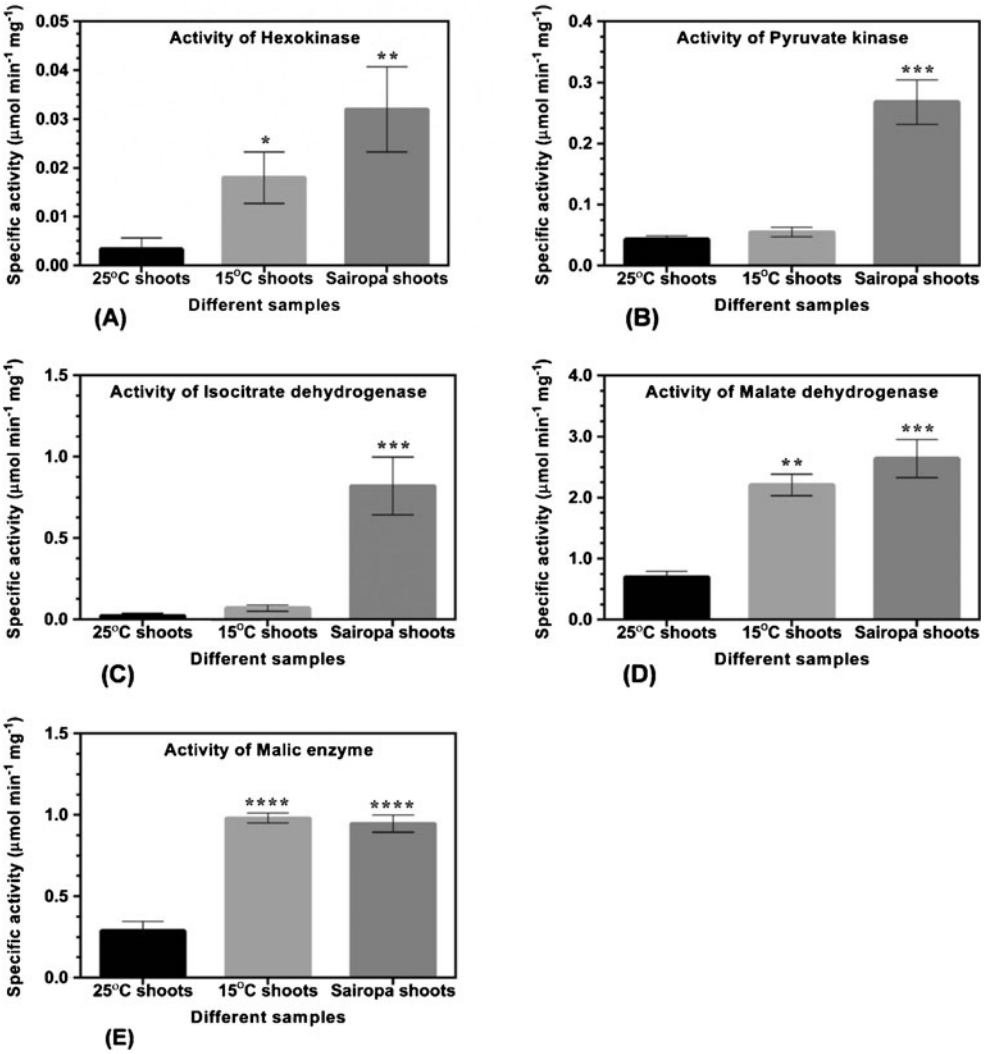

Fig. 2 Representation for catalytic activities of selected glycolytic (HK, PK), TCA cycle (ICDH, MDH) and NADP+-ME enzymes' in *P. kurroa* shoots grown in field (Sairopa) and in tissue culture conditions (15 and 25 °C). The vertical axis represents the specific activity and horizontal axis represents the different samples. *Bar graphs* show means ± SD of three separate experiments. Significance was evaluated between 15 °C, field grown (Sairopa) samples and 25 °C condition sample control (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

plants growing under varying conditions was different (Fig. 2). It was observed that the enzyme activities were much lower in the shoots of plants growing at 25 °C as compared to those at 15 °C and Sairopa. For glycolytic enzymes, specific activity of HK was 0.032 (p<0.01), 0.0176 (p<0.05) and 0.004 µmoles $NADP^+$ reduced min^{-1} mg^{-1} protein whereas that of PK was 0.268 (p<0.001), 0.055 and 0.043 µmoles of NADH oxidized min^{-1} mg^{-1} protein in the shoots of Sairopa, tissue culture plants at 15 °C and at 25 °C, respectively. The activity profiles of TCA cycle enzymes showed that in Sairopa shoots, specific activity of ICDH was 11.7-fold and 35.7-fold (p<0.001) higher whereas that of MDH was 1.2-fold and 4.0-fold (p<0.001) greater than in plant shoots at 15 °C and at 25 °C, respectively. The pattern of $NADP^+$-ME activity was somewhat different; its activity being maximum in the plants growing at 15 °C followed by Sairopa and plants at 25 °C. It was evident from the results that specific activities of all the five enzymes were the lowest in plants growing at 25 °C. The results of this study revealed a positive correlation between the specific activity of glycolysis (HK and PK), TCA cycle (ICDH and MDH) enzymes and $NADP^+$-ME with the levels of picroside-I under different conditions of plant growth, indicating the importance of these primary metabolic pathway enzymes in the accumulation of picrosides.

Gene expression profiles of the selected enzymes under different growth conditions

The genes of most of the selected enzymes of primary metabolic pathways showed relatively high expression in *P. kurroa* shoots, particularly in Sairopa shoots and tissue culture plants grown at 15 °C. However, the gene expression was much lower in plants grown at 25 °C as compared to other conditions (Fig. 3). The expression of genes encoding HK, PK, ICDH and MDH was recorded as the highest in Sairopa shoots followed by tissue culture grown *P. kurroa* plants at 15 and at 25 °C, whereas the expression of $NADP^+$-ME gene was maximum in plants grown at 15 °C followed by Sairopa and plants grown at 25 °C (Fig. 3). The gene expression of HK in Sairopa shoots was 8- and 87-fold (p<0.001) greater than plants

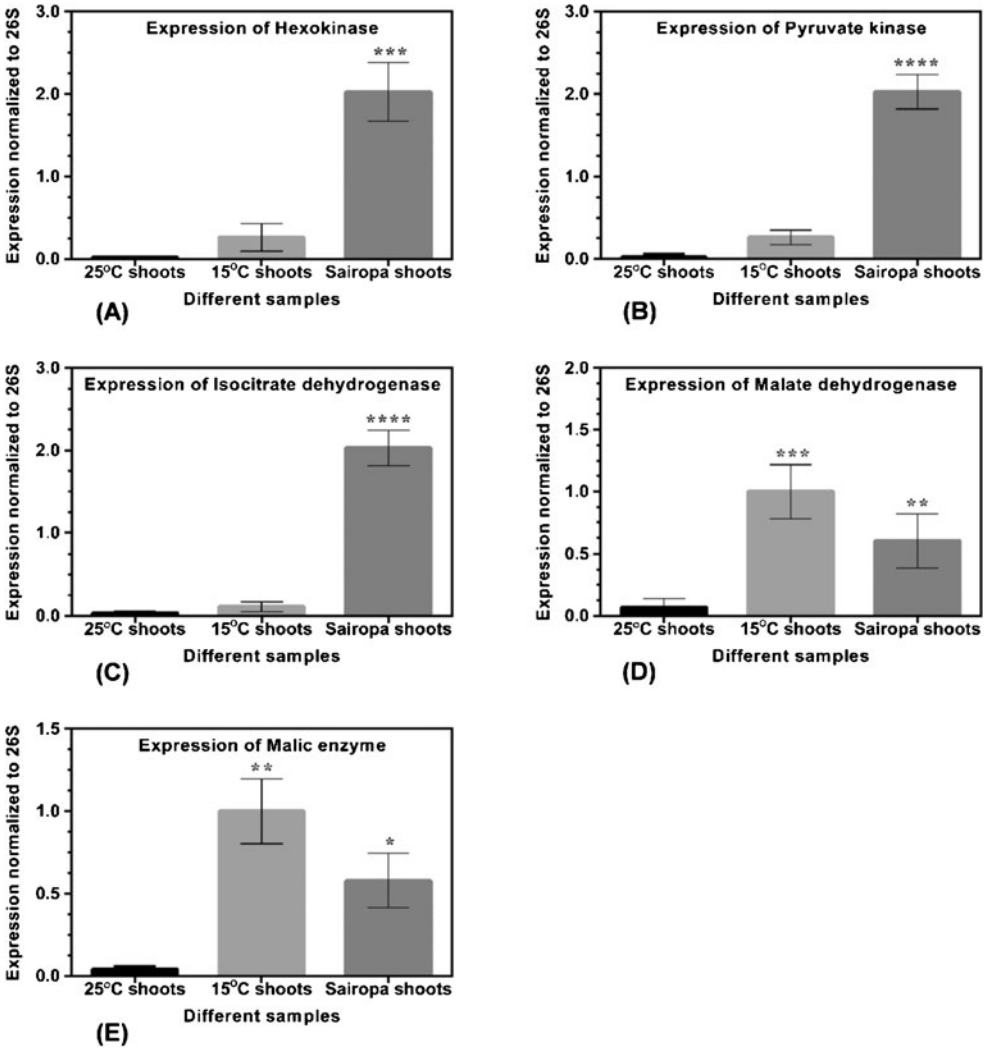

Fig. 3 Representation for quantitative gene expression of selected glycolytic (HK, PK), TCA cycle (ICDH, MDH) and NADP+-ME enzymes' in *P. kurroa* shoots grown in field (Sairopa) and in tissue culture conditions (15 and 25 °C). The *vertical axis* represents the gene expression and *horizontal axis* represents the different samples. Expression level was normalized to housekeeping gene *i.e.* 26S. *Bar graphs* show means ± SD of three separate experiments. Significance was evaluated between 15 °C, field grown (Sairopa) samples and 25 °C condition sample control (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$)

grown at 15 and 25 °C, respectively. Similarly, level of PK transcript in Sairopa was 12.6- and 81.2-fold ($p<0.0001$) greater than plants grown at 15 and 25 °C, respectively. For the TCA cycle enzymes, the expression of ICDH gene was 18-fold and 54.3-fold ($p<0.0001$) higher whereas that of MDH gene was 1.7-fold and 13.9-fold ($p<0.001$) superior in 15 °C shoots as compared to *P. kurroa* plants at Sairopa and at 25 °C, respectively. On the contrary, the transcript level of $NADP^+$-ME in plants grown at 15 °C was 1.7-times greater than in Sairopa shoots but 23.6-times ($p<0.01$) greater than in plants grown at 25 °C.

It was evident from the results that the gene expression levels of all the five enzymes of primary metabolism under different growth conditions showed a positive correlation with the profiles of enzyme activities, implying that the higher activities of enzymes were due to stimulation of their gene expression. Further, the transcript levels were greater under the growth conditions which resulted in maximum accumulation of picroside-I, indicating that an enhancement in the gene expression of the enzymes might be responsible for elevated content of picrosides.

Correlation analysis

HK, PK, ICDH, MDH and $NADP^+$-ME activities in field grown (Sairopa) as well as tissue culture grown (15 and 25 °C) shoots of *P. kurroa* were compared with their gene expression levels in the respective plants. The significant positive correlations (Pearson's correlation coefficient, $r>0.79$) were observed between enzyme activity and gene expression in all the selected five enzymes as shown in Table 2. The Pearson's correlation coefficient of ICDH ($r=0.9994$) was observed as the highest followed by PK ($r=0.9977$), $NADP^+$-ME ($r=0.9161$) and HK ($r=0.8837$); whereas MDH ($r=0.7925$) showed minimum correlation coefficient.

The significant positive correlations obtained between enzyme's activities and gene expression under differential

Table 2 The correlation between enzyme activity and their gene expression in selected enzymes. The correlation coefficient r was calculated using the Pearson's correlation

Gene	Correlation coefficient
HK	0.8837**
PK	0.9977**
MDH	0.7925*
ICDH	0.9994**
ME	0.9161**

*significant at ($p<0.05$) **significant at ($p<0.01$)

conditions of picroside biosynthesis indicated the involvement of these enzymes in picroside accumulation.

Discussion

Plant primary carbohydrate metabolism is highly variable and susceptible to many perturbations. However, its response to the differential accumulation of secondary metabolites is largely unknown. In the present study, changes in catalytic activities and gene expression levels of the selected glycolytic (HK and PK), TCA cycle (ICDH and MDH) enzymes and $NADP^+$-ME under differential experimental conditions were positively correlated with each other as well as with the picroside content, indicating that these enzymes were likely to trigger alterations in picroside levels in *P. kurroa* shoots. The levels of enzyme activities and gene expression of all the five enzymes were the lowest in tissue culture plants grown at 25 °C, which also contained a very low content of picroside-I. It could be inferred from this observation that lower accumulation of picrosides at 25 °C than at 15 °C was due to the adverse effect of this growth temperature on the activities of enzymes involved in primary metabolic pathways. The correlation between the activities of enzymes and their transcript levels could have a high impact on metabolic fluxes as the variations in the genes lead to alterations in their encoding enzyme activity (Keurentjes et al. 2008). Thus, the variation observed in the transcript levels of the selected genes most likely contributed to the difference observed in the enzyme activity, as indicated by a high correlation coefficient (Table 2). The correlation between enzyme activity and gene expression also implied that higher enzyme activity at 15 °C and field-grown plants was due to an increase in the enzyme concentration. This is supported by a previous study which also showed a strong positive correlation (Pearson's correlation coefficient, $r>0.8$) between glycolytic enzyme activities compared with the expression of their genes (Mutuku and Nose 2010) in order to investigate the coarse and fine controls in the regulation of glycolysis (Mutuku and Nose 2012).

HK catalyzes the primary step in the glycolytic pathway, *i.e.* phosphorylation of glucose to form glucose-6-phosphate, which is situated at the entry point into pentose phosphate pathway. In this study, an increased activity and gene expression of HK was observed in picroside accumulating condition *i.e.* 15 °C as compared to picroside non-accumulating condition *i.e.* 25 °C. An increase in HK activity would enhance the concentration of glucose-6-phosphate concentration leading to the activation of oxidative pentose-phosphate pathway under stress conditions, which in turn, perhaps contributed to picroside-I accumulation. Savitch et al. (2001) also reported that cold acclimation resulted in an increase in the reduction state of the stroma, indicated by increased total activity along with light-dependent activities of the major regulatory enzymes of the oxidative pentose-phosphate pathway. The activated oxidative pentose-phosphate pathway might result in an increased level of erythrose-4-phosphate, which is a precursor for the shikimate pathway leading to phenylpropanoid metabolism. Henkes et al. (2001) also showed that precursors of primary metabolism colimit flux into the shikimate pathway and phenylpropanoid metabolism. The connection of phenylpropanoid metabolism to picroside biosynthesis was previously documented by various studies (Gahlan et al. 2012; Kumar et al. 2013). This may imply the role of HK in picroside biosynthesis.

Pyruvate kinase catalyzes the rate limiting step in the glycolytic pathway, *i.e.* dephosphorylation of PEP to form pyruvate, which acts as the precursor for MVA and MEP pathways. This study revealed higher activity and gene expression of pyruvate kinase in picroside accumulating condition as compared to picroside non-accumulating condition. This increase might result in an increased level of pyruvate, which colimits flux into mevalonate (MVA) and non mevalonate pathway (MEP) leading to picroside biosynthesis. The elevated level of pyruvate is likely to boost the activity of 1-deoxy-D-xylulose 5-phosphate synthase (DXPS), which uses pyruvate as its substrate and catalyzes the rate limiting step in the MEP pathway (Cordoba et al. 2009), leading to the enhanced levels of secondary metabolites. Enfissi et al. (2005) recorded 1.6-fold increase in carotenoid content in transgenic lines containing a bacterial *dxs* gene targeted to the plastid with the tomato *dxs* transit sequence.

In addition to the glycolytic enzymes, ICDH, which catalyses the rate limiting step in the TCA cycle *i.e.* the oxidative decarboxylation of isocitrate producing α-ketoglutarate, CO_2 and NADH, also showed an enhanced activity and gene expression in picroside accumulating condition as compared to picroside non-accumulating condition. The increase in ICDH activity might result *via* TCA cycle reactions in an increase in the concentration of malate, which is converted into pyruvate by $NADP^+$-ME under stress conditions (Doubnerova and Ryslava 2011). MDH, which catalyses the reversible oxidation of malate to oxaloacetate along with the reduction of NAD^+ to NADH, showed much higher increase in gene expression over enzyme activity in picroside accumulating condition as compared to picroside non-accumulating condition

as well as the field grown Sairopa shoots. An increase in the activities of ICDH and MDH is likely to affect the picroside biosynthesis by generating TCA cycle intermediates in greater concentration in addition to NADH which could be used for ATP production. $NADP^+$-ME showed much higher increase both in gene expression and enzyme activity in picroside accumulating condition as compared to picroside non-accumulating condition as well as the field grown Sairopa shoots. This elevation might result in higher levels of pyruvate leading to the enhanced picroside accumulation under stress. Beck et al. (2012) also reported an increased production of mevalonate and isoprenoids in microorganisms by engineering a microorganism for increased carbon flux towards mevalonate production by over expressing malic enzyme activity.

Overall, this study showed enhanced expression and activity of all the five selected enzymes of glycolysis and TCA cycle in 15 °C shoots (stress condition) as compared to 25 °C shoots (non- stress condition) in relation to picroside-I content in *P. kurroa*. A good correlation observed between gene expression and enzyme activity as well as picroside-I concentration indicated the involvement of these enzymes in picroside accumulation. This suggests that the integrative genetic analysis of gene expression and enzyme activity in order to decipher connectivity between primary metabolism and accumulation of secondary metabolites, described here for the first time, can assist in determining the regulatory steps in primary carbohydrate metabolism. These steps of primary metabolism are likely to be amenable targets for metabolic engineering aiming at the accumulation of secondary metabolites.

Glycolysis is at the core of carbon allocation to secondary metabolic pathways and the integrative analyses of gene expression and enzyme activity have revealed connectivity between primary metabolism and accumulation of the secondary metabolite picroside. So, targeting primary metabolism in plants can help to detect key spots for metabolic engineering in order to achieve enhanced secondary metabolite production.

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Conflict of interest The authors declare that they do not have any conflict of interest.

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