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RESEARCH NOTE

An insight into conflux of metabolic traffic leading to picroside-I biosynthesis by tracking molecular time course changes in a medicinal herb, *Picrorhiza kurroa*

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Abstract Picroside-I (P-I) is a member of the iridoid glycosides family of natural products, which are used to treat liver disorders. The growing medicinal need for this benign compound has stimulated the present study to identify genes important for the biosynthesis of P-I. In this study, molecular screens have been generated using gene expression patterns obtained by quantitative RT-PCR which have extended the knowledge of genes associated with P-I biosynthesis. A total of 13 genes encoding the rate limiting enzymes of different pathways, were analyzed by qRT-PCR in *Picrorhiza kurroa* shoots collected at 0, 10, 20, 30 and 40 days. The results showed that five of the genes (HK, DXPS, ISPD, HMGR and PMK) are supposed to be essential for P-I biosynthesis up to 20 days while DAHPS and G-10-H, in conjunction might assist P-I biosynthesis between 20 and 30 days of *P. kurroa* growth. This is apparently the first report on the molecular aspects of different pathways integrated in P-I biosynthesis. Moreover, principal component analysis prediction also corroborated the genes selection by identifying genes signatures for different samples (collected at different time intervals) and supported the link between samples and gene expression patterns. Overall, this study capitalizes on dynamic gene expression patterns obtained in response to the P-I stimuli under different stages of *P. kurroa* growth which are likely to define the regulatory steps in P-I biosynthesis.

Keywords P-I · G-10-H · DAHPS · qPCR · PCA · Biosynthesis · Gene expression

Introduction

Picrorhiza kurroa, a medicinally important plant belonging to family Plantaginaceae (formerly known as Scrophulariaceae), is distributed between 3000 and 5000 m above mean sea level in the Himalayan region (Chettri et al. 2005). *Picrorhiza*-based formulations viz. Picroliv, Livocare, Livplus and Arogya, have been accepted worldwide due to their demonstrated pharmacological potential in the treatment of liver ailments. These pharmacological activities are attributed to the presence of iridoid glycosides mainly picroside-I (P-I) and picroside-II (P-II) (Dwivedi et al. 1993; Kumar et al. 2014). P-I is produced in the shoots and rhizomes whereas P-II is found in the roots and rhizomes of *P. kurroa* (Pandit et al. 2013a). Despite the pronounced pharmacological activities of picrosides, their biosynthetic pathways are still not completely validated. Moreover, an unregulated massive surge in the harvesting of *P. kurroa* has led it to be listed as 'endangered' that opened our eyes to investigate the biosynthetic pathway of picrosides.

Recently, in addition to the documentation of proposed biosynthetic pathway of picrosides (Kumar et al. 2013), the major progress in P-I biosynthesis pertains to modulation in

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expression patterns of genes involved in the mevalonate, non-mevalonate and primary metabolic pathways vis-a-vis P-I content under different growth conditions of *P. kurroa* (Kumar et al. 2015; Pandit et al. 2013b; Singh et al. 2013). Although these reports have given us valuable insights into P-I biosynthesis yet these remain silent in the absence of a specific trigger. Hence, proper execution of P-I biosynthesis requires the coordination of a more number of primary and secondary pathways but the challenge is to filter these numerous enzymes in order to select the suitable targets for its enhanced production in *P. kurroa*. For unveiling regulatory steps, tracking events occurring at the molecular level has become a major focus for modern molecular biology (Zhu et al. 2007).

The quantitative RT-PCR (qPCR) technique provides a powerful tool which can estimate and compare the changes in gene expression patterns over a wide range of experimental conditions and deliver amazed sensitivity and accuracy (Asters et al. 2014). Even though, qPCR data can provide valuable information but execution of appropriate statistical analysis can make significant inferences from the gene expression data. Principal component analysis (PCA) is an appropriate statistical method which has been frequently used to explore the gene expression data in a low dimensional space (Shankar et al. 2013; Asters et al. 2014). Further, by taking in account the flexibility of this technique to easily cope with large datasets, we brought secondary metabolite precursors back into the spotlight and assembled different pathways for the identification of regulatory steps in P-I biosynthesis.

Thus, the objective of present study is to determine the gene expression patterns along with the P-I content at different stages of *P. kurroa* growth viz. 0, 10, 20, 30 and 40 days in order to identify regulatory steps that provides insight into the metabolic basis controlling the biosynthesis of P-I.

Materials and methods

Plant material

Field grown plantlets of *P. kurroa* were collected from nursery (Jagatsukh, Himachal Pradesh; 32°12′0N, 77°12′0E; 2193 m altitude) and maintained at Jaypee University of Information Technology (Waknaghat, Himachal Pradesh; 31°0′58.55″N, 77°4′12.63″E; 1700 m in altitude). The plantlets were cultured in an optimized Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 3 mg/L indole-3-butyric acid and 1 mg/L kinetin and incubated in a plant tissue culture chamber maintained at 25 ± 1 °C with 16 h photoperiod provided by cool white fluorescent light (3000 lux). It was previously reported that 25 °C temperature hinders the P-I biosynthesis and accumulation whereas, 15 °C increases the P-I accumulation approx. 20-fold in an in vitro grown *P. kurroa* shoots (Sood and Chauhan 2010). That's why, after 6 weeks of incubation at 25 °C, the in vitro grown *P. kurroa* shoots were taken as an explant for carrying out further experimentation at 15 °C where the in vitro grown shoots were incubated and the shoot samples were collected at 0, 10, 20, 30 and 40 days after the start of the experiment and were immediately stored at −80 °C for isolation of total RNA and quantification of P-I. The experiment was performed in quadruplicates.

Selection of genes

Various genes encoding the enzymes catalyzing the rate limiting steps (that control and mediate the overall flux of the pathway) in primary and secondary metabolism were selected by examining the participating pathways in P-I biosynthesis for monitoring their expression at different stages of *P. kurroa* growth (Fig. 1). The selected genes encoded the enzymes of glycolysis (hexokinase, HK; pyruvate kinase, PK), TCA cycle (isocitrate dehydrogenase, ICDH; malate dehydrogenase, MDH), pentose phosphate pathway (glucose-6-phosphate dehydrogenase, G-6-PDH), shikimate/phenylpropanoid pathway (3-deoxy-D-arabinoheptulosonate 7-phosphate synthase, DAHPS; phenylalanine ammonia lyase, PAL), mevalonate pathway (hydroxymethylglutaryl-CoA reductase, HMGR) and non-mevalonate pathway (1-deoxy-D-xylulose-5-phosphate synthase, DXPS). In addition, the genes encoding the enzymes, 4-diphosphocytidyl-2C-methyl-D-erythritol synthase (ISPD) and phosphomevalonate kinase (PMK) were also selected which previously showed enhanced elevation vis-à-vis P-I content under different growth conditions of *P. kurroa* (Pandit et al. 2013b). The gene coding geraniol synthase (GS) was also selected as it showed significant fold increase in shoots of *P. kurroa* exposed to 15 °C as compared to 25 °C (Gahlan et al. 2012). Finally, geraniol-10-hydroxylase (G-10-H) gene was chosen as it is one of the first two genes in P-I biosynthesis which was used as a bait to decipher the enzymes of seco-iridoid pathway in *Catharanthus roseus* (Miettinen et al. 2014). The primers of mevalonate and non-mevalonate pathway genes (DXPS, ISPD, HMGR and PMK) were procured from Pandit et al. (2013b) while glycolysis (HK, PK), TCA cycle (ICDH, MDH) and pentose phosphate pathway (G6PDH) were procured from Kumar et al. (2015). The primers for rest of the selected genes were designed from transcriptomic sequences of *P. kurroa* by using Primer3 software. The details of the primers with annealing temperatures are provided in the Supplementary Table 1.

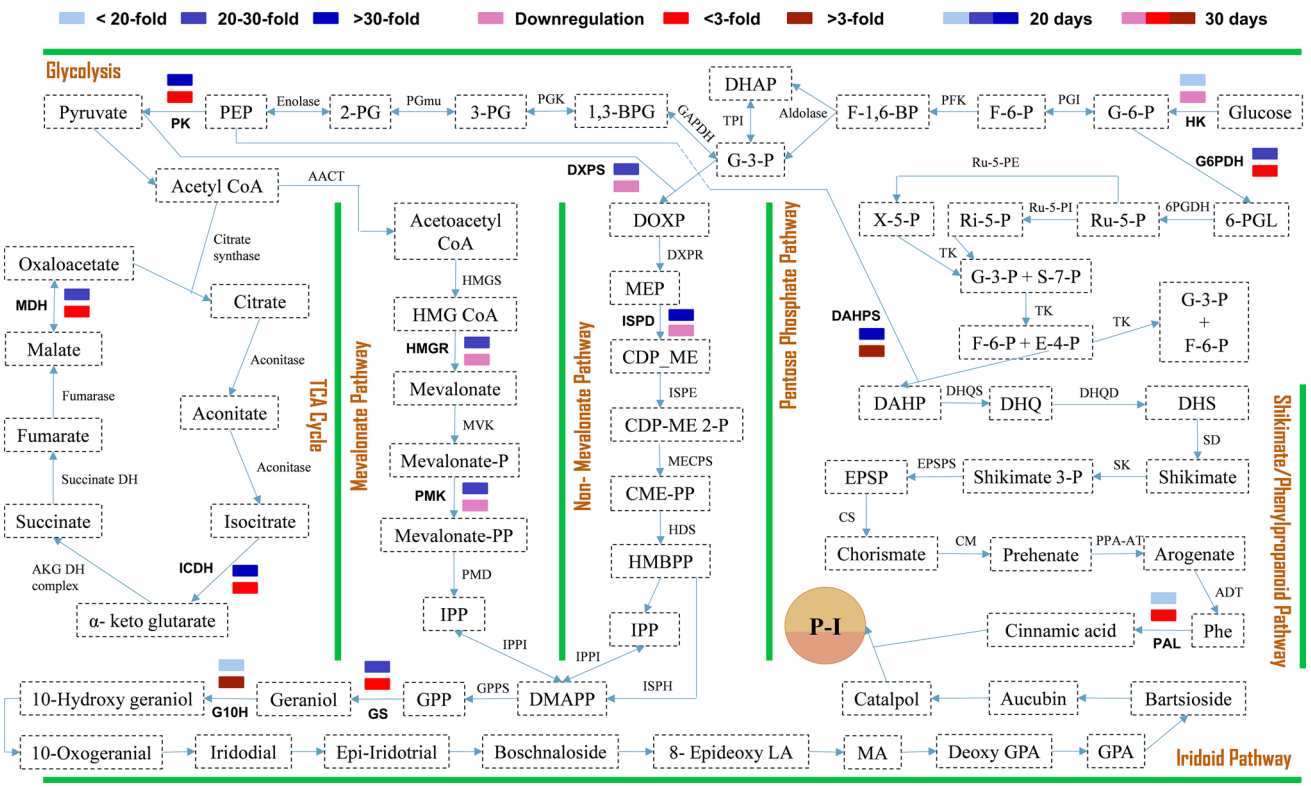

Fig. 1 Integration of primary and secondary metabolic pathways. An outline of the primary and secondary metabolism which integrates into picroside-I biosynthesis. The metabolic network has been reconstructed by including glycolysis, TCA cycle, pentose phosphate pathway, shikimate/phenylpropanoid, mevalonate, non-mevalonate and iridoid pathway (Kumar et al. 2015). *Squares coded with different colors* highlighted the expression of genes encoding enzymes examined in the primary and secondary metabolism at different time intervals of *P. kurroa* growth; after 20 days (*blue color*) and, after 30 days (*red color*). Abbreviations are elaborated in supplementary Table 3. (Color figure online)

Extraction and quantification of P-I

P-I from *P. kurroa* shoots collected at 0, 10, 20, 30 and 40 days of growth was extracted as described in Kumar et al. (2015) and quantified by HPLC as per the method of Pandit et al. (2013a). The experiment was performed three times in replicates.

RNA extraction

Four culture jars containing *P. kurroa* shoot samples were collected at each 0, 10, 20, 30 and 40 days and were homogenized in prechilled pestle and mortar (−20 to −80 °C) using liquid nitrogen. Total RNA was isolated by using TRIzol reagent (Ambion) as per the manufacturer's instructions. The resulting RNA was quantified with the help of a ND-2000 UV spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The RNA quality was checked on 1 % (w/v) agarose gel which was stained with ethidium bromide and visualized by observing UV fluorescence using gel documentation system (Alpha Innotech, USA).

Preparation of cDNA and quantitative RT-PCR analysis

Complementary DNA (cDNA) was synthesized from 5 µg of total RNA using Verso cDNA synthesis kit (Thermo scientific, US) as per the manufacturer's instructions. The quantification of resulting cDNA was carried out by using ND-2000 UV spectrophotometer. The gene expression analysis was conducted in quadruplicates on CFX96 system (Bio-Rad Laboratories; Hercules CA) with the standard 96 well block. The reaction mixture (12.5 µL) used for qPCR analysis contained: 0.5 µL forward primer (10 pmol), 0.5 µL reverse primer (10 pmol), 6.25 µL iQ^{TM} SYBR Green Supermix (BIO-RAD), 1 µL cDNA (100 ng) and 4.25 µL nuclease free H_2O. The qPCR programme was: (1) 1 cycle of 95 °C for 3 min; (2) 39 cycles of 95 °C for 10 s, 49–60 °C for 20 s and 72 °C for 20 s followed by a final melt curve analysis. The housekeeping genes, 26S and GAPDH were used as the reference genes in this study.

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 one-way ANOVA and followed by a Bonferroni test using GraphPad prism software version 6.0. The principle component analysis (PCA) was performed by using XLSTAT-Pro 7.5 software (Addinsoft, New York, USA). The heat map was generated by using R-language from quadruplicate data.

Results and discussion

To address the metabolic basis of P-I biosynthesis, genes encoding the rate limiting enzymes of metabolic pathways providing precursors for biosynthesis of P-I were selected and analysed for modulation vis-à-vis P-I content in 0–40 days old shoots of *P. kurroa*. The data revealed that *P. kurroa* shoots exhibited a lot of variation in P-I content at 0−40 days of plant growth (Supplementary Fig. 1 and 2) with a progressive enhancement till 30 days and then remained unchanged. Further, time course expression of HK, PK, ICDH, MDH, G-6-PDH, DXPS, ISPD, HMGR, PMK, DAHPS, PAL, GS and G-10-H genes showed significant changes in congruence with P-I content between 0 and 40 days of plant growth at 15 °C.

At 10 days stage, the P-I content increased from 0 to 0.19 µg/mg but expression of all the above selected genes appeared to be similar to 0 day stage (Fig. 2). This might indicate that already accumulated metabolites in the shoots of *P. kurroa* were responsible for initial biosynthesis of P-I. It was likely that during this period plant cells adapted to the new culture conditions as inferred from the growth and gene expression analysis. This was supported by further evidence of PCA which represented that none of the genes were distributed around 10 days growth condition in the biplot as shown in Fig. 3.

Further, an increase in P-I content (2.16-fold) was observed between 10 and 20 days of *P. kurroa* growth which might be due to elevated expression of all the selected genes during this period as compared to the 10 days stage. The expression of HK, PAL and G-10-H genes showed <20-fold increase over the 10 days stage while the expression of G-6-PDH, MDH, DXPS, HMGR, PMK and GS genes augmented between 20- and 30-folds. Moreover, the transcript levels of DAHPS, ISPD, PK and ICDH registered a marked increase of 44-, 77- ($p < 0.001$), 81.5- and 130-fold ($p < 0.01$), respectively (Fig. 2). Glycolysis is the carbon core for central metabolic pathways and activated by an increase in the transcript level of glycolytic enzymes i.e. HK and PK (Fig. 1). The enhanced expression of HK gene would produce higher levels of G-6-P, the substrate for G-6-PDH. The elevated expression of G-6-PDH gene resulting in activation of the pentose phosphate pathway, which might produce higher levels of erythrose-4-phosphate. The later one is the substrate for the enzyme DAHPS, considered to be rate-limiting enzyme of the shikimate/phenylpropanoid pathway (Tzin et al. 2012). Further, PAL is supposed to be the entry point into P-I biosynthesis via the formation of cinnamoyl CoA (Kumar et al. 2013; Gahlan et al. 2012). This statement was corroborated by the enhanced expression of G-6-PDH, DAHPS and PAL genes observed in the study. On the other hand, the increased PK gene expression might be associated with enhanced allocation into the TCA cycle, mevalonate and non-mevalonate pathways (Lange et al. 2000; Mutuku and Nose 2012). ICDH, catalysing the rate limiting step in the TCA cycle, showed augmented expression which could produce higher concentrations of TCA cycle intermediates along with the increased ATP production required for *P. kurroa* growth and thus, might not affect the P-I biosynthesis. This was also supported by PCA analysis which showed that ICDH did not make any significant contribution in the biosynthesis of P-I (Supplementary Table 2). The increased transcript level of ICDH might be correlated with elevated expression of MDH, which in addition to ATP generation could contribute to the P-I biosynthesis by producing higher levels of pyruvate through $NADP^+$-Malic enzyme (Kumar et al. 2015). This was in agreement with the PCA analysis results which showed that MDH made highly significant contribution in the biosynthesis of P-I (Supplementary Table 2). Moreover, the mevalonate and non-mevalonate pathways were activated via modulations in the expression of DXPS, ISPD, HMGR and PMK genes. Astonishingly, the observed greater elevation in the transcript levels of ISPD as compared to DXPS, HMGR and PMK genes indicated that the non-mevalonate pathway possibly played a major role in the biosynthesis of P-I. Further, PCA analysis also showed that the ISPD significantly contributed to the biosynthesis of P-I inevitably over HMGR and PMK (Supplementary Table 2). Our observations are in agreement with another study which revealed that the non-mevalonate or MEP pathway, localized in plastids, provides C5 units (IPP and DMAPP) for monoterpene, hemiterpene and diterpene biosynthesis, whereas mevalonate pathway, localized in cytosol, provides C5 units for sesquiterpene biosynthesis (Dudareva et al. 2005). Thus, elevated transcript levels of ISPD between 10 and 20 days of plant growth would be associated with the supply of GPP, which is a precursor for the iridoid biosynthesis. Further, we also observed that transcripts encoding the GS and G-10-H enzymes were up-regulated between 10 and 20 days of plant growth which suggests their possible role

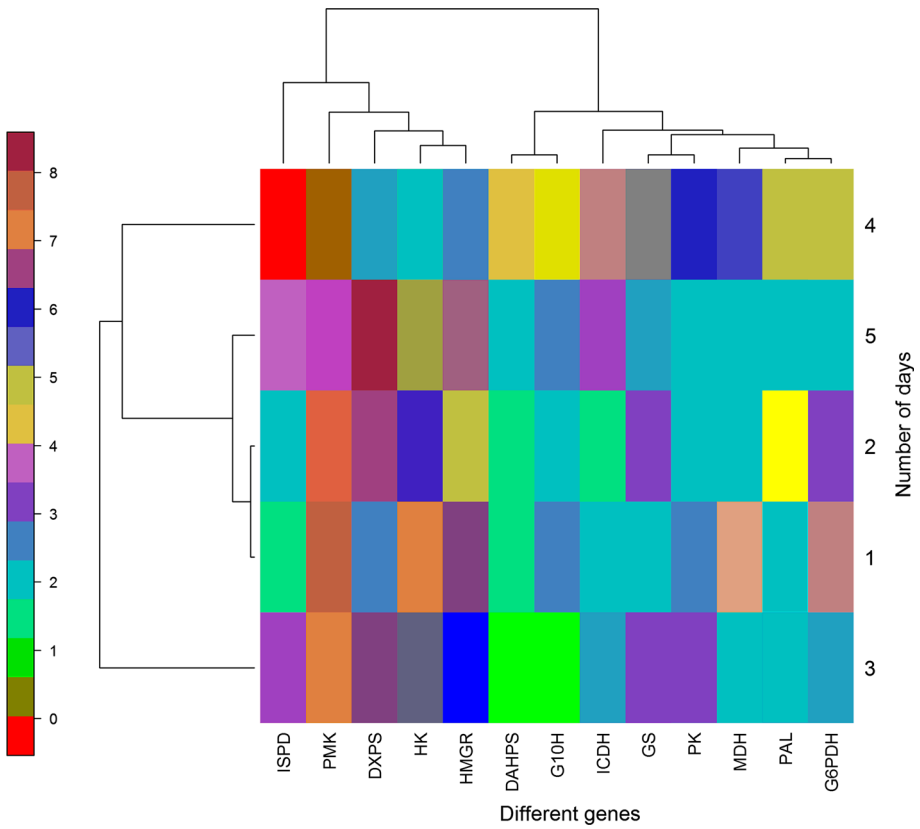

Fig. 2 Quantitative RT-PCR analysis of selected genes at different time intervals of *P. kurroa* growth. Thirteen genes were selected and their expression profiles were assessed at different time intervals of *P. kurroa* growth i.e. 0, 10, 20, 30 and 40 days. The expression for each selected gene in metabolic pathways in all the above samples is demonstrated by using heat map. Expression levels were calculated from data in quadruplicate and normalized to average value of housekeeping genes i.e. 26S and GAPDH. *1* 0 day, *2* 10 days, *3* 20 days, *4* 30 days and *5* 40 days

in the accumulation of P-I (Fig. 1). Taken together, these results suggested that enhanced expression of a set of genes between 10 and 20 days of plant growth may play an important role in the biosynthesis of P-I.

Upon analyses of 30 days grown *P. kurroa* shoots, 4.07-fold ($p < 0.001$) rise in P-I content was observed and transcripts encoding HK, DXPS, ISPD, HMGR and PMK enzymes were down-regulated whereas the expression of PAL, G-6-PDH, PK, ICDH, MDH and GS genes was up-regulated by <3-fold in comparison to 20 days grown *P. kurroa* shoots (Fig. 2). The results indicated that HK, DXPS, ISPD, HMGR and PMK genes are likely to be involved in P-I biosynthesis for the first 20 days of plant growth and thereby might result in the accumulation of precursors required for the biosynthesis of P-I. Further, evidence and supportive data of PCA analysis also represented the distribution of DXPS, HMGR, HK and PMK genes in the cluster around 20 days stage of *P. kurroa* growth in the biplot (Fig. 3). Of more significance is the demonstration that out of the genes up-regulated in 30 days grown *P. kurroa* shoots, transcripts encoding DAHPS (4.68-fold; $p < 0.01$) and G-10-H (6.28-fold; $p < 0.001$) showed a marked elevation in the expression levels as compared to the other studied genes (Fig. 2). It might be inferred that the DAHPS was likely to be associated with the biosynthesis of P-I possibly by activating shikimate/phenylpropanoid pathway, while G-10-H transcript is proposed to be involved in the biosynthesis of P-I via a series of hydroxylation reactions supposed to be catalysed by cytochrome p450 enzymes (Gahlan et al. 2012; Kumar et al. 2013). Thus, it is striking that G-10-H and DAHPS might act as a regulator of P-I biosynthesis (Fig. 1). An empirical evidence of PCA analysis also inferred that G-10-H and DAHPS significantly contributed to the biosynthesis of P-I (Supplementary Table 2).

At 40 days of growth, P-I content showed no further increase with respect to 30 days stage while a striking exhibition of reversed gene expression was observed i.e. restoration of expression levels as seen during the initial growth stage. This difference in gene expression might indicate the possible association of the above selected rate limiting genes with the biosynthesis of P-I and also the flexibility of biochemical pathways to quickly adapt according to the changes in the plant growth stages.

Overall, in an attempt to provide the regulatory steps for biosynthesis of P-I, this work clearly highlights that HK, DXPS, ISPD, HMGR and PMK genes related to glycolysis, non-mevalonate and mevalonate pathways might contribute to the P-I biosynthesis for the first 20 days of plant growth with the major role of non-mevalonate pathway as compared to the mevalonate pathway. In addition, DAHPS and G-10-H related to shikimate/phenylpropanoid and iridoid pathway, respectively might be responsible for the P-I biosynthesis between 20 and 30 days of *P. kurroa* growth and thus, hinted

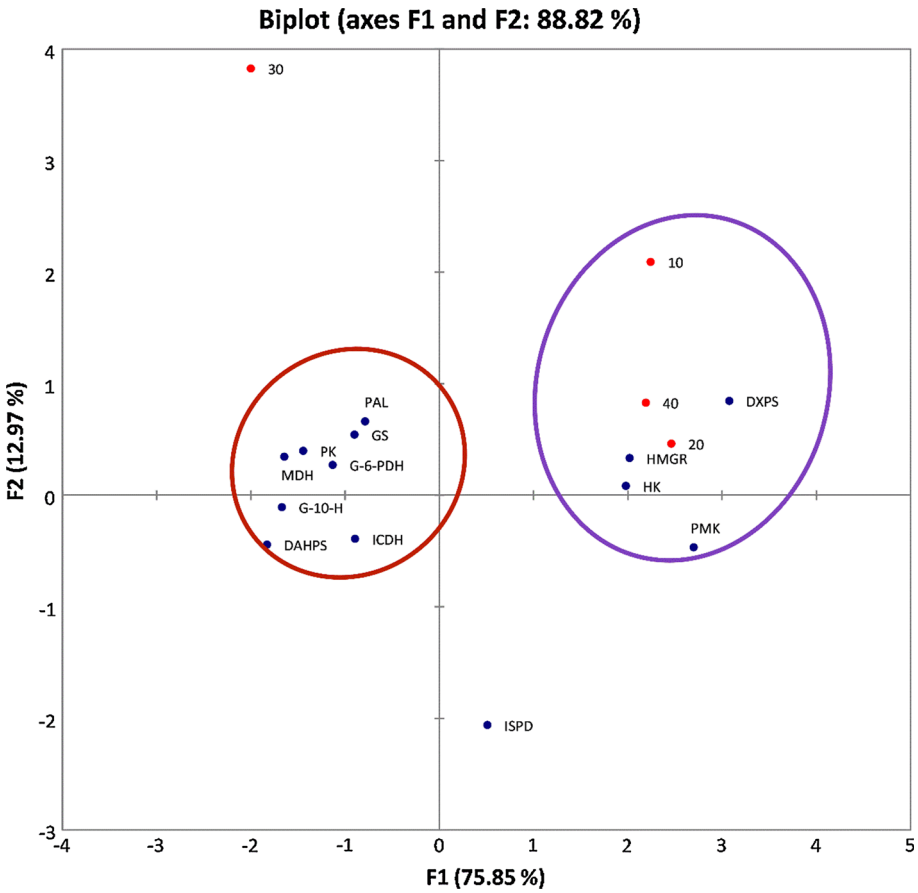

Fig. 3 Biplot (F1 and F2) showing results of the principal component analysis (PCA). The presenting data incorporated the four different conditions i.e. 10, 20, 30, and 40 days as observations in *red dots* and thirteen genes as loadings in *blue dots*. Genes loaded in *red circle* are highly correlated in terms of 30 days observation, whereas genes that are loaded in *purple circle* are highly correlated in terms of 20 days observation. (Color figure online)

as the possible regulators of P-I biosynthesis. This study provides a platform for the exploitation of geraniol-10-hydroxylase and DAHPS as regulatory steps in order to elevate the biotechnological production of P-I in *P. kurroa*.

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Author contribution statement VK, NS and HS conducted tissue culture experiments. VK and KS conducted molecular experiments. TRS conducted PCA analysis. VK, HS, CT and RSC conceived and designed research. VK, RSC, and CT analyzed data. VK, NS and CT wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

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

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