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ORIGINAL ARTICLE

Discerning picroside-I biosynthesis via molecular dissection of in vitro shoot regeneration in *Picrorhiza kurroa*

Neha Sharma[1] · Rajinder Singh Chauhan[1] · Hemant Sood[1]

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Abstract

Key message **Expression analysis of primary and secondary metabolic pathways genes vis-à-vis shoot regeneration revealed developmental regulation of picroside-I biosynthesis in *Picrorhiza kurroa*.**

Abstract Picroside-I (P-I) is an important iridoid glycoside used in several herbal formulations for treatment of various disorders. P-I is synthesized in shoots of *Picrorhiza kurroa* and *Picrorhiza scrophulariiflora*. Current study reports on understanding P-I biosynthesis in different morphogenetic stages, viz. plant segment (PS), callus initiation (CI), callus mass (CM), shoot primordia (SP), multiple shoots (MS) and fully developed (FD) stages of *P. kurroa*. Expression analysis of genes involved in primary and secondary metabolism revealed that genes encoding HMGR, PMK, DXPS, ISPE, GS, G10H, DAHPS and PAL enzymes of MVA, MEP, iridoid and shikimate/phenylpropanoid pathways showed significant modulation of expression in SP, MS and FD stages in congruence with P-I content compared to CM stage. While HK, PK, ICDH, MDH and G6PDH showed high expression in MS and FD stages of *P. kurroa*, RBA, HisK and CytO showed high expression with progress in regeneration of shoots. Quantitative expression analysis of secondary metabolism genes at two temperatures revealed that 7 genes HMGR, PMK, DXPS, GS, G10H, DAHPS and PAL showed high transcript abundance (32–87-folds) in FD stage derived from leaf and root segments at 15 °C compared to 25 °C in *P. kurroa*. Further screening of these genes at species level showed high expression pattern in *P. kurroa* (6–19-folds) vis-à-vis *P. scrophulariiflora* that was in corroboration with P-I content. Therefore, current study revealed developmental regulation of P-I biosynthesis in *P. kurroa* which would be useful in designing a suitable genetic intervention study by targeting these genes for enhancing P-I production.

Keywords *Picrorhiza kurroa* · *Picrorhiza scrophulariiflora* · Gene expression · Primary metabolism · Secondary metabolism · Morphogenetic stages · P-I

Abbreviations

26S	26S rRNA
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HMGR	3-Hydroxy-3-methylglutaryl-CoA reductase
PMK	Phosphomevalonate kinase
DXPS	1-Deoxy-D-xylulose-5-phosphate synthase
ISPD	2-C-methylerythritol 4-phosphate cytidyl transferase
ISPE	4-(Cytidine-5-diphospho)-2-C-methylerythritol kinase
GS	Geraniol synthase
G10H	Geraniol-10-hydroxylase
10-HGO	10-Hydroxygeraniol dehydrogenase
IS	Iridoid synthase
DAHPS	3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase

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✉ Hemant Sood
hemant.sood@juit.ac.in

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

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PAL	Phenylalanine ammonia lyase
HK	Hexokinase
PK	Pyruvate kinase
ICDH	Isocitrate dehydrogenase
MDH	Malate dehydrogenase
G6PDH	Glucose-6-phosphate dehydrogenase
RBA	RuBisCO activase
ARP	Auxin response protein
ARF7	Auxin response factor 7
HisK	Histidine kinase
CytO	Cytokinin oxidase
MVA	Mevalonate
MEP	Non-mevalonate
PS	Plant segment stage
CI	Callus initiation stage
CM	Callus mass stage
SP	Shoot primordia stage
MS	Multiple shoots stage
FD	Fully developed stage
MS medium	Murashige and Skoog medium
P-I	Picroside-I

Introduction

Picrorhiza kurroa Royle ex Benth and *Picrorhiza scrophulariiflora* Pennel of family Scrophulariaceae are the two endangered species of genus *Picrorhiza* which have been widely used in traditional as well as in modern medicinal system in India, China, Tibet, Nepal and Sri Lanka (Sah and Varshney 2013). Both species are rich source of picroside-I (P-I) and picroside-II (P-II)—the two major iridoid glycosides contributing to their medicinal properties like hepatoprotective, anti-malarial, anti-microbial, anti-inflammatory, anti-oxidant, anti-allergic, anti-anaphylactic, immune modulator and are also used for the treatment of fever, asthma, jaundice, leukoderma, snake bite, scorpion sting, gastrointestinal and urinary disorders (Bhandari et al. 2008; Sidiq et al. 2010; Singh and Banyal 2011; Sah and Varshney 2013).

The past decade has seen research focused towards *P. kurroa* in a large number of studies due to its economic as well as therapeutic importance than *P. scrophulariiflora*. *P. kurroa* is one of the top 15 plant species traded in India (Shitiz et al. 2013). It is used in a number of commercially available drug formulations like livocare, livomap, livplus, katuki, arogya, etc. for various ailments (Bhandari et al. 2008). The biosynthesis of P-I occurs in shoots (Sood and Chauhan 2010; Pandit et al. 2013a), therefore, many studies including micropropagation (Sood and Chauhan 2009, 2010; Patial et al. 2012), effects of light and temperature (Kawoosa et al. 2010), use of elicitors (Sharma et al. 2015), etc., have been taken up to increase P-I content in shoots but limited success has been achieved under in vitro conditions. Secondary metabolites are produced in small amounts in plants naturally, therefore, understanding the molecular basis of their biosynthesis is prerequisite for the metabolic engineering of iridoid glycosides in *P. kurroa*. P-I is a monoterpenoid glycoside derived from combined biosynthetic route involving plastidic non-mevalonate (MEP), cytosolic mevalonate (MVA), phenylpropanoid and iridoid pathways (Kumar et al. 2013; Singh et al. 2013; Shitiz et al. 2015). Recently, studies related to the involvement of primary metabolic enzymes with the picrosides levels and modulation of gene expression level in subcultured shoots of *P. kurroa* at 0–40 days have been carried out (Kumar et al. 2015b, c), but complete overview of P-I biosynthesis has not been reported. Therefore, it is necessary to understand the dynamics of P-I biosynthesis at different developmental stages of *P. kurroa*.

The environmental factors such as high altitude and seasonal variations complicate the process of understanding biology of P-I biosynthesis in *P. kurroa*; hence, cell cultures offer a suitable biological system under homeostasis wherein the morphogenetic events can be regulated by manipulating the levels of growth hormones in the nutrient medium for identification of genes responsible for P-I biosynthesis at different developmental stages. The P-I biosynthesis occur in differentiated tissue (shoot) rather than the undifferentiated mass of cells suggesting stages of growth and development in cell cultures playing imperative role in the regulation of biosynthetic pathways (Sood and Chauhan 2010). The morphogenetic event and developmental fate of regenerating tissue can provide the clear picture of P-I biosynthesis. Till date, there is no report of expression analysis of genes involved in primary and secondary metabolism for P-I biosynthesis in different morphogenetic stages of *P. kurroa*. Moreover, many studies have limited their interest towards *P. kurroa*, thereby neglecting the species diversity and leaving *P. scrophulariiflora* unexplored. Studies on micropropagation, somatic embryogenesis and organogenesis have been reported (Bantawa et al. 2010, 2011), but very limited progress has been made for extracting, enhancing and understanding P-I biosynthesis in *P. scrophulariiflora.*

Therefore, current study reports identification of genes responsible for P-I biosynthesis in different developmental stages of *P. kurroa* beginning from plant segment (leaf or root segments) of in vitro grown plants through de-differentiation of these segments into callus mass and then re-differentiation of callus into shoot primordia and fully developed shoots under tissue culture conditions. Furthermore, effect of temperature and plant segments were also studied to ascertain the role of key genes involved in P-I biosynthesis at different developmental stages. This is the

first report where *P. kurroa* and *P. scrophulariiflora* have been compared at molecular level for P-I biosynthesis.

Materials and methods

Plant material

Picrorhiza kurroa and *P. scrophulariiflora* were procured from Himalayan Forest Research Institute, Jagatsukh, Manali, H.P., India and National Bureau of Plant Genetic Resources, New Delhi, India, respectively. Shoot apices were cultured in an optimized Murashige and Skoog medium (MS medium) (Murashige and Skoog 1962) supplemented with indole-3-butyric acid (IBA) (3 mg/L), kinetin (KN) (1 mg/L), sucrose (30 g/L) and agar (9 g/L). The cultures were maintained in plant tissue culture chambers at 25 ± 2 and 15 ± 2 °C with 70 % relative humidity, 16 h photoperiod at photosynthetic photon flux density of 40 μmol/m^2/s provided by cool white fluorescent tubes (Philips, India) with subculturing after every 4 weeks (Sood and Chauhan 2009).

Callus induction

Leaf and root segments (0.5–1 cm each) from 4 to 5 weeks old in vitro grown *P. kurroa* and *P. scrophulariiflora* cultures were used for callus induction. MS media having different growth hormone combinations, sucrose (30 g/L) and agar (9 g/L) were prepared. The pH was adjusted to 5.7 using 0.1 N HCl or 0.1 N NaOH prior to the addition of agar and autoclaved at 121 °C, 15 lb/$inch^2$ pressure for 20 min. Different growth hormones used were: 2, 4-dichlorophenoxy acetic acid (2, 4-D) (0.50, 1.00, 1.50, 2.00 mg/L) and/or IBA (0.50, 1.00, 1.50, 2.00 mg/L) and thidiazuron (TDZ) (0.25, 0.50 and 1.00 mg/L). In all the experiments, 5–6 leaf (abaxial side touching the medium) and root segments were taken from both plant species maintained in two plant growth chambers at 25 ± 2 and 15 ± 2 °C incubation temperatures. These plant segments were inoculated on different media combinations at aforementioned incubation temperatures, respectively for 4 weeks. Medium without growth hormones served as control. Callus initiation started in TDZ containing media within 10–15 days and callus mass was achieved in next 15 days for both species at 25 ± 2 and 15 ± 2 °C. Cultures were further transferred to shoot induction media. The experiment was performed in triplicates and repeated thrice. Samples of both species at 25 ± 2 and 15 ± 2 °C for different stages viz. plant segment representing leaf and root segments (PS), callus initiation (CI) and callus mass (CM) stages were frozen and kept in −80 °C for further experimentation.

Plant regeneration

The calli initiated from leaf and root segments at 25 ± 2 and 15 ± 2 °C from both species were then transferred to MS media supplemented with IBA (1.0, 2.0 mg/L) and/or 6-benzylaminopurine (BAP) (0.5, 2.0 mg/L) and/or KN (0.2, 0.5, 1.0, 2.0, 3.0 mg/L) for regeneration and multiple shoot formation. Sucrose (30 g/L) and agar (9 g/L) were invariably added to the media. Cultures were incubated at same culture conditions as described earlier for 4–6 weeks. Medium without growth hormones served as control. Regenerated multiple shoots were then transferred to MS medium supplemented with IBA (3 mg/L), KN (1 mg/L), sucrose (30 g/L) and agar (9 g/L) for shoot elongation, root induction and full growth of both plant species. The experiment was performed in triplicates and repeated thrice. Samples of both species at 25 ± 2 and 15 ± 2 °C for different stages viz. shoot primordia (SP), multiple shoots (MS) and fully developed (FD) stages were frozen and kept in −80 °C for further experimentation.

Quantification of P-I by HPLC

Samples from all stages derived from leaf and root segments of *P. kurroa* and *P. scrophulariiflora* at 25 ± 2 and 15 ± 2 °C were subjected to P-I estimation. Samples were ground in liquid nitrogen and 100 mg of powdered sample was percolated in 10 mL 80 % methanol. They were further vortexed, sonicated for 30 min at room temperature and filtered through 0.22 μm filter (Millipore). The filtrate was diluted 1:10 for estimation of P-I content using RP-HPLC following the method described by Sood and Chauhan (2010). The experiment was performed in triplicates. The significant differences between replicates were statistically evaluated by standard deviation.

Selection of genes

Total 21 genes were selected on the basis of literature survey and/or their role as rate limiting enzymes in primary and secondary metabolic pathways (Supplementary Table 1). Various genes encoding enzymes, including 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), phosphomevalonate kinase (PMK) of MVA pathway; 1-deoxy-D-xylulose-5-phosphate synthase (DXPS), 2-C-methylerythritol 4-phosphate cytidyl transferase (ISPD), 4-(cytidine-5-diphospho)-2-C-methylerythritol kinase (ISPE) of MEP pathway; geraniol synthase (GS), geraniol-10-hydroxylase (G10H), 10-hydroxygeraniol dehydrogenase (10-HGO), iridoid synthase (IS) of iridoid pathway and 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS), phenylalanine ammonia lyase (PAL) of phenylpropanoid

pathway were selected to analyze their expression in *P. kurroa* and *P. scrophulariiflora* for P-I production in different developmental stages (Fig. 1). In addition, various genes encoding the enzymes of primary metabolic pathways including hexokinase (HK), pyruvate kinase (PK) of glycolysis, isocitrate dehydrogenase (ICDH), malate dehydrogenase (MDH) of tricarboxylic acid (TCA) cycle and glucose-6-phosphate

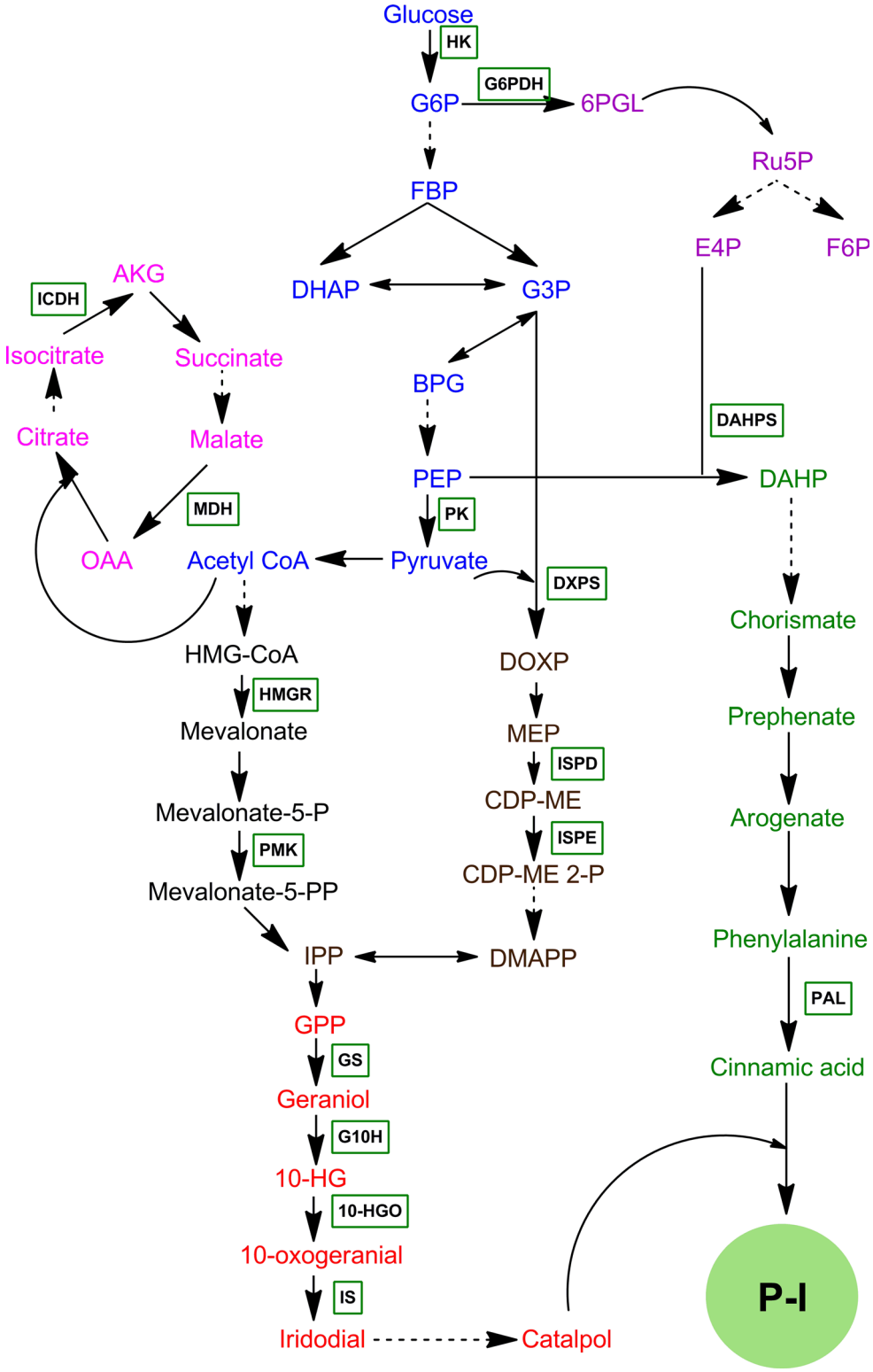


Fig. 1 Linking of primary and secondary metabolic pathways integrating to P-I biosynthesis. The metabolic network has been reconstructed by including glycolysis (*blue color*), TCA cycle (*pink color*), pentose phosphate pathway (*purple color*), MVA (*black color*), MEP (*brown color*), shikimate/phenylpropanoid (*green color*) and iridoid pathways (*red color*). Single and multiple steps were indicated by *solid* and *dotted lines*, respectively. Abbreviations have been elaborated in Supplementary Table 3 (color figure online)

dehydrogenase (G6PDH) of pentose phosphate pathway were selected to monitor their expression on tissues of different developmental stages of *P. kurroa* derived from leaf segments at 15 ± 2 °C for their involvement in P-I production (Fig. 1). Additionally, RuBisCO activase (RBA), auxin response protein (ARP), auxin response factor 7 (ARF7), histidine kinase (HisK) and cytokinin oxidase (CytO) were also selected to study the different developmental stages of *P. kurroa* under in vitro conditions.

RNA isolation and cDNA synthesis

Total RNA was isolated using TRIzol® Reagent (Life Technologies, USA) according to the manufacturer's instructions and quality was assessed in 1 % (w/v) ethidium bromide-stained agarose gel. RNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). cDNA synthesis was done using Verso cDNA synthesis kit (Thermo Scientific) from total RNA (1 µg) as per manufacturer's instructions. Concentration of each cDNA sample was adjusted to 100 ng/µL for expression analysis.

Quantitative real time-PCR (qRT-PCR) analysis

Primer pairs for HMGR, PMK, DXPS, ISPD, ISPE, HK, PK, GS, G10H, 10-HGO, IS, DAHPS, PAL, MDH, ICDH and G6PDH were procured from Pandit et al. (2013b), Kumar et al. (2015b) and Shitiz et al. (2015) while RBA, ARP, ARF7, HisK and CytO genes primers were designed from transcriptomic sequences of *P. kurroa* (data not published) using Primer3 software (Koressaar and Remm 2007) (Supplementary Table 2). The reaction was performed in triplicates on CFX96 system (Bio-Rad Laboratories, Hercules CA, USA) with protocol: denaturation for 5 min at 94 °C, followed by 40 cycles each of denaturation for 20 s at 94 °C, annealing for 30 s at 48–60 °C, followed by one elongation step for 20 s at 72 °C. The housekeeping genes, 26S and GAPDH were used as the internal controls for calculating transcript abundance. The significant differences between replicates were statistically evaluated by standard deviation in the form of error bars as mean ± SD for data recorded in triplicates (repeated thrice).

Statistical analysis

The expression analysis of the selected genes of primary and secondary metabolic pathways in different stages viz. PS, CI, CM, SP, MS and FD stages was done in triplicates and demonstrated by heat map. The heat map was generated using GenEx software (V 1.1).

Results and discussion

Callus initiation and shoot regeneration

Different developmental stages passing through different morphogenetic events wherein de-differentiation of leaf and root segments into callus mass followed by re-differentiation of callus into shoot primordia and fully developed plant were obtained using tissue culture techniques. In this study, callus initiation was observed in both leaf and root segments of *P. kurroa* while only leaf segments of *P. scrophulariiflora* responded to callus induction in all the tested media combinations at 25 ± 2 and 15 ± 2 °C. This might be attributed to the internal status of plant growth regulators towards different plant species and plant segments under same in vitro conditions. Growth hormones like 2, 4-D, IBA, NAA and BAP have already been reported for callus initiation in *P. kurroa* and *P. scrophulariiflora* (Sood and Chauhan 2009; Bantawa et al. 2011). Our results showed creamish and friable calli on MS media supplemented with different concentrations and combinations of 2, 4-D and/or IBA while greenish and friable calli were obtained in both species at 25 ± 2 and 15 ± 2 °C on MS media containing TDZ (0.5 mg/L). Callus initiation was not observed in leaf and root segments of both species on MS basal media without any growth hormone at 25 ± 2 and 15 ± 2 °C. Leaf and root segments of *P. kurroa* showed callus initiation within 10–12 and 12–15 days at 15 ± 2 and 25 ± 2 °C, respectively (Fig. 2), while *P. scrophulariiflora* leaf segments showed callus initiation in 14–15 days at 25 ± 2 and 15 ± 2 °C (Fig. 3). Further, callus mass was observed in next 10–15 days in both species at 25 ± 2 and 15 ± 2 °C on MS media supplemented with TDZ (0.5 mg/L). TDZ has been previously reported for callus initiation and plant regeneration in various plants species like *P. kurroa*, *Scutellaria baicalensis* and *Linum usitatissimum* L. by regulating the morphogenetic systems depending upon its exposure time (Zhang et al. 2005; Rosu et al. 2010; Guo et al. 2011; Patial et al. 2012).

These calli were then transferred to shoot regeneration media containing MS basal media with different concentrations and combinations of IBA, BAP and KN. KN (0.5 mg/L) was found to be best for shoot primordia formation in 15–20 days followed by multiple shoot formation within next 10–15 days in both species at 25 ± 2 and 15 ± 2 °C. Lower concentration of KN was found better for shoot initiation and multiple shoot formation. Patial et al. (2012) have also suggested the use of KN at low concentration for shoot initiation from in vitro grown leaves of *P. kurroa*. These multiple shoots were then

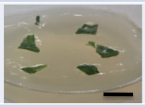
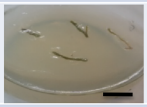
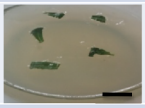
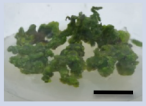
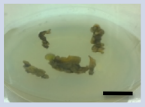
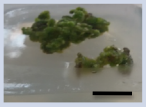
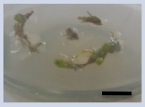
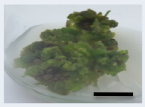
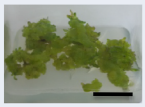
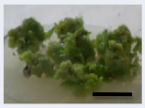
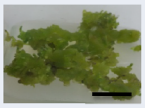
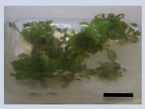
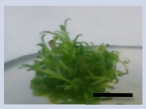
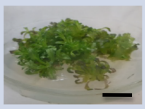
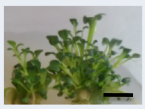
S. No.	Developmental stage	25^{0}C		15^{0}C	
		Leaf segment	Root segment	Leaf segment	Root segment
1	Plant segment (PS) (0 day) (TDZ = 0.5 mg/L)				
2	Initiation of callus formation (CI) (1-15 days) (TDZ = 0.5 mg/L)				
3	Callus mass formation (CM) (16-30 days) (TDZ = 0.5 mg/L)				
4	Shoot primordia formation (SP) (31-50 days) (KN = 0.5 mg/L)				
5	Multiple shoot formation (MS) (51-65 days) (KN = 0.5 mg/L)				
6	Shoot elongation, growth and full development (FD) (66-80 days) (IBA = 3mg/L + KN = 1 mg/L)				

Fig. 2 Different developmental stages of *P. kurroa* at 25 ± 2 and 15 ± 2 °C on MS media containing different growth hormones: leaf and root segments on TDZ = 0.5 mg/L represent PS stage (*1*); callus initiation and callus mass formation on TDZ = 0.5 mg/L represent CI and CM stages, respectively (*2* and *3*); shoot primordia and multiple shoot formation on KN = 0.5 mg/L represent SP and MS stages, respectively (*4* and *5*); shoot elongation, full growth and development on IBA = 3 mg/L and KN = 1 mg/L represent FD stage (*6*) (*scale bar* = 1 cm)

transferred to rooting medium viz. MS basal medium supplemented with IBA (3 mg/L) and KN (1 mg/L) since rooting is required for plant development. Fully developed plants with rooting were obtained within next 15–20 days. Leaves of fully developed plants of both species were found to be thicker and longer at 15 ± 2 °C compared to 25 ± 2 °C. This could be attributed to the accumulation of hemicelluloses at low temperature which causes thickening of leaves, thereby increasing plant strength (Patial et al. 2012).

Expression analysis of pathways genes vis-à-vis P-I biosynthesis in different developmental stages

To study the molecular basis of P-I biosynthesis, various genes of primary and secondary metabolic pathways were selected and analyzed for modulation of their expression vis-à-vis P-I content at different developmental stages. The amount of P-I reduced from 2.51 µg/mg to non-detectable level during de-differentiation in CI and CM stages obtained from leaf segments of *P. kurroa* grown at 15 ± 2 °C. With the progress of re-differentiation, levels of P-I increased consistently during SP, MS and FD stages of *P. kurroa* (Fig. 4). Our results were found to be in congruence with study of Sood and Chauhan (2010) which highlighted the site of P-I biosynthesis in in vitro grown shoots rather than undifferentiated callus cultures which could be attributed to the absence of cell programming machinery in callus cultures for biosynthesis of metabolites in *P. kurroa*.

The information on expression pattern of genes contributing to P-I biosynthesis at different developmental stages is a pre-requisite for understanding the metabolic basis of P-I biosynthesis. Results revealed that out of 11 genes of MVA, MEP, iridoid and shikimate/phenylpropanoid pathways, genes encoding enzymes HMGR,

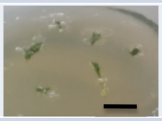
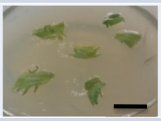
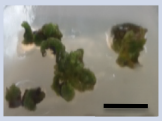
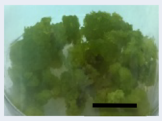
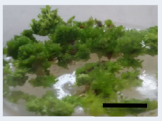
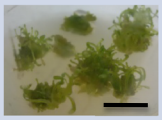
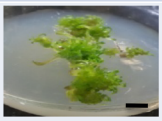
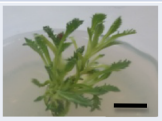
S. No.	Developmental stage	Leaf segment	
		25^{0}C	15^{0}C
1	Plant segment (PS) (0 day) (TDZ = 0.5 mg/L)		
2	Initiation of callus formation (CI) (1-15 days) (TDZ = 0.5 mg/L)		
3	Callus mass formation (CM) (16-30 days) (TDZ = 0.5 mg/L)		
4	Shoot primordia formation (SP) (31-50 days) (KN = 0.5 mg/L)		
5	Multiple shoot formation (MS) (51-65 days) (KN = 0.5 mg/L)		
6	Shoot elongation, growth and full development (FD) (66-80 days) (IBA = 3mg/L + KN = 1 mg/L)		

Fig. 3 Different developmental stages of *P. scrophulariiflora* at 25 ± 2 and 15 ± 2 °C on MS media containing different growth hormones: leaf segments on TDZ = 0.5 mg/L represent PS stage (*1*); callus initiation and callus mass formation on TDZ = 0.5 mg/L represent CI and CM stages, respectively (*2* and *3*); shoot primordia and multiple shoot formation on KN = 0.5 mg/L represent SP and MS stages, respectively (*4* and *5*); shoot elongation, full growth and development on IBA = 3 mg/L and KN = 1 mg/L represent FD stage (*6*) (*scale bar* = 1 cm)

PMK, DXPS, ISPE, GS, G10H, DAHPS and PAL showed significant modulation of expression in agreement with P-I content at different morphogenetic stages in comparison to CM stage. At day 0, these genes showed 16–47 folds transcript abundance in PS stage, which declined to 0–3 folds by the end of day 50 corresponding to CI and CM stages of *P. kurroa* (Fig. 5). This might be due to the de-differentiation of leaf segments into callus mass or loss of organogenesis which decreased the expression of genes involved in secondary metabolism, leading to non-detectable amount of P-I in CI and CM stages. Conner (1987) also reported low level of alkaloid in *Solanum laciniatum* due to alteration in gene expression level in undifferentiated cells which was attributed to their heterotrophic mode of nutrition. Further, as re-differentiation progresses, HMGR, DXPS, ISPE and G10H showed 2–7 folds increase while DAHPS showed marked elevation of 28 fold during SP stage. Formation of shoot primordia might lead to activation of important regulatory enzymes of all four biosynthetic pathways involved in P-I production. HMGR and DXPS are the rate limiting enzymes of MVA and MEP pathways, which along with ISPE, have been reported to be involved in P-I biosynthesis (Kawoosa et al. 2010; Pandit et al. 2013b; Bhat et al. 2014). G10H and DAHPS have also been reported as key regulatory enzymes for secondary metabolism in Arabidopsis and *Catharanthus roseus* (Tzin et al. 2012; Cui et al. 2015). Increased P-I content (1.55 µg/mg) was observed in MS stage between

Duration (Days)	*P. kurroa*				*P. kurroa / P. scrophulariiflora*	*P. scrophulariiflora*	
	15⁰C		25⁰C			15⁰C	25⁰C
	L	R	L	R		L	L
0	2.51± 0.11	0.00±0.00	0.34±0.05	0.00±0.00	Plant segment	2.12±0.09	0.23±0.06
					TDZ=0.5 mg/L		
1-15	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	Initiation of callus formation	0.00±0.00	0.00±0.00
					TDZ=0.5 mg/L		
16-30	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	Callus mass formation	0.00±0.00	0.00±0.00
					KN=0.5 mg/L		
31-50	0.42±0.09	0.31±0.04	0.00±0.00	0.00±0.00	Shoot primordia formation	0.21±0.04	0.00±0.00
					KN=0.5 mg/L		
51-65	1.55±0.11	1.21±0.07	0.30±0.04	0.22±0.01	Multiple shoot formation	0.63±0.08	0.13±0.02
					IBA=3 mg/L KN=1 mg/L		
66-80	3.65±0.13	2.87±0.09	0.44±0.05	0.31±0.03	Shoot elongation, rooting and full growth	2.35±0.09	0.39±0.02

Fig. 4 P-I content (μg/mg) in different developmental stages of *P. kurroa* and *P. scrophulariiflora* obtained from leaf (L) and root (R) segments at 15 ± 2 and 25 ± 2 °C. Data has been recorded in triplicates (repeated thrice) and represented as mean ± SD

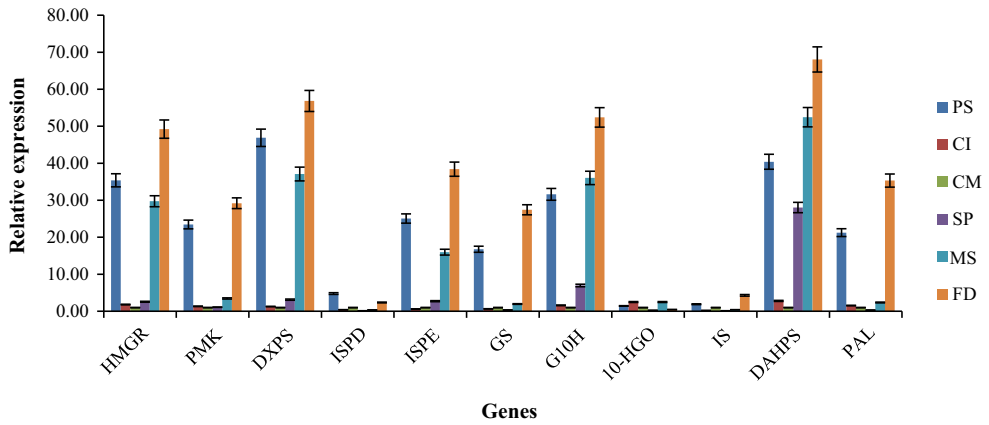


Fig. 5 Expression pattern of secondary metabolic pathways genes in different developmental stages of *P. kurroa* obtained from leaf segments at 15 ± 2 °C. These include: plant segment (PS), callus initiation (CI), callus mass formation (CM), shoot primordia formation (SP), multiple shoot formation (MS) and fully developed plant (FD) stages. Transcript abundance has been normalized with average value of 26S and GAPDH housekeeping genes that served as internal controls. Relative expression of genes in different developmental stages was calculated in relation to CM stage. *Error bars* represent mean ± SD for data recorded in triplicates (repeated thrice)

day 51–65 due to elevated expression levels of genes encoding enzymes HMGR, DXPS, ISPE, G10H and DAHPS, showing 15–52 folds high expression in MS stage compared to CM stage. Upregulation of HMGR, DXPS and ISPE might be associated with the supply of GPP, which acts as precursor for iridoid biosynthesis (Qu et al. 2015). Pandit et al. (2013b) have also reported the positive correlation of HMGR, DXPS and ISPE enzymes of MVA and MEP pathways with P-I biosynthesis in *P. kurroa*. Higher expression of G10H and DAHPS demonstrated their possible roles in activating iridoid and shikimate/phenylpropanoid pathways (Gahlan et al. 2012; Kumar et al. 2013). Finally, a drastic increase in expression of genes encoding HMGR, PMK, DXPS, ISPE, GS, G10H, DAHPS and PAL from 27–68 folds vis-à-vis P-I production was observed in fully developed *P. kurroa* shoots in FD stage obtained between day 66–80 (Fig. 5). In addition to genes elevated in MS stage, genes such as PMK, GS and PAL showed high expression in FD stage compared to CM stage. GS has been reported to initiate monoterpenoid branch of monoterpene indole alkaloid (MIA) pathway in *C. roseus* (Simkin et al. 2013), while PAL acts as an

important regulatory enzyme of shikimate/phenylpropanoid pathway (Klee et al. 1987; Herrmann 1995; Bauer et al. 2011). High expression of these genes in FD stage suggested their possible role in the accumulation of P-I in *P. kurroa*. Therefore, genes encoding HMGR, PMK, DXPS, ISPE, GS, G10H, DAHPS and PAL enzymes of all four pathways seemed to be associated with P-I biosynthesis in *P. kurroa*. Previous studies have suggested the involvement of multiple genes of biosynthetic pathways in terpenoid biosynthesis such as artemisinin in *Artemisia annua* (Olofsson et al. 2011), MIAs in *C. roseus* (Peebles et al. 2011) and lignin biosynthesis in *Arabidopsis thaliana* (Ali and McNear 2014).

P-I biosynthesis increased with developmental stages of shoot formation starting from SP to FD stages; therefore, identification and expression analysis of genes involved in shoot development vis-à-vis P-I biosynthesis was important to understand the link between primary and secondary metabolic pathways. Secondary metabolic pathways are dependent on primary metabolic pathways for the supply of precursors (Kumar et al. 2015b, c). Tzin et al. (2012) have shown that DAHPS enzyme of shikimate pathway bridged plant primary and secondary metabolism by conversion of primary carbon metabolites via chorismate to various secondary metabolites such as alkaloids, flavonoids, lignin and other phenolic compounds. So, expression analysis of 10 selected enzymes viz. HK, PK, ICDH, MDH, G6PDH, RBA, ARP, ARF7, HisK and CytO was studied and correlated with P-I content in different developmental stages of *P. kurroa* as compared to CM stage. Results revealed that genes of glycolysis, TCA cycle and pentose phosphate pathway involved in primary metabolism viz. HK, PK, ICDH, MDH and G6PDH showed 2–5 folds and 5–11 folds transcript abundance in MS and FD stages, respectively while marginal fold variation was observed between CI, CM and SP stages (Fig. 6). The expression of these genes showed positive correlation with shoot development and P-I biosynthesis in different developmental stages of *P. kurroa*. HK and PK are the rate limiting enzymes of glycolysis and elevation in expression of HK gene might lead to higher levels of G6P, the substrate for G6PDH, resulting in activation of the pentose phosphate pathway (Bolaños et al. 2008). Wakao et al. (2008) showed the importance of glycolysis in seed oil biosynthesis and also reported increase in supply of precursors for fatty acid biosynthesis by increasing the glycolytic flux in Arabidopsis. Increased transcript levels of ICDH and MDH might activate the TCA cycle and could produce higher concentrations of their intermediates. Therefore, elevation in the transcript level of these genes during MS and FD stages resulted in the accumulation of precursors required for plant growth and P-I biosynthesis. Bouthour et al. (2012) also showed the role of ICDH and MDH in controlling TCA cycle, thereby regulating growth and developmental processes. Kumar et al. (2015c) also studied the catalytic activities and gene expression profiles of HK, PK, ICDH and MDH enzymes which were in congruence with P-I content under different conditions of *P. kurroa* plants.

Further, to get an insight into the molecular basis of regeneration of shoots from callus mass, quantitative expression of RBA, ARP, ARF7, HisK and CytO genes was studied in different developmental stages of *P. kurroa*. qRT-PCR analysis revealed that RBA, HisK and CytO transcript levels increased constantly with the progress of shoot development, while ARP and ARF7 showed decreased transcript levels in SP, MS and FD stages compared to CM stage. RBA, HisK and CytO showed 2–4, 6–9 and 7–12 folds increased expression in SP, MS and FD stages, respectively (Fig. 6). This might be due to the involvement of these genes in shoot development and P-I biosynthesis in *P. kurroa* since RBA is involved in photosynthetic function, HisK serve as cytokinin receptor and CytO is responsible for cytokinin

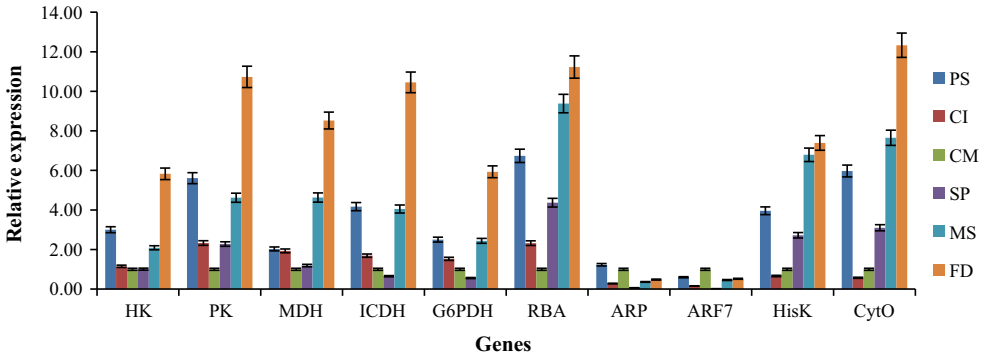


Fig. 6 Expression pattern of primary metabolic pathways genes in different developmental stages of *P. kurroa* obtained from leaf segments at 15 ± 2 °C. These include: plant segment (PS), callus initiation (CI), callus mass formation (CM), shoot primordia formation (SP), multiple shoot formation (MS) and fully developed plant (FD) stages. Transcript abundance has been normalized with average value of 26S and GAPDH housekeeping genes that served as internal controls. Relative expression of genes in different developmental stages was calculated in relation to CM stage. *Error bars* represent mean ± SD for data recorded in triplicates (repeated thrice)

catabolism, thereby contributing to the regulation of cytokinin-dependent processes (Schmülling et al. 2003; Che et al. 2006; Lee et al. 2014). Similarly, Che et al. (2002) also showed the upregulation of photosynthetic genes and histidine kinases during shoot commitment in shoot induction media. Matt et al. (2002) have showed that levels of chlorogenic acid and nicotine reduced with drop in RuBisCO activity leading to decreased levels of primary metabolites, thus hampering the content of secondary metabolites. Lloyd and Zakhleniuk (2004) have showed the role of primary metabolites in regulating secondary metabolism by studying the transcript levels of genes encoding enzymes and regulatory proteins involved in primary carbon assimilation in mature rosette leaves of wild-type and mutant Arabidopsis plants. Henkes et al. (2001) have also showed that reduction in the oxidative pentose phosphate pathway enzymes affected metabolic flux involved in shikimate and phenylpropanoid pathways. Hence, RBA, HisK and CytO genes were found to be involved in shoot development of *P. kurroa*.

Overall, multiple genes of secondary metabolic pathways showed higher expression in different developmental stages of *P. kurroa* compared to those from primary metabolic pathways showing slightly higher expression for P-I biosynthesis. A representative heat map highlighted the involvement of secondary metabolic pathways genes such as HMGR, PMK, DXPS, ISPE, GS, G10H, DAHPS and PAL for P-I biosynthesis in FD stage of *P. kurroa* obtained from leaf segments at 15 °C (Fig. 7).

Effect of temperature, plant segments and species on P-I biosynthesis

Secondary metabolism in plants is influenced by various factors such as temperature, plant segments, species etc. (Ramakrishna and Ravishankar, 2011; Zhang et al. 2011; Kumar et al. 2015a). Previous studies on *P. kurroa* showed higher P-I content in in vitro grown shoots at 15 °C than 25 °C while shoots regenerated from leaf segments accumulated higher P-I content compared to stem and root segments (Sood and Chauhan 2010; Kawoosa et al. 2010). These reports suggested optimal growth conditions for enhanced P-I biosynthesis but mechanism underlying their effects is not clear. Therefore, effect of temperature, plant segments and species vis-à-vis transcript abundance of P-I biosynthetic pathway genes was studied in different morphogenetic stages of *P. kurroa*.

Higher P-I content was observed in *P. kurroa* shoots developed from leaf (3.65 µg/mg) and root (2.87 µg/mg) segments at 15 °C compared to 25 °C (negligible amount) (Fig. 4). Seven out of 11 genes showed 32–87 folds expression in fully developed shoots originated from leaf and root segments at 15 °C compared to 25 °C. In FD stage, shoots regenerated from leaf derived callus showed elevation in transcript levels of HMGR, PMK, DXPS, GS, G10H, DAHPS and PAL with 47, 57, 87, 52, 77, 59 and 79 folds, respectively at 15 °C compared to 25 °C (Fig. 8). Similarly, shoots developed from root derived callus showed an increase of 45, 44, 71, 32, 53, 48 and 57 folds in genes encoding HMGR, PMK, DXPS, GS, G10H, DAHPS and PAL, respectively at 15 °C compared to 25 °C (Fig. 9). These results revealed that development of shoots at low temperature up-regulated the expression of genes involved in secondary metabolism leading to enhanced P-I accumulation in *P. kurroa*. Hannah et al. (2005) also showed that low temperature increased the secondary metabolite production in Arabidopsis by transcriptional up-regulation of genes involved in secondary metabolism.

Plant tissues respond variably from each other for organogenesis on similar combinations of growth regulators (Hosokawa et al. 1996; Mikula and Rybczynski 2001;

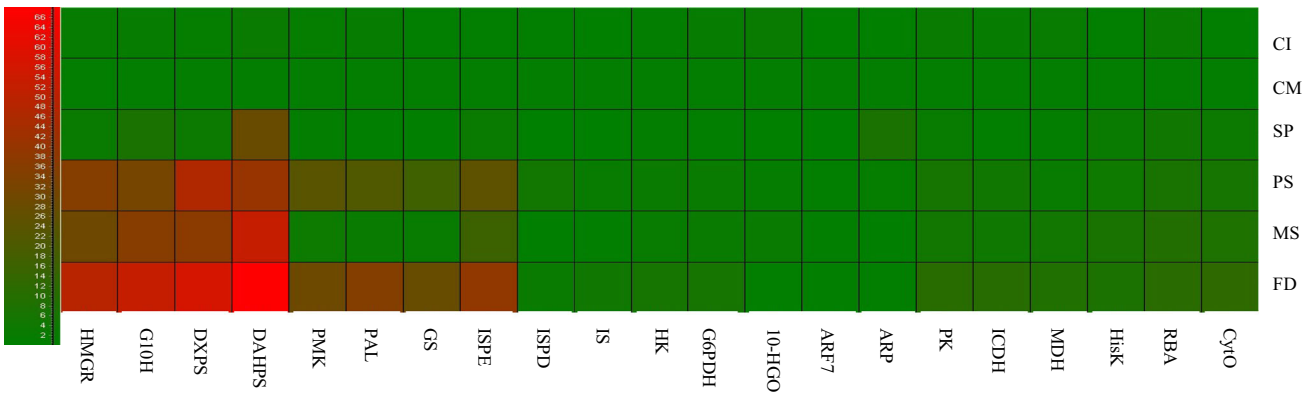


Fig. 7 A representative heat map demonstrating the differential expression pattern of genes involved in primary and secondary metabolic pathways at different developmental stages of *P. kurroa* obtained from leaf segments at 15 ± 2 °C. These include: plant segment (PS), callus initiation (CI), callus mass formation (CM), shoot primordia formation (SP), multiple shoot formation (MS) and fully developed plant (FD) stages

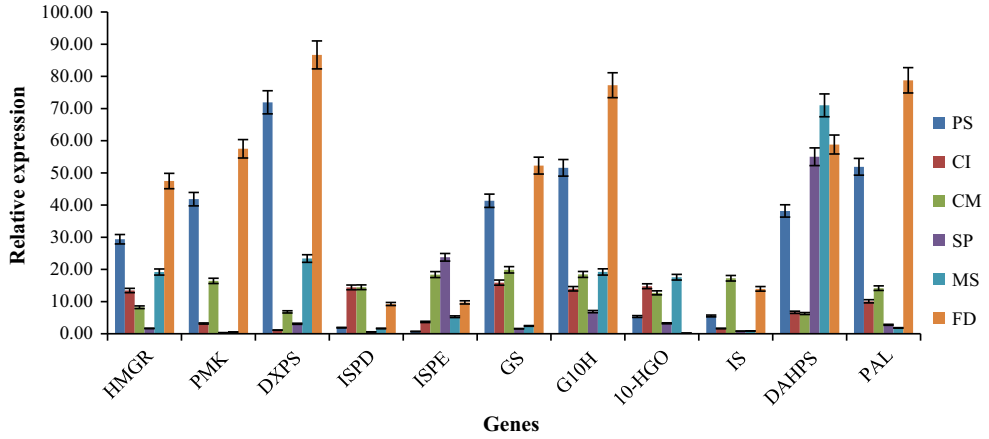


Fig. 8 Effect of temperature on transcript levels of selected genes of MVA, MEP, iridoid and shikimate/phenylpropanoid pathways at different developmental stages of *P. kurroa* derived from leaf segments at 15 ± 2 and 25 ± 2 °C. These include: plant segment (PS), callus initiation (CI), callus mass formation (CM), shoot primordia formation (SP), multiple shoot formation (MS) and fully developed plant (FD) stages. Transcript abundance has been normalized with average value of 26S and GAPDH housekeeping genes that served as internal controls. Relative expression of genes was calculated by comparing the transcript abundance of genes in different developmental stages at 15 ± 2 °C with 25 ± 2 °C. *Error bars* represent mean ± SD for data recorded in triplicates (repeated thrice)

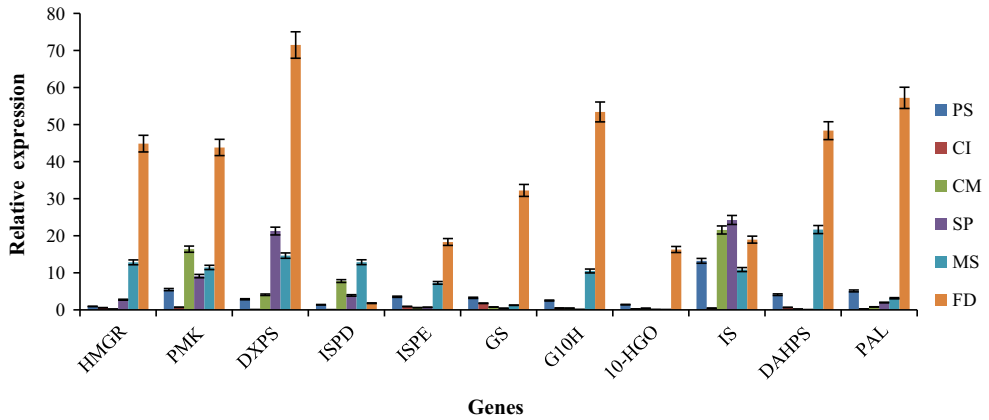


Fig. 9 Effect of temperature on transcript levels of selected genes of MVA, MEP, iridoid and shikimate/phenylpropanoid pathways at different developmental stages of *P. kurroa* derived from root segments at 15 ± 2 and 25 ± 2 °C. These include: plant segment (PS), callus initiation (CI), callus mass formation (CM), shoot primordia formation (SP), multiple shoot formation (MS) and fully developed plant (FD) stages. Transcript abundance has been normalized with average value of 26S and GAPDH housekeeping genes that served as internal controls. Relative expression of genes was calculated by comparing the transcript abundance of genes in different developmental stages at 15 ± 2 °C with 25 ± 2 °C. *Error bars* represent mean ± SD for data recorded in triplicates (repeated thrice)

Sharma et al. 2014a), thereby influencing the metabolite production during shoot regeneration. Our results showed marginal variation in P-I content between shoots derived from different plant segments viz. 3.65 and 2.87 µg/mg P-I content in shoots developed from leaf and root segments, respectively at 15 °C (Fig. 4). So, to study the effect of plant segments on P-I production at molecular level, expression analysis was carried out in different morphogenetic stages derived from leaf and root segments at 15 °C. Out of 11 genes, 7 genes encoding HMGR, PMK, DXPS, GS, G10H, DAHPS and PAL showed 4–9 and 2–8 folds elevated expression in leaf segment derived CI and CM stages, respectively compared to root segment derived stages which could be correlated to biosynthesis of P-I in leaf tissue (Fig. 10). Expression levels remained unaltered in SP, MS and FD stages as same machinery was activated for P-I biosynthesis in the shoots developed from leaf and root segments. Kurz and Constabel (1985) also suggested that origin of tissue become irrelevant after de-differentiation, and subsequent regenerated plants inherit physiological capability of the source plant to express all the biosynthetic pathways under permissive conditions.

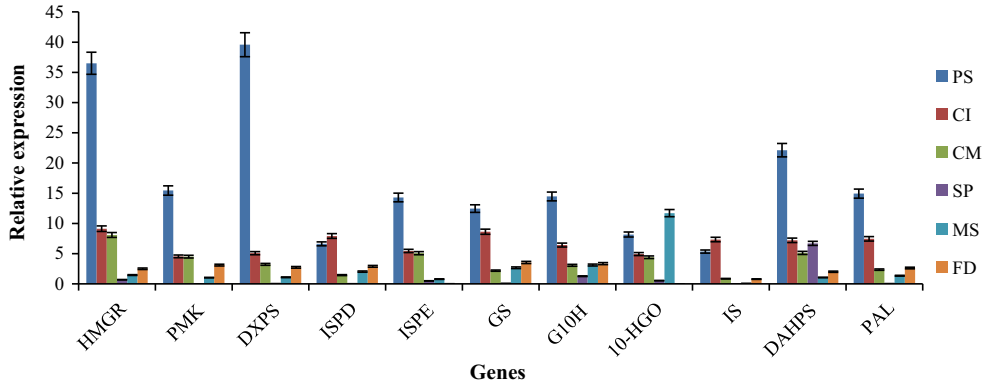


Fig. 10 Effect of different plant segments on transcript levels of selected genes of MVA, MEP, iridoid and shikimate/phenylpropanoid pathways at different developmental stages of *P. kurroa* at 15 ± 2 °C. These include: plant segment (PS), callus initiation (CI), callus mass formation (CM), shoot primordia formation (SP), multiple shoot formation (MS) and fully developed plant (FD) stages. Transcript abundance has been normalized with average value of 26S and GAPDH housekeeping genes that served as internal controls. Relative expression of genes was calculated by comparing the transcript abundance of genes from leaf segment derived stages with root segment derived stages. *Error bars* represent mean ± SD for data recorded in triplicates (repeated thrice)

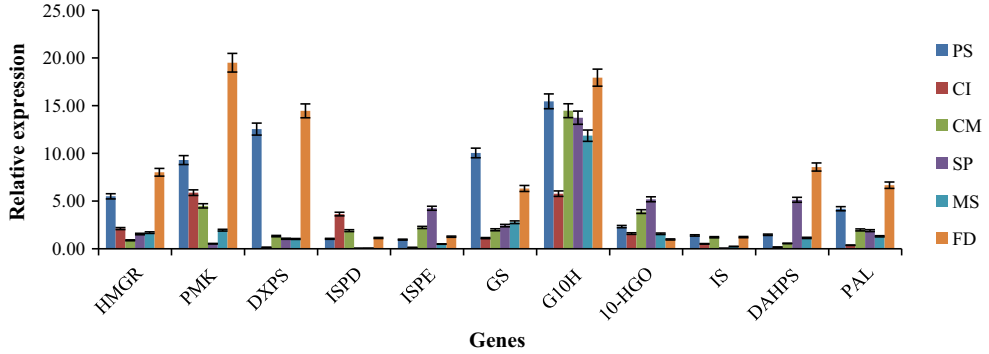


Fig. 11 Expression pattern of selected genes of MVA, MEP, iridoid and shikimate/phenylpropanoid pathways for P-I biosynthesis at different developmental stages of *P. kurroa* and *P. scrophulariiflora* derived from leaf segments at 15 ± 2 °C. These include: plant segment (PS), callus initiation (CI), callus mass formation (CM), shoot primordia formation (SP), multiple shoot formation (MS) and fully developed plant (FD) stages. Transcript abundance has been normalized with average value of 26S and GAPDH housekeeping genes that served as internal controls. Relative expression of genes was calculated by comparing the transcript abundance of genes in different developmental stages derived from leaf segments of *P. kurroa* with *P. scrophulariiflora*. *Error bars* represent mean ± SD for data recorded in triplicates (repeated thrice)

Previous reports on metabolic profiling of important phytochemicals such as podophyllotoxin in *Podophyllum hexandrum* and *P. peltatum*, rutin in *Fagopyrum tataricum* and *F. esculentum*, aconitine in *Aconitum* species have shown variation in their biosynthesis from one species to another (Gupta et al. 2011; Kumar et al. 2015a; Nagarajan et al. 2015). Therefore, P-I biosynthesis was also studied between *P. kurroa* and *P. scrophulariiflora*. These two species are rich source of P-I but higher P-I content has been observed in *P. scrophulariiflora* than *P. kurroa* under field conditions (Bantawa et al. 2010). However, our results showed slightly higher P-I content in *P. kurroa* shoots compared to *P. scrophulariiflora* in different developmental stages under in vitro conditions (Fig. 4). Sharma et al. (2014b) have also showed slightly higher P-I content in *P. kurroa* compared to *P. scrophulariiflora* under controlled conditions. So, to understand the molecular basis of P-I biosynthesis in both species, expression analysis of selected genes involved in secondary metabolism was carried out in different developmental stages obtained from leaf segments of *P. kurroa* and *P. scrophulariiflora* at 15 °C. As described for *P. kurroa*, similar pattern of P-I biosynthesis vis-à-vis morphogenetic stages was observed in *P. scrophulariiflora* (Fig. 4). Out of the selected genes, seven genes viz. HMGR, PMK, DXPS, GS, G10H, DAHPS and PAL showed 6–19 folds high expression in FD stage of *P. kurroa* compared to *P. scrophulariiflora* which was in corroboration with P-I content (Fig. 11). Al-Ghazi et al. (2009) have also observed stage specific and species specific expression of genes involved in phenylpropanoid and flavonoid pathways for the production of flavonoids, phenylpropanoids, terpenes and waxes in *Gossypium*

hirsutum L. and *G. barbadense* L. Therefore, this study suggested potential gene targets at FD stage for their utilization in genetic improvement of *P. kurroa*.

Overall, temperature and species influenced P-I production by regulating the genes involved in MVA, MEP, iridoid and shikimate/phenylpropanoid pathways under in vitro conditions.

Conclusion

The study concluded that P-I biosynthesis is developmentally regulated during different stages of differentiation in *P. kurroa.* Expression analysis of multiple genes of primary and secondary metabolic pathways at different morphogenetic stages of *P. kurroa* confirmed their involvement in P-I biosynthesis with shoot development. Modulation in expression of HMGR, PMK, DXPS, GS, G10H, DAHPS and PAL genes during different conditions including temperature and species has confirmed their role in P-I biosynthesis. These findings would be helpful in successful exploitation of plant metabolism for enhancement of P-I production and metabolic engineering of *P. kurroa*. The higher expression of pathways genes in relation to P-I content revealed *P. kurroa* as a better target compared to *P. scrophulariiflora*, although further validation of these genes by gene function approaches will fully ascertain their role in P-I biosynthesis.

Author contribution statement NS, HS and RSC conceived and designed research. NS conducted the tissue culture and molecular experiments. NS and HS wrote the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

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

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